A Discontinuous SNAP-25 C-terminal Coil Supports Exocytosis*

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The ability to fuse two biological membranes is one of the essential features of every cell. Membrane fusion has been particularly well studied in the context of Ca\(^{2+}\)-dependent exocytosis of neurotransmitters and hormones in neurons and neuroendocrine cells (1, 2). Investigation of the mechanism of membrane fusion showed that the soluble N-ethylmaleimide-sensitive fusion protein receptor (SNARE)\(^1\) protein family plays essential roles in this process. It is hypothesized that the v- or B-SNARE vesicle-associated membrane protein (VAMP), also known as synaptobrevin, the plasma membrane t- or Q-SNAREs syntaxin, and SNAP-25 (3) assemble into a highly stable four-helical bundle, and in doing so bring the two membranes into close proximity and promote lipid mixing (4–6).

Membrane fusion requires the formation of four-helical bundles comprised of the SNARE proteins syntaxin, vesicle-associated membrane protein (VAMP), and the synaptosomal-associated protein of 25 kDa (SNAP-25). Botulinum neurotoxin E cleaves the C-terminal coil of SNAP-25, inhibiting exocytosis of norepinephrine from permeabilized PC12 cells. Addition of a 26-mer peptide comprising the C terminus of SNAP-25 that is cleaved by the toxin restores exocytosis, demonstrating that continuity of the SNAP-25 C-terminal helix is not critical for its function. By contrast, vesicle-associated membrane protein peptides could not rescue botulinum neurotoxin D-treated cells, suggesting that helix continuity is critical for VAMP function. Much higher concentrations of the SNAP-25 C-terminal peptide are required for rescuing exocytosis (\(K_{\text{assembly}} = 460 \mu M\)) than for binding to other SNAREs \textit{in vitro} (\(K_d < 5 \mu M\)). Each residue of the peptide was mutated to alanine to assess its functional importance. Whereas most mutants rescue exocytosis with lower efficiency than the wild type peptide, D186A rescues with higher efficiency, and kinetic analysis suggests this is because of higher affinity for the cellular binding site. This is consistent with Asp-186 contributing to negative regulation of the fusion process.

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\(K_{\text{assembly}}\), apparent affinity of binding to cellular SNAREs.

Experimental support for this hypothesis includes structural data demonstrating a parallel organization of the SNARE coiled-coil bundle (4, 7–9), biophysical and biochemical data showing that the SNARE complex formed \textit{in vitro} is SDS-resistant and highly thermostable (10–12), and functional data obtained using synthetic liposome, cracked PC12 cell, and adrenal chromaffin cell systems (6, 13–15). After membrane fusion is completed, cis-SNARE complexes (with their transmembrane domains in a single membrane) require α-SNAP and the ATPase NSF for disassembly, so that SNAREs can be re-used for the next round of fusion (16–18).

SNAREs are thought to drive or catalyze membrane fusion. Recent studies using purified SNARE proteins in synthetic liposomes have shed light on the potential mechanical driving mechanism of SNAREs. Increasing the length of the linker between the transmembrane domain and the conserved helical domain of SNAREs decreased the liposome fusion efficiency (19). Furthermore, replacement of transmembrane anchors of syntaxin and VAMP with short covalently attached lipids prevented lipid mixing without detriment to the liposome docking, suggesting that membranes do not simply fuse passively when held together (6). Thus SNAREs appear to exert force on their membrane anchors by pulling on the linker and transmembrane domains, thus actively promoting lipid rearrangement and fusion. This force is almost certainly derived from the assembly of the parallel core complex bridging the two membranes. It was shown in the cracked PC12 cell system that mutating the conserved hydrophobic residues that are important for core complex formation resulted in decreased thermostability of core complexes and also dramatically impaired the ability to function in exocytosis (14).

Despite general agreement as to the major function of SNARE proteins, the sequence of events leading to complex assembly and bilayer fusion remain unclear. Some recent reports suggest that SNAREs interact prior to lipid mixing, perhaps as a partially assembled complex. Studies using synthetic liposomes and synaptosomes led to the same conclusion that SNARE complexes exist prior to the actual lipid and content mixing, although opposite conclusions were drawn as to whether NSF and α-SNAP disrupt such complexes (20, 21). Electrophysiological studies using adrenal chromaffin cells showed that SNARE complexes exist in a dynamic equilibrium between a loose and a tight form before the arrival of the Ca\(^{2+}\) trigger, with the two forms leading to fusion with rate constants of 300 and 30 ms, respectively (15). In crayfish neurons, an N-terminally zippered, C-terminally unzipped SNARE complex was suggested to exist prior to the fusion trigger (22). Our recent results using the cracked PC12 cell system predict the existence of a reversibly assembled SNARE complex, with which the syntaxin coil is perhaps only loosely associated before the arrival of Ca\(^{2+}\) (23). If the full assembly of the SNARE complex is mechanically coupled to lipid fusion, a completely assembled four-helical bundle as in the crystal structure (9)
should not exist before the fusion event itself and should only be the end product of the fusion event. Thus a clear distinction has to be made between a tight helical bundle complex and a partially assembled complex, as more than one kind of SNAPRE complex likely exists.

A useful tool in studying exocytosis is a series of potent neurotoxins, botulinum and tetanus toxins, that proteolytically cleave SNARE (24). Botulinum neurotoxin E (BoNT/E) cleaves the SNAP-25 C-terminal helix (aa 142–206) between residues 180 and 181, removing a 26-amino acid peptide from the C terminus of SNAP-25 (25). BoNT/E-cleaved SNAP-25 is incapable of forming an SDS-resistant SNAPRE complex (10), and exocytosis in neurons and neuroendocrine cells is abolished. We have shown previously that this inhibition can be reversed by adding a recombinant 65-mer SNAP-25 C-terminal coil (S25C) (14). The added coil displaces the remaining half of the endogenous S25C coil (aa 142–180) and participates in SNAPRE complex formation as a complete new S25C coil. In the current study, we show that a peptide encompassing only aa 181–206 of SNAP-25, the 26-aa C terminus that is cleaved by BoNT/E, is also able to rescue exocytosis from BoNT/E-treated PC12 cells, albeit at higher concentrations than the full-length coil. This surprising finding prompted us to investigate the in vitro requirements for a functional SNAPRE complex, providing further insight into the mechanism of SNAPRE-mediated fusion. We also mutated each residue to alanine to study the functional contribution of each amino acid in this 26-aa region to SNAP-25 function.

**EXPERIMENTAL PROCEDURES**

**Cracked Cell Assay**—PC12 cells were labeled with [3H]NE, cracked, toxin-treated, and washed as described (14, 26). Regular release reactions contained ~10⁶ cells, 2 mM MgATP, 0.5–1 mg/ml rat brain cytosol (prepared as described in Ref. 26), 1.6 ml total Ca²⁺ (15 μM free Ca²⁺ measured by flura-2 fluorescence as described in Ref. 27), and indicated concentrations of proteins or peptides in a total volume of 200 μl of KGlu buffer (50 mM Hepes, pH 7.2, 105 mM potassium glutamate, 20 mM potassium acetate, 2 mM EGTA). Release was initiated by incubation at 30 °C and terminated by chilling to 0 °C. The data shown are representative of experiments performed at least three times. In kinetic assays, toxin-treated cells were first primed with 2.6 mM MgATP and 0.65 mg/ml cytosol for 15 min at 30 °C in the absence of rescuing peptide. The primed cells were then chilled for 3 min and immediately aliquoted into ice-cold pool tubes containing the indicated amounts of peptide (0–1000 μM), ~1 μM free calcium, plus extra MgATP and cytosol to reach the final concentrations of a typical release reaction. These pools were then divided into 6 equal aliquots and either left on ice (0 min) or incubated at 30 °C for 3, 6, 12, 20, or 30 min prior to chilling. NE release is calculated as a percentage of the total [3H]NE in the 2500 x g supernatant after scintillation counting.

**Preparation of Recombinant Proteins and SNAPRE Complexes**—Rat syntaxin 1a H3 domain (aa 191–266), rat VAMP2 coil domain (aa 25–94), rat VAMP2 fragment (aa 1–59), and mouse SNAP-25 (as described in Ref. 26), were purified by gel filtration on a Superdex 75-HiLoad 16/60 column (Amersham Pharmacia Biotech) in 20 mM Hepes, pH 7.2, 150 mM KCl. The base complex was judged to be free of potentially contaminating binary complexes because it eluted in a completely separate peak to a binary (syntaxin:BoNT/E-cleaved SNAP-25) complex, and it melted with a single transition higher than that of the binary complex (see Fig. 3). The base complex was judged to be free of potentially contaminating binary complexes because it eluted in a completely separate peak to a binary (syntaxin:BoNT/E-cleaved SNAP-25) complex, and it melted with a single transition higher than that of the binary complex (see Fig. 3).

**Circular Dichroism (CD) Spectroscopy**—Data were recorded with an Aviv 62DS CD spectrophotometer and a 0.1-cm path length quartz cuvette. Thermal unfolding experiments were performed by measuring the CD signal at 222 nm (30-s averaging time) in 1 °C steps (1-min equilibration at each temperature). Data were normalized to % unfolded by fitting the lower and upper signals to 0 and 100% unfolded, respectively. The Tm values were taken as the temperature at which 50% of the signal remained. To study peptide binding, CD spectra were collected scanning from 250 to 200 nm in 1-nm steps at 30 °C (averaging time 30 s). The average signal at 222 nm from three such consecutive scans was used. Neither the buffer nor the SNAP-25 C-terminal peptide alone gave a signal in the above wavelength range. However, the signal due to 5 μM coil complex lacking the peptide region (the base complex) significantly increased upon addition of increasing amounts (0–10 μM) of the peptide, suggesting the formation of additional α-helical structures.

**Peptides**—All SNAP-25 C-terminal peptides (aa 181–206) and VAMP2 peptide (aa 25–59) were synthesized by the Stanford PAN Facility. Analytical high performance liquid chromatography and mass spectroscopy were performed by the Facility to ensure the quality of each peptide. For use in the cracked cell assay, peptides were weighed and dissolved in KGlu buffer to a final concentration of 5 or 2 mM, depending on solubility. In a few cases where peptides could not fully dissolve in KGlu at 2 mM concentration, H₂O was used instead, followed by addition of 5 X KGlu buffer to 1 X final concentration. Amino acid analysis (AAA Laboratory, Mercer Island, WA) was used to verify the peptide concentrations used in the kinetic analysis.

**Kinetic Analysis**—The percent of [3H]norepinephrine release was calculated and plotted versus time for each concentration of rescuing peptide, with DeltaGraph® 4.0.5 software. The data were fitted with exponential curves given by y = a x (1 − e⁻ᵃᵇˣ) + c (r² is typically 0.99–0.999). The initial rate of release (initial slope of each time course curve), V₀, is equal to “a” x “b”. The V₀ value in the absence of S25C (0 μM) represents the background leakage and so was subtracted from all the V₀ values. V₀ (minus background) was then plotted versus peptide concentration. Six independent experiments were carried out for the wild-type peptide and the mutant D186A. Data points at each peptide concentration were averaged across six experiments, and the mean ± S.D. were plotted in Fig. 6C. Curve-fitting to the equation y = Vmax x x/((Vmax + Km)), where Km reflects the apparent affinity of the peptide for the endogenous SNAPREs, was carried out using the mean values. The Vmax and Km values predicted from each experiment were averaged to give the mean ± S.D. reported. For derivation of the Vmax and Km values, see Ref. 23.

**RESULTS**

A SNAP-25 C-Terminal Peptide Rescues Exocytosis in BoNT/E-treated PC12 Cells—BoNT/E inhibits NE exocytosis in cracked PC12 cells by cleaving 26 residues (aa 181–206) from the C terminus of SNAP-25. We showed previously that addition of a 65-aa SNAP-25 C-terminal coil (S25C, aa 142–206) rescues exocytosis in toxin-treated cells (Fig. 1; see Ref. 14). It would be interesting to see whether addition of a 26-mer peptide, encompassing the region that is removed by the toxin, would also be sufficient to rescue exocytosis. A peptide of the 26 C-terminal
residues of SNAP-25 was therefore synthesized and tested for its ability to rescue BoNT/E-treated cells. As shown in Fig. 1, the peptide did rescue exocytosis as did S25C, although higher concentrations were required (EC_{50} \geq -150 \mu M, compared with EC_{50} = -30 \mu M for S25C). The maximal level of release achieved by the peptide (up to 1 mM concentration) is similar to that achieved by the S25C coil (68.8% of total [H][H]NE released), which is also similar to that in non-toxin-treated cells. The observation that the peptide rescues exocytosis does not contradict the fact that BoNT/E inhibits exocytosis, as the concentration of the soluble peptide generated by toxin cleavage of endogenous SNAP-25 is likely to be less than 10 \mu M, too low to support exocytosis (Fig. 1).

The ability of the peptide to rescue exocytosis indicates that a discontinuous SNAP-25 C-terminal coil that is comprised of two helices, aa 142–150 from endogenous SNAP-25 and aa 181–206 from the added peptide, is able to support exocytosis. Therefore a functional SNARE complex does not require continuity in the SNAP-25 C-terminal coil.

**VAMP Peptides Do Not Rescue Exocytosis in BoNT/D-treated Cells**—We also tested whether a VAMP peptide could rescue cracked BoNT/D-treated cells. Because BoNT/D cleaves between residues 59 and 60 of the VAMP coil (aa 25–94; Ref. 29), releasing aa 1–59 of VAMP into the buffer, we reasoned that high concentrations of VAMP peptides (aa 1–59 or 25–59) might be able to rescue exocytosis. However, neither peptide had any rescuing ability up to 200 and 500 \mu M concentration, respectively (data not shown). One possible reason for this result is that the continuity of the VAMP coil is required for its function. Unlike SNAP-25, which is only anchored to the membrane by palmitoylation, VAMP is a transmembrane SNARE. As the formation of the helical bundle core complex is thought to exert force on the transmembrane domains of syntaxin and VAMP, thus promoting membrane fusion (4, 6, 7, 9), the continuity of the coil might be more important for VAMP than for SNAP-25.

Another possible reason for the lack of efficacy of the soluble VAMP peptides may be that they comprise the membrane-distal N-terminal region of the coil, whereas the rescuing SNAP-25 peptide comprises the membrane-proximal C-terminal end of the coil. If the initial assembly of SNARE complexes requires the N-terminal portion of VAMP and the zipper occurs from the N- to C-terminal end as proposed (7, 22), then the cleaved endogenous VAMP may be unable to position itself for full assembly without its N terminus.

**The SNARE Complex with the Discontinuous S25C Coil Is neither SDS-resistant nor Thermostable**—It was shown previously that ternary SNARE core complexes formed in vitro are resistant to SDS denaturation and are thermostable at temperatures above 90 °C (10, 11). These properties are thought to be important for the membrane fusion activity of SNAREs (14). Because we found that a SNARE complex composed of a discontinuous S25C coil is also functional, we tested whether such a complex is SDS-resistant. A purified “normal” ternary core complex (comprised of full-length SNAP-25 (actually SNAP25ACys in which the four palmitoylatable cysteines are mutated to alanine), VAMP coil, and syntaxin H3 domains) runs as an ~40-kDa complex by SDS-polyacrylamide gel electrophoresis unless boiled, which reduces it to monomers (Fig. 2, lanes 1 and 2). In contrast, a SNARE complex composed of the VAMP coil domain, the syntaxin H3 domain, BoNT/E-cleaved SNAP-25, and the SNAP-25 C-terminal peptide appears as individual monomers whether or not the sample is boiled (lanes 7 and 8), indicating that the core complex containing clipped SNAP-25 is not SDS-resistant. Likewise, a complex containing VAMP, the syntaxin H3 domain, BoNT/E-cleaved SNAP-25, but lacking the peptide, is also sensitive to SDS (lanes 5 and 6), as is the binary complex containing just BoNT/E-cleaved SNAP-25 and the syntaxin H3 domain (lanes 3 and 4).

We then used circular dichroism (CD) spectroscopy to monitor the unfolding of the α-helical structure of core complexes with temperature (Fig. 3). As expected, the normal intact ternary complex (as in Fig. 2, lane 1) was extremely stable, with only minimal unfolding up to 92 °C. Also as expected, the binary complex consisting of cleaved SNAP-25 and the syntaxin H3 domain (as in Fig. 2, lane 3) was relatively unstable (T_m = 67 °C). A ternary complex lacking the SNAP-25 26-aa C terminus (henceforth referred to as “the base complex,” as in Fig. 2, lane 5; 0 \mu M pep in Fig. 3) was slightly more stable (T_m = 76 °C). Adding 100 \mu M SNAP-25 peptide (a 10-fold molar excess of peptide over the base complex) did not increase the T_m. However, very high concentrations of peptide did increase the T_m, but only to 84 °C with 1 mM peptide, still a much lower unfolding transition than the intact ternary complex (~99 °C).

Because 1 mM peptide was sufficient to rescue exocytosis to a maximal level (Fig. 1) and the cellular SNARE concentration is likely to be less than 10 \mu M, it follows that high thermostability of a SNARE complex is not necessarily required for function.

**Effect of Mutations on the Rescuing Ability of SNAP-25 Peptides**—Many residues in the 26-aa C terminus of mouse SNAP-25 are highly conserved across species and homologues (Fig. 4). To assess the functional importance of each residue, 23 peptides were synthesized, with every non-alanine residue singly mutated to alanine. The ability of these mutant peptides to rescue exocytosis from BoNT/E-treated cells compared with the wild-type peptide was tested in at least three independent experiments and the relative magnitude of release compared with the wild-type peptide was reproducible. Figs. 4 and 5 summarize the results from one such representative experiment. Although the absolute values, background release, and maximal rescue levels vary, the relative values for each mutant are consistent across numerous experiments. Fig. 5 shows the amount of exocytosis achieved by different concentrations of each mutant during 30 min of stimulation. Only results obtained with mutants that rescued in a manner significantly different from the wild-type peptide are shown. The relative rescuing ability (expressed as % of wild-type) of these mutants at 100 \mu M and 1 mM concentrations (see Fig. 5) is summarized.
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FIG. 3. SNAP-25 peptide only slightly increases the stability of a core complex containing BoNT/E-cleaved SNAP-25 (the base complex). The structural unfolding of SNARE complexes with increasing temperature was monitored by CD spectroscopy. The CD signal was normalized to percent unfolded by fitting the lower and upper levels to 0 and 100%, respectively. Cleaved binary, complex as in Fig. 2, lane 3, at 5 μM concentration; 0 μM pep, complex as in Fig. 2, lane 5 (the base complex) at 10 μM; 100 μM, 500 μM, 1 mM SNAP-25 peptide added to the 10 μM base complex; respectively; intact ternary, complex as in Fig. 2, lane 1, at 10 μM.

FIG. 4. Sequence of the SNAP-25 C-terminal 26 aa compared with various homologues and summary of the rescuing ability of mutants. Amino acid sequences of mouse SNAP-25 C terminus (mS25, with residue numbers marked below) and the homologous region from other species or homologues were compared using the program Pileup and Boxshade. Black boxes highlight residues that are identical in over one-half of the sequences, whereas gray boxes indicate conservative substitutions. aS25, Arabidopsis SNAP-25; hS25, human SNAP-25; Sec9, Saccharomyces cerevisiae Sec9; hS23, human SNAP-23; dS25, Drosophila SNAP-25; hS25, human SNAP-25. The sequences are grouped into heptad blocks, with residues at “a” and “d” positions, which are involved in core interactions of the SNARE complex, being marked with bullets and the hydrophobic layer number (1 to 8) that indicates their relative position in the core complex (as in Sutton et al. (9)). Each non-alanine residue in the SNAP-25 C-terminal 26-aa peptide was mutated to alanine, and the amount of rescue (expressed as % of wild-type rescue) at 100 μM and 1 mM concentrations (calculated from the experiment shown in Fig. 5) is shown. Where no number appears below a given non-alanine residue, the level of rescue was not significantly different from wild-type. The amount of rescue (with the background subtracted) at 100 and 1000 μM was divided by those afforded by the wild-type peptide and presented as % wild-type rescue in Fig. 4.

in Fig. 4. Where no number is listed beneath a non-alanine residue, mutation of that residue did not significantly affect its ability to rescue at either concentration.

Consistent with earlier results obtained in the S25C rescue system (14), mutation of the highly conserved hydrophobic residues residing at “a” or “d” positions in the heptad repeats had the greatest effect on rescue (I181A and I192A, Figs. 4 and 5A). Peptide M182A also rescued very poorly, although the same mutation in S25C did not significantly alter rescue (Fig. 5A and data not shown). Perhaps the M182A mutation in the 26-mer peptide, which is very close to the beginning of the peptide, indirectly affects the function of the adjacent residue, Ile-181, hindering proper binding of the peptide to the base complex. Interestingly, mutation of the asparagine at a “d” position to alanine (N188A, Fig. 5B) resulted in increased rescue compared with wild-type at low concentrations of the pep-

tide, suggesting that this polar residue may play a negative role in the overall hydrophobic core interactions of the SNARE complex.

The other residues that affected rescue were all charged or polar residues at non-“a/d” positions in the heptad repeats. Mutations D186A (a “b” position, which disrupts a salt bridge to Arg-66 of VAMP) and R198A (a “g” position, which disrupts a potential salt bridge to Glu-78 of VAMP) most significantly increased and decreased exocytosis, respectively (Fig. 5, B and C), consistent with earlier S25C rescue results (14). This is perhaps not surprising, as these two residues, along with Ile-181 and Ile-192, are the most conserved in the whole peptide (Fig. 4). Whereas the mechanism of differential rescue of these non-“a/d” mutants is not yet understood, interactions of these residues with potential SNARE complex regulator(s) might be involved.

Kinetic Analysis of Peptide-mediated Rescue—To quantify the ability of certain SNAP-25 peptides to support exocytosis in the cracked PC12 cells, we performed kinetic analysis with the wild-type, D186A, and I192A peptides. As shown above, Asp-186 and Ile-192 reside at a surface “b” position and a hydrophobic core “a” position of a heptad helical wheel, and mutation to alanine positively and negatively affected exocytosis, respectively (Figs. 4 and 5, A and B). Unfortunately, the I192A mutant afforded too little release to allow accurate determination of kinetic parameters; thus only data obtained with the wild-type and D186A peptides are presented.
The time course of release afforded by different concentrations of rescuing peptides was measured, and the data points were fitted to single exponential curves to allow determination of the initial release rate (Fig. 6, A and B; see “Experimental Procedures” for details). The initial rate of release was then plotted versus the concentration of the peptide and fitted to the equation $y = V_{\text{max}} \times x/(x + K_{\text{assembly}})$ (Fig. 6C). According to our previous kinetic model of the vesicle release reaction (23), $K_{\text{assembly}}$ in the equation defines the apparent affinity of the rescuing peptide for its binding site in the cell, whereas $V_{\text{max}}$, which is the maximal velocity of the fusion reaction, relates to the forward rate constant of the final irreversible assembly of SNARE complexes.

The $V_{\text{max}}$ determined from the data shown in Fig. 6C was $14.8 \pm 3.0$ (% $^3$H/min) for wild-type and $9.9 \pm 1.3$ (% $^3$H/min) for D186A-mediated release. The initial rate of release from control non-toxin-treated cells in these experiments was $10.0 \pm 1.7$ (% $^3$H/min). Thus both peptides are able to support a fusion reaction that is as fast as the normal rate of exocytosis. Previously, we showed that the $V_{\text{max}}$ of wild-type S25C-dependent exocytosis is also indistinguishable from the initial rate of exocytosis in non-toxin-treated cells (23).

The determined $K_{\text{assembly}}$ (the concentration of peptide at which the rate of fusion is one-half $V_{\text{max}}$) was $460 \pm 180$ $\mu$M and $126 \pm 56$ $\mu$M for wild-type- and D186A-mediated release, respectively. Previously, we showed that the $K_{\text{assembly}}$ of wild-type 65-mer S25C and S25C/D186A-rescued exocytosis was $176 \pm 23$ $\mu$M and $115 \pm 23$ $\mu$M, respectively. Therefore the 26-mer wild-type peptide, compared with the 65-mer S25C, has an ~3-fold higher $K_{\text{assembly}}$, indicating a reduced affinity for the other SNAREs in the cell, most likely because of the diminished number of contacts. This is consistent with the dose-response curves shown in Fig. 1. That the D186A mutation results in a decreased $K_{\text{assembly}}$ in both the 65-mer and 26-mer peptide contexts is also consistent with the idea that residue Asp-186 normally plays a negative role in the binding of the SNAP-25 C-terminal coil to the other SNAREs (23).

$K_{\text{assembly}}$ defines the affinity of the SNAP-25 C-terminal peptide for the functional base complexes in PC12 cells (the ones leading to fusion), which are trans-complexes. We therefore attempted to determine the affinity of the same peptide for the cis-base complex in vitro (the $K_d$) so as to obtain an estimate of the energy change that occurs upon conversion of the trans-complex into the cis-complex. Because we were unable to obtain results by anisotropy of fluorescently labeled peptide, we attempted to measure the increase in $\alpha$-helicity of the base complex upon addition of increasing concentrations of peptide by CD. The increase in signal at 222 nm due to binding of the peptide to $5 \mu$M base complex saturated at an equivalent concentration of peptide (data not shown). It was not possible to use lower concentrations of base complex because of the low level of signal obtained. A similar value was obtained by monitoring the binding of the base complex to the S25C peptide coupled to beads by Western blotting, but this technique was also not sensitive enough to permit the use of lower peptide concentrations. However, although we could not determine the $K_d$ of the peptide for the base complex accurately, we can set $5 \mu$M as its absolute upper limit, which is still ~100-fold lower than the $K_{\text{assembly}}$ determined for the trans-complexes. The real $K_d$ is likely to be much lower still.

**DISCUSSION**

We show that the SNAP-25 C-terminal 26-aa peptide can rescue BoNT/E-inhibited exocytosis. That a discontinuous S25C coil is able to function was initially surprising. Combined with the finding that the VAMP N-terminal peptide does not rescue BoNT/D-inhibited exocytosis, these results suggest that SNAP-25 likely promotes membrane fusion by contributing two cytoplasmic $\alpha$-helices to stabilize the core complex, without directly doing work on the membranes. The lipid reorganization required for bilayer fusion is perhaps carried out by the two transmembrane SNAREs, syntaxin and VAMP, which can pull their membrane anchors upon core complex zippering (6).
Presumably the fact that two separate fragments of S25C with correct side chain arrangements can effectively rescue exocytosis means that no such force is transduced along the SNAP-25 C-terminal coil.

The in vitro properties of the SNARE core complexes, most often SDS resistance and thermostability, have been used to predict their function inside cells. Whereas the normal core complex is known to be SDS-resistant and highly thermostable, we found that the core complex containing a discontinuous SNAP-25 coil is no longer SDS-resistant and much less stable. This is perhaps to be expected, given the structural disruption to the carbon backbone. We also found previously that if a core complex contains noncoenate SNAREs or SNAP-25 mutations at non-"aid" positions, the thermostability of purified SNARE complexes. In the context might be affected, which would not be reflected in the measured thermostability of purified SNAP-25 complexes. In the case of the discontinuous SNAP-25 coil, the structural discontinuity probably causes a particularly dramatic effect on the interaction of SNAREs with essential regulator(s) in a cellular surface residue of the core complex is mutated, the potential for the complex to activate fusion is significantly reduced.

It appears, then, that thermostability should only be used as an indicator of SNARE function in the case of mutating hydrophobic core residues involved in inter-SNARE interactions. When a surface residue of the core complex is mutated, the potential interaction of SNAREs with essential regulator(s) in a cellular surface residue of the core complex is mutated, the potential for the complex to activate fusion is significantly reduced.

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A Discontinuous SNAP-25 Coil Supports Exocytosis

The striking difference between the binding affinities of the wild-type SNAP-25 peptide in vitro and in PC12 cells (<5 μM versus ~460 μM, an ~100-fold or more difference) suggests that an energy-expensive reaction, likely membrane fusion, is coupled to the peptide binding. Because the peptide is only 26 residues long, it is unlikely that accessibility to a binding site accounts for the low rescuing efficiency, especially because the longer 65-mer S25C rescues more effectively at an equivalent concentration. It is likely that the free energy difference between peptide binding in vitro and in the cells is used by the SNAP-25s to lower the activation energy barrier. A future challenge in the field will lie in the regulation of SNARE complex formation, in particular the regulation by Ca2+.

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A Discontinuous SNAP-25 C-terminal Coil Supports Exocytosis
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