Defining the SNARE Complex Binding Surface of α-SNAP
IMPLICATIONS FOR SNARE COMPLEX DISASSEMBLY

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N-Ethylmaleimide-sensitive factor (NSF) and its adaptor protein α-soluble NSF attachment protein (α-SNAP) sustain membrane trafficking by disassembling soluble NSF attachment protein receptor (SNARE) complexes that form during membrane fusion. To better understand the role of α-SNAP in this process, we used site-directed mutagenesis to identify residues in α-SNAP that interact with SNARE complexes. We find that mutations in charged residues distributed over a concave surface formed by the N-terminal nine α-helices of α-SNAP affect its ability to bind synaptic SNARE complex and promote its disassembly by NSF. Replacing basic residues on this surface with alanines reduced NSF activity in both assays. These findings show that the ability of NSF to take apart SNARE complexes depends upon electrostatic interactions between α-SNAP and the acidic surface of the SNARE complex and provide insight into how NSF and α-SNAP work together to drive disassembly.

Intercompartmental transport and exocytosis depend upon membrane fusion. This fusion requires membrane-associated helical proteins known as soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs), which coil together into SNARE complexes that are thought to bring membranes together and promote membrane fusion (1–3). After fusion, SNARE complexes must be disassembled to regenerate individual SNAREs for use in subsequent fusion reactions. Proteins responsible for this disassembly are the ATPase NSF and its adaptor protein soluble NSF attachment protein (SNAP). Insight into this process is important to understanding how membrane trafficking is controlled in the cell.

α-SNAP (a ubiquitous SNAP isoform) serves as the requisite link between NSF and SNARE complexes. It binds to SNARE complexes, and together they recruit NSF (4). SNARE-bound α-SNAP stimulates ATP hydrolysis by NSF, leading to conformational changes and concomitant disassembly of the SNARE complex (2, 4). Scanning transmission electron microscopy has been used to define the probable composition of the complex containing α-SNAP, NSF, and SNAP-complex, also referred to as 20 S complex (5). Each 20 S complex consists of one SNAP complex, three α-SNAPs, and one NSF hexamer. Interestingly, α-SNAP only binds NSF in the absence of SNAP-complex when forced into an oligomeric form by fusion to an intrinsically trimeric protein (5) or when it is immobilized on plastic (6). Electron micrographs of α-SNAP bound to SNAP-complex and an antibody specific for the N terminus of α-SNAP show that α-SNAP binds SNAP-complex in an antiparallel orientation. This positions its N terminus near the membrane and its C terminus away from the membrane, where it can interact with NSF (2, 7). Consistent with this, the α-SNAP C terminus has been shown to play a critical role in stimulating NSF ATPase activity and promoting SNAP-complex disassembly (8).

Despite limited overall sequence similarity among SNAP-complexes on different membranes, all SNAP-complexes so far examined bind α-SNAP and can be disassembled by NSF (9–14). SNAP-complexes are stable α-helical structures containing specific combinations of four SNAP-helices (for review, see Ref. 15). Crystal structures of the synaptic and late endosomal SNAP-complexes show that these two complexes are rod-shaped and held together by interactions among conserved, mostly hydrophobic residues within the core of the complex (16–18). Although both complexes have largely acidic surface potentials, there are few specifically conserved residues on the outer, solvent-exposed, surfaces (19, 20).

Little is known about how α-SNAP recognizes the variety of SNAP-complexes involved in different membrane fusion reactions. Based on deletion studies (8, 21–24) and electron micrographs in which α-SNAP molecules appear to ensheathe the SNAP-complex (7), a significant portion of α-SNAP is thought to participate in its interaction with the SNAP-complex. The structure of the Saccharomyces cerevisiae α-SNAP orthologue, Sec17p (25), provides some clues to where the interfaces between SNAPs and SNAP-complexes might be. Sec17p is a 14-helix α/α protein with two principal domains, an N-terminal twisted sheet (nine α-helices arranged in antiparallel to form a sheet-like structure) and a C-terminal globular bundle (25). Each helix in the twisted sheet is slightly askew from its neighbors, giving the domain a concave face whose curvature complements that of the convex surface of the SNAP-complex. One edge of the twisted sheet is longer than the other and curved in a way that could allow it to fit into the shallow grooves that lie between individual helices of the SNAP-complex (25). Both the concave face and the longer edge of the
twisted sheet contain residues that are conserved among SNAP orthologues (25). Shape complementarity, sequence conservation, and overall surface charge distribution led to the proposal that either the concave face or longer edge of the Sec17p (or the α-SNAP) twisted sheet domain might be responsible for SNAP complex binding (25). Whether this is the case and, if so, how α-SNAP actually contacts SNAP complexes has not been clearly examined.

In this report, we present evidence showing that the SNAP complex binding surface α-SNAP is the concave face of its twisted sheet domain. In a coinmunoprecipitation-based binding assay, point mutations in charged residues on this surface strongly affect SNAP complex binding, whereas mutations elsewhere do not. Most of these mutations similarly affect the ability of α-SNAP to promote SNAP complex disassembly by NSF. Based on these results we present a model of how α-SNAP interacts with SNAP complexes and discuss its implications for SNAP complex disassembly.

EXPERIMENTAL PROCEDURES

Materials—Bovine α-SNAP in pQE-9 was a gift from J. E. Rothman (Memorial Sloan-Kettering Cancer Center, New York, NY). All chemicals were reagent grade or better from Sigma or Fisher unless otherwise noted.

Cloning and Site-directed Mutagenesis—Bovine α-SNAP DNA (from pQE-9-α-SNAP) was cloned into NdeI/HindIII-digested pET28 (Noverga) to generate a His6-α-SNAP expression construct. The resulting protein had the sequence MGSSHRRHHRHHRHHRHRHHRHRHRHHRHRHHRHRHHRHRHHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRH

α-SNAP mutants (F32G, E39A, E43A, K65A, D87A, K93A, R116A, K140A, E155A, K163A, and Y200K) were generated by PCR using an overlap extension method with pQE-9-α-SNAP as template and subcloned into pET28. The remaining mutants (E99K/E109K, R47A, K53A, K53E, Q47A, D80A, K94A, E99A, E109A, K122A, E129A, E132A, E134A, D150A, E156A, K167A, K203A, and D273R) were generated using the QuikChange mutagenesis kit (Stratagene) with wild-type pET28 α-SNAP as template.

Because of a PCR error, some of the α-SNAP mutants (E99K/E109K, Q47A, K94A, E99A, E109A, E129A, D150A, K203A, and D273R) contained a C84S mutation. However, wild-type and C84S α-SNAP were functionally equivalent in our assays (data not shown), and the mutation was, therefore, left in place.

Protein Expression and Purification—α-SNAP was expressed in Escherichia coli BL21 (DE3) cells. 1–2 liters of Terrific Broth cultures were grown 10–14 h at 30 °C to A600 = ~2 and induced for 2–4 h with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside. Bacterial pellets were resuspended in lysis buffer (20 mM Tris, 250 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol, pH 7.4) and stored at −20 °C. After thawing, pellets were sonicated (20 mg/ml) for 30 s), Triton X-100 was added to a final concentration of 0.1%, and lysates were centrifuged for 20 min at 18,500 g. After thawing, pellets were sonicated (20 min at 18,500 g) to generate a His6-α-SNAP solution. The resulting protein had the sequence MGSSHRRHHRHHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRHHRHRH

addition was, therefore, left in place. Circular Dichroism Spectroscopy—Circular dichroism (CD) spectra were collected on a Jasco J600 spectropolarimeter; each spectrum was the average of five scans from 190 to 250 nm in 0.4-nm increments at 50 nm/min. Wild-type and mutant α-SNAP proteins were diluted to 2.9 μM in 20 mM Tris, 100 mM NaCl, pH 7.4. Measurements were taken at room temperature using a quartz cell with a 1-mm path length. Mean residue ellipticity (degree × cm2/mg) was calculated after subtracting the signal from buffer alone. α-Helical content was calculated using the mean residue ellipticity at 222 nm (θ222 nm) and the assumption that for 100% α-helix, θ222 nm = −39,500 (1 − 2.57/n), where n is the average number of residues per helix (27). Based on homology with the Sec17p structure (25), n for α-SNAP is 15 residues.

Fluorescence Resonance Energy Transfer (FRET)-based SNAP Complex Disassembly Assay—Fluorescently tagged SNAP proteins were used to build a SNAP complex in which FRET could be used to monitor SNAP complex assembly and disassembly. Briefly, SNAP- and SNAP-negative complex were pooled, quantitated using a Bradford assay with BSA as standard, and snap-frozen for storage.
is the ratio of
complex in the presence of 100 mM NaCl. Proteins were visualized by
SNARE complex consisted of syntaxin H3
(syntaxin 1a (184–265)), SNAP-25b-Myc, and synaptobrevin
(synaptobrevin II (1–96)). B, quantitation of SNARE complex binding in the
presence of 100 mM (closed circles) or 500 mM NaCl (open circles). Shown
is the ratio of α-SNAP to SNAP-25b-Myc SYPRO Red fluorescence
(normalized for molecular weight); each point represents the average of
three binding reactions with S.D. except where marked (*, two reac-
tions; **, one reaction).

RESULTS

α-SNAP Binding to Synaptic SNARE Complex—α-SNAP binds to an array of SNARE complexes (9–14) and is thought to serve as an essential link between these complexes and NSF (9). To study α-SNAP interaction with the SNARE complex, we monitored α-SNAP binding to a synaptic SNARE complex containing the SNARE motifs from syntaxin 1a and synaptobrevin II together with full-length SNAP-25 (Fig. 1). This SNARE complex lacks the N-terminal Habc domain of syntaxin as well as the transmembrane domains of syntaxin and synaptobrevin. After combining α-SNAP and SNARE complex, we immobilized the SNARE complex on protein G-Sepharose using a C-terminal c-Myc epitope tag on SNAP-25 and an anti-Myc antibody. Unbound α-SNAP was washed off, and immunoprecipitated proteins were visualized by SDS-PAGE and SYPRO Red staining. SYPRO dyes fluoresce upon interaction with protein-bound SDS; α-SNAP binding to the SNARE complex was, therefore, defined as the molar ratio of α-SNAP to SNAP-25 fluorescence in each lane. The EC_{50} of α-SNAP binding to SNARE complex in this assay was ~5 μM.

At high α-SNAP concentrations, approximately three α-SNAPs were bound per SNARE complex (Fig. 1). SYPRO dyes stain different proteins differently, rendering measurements of stoichiometry only semiquantitative (31, 32). It is, however, clear that more than one, and most likely three, α-SNAPs bind to a single SNARE complex. This is consistent with early estimates of the stoichiometry (22) and with calibrated scanning transmission electron microscopy analysis of the 20 S complex, which suggests that it contains one SNARE complex, one NSF, and three molecules of α-SNAP (5). Differences between the three α-SNAP binding sites on the SNARE complex are not apparent in our binding data.

Interestingly, binding of α-SNAP to SNARE complex was sharply reduced when the concentration of NaCl in the assay was increased from 100 to 500 mM (Fig. 1). This demonstrates that electrostatic interactions are important for α-SNAP binding to the SNARE complex. It also confirms the specificity of binding detected in our assay, even at the highest concentrations of α-SNAP.

Design and Production of α-SNAP Mutants—To define functionally important contacts between α-SNAP and the synaptic SNARE complex, we generated a series of α-SNAP mutants based on a homology model of α-SNAP. We built this model using the crystal structure of the S. cerevisiae α-SNAP orthologue, Sec17p (25). Sec17p is 35% identical and 15% similar to α-SNAP and can functionally replace α-SNAP in in vitro Golgi transport assays (6). To provide evidence that the homology model reasonably predicts the actual α-SNAP structure, we used circular dichroism (CD) spectroscopy to estimate α-SNAP helical content (Fig. 2). Based on its mean residue ellipticity at 222 nm, α-SNAP is ~70% helical. The helical content of Sec17p predicted from its crystal structure (25) is 73%, and that of α-SNAP from the homology model is 74%. The close correlation between the observed and predicted helical content of these proteins both substantiates the α-SNAP homology model and provides independent evidence that the Sec17p crystal structure reflects its structure in solution.

As discussed in the introduction, the nine N-terminal α-helices in Sec17p form a twisted sheet domain which has two surfaces that are good candidates for SNARE complex binding. These two surfaces, the concave face and longer edge, are also found on the α-SNAP homology model. The concave face, which is part of the front face of the protein, is complementary in shape and charge to the convex, mostly acidic surface of the...
SNARE complex, and many of its exposed residues are strongly conserved among SNAP orthologues (25). The longer edge of the twisted sheet also has several conserved residues, and its curvature complements that of the shallow grooves on the surface of the SNARE complex that run between the SNARE helices.

The SNAP-SNARE complex interface is, therefore, likely to involve either the front face or the longer edge of the SNAP twisted sheet domain. Because of this and because the salt sensitivity of binding (Fig. 1) indicated that electrostatic interactions are important for SNAP binding to the SNARE complex, we introduced point mutations at all conserved charged residues on these two surfaces (Fig. 3). In addition, we generated mutations at three conserved residues (Tyr-200, Lys-203, and Asp-273) on the edge of the globular bundle near the twisted sheet and at one conserved hydrophobic residue (Phe-32) on the longer edge of the twisted sheet. Conservation on the back face is less extensive than on the front face, but several charged and polar residues are moderately conserved. To examine the role of the back face in interaction with the SNARE complex, we mutated five of these. Most mutations were to alanine to remove the charge from the side chain without imposing new structural constraints.

Because we introduced mutations only at positions predicted to be solvent-exposed, we did not expect the mutations to cause significant structural disturbances. Indeed, as with wild-type SNAP, mutant proteins could be expressed at high levels in E. coli and were not proteolyzed during growth or purification. All were soluble at high concentrations (50–100 mM). The one exception was SNAP(D273R), which was largely insoluble in E. coli and was, therefore, not included in the rest of this study.

To further confirm that the mutations did not change SNAP structure, we compared CD spectra of wild-type SNAP and each of the mutants used in this study (Fig. 2). All of the proteins showed similar helical content, with an average mean residue ellipticity at 222 nm of 24160 ± 1280 degree cm²/dmol. In addition, we treated a subset of the mutants with limited concentrations of trypsin. There were no detectable differences in the proteolytic patterns of wild-type, F32G, R116A, and E155A-SNAP (data not shown).

**SNARE Complex Binding by α-SNAP Surface Mutants**—To compare binding of wild-type and mutant α-SNAP to SNARE
complex, we carried out binding reactions as described in Fig. 1. A representative experiment is shown in Fig. 4. α-SNAP(K122A) did not bind appreciably to SNARE complex at concentrations of up to 15 μM. In contrast, α-SNAP(D80A) bound with higher affinity than wild-type α-SNAP to the SNARE complex, with an EC50 of ~2.5 μM. Both decreased and increased binding were readily apparent when 7.5 μM α-SNAP was added to the complex in this and similar experiments. We, therefore, used this concentration to compare SNARE complex binding by all α-SNAP mutants to that of wild-type α-SNAP (Fig. 5).

The effects of the mutations fell into three categories: depressed binding (<50% of wild-type α-SNAP binding; colored blue in Fig. 5), enhanced binding (>130% that of wild-type α-SNAP; colored red in Fig. 5), and binding that was not very different from that of wild-type α-SNAP (colored yellow in Fig. 5). Nine of the 11 mutations that reduced binding were of basic residues on the front face of α-SNAP. In contrast, mutations of seven acidic residues on the front face enhanced SNARE complex binding (Fig. 5).

**Fig. 4.** Binding of wild type, D80A, and K122A α-SNAP to SNARE complex. A, representative gel lanes showing binding of α-SNAP (top panel), α-SNAP(D80A) (middle panel), and α-SNAP(K122A) (bottom panel) to immunoprecipitated SNARE complex. Proteins were visualized by staining with SYPRO Red. B, quantitation of binding reactions. Closed squares, wild-type α-SNAP; open triangles, α-SNAP(D80A); open circles, α-SNAP(K122A). Shown is the ratio of α-SNAP to SNAP-25b-Myc SYPRO Red fluorescence (normalized for molecular weight); each point represents a single binding reaction. For each mutant, each concentration of α-SNAP was assayed in duplicate.

**Fig. 5.** Mutant α-SNAP binding to the SNARE complex. A, binding of 7.5 μM wild-type or mutant α-SNAP to immunoprecipitated SNARE complex expressed as a percentage of wild-type α-SNAP binding. Each bar represents the average of three to seven assays, each done in triplicate. Color coding indicates relative binding: blue, <50% wild type (wt); yellow, 50–130% wt; and red, >130% wt. EEEK is an abbreviation for α-SNAP/E39K/E40K. Error bars show S.D. B, an α-SNAP homology model generated using SWISS-PROT and the Swiss PDB Viewer (30) based on the structure of Sec17p (PDB code 1QQE). Effects of mutations on binding are mapped onto the homology model using the same coloring as in panel A.
plex binding. Mutations that did not affect SNARE complex binding included changes in all residues on the back face and four of the five residues on the longer curved edge of α-SNAP. Together these data indicate that much of the α-SNAP front face and, in particular, the concave face of the twisted sheet is involved in SNARE complex binding. The extensive binding surface defined here is consistent with the large minimal binding domain predicted by previous studies of truncation mutants (8, 21–24).

Although most mutations were alanine substitutions, three mutations were different. Two of these included mutations to lysine that had opposite effects on binding. Changing Glu-39 and Glu-40 to lysine (E39K/E40K) enhanced binding as had alanine mutations at acidic residues on the front face. In contrast, a Y200K mutation caused α-SNAP to bind poorly to the SNARE complex, suggesting that nonionic interactions involving this residue are important for SNARE complex binding. Finally, a glycine mutation at Phe-32 led to only a small decrease in SNARE complex binding. This was unexpected because Phe-32 is the only conserved residue not on the back face among the first 35 amino acids of α-SNAP, a region reported to be critical for SNARE complex binding (24).

**SNARE Complex Disassembly Directed by α-SNAP Mutants—**α-SNAP recruits NSF to disassemble SNARE complexes. It is, therefore, likely that mutations that affect α-SNAP binding to SNARE complex will also affect its ability to mediate SNARE complex disassembly. However, because NSF actually promotes disassembly it is unclear, it is possible that individual mutations in α-SNAP could change SNARE complex disassembly differently from how they change binding. Identifying such mutations in α-SNAP could yield insight into the role of α-SNAP in SNARE complex disassembly.

We monitored SNARE complex disassembly by measuring FRET between CFP and YFP tags that were attached to the C termini of the SNAREs syntaxin 1a and synaptobrevin II in place of their transmembrane domains. FRET between the CFP and YFP moieties is lost when SNAREs in the SNARE complex are separated by NSF and α-SNAP. To determine how mutations in α-SNAP affected its ability to promote disassembly, we measured the initial velocity of the reaction using an α-SNAP concentration determined to be approximately the EC_{50} for disassembly by wild-type α-SNAP (Table I). In most cases, mutations affected disassembly similarly to how they affected binding, thereby confirming the results of the binding assays (Fig. 6). On the front face of α-SNAP, where all mutations that changed binding were located, mutations that reduced binding also reduced disassembly. Among the mutations that enhanced binding, most also enhanced disassembly, although less so than binding. Interestingly, several mutations that did not increase binding did enhance disassembly (E99A, E109A, and K140A; Fig. 6). These residues are on the back face of the α-SNAP twisted sheet and may contribute to the SNAP-NSF interface.

**DISCUSSION**

In this report, we have further explored the role of α-SNAP in SNARE complex disassembly by NSF. We used site-directed mutagenesis to examine the importance of different α-SNAP surfaces in both SNARE complex binding and NSF-promoted disassembly. Effects of mutations clearly show that SNAP-α-SNAP complex interactions involve charged α-SNAP residues distributed over the majority of the α-SNAP front face, in particular over the concave surface of its twisted sheet domain (Table I, Figs. 5 and 6). The similarity in effects of α-SNAP mutations on SNARE complex binding and NSF-promoted SNARE complex disassembly confirm that α-SNAP interaction with SNARE complexes is intimately coupled to the ability of NSF to disassemble the complexes. The location of the α-SNAP residues that affect binding and disassembly together with those of potentially complementary residues on the SNARE complex provides a framework for predicting how these proteins fit together to enable NSF activity.

Mutating basic residues on the front face of α-SNAP reduced
SNARE complex binding by up to approximately 20-fold (Fig. 5). The effects of these mutations were specific since similar mutations on the back face of H9251-SNAP did not affect SNARE complex binding. Similar effects of alanine mutations have been observed at other protein-protein interfaces where the interactions are primarily electrostatic, including the interface between tubulin and kinesin (33) and that between barnase and barstar (34, 35). Interestingly, in vitro phosphorylation of H9251-SNAP by protein kinase A reduces SNARE complex binding by 10-fold (36), demonstrating in another way that positive charge on the surface of H9251-SNAP is important for its binding to SNARE complex. The EC50 of wild-type H9251-SNAP binding to SNARE complex (Fig. 1) is consistent with data in previous studies that examine binding between α-SNAP and SNARE complexes using affinity matrix-based binding assays (22, 36, 37). This EC50 is nevertheless surprisingly low given that the EC50 of α-SNAP for mediating SNARE complex disassembly is ~0.2 μM (Table I). 0.2 μM α-SNAP is closer to what is needed for functional reconstitution of NSF- and SNAP-dependent processes in vitro (24, 38, 39). That co-precipitation-based assays inherently underestimate affinity may in part explain this discrepancy. In addition, NSF may enhance SNAP-SNARE complex binding (4).

The fact that binding to the synaptic SNARE complex was readily enhanced by mutagenesis (Fig. 5) is probably not surprising given the diversity among SNARE complexes throughout the cell to which α-SNAP must bind; optimized binding to one SNARE complex could result in impaired binding to another. Because dissociation of α-SNAP from the SNARE complex is a step in disassembly, one might imagine that enhancing binding would eventually impair disassembly. In fact, excess α-SNAP (38) and Sec17p (40) have been found to impair SNARE complex-dependent processes in vitro. Although they

**Fig. 7. Model of α-SNAP binding to SNARE complex.** A, putative α-SNAP binding sites on the SNARE complex. Left, ribbon diagram of α-SNAP homology model (see Fig. 5 and “Experimental Procedures”), showing basic residues (blue) whose mutation reduces SNARE complex binding. Right, conserved acidic residues on the SNARE complex define three potential binding sites for α-SNAP. These sites are designated Site 1 (red, syntaxin Asp-214, Asp-218; synaptobrevin Asp-51, Glu-55; SNAP-25 C-terminal helix Glu-183), Site 2 (orange, SNAP-25 N-terminal helix Glu-38, Asp-41; syntaxin Glu-228; synaptobrevin Asp-65), and Site 3 (yellow, SNAP-25 C-terminal helix Asp-166; SNAP-25 N-terminal helix Asp-51, Glu-52, Glu-55; syntaxin Glu-238). B, proposed SNAP-SNARE binding model (left) showing three α-SNAP twisted sheet domains bound to SNARE complex. Each α-SNAP is color-coded according to the SNARE complex site it binds. Lines through the model show where slices were made to generate axial views (i) and (ii), views are from the N-terminal, cytoplasmic end of the SNARE complex. Basic residues whose mutation reduces SNARE complex binding are colored blue. The SNARE complex ionic layer is colored black. Diagrams were generated using RasMol (46).
were not inhibitory, most mutations that enhanced binding only slightly enhanced disassembly, and two did not enhance disassembly at all (Fig. 6).

A group of mutations that did not enhance SNARE complex binding but did enhance its disassembly included residues on the back face of α-SNAP (E99A, E109A, and K140A). These residues are likely to remain accessible to NSF when α-SNAP is bound to the SNARE complex and may, therefore, play a role in coupling conformational changes in NSF to SNARE complex disassembly.

SNAP-SNARE Complex Binding Model—To develop a model for how α-SNAP binds SNARE complexes we examined shape and charge complementarity between the α-SNAP surface shown in this study to be critical for binding and the surface of the SNARE complex. Six of the 10 residues whose mutation reduced SNARE complex binding (Lys-56, Lys-93, Lys-94, Lys-122, Lys-163, and Lys-167; Fig. 5) are basic and form a diagonal band across the front face of the α-SNAP twisted sheet domain (Fig. 7). Diagonal bands of basic residues on the SNARE complex surface define three possible binding sites; similar groups of residues are also present in other well characterized SNARE complexes (17, 20, 41). Pairing basic and acidic residues allowed us to manually align three α-SNAP twisted sheet domains with a single SNARE complex (Fig. 7). In this arrangement, shape complementarity between α-SNAP and the SNARE complex is maximized and is substantially greater than when SNAPs are placed directly parallel to individual SNARE helices within the complex (not shown). Each α-SNAP interacts with three SNAREs. This may explain why α-SNAP binding to individual SNAREs is weaker than to the SNARE complex (21, 42) and why α-SNAP dissociates after SNARE complex disassembly. Each putative α-SNAP binding site contains 4 or 5 acidic residues close enough to α-SNAP residues Lys-56, Lys-93, Lys-94, Lys-163, and/or Lys-167 to form salt bridges (Fig. 7). Mutation of any one of these α-SNAP residues significantly reduced SNARE complex binding (Fig. 5). Whether α-SNAP mutations directly impair binding to one, two, or all three of the sites or whether the mutations affect one critical site and thereby disrupt cooperative interactions with other sites cannot be distinguished in our binding assay. α-SNAP binding to the site designated Site 1 positions it over the groove between syntaxin and synaptobrevin in which complexin binds (43, 44). This would be expected to interfere with complexin binding and is, therefore, consistent with the reported competition between α-SNAP and complexin for binding to the SNARE complex (45). The largest gap between α-SNAP molecules is between sites 3 and 1. The interhelical linker that connects the two helices of SNAP-25 could fit between these two α-SNAPs.

The model shown in Fig. 7 accounts for most of the experimental data described in this report. There are, however, a few observations it does not presently explain that raise questions for further study. Four basic residues on the twisted sheet of α-SNAP (Arg-47, Lys-53, Arg-116, and Lys-122) appear to be important for SNARE complex binding (Fig. 5) but are not predicted to directly interact with acidic residues on the SNARE complex. These residues may play other roles; for example, Lys-53 is likely to be involved in an interhelical salt bridge within α-SNAP, and Arg-47 may counteract the charge of a nearby acidic residue. Second, to dock the α-SNAP twisted sheet domain closely against the SNARE complex, the N-terminal globular bundle domains have been left out of the model shown in Fig. 7. If kept in the model these domains would collide with the SNARE complex. The Sec17p crystal structure is of isolated protein (25) and does not necessarily reflect the conformation of SNARE complex-bound α-SNAP. The globular bundle domain may, therefore, move in order for the twisted sheet domain to effectively bind SNARE complex. Experiments to examine this possibility are in progress.

Implications for SNARE Complex Disassembly—Our model of the SNAP-SNARE complex interaction is compatible with a variety of mechanisms for SNARE complex disassembly. Importantly, it implies that α-SNAP molecules are the primary connection between NSF and the SNARE complex. The simultaneous binding of three α-SNAP molecules to the SNARE complex suggests that NSF may use its nucleotide-driven conformational changes to promote disassembly by affecting some property of all three SNAP-SNARE interfaces. An attractive target for NSF action is found at the level of the SNARE complex ionic layer, a conserved point of interaction among the four SNARE helices (19). This layer is probably the least stable region of the SNARE complex (18, 44) and has been proposed to be essential for efficient SNARE complex disassembly (47). A number of α-SNAP mutations that affected binding and disassembly lie near this layer. These mutations may alter critical connections between the region of the SNARE complex surrounding the ionic layer, α-SNAP, and NSF, which in turn impair the ability of NSF to effect complex disassembly. Further studies to explore these possibilities are in progress.

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