The structure of a truncated SNARE complex has been solved to 1.4-Å resolution revealing a stabilizing salt bridge, sites of hydration, and conformational variability of the ionic central layer that were not observed in a previously published structure at 2.4-Å resolution (1). The crystallization method used here is the same as that used in our previous study (1). In the final stages of fusion, neurotransmitter release is probably regulated by the Ca2+-binding protein synaptotagmin (8). Each SNARE protein contains at least one core domain that binds to other SNAREs to form a four-helix bundle (8). The four-helix bundle is composed of 16 layers transverse to the helical axes including a buried ionic core domain that binds to other SNARE proteins to form a synaptotagmin I (8). Each SNARE protein contains at least one core domain that binds to other SNAREs to form a four-helix bundle (8). The four-helix bundle is composed of 16 layers transverse to the helical axes including a buried ionic core domain that binds to other SNARE proteins to form a synaptotagmin I (8).

The neuronal SNARE complex consists of three SNAREs: synaptobrevin, syntaxin, and SNAP-25 (Synaptosome-associated protein, 25 kDa) (Fig. 1). Synaptobrevin (also referred to as vesicle-associated membrane protein) is a 12-kDa protein with a SNARE binding domain and a single spanning transmembrane domain (10, 11). Syntaxin is a 35-kDa protein with a three-helix bundle regulatory domain, a SNARE binding domain, and a single spanning transmembrane domain (1, 12, 13). SNAP-25 is a 25-kDa protein with two SNARE binding domains and a linker domain of 45 amino acids. SNAP-25 is targeted to the plasma membrane by its association with syntaxin via palmitoylation of three cysteine residues in the linker domain (14, 15).

Members of the conserved family of SNARE proteins play an important role in protein-assisted vesicle membrane fusion (1–7). SNARE complex formation juxtaposes synaptic vesicle and plasma membranes and thus may set the stage for vesicle membrane fusion. In the final stages of fusion, neurotransmitter release is probably regulated by the Ca2+-binding protein synaptotagmin (8). Each SNARE protein contains at least one core domain that binds to other SNAREs to form a four-helix bundle (8). The four-helix bundle is composed of 16 layers transverse to the helical axes including a buried ionic core domain that binds to other SNARE proteins to form a synaptotagmin I (8). The crystal structure of the neuronal SNARE complex revealed a conserved buried ionic layer at the center of the four-helix bundle (9) whose function is still uncertain (16). Most probably, it plays a role during N-ethylmaleimide-sensitive factor (NSF) driven disassembly of the SNARE complex, because mutations of this central layer can disrupt this process (17).

Here we present the crystal structure of the neuronal SNARE complex at a 1.4-Å resolution. To obtain this high resolution crystal structure, the individual SNAREs were truncated in comparison with the corresponding constructs used in the previously published crystal structure solved at a 2.4-Å resolution (9). This high resolution structure reveals new sites of hydration and stabilizing intermolecular interactions. We further characterize the thermal stability of this SNARE complex by CD and SDS melts, its oligomerization state, and its binding properties to synaptotagmin in the presence of Ca2+ and EDTA.

MATERIALS AND METHODS

Constructs

Constructs encoding sequences for the “minimal” complex (Fig. 1), rat syntaxin 1a residues 180–262 (SXa), synaptobrevin II residues 1–96 (SBa), SNAP-25 B residues 1–83 (SN1a), and SNAP-25 B residues 120–206 (SN2a) were described elsewhere (18). The cDNA-encoding sequences for the N-terminally truncated minimal complex and the microcomplex (Fig. 1), rat syntaxin 1a residues 188–262 (SBx) and residues 191–256 (Sxa), synaptobrevin II residues 25–96 (SBb) and residues 28–89 (Sbc), SNAP-25 B residues 7–83 (SN1b), and SNAP-25 B residues 132–204 (SN2b) and 141–204 (SN2c) were subcloned from these constructs into the expression plasmid pET28a (Novagen) or pGEX-3T (Amersham Biosciences) (SN2b only). The cDNA encoding the sequence for rat synaptotagmin I (139–421) were subcloned from synaptotagmin I cDNA into the pGEX-2T expression vector. The GST74 sequence variant of synaptotagmin (19) was generated using the QuikChange mutagenesis kit (Stratagene) using the oligomers 5′-TGTAACCAAGGAGCATGCGCGATGCCGTCGGTTGGCC-3′ and 5′-GGCAAGAAGGACGCGCTGCCAATCTGCTTGGTTAACA-3′. The correct sequences of all of the constructs were verified by DNA sequencing (Biocore Inc., Palo Alto, CA, or Keck facility, Yale University, New Haven, CT).

The pET28a expression plasmids were transformed into E. coli BL21(DE3) competent cells using standard protocols (20). Cells were grown at 37 °C in terrific broth supplemented with 100 μg/ml ampicillin media in 4-liter flasks. At an A600 of ~1, the temperature was reduced to 25 °C and expression was induced for 3 h using 1 mM isopropyl-1-thio-β-D-galactopyranosidase at an A600 of 20. The pGEX-2T expression plasmids were transformed into E. coli BL21-competent cells using standard protocols. Cells were grown at 37 °C in terrific broth supplemented with 100 μg/ml ampicillin media in 4-liter flasks. At an A600 of ~1, the temperature was reduced to 25 °C and expression was induced for 3 h using 1 mM isopropyl-1-thio-β-D-galactopyranosidase. Approximately 3 h after induction, cells were harvested by centrifugation for 20 min at 4200 rpm in a Beckman J6-HC Centrifuge using a JS-4.2 rotor. Cells were immediately frozen in liquid nitrogen and stored at ~80 °C.

Cells containing histidine-tagged SNARE proteins were resuspended in 1:10 denaturing lysis buffer (7 M guanidine, 50 mM Tris, pH 8.2, 10

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2 The abbreviations used are: SNARE, soluble NSF-attachment protein receptor; SNAP-25, Synaptosome-associated protein, 25 kDa; GST, glutathione S-transferase; DTT, diithiothreitol; MALLS, multi-angle laser light scattering; MPD, (±)-2-methyl-2,4-pentanedione; MES, 4-morpholineethanesulfonic acid.

3 The expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranosidase (Novagen) or pGEX-3T (Amersham Biosciences) (SN2b only). The cDNA encoding the sequence for rat synaptotagmin I (139–421) were subcloned from synaptotagmin I cDNA into the pGEX-2T expression vector. The GST74 sequence variant of synaptotagmin (19) was generated using the QuikChange mutagenesis kit (Stratagene) using the oligomers 5′-TGTAACCAAGGAGCATGCGCGATGCCGTCGGTTGGCC-3′ and 5′-GGCAAGAAGGACGCGCTGCCAATCTGCTTGGTTAACA-3′. The correct sequences of all of the constructs were verified by DNA sequencing (Biocore Inc., Palo Alto, CA, or Keck facility, Yale University, New Haven, CT).

4 The abbreviations used are: SNARE, soluble NSF-attachment protein receptor; SNAP-25, Synaptosome-associated protein, 25 kDa; GST, glutathione S-transferase; DTT, diithiothreitol; MALLS, multi-angle laser light scattering; MPD, (±)-2-methyl-2,4-pentanedione; MES, 4-morpholineethanesulfonic acid.
The SNARE complex was formed by mixing SNAP-25, synaptobrevin II, syntaxin-1A, and SNAP-25 B (gi:2116627). The sequence for the Constructs used for the 2.4Å crystal structure of the minimized complex (9) is shown in italics. In text, they are referred to as constructs SBa, SXa, SN1a, and SN2a. The N-terminal truncations of these constructs are underlined. In text, they are referred to as constructs SBb, SXb, SN1b, and SN2b. The constructs of the microcomplex are indicated by boxes. In the text, these constructs are referred to as Sbc, Sxc, Snc, and Snd. The SNARE complex and the ionic central (zero) layer is shown in red. Sequences displayed are rat synaptobrevin II (gi:6981613), rat syntaxin-1A (gi:207126), rat SNAP-25 B (gi:2116627).
above the mean in a test set comprising 10% of the data. The sites of hydration were identified by inspection of $F_o - F_c$ composite omit maps. At various points during refinement, $\sigma_A$-weighted electron density maps. At various points during refinement, $\sigma_A$-weighted, annealed $F_o - F_c$ composite omit maps were used to minimize the effects of model bias. All of the refinements were carried out using the program CNS (25). Statistical linear least-squares superposition of the structures was performed using the LSQMAN (31)

validation using $R_{free}$ (30), which was computed from a randomly chosen test set comprising 10% of the data. The sites of hydration were placed by inspection of peaks larger than three standard deviations above the mean in $F_o - F_c$, $\sigma_A$-weighted electron density maps. Only those sites were kept that exhibited reasonable protein solvent hydrogen-bonding distances without sterie conflict and whose $B$-value refined to $<55 \text{ Å}^2$. MPD and Ca$^{2+}$ were identified by inspection of $F_o - F_c$, and $2F_o - F_c$, $\sigma_A$-weighted electron density maps. At various points during refinement, $\sigma_A$-weighted, annealed $2F_o - F_c$ composite omit maps were used to minimize the effects of model bias. All of the refinements were carried out using the program CNS (25). Statistical linear least-squares superposition of the structures was performed using the LSQMAN (31)

from the Uppsala software factory suite. Graphical images were prepared using PyMOL (Fig. 5) (32) or GRASP (Fig. 4, a and b) (33).

RESULTS

Oligomeric State of the SNARE Complex—The neuronal SNARE complex has a tendency to oligomerize as shown by analytical ultracentrifugation and MALLS (18). The minimal SNARE complex obtained by limited proteolysis that was used in the 2.4-Å crystal structure had an apparent molecular mass of 60–90 kDa compared with a calculated molecular mass of 41 kDa (18). The C-terminal truncations of synaptobrevin by botulinum toxin B or tetanus toxin produced a monomeric SNARE complex (34). Furthermore, the C-terminal truncation of endobrevin (vesicle-associated membrane protein 8) in the endosomal SNARE complex produced a monodisperse sample (35). Therefore, we truncated the neuronal synaptobrevin at Trp-89 and lanes 1 and 2) or 2 μM GST alone (lanes 3 and 4) and glutathione resin. Experiments were performed in the presence of 0.5 mM Ca$^{2+}$ (lanes 1 and 3) or 1 mM EDTA (lanes 2 and 4). The microcomplex is indicated by an asterisk, and GST-synaptotagmin is indicated by an arrow.

Fig. 2. Stability of SNARE complexes. A, SDS stability of the minimal complex, the N-terminally truncated minimal complex, and the microcomplex. Experiments were performed as described previously (49). SNARE complex was mixed with SDS to a final concentration of 0.67%, heated at the indicated temperature for 5 min, and immediately run on a 10–15% SDS-PAGE gel. B, CD thermal melts of SNARE complexes as monitored at 220 nm. The minimal SNARE complex is shown in blue, the N-terminal truncation of the minimal SNARE complex is shown in red, and the microcomplex is shown in green.
**Fig. 4.** Superposition of neuronal and endosomal SNARE complex. A, C, superposition of the micro-SNARE complex and the endosomal SNARE complex (Protein Data Bank code 1GL2). The color code is as follows: synaptobrevin is shown in blue; syntaxin is shown in red; SNAP-25 is shown in green; endobrevin is shown in light blue; vti1b is shown in magenta; and syntaxin-8 and syntaxin-7 are shown in yellow. B, C, superposition of the micro-SNARE complex with the three complexes in the 2.4-Å crystal structure of the minimal SNARE structure (Protein Data Bank code 1SFC). The color code is as follows: the microcomplex is shown in red; the first molecule of the minimal complex crystal structure (chains a–d) (see Ref. 9) is shown in dark blue; the second molecule (chains e–h) is shown in medium blue; and the third molecule (chains i–l) is shown in light blue. C, B-value plot for the C residues of the micro-SNARE complex. The color code is as follows: synaptobrevin is shown in blue; syntaxin is shown in red; SNAP-25 SN1c is shown in light green; and SNAP-25 SN2c is shown in dark green.
thermal stability of the various SNARE complexes under native conditions by CD. Both the minimal and the N-terminally truncated SNARE complexes have a T_m of 94 °C, whereas the micro-SNARE complex has a reduced T_m of 89 °C (Fig. 2b).

Synaptotagmin Binding—Having demonstrated that the micro-SNARE complex forms a quantitative and stable complex, albeit with somewhat reduced T_m, we investigated whether it would retain its ability to interact with the C_2 domains of synaptotagmin I. GST pull-down experiments were conducted in the presence of both 1 mM EDTA and 0.5 mM CaCl_2. As shown in Fig. 3, synaptotagmin I is capable of binding the micro-SNARE complex in both the presence and absence of Ca^{2+}. These findings are consistent with prior reports using the C_2AB domain of synaptotagmin III and the minimal SNARE complex (37).

Microcomplex Structure—We next determined the crystal structure of the microcomplex. Crystals were obtained in space group P2_12_12_1 in the presence of MPD and CaCl_2 at 4 °C. These conditions are similar to the previous crystallization conditions used for the minimal SNARE complex (9). The crystal structure contained only one copy of the complex per asymmetric unit in contrast to the minimal SNARE complex that crystallized in a different space group (I222) with three complexes per asymmetric unit. Most importantly, the crystals of the microcomplex diffracted to 1.4 Å, making this the highest resolution crystal structure of a SNARE complex available to date. All of the residues of the microcomplex were visible in the final model, which refined to a R_crys value of 19.8% and a R_free value of 22.4%. The statistics of the diffraction data and the final refined model are shown in Tables I and II. The electron density maps are of excellent quality (Fig. 5b) and allowed assignments of nearly all of the side-chain rotamers.

Three Ca^{2+} sites were found that are coordinated by symmetry-related molecules. These sites were visible as 8 σ peaks in 2F_o − F_c maps. The coordinating oxygen atoms are located on SNAP-25 Gln-20 and Glu-27 of a SNARE complex and synaptobrevin Asp-80, Lys-83 of SNAP-25, Tyr-88, Trp-89, and syntaxin Lys-226 of a symmetry-related complex. Several water molecules complete the coordination spheres around the Ca^{2+}. Because these Ca^{2+} sites are located at the artificially truncated C terminus of the microcomplex, it is probable that these binding sites are the result of crystallization conditions.

As expected, the micro-SNARE complex forms a four-helix bundle. The C_o atoms of the microcomplex were superimposed on that of the minimal complex structure and on all of the homologous residues of the endosomal complex structure. The results of these superpositions are shown in Fig. 4. a and b, and Table III. It is interesting to note that the root mean square (r.m.s) difference between the microcomplex structure and the endosomal structure is larger than the root mean square difference observed when comparing either structure to the minimal complex structure. In contrast to the endosomal structure, the microcomplex displays little variation in B-values over most of the four-helix bundle (see Fig. 4c). Only the second α-helix of SNAP-25 between layers 2 and 8 and synaptobrevin between layers −7 and 0 display any systematic increase in C_o B-values.

To further compare the various SNARE complex crystal structures, we superimposed the residues around the ionic central layer (Fig. 1) with the corresponding residues of the previously solved structures. For the neuronal SNAREs, the layer consists of synaptobrevin Arg-56, syntaxin Gln-226, SNAP-25 Gln-53, and SNAP-25 Gln-174. For the endosomal complex, the corresponding residues are endobrevin Arg-76, syntaxin-7 Gln-199, vti1b Gln-170, and syntaxin-8 Gln-179. The root mean square differences for residues at this layer between the microcomplex and the endosomal complex are
0.315 and 0.509 Å for Cα and all atoms, respectively. Several of the SNARE crystal structures show the presence of a bifurcated hydrogen bond between synaptobrevin Arg-56 and SNAP-25 Gln-53 and Gln-174 (Fig. 5a). However, in one of the molecules of the minimal complex crystal structure (Fig. 5a, cyan), Arg-56 exhibits a rotamer that allows direct hydrogen bonding from each of the side-chain nitrogen atoms of Arg-56 to each of the buried glutamines. This Arg-56 rotamer is also visible in the structure of the squid neuronal SNARE complex with complexin (38). Thus, Arg-56 exhibits significant conformational variability among the different structures, whereas the three glutamines exhibit very similar conformations (Fig. 5a). The observed conformational variability of the central layer may suggest a possible functional role in the disassembly process (39).

The quality of our diffraction data allowed us to assign numerous sites of hydration that were previously unobservable (Fig. 5b). Of particular interest is a buried water molecule (Fig. 5b, H2O 89) at the ionic central layer. This water molecule is located 3.10 Å from the ε-nitrogen of Arg-56, satisfying the hydrogen bond requirements of this nitrogen. It is possible that this water molecule is not present when Arg-56 adopts alternate conformations observed in some of the other crystal structures.

The formation of salt bridges on the surface of proteins is known to stabilize exposed structural elements. The presence of surface salt bridges positioned to stabilize buried structural elements is less common. However, a carboxylic acid ε-oxygen of SNAP-25 Glu-170 shows just such an interaction buttressing SNAP-25 Gln-174 at 2.79 Å through the Gln-174 ε-nitrogen (Fig. 5b). This interaction stabilizes the SNAP-25 Gln-174 ε-oxygen as compared with the Arg-56 Nε1 and syntaxin Gln-226 interaction probably reflects the proximity of the negatively charged carboxylic group from SNAP-25 Glu-170. We note further that the three glutamines exhibit very similar conformations (Fig. 5b). The observed conformational variability of the central layer probably reflects the proximity of the negatively charged carboxylic group from SNAP-25 Glu-170. We note further that the three glutamines exhibit very similar conformations (Fig. 5b). Such structural details offer important new information for the design of future experiments to study SNARE complex function.

REFERENCES