Chapter

Mechanism of SNARE Assembly and Disassembly

Ulrike Winter* and Dirk Fasshauer

Abstract

The formation of SNARE (soluble NSF attachment protein receptor) complexes is the driving force for most types of biological membrane fusion reactions. An extremely fascinating feature of this reaction is its potency to couple the assembly energy to the mechanical process of pulling two membranes into close apposition and eventually making them fuse. By now a basic picture of how this transition of energy is achieved via structural changes has evolved with the help of studies using several experimental systems and organisms. Since membrane fusion plays an important role in as diverse physiological processes as secretion in yeast and neurotransmission it must underlie various levels and types of regulation, which should all influence SNARE assembly kinetics. First, features intrinsic to the SNAREs themselves as well as local concentrations, second their way of insertion into the membrane and last but not least additional regulatory proteins control the speed of the reaction. These can on the one hand act on individual SNAREs or partially assembled intermediates to change reaction rates, on the other hand on fully assembled or unproductive complexes by reversal of the reaction. This so called disassembly of SNARE complexes is mediated by the ATPase NSF which fuels the thermodynamically unfavorable reaction by ATP-hydrolysis. All the factors mentioned do obviously not act independently from each other but rather add up to a complex interplay which leads to an impressively well functioning organization of the various membranous compartments which make up living cells.

Introduction

To fulfill their specific function most if not all proteins interact with other proteins in some way or another. The assembly of protein complexes is therefore nothing unusual and one might ask why the mechanism of SNARE-assembly should be interesting enough to be explained in great detail.

The assembly of SNARE-complexes is said to mediate fusion of biological membranes. But why is that so fascinating?

How does the cell succeed to fuse membranes? How the right ones, at a sufficient speed, at the right times? Which are the key players, what is their mode of action and how are they being regulated?

The main purpose of intracellular membranes being the enclosure and thereby division of different sub-cellular environments, they are usually spatially separated from each other which keeps them from interacting spontaneously. Furthermore, even if two membranes are in close apposition, the repulsion of charges between them still prevents fusion of different compartments from occurring—as long as no additional input of energy is provided. Therefore a reaction bearing the potential to drive membrane fusion obviously needs to fulfill at least two critical criteria: First,

*Corresponding Author: Ulrike Winter—Max-Planck-Institute for Biophysical Chemistry Am Fassberg 11, 37077 Göttingen, Germany. Email: uwinter@gwdg.de

SNARE Proteins, edited by Francesco Filippini and David Banfield. ©2007 Landes Bioscience.

it needs to be able to mechanically draw the membranes close to each other, second it needs to generate enough energy to overcome the energetic barrier.

These preconditions given, an additional level of complexity is added when we start to think of regulatory functions providing specificity. Tight regulation concerning the amount, speed and type of vesicles prone to fuse is also required to add to the quality of the fusion reaction. Nevertheless these regulatory mechanisms do not necessarily have to be inherent to the basal fusion machinery but could theoretically also be provided by other factors.

Keeping these prerequisites in mind the underlying mechanisms bear some fascination. If the two main criteria, pulling membranes together and overcoming the energetic barrier were to be met by proteins—how should these proteins be structured, how would they interact? Since, as stated in the beginning, the SNAREs actually represent a family of proteins capable to fulfill this difficult task, studying their structure and mechanisms of interaction has helped to gain insights into membrane fusion during the last decades and probably will continue to do so in the future.

Function of SNARE-Assembly

All intracellular transport processes ranging from secretion in yeast to neurotransmitter release in the brain, depend on the ability of membranes to fuse with each other. Due to the high variability regarding time-scale and function of these various processes the group of proteins mediating membrane fusion must on the one hand show common characteristics which enable them to execute the event of fusion. On the other hand however, there must be diversities allowing for tight spatial and temporal regulation.

SNAREs can be found in all eukaryotic organisms and their involvement in many steps of intracellular transport has been proven using various experimental systems. It is now commonly believed that they are involved in all vesicular fusion events, with different sets of SNAREs being responsible for different trafficking steps.[1]

The SNAREs are usually short and C-terminally attached to the membrane by either a transmembrane region or a membrane anchor. One set of SNAREs typically consists of four proteins, which when combined in appropriate solutions spontaneously form extremely stable complexes via a region common to all SNAREs called the "SNARE-motif" or "core-domain". The association takes place along a highly exothermic reaction pathway which already early in SNARE-research made it tempting to speculate that the assembly of SNARE complexes provides the driving force for membrane fusion.[2-4]

Indeed, later they were shown to be able to autonomously catalyze liposome fusion in vitro without any additional factors.[5]

Mechanism of SNARE Assembly

But how can the assembly of proteins lead to the merger of two membranes?

Even though the complete answer to this question has not been conclusively found, some by now well understood basic mechanistic principles of SNARE assembly have been of help to establish different models. One of the concepts of how SNARE assembly actually proceeds, the so called "zipper"-mechanism, has gained more and more support over the years.

The "Zipper" Model—How Much Evidence Do We Have?

The zipper model proposes that SNARE-proteins residing on opposing membranes first interact at their membrane distal termini to form a loose complex. Starting from this point of interaction they consequently wind up towards their C-terminal membrane anchors in a zipper-like fashion to form the four-helix-bundle, bringing these membranes into close proximity. The amounts of energy generated during assembly eventually suffice to allow for the membrane merger.

In other words, SNAREs directly function as fusion catalysts.[6-9]

The Driving Force—Spontaneous Formation of Extremely Stable Complexes

Evidence for this model comes from various kinds of experimental systems. The first mechanistic insights resulted from experiments with recombinant neuronal SNAREs in solution. Most

of the early experiments were performed using only the SNARE-core domains, which were found to be sufficient to mediate SNARE assembly displaying similar biophysical characteristics as the full-length SNARE complex. A common characteristic of these motifs is a lack of secondary structure as long as they are in a monomeric state.[3,4,6,10-12] Only upon mixing with other SNAREs do certain combinations lead to the spontaneous association into complexes accompanied by major conformational and free-energy changes. Even though the two neuronal SNAREs Syntaxin1 and SNAP-25 (synaptosome-associated protein of 25kDa) alone form complexes displaying high alpha-helical content, combining all three neuronal SNAREs amongst other side-products produces one uniquely stable outcome, which consists of all three neuronal SNAREs in a 1:1:1 stoichiometry. Not only is it resistant to higher amounts of SDS than all other resulting complexes and extremely high temperature—it also shows a pronounced hysteresis meaning that there is basically no spontaneous dissociation under assembly conditions.[13] Since all other side products are in equilibrium with the reactants, the degree of this respective complex increases with reaction time. Furthermore this ternary SNARE-complex can be isolated from brain extracts indicating its existence in vivo[14] making it the most probable end product of the assembly pathway.

The Logistics—SNAREs Switch from "trans" to "cis" during Membrane Fusion and Align in a Parallel Fashion

The SNAREs are in a "trans"-state as long as fusion is incomplete (residing on opposite membranes). As soon as the membrane-merger has taken place and all helices are anchored in one membrane the complex is considered to be in a "cis"-configuration (Fig. 1). Originally the SNAREs were classified into vesicle (v-) and target (t-)-SNAREs, according to their location on the membrane before fusion.[15] The drawback of that nomenclature is however that the functional distribution of some SNARE sets has not been characterized sufficiently to assign vesicle- or target-function and especially when it comes to homotypic membrane fusion this classification meets its limits.

By comparison of the neuronal ternary complex-crystal structure to those of two other, only distantly related (endosomal) SNARE-complexes, it became evident that all three complexes resemble an intertwined α-helical bundle of parallel coiled coil domains consisting of four helices per complex.[16-18] The parallel alignment of SNAREs in the ternary complex mentioned in the context of the crystal structure was originally observed with the help of FRET—as well as EM-experiments.[19,20]

These SNARE-complexes show a remarkable degree of conservation which led to a reclassification of SNAREs based on a structural property: The centre of the bundle contains 16 stacked "layers" of interacting side chains which are largely hydrophobic, except for the very central one which contains three highly conserved glutamine (Q) residues and one highly conserved arginine (R) residue. Hence the SNAREs with a glutamine in this so called "0"-layer were grouped Q- and the ones with arginines R-SNAREs. The Q-SNAREs can further be divided into Qa-, Qb- and Qc-SNAREs, each of these classes contributing one helix to the four helix bundle.[7] The neuronal SNAREs belong to a unique subset of SNAREs in the sense that rather than being part of two independent proteins, the Qb and Qc-SNARE-motifs are accommodated in one SNARE called SNAP-25 (synaptosome-associated protein of 25kDA). It is tempting to speculate that it represents a fusion product of two reactants of the assembly reaction which has evolved because it may have led to a kinetic advantage over a four-component reaction. Thinking along these lines neuroexocytic events might profit from this advantage if one imagines the high speed of fast firing neurons. Syntaxin and Synaptobrevin represent the second neuronal Q-SNARE and the R-SNARE, respectively.

The neuronal SNAREs are distributed such that the R-SNARE is primarily located on the vesicle whereas the Q-SNAREs are predominantly located on the cell membrane. If the structural arrangement is a general feature of all SNARE-complexes, their localization to the different membranes might also be conserved, but since characterization of different SNARE-topologies has not come to an end yet, this at the moment is mere speculation.

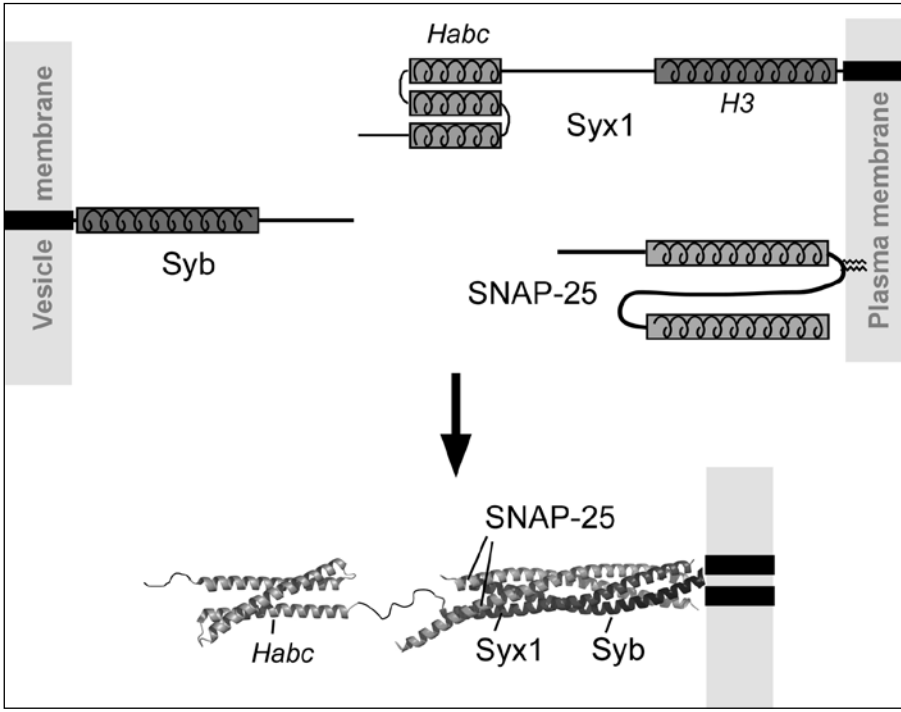

Figure 1. Function of SNARE assembly. Figure one exemplary shows the distribution of the neuronal SNAREs before and after fusion. Before fusion, the Q-SNAREs reside on the plasma membrane whereas Synaptobrevin (Syb) is found on the vesicle. After fusion of the two membranes into one, all three SNAREs are depicted in complex, indicating that the complex has assembled during fusion. As described in the text, assembly does not only temporally coincide with but rather is instrumental to drive the fusion reaction. The driving force results from conformational changes which are schematically shown: Before fusion the SNAREs are mostly unstructured whereas during fusion a tight helical bundle is formed.

According to this hypothesis one membrane would typically contribute three SNARE-motifs whereas the second would contribute the fourth helix. On the one hand experiments on neuronal SNAREs and in yeast have led to this hypothesis. On the contrary homotypic in vitro-fusion of liposomes mediated by early-endosomal SNAREs has been shown to proceed with different SNARE topologies regardless of whether all Q-SNAREs are contributed by the same membrane or not.[18] Whether a 3Q/1R-rule holds true for all SNARE sets in a physiological context therefore remains a matter of debate and yet needs to be elucidated.

Taken together, these findings are all in accordance with the zipper model: First of all the assembly reaction is exogenous and hence a source of energy, which spontaneously proceeds without additional factors. Second, the SNAREs reside on opposing membranes before, whereas they are found on the same membrane after fusion. Third, the helices are aligned in parallel after assembly, thus the N-termini interact with the N- whereas the C-termini interact with the C-termini of the partner SNAREs.

Random Collision or Directed Zippering?

Do the SNAREs really need to interact at their N-termini first and zipper up from there? Evidence for this came from studies in which SNARE-proteins were specifically mutated to

see whether these mutations will change assembly kinetics. N-terminally deleted SNAP-25 and Syntaxin1a prohibited assembly whereas C-terminal mutations did not. Conclusively SNARE-assembly most likely depends on a nucleation-event which requires the interaction of the N-termini of the Q-SNAREs.[21] In a subsequent study effects of impaired SNARE-assembly were correlated with electrophysiological events. C-terminally mutated complexes still allowed for complex assembly in vitro although the formed complexes appeared to have two unequally stable parts and the mutants nevertheless affected the final triggering of fusion. N-terminal mutations had no effect and central mutations had a negative effect on priming but did not affect the fusion rate constant of already primed vesicles.[22] This can be explained in the context of the zipper model: Partially zippered SNARE-complexes could be arrested in a position of incomplete assembly and proceed to full zippering as soon as a trigger is being pulled. As long as completion of zippering were the limiting factor, differences in priming efficiency would not change the outcome of the measurements. All this led to the conclusion, that assembly proceeds in the N- to C-terminal direction along a pathway which can be interrupted at a partially zippered state.

Kinetics of Assembly

As mentioned above, SNAREs alone can mediate fusion of liposomes.[5] Even though this certainly is a fascinating observation it does not suffice to solve the complete "mystery of membrane fusion". The complexity of intracellular transport naturally implies that membrane fusion has many faces, ranging from slow processes like secretion in yeast to extremely fast and tightly regulated processes like the exocytosis of neurotransmitters in our brain, a process completed on a millisecond timescale. To a certain extent it is conceivable that these time differences result from different "zippering"-speeds structurally encoded in the SNAREs responsible for a particular fusion step. Nevertheless the fusion of liposomes, even when using the neuronal exocytic SNAREs, requires hours for completion.[5] The speed of assembly can therefore not be entirely stored intrinsically in the sequence of the respective set of SNAREs. This rises the question, whether the in vitro reaction is slow due to nonphysiological side reactions and/or whether in vivo there are additional factors involved which either speed up assembly or lock partially assembled SNARE complexes in a state prior to complete fusion from which complete assembly can occur in a millisecond timescale as soon as these factors are released. Furthermore the anchorage of the SNAREs in the membrane as well as the membranous environment could have an impact on assembly kinetics.

Intramolecular Regulation Via the SNAREs Themselves—"Open/Closed" Syntaxin

As a general mechanism conformational particularities can influence protein/protein interactions. Considering the unstructured nature of the individual SNARE-core domains, this does not seem to apply to them. Nevertheless some of the SNAREs carry additional domains, which do not participate in the core (4-helix-bundle) complexes but may influence assembly rates. As a matter of fact such intramolecular regulation occurs in the Syntaxin molecule where an N-terminal globular domain can fold back onto the core domain. Syntaxin is then considered to be in a "closed" conformation which due to the occupied binding site is unable to interact with its partner-SNAREs.[23] A protein known to bind the "closed" Syntaxin molecule is Munc-18 whose exact role in regulating SNARE assembly is yet unknown. What is known is that Syntaxin somehow switches to an "open" conformation either before or during SNAP-25 binding.[24]

Four Reactants, but Only One Pathway? Directing the Reaction Sequence Via *"Acceptor Complexes"*

The fact that (at least) three to four reaction partners have to encounter each other along the reaction pathway for a certain set of SNAREs to form the complex gives rise to a range of putative paths of reaction. Do they assemble in a given sequential order or collide randomly? Is only the formation of four-helical complexes fusogenic or do other combinations of SNAREs also suffice?

Since Syntaxin and SNAP-25 are located in one membrane, it is likely that they provide the binding site for Synaptobrevin. For a long time people were not aware of the fact, that simply adding these two proteins together does not automatically result in a readily available Synaptobrevin binding site, but rather leads to a mixture of two different Q-SNARE-complexes, one of them being unable to bind Synaptobrevin. Only after detailed in vitro studies were performed on exocytic *S. cerevisiae* and neuronal SNARE-complexes a better understanding of the reaction sequence could be obtained (Fig. 2). The determination of reaction speeds as well as on and off-rates of putative reaction intermediates led to the conclusion, that the formation of a partially helical Qabc (or 1:1) intermediate, consisting of one syntaxin and one SNAP-25 molecule, is the rate-limiting step of SNARE-assembly.[6,21,25] This intermediate is rate-limiting because it quickly reacts with a second Syntaxin molecule to form a four-helix-bundle (Qaabc or 2:1 intermediate) which obviously is unproductive in terms of membrane fusion considering that no vesicle SNARE is involved. None of the other SNARE-sets have been characterized to such an extent making it difficult to draw general conclusions, but at least for the exocytic SNARE-complex, changing the availability of the rate limiting 1:1 intermediate should influence assembly kinetics and therefore membrane fusion.

Due to this fast binding of a second Syntaxin molecule the Qabc intermediate is difficult to isolate. Synaptobrevin competes for the same binding site in solution but cannot actively replace the Syntaxin molecule when it has already bound. Thus no matter how much Synaptobrevin is present, the slow off-rate of the second Syntaxin molecule will always slow down the rate of

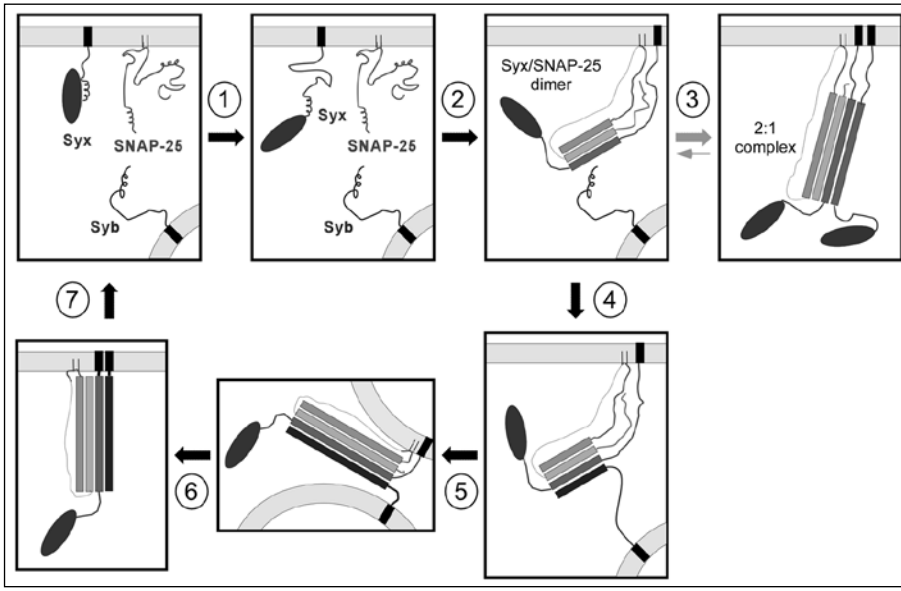

Figure 2. Reaction pathway of SNARE assembly. SNARE assembly involves four different SNARE-motifs which are accommodated in three proteins in case of the neuronal SNAREs. The reaction sequence determined in vitro is as follows: First, Syntaxin (Syx1) switches from the "closed" to the "open" conformation (1) to allow for interaction with SNAP-25. Via Syntaxin/SNAP-25 interaction a highly reactive Qabc intermediate is created (2) and then quickly bound by a second Syntaxin molecule to form the Qaabc intermediate (3) which is unproductive in terms of fusion. The two intermediates are in equilibrium with each other, allowing Synaptobrevin to compete for the binding site on the Qabc-intermediate to form a productive ternary complex. Interaction starts at the N-terminus (4) and proceeds in the C-terminal direction (5) in a "zipper"-like fashion, thereby pulling the membranes together and making them fuse (6). Finally they are dissociated into the monomers and redistributed to their respective membranes again (7).

ternary complex assembly (Fig. 2). When this became apparent the following question arose. Do these unproductive complexes occur in vivo or do they merely represent an in vitro artifact? If the latter were true, their formation could maybe lead to the observed time discrepancy. Slow in vitro-fusion could simply be due to a low success-rate of fusogenic Synaptobrevin binding caused by occlusion from its binding site. The key-problem of investigating whether this hypothesis holds true was to stabilize a Qabc-intermediate which still provides a site for naturally fast Synaptobrevin binding. Here the knowledge about N- to C-terminal zippering discussed earlier came in handy: If an N-terminally deleted version of Synaptobrevin were able to form stable ternary complexes with Syntaxin and SNAP-25, it should be possible to generate such mutant complexes with a high degree of purity in vitro. If further this deletion would suffice to render the Synaptobrevin binding site of these complexes accessible to full length Synaptobrevin they could serve to study the speed of unhindered Synaptobrevin binding.

Indeed using this strategy it was recently shown in liposome fusion experiments with neuronal SNAREs, that in vitro-fusion can be dramatically accelerated by stabilizing this limiting intermediate.[26] These findings show, that presentation of an "acceptor"-site which can readily bind Synaptobrevin does influence fusion and SNAREs do have the capacity to execute membrane fusion at very fast speeds. In that context it is conceivable that the maintenance of acceptor-sites of such kind represents a critical step in biological fusion reactions. Thinking along these lines, controlling the life-time of the Synaptobrevin acceptor-site would be ideally suited to control the fusion process. At this point SNARE-interacting proteins could play a role in priming the complexes, e.g., by regulating the availability of the rate-limiting intermediate. One possible candidate is the neuronal protein Tomosyn, which might via its R-SNARE motif act as a "placeholder" in the sense that it occupies the Synaptobrevin binding site on the acceptor-complex until a Synaptobrevin-molecule is presented by a fusogenic membrane.

The Membrane—Another Check-Point of SNARE-Assembly?

Even though SNARE-complexes readily assemble in solution they are naturally located on membranes, which might also influence their mechanism as well as speed of assembly.

SNAREs have been inserted into membranes but only little has been done to dissect the process on membranes in detail. What has been done up to now has led to contradictory results. EPR-measurements have attributed a possible role of the membrane to control the capability of SNARE motifs to enter SNARE complexes. They suggested that a short membrane proximal region of synaptobrevin-2 is dipped into the membrane and thereby inhibits the formation of SNARE-complexes,[27] a regulatory mechanism they called "Synaptobrevin restriction" which could in their hands be overcome by mutation of two membrane proximal tryptophan residues. This scenario would to some extend contradict the results mentioned above, which suggested that SNARE-zippering proceeds in an N- to C-terminal direction. Notwithstanding these findings other groups have witnessed fusion of native Synaptobrevin membranes and Synaptobrevin has recently been shown to be constitutively active, regardless of whether incorporated into membranes or not.[28] Here Synaptobrevin could be driven into SNARE-complexes both in isolated synaptic vesicles and in proteoliposomes.

To sum it up, quite some progress has been made in the process of unraveling SNARE assembly which can proceed fast and independent of additional factors in solution as well as on liposome membranes. Nevertheless a wealth of SNARE interacting proteins has been described, some of which might also have a direct or indirect influence on assembly kinetics. Some of these proteins catalyze the reverse of the assembly reaction which will be explained in greater detail in the following section.

Postfusion—Time for Recycling of SNARE-Complexes

After assembly, the complexed R- and Q-SNAREs reside in the same membrane and are no longer free to act in further rounds of fusion. As much as the above described characteristics like pronounced hysteresis, exothermal driving force of the reaction and the high level of complex-stability

push the reaction into the direction of assembly they of course prevent the complex from voluntary disassembly under physiological conditions. Hence basically no spontaneous dissociation occurs.[13] If there were no counteracting mechanism all fusion events would come to an end as soon as all free SNAREs were used once. Of course the cell could constantly dispose of the fully assembled SNARE complexes and synthesize new free SNAREs in a single-use fashion, but keeping in mind that during assembly the SNAREs only undergo structural but no degradational changes, there is a faster and probably energetically more favorable solution to this problem: A factor which tears the complexes apart despite their apparent stability, e.g., by lowering the activation energy of this thermodynamically unfavorable reaction. The cell has exploited a family of ATPases, which due to their functional variability have been assigned "ATPases associated with various cellular activities (AAA ATPases)", to lower the activation energies of various reactions, mostly connected to folding or unfolding of proteins. One of these ATPases called NSF (N-ethylmaleimide sensitive fusion protein), with the help of its cofactors, the so called SNAPs (soluble NSF-attachment proteins), mediates the disassembly of the stable SNARE complexes and thereby "recycles" the fusion machinery.[29] To do so NSF manages to couple ATP hydrolysis to the highly endothermic dissociation of the 4-helix bundle.

NSF and the SNAPs—The Disassembly Machinery

NSF is ubiquitously expressed and therefore most likely capable of disassembling all existing SNARE-complexes of a certain species.[29] Its universal mechanism is also underlined by the fact that to a certain extent functionality even between different species, seems to be conserved. As an example the NSF yeast homologue sec18p has been shown to stimulate exocytosis in permeabilized adrenal chromaffin cells.[30]

NSF consists of three domains, two of which, termed the D1- and D2-domain, can bind ATP.[31] At one of these binding sites ATP is actively being hydrolyzed to provide energy during disassembly, whereas ATP-binding to the second site functions in oligomerization of the protein,[32,33] which is largely hexameric under equilibrium conditions.[34]

SNARE complexes do not display any direct binding sites for NSF. In order for it to disassemble the complex SNAPs are required which provide high affinity binding sites for both, the enzyme as well as its substrate.[35] SNAPs therefore serve as connectors between the SNARE-complex and NSF which can disassemble the SNARE complex in the presence of Mg2+ATP as soon as one or more, most likely three, SNAPs have bound (Fig. 3). Even though α-SNAP alone is sufficient to serve as a cofactor for disassembly in in vitro experiments, it remains unclear whether the other known SNAP isoforms β- and γ-SNAP function as positive or negative regulators or simply provide redundancy despite relatively low sequence homology.[36-39]

In fact so little is known about the mechanism, that the number of αSNAPs required is still debated. Three might be needed, but alternatively one or two might also suffice. In any case participation of more than three can be excluded, creating difficulties in interpreting how one SNARE complex, one to three αSNAPs but six NSF molecules (meaning one hexamer) can reasonably be put together. No structure of the complete disassembly machinery, also termed the "20S"-complex, has been solved leaving room for speculations.

How Does This Transition of Energy Take Place?

Compared to the assembly reaction the molecular details of disassembly are much less understood. Electron microscopic images of NSF gave some insight into the mechanism in the sense that clearly distinguishable structures were visible, depending on the respective nucleotide bound.[20] These pictures suggest that hydrolyzing ATP to ADP + Pi leads to conformational changes in the whole NSF-hexamer which might exert mechanical force onto the complex it is bound to.

Hexamers have a donut-like shape, which gives rise to the speculation that the hole in the middle might fulfill some kind of function, especially since other likewise hexameric AAA ATPases are known to use their holes to pull interaction partners through.[40,41] On the one hand, it is well imaginable that NSF drags one of the SNAREs through its center pore during disassembly, thereby separating it from the other SNAREs. On the other hand the size of the pore in an NSF-hexamer

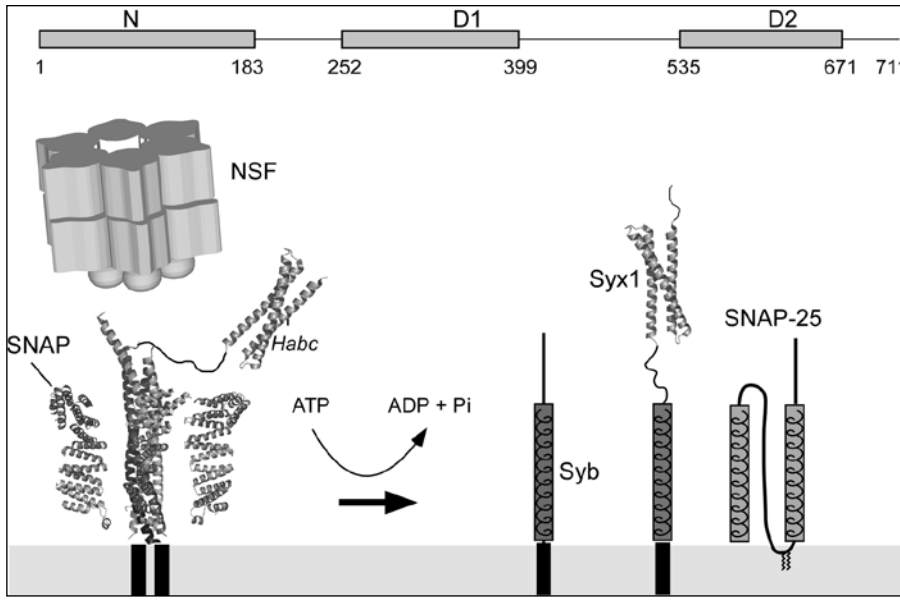

Figure 3. Disassembly of SNARE complexes. Top: The NSF-molecule consists of three subdomains which are schematically depicted here. The N-domain includes the binding region for *a*SNAP, the D1 domain actively hydrolyses ATP during disassembly and the D2 domain plays a role in oligomerization of the molecule, which is mostly hexameric under equilibrium conditions. Bottom: Most likely three *a*SNAPs bind to one SNARE complex to form a recognition site for one hexamer of NSF. Since no high resolution structure of NSF in its hexameric state has been solved it is shown schematically. In the presence of Mg^{2+} NSF then disassembles the SNARE-complex, a process which is fuelled by hydrolysis of ATP into ADP + Pi.

would theoretically be large enough to accommodate an unfolded protein, like e.g., monomeric synaptobrevin, but according to EM-data meet its limitations as soon as folded domains were to fit through.[20] In some sets of SNAREs each of the four proteins includes one domain with inherent tertiary structure which makes this model improbable unless drastic conformational changes facilitating pore enlargement take place during ATP-hydrolysis.

Alternatively, the NSF-hexamer might, while sitting on the threshold provided by SNAPs, create a rotational force through conformational changes which moves the SNAPs in a circular manner. The SNAPs being connected to the SNARE-complex would insert the same rotational force onto the complex. Since this again is rigidly attached to the membrane it is not difficult to picture that as a consequence the complex would be unwound into its individual SNAREs, like operating a mandrel in the opposite direction would unwind a thread into its individual fibers. Originally it was widely believed that disassembly proceeds in a "symmetric" fashion, all six NSF-molecules synchronously hydrolyzing ATP and only then leading to conformational changes in the ring-like structure which consequently enable dismantling of the SNARE complex. More recently an alternative possibility has been raised, which is based on studies undertaken on another AAA-protein called ClpX.[42]

Here the authors could show, that the single subunits of one ClpX-hexamer were capable to exert their catalytic function independently from each other, whereas a concerted action of several subunits apparently increased the enzymatic efficiency in a proportional dependence. If a comparable mechanism were to be utilized by NSF, it would also be conceivable that, depending on the number of NSF-subunits participating in a specific reaction the amount of αSNAPs per disassembly event is similarly flexible. Nevertheless no attempts have been made to investigate

whether NSF-activity also works in an asymmetric manner, or whether all six subunits need to bind and hydrolyze ATP in concerted fashion.

Input and Output of the Machinery—Which Other Substrates or Products Are Feasible?

Neither all substrates nor products of the disassembly-reaction are known. For instance SNAREs could theoretically be fully disassembled into monomers but alternatively might also only be partially disassembled up to an intermediate stage. SNAREs contributed by one membrane could for example be kept together in order to decrease the complexity of the subsequent fusion reaction.

Likewise even though there is no doubt that the ternary SNARE complex is a target of NSF, it cannot be excluded that othe r complexes e.g., assembly intermediates or unwanted dead-end-complexes also serve as substrates for NSF-mediated disassembly. If this were to be the case, NSF might in addition to its indirect effect on SNARE-assembly, in terms of SNARE-recycling, also directly regulate complex assembly by influencing the steady-state concentrations of complex intermediates.

References

1. Bonifacino JS, GB. The mechanisms of vesicle budding and fusion. Cell 2004; 116:153-166.
2. Faix J et al. Cortexillins, major determinants of cell shape and size, are actin-bundling proteins with a parallel coiled-coil tail. Cell 1996; 86:631-642.
3. Fasshauer D et al. A structural change occurs upon binding of syntaxin to SNAP-25. J Biol Chem 1997; 7:4582-4590.
4. Fasshauer D et al. Structural changes are associated with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. J Biol Chem 1997; 272:28036-28041.
5. Weber T et al. SNAREpins: minimal machinery for membrane fusion. Cell 1998; 92:759-772.
6. Fiebig KM et al. Folding intermediates of SNARE complex assembly. Nature Struct Biol 1999; 6:117-123.
7. Fasshauer D et al. Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci USA 1998; 95:15781-15786.
8. Hanson PI et al. Neurotransmitter release—four years of SNARE complexes. Curr Opin Neurobiol 1997; 7:310-315.
9. Hay JC, Scheller RH. SNAREs and NSF in targeted membrane fusion. Curr Opin Cell Biol 1997; 9:505-512.
10. Fasshauer D et al. Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. Biochemistry 1998; 37:10354-10362.
11. Hazzard J et al. NMR analysis of the structure of synaptobrevin and of its interaction with syntaxin. J Biomol NMR 1999; 14:203-207.
12. Margittai M et al. Homo- and heterooligomeric snare complexes studied by site-directed spin labeling. J Biol Chem 2001; 276:13169-13177.
13. Fasshauer D et al. SNARE assembly and disassembly exhibit a pronounced hysteresis. Nat Struct Biol 2002; 9:144-151.
14. Otto H et al. Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin and SNAP-25 in the membrane of synaptic vesicles. Proc Natl Acad Sci USA 1997; 94:6197-201.
15. Rothman JE. Intracellular membrane fusion. Advances in Second Messenger & Phosphoprotein Research 1994; 29:81-96.
16. Antonin W et al. Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. Nat Struct Biol 2002; 9:107-111.
17. Antonin W et al. A SNARE complex mediating fusion of late endosomes defines conserved properties of SNARE structure and function. EMBO J 2000; 19:6453-6464.
18. Zwilling D et al. Early endosomal SNAREs form a structurally conserved SNARE complex and fuse liposomes with multiple topologies. Embo J 2007; 26:9-18.
19. Hwang SB, Lee J. Neuron cell type-specific SNAP-25 expression driven by multiple regulatory elements in the nematode Caenorhabditis elegans. J Mol Biol 2003; 333:237-247.
20. Hanson PI et al. Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. Cell 1997; 90:523-535.
21. Fasshauer D, Margittai M. A Transient N-terminal Interaction of SNAP-25 and Syntaxin Nucleates SNARE Assembly. J Biol Chem 2004; 279:7613-7621.
22. Sorensen JB et al. Sequential N- to C-terminal SNARE complex assembly drives priming and fusion of secretory vesicles. Embo J 2006; 25:955-966.

23. Calakos N et al. Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. Science 1994; 263:1146-1149.
24. Margittai M WJ, Schweinberger E, Schroder GF et al. Single-molecule fluorescence resonance energy transfer reveals a dynamic equilibrium between closed and open conformations of syntaxin 1. Proc Natl Acad Sci USA 2003; 100:15516-15521.
25. Nicholson KL et al. Regulation of SNARE complex assembly by an N-terminal domain of the t-SNARE Sso1p. Nature Struct Biol 1998; 5:793-802.
26. Pobbati A et al. N- to C-terminal SNARE complex assembly promotes rapid membrane fusion. Science 2006; 313:673-676.
27. Hu K et al. Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. Nature 2002; 415:646-650.
28. Siddiqui T et al. Determinants of synaptobrevin regulation in membranes. Mol Biol Cell 2007; 18:2037-2046.
29. Jahn R, Südhof TC. Membrane fusion and exocytosis. Annu Rev Biochem 1999; 68:863-911.
30. Steel GJ et al. Biochemical analysis of the Saccharomyces cerevisiae SEC18 gene product: implications for the molecular mechanism of membrane fusion. Biochemistry 1999; 38:7764-7772.
31. Tagaya M et al. Domain structure of an N-ethylmaleimide-sensitive fusion protein involved in vesicular transport. J Biol Chem 1993; 268:2662-2666.
32. Whiteheart SW et al. N-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion. Journal of Cell Biology 1994; 126:945-954.
33. Nagiec EE et al. Each domain of the N-ethylmaleimide-sensitive fusion protein contributes to its transport activity. J Biol Chem 1995; 270:29182-29188.
34. Fleming KG et al. A revised model for the oligomeric state of the N-ethylmaleimide-sensitive fusion protein, NSF. J Biol Chem 1998; 273:15675-15681.
35. Wilson DW et al. A multisubunit particle implicated in membrane fusion. Journal of Cell Biology 1992; 117:531-538.
36. Stenbeck G. Soluble NSF-attachment proteins. Int J Biochem Cell Biol 1998; 30:573-577.
37. Sudlow AW, MB, Bodill H et al. Similar effects of alpha- and beta-SNAP on Ca(2+)-regulated exocytosis. FEBS Lett 1996; 393:185-188.
38. Xu J, XY, Ellis-Davies GC GJ et al. Differential regulation of exocytosis by alpha- and beta-SNAPs. J Neurosci 2002; 22:53-61.
39. Whiteheart SW et al. SNAP family of NSF attachment proteins includes a brain-specific isoform [see comments]. Nature 1993; 362:353-355.
40. Egelman E et al. Bacterial helicases. J Struct Biol 1998; 124:123-128.
41. Schmidt M, LAN, Finley. Structure and mechanism of ATP-dependent proteases. Curr Opin Chem Biol 1999; 3:584-591.
42. Martin A, BT, Sauer RT. Rebuilt AAA + motors reveal operating principles for ATP-fuelled machines. Nature 2005; 437:1115-1120.