SNARE proteins mediate intracellular membrane fusion by forming a coiled-coil complex to merge opposing membranes. A “fusion-active” neuronal SNARE complex is a parallel four-helix bundle containing two coiled-coil domains from SNAP-25 and one coiled-coil domain each from syntaxin-1a and VAMP-2. “Prefusion” assembly intermediate complexes can also form from these SNAREs. We studied the N-terminal coiled-coil domain of SNAP-23 (SNAP-23N), a non-neuronal homologue of SNAP-25, and its interaction with other coiled-coil domains. SNAP-23N can assemble spontaneously with the coiled-coil domains from SNAP-23C, syntaxin-4, and VAMP-3 to form a heterotetrameric complex. Unexpectedly, pure SNAP-23N crystallizes as a coiled-coil homotetrameric complex. The four helices have a parallel orientation and are symmetrical about the long axis. The complex is stabilized through the interaction of conserved hydrophobic residues comprising the α and δ positions of the coiled-coil heptad repeats. In addition, a central, highly conserved glutamine residue (Gln-48) is buried within the interface by hydrogen bonding between glutamine side chains derived from adjacent subunits and to solvent molecules. A comparison of the SNAP-23N structure to other SNARE complex structures reveals how a simple coiled-coil motif can form diverse SNARE complexes.

Endosomal trafficking and vesicle secretion in eukaryotic cells requires fusion of membrane compartments. Synaptic vesicle secretion in neurons has served as a paradigm for understanding the molecular basis for membrane fusion (1). In general, a v-SNARE1 called VAMP (synaptobrevin) is associated with the vesicle membrane and two t-SNAREs called SNAP-25 and syntaxin are associated with the plasma or target membrane. According to the SNARE hypothesis, specific pairing between v- and t-SNAREs brings together two membranes for fusion (2). Following fusion, NSF and SNAPs dissociate the SNARE complex in an ATP-dependent reaction (3).

SNARE proteins contain coiled-coil regions that mediate complex formation (4). VAMP and syntaxin proteins each have a single SNARE-interacting coiled-coil domain followed by a C-terminal transmembrane domain, whereas SNAP-25 proteins have N- and C-terminal coiled-coil domains separated by a loop that attaches to membranes via palmitoylated cysteines (5). The coiled-coil sequences are highly conserved among SNARE families (4). The homologous regions are made up of ~8 heptad repeats in which the hydrophobic residues in the α and δ positions of each repeat align in 16 layers (4). The central “0” layer is uniquely polar and consists of a glutamine in syntaxin and the SNAP-25 N- and C-terminal coiled-coil domains and an arginine in the VAMP coiled-coil domain. The structure of a core SNARE complex from neurons revealed a parallel four-helix bundle composed of two coiled-coil domains from SNAP-25, and one coiled-coil domain each from syntaxin-1a and VAMP-2 (6, 7). The parallel orientation of SNARE proteins attached to opposite membranes suggests a “zipper-like” mechanism for membrane fusion (7). A distantly related endosomal SNARE complex has a very similar structure, suggesting a common fusion mechanism mediated by neuronal, endosomal, and possibly other SNARE complexes (8).

SNARE isoforms SNAP-23, syntaxin-4, and VAMP-3 (Cellubrevin) are present in many non-neuronal cell types (9–12). Foster et al. (13) found that soluble forms of SNAP-23, syntaxin-4, and VAMP-2 (present in neurons and other cells) formed binary complexes but not a native-like ternary complex. In contrast, Yang et al. (14) found that the coiled-coil domains of these proteins could form a heterotetrameric complex. However, the stability of this non-neuronal SNARE complex was decreased relative to a neuronal one composed of SNAP-25, syntaxin-1a, and VAMP-2. These studies suggest that this group of non-neuronal SNARE proteins may function differently than the neuronal ones.

The structures of non-neuronal SNAREs and their complexes are not well characterized. We used a structural approach to understand the function of non-neuronal SNARE proteins involved in membrane fusion. Interestingly, we find that SNAP-23N (Fig. 1) can form a parallel four-helix bundle in the absence of other SNARE proteins. The 2.3 Å crystal structure of SNAP-23N reveals important similarities and differences with other SNARE complex structures. Comparison of these structures extends our understanding of how SNARE proteins assemble and function.
SNAP-23N Structure

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—PCR DNA fragments encoding amino acids 19–71 of rat VAMP-3 (100% identical to human), 210–292 of rat syntaxin-4 (94% identical to human), and 23–76 and 158–208 of human SNAP-23 were cloned into the BamHII and EcoRI sites of a pET vector (Novagen) that expresses glutathione S-transferase (GST) fusion proteins containing a thrombin protease site. The expression plasmid introduces a vector-derived Gly-Ser at the N terminus of each peptide that remains after thrombin cleavage. The constructs were transformed individually into BL21 (DE3) Escherichia coli cells. To prepare selenium-methionine-substituted SNAP-23N (SeMet-SNAP-23N), the SNAP-23N-expressing plasmid was transformed into B834 (DE3) methionine auxotroph E. coli cells and grown in M9 minimal medium containing a complete medium of amino acids except for methionine substituted by selenomethionine (Sigma). Bacteria were grown in 50 μM Tris-HCl, pH 7.5, 100 mM NaCl, and 50 μM SeMet, and their final concentrations were determined using the Coomassie plus protein assay reagent (Pierce) with bovine serum albumin as a standard. Purity was determined by SDS-PAGE and MALDI-TOF mass spectrometry.

**SNARE Binding Assay**—Approximately equimolar amounts (20 μM) of VAMP-3, syntaxin-4, SNAP-23N, and SNAP-23C-coiled-coil peptides were mixed together in the indicated combinations and incubated at 4 °C for 2 h. The binding buffer contained 50 mM Tris-HCl, pH 7.5, and 50 mM NaCl. Analysis of each binding reaction was analyzed by non-denaturing electrophoresis on 10% polyacrylamide gels.

**Crystallization, X-ray Diffraction Data Collection, and Phasing**—Underivatized SNAP-23N was crystallized by the hanging drop vapor diffusion method at 23 °C. SNAP-23N was crystallized at a concentration of 1–4 mg/ml by combining 1 μl of purified protein in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl with 1 μl of reservoir solution containing 0.1 M Hepes, pH 7.5, 0.2 M CaCl₂, and 28% polyethylene glycol (PEG) 400. Typically, crystals were observed within 24 h and grew to maximal size by 48–72 h. SeMet-SNAP-23N was crystallized using the same buffer conditions, but at 4 °C.

Diffraction data were collected by flash-freezing crystals from the molybdenum in the cryostream at 100 K. Native diffraction data were recorded on a 300 mm MarResearch image plate detector using a rotating anode source with mirror optics (Osmic™). Multiwavelength anomalous diffraction (MAD) data were recorded on a Brandeis four-module CCD detector at beamline X-12C of the National Synchrotron Light Source of Brookhaven National Laboratories. Data were processed and scaled using DENZO SCALEPACK (15) for home data and HKL-2000 (15) for MAD data. Native crystals diffracted x-rays on the home detector to 2.5-Å resolution and SeMet crystals diffracted at the synchrotron source to 2.3 Å resolution. Crystallographic data are summarized in Table I.

**Structure Determination**—Molecular replacement with COMO (16) using the coordinates for rat SNAP-25N (7) as a search model yielded clear rotation and translation solutions for the peptide. The resulting 2Fₒ – Fᵣ electron density map was readily interpretable for the N-terminal region of SNAP-23N, but weak density for the more C-terminal region of the structure complicated rebuilding and refinement. Therefore, we recorded a three-wavelength MAD data set with SeMet-substituted peptide. An electron density map generated from the MAD data confirmed the molecular replacement solution and revealed clear density for most of the peptide sequence. However, density for the C-terminal 8 residues was relatively weak. The selenium positions of the 2 SeMet residues were located and refined with SOLVE (17) using data from 20 to 3 Å. Manual rebuilding with O (18) and refinement with CNS (19) to the higher resolution SeMet data produced a final model with an R value of 21.6% and a free R value of 31.6%. The higher than expected final R factor may be a consequence of the relatively weak density and high thermal parameters for the C-terminal 8 residues in the structure. Similar R factors were reported for a related SNARE complex for similar reasons (20). The final model contains residues 23–76 of SNAP-23N and 43 solvent molecules.

**RESULTS**

Non-neuronal SNARE peptides from SNAP-23, syntaxin-4, and VAMP-3 were expressed recombinantly in bacteria and purified as described under “Experimental Procedures.” The N- and C-terminal boundaries were chosen based on homology to...
the coiled-coil regions in the neuronal SNARE complex (4). Each peptide is a 55-mer (including the vector-derived N-terminal Gly-Ser) that corresponds to 7.5 heptad repeats. All peptides except the SNAP-23C peptide were soluble in the milli-}
of
electrolyte; SNAP-23C has a pI of 9.39, which may contribute to its modest solubility. A binding assay using native polyacrylamide gel electrophoresis was performed to determine whether the SNARE peptides could interact with each other. Mixing equimolar amounts of all four peptides yielded a new complex band not observed with any of the three-way combinations of peptides (Fig. 2A). In another binding experiment, GST-SNAP-23C attached to glutathione beads was mixed with the other three SNARE peptides, and a pure complex confirmed this prediction (see below).

TABLE I

<table>
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<tr>
<th>Data collection statistics</th>
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<td>Data set</td>
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<td>Rmerge (^{a})</td>
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Refinement and model statistics\(^{b}\)

| R-factor (P-factor) \(^{c}\) | 0.640/0.72          |
| Resolution range (Å) \(^{a}\) | 27.3/31.6          |
| Average B-factors (Å\(^2\)) | 20–2.3             |
| Main chain                 | 29.1                |
| Side chain                  | 32.7                |
| Solvent                     | 41.3                |
| r.m.s.d. from ideality\(^f\) | 0.005              |
| Bonds (Å)                  | 1.00                |

\(^{a}\) Rmerge = \(\Sigma |I_{obs} - I_{ave}| / \Sigma I_{ave}\).

\(^{b}\) Based on MAD data.

\(^{c}\) FOM, figure of merit \((1/\langle\cos^2\rangle^2 + \langle\sin^2\rangle^2\rangle^{1/2}; 20–3 Å\).

\(^{d}\) \(R\)-factor = \(|F_{obs} - F_{calc}| / \Sigma |F_{obs}|\), where free \(R\) is calculated for 10\% of reflections.

\(^{f}\) No data cutoff.

\(^{f}\) r.m.s.d., root mean square deviation.

The electron density map shows excellent main chain connectivity along its entire length from Ser-23 to Leu-76 (Fig. 3). However, the side chain conformations of surface-exposed residues between Lys-64 and Leu-81 are less well defined. Four molecules of SNAP-23N produce a parallel four-helix bundle straits resulting from the 4-fold symmetry of the space group suggested that SNAP-23N molecules were organized as a parallel homotetrameric complex. The human SNAP-23N sequence is shown aligned with homologous sequences in Fig. 1.

Purified SNAP-23N crystallized in the presence of divalent metal ions (e.g. CaCl\(_2\) or MgCl\(_2\)). The native crystals are rectangular prisms with approximate dimensions of 0.08 × 0.02 × 0.02 mm\(^3\), whereas the SeMet crystals are square plates with approximate dimensions of 0.13 × 0.13 × 0.01 mm\(^3\). Both crystal forms have the same tetragonal space group with one copy of SNAP-23N in the asymmetric unit. Symmetry con-
with overall dimensions of \(-80 \times 24 \times 24 \, \text{Å}^3\) (Fig. 4, A and B). Each peptide in the complex has the same conformation, and thus, the tetramer has exact 4-fold symmetry as required by the tetragonal crystal lattice. The SNAP-23N peptide is acidic (pI = 4.70), and the complex displays a mostly electronegative solvent-exposed surface; in contrast, the complex interface is mostly neutral (Fig. 4C).

The homotetrameric complex is held together primarily through hydrophobic interactions (Fig. 5A). Hydrophobic residues occupy the \(a\) and \(d\) positions of the coiled-coil heptad repeats, and their side chains face the interior of the complex (Fig. 1). The only exception is Gln-48 whose polar side chain is also buried in the interface (Fig. 5B). Thus, planes of buried residues occur at regular intervals along the chain and create 16 layers of intermolecular contacts. Gln-48 is located in the central or 0 layer as in other SNARE complexes. Our structure shows that the polar groups of the Gln-48 side chains point toward the center of the complex and are only 2.5 Å apart from one another (Fig. 5B). Although it is a deviation from the strict crystallographic symmetry, we have modeled the four Gln-48 residues with alternating side chain rotamers as it allows chemically reasonable hydrogen bonding in the tetramer. In addition, a water molecule hydrogen bonds to the free polar group not coordinated by a Gln-48 side chain in an adjacent molecule (Fig. 5B).

Although Ca\(^{2+}\) is required for secretion in a variety of cell types (21) and divalent cations were required for crystallization, Ca\(^{2+}\) ions were not observed in the electron density map in the crystal structure. Thus, Ca\(^{2+}\) ions may have a nonspecific stabilizing effect on the SNARE complex structure.

**DISCUSSION**

We found that SNAP-23N can associate with other non-neuronal SNARE coiled-coil domains to form a minimum heterotetrameric complex. This result is in agreement with the findings of Yang et al. (14) who used coiled-coil domains from SNAP-23, syntaxin-4, and VAMP-2 (98% identical to VAMP-3) but conflicts with the work of Foster et al. (13) who could not detect a ternary complex using GST-fused cytoplasmic regions of the same proteins. It is possible that the assay conditions of the latter study were not suitable for creating a ternary complex.

Work by others (22, 23) indicated that the neuronal SNAP-25N homologue, SNAP-25N, is mostly unstructured in solution. SNAP-23N and SNAP-25N are 72% identical and so might be expected to behave similarly. On the other hand, heterotetrameric coiled-coil complexes containing SNAP-25 have a higher stability compared with those containing SNAP-23 (14); perhaps the driving force for SNAP-23N self-association destabilizes its interaction with other SNARE coiled-coil domains.

Comparison of the SNAP-23N structure to other SNARE complexes reveals important similarities and differences. Like the SNAP-23N homotetramer, the neuronal heterotetramer and complexes between the SNAP-25 coiled-coil domains with the syntaxin-1a coiled-coil domain are arranged as parallel four-helix bundles (7, 23–25). In contrast, a syntaxin-1a homotetramer contains two pairs of helices that run antiparallel to one another (26). The neuronal heterotetrameric complex has a much larger left-handed helical twist compared with the SNAP-23N homotetramer, because each chain in the heterotetramer has a slightly different conformation. Despite these differences the SNAP-23N homotetramer and neuronal heterotetramer appear remarkably similar (Fig. 6B). In fact, superimposition of the SNAP-23N and SNAP-25N chains re-
rules proposed by Harbury bundle can be predicted from the SNAP-23N sequence using the 0 layer (Fig. 5). This structural difference may stem from an inherent difference between SNAP-23 and SNAP-25, the conformational lability of the SNAP-23N C-terminal region, or the fact that SNAP-23N in a homotetrameric complex is being compared with SNAP-25N in a heterotetrameric complex.

Hydrophobic intermolecular contacts are found in all SNARE complex structures. Interestingly, the packing of hydrophobic a and d residues (Fig. 1) to form a parallel four-helix bundle can be predicted from the SNAP-23N sequence using rules proposed by Harbury et al. (27). An enrichment of β-branched residues at the d position but not the a position favors coiled-coil tetramers, whereas the reverse pattern favors coiled-coil dimers. Each intermolecular layer of our structure has the same four hydrophobic side chains as a result of the 4-fold symmetry, whereas other SNARE complex structures have different hydrophobic side chains in each layer. The latter is also true for the syntaxin-1a homotetrameric structure, because the chains in this complex are antiparallel (26). Bulky hydrophobic side chains are accommodated by complementary smaller hydrophobic ones in each layer of these other SNARE complex structures (4). The SNAP-23N sequence lacks bulky hydrophobic side chains that would clash with one another as a result of parallel packing. Therefore, SNAP-23N may be unique among SNARE coiled-coil domains in its ability to form a parallel homotetrameric complex.

The SNAP-23N structure has buried glutamites in the 0 layer like other SNARE complexes (7, 24, 26). Buried polar residues at the center of coiled-coil complexes are thought to be important for orienting the direction of coiled-coil complexes (28, 29). The molecular arrangement of the 0 layer varies among SNARE complex structures. The three buried glutamates in the neuronal heterotetrameric complex structure are stabilized by hydrogen bond contacts between their carbonyl groups and the guanidinium side chain of an arginine from VAMP-2 (7). A network of water molecules coordinates the four buried glutamites in the SNAP-25N/syntaxin-1a tetrameric complex (24). The buried glutamites in the syntaxin-1a homotetramer hydrogen bond directly to each other as in our structure (26). However, in contrast to the SNAP-23N structure, the four glutamites in the syntaxin-1a structure are arranged in pairs on opposite sides of the complex due to the antiparallel topology of the helices. Therefore, the hydrogen bonding arrangement of the four buried glutamites in the SNAP-23N homotetramer is a new variant to the SNARE complex 0 layer (Fig. 5B).

Finally, the surface of the SNAP-23N structure is mostly acidic like other SNARE complexes (Fig. 4C) (7, 24, 26). It was speculated that SNARE regulatory molecules, NSF and SNAPs, have diverse recognition properties for different SNARE complexes by surface complementarity rather than for a specific sequence (30).

The biological significance of the homotetramer we observe is unclear. To date, homooligomerization of SNAP-23 has not been demonstrated in vivo. However, SNARE complex assembly is difficult to study in cells, and therefore purified SNARE proteins are used as a surrogate for this process (23, 31). What is the conformation of a v-SNARE or t-SNARE prior to vesicle fusion? One possibility is that SNAREs are disordered on membranes before interacting to form a membrane fusion complex. On the other hand, this seems unlikely given the growing data base of assembly intermediate structures. Furthermore, the high local concentrations of SNARE proteins on membranes should favor oligomerization. We propose that the SNAP-23N homotetramer and other assembly intermediates may represent low stability “storage forms.” This could prevent proteolytic degradation of SNAREs yet allow rapid reorganization into high affinity heterotetrameric “fusion-active” forms. Future studies will be necessary to demonstrate SNARE complex intermediates within cells.

In summary, we have shown that SNAP-23N forms a heterotetrameric complex with other non-neuronal SNARE coiled-coil domains and, independently, self-associates to form a parallel homotetramer. The homotetrameric SNAP-23N structure has common and unique features compared with other reported SNARE complex structures and extends our understanding of SNARE coiled-coil complex diversity and SNAP-23 function.

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