Probing Domain Swapping for the Neuronal SNARE Complex with Electron Paramagnetic Resonance†

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ABSTRACT: Highly conserved soluble *N*-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins control membrane fusion at synapses. The target plasma membrane-associated SNARE proteins and the vesicle-associated SNARE protein assemble into a parallel four-helix bundle. Using a novel EPR approach, it is found that the SNARE four-helix bundles are interconnected via domain swapping that is achieved by substituting one of the two SNAP-25 helices with the identical helix from the second four-helical bundle. Domain swapping is likely to play a role in the multimerization of the SNARE complex that is required for successful membrane fusion. The new EPR application employed here should be useful to study other polymerizing proteins.

Neurotransmitter release at synapses requires fusion of synaptic vesicles with the presynaptic plasma membrane. In this process, the target plasma membrane proteins syntaxin 1A and SNAP-25[1] (synaptosome-associated protein of 25000 Da), and the VAMP2 (vesicle-associated membrane protein 2) interact with each other to form the ternary SNARE complex (*1*, *2*). The C-terminal domain of syntaxin, two separate helix domains from SNAP-25, and a soluble domain of VAMP2 are assembled into a core structure that is a 110 Å long parallel four-stranded coiled coil (*3*, *4*). In full-length SNAP-25 two helix domains are, however, flanked by a 56 amino acid region. This putative loop appears to be sufficiently long to transverse the coiled coil, connecting the two SNAP-25 helices from tail to head. Interestingly, when the flanking loop region of SNAP-25 is present in the complex, several higher molecular mass bands are observed in the SDS−PAGE than that of the core complex (*5*, *6*) (Figure 1a). The flanking loop region might play an essential role in causing the oligomerization of the SNARE complex. Multimerization has been observed for the native SNARE complex as well (*7*). Multimerization of the SNARE complex is required for membrane fusion as it is for viral membrane fusion systems (*8*, *9*).

One possible mechanism for the SNARE complex oligomerization is domain swapping (*10*, *11*): a domain in one molecule is replaced with the identical domain from the second molecule (Figure 1b). Such swapping scheme may propagate to make a polymeric protein chain (Figure 1b). The polymerization via domain swapping has been proposed as the main mechanism for promoting the disease-causing fibril formation for proteins including human cystatin C (*12*) and prion proteins (*13*). Thus, what is learned from the SNARE oligomerzation might help to understand better the mechanisms of protein polymerization.

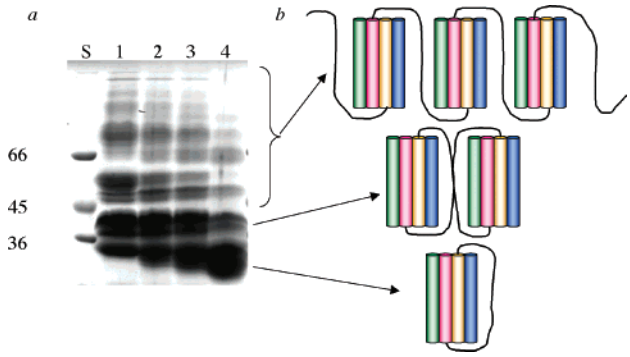

FIGURE 1: (a) Sodium dodesyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) analysis of the SNARE complexes. Ternary SNARE complexes are resistant to SDS. Lane S is the size marker. The lane 1 shows the appearance of the high molecular weight bands for the SNARE complex made of VAMP2 (amino acids 1−94), syntaxin 1A (amino acids 191−266), and SNAP-25 (amino acids 1−206), consistent with the western blot analysis in a previous report (*3*). Lanes 2−4 show SDS−PAGE analysis of the trypsin-digested SNARE complex for 3, 10, and 30 min, respectively. The trypsin-treated core SNARE complex migrates near 35 kDa, similar to the previous report (*3*). (b) Hypothetical models of the SNARE complex. Bottom: nondomain swapped SNARE complex. Middle: domain swapped dimer. Top: polymerized SNARE complex. The components of the four-helix bundle are color coded {green, syntaxin 1A; blue, VAMP2; red, N-terminal helix of SNAP-25 [SNAP-25(N)]; yellow, C-terminal helix of SNAP-25 [SNAP-25(C)]}.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Mutagenesis. Recombinant glutathione *S*-transferase (GST) fusion proteins were ex-

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[1] Abbreviations: EPR, electron paramagnetic resonance; GST, glutathione *S*-transferase; IPTG, isopropyl *β*-D-galactopyranoside; LB, Luria−Bertini broth; MTSSL, (1-oxy-2,2,5,5-tetramethylpyrroline-3-methyl)methanethiosulfonate; OD_{600}, optical density at 600 nm; PBST-Met, phosphate-buffered saline, pH 7.4, 0.05% (v/v) Tween-20, and 10 mM L-methionine; SNAP-25, synaptosome-associated protein of 25000 Da; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptor; VAMP2 (or v-SNARE), vesicle-associated membrane protein 2.

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pressed in *Escherichia coli* from the pGEX-KG vector (*14*), a derivative of the pGEX-2T vector (Amersham Pharmacia Biotech, Uppsala, Sweden). Plasmids encoding syntaxin 1A (amino acids 191−266), VAMP2 (amino acids 1−94), and SNAP-25 (amino acids 1−206) were prepared as previously described (*5*, *15*). The four native cysteines in the loop region of SNAP-25 were changed to alanines. All cysteine mutants were generated by a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with some modifications and confirmed by DNA sequencing (Iowa State University DNA Sequencing Facility).

Protein Expression, Purification, and Spin Labeling. GST fusion proteins were expressed in *E. coli* BL21(DE3) Codon Plus RIL (Stratagene, La Jolla, CA) and purified using glutathione−agarose chromatography. The cells were grown at 37 °C in LB with 2 g/L glucose, 100 μg/mL ampicillin, and 50 μg/mL chloramphenicol until the OD_{600} reached 0.6−0.8. Protein expression was induced by addition of IPTG (0.3 mM), and the cells were grown for 6 h at 30 °C for SNAP-25, at 22 °C for VAMP2, and at 16 °C for syntaxin 1A. After being washed with an excess volume of PBST-Met buffer, a 10-fold molar excess of MTSSL spin label was added, and the sample was incubated at room temperature for 4 h and incubated further at 4 °C overnight. After unreacted free spin labels were washed out, the spin-labeled protein was cleaved off from GST bound to the resin with thrombin. To prepare the ternary SNARE complex, equimolar amounts of syntaxin 1A, VAMP2, and SNAP-25 were mixed and incubated at 4 °C overnight. The ternary complex was purified with a Bio-Rad UNO Q1 perfusion column, equipped in the Bio-Rad Duoflo system, in 15 mM Tris-HCl (pH 8.4) buffer using a NaCl gradient from 15 mM to 0.5 M. Protein concentrations were estimated by Bio-Rad protein assay using BSA as a standard.

EPR Data Collection and Analysis. EPR spectra were obtained using a Bruker ESP 300 spectrometer (Bruker, Germany) equipped with a low-noise microwave amplifier (Miteq, Hauppauge, NY) and a loop-gap resonator (Medical Advances, Milwaukee, WI). 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. For the composite EPR spectrum the Fourier deconvolution method was used to decompose the noninteracting spectral component and the *J*-coupling spectral component (*16*).

RESULTS AND DISCUSSION

EPR Strategy To Detect Domain Swapping. The domain swapping hypothesis for the neuronal SNARE complex can be tested using site-directed spin-labeling EPR (*17*). When two nitroxide spin labels are placed sufficiently close to one another (less than 7 Å), the spin exchange or the *J*-coupling interaction would be turned on and becomes the dominant interaction. This spin exchange is observable only when the two nitroxides are physically in contact, and it decays exponentially as a function of the distance. Often, the *J*-coupling has been observed for two spin labels attached to two tertiary-contacting internal positions (*3*).

The short-range *J*-coupling interaction is theoretically different from the long-range dipolar interaction, although the two contributions are hardly separable. The dipolar

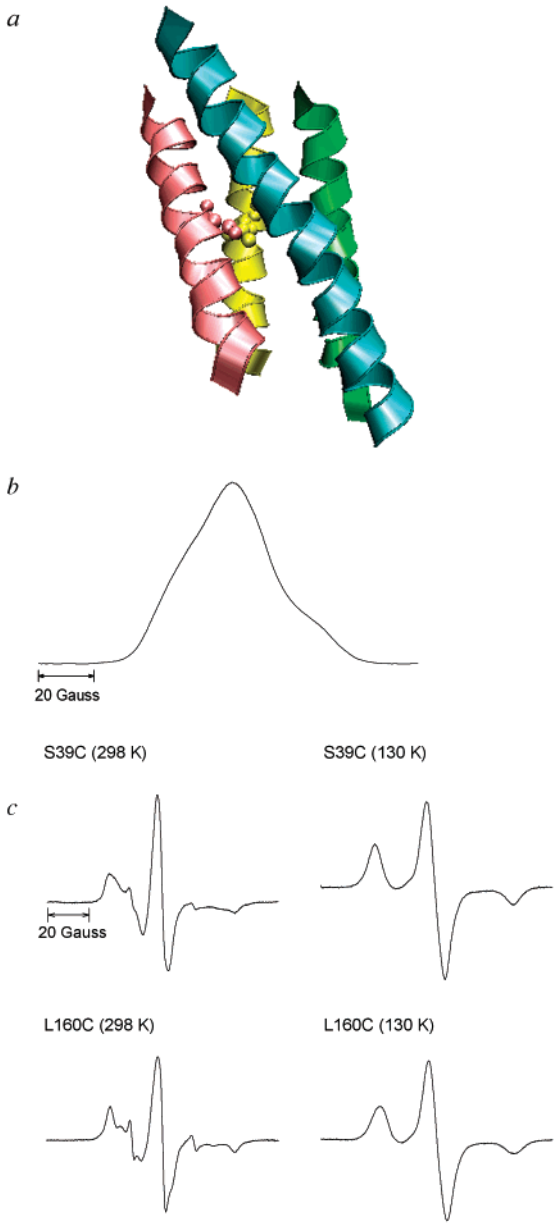

FIGURE 2: (a) The selected test pair in the SNARE core complex. Position 39 on SNAP-25(N) (red) and position 160 on SNAP-25-(C) (yellow) are spatially close from one another in the crystal structure (*4*). (b) Low-temperature absorbance EPR spectrum of the SNARE core complex made of SNAP-25(N) S39C, SNAP-25-(C) L160C, syntaxin 1A, and VAMP2 (*5*). (c) Room temperature and low-temperature derivative mode EPR spectra for SNARE complexes containing singly labeled SNAP-25.

interaction has the inverse cube dependence on the distance, and it stretches up to 25 Å (*17*). The dipolar interaction causes a simple line broadening and is frequently used to estimate the interspin distance (*18*, *19*).

Typically at low temperatures, the spin exchange causes the smearing of the hyperfine features of the EPR spectrum, resulting in a bell-shaped absorption line (Figure 2b) that is distinctly different and easily separable from the noninteracting EPR spectra.

Here, the experimental strategy is to utilize a selected pair of positions that are physically in contact and could be *J*-coupled when spin labeled (Figure 2a). However, if domain swapping occurs, two spin labels separately enter into two adjacent molecules, losing the *J*-coupling interactions when

the sample is mixed with the wild type. Conversely, if we individually prepare two singly labeled polypeptide chains at the corresponding positions, the domain swapping would bring two spin labels from separate polypeptide chains into one molecule so that the *J*-coupling is turned on.

For the SNARE proteins positions 39 and 160 in SNAP-25 qualify well as the test pair (Figure 2a). For this pair, the *J*-coupling interaction has been previously observed in the ternary SNARE complex (*3*) as well as in the binary SNARE complex (*20*) of sytaxin 1A and SNAP-25. In those cases, the N-terminal and the C-terminal fragments of SNAP-25 were separately prepared and assembled into the core four-helix bundle, and the putative loop region was not present.

For the EPR investigation the SNAP-25 construct representing amino acids 1−206, the C-terminal domain of syntaxin 1A of amino acids 191−266, and VAMP2 (amino acids 1−94) were expressed in bacteria with a glutatione *S*-trasferase (GST) affinity tag. Single cysteine mutants S39C and L160C and the double cysteine mutant S39C/L160C of SNAP-25 were made, and the mutants were derivatized with methanethiosulfonate spin label (MTSSL).

Spin Labels from Separate SNAP-25 Polypeptides Assemble into One SNARE Complex. First of all, it is important to establish that spin labels attached to position 39 and position 160 of SNAP-25 interact with one another only when they are within the same core four-helix bundle.

The SNARE complex containing the singly labeled SNAP-25 S39C mutant does not show any indication of spectral broadening due to either the *J*-coupling or the dipolar interaction (Figure 2c) when compared with several known noninteracting spectra (data not shown). The same is true for the SNARE complex containing the singly labeled SNAP-25 L160C mutant (Figure 2c). Further, when we mixed the former SNARE complex and the latter, we observed an EPR spectrum that is identical to the sum of two spectra taken individually for two singly labeled complexes. This indicates that the individual four-helix bundles are sufficiently separated, despite the possibility of the interconnection due to domain swapping.

However, when we used the 1:1 mixture of singly labeled SNAP-25 S39C and singly labeled SNAP-25 L160C to form the SNARE complex with VAMP2 and syntaxin 1A, we observed some significant spectral broadening in the EPR spectrum (Figure 3a). It is most likely that the intermolecular domain swapping played a role. The N-terminal helix carrying the nitroxide at position 39 in one SNAP-25 polypeptide chain and the C-terminal helix carrying the nitroxide at position 160 in another SNAP-25 polypeptide chain might have assembled into the same four-helix bundle (inset of Figure 3a).

The EPR spectrum of the mixed SNARE complex is a composite one consisting of two components, one noninteracting and the other bell-shaped and *J*-coupled. The Fourier deconvolution method has been developed to effectively resolve such two spectral components (*16*). Using this method, we found that the broad bell-shaped component (red line in Figure 3b) is nearly identical to the EPR spectra from the S39C/L160C doubly labeled core complex (Figure 2b). This suggests that the two nitroxides are *J*-coupled and physically in contact in a single four-helix bundle, although they are from two separate SNAP-25 polypeptide chains.

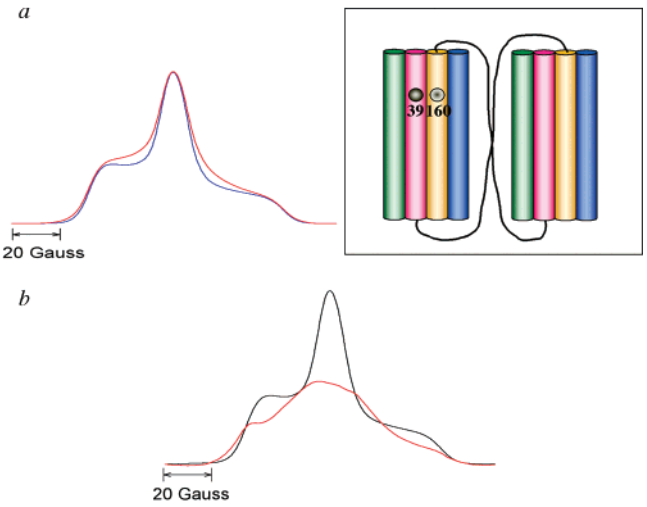

FIGURE 3: (a) The absorbance spectrum of the SNARE complex made of the 1:1 mixture of SNAP-25 S39C and L160C, syntaxin 1A, and VAMP2 collected at 130 K (red line) is compared with the sum of the absorbance spectrum from the SNARE complex containing SNAP-25 S39C and that from the SNARE complex containing SNAP-25 L160C (blue line). (Inset) Schematic model of the domain swapped SNARE complex dimer. Two four-helix bundles are intertwined by sharing two SNAP-25 polypeptide chains while maintaining the all-parallel coiled-coil structure. The N-terminal helix with the spin label at position 39 from one SNAP-25 and the C-terminal helix with the spin label at position 160 from another come to the same four-helical bundle and turn on the spin exchange *J*-coupling. (b) The absorbance spectrum of the SNARE complex containing the 1:1 mixture of SNAP-25 S39C and L160C was decomposed into the noninteracting component (black line) (66%) and the *J*-coupled component (red line) (33%) using the Fourier deconvolution method and spectral subtraction.

These EPR data strongly support the intermolecular domain swapping for the SNARE complex.

The Spin Dimer in a Single SNAP-25 Chain Splits into Two Separate Four-Helical Bundles. The domain swapping hypothesis for the SNARE complex can be tested further using the S39C/L160C doubly labeled mutants of SNAP-25. As expected, the SNARE complex containing SNAP-25 S39C/L160C shows a strongly *J*-coupled EPR spectrum, except for some slight contamination of the singly labeled species due to the less than quantitative (∼90%) spin labeling (red line in Figure 4a). However, when we made the SNARE complex with the 1:2 mixture of SNAP-25 S39C/L160C and unlabeled SNAP-25, a dramatic decrease of spin−spin interactions was observed (blue line in Figure 4a), likely due to the domain swapping. If the SNARE four-helix bundles are linked from one another by sharing helix domains of a SNAP-25 peptide chain (inset of Figure 4a), the spin dimer from a doubly labeled SNAP-25 S39C/L160C would dissociate. The unlabeled SNAP-25 would then fill the remaining helix positions in a "give and take" manner. This would result in the decrease of the population of the *J*-coupled spin dimer and the increase of the spin monomer.

We note that adding the preformed unlabeled complex to the preformed complex containing labeled SNAP-25 S39C/L160C did not change the EPR spectrum in the period of 24 h, suggesting that the component exchange hardly occurs once the complex is formed.

Using the Fourier deconvolution analysis, we found that this EPR spectrum is a composite one, composed of a noninteracting spectral species and the *J*-coupled spectral

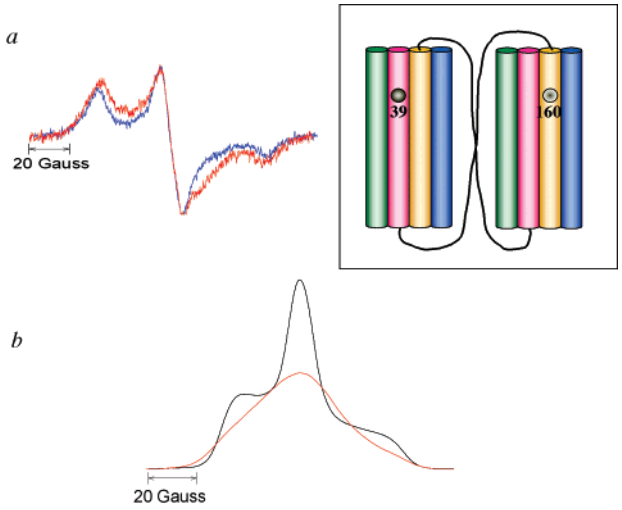

FIGURE 4: (a) The derivative mode EPR spectrum from the SNARE complex containing a 1:2 mixture of SNAP-25 S39C/L160C and unlabeled SNAP-25 (blue line), collected at 130 K, is compared with that from the complex containing only SNAP-25 S39C/L160C (red line). (Inset) A domain swapped dimer containing SNAP-25 S39C/L160C. Two spin labels attached to a single SNAP-25 peptide chain divide into two separate four-helix bundles. (b) The absorbance EPR spectrum from the SNARE complex containing a 1:2 mixture of SNAP-25 S39C/L160C and unlabeled SNAP-25 was decomposed into two components (50% noninteracting and 50% *J*-coupled) using the Fourier deconvolution method and spectral subtraction. The *J*-coupled component is essentially the same as that in Figure 2b.

species (red line in Figure 4b). This is consistent with the diminished *J*-coupled spin pair caused by the domain swapping with the unlabeled SNAP-25.

Multimerization May Be Important for SNARE-Induced Membrane Fusion. For viral membrane fusion proteins such as influenza hemagglutinin (HA) the clustering of HA at the fusion site is essential for the successful fusion (*8*, *9*). Likewise, we expect that a similar molecular clustering is perhaps required for the SNARE-induced membrane fusion. For the native SNARE proteins multiple SDS-resistant high molecular mass bands were observed in the SDS−PAGE analysis (*7*). This can occur through several mechanisms such as the interaction between transmembrane domains. However, we speculate that the domain swapping can also play a role in the multimerization of the SNARE complex on the membrane surface. The domain swapping perhaps induces ring structures of several four-helix bundles to be interconnected. Conceivably, the ring structure on the membrane surface could direct the effort of individual molecules toward membrane fusion collectively onto the fusion site. It is now necessary to establish the exact functional roles of the interhelical loop of SNAP-25. The membrane fusion assays based on PC-12 cells (*21*) and the in vitro assay developed by Weber and co-workers (*22*) should be adequate for this task.

New EPR Method for Studying the Protein Polymerization. Three-dimensional (3D) domain swapping has emerged as a mechanism for protein polymerization. This has been observed for other oligomeric proteins in the formation of highly ordered aggregates such as the fibrils characteristic of amyloid plaques. Domain swapping has also been proposed to play a role in mutimerization of the G-protein-coupled receptors (GPCR) (*23*). Despite the emerging biological significance of domain swapping, studying this phenomenon in polymerized forms or aggregates has been technically challenging.

In this work the EPR strategy employing the *J*-coupled nitroxide pair has proven highly effective in verifying the domain swapping in the neuronal SNARE complex. The *J*-coupling is a short-range interaction, only observable for physically contacting nitroxide pairs. Experiments can be judiciously designed to turn on or to turn off this specific interaction in accordance with the pattern of domain swapping. This EPR approach should be generally applicable to other protein systems, including proteins directly involved in neurodegenerative diseases. Although the short-range *J*-coupling has been utilized in the present study, the same approach employing the dipolar broadening should also be effective for other cases.

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