Is Assembly of the SNARE Complex Enough to Fuel Membrane Fusion?

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The three key players in the exocytic release of neurotransmitters from synaptic vesicles are the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins synaptobrevin 2, syntaxin 1a, and SNAP-25. Their assembly into a tight four-helix bundle complex is thought to pull the two membranes into close proximity. It is debated, however, whether the energy generated suffices for membrane fusion. Here, we have determined the thermodynamic properties of the individual SNARE assembly steps by isothermal titration calorimetry. We found extremely large favorable enthalpy changes counterbalanced by positive entropy changes, reflective of the fact that ternary complex formation is essentially irreversible. We used a stabilized syntaxin-SNAP-25 heterodimer to study synaptobrevin binding. This strategy revealed that the N-terminal synaptobrevin coil binds reversibly with nanomolar affinity. This suggests that individual, membrane-bridging SNARE complexes can provide much less pulling force than previously claimed.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3 and Table 1.

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3 The abbreviations used are: SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, SNAP receptor; SNAP-25, synaptosomal-associated protein of 25 kDa; ITC, isothermal titration calorimetry; SFA, surface force apparatus.
Energetics of SNARE Complex Formation

As the folding and unfolding transitions of the ternary SNARE complex exhibit a marked hysteresis (25), the question of how much energy is released during complex formation has been difficult to answer as well. To avoid the quasi-irreversibility of the process, the problem has been elegantly tackled by atomic force microscopy by two different research groups (26–28). In these experiments individual complexes affixed to solid supports were ruptured, yielding energy values of 43 and 33 k_BT. In another approach, which used a surface-force apparatus (SFA), a comparable energy of 35 k_BT has recently been determined (29). Strikingly, these values appear to correspond closely with the activation energy needed to fuse two membranes, substantiating the view that SNAREs are nano-fusion machineries. However, one should be cautious about the conclusion that these sophisticated procedures in fact yield the genuine SNARE assembly energy. For example, with the SFA approach, the number of complexes had to be deduced rather indirectly to estimate the free energy. Moreover, these approaches offered only indirect information about the reaction pathway.

In this study we set out to determine the SNARE assembly energy more directly by using isothermal titration calorimetry (ITC) complemented by kinetic measurements. ITC is a powerful technique for studying the thermodynamics of macromolecular interactions by directly measuring the heat changes associated with complex formation, which at constant pressure is equal to the enthalpy change (ΔH). The titration approach also yields the stoichiometry (n), the entropy change (ΔS), and the association constant (K_A) of the reaction. We studied the consecutive reaction steps individually to gain deeper insights into the rugged energy landscape of complex formation. To study synaptobrevin binding in isolation, we used a stabilized syntaxin-SNAP-25 heterodimer, which has been shown to greatly accelerate liposome fusion rates (30). This strategy revealed that the N-terminal coil of synaptobrevin binds reversibly, making it feasible to access the free energy of SNARE assembly. Overall, our results suggest that individual SNARE complexes might provide much less pulling energy than previously claimed.

EXPERIMENTAL PROCEDURES

Protein Constructs—All recombinant proteins were derived from cDNAs from rat. The basic SNARE expression constructs cysteine-free SNAP-25A (residues 1–206), the syntaxin 1A SNARE motif (SyxH3, residues 180–262), the soluble domain of synaptobrevin 2 (Syb-(1–96)), and a few shortened synaptobrevin constructs have been described before; Syb-(49–96) and Syb-(49–96) contain a single cystein residue at position 79 (Syb-(49–96) 979) for fluorescence labeling (25, 30, 31). Additional truncated versions of synaptobrevin were cloned into the pET28a vector: Syb-(1–87), Syb-(1–70), Syb-(1–65), Syb-(1–52). Note that the constructs Syb-(1–87) and Syb-(1–52) each contain an additional C-terminal Cys residue, residues 88 and 53, respectively, for fluorescence labeling.

Protein Purification—All proteins were expressed in Escherichia coli strain BL21 (DE3) and purified by Ni2++-nitrilotriacetic acid chromatography followed by ion exchange chromatography on an Äkta system (GE Healthcare) essentially as described (57). His tags were generally removed using thrombin. All SNARE complexes were purified using a Mono Q column (GE Healthcare) after overnight assembly of the purified monomers. The following ternary complexes were employed: Syb-(49–96)SyxH3-SNAP-25 (ΔN complex), Syb-(49–96) SyxH3-SNAP-25, Syb-(1–52)SyxH3-SNAP-25 (Syb-(1–52) complex), and Syb-(1–70)·SyxH3-SNAP-25 (Syb-(1–70) complex). The peptide comprising the C-terminal region of synaptobrevin (Syb-(71–96)) and a similar peptide containing an additional C-terminal Cys residue (Syb-(71–96) 979) were synthesized. Protein concentrations were determined by absorption at 280 nm in 6 M guanidine hydrochloride and/or using the Bradford assay.

ITC—ITC was performed on a VP-ITC instrument (Microcal) at 25 °C essentially as described (51). Samples were dialyzed twice against a degassed phosphate buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol). Typically, an initial 5-μl injection was followed by several 15-μl injections. The heat change per injection was integrated to yield the molar enthalpy for each injection. Blank titrations, which were carried out by injection of the ligand into a buffer, were subtracted from each data set. Note that we had to use relatively long equilibration times (300–1500 s) to record the slow binding reaction between SNARE proteins. In addition, we noticed that with further injections, the time required for the ΔT signal to return to the base line after an injection peak deflection was getting longer, rendering it necessary to extend the spacing between injections. All ITC experiments were carried out at least twice. The resulting binding isotherms were analyzed using the Microcal Origin ITC software package to obtain the binding enthalpy (ΔH), the stoichiometry (n), and the association constant (K_A). Depending on the reaction investigated, we used either a “one-set-of-sites” binding model that assumes that one or more ligands bind with similar affinities or a “two-sets-of-sites” binding model that assumes two sites with different values of K_A and ΔH. The dissociation constant (K_D) and the binding free energy (ΔG) were calculated using the basic thermodynamic relationships K_D = K_A−1, ΔG = −RT ln K_A, and ΔG = ΔH − TΔS. Note that because of the limitations of the standard ITC analysis method, the contributions of sequential reaction steps cannot be resolved completely. ΔH versus T plots were fitted to the equation ΔH = ΔCp (T − TΔH = 0), where ΔCp is the heat capacity change of binding, and TΔH is a reference temperature at which ΔH = 0.

Fluorescence Spectroscopy—All measurements were carried out in a Fluorolog 3 spectrometer in T-configuration equipped for polarization (Model FL322, Horiba Jobin Yvon). Single cysteine variants were labeled with Texas Red C5 bromoacetamide or Alexa-488 C5 maleimide according to the manufacturer’s instructions (Invitrogen). All experiments were performed at 25 °C in 1-cm quartz cuvettes (Hellma) in a phosphate buffer. Measurements of the fluorescence anisotropy, which reports the local flexibility of the labeled residue and which increases upon complex formation and decreases upon dissociation, were carried essentially as described (30, 31). The G factor was calculated according to G = I_{HH}/I_{HV}, where I is the fluorescence intensity, the first subscript letter indicates the direction of the exciting light, and the second subscript indicates the let-
after the direction of emitted light. The intensities of the vertically (V) and horizontally (H) polarized emission light after excitation by vertically polarized light were measured. The anisotropy (r) was determined according to \( r = (I_{VH} - G I_{HH})/(I_{VV} + 2 G I_{HH}) \).

**Circular Dichroism (CD) Spectroscopy**—Measurements were performed essentially as described (25, 30, 31) using a Chirascan instrument (Applied Photophysics). Hellma quartz cuvettes with a path length of 0.1 cm were used. The far-UV spectra were obtained using steps of 1 nm with a scan rate of 60 nm/min and an averaging time of 0.5–2 s. For spectral measurements, different protein combinations at a concentration of about 5–10 \( \mu M \) in phosphate buffer were incubated overnight. The measurements were carried out at 25 °C. For thermal denaturation experiments, the purified complexes were dialyzed against phosphate buffer. The ellipticity at 222 nm was recorded between 25 and 95 °C at temperature increments of 30 °C/h.

**RESULTS**

In ITC experiments, two macromolecules, one in a thermally insulated cell and the other in a syringe, are sequentially mixed at a constant temperature. ITC is, therefore, well suited to monitor reversible bimolecular reactions. Because neuronal SNARE complex formation occurs by interaction of the three molecules, it was necessary to investigate each individual binary binding step first. The assembly pathway of the ternary SNARE complex is illustrated in Fig. 1a. This information then served as a basis to dissect the ternary SNARE complex reaction further by using a stabilized syntaxin-SNAP-25 heterodimer as a binding site for synaptobrevin (30). Eventually, this stabilized acceptor complex allowed the quasi-irreversibility (25) of the whole reaction to be avoided.

Large Enthalpic Change Observed within Assembly of the SNARE Complex—At first we investigated all possible binary combinations of the three neuronal SNARE proteins. For the experiments, we used full-length SNAP-25A and the entire cytosolic portion of synaptobrevin, Syb-(1–96). As the N-terminal autonomously folded region of syntaxin 1a is not part of the core SNARE complex, we employed a construct bearing only the SNARE motif (also referred to as the H3-domain) of syntaxin (SyxH3) for our studies. Titrations of synaptobrevin into SyxH3 (step 1.1) or into SNAP-25 (step 1.3) produced only background heat of dilution, whereas large heat changes were detected upon titration of SNAP-25 and SyxH3 (step 1.2 and 2.1, Fig. 1b). This is consistent with earlier CD and fluorescence measurements that had shown that only syntaxin and SNAP-25 form a stable binary complex that exhibits a 2:1 stoichiometry (8, 31). The result of a typical ITC experiment, in which SNAP-25 was titrated into syntaxin, is shown in Fig. 1b. With progressive injections, the heat signal diminished, saturating at a molar ratio of \( \approx 0.65 \), corroborating the formation of a complex with 2:1 stoichiometry. A striking feature of this interaction is the enormous favorable binding energy per mole of injected SNAP-25, suggesting that a large number of binding interactions are established. This enormous enthalpic change was counterbalanced by a large positive entropy change, reflecting the disorder-to-order transition during complex formation.

As this approach did not clearly disclose two consecutive binding steps, we next swapped the contents of the cell and the syringe by injecting SyxH3 into SNAP-25. In this manner, a transient 1:1 heterodimer is more likely to be detected, as SyxH3 is first injected into a large excess of SNAP-25 that shifts the equilibrium toward the formation of the 1:1 heterodimer. Indeed, the resulting ITC profile of this titration exhibited two distinct binding steps (Fig. 1c). This binding equilibrium was approximately evaluated with the two-sets-of-sites model that assumes the two binding sites have different affinities and enthalpies. Binding of the first SyxH3 molecule to SNAP-25 occurred with high affinity (\( K_D \approx 5 \text{ nM} \), step 1.2), whereas the second SyxH3 associates at moderate affinity (\( K_D \approx 234 \text{ nM} \), step 2.1). This agrees well with our earlier kinetic investigations that had suggested that the first SyxH3 molecule binds with a \( K_D \approx 16 \text{ nM} \) to SNAP-25 (Fig. 1a) (31).

In the next set of experiments we monitored the formation of the ternary SNARE complex by injecting SNAP-25 into a stoichiometrically mixed 1:1 solution of the non-interacting constituents syntaxin and synaptobrevin (step 1.2 and 2.2). Fig. 1d shows a typical ITC titration of SNAP-25 into a premix of SyxH3 and Syb-(1–96). These data reveal that upon ternary SNARE complex formation, even larger enthalpic changes occurred (\( \Delta H \approx 110 \text{ kcal/mol SNAP-25} \)) than were observed for the interaction of syntaxin and SNAP-25. This very probably reflects the even more pronounced structural changes coupled to assembly of the ternary SNARE complex as compared with the assembly of syntaxin and SNAP-25. As we observed in the formation of the syntaxin-SNAP-25 complex, the large enthalpic changes upon assembly of the ternary complex were balanced by large entropic changes. This is also reflected in the large negative heat capacity change of binding (\( \Delta C_p \), supplemental Fig. 1). In agreement with the composition of the ternary SNARE complex, saturation of binding occurred at a molar ratio of \( \approx 1 \), consistent with a 1:1:1 composition of the complex. We also found clear evidence for a two-step assembly process for the ternary interaction when we titrated a mix of SyxH3 and Syb-(1–96) into SNAP-25 (Fig. 1e), confirming that synaptobrevin binds to the preassembled 1:1 syntaxin-SNAP-25 heterodimer (30, 31).

**Isolating the Synaptobrevin Binding Step**—So far, our ITC measurements have revealed that SNARE complex formation is accompanied by major enthalpic changes but did not reveal the free energy of the process. In fact, as the assembly is quasi-irreversible (25), a conventional thermodynamic analysis of the ITC data is not possible. However, an estimate of the \( K_D \approx 10 \text{ nM} \) was achieved by fitting the data provisionally to a one-site binding model. It should be kept in mind, however, that the actual affinity of the ternary SNARE complex might be higher. To overcome this problem, we set out to investigate the association of synaptobrevin (step 2.2) in more detail, as this step of the reaction cascade seems to establish the irreversibility of the assembly process. However, synaptobrevin binding is difficult to study individually because the complex of the two proteins syntaxin and SNAP-25, as shown in the previous section, resides in a dynamic equilibrium between a 1:1 and a 2:1 stoi-
chiometry, depending on the mixing ratio. As an alternative, the syntaxin-SNAP-25 heterodimer can be stabilized artificially by a short C-terminal fragment of synaptobrevin. This so-called “ΔN” complex can be purified. As we have demonstrated earlier, the ΔN complex offers an accessible binding site for synaptobrevin but does not allow for binding of a second syntaxin. In a second step, the C-terminal peptide is quickly displaced from the ΔN complex (30). The assembly pathway using the ΔN complex is illustrated in Fig. 2a.

For our ITC experiments, we purified a ΔN complex containing a fragment of synaptobrevin encompassing the residues 49–96 (Syb(49–96)-SyxH3-SNAP-25 complex). When we titrated Syb(1–96) into the ΔN complex, we detected favorable enthalpic changes of ∼30 kcal/mol (Fig. 2b). This enthalpic change was markedly reduced in comparison to the ITC titrations in which all three constituents were mixed (Fig. 1c). Because synaptobrevin in this setting probably binds to a prestructured binding site in the ΔN complex, the enthalpic changes might to a large degree reflect coupled folding and binding of the synaptobrevin coil. We fitted the data to a one-site binding model, yielding an apparent affinity of ∼2 nM. However, it should be pointed out again that this value might not correspond completely with the genuine affinity of the SNARE complex. The reason is that binding of synaptobrevin is followed by a second reaction by which the C-terminal fragment is displaced. Thus, at the end of this assembly pathway, the same quasi-irreversible product is formed (Fig. 2a). Moreover, it seemed possible the displacement of the fragment might render the assembly pathway via the ΔN complex energetically less efficient. On the other hand, greatly enhanced fusion rates are observed using the ΔN complex compared with the use of the preassembled binary syntaxin-SNAP-25 complex, dem-
It seemed possible that this step does not significantly contribute to the thermal profile of the reaction, as the C-terminal portion of the displacing Syb-(1–96) is virtually identical to the displaced fragment (Syb-(49–96)). We, thus, sought an experimental strategy to isolate the thermodynamic parameters of the binding event. When we examined several C-terminally truncated synaptobrevin constructs, we found that a fragment comprising only the amino acids 1–52 (Syb-(1–52)) was incapable of displacing the Syb-(49–96) fragment (Fig. 2c). This observation suggests that both N- and C-terminal fragments of synaptobrevin can bind simultaneously, forming a Syb-(1–52)-Syb-(49-96) complex, although few amino acids overlap in this assembly. To rule out the possibility that the labeled Syb-(49–96) was not removed from its position simply because Syb-(1–52) was not able to bind to the ΔN complex, we labeled Syb-(1–52) at its extra C-terminal cysteine residue with the fluorescent dye Alexa-488. Upon mixing of the labeled Syb-(1–52) with the ΔN complex, a rapid change in fluorescence was monitored, demonstrating that Syb-(1–52) is able to bind efficiently. At pseudo-first-order conditions, the rate of assembly was $\approx 123,000$ M$^{-1}$s$^{-1}$ (supplemental Fig. 2a). These findings are consistent with our previous observation that two non-overlapping synaptobrevin fragments can bind to the same site, forming a complex that structurally resembles the extended four-helix SNARE bundle (30). Note, however, that in the earlier study we used different fragments, Syb1–59 and Syb60–96 (see supplemental Fig. 2a). These findings are consistent with our previous observation that two non-overlapping synaptobrevin fragments can bind simultaneously, forming a complex that structurally resembles the extended four-helix SNARE bundle (30).
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TABLE 1
Thermodynamic parameters of the interaction of the neuronal SNARE proteins measured by ITC at 25 °C

| Cell                  | Syringe      | Fit model | $K_D$ (nM) | $\Delta H$ (kcal mol$^{-1}$) | $-T\Delta S$ (kcal mol$^{-1}$ K$^{-1}$) | $\Delta G$ (kcal mol$^{-1}$) | N |
|-----------------------|--------------|-----------|------------|------------------------------|----------------------------------------|-------------------------------|---|
| SyxH3                 | SNAP-25      | 1-site    | 64.9 ± 17.1| $-14.3 ± 1.0$                | 31.5                                   | $-9.8$                        | 1.88 |
| SNAP-25               | SyxH3        | 2-site    | 4.3 ± 2.2  | $-46.9 ± 7.2$                | 35.5                                   | $-11.4$                       | 0.21 |
| SyxH3/Syb-(1–96)      | SNAP-25      | 1-site    | 233.6 ± 64.3| $-31.0 ± 1.8$               | 22.0                                   | $-9.0$                        | 1.21 |
| SNAP-25               | SyxH3/Syb-1–96| 2-site  | 22.8 ± 6.1 | $-112.8 ± 2.5$              | 102.4                                  | $-10.4$                       | 0.97 |
| $\Delta N$ complex   | Syb-1–96     | 1-site    | 2.1 ± 0.6  | $-29.9 ± 0.3$               | 18.1                                   | $-11.8$                       | 1.05 |
| $\Delta N$ complex   | Syb-1–87     | 1-site    | 5.4 ± 1.8  | $-26.3 ± 0.4$               | 15.0                                   | $-11.3$                       | 0.96 |
| $\Delta N$ complex   | Syb-1–65     | 1-site    | 5.7 ± 1.2  | $-18.1 ± 0.2$               | 6.9                                    | $-11.2$                       | 0.97 |
| $\Delta N$ complex   | Syb-1–52     | 1-site    | 18.3 ± 2.0 | $-27.2 ± 0.2$               | 12.1                                   | $-10.6$                       | 1.03 |
| Syb-(1–70) complex   | Syb7-1–96    | 1-site    | 13680.0 ± 293.0 | $-6.0 ± 0.3$           | $-2.0$                                 | 0.8                      | 8.90 |

Reversible Binding of the N-terminal Coil of Synaptobrevin to the $\Delta N$ Complex—The binding of different synaptobrevin fragments to the $\Delta N$ complex produced favorable enthalpic changes that saturated at a 1:1 molar ratio (Table 1; for comparison, the ITC results for the titration of SNAP-25 into a mix of the synaptobrevin fragments with SyxH3 are given in supplemental Table 1; see also supplemental Fig. 3). Interestingly, the apparent affinity and enthalpy change was somewhat reduced for the Syb-(1–87) fragment as compared with Syb-(1–96). The binding of the shorter Syb-(1–65) fragment to the $\Delta N$ complex was even somewhat less affine and resulted in less pronounced enthalpic changes compared with the Syb-(1–87) fragment. Similar results were obtained for the binding of the non-replacing Syb-(1–52) fragment (Fig. 2b), which took place at 23 nm affinity (Table 1). We have shown above that the Syb-(1–52) fragment does not displace the bound C-terminal fragment, Syb-(49–96) (Fig. 2c). Consequently, the ITC data reflect only binding to the preformed N-terminal binding site of the $\Delta N$ complex. Surprisingly, we observed no clear difference in the ITC profiles of Syb-(1–65) compared with Syb-(1–52) (Table 1; supplemental Fig. 3, h and i). It might be tempting to speculate that the thermodynamic profiles of the synaptobrevin fragments reflect an incremental zippering process. However, because the shorter fragment Syb-(1–65) displaces the bound Syb-(49–96) fragment from the $\Delta N$ complex more slowly than the longer Syb-(1–96) and Syb-(1–87) (Fig. 2c), it is likely that the ITC approach did not record the slow heat change of the displacement step for these fragments.

Up to this point we have assumed that ITC did not provide the true energetics of the synaptobrevin binding step because the assembly process is essentially irreversible. Now, however, the question arose of whether the binding of Syb-(1–52) to the $\Delta N$ complex may be reversible, as the C-terminal Syb-(49–96) fragment can remain bound, thereby keeping the entire complex together. To record this step, we used labeled Syb-(1–52), which dissociated at a rate of about 0.0006 s$^{-1}$ from the $\Delta N$ complex (supplemental Fig. 2b). The ratio of off- and on-rates result in an affinity of about 5 nM, confirming the affinity determined by the ITC measurements.

Energetics of the C-terminal Zippering Step—Finally, to investigate the energetics of the C-terminal zippering step, we used a ternary SNARE complex containing a C-terminal-truncated synaptobrevin fragment, Syb-(1–70), and monitored binding of the Syb-(71–96) peptide to the free C-terminal portion of complex. Previous investigations had suggested that the C-terminal portion of the Syb-(1–70) complex is less structured (30). We employed CD spectroscopy to test whether the association of the Syb-(71–96) peptide to the Syb1–76 complex would induce additional structure. Indeed, we observed a small increase in $\alpha$-helical content upon mixing of the Syb-(1–70) complex and Syb-(71–96) peptide (Fig. 3a). We then used ITC to assess the energetics of the reaction. Compared with the ITC measurements described so far, we observed a relatively small enthalpy of binding ($\sim$ 6 kcal/mol) upon titrating the Syb-(71–96) peptide into the purified Syb-(1–70) complex. The affinity of the association was about 1.4 μM. This suggests that zippering of the very C-terminal end of the bundle contributes only moderately to the force of the process. However, it should
be kept in mind that the energy contribution of this portion might be considerably higher with an intact synaptobrevin coil.

**DISCUSSION**

In the synapse the core of the membrane fusion machinery consists of the three SNARE proteins, syntaxin 1a, SNAP-25, and synaptobrevin 2. Although these proteins have been intensively studied, the central question of how their sequential assembly into a trans-complex drives the fusion of the vesicle with the plasma membrane still remains unanswered (for review, see Refs. 1–7). Here, we have investigated the energetics of complex formation involving the soluble portion of the neuronal SNARE proteins using ITC. The major goal of this work was to build a solid thermodynamic foundation for improved understanding of how the energy released during complex formation can be harnessed for membrane fusion. Using kinetic investigations as a basis, the following assembly pathway has previously been put forward; in an initial, rate-limiting step, the two plasma membrane SNAREs, syntaxin and SNAP-25, assemble into a transient 1:1 heterodimer, which in turn provides a high affinity binding site for the vesicular synaptobrevin (30, 31). Indeed, this assembly pathway is strongly corroborated by our thermodynamic investigations, suggesting that it corresponds to the series of intermediate stages through which the assembling SNAREs pass in vivo.

**Large Enthalpic Changes Drive SNARE Complex Formation**—Earlier spectroscopic investigations have shown that SNARE complex formation is attended by large structural rearrangements. As the interacting domains of the individual proteins are mostly disordered and the resulting complexes, either the 2:1 complex or the ternary SNARE complex, have an extended four-helix bundle structure (8–10, 32–34), the assembly reaction can be described as coupled folding and binding. Our ITC investigations revealed that assembly is driven by large favorable enthalpic changes (\(\Delta H\)), reflecting the extensive intermolecular interface established during the reaction. The enthalpic change is counterbalanced by the large entropic costs (\(-T\Delta S\)) associated with the disorder-to-order transition. Although it has long been thought that proteins have a three-dimensional fold dictated by their amino acid sequence, it has been established more recently that numerous proteins contain long disordered segments under physiological conditions (for review, see Refs. 35 and 36). Many intrinsically disordered proteins fold into stable structures only upon binding to their targets. Their inherent flexibility enables these proteins to form extensive binding surfaces, often by binding to multiple partners in a multiprotein complex. Coupled folding and binding has been found in molecules with various functions. The SNARE complex assembly reaction, which uses assembly energy to bring two membranes into close apposition, adds a new facet to the versatility of unfolded proteins.

Notably, to the best of our knowledge the enthalpic changes of \(-110\) kcal/mol recorded for the formation of the ternary SNARE complex are among the largest observed for protein-protein interactions so far. Remarkably, large enthalpic changes also occur within the interaction of the human immunodeficiency virus envelope glycoprotein with the cellular CD4 receptor (37, 38), a reaction that initiates the fusion of the viral membrane with the cellular plasma membrane. Envelope glycoprotein is composed of the surface (gp120) and the transmembrane (gp41) subunits. The binding of the gp120 glycoprotein to CD4 is coupled to an extensive structural rearrangement that leads to the activation of the co-receptor binding site (39, 40). Upon binding to the coreceptor (41), this conformational change of gp120 is transmitted to gp41, which then reorganizes itself into a fusogenic configuration. The actual driving force for viral membrane fusion is thought to be the formation of a highly stable six-helical bundle structure by gp41 (42, 43). The assembly passes through a “prehairpin” intermediate that is thought to consist of a central trimeric coiled coil formed by N-terminal heptad repeats; note that gp41 is present as a trimer. Antiparallel packing of the three C-terminal heptad repeats to the trimeric core pulls the viral and cellular membranes together (for review, see Refs. 44–47). Hence, both membrane fusion machineries, the SNAREs and envelope glycoprotein, undergo large conformational changes that culminate in the formation of a tight helix bundle structure that pulls two membranes together.

On the surface it seems that both machineries employ the same “energetic strategy” to fuse membranes, but it should be noted that there are also fundamental differences in both mechanisms. First, the topology of SNARE proteins relative to the opposing membranes is different from that of viral fusion proteins, which reside only in the viral membrane. Then, viral fusion proteins like envelope glycoprotein store their energy in a so-called metastable configuration, which arises from the proteolytic cleavage of the precursor, gp160. Consequently, the viral fusion proteins can undergo the conformational change only once. Conversely, the SNAREs remain in an unfolded configuration that can snap into a tight complex upon contact. The energy set free during the SNARE assembly process is “recycled” by the disassembly reaction catalyzed by the ATPase N-ethylemaleimide. Hence, in contrast to the viral fusion machinery, the cellular SNARE machinery can be re-used. Still, it is striking that in both fusion reactions, highly stable helix bundles are used, although they seem to be the product of convergent evolution. This suggests that this structure constitutes a relatively simple assembly mechanism to generate force.

**A Transient Syntaxin-SNAP-25 Heterodimer Serves as Intermediate**—During SNARE complex formation, the two plasma membrane proteins syntaxin and SNAP-25 are believed to provide the binding site for the vesicular synaptobrevin. Indeed, of all the possible binary combinations, only these two proteins were found to form a tight complex (8, 9). Hence, synaptobrevin binding requires a structural rearrangement of syntaxin and SNAP-25. However, in vitro they tend to reside in a complex with 2:1 stoichiometry in which the binding site for synaptobrevin is occupied by a second syntaxin molecule (8, 31). Our ITC investigations show that the 2:1 complex is the predominant entity at higher nanomolar concentrations, whereas the 1:1 heterodimer forms at lower nanomolar concentrations. The large enthalpic changes suggest that the formation of the 1:1 heterodimer is accompanied by major conformational changes. It should be noted that because of the limitations of the standard ITC analysis method, the contributions of both reaction steps to the enthalpy of binding were not determined more recently that numerous proteins contain long disordered segments under physiological conditions (for review, see Refs. 35 and 36). Many intrinsically disordered proteins fold into stable structures only upon binding to their targets. Their inherent flexibility enables these proteins to form extensive binding surfaces, often by binding to multiple partners in a multiprotein complex. Coupled folding and binding has been found in molecules with various functions. The SNARE complex assembly reaction, which uses assembly energy to bring two membranes into close apposition, adds a new facet to the versatility of unfolded proteins.

Notably, to the best of our knowledge the enthalpic changes of \(-110\) kcal/mol recorded for the formation of the ternary SNARE complex are among the largest observed for protein-protein interactions so far. Remarkably, large enthalpic changes also occur within the interaction of the human immunodeficiency virus envelope glycoprotein with the cellular CD4 receptor (37, 38), a reaction that initiates the fusion of the viral membrane with the cellular plasma membrane. Envelope glycoprotein is composed of the surface (gp120) and the transmembrane (gp41) subunits. The binding of the gp120 glycoprotein to CD4 is coupled to an extensive structural rearrangement that leads to the activation of the co-receptor binding site (39, 40). Upon binding to the coreceptor (41), this conformational change of gp120 is transmitted to gp41, which then reorganizes itself into a fusogenic configuration. The actual driving force for viral membrane fusion is thought to be the formation of a highly stable six-helical bundle structure by gp41 (42, 43). The assembly passes through a “prehairpin” intermediate that is thought to consist of a central trimeric coiled coil formed by N-terminal heptad repeats; note that gp41 is present as a trimer. Antiparallel packing of the three C-terminal heptad repeats to the trimeric core pulls the viral and cellular membranes together (for review, see Refs. 44–47). Hence, both membrane fusion machineries, the SNAREs and envelope glycoprotein, undergo large conformational changes that culminate in the formation of a tight helix bundle structure that pulls two membranes together.

On the surface it seems that both machineries employ the same “energetic strategy” to fuse membranes, but it should be noted that there are also fundamental differences in both mechanisms. First, the topology of SNARE proteins relative to the opposing membranes is different from that of viral fusion proteins, which reside only in the viral membrane. Then, viral fusion proteins like envelope glycoprotein store their energy in a so-called metastable configuration, which arises from the proteolytic cleavage of the precursor, gp160. Consequently, the viral fusion proteins can undergo the conformational change only once. Conversely, the SNAREs remain in an unfolded configuration that can snap into a tight complex upon contact. The energy set free during the SNARE assembly process is “recycled” by the disassembly reaction catalyzed by the ATPase N-ethylemaleimide. Hence, in contrast to the viral fusion machinery, the cellular SNARE machinery can be re-used. Still, it is striking that in both fusion reactions, highly stable helix bundles are used, although they seem to be the product of convergent evolution. This suggests that this structure constitutes a relatively simple assembly mechanism to generate force.

**A Transient Syntaxin-SNAP-25 Heterodimer Serves as Intermediate**—During SNARE complex formation, the two plasma membrane proteins syntaxin and SNAP-25 are believed to provide the binding site for the vesicular synaptobrevin. Indeed, of all the possible binary combinations, only these two proteins were found to form a tight complex (8, 9). Hence, synaptobrevin binding requires a structural rearrangement of syntaxin and SNAP-25. However, in vitro they tend to reside in a complex with 2:1 stoichiometry in which the binding site for synaptobrevin is occupied by a second syntaxin molecule (8, 31). Our ITC investigations show that the 2:1 complex is the predominant entity at higher nanomolar concentrations, whereas the 1:1 heterodimer forms at lower nanomolar concentrations. The large enthalpic changes suggest that the formation of the 1:1 heterodimer is accompanied by major conformational changes. It should be noted that because of the limitations of the standard ITC analysis method, the contributions of both reaction steps to the enthalpy of binding were not
resolved precisely. Thus, it remains unclear how much surface is buried during this step.

Although the general outline of the assembly pathway of the neuronal SNARE proteins is widely accepted, some few aspects are controversial, one of them being the configuration of the syntaxin-SNAP-25 assembly in the membrane. Although structural investigations have demonstrated that they form a 2:1 complex in solution, it has been claimed that the two proteins form a 1:1 entity when co-expressed and inserted into artificial membranes (48). It should be noted, however, that if co-expressed, syntaxin and SNAP-25 in fact resided in a 1:1 configuration, much faster liposome fusion rates should be observable when this complex is used (14). One, therefore, has to assume that the coexpressed complex does not reside in a conformation that supports immediate synaptobrevin binding. It should also be noted that earlier EPR studies have shown that the membrane-inserted syntaxin-SNAP-25 complex has a 2:1 configuration as well (49), although in this study only the H3 region of syntaxin was used. In the 2:1 configuration, the slow fusion rates can be explained easily by competition between the second syntaxin and synaptobrevin for binding. That the 2:1 complex does not actually offer a reactive synaptobrevin binding site was demonstrated recently when its liposome fusion activity was compared with the ΔN complex, which allowed for much quicker fusion (30).

Micro-reversibility of Synaptobrevin Binding—According to our earlier investigations, the SNARE assembly process essentially becomes irreversible upon association of the synaptobrevin (25). So far, it has been unclear at which point synaptobrevin binding becomes quasi-irreversible. Previous investigations showed that the C-terminal portion of synaptobrevin can bind reversibly as long as the complex is held together by the N-terminal portion (30). Remarkably, using the ΔN complex we have demonstrated that binding of the N-terminal coil of synaptobrevin is reversible as well. One should bear in mind, however, that reversibility was found only when fragments were used rather than when the entire coil of synaptobrevin was used. Obviously, the intact synaptobrevin helix is much more stably bound than the fragments, and consequently, its dissociation would be too slow to be measured. In addition, our approach is not able for detecting when only a portion of the synaptobrevin coil detached. Single molecule techniques and molecular dynamics (see e.g. Ref. 50) would provide better means to observe such events.

Note that reversible binding of the N-terminal portion of synaptobrevin was found only when the C-terminal region of the complex was held together by a fragment of synaptobrevin. For topological reasons, this setting probably does not occur in vivo. Hence, it might be argued that our reductionist approach, which assesses the thermodynamic and kinetic properties of the molecules involved, does not reflect the cellular situation. However, a situation can be envisioned in which the binding site of synaptobrevin in the 1:1 heterodimer is maintained by the action of other molecules. For example, we have recently shown that the protein Munc18 tightly controls the formation of the syntaxin-SNAP-25 complex (51). Along with other factors, the task of Munc18 might be to organize and control the formation of the synaptobrevin binding site. In fact, the interaction of syntaxin and SNAP-25 is very slow, suggesting that in vivo “priming” factors are required to orchestrate the reaction so it can overcome its large activation barrier. At first glance, this appears to be a major investment, but it should also be considered that a relatively labile synaptobrevin acceptor site ensures that the fusion reaction can be tightly controlled in vivo.

Assessing the Free Energy of SNARE Complex Formation—The free energy of SNARE complex formation cannot be assessed conventionally (25). However, the energy stored in the SNARE bundle (or more precisely in about three-quarter of the bundle) has recently been estimated to be around 35 k_BT (equivalent to about 20 kcal/mol) by either atomic force microscopy or SFA measurements (29). This extraordinarily high energy would imply that the affinity of the SNARE complex is in the low femtomolar range and is one of the most stable protein complexes observed so far. When we investigated the SNARE assembly by ITC, however, we only observed affinities in the range of about 1 nm (equivalent to about 11 kcal/mol; note that a 10-fold change in binding constant corresponds to ≈1.4 kcal/mol) no matter which reaction pathway we examined. How can this large difference be explained?

It is well known that atomic force microscopy experiments are carried out under non-equilibrium conditions and, thus, do not measure the bona fide free energy of SNARE complex formation. Nonetheless, it is puzzling why, for example, vastly different rates of spontaneous dissociation of ternary SNARE complexes of ≈158 years (26) and ≈2.1 s (27, 28) were reported or why very similar high stabilities were measured for the interaction of syntaxin and synaptobrevin, although these two SNARE proteins do not form a tight complex (Fig. 1b) (9). These inconsistencies raise the possibility that handling of SNARE proteins might be still very critical for single-molecule approaches (for a comprehensive assessment of the different approaches see Ref. 52). Henceforth, we will, thus, compare only the SFA and ITC equilibrium approaches.

Previous investigations have shown that the folding equilibrium of SNARE complexes cannot be reached within an experimentally affordable time, suggesting that the ITC measurements underestimated the binding constant. We noticed, however, that the SFA measurements might have been affected by the same problem. These experiments measured approach/ separation cycles involving two lipid surfaces, one of which had the synaptobrevin anchored within it, whereas the other provided the anchor for the coexpressed syntaxin-SNAP-25 complex (29, 53). Strikingly, these revealed diverging profiles of the approach and separation phases that bear strong resemblance to the non-coinciding folding and unfolding transitions observed by standard denaturation protocols (25). Although the proteins appear to assemble progressively during the approaching phase, once the complex is assembled, it seems to resist separation until it suddenly breaks apart. Notably, a plateau was reached after about 60 min. This time period does not correspond to equilibrium but, rather, suggests that after this time, the SFA instrument, like the ITC instrument, is not sensitive enough beyond this point to detect further subliminal changes. Nonetheless, it remains unclear why the energetics obtained by ITC and by SFA measurements are vastly different.
We noted, however, that with the SFA approach the number of pulling complexes had to be deduced rather indirectly to calculate the free energy assigned to a single SNARE complex. Hence, although the SFA measurements describe the SNARE assembly and disassembly cycle between membranes very well, it seems possible that the energetic contribution of the SNARE assembly is overestimated.

How Do SNAREs Catalyze Membrane Fusion?—According to the zipper model, progressive assembly of SNARE complexes may culminate in a release of energy that is sufficient to drive membrane merging. The earliest step at which SNARE complex formation would be able to provide energy for pulling the membranes together is upon engagement of synaptobrevin with the 1:1 heterodimer. It should be kept in mind, however, that the affinity of the 1:1 heterodimer formation gives an upper limit for the subsequent engagement of synaptobrevin. Our data suggest that the N-terminal coil of synaptobrevin can bind reversibly to the N-terminal region of the syntaxin-SNAP-25 heterodimer. It is conceivable that this configuration corresponds to a stable assembly that the SNAREs form between two opposing membranes. N-terminal association is then followed by assembly of the C-terminal portion of the four-helix bundle. Interestingly, our measurements suggest that the energy released during the very C-terminal zipper process is smaller than during N-terminal assembly. Thus, our data corroborate the notion that C-terminal zipper might be reversible, suggesting that assembly of the trans-SNARE complex might be balanced by the repulsive forces of the two membranes.

In addition, the question of how many SNARE complexes are needed to fuse two membranes has been discussed intensively over the years. As outlined before, according to the stalk hypothesis, a large activation energy of about 40 k_BT needs to be provided by the SNARE assembly process. The extraordinary stability measured by the SFA implies that only a few, maybe even only one, complex would need to join forces to fuse membranes (29), whereas the lower stability measured here by ITC appears to imply that a larger number of SNARE complexes have to cooperate. In this regard, it is interesting to note that a single human immunodeficiency virus fusion protein releases a similar amount of energy to the SNARE complex during zipperung as the affinity of the outer-layer peptide for a trimeric inner core of gp41 was determined to be around 3.6 nM (54). Moreover, it should be kept in mind that the high free energy of the transition state of membrane fusion inferred by the stalk model is controversial, as the model does not account for the activity of proteins, which probably modulate the fusion pathway (23, 24). Also, the stalk model treats lipid membranes as an elastic surface, although biological membranes are crowded with proteins (55). For example, in synaptic vesicles (the organelle in which synaptobrevin 2 is located) about a quarter of the entire membrane volume is taken up by transmembrane domains, suggesting that the majority of lipid molecules are not free (56).

The putative energy landscape of SNARE complex formation between membranes is schematically drawn in Fig. 4. Clearly, this scenario is speculative, as our results shed light only on the assembly energy of the soluble portions of the neuronal SNAREs. Thus, to understand whether SNARE assembly indeed provides the mechanical force to fuse membranes, it is necessary to understand how the assembly force is transmitted through the transmembrane domains into the membrane. Unfortunately, little is known about the structure of the linker region between the core SNARE complex and the transmembrane domains. It should also be kept in mind that although the SNARE proteins were anchored in lipid bilayers in the SFA experiments, anchoring was achieved by an artificial lipid anchor attached C-terminally. Hence, the approach is not suited to determine the force transmitted across lipid bilayers. Clearly, although we do not exactly understand how the SNARE assembly force is harnessed for membrane fusion, models of this process will remain speculative.

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