Membrane-anchored SNAREs assemble into SNARE complexes that bring membranes together to promote fusion. SNARE complexes are parallel four-helix bundles stabilized in part by hydrophobic interactions within their core. At the center of SNARE complexes is a distinctive zero layer that consists of one arginine and three glutamines. This zero layer is thought to play a special role in the biology of the SNARE complex. One proposal is that the polar residues of the zero layer enable N-ethylmaleimide-sensitive factor (NSF)-mediated SNARE complex disassembly. Here, we studied the effects of manipulating the zero layer of the well studied synaptic SNARE complex in vitro and in vivo. Using a fluorescence-based assay to follow SNARE complex disassembly in real time, we found that the maximal rate at which NSF disassembles complexes was unaffected by mutations in the zero layer, including single replacement of the syntaxin glutamine with arginine as well as multiple replacement of all four layer residues with non-polar amino acids.

To determine whether syntaxin with arginine instead of glutamine in its zero layer can support SNARE function in vivo, we introduced it as a transgene into a Caenorhabditis elegans syntaxin-null strain. Mutant syntaxin rescued viability and locomotory defects similarly to wild-type syntaxin, demonstrating that SNARE complexes with two glutamines and two arginines in the zero layer can support neuronal transmission. These findings show that residues of the zero layer do not play an essential role in NSF-mediated disassembly.

Intracellular membrane trafficking depends on the ordered formation and consumption of transport intermediates and requires that membranes fuse with each other in a tightly regulated and highly specific manner. Membrane-anchored helical proteins known as SNAREs have emerged as central players in membrane fusion. SNAREs assemble into coiled-coil-like complexes that are thought to pull membranes together and thereby promote fusion (1–4). After fusion, SNARE complexes must disassemble to regenerate individual SNAREs for use in subsequent fusion reactions. Proteins responsible for disassembly are the AAA+-ATPase associated with a variety of cellular activities) providing the R helix; the N- and C-terminal ends of SNAP-25 providing the QA helix; and R-SNAREs to reflect both the residue that each SNARE contributes to the zero layer and the position that it occupies in an assembled complex (8, 9, 13). The founding members of each of these groups are the SNAREs of the synaptic SNARE complex, with syntaxin-1 providing the Qₜ helix; the N- and C-terminal ends of SNAP-25 providing the Qₐ and Qₚ helices, respectively; and synaptobrevin (vesicle-associated membrane protein) providing the R helix.

Various ideas for how polar zero layer residues might impact SNARE function have been put forward. One attractive possibility is that favoring membrane protein) providing the R helix.
Contributions of individual core residues, including those of the zero layer, to the function of the synaptic SNARE complex have been extensively examined using in vitro assays of PC12 cell secretory granule exocytosis (22–24) as well as in more limited ways in a variety of living preparations (25–29). For in vitro exocytosis studies, analysis has been limited to changes in the SNAP-25 Q9 and Qc helices because these do not need to be anchored to the membrane to function. Not surprisingly, mutations of hydrophobic core residues in either the Q9 or Qc helix disrupted the apparent stability of the SNARE complex as well as its ability to promote exocytosis (22–24). When examined in living preparations, mutations of hydrophobic layer residues in syntaxin or synaptobrevin have been similarly disruptive (25–27). Somewhat surprisingly, changes in the residue(s) that SNAP-25 brings to the zero layer have relatively minor effects on in vitro secretory granule exocytosis, particularly when compared with the effects of mutations in adjacent hydrophobic layers that have similar effects on apparent complex stability (22–24). SNAP-25 zero layer mutants introduced into living cells again have little or no effect on fusion per se (28, 29), although expressing SNAP-25 with both its glutamines changed to leucine slows the kinetics of vesicle pool refilling in adrenal chromaffin cells (29).

Because of the minimal effects of synaptic SNARE complex zero layer mutations on secretory granule exocytosis, Scheller and co-workers (16) investigated whether these changes might instead affect a SNARE reaction not directly involved in fusion such as NSF-mediated complex disassembly. They found that disassembly appeared to be inhibited by some zero layer mutations, with the most dramatic effects seen when the QA (syntaxin) zero layer glutamine was changed to any other amino acid (16). Neher and co-workers (29) considered slowing of disassembly as a possible explanation for the delay in pool replenishment caused by expressing the SNAP-25 zero layer leucine mutant in adrenal chromaffin cells, but dismissed it based on unpublished data showing that a SNAP complex with four leucines in its zero layer was disassembled in vitro as fast or faster than the wild-type complex. Disassembly of SNARE complexes in yeast expressing zero layer mutants has not been directly examined, but the range of phenotypes observed (17–21) and, in particular, the ability to restore function by rotating the arginine within the layer indicate that a general requirement for a QA glutamine in disassembly is unlikely.

To understand more specifically how NSF disassembles SNARE complexes, we were interested in further exploring the proposed role of the zero layer in disassembly of the synaptic SNARE complex. Here, we revisited the question of how manipulating the zero layer affects complex disassembly in vitro and also investigated how a zero layer mutation in syntaxin affects the normal function of this QA-SNARE in vivo in Caenorhabditis elegans. We found that changes in the zero layer, either single replacement of the syntaxin glutamine with arginine or multiple replacement of all four layer residues with non-polar amino acids, did not significantly affect NSF-mediated disassembly in vitro. Furthermore, replacing the syntaxin glutamine with arginine did not impair syntaxin function in C. elegans. This suggests that, at least in the case of the synaptic SNARE complex, there is no special role for polar zero layer residues and, in particular, for the syntaxin glutamine in NSF-mediated disassembly. Our results confirm that the function of the synaptic SNARE complex is remarkably tolerant to changes in its zero layer and open the way to further studies aimed at defining the contribution of this layer to the normal functioning of SNARE complexes.

**Experimental Procedures**

**Protein Expression Vectors**—pET28-CFP and pET28-YFP vectors were created by moving enhanced CFP or YFP (Invitrogen) from pECP-N1 or pEYFP-N1, respectively, as a BamHI-NotI fragment into pET28a. His5-syntaxin-CFP was created by introducing residues 1–265 of rat syntaxin-1a as a Ndel-Agal fragment into pET28a-CFP. The resulting construct contains a His5 tag, syntaxin-1a, and CFP with residues VPVAT between syntaxin and CFP. The syntaxin also contains a C145S mutation. His5-synaptobrevin-YFP was similarly constructed by introducing residues 1–96 of rat synaptobrevin II into the Ndel-BamHI fragment of pET28a-YFP with residues VPVAT between synaptobrevin and YFP. All fragments were generated by PCR using oligonucleotide primers containing the indicated restriction sites. The sequences of all constructs were confirmed by ABI PRISM BigDye terminator cycle sequencing (Applied Biosystems) at the Protein and Nucleic Acid Chemistry Laboratory of the Washington University School of Medicine. SNAP-25b in pH40d, syntaxin-(1–265) in pH40d, and synaptobrevin-(1–96) in PET15b were as described previously (10). Mutations in the SNAP-25 zero layers (syntaxin(Q226R), syntaxin(Q226L), SNAP-25(Q53A/Q174L), synaptobrevin(R56Q), and synaptobrevin(R56A)) were introduced by QuikChange site-directed mutagenesis (Stratagene) into the indicated plasmids. For expression in C. elegans, a Q227R mutation was similarly introduced into pTX12, an unc-64 syntaxin minigene expressed under the control of the unc-64 promoter (25).

An expression vector for NSF was created by amplifying full-length Chinese hamster NSF from pQe9-NSF (5) and inserting it as a BamHI-Xhol fragment into pET28a. The resulting protein has N-terminal His5 and T7 tags. Bovine α-SNAP in pET28a was as described (30).

**Protein Purification and Complex Preparation**—Proteins were purified essentially as described (10, 30). NSF, syntaxin, SNAP-25, and synaptobrevin were quantified using Bradford reagents (Bio-Rad) with bovine serum albumin as a standard. α-SNAP was quantified by measuring absorbance at 280 nm using ε = 40,200 (30). All proteins and complexes were flash-frozen in liquid nitrogen and stored at −80 °C.

A SNARE complex was assembled by combining syntaxin-CFP, SNAP-25, and synaptobrevin-YFP (8–10 µM final concentration of each); dialyzing overnight into 20 mM Tris (pH 7.4), 100 mM NaCl, and 1 mM dithiothreitol; and then separating the mixture on a Mono Q anion exchange column. Fractions containing the highest concentrations of SDS-resistant complex relative to unincorporated individual proteins were pooled, aliquoted, and stored as described above. At least three independent preparations of each complex were purified and studied.

**Quick-freeze Deep-etch Electron Microscopy**—SNARE complexes adsorbed to mica chips were replicated and viewed by transmission electron microscopy as described previously (10).

**Temperature-dependent Dissociation in SDS**—The SNARE complex (30 µg) was brought to a final volume of 150 µl in 1× Laemmli buffer (50 mM Tris (pH 6.8), 1% SDS, 10% glycerol, and 0.1% bromphenol blue). Aliquots (15 µl) were incubated at the indicated temperatures for 5 min, returned to room temperature, and loaded onto a 10% SDS-polyacrylamide gel. Proteins in the gel were stained with SYPRO Red and visualized and quantified using a STORM 860 imaging system (30).

**SNARE Complex Disassembly Reactions**—Disassembly reactions were performed in 30 mM HEPES (pH 7.6), 100 mM potassium glutamate, 10 mM MgCl2, 2 mM ATP, and 1 mg/ml bovine serum albumin supplemented where indicated with an ATP-regenerating system of 1 mM creatine phosphate and 20 µg/ml creatine phosphokinase. Bovine serum albumin was omitted from disassembly reactions analyzed by gel electrophoresis. Unless indicated otherwise, all reactions were performed at 30 °C.

For FRET analysis, disassembly reactions were carried out in a final volume of 500 µl. Buffer, SNARE complex (100 nM unless indicated otherwise), and α-SNAP (2 µM or as indicated) were premixed and
transferred into a 10 × 2-mm quartz cuvette. An initial emission scan was acquired (λex = 434 nm and λem = 450–600 nm with slits at 3 nm and measurements every 1 nm), followed by two kinetic scans (λex = 434 nm and λem = 473 and 523 nm in separate photomultiplier tubes). The first scan was used to confirm a stable fluorescence baseline. In the second scan, NSF was added after 60 s, and the reaction was followed for another 500 s. Fluorescence measurements were performed using a Spex FluoroLog-3 operating in T-format.

For monitoring disassembly of SDS-resistant complexes by gel electrophoresis, α-SNAP was premixed with the SNARE complex (100–800 nM, 150-μl reaction volume) prior to initiating disassembly with NSF. Aliquots were removed at the indicated times and quenched by addition of 5 mM EDTA and Laemmli buffer. Samples were immediately separated on a 10% SDS-polyacrylamide gel. Protein was visualized and quantified by staining with SYPRO Red as described above.

**Data Analysis**—All scans were corrected for buffer background. Apparent FRET efficiency was calculated from corrected CFP emission (477 nm) as 1 − (FDA/F0), where FDA is the measured CFP fluorescence of the complex at a given time and F0 is the CFP fluorescence of the fully disassembled complex. The FRET signal measured for each complex was taken to represent 100% assembled complex, whereas that for the fully disassembled complex was set to 0%. The initial rate of complex disassembly was calculated from the slope of a line fit to the first 20 s of data collected after addition of NSF (data rejected if the line had R2 < 0.98), taking into account the amount of SNARE complex (in pmol) and NSF (in μg) in the reaction. Rate data for disassembly at different α-snap concentrations (see Fig. 6) were fit directly to a modified Hill equation: \[ v = \frac{V_{\text{max(app)}} [\alpha\text{-SNAP}]^n}{K_{\text{Hill}} + [\alpha\text{-SNAP}]^n} \], assuming equivalent binding sites for α-SNAP on the SNARE complex. Curve fitting and plotting were carried out using KaleidaGraph (Synergy Software, Reading, PA) and GraphPad Prism (GraphPad Software, San Diego, CA).

**Growth and Culture of C. elegans**—C. elegans was grown at 22.5 °C on solid medium as described (31). Aldicarb (2-methyl-2-(methylthio)propiionaldehyde O-(methylcarbamoyl)oxime) was obtained from Chem Services, Inc. (West Chester, PA). The strains used in this study were wild-type N2, NM2707 (unc-64(js115), jsEx822 (pTX12(Q227R), pPD118.33)), NM2708 (unc-64(js115), jsX822 (pTX12, pPD118.33)), NM2709 (unc-64(js115), jsEx824 (pTX12(Q227R), pPD118.33)), NM2710 (unc-64(js115), jsEx825 (pTX12(Q227R), pPD118.33)), NM204 (wnt-1(md290), NM467 (snb-1(md247)), NM464 (unc-64(md130)), NM979 (unc-64(js115)/ bli-5(e518)), and NM318 (unc-64(e246)).

**Construction of Syntaxin(Q227R) Transgenic Strains**—C. elegans strains were transformed using a standard microinjection protocol (32). Plasmid pH118.33 (a gift of Andy Fire) expressing green fluorescent protein in the pharynx under the control of the myo-2 promoter was used as a cotransformation marker. Transgenic strains containing unc-64(js115) in the homozygous state were created by injecting unc-64(js115) into larvae and screened for fluorescent transgenic animals that failed to segregate the bli-5(e518) chromosome. Progeny of the resulting animals consisted only of green fluorescent protein-positive viable animals and green fluorescent protein-negative Unc-64. L1-arrested paralyzed animals. Three independent lines were analyzed for each transgene.

**Immunoblotting**—C. elegans lysates were created as described previously (33). Blots were blocked for 1 h, incubated overnight with primary antibody at 4 °C, and developed using enhanced chemiluminescence and a STORM 860 imaging system. Antisera directed against UNC-64 syntaxin (antibody 939) (25), SNB-1 synaptobrevin/vesicle-associated membrane protein (antibody 1092) (26), and SNT-1 synaptotagmin (antibody 1095) (34) were used at 1:5000, 1:10,000, and 1:2000 dilutions, respectively.

**RESULTS**

**Real-time Assay of NSF-mediated SNARE Complex Disassembly**—To study the assembly and disassembly of SNARE complexes *in vitro*, we created a reporter system in which fluorescent proteins (enhanced CFP and YFP) are fused to synaptic SNARE proteins, making it possible to directly compare the behavior of SNARE complexes. Two early studies using blue fluorescent protein and YFP fused to syntaxin and synaptobrevin to test their relative orientation within a SNARE complex demonstrated that, in principle, this approach will work (37, 38).
Normal Function of SNARE Complex Zero Layer Mutants

To generate a tagged SNARE complex, we combined equimolar amounts of syntaxin-CFP, synaptobrevin-YFP, and non-fluorescent SNAP-25. Following overnight incubation, we separated the SNARE complex from unincorporated component proteins by ion exchange chromatography. The resulting complex contained the three SNAREs in an ~1:1:1 molar ratio and was resistant to dissociation by SDS unless boiled (Fig. 1B), as is typical of the synaptic SNARE complex (39). To confirm that the complexes were properly assembled, we carried out quick-freeze deep-etch electron microscopy and looked at the morphology of individual particles (Fig. 1C). Similar to what we reported in a previous study of SNARE complexes tagged with maltose-binding protein (10), two protein tags (in this case, CFP or YFP, which appeared as ~4-nm diameter balls) were present where expected on one end of each ~12–14-nm rod-like SNARE complex.

We next compared the fluorescence emission spectra of an assembled SNAP-SNARE complex mixture. Apparent FRET efficiency was calculated from CFP emission of the synaptic SNARE complex. The inserted schematic illustration shows the arrangement of amino acids within the wild-type SNARE complex zero layer. The inserted schematic illustration shows the arrangement of amino acids within the wild-type SNARE complex zero layer. Individual curves show disassembly with 0.25, 0.5, 0.75, or 1 μg of NSF under standard conditions of 2 μM α-SNAP and in control reactions in which α-SNAP was omitted or 10 μM EDTA was added along with 1 μg of NSF. All reactions were at 30 °C. The initial rate of disassembly was 18.6 ± 2.1 pmol of SNAP-SNARE complex μg of NSF -1 min-1 (n = 25).
On the basis of the crystal structures of SNARE complexes (11, 12, 14) and molecular dynamics simulations of the effects of zero layer mutations on the structure of the synaptic SNARE complex (17), it is clear that the zero layer cannot accommodate two arginines without some distortion. Consistent with this, a previous study of zero layer mutations in the synaptic SNARE complex found that the syntaxin(Q226R) mutation decreased the apparent stability of the complex, with an 

$\Delta H_{11011}^{20 \degree C}$ reduction in the melting temperature measured by circular dichroism and an $\Delta H_{11011}^{10 \degree C}$ reduction in the temperature needed to dissociate the complex in 1% SDS (16). To confirm that the CFP/YFP-tagged complex was similarly affected by a syntaxin(Q226R) mutation, we incubated the wild-type or mutant complex in 1% SDS at varying temperatures prior to electrophoresis and measured the amount of remaining SDS-resistant complex (Fig. 3B). As expected, the complex containing syntaxin(Q226R)-CFP was half-dissociated at an $\sim 10 \degree C$ lower temperature compared with the wild-type complex. Because of the hysteresis observed in studies of SNARE unfolding and refolding (42), it is not clear whether this change in apparent stability reflects a change in the kinetics of complex dissociation or in the free energy of the complex itself, but the change confirms that a structural change has occurred.

On the basis of the previous study of synaptic SNARE complexes with zero layer mutations (16), we expected that changing the syntaxin glutamine to an arginine (Q226R), despite decreasing the apparent stability of the complex, would slow the rate at which NSF disassembles the complex. We again monitored complex disassembly using FRET between attached CFP and YFP tags. Surprisingly, FRET was completely lost within 10 min of addition of NSF just as for the wild-type complex (Fig. 4B).
Normal Function of SNARE Complex Zero Layer Mutants

A

B

FIGURE 5. Monitoring complex disassembly by SDS resistance shows no difference between complexes containing wild-type syntaxin and syntaxin(Q226R). A, SDS-resistant SNARE complex containing wild-type (wt) syntaxin or syntaxin(Q226R) at different times after addition of NSF (800 nM complex and 3 μg of NSF). Reactions were stopped by addition of EDTA and then separated on an SDS-polyacrylamide gel without heating. Proteins were stained with SYPRO Red and visualized with a STORM 860 imaging system. B, quantification of protein remaining in the wild-type (*) and Q226R mutant (○) SDS-resistant complexes. The last points show boiled samples.

FIGURE 6. Requirement for α-SNAP in disassembly of wild-type syntaxin- and syntaxin(Q226R)-containing SNARE complexes. The rate data are from standard reactions measuring disassembly of wild-type syntaxin-containing (■) and syntaxin(Q226R)-containing () SNARE complexes in the presence of the indicated α-SNAP concentration. The curves shown were fit to the Hill equation with an apparent Hill coefficient of 1.3 for both complexes. The K_{0.5} values for α-SNAP in disassembly of the wild-type and Q226R mutant complexes were 0.22 ± 0.07 and 0.50 ± 0.12 μM, respectively.

α-SNP in disassembly of the two complexes were similar, with dose-response curves for both showing a small degree of positive cooperativity (apparent Hill coefficient of 1.3). More α-SNAP was needed to promote half-maximal disassembly of the mutant complex compared with the wild-type complex (wild-type complex K_{0.5} = 0.22 ± 0.07 μM and two-glutamine/two-arginine mutant complex K_{0.5} = 0.50 ± 0.12 μM), consistent with a small decrease in the affinity of the two-glutamine/two-arginine mutant complex for α-SNAP. This (together with Fig. 3B) confirms that the Q226R mutation affects the structure of the SNARE complex, but not its overall susceptibility to NSF-mediated disassembly. These findings also reinforce the essential role of α-SNAP in selecting SNARE complexes for disassembly.

Hydrophobic Repacking of the Zero Layer Does Not Affect Disassembly—If the syntaxin zero layer glutamine is not essential for efficient SNARE complex disassembly, we wondered whether the presence of a polar layer near the center of the complex might nonetheless be important for the reaction. We therefore repacked the zero layer of the synaptic SNARE complex with non-polar residues and studied disassembly of the resulting complex. The SNARE complex containing syntaxin(Q226L)-CFP, SNAP-25(Q53A/Q174L), and synaptobrevin(R56A)-YFP had a fluorescence emission profile similar to that of the wild-type SNARE complex (Fig. 7A). FRET was once again completely lost following a 10-min incubation with NSF. The progress of this reaction was similar to that for the wild-type complex (Fig. 7B).

Syntaxin Zero Layer Mutant Restores Function in a C. elegans Syntaxin-null Strain—To determine whether the three-glutamine/one-arginine composition of the zero layer is important for function of the synaptic SNARE complex in neurotransmitter release, we took advantage of the high degree of conservation between rat and C. elegans SNAREs (25, 26) and the ability to rescue mutant strains of C. elegans with appropriate transgenes. We investigated whether syntaxin (UNC-64) containing arginine in place of its usual zero layer glutamine (Q227R) rescues movement in a strain lacking UNC-64 syntaxin (25). Worms expressing comparable levels of wild-type or Q227R mutant UNC-64 from transgenes were generated (Fig. 8A). The overall movements of parental, wild-type, and mutant transgenic strains were indistinguishable from each other (Fig. 8B), showing that the overall func-
Normal Function of SNARE Complex Zero Layer Mutants

Although the SNARE complex conserved zero layer has attracted much attention, its actual function remains unknown. As part of an effort to understand how NSF disassembles SNARE complexes, we tested the hypothesis that polar zero layer residues and, in particular, the glutamine in the Q,α-SNARE syntaxin are important for efficient SNARE complex disassembly (11, 16). Surprisingly, we found that changing the composition of the zero layer had no significant effect on the rate at which synaptic SNARE complexes could be disassembled by NSF. Furthermore, syntaxin containing arginine instead of glutamine in its zero layer position remained functional in living worms. Based on the literature and our previous studies, our findings suggest that the true function(s) of the zero layer remain to be discovered. This latter result is consistent with the relatively benign effects previously associated with mutations of SNAP-25 zero layer residues in semi-intact and intact cell systems (22, 23, 28, 29) and seems to be inconsistent with a major change in the ability of NSF to disassemble SNARE complexes with mutant zero layers.

Where did the idea that disassembly depends on polar residues in the core of the zero layer come from? It was first proposed as a possible explanation for the layer’s high degree of conservation and for the seemingly unique sequestration of the layer’s polar residues from surrounding solvent (11). It re-emerged in efforts to explain the weak effects of perturbative zero layer mutations in SNAP-25 SNARE motifs on exocytosis (22, 23, 28, 29). Scales et al. (16) further developed the hypothesis in a study examining the effects of zero layer mutations on features of the SNARE complex cycle not directly linked to membrane fusion, including overall stability and NSF-mediated disassembly. Like us, they found that changes in the zero layer reduced the apparent stability of the synaptic SNARE complex. However, their primary conclusion that changes in the zero layer and especially in the glutamine of syntaxin severely impair NSF-mediated disassembly draws attention to other features of the SNARE complex that NSF might utilize in initiating its disassembly reaction, as discussed further below.

FIGURE 7. SNARE complex with a non-polar zero layer is efficiently disassembled by NSF. A, fluorescence emission (λem = 434 nm) of a complex containing syntaxin(Q226L)/CFP, SNAP-25(Q53A/Q174L), and synaptobrevin(R56A)/YFP before (solid line) and after (dashed line) 10 min of exposure to NSF (2 μg). The inserted schematic illustration shows the arrangement of amino acids within the mutant SNARE complex zero layer. B, kinetic scan of disassembly of the hydrophobic zero layer complex by NSF (1.5 μg). The arrow shows the point at which NSF was added to the SNAP/SNARE complex mixture. FRET efficiency was calculated from CFP emission as described under “Experimental Procedures.”

To look for more subtle impairment of neurotransmission in worms expressing this mutant syntaxin, we measured the sensitivity of various strains to the acetylcholinesterase inhibitor aldicarb. Mutant worms that release less acetylcholine than normal are typically resistant to aldicarb, whereas those that release more are hypersensitive (35, 44–46). Disruptive mutations of hydrophobic layer residues in the SNARE motifs of syntaxin (unc-64) and synaptobrevin (snb-1) have been shown previously to generate worms resistant to aldicarb, whereas a syntaxin mutation that is “open” and thus more readily incorporated into SNARE complexes creates worms with a hypersensitive phenotype (25, 26, 47). Eliminating tomosyn, a proposed negative regulator of SNARE function, also leads to hypersensitivity (46, 48). As expected, mutant syntaxin worms expressing wild-type UNC-64 responded to aldicarb similarly to the parental strain (N2), whereas known syntaxin-deficient worms were resistant (Fig. 8C). Surprisingly, mutant worms rescued with transgenic Q227R mutant UNC-64 were paralyzed sooner than those expressing wild-type syntaxin, indicating a hypersensitivity to aldicarb. Although the basis for this hypersensitivity merits further exploration, the fact that worms expressing the syntaxin zero layer mutant are not resistant to aldicarb confirms that synaptic SNARE complexes with a zero layer of two glutamines and two arginines are functional in C. elegans.

DISCUSSION

Where did the idea that disassembly depends on polar residues in the core of the zero layer come from? It was first proposed as a possible explanation for the layer’s high degree of conservation and for the seemingly unique sequestration of the layer’s polar residues from surrounding solvent (11). It re-emerged in efforts to explain the weak effects of perturbative zero layer mutations in SNAP-25 SNARE motifs on exocytosis (22, 23, 28, 29). Scales et al. (16) further developed the hypothesis in a study examining the effects of zero layer mutations on features of the SNARE complex cycle not directly linked to membrane fusion, including overall stability and NSF-mediated disassembly. Like us, they found that changes in the zero layer reduced the apparent stability of the synaptic SNARE complex. However, their primary conclusion that changes in the zero layer and especially in the glutamine of syntaxin severely impair NSF-mediated SNARE complex disassembly differs from our results. We found that SNARE complexes containing arginine instead of glutamine in the syntaxin zero layer position were readily disassembled under a variety of assay conditions using two distinct reporter systems. Furthermore, we were able to show that syntaxin with this potentially perturbative zero layer mutation remained functional in living worms. This latter result is consistent with the relatively benign effects previously associated with mutations of SNAP-25 zero layer residues in semi-intact and intact cell systems (22, 23, 28, 29) and seems to be inconsistent with a major change in the ability of NSF to disassemble SNARE complexes with mutant zero layers.

Although there are a number of minor differences in the recombinant...
proteins and assays used here versus those used by Scales et al. (16), none clearly explain the different results obtained. One way in which changes in the SNARE complex can inhibit NSF-mediated disassembly is to decrease α-SNAP binding. As shown in Fig. 6, the one difference we found in disassembly of wild-type and zero layer mutant SNARE complexes was a small shift (K<sub>0.5</sub> = 0.2 μM to K<sub>0.5</sub> = 0.5 μM) in the amount of α-SNAP needed to reach the half-maximal disassembly rate. This shift is readily explained by the likely effects of changes in the shape of the SNARE complex around the zero layer upon α-SNAP binding (17, 30, 43). If active α-SNAP was limiting in the assays of Scales et al. (16), then changes in the affinity of different mutant complexes for α-SNAP could explain some of the reported effects of zero layer mutations on disassembly. A more recent study in which arginine in the synaptobrevin zero layer was replaced with a highly disruptive proline residue found that cells expressing this mutant accumulated SNARE complexes in their plasma membrane (41). Although the effects of this mutation on α-SNAP binding were not examined, our studies of α-SNAP binding to the SNARE complex predict that such a disruptive mutation will impair α-SNAP binding and thus slow disassembly at physiologic concentrations of α-SNAP (30).

Given the high degree of conservation of the zero layer and the common ability of NSF/α-SNAP to disassemble all SNARE complexes, it seems likely that any essential role for residues of the zero layer in NSF-driven disassembly would apply to more than just the synaptic SNARE complex. Studies examining the effects of zero layer mutations in other SNARE complexes have not precisely addressed this question, but they are at the least inconsistent with an obligate role for the QA glutamine in ongoing complex function. In particular, whereas yeast exocytic and endoplasmic reticulum/Golgi complexes containing two-glutamine/two-arginine zero layers were found to be seriously impaired or nonfunctional in vivo, their function could be restored by replacing the arginine in the original R-SNARE with glutamine to create a rotated three-glutamine/one-arginine layer (17–19). Because none of the SNARE complexes so far studied in yeast have the convenient property of resisting dissociation by SDS, the precise quantita-
tion of the steady-state levels of the SNARE complex will require the development of better methodology.

If the zero layer is not conserved to facilitate disassembly of complexes by NSF, what role(s) does it play? Among the more obvious possibilities that have been considered are that (a) it helps SNAREs assemble with each other in the proper register and orientation to avoid off-pathway assembly intermediates; (b) it contributes to establishing the desired (four-helix) oligomeric state; and (c) it provides an intermediate "stopping point" at the stage at which complexes are half-way zippered up between membranes (partially assembled \textit{"trans-complexes"}). This latter role would not be obvious in studies of solubilized SNAREs because half-assembled SNARE complexes are stable only when anchored in two membranes. Buried polar residues are known from studies of other coiled-coil systems to affect assembly kinetics as well as the orientation and oligomeric state of the resulting complexes, making all of these reasonable possibilities. Which of these or other roles the zero layer plays will require systematic study of SNARE complexes in which the zero layer polar residues are replaced with structurally appropriate hydrophobic residues.

Our finding that worms expressing the zero layer mutant syntaxin(Q227R) are hypersensitive to aldicarb provides evidence for a previously unappreciated role for the syntaxin zero layer glutamine in negatively regulating syntaxin function. Further studies will be needed to establish what this is, but three possibilities are that (a) the Q227R mutation could affect the ratio between open and "closed" syntaxin, thereby making all of these reasonable possibilities. Which of these or other possibilities that have been considered are that (a) it helps SNAREs assemble with each other in the proper register and orientation to avoid off-pathway assembly intermediates; (b) it contributes to establishing the desired (four-helix) oligomeric state; and (c) it provides an intermediate "stopping point" at the stage at which complexes are half-way zippered up between membranes (partially assembled \textit{"trans-complexes"}). This latter role would not be obvious in studies of solubilized SNAREs because half-assembled SNARE complexes are stable only when anchored in two membranes. Buried polar residues are known from studies of other coiled-coil systems to affect assembly kinetics as well as the orientation and oligomeric state of the resulting complexes, making all of these reasonable possibilities. Which of these or other roles the zero layer plays will require systematic study of SNARE complexes in which the zero layer polar residues are replaced with structurally appropriate hydrophobic residues.

If NSF and α-SNAP do not initiate disassembly from the zero layer, what do they disrupt to promote SNARE complex disassembly? The original goal of this study was to use the differences between disassembly of wild-type \textit{versus} Q226R mutant complexes as a starting point for understanding how interactions between α-SNAP and SNAREs are modified by NSF to promote disassembly. With the finding that mutations in the zero layer have no effect on disassembly, we turn our attention to other features of the SNARE complex that α-SNAP and NSF might perturb. Our previous studies of the interaction between α-SNAP and the SNARE complex have defined a critical role for charged residues (30, 50). Within the SNARE complex, interhelical interactions fall into two categories. First are the interactions within the core of the complex that are hydrophobic except for the hydrogen-bonded network of the zero layer. Second are a large number of interhelical hydrogen bonds and salt bridges between partially surface-exposed residues. These interactions extend the length of the complex and include approximately 24 interhelical salt bridges in the synaptic SNARE complex and somewhat fewer in other complexes (11, 12). In two-stranded coiled coils, stability often depends on interhelical salt bridges. We propose that destabilizing salt bridges would be one way for NSF and α-SNAP to disassemble the SNARE complex. This could happen by using the charged SNARE-binding surface of α-SNAP to provide alternative salt bridge partners for residues of the SNARE complex involved in interhelical salt bridges. Conformational changes in NSF driven by ATP binding and/or hydrolysis could place α-SNAP in position to promote "salt bridge switching," leading to destabilization and disassembly of the SNARE complex. Insight into how efficiently this might happen will come from direct measurement of the amount of ATP needed to disassemble a SNARE complex. Experiments to define the energy requirements of the reaction and to test this idea are currently in progress.

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Normal Function of SNARE Complex Zero Layer Mutants

1783–1791

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