High Resolution Structure, Stability, and Synaptotagmin Binding of a Truncated Neuronal SNARE Complex*

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 (SXb) 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-2T (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 G374 sequence variant of synaptotagmin (19) was generated using the QuikChange mutagenesis kit (Stratagene) using the oligomers 5'-TAACCAAGAAGACTTTGCCGATGGCGTCGTTCTTGCC-3' and 5'-GGCAAGACGCGCATGGCGAATGTTCTTGCTGCCC-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 g/ml ampicillin media in 4-liter flasks. Cells were harvested at 37 °C in terrific broth supplemented with 100 g/ml ampicillin media in 4-liter flasks. At an absorbance of 0.5, the temperature was reduced to 25 °C and expression was induced for 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. The pET28a expression plasmids were transformed into E. coli BL21(DE3) competent cells using standard protocols. Cells were grown at 37 °C in a BIOFLO 3000 fermentor (New Brunswick, NJ) using ECPYM1 medium (21) in the presence of 50 g/ml kanamycin sulfate. The expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside 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 g/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 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.

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sequence alignment of neuronal SNAREs. The sequence for the constructs used for the 2.4-Å 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.

The concentration of the proteins was calculated by using a Bradford protein assay kit (Bio-Rad) and expressed in mg/ml. Light scattering was performed in 150 mM NaCl, 10 mM HEPES, pH 7.8, and 5 mM DTT. The elution profile was monitored by UV absorption at 280 nm, light scattering at 690 nm, and differential refractometry. Light scattering and differential refractometry were carried out using the Dawn and OptiLab instruments (Wyatt Technology). Analysis was carried out using the Astra software (22).

Crystallographic Data—Diffraction data were collected at the Lawrence Berkeley National Laboratory Advanced Light Source beamline 8.2.1 from a single crystal in one pass at 100 K using an Area Detector System Quantum 210 2D detector. Diffraction data were collected to a 1.4-Å resolution. The phases for the diffraction data were obtained by molecular replacement using the program ENIGMA (26). Statistics of the diffraction data are shown in Table 1. The crystals formed with one copy of the SNARE complex per asymmetric unit in space group P212121.

Phases—The phases for the diffraction data were obtained by molecular replacement using the direct rotation search (24) as implemented in the program CNS (version 1.1) (25) using diffraction data from 15 to 4-Å resolution and a single crystal. The search model consisted of one of the three non-crystallographically related copies of the neuronal SNARE solved at 2.4 Å (9). It was truncated to contain only those residues present in the microcomplex.

Biochemistry—CD experiments were performed on a Aviv 62DS spectrometer at 150 mM NaCl, 20 mM Na2HPO4, pH 7.8, and 10 mM SNARE complex. Temperature scans were performed between 37 and 97 °C at two-degree intervals with 1-min equilibration between temperature changes and 1-min acquisition with data averaging at each temperature point. The elution profile was monitored by UV absorption at 280 nm, light scattering at 690 nm, and differential refractometry. Light scattering and differential refractometry were carried out using the Dawn and OptiLab instruments (Wyatt Technology). Analysis was carried out using the Astra software (22).

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Model Building—Model building was performed using the program O (26). The initial model was optimized by rigid body refinement followed by simulated annealing with torsion angle dynamics (27), restrained B-value refinement (28), and conjugate gradient minimization using the MLF target function (29). Overall anisotropic scale factors and bulk solvent correction were applied to the diffraction data. The progress of model rebuilding and refinement was monitored by cross-
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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 to 90 kDa compared with a calculated molecular mass of 41 kDa (18). We performed temperature-dependent SDS and CD melts of the micro-SNARE complex and compared the results to both the minimal SNARE complex (36) and a SNARE complex that was obtained from the minimal complex by truncation at the N terminus (Fig. 2). Our experiments revealed a roughly 20 °C reduction in the stability of the micro-SNARE complex in SDS relative to both the minimal and N-terminally truncated SNARE complexes (Fig. 2a). In light of this reduction in SDS stability, we further investigated the

validation using $R_{true}$ (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 $F_o - F_e$, $\lambda$-weighted electron density maps. Only those sites were kept that exhibited reasonable protein solvent hydrogen-bonding distances without steric conflict and whose $B$-value refined to $<35$ Å$^2$. MPD and Ca$^{2+}$ were identified by inspection of $F_o - F_e$ and $2F_o - F_e$, $\lambda$-weighted electron density maps. At various points during refinement, $\lambda$-weighted, annealed $2F_o - F_e$ 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).

Table I

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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 (43). 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. 3. Synaptotagmin I binding of microcomplex. Shown is a GST pull-down assay of the micro-SNARE complex using the GST-tagged C2AB fragment of synaptotagmin I (residues 139–421). In each lane, C2AB microcomplex was incubated with 2.0 μM GST-synaptotagmin I C2AB (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. 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$_{2A}$B 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$_1$2$_1$2$_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 (9). The crystal structure is of excellent quality (Fig. 5) 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 peaks in 2$F_o$ – $F_c$ 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$_2$ 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 $\gamma$-helix of SNAP-25 between layers 2 and 8 and synaptobrevin between layers 7 and 0 display any systematic increase in C$_2$ 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 are shown in Table III.
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0.315 and 0.509 Å for C 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, H atoms) at the ionic central layer. This water molecule is located 3.1 Å from the 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 --oxygen of SNAP-25 Gln-174 shows just such an interaction buttressing SNAP-25 Gln-174 at 2.79 Å through the Gln-174 --oxygen (Fig. 5b). This interaction stabilizes the SNAP-25 Gln-174 --oxygen which in turn interacts with synaptobrevin Arg-56 N2 at a distance of 2.65 Å (Fig. 5b). The close interaction of synaptobrevin Arg-56 N2 with the SNAP-25 Gln-174 --oxygen as compared with the Arg-56 N1 and syntaxin Glu-226 interaction probably reflects the proximity of the negatively charged carboxylic group from SNAP-25 Gln-174. We note further that some of the residues that are involved in phospholipid binding are also involved in oligomerization.

We have shown that C-terminal truncation of synaptobrevin, C-terminal to residue 89 (see Fig. 1), along with C-terminal truncation of syntaxin produces a SNARE complex that is both monomeric and monodisperse. Interestingly, the fragments of synaptobrevin containing residues 77–90 bind to phospholipids (40, 41). Thus, it is possible that some of the residues that are involved in phospholipid binding are also involved in oligomerization.

REFERENCES