A Conserved Membrane Attachment Site in α-SNAP Facilitates N-Ethylmaleimide-sensitive Factor (NSF)-driven SNARE Complex Disassembly**

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The ATPase NSF (N-ethylmaleimide-sensitive factor) and its SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein) cofactor constitute the ubiquitous enzymatic machinery responsible for recycling of the SNARE (SNAP receptor) membrane fusion machinery. The enzyme uses the energy of ATP hydrolysis to dissociate the constituent proteins of the SNARE complex, which is formed during the fusion of a transport vesicle with the acceptor membrane. However, it is still unclear how NSF and the SNAP adaptor work together to form the tight SNARE bundle apart. SNAPs have been reported to attach to membranes independently from SNARE complex binding. We have investigated how efficient the disassembly of soluble and membrane-bound substrates are, comparing the two. We found that SNAPs support disassembly of membrane-bound SNARE complexes much more efficiently. Moreover, we identified a putative, conserved membrane attachment site in an extended loop within the N-terminal domain of α-SNAP. Mutation of two highly conserved, exposed phenylalanine residues on the extended loop prevent SNAPs from facilitating disassembly of membrane-bound SNARE complexes. This implies that the disassembly machinery is adapted to attack membrane-bound SNARE complexes, probably in their relaxed cis-configuration.

All intracellular vesicle transport processes, ranging from secretion in yeast to neurotransmitter release in the brain, depend on the ability of membranes to fuse with each other. Intracellular fusion is mediated by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)3 proteins on opposing membranes, which assemble in trans-configuration into four-helix bundle complexes, bringing the membranes into close proximity (for an overview see Refs. 1–4). After fusion, all SNAREs constituting one complex are anchored in a relaxed cis-configuration to one membrane and are no longer free to act in further cycles of fusion. These cis-SNARE complexes were found to be extremely stable, with basically no spontaneous dissociation detectable (5–7). Thus, in order to recycle the fusion machinery, these complexes need to be dissociated actively. To achieve this, the cell exploits the AAA ATPase NSF (N-ethylmaleimide-sensitive factor), which couples ATP hydrolysis to the thermodynamically unfavorable dissociation of the SNARE bundle.

Despite its fundamental role in the cell, surprisingly little progress has been made in deciphering the molecular details of NSF action since its discovery about 30 years ago (8). It is thought that for disassembly, ring-shaped NSF hexamers (9–11) attack SNARE bundles standing upright in the membrane at their N-terminal side (12). As SNARE complexes do not display a direct binding site for NSF, SNAPs (soluble NSF attachment proteins) (13), very probably in three copies (14), have to serve as adaptors between the SNARE complex and the enzyme. The entire disassembly complex is referred to as the 20S complex (15, 16). Although high-resolution structures of several components and domains are available, neither the structure of the complete NSF molecule nor of the 20S complex is known in sufficient detail. Hence, the molecular details of the arrangement of the molecules in the 20S particle are elusive (17–19).

A milestone on the way toward rebuilding SNARE disassembly from scratch has recently been achieved (20, 21). A new in vitro assay with high time resolution has been established. This assay uses recombinant SNAREs fused to GFP (green fluorescent protein) analogs as substrates and monitors the disassembly reaction spectroscopically. Interestingly, even though the amounts of NSF the authors reported for efficient disassembly were reasonably low for enzymatic reactions, α-SNAP (i.e. one of the three SNAP isoforms in vertebrates) was required in surplus concentrations. Because this assay does not allow for direct quantification of α-SNAP/SNARE binding, the α-SNAP/SNARE complex affinity was determined using GST (glutathione S-transferase)-coupled SNARE complexes. This led to an EC50 of 5 μM at a complex concentration of only 100 nm, suggesting that α-SNAP has a very low affinity for the SNARE complex (20). Comparably low α-SNAP affinities have also been observed by other groups pursuing in vitro matrix-based studies of the affinity between α-SNAP and SNARE com-
plexes (22, 23). Besides the fact that this high demand for α-SNAP for disassembly probably has no physiological basis, it also is at odds with findings from other experiments (24, 25), in which optimal function was reconstituted using much lower amounts of α-SNAP (~0.6 μM). For example, in a study we published recently, we found that disassembly reached optimal speed at an α-SNAP concentration of ~100 nM when monitoring disassembly in a more physiological system, the so-called membrane sheets (26).

So why are there such marked differences between different systems and studies? One possible explanation for variances in α-SNAP efficiency may be experimental constraints, e.g. a loss of α-SNAP functionality under certain conditions. Alternatively, an additional and as yet unknown player might be important for optimal α-SNAP function that is only present in physiological systems. To investigate whether a hitherto unrecognized factor contributes to optimal SNAP reactivity, we therefore chose to establish a reductionist approach based on fluorescence spectroscopy, which directly monitors SNAP/SNARE and SNARE/SNARE interactions in a time-resolved manner.

EXPERIMENTAL PROCEDURES

Protein Constructs—Besides the expression constructs for the yeast disassembly proteins Sec17 and Sec18 in pQE19-vectors (27) (kindly provided by C. Ungermann, Osnabrück, Germany), and β-SNAP in pGEX-2T, all other recombinant proteins were in a PET28a or PET15b vector (Novagen, Schwabach, Germany), which encode for an N-terminal His6 tag that can be cleaved by thrombin. The following constructs derived from rat cDNAs have been described earlier (28–31): cysteine-free SNAP25a (aa 1–206), soluble synaptobrevin 2 (aa 1–96, Syb), synaptobrevin 2 full-length (aa 1–116, SybTMD), the soluble SNARE domain of syntaxin 1a (aa 180–262, SxH3), full-length syntaxin 1a (aa 1–288), and complexin 1 (Cpx1). We also used the single cysteine variants of SNAP-25, SNAP25C130 (32), and synaptobrevin (aa 1–96, SybC28 and aa 1–116, SybTMD28) that have been described previously (33). The recombinant proteins encoding for bovine α-SNAP (aa 1–295) and β-SNAP (aa 1–298) were recloned into the pET28a vector as described earlier (34), as were recombinant proteins encoding for NSF in Chinese hamster (kindly provided by J. E. Rothman, New Haven, CT). All these recombinant proteins were originally in pQE9 vectors. In addition, we used the single point mutation Sec18, followed by thrombin cleavage of either the His6 tag that is only present in physiological systems. To investigate whether a hitherto unrecognized factor contributes to optimal SNAP reactivity, we therefore chose to establish a reductionist approach based on fluorescence spectroscopy, which directly monitors SNAP/SNARE and SNARE/SNARE interactions in a time-resolved manner.

Disassembly Reaction—For disassembly, a buffer containing KGl/KAc at concentrations of 120 mM and 20 mM respectively, as well as 2 mM ATP, 5 mM MgCl2, and 50 mM HEPES (pH 7.4) was used. Covalent attachment of the sulphydryl-reactive fluorophores Oregon Green 488 iodoacetamide (OG) or Texas Red C5 bromoacetamide (TR) or Alexa594 C5 maleimide (Invitrogen) to the respective SNAP proteins was performed according to the manufacturer’s instructions. Typically, SNAP complexes used for fluorescence anisotropy measurements were purified by ion-exchange chromatography and labeled afterward at the single cysteine residue. SNAP complex formation was then monitored spectroscopically. The disassembly enzymes were added at the concentrations indicated.

Fluorescence Measurements—Measurements were carried out in a Fluorolog 3 spectrometer in T-configuration equipped with the detergent n-dodecyl-β-d-maltoside (DM) was monitored according to the manufacturer’s instructions. Typically, SNAP complexes used for fluorescence anisotropy measurements were purified by ion-exchange chromatography and labeled afterward at the single cysteine residue. SNAP complex formation was then monitored spectroscopically. The disassembly enzymes were added at the concentrations indicated.
for polarization (Model FL322, Horiba Jobin Yvon) or a Fluoro-
max 2 Instrument (Horiba Jobin Yvon). All measurements were
carried out at 37 °C in quartz cuvettes (Hellma) in a disassembly
buffer. FRET experiments were recorded by excitation at 488
nm, monitoring donor (OG) fluorescence emission at 520 nm
and acceptor (TR or Alexa594) fluorescence emission at 610
nm. Typically, the slit widths were set to 1 nm for the excitation
wavelength, 2 nm for the donor and 4 nm for the acceptor, and
the integration time was set to 1 s. Fluorescence anisotropy was
measured using Texas Red-labeled proteins and a slit width of 3
nm for the excitation wavelength (520 nm) and 5 nm for the
emission wavelength (610 nm), respectively. The G factor was
calculated according to $G = I_{H V}/I_{V V}$, where $I$ is the fluores-
cence intensity, the first subscript letter indicates the direction
of the exciting light, and the second subscript letter direction
of emitted light. The intensity of the vertically (V) and
horizontally (H) polarized emission light after excitation by ver-
tically polarized light was measured. The anisotropy ($r$) was
determined according to $r = (I_{V V} - G I_{V H})/(I_{V V} + 2 G I_{V H})$.

RESULTS

FRET and Fluorescence Anisotropy Can Monitor SNARE Complex Disassembly in Vitro—To monitor the assembly of the
neuronal SNARE complex in real time, we had previously
developed fluorescence assays (30). We took advantage of these
assays to investigate the reverse reaction, the breaking up of the
four-helix bundle SNARE complex by NSF and its SNAP cofactor.

For FRET measurements, we generally mixed the three
SNARE proteins, two of which were specifically labeled, in stoi-
chiometric amounts. SNARE complex formation was readily
identified by a marked increase in donor fluorescence (30).
Upon addition of NSF, α-SNAP and free Mg$^{2+}$, a reduction of
energy transfer occurred. Fig. 1 shows a typical FRET measure-
ment, in which a FRET pair in the N-terminal region of the
complex was used, i.e. synaptobrevin labeled at position 28 with
Texas Red (Syb$^{C28TR}$) and SNAP-25 labeled at position 130
with Oregon Green (SNAP25$^{C130OG}$).

For fluorescence anisotropy measurements, we routinely
used a SNARE complex labeled with Texas Red at Syb$^{C28}$. The
readout here depends on the flexibility of the dye, which
changes upon interaction of the labeled protein with other pro-
teins. Indeed, addition of α-SNAP alone caused a significant
increase of fluorescence anisotropy before disassembly was
even initiated. The same increase in anisotropy was observed
when α-SNAP$^{wt}$ was substituted by the mutant α-SNAP$^{L294A}$,
which was not able to support disassembly (35). Interestingly,
when enzymatic amounts of NSF were subsequently added, no
further signal change was observed. However, when much
higher concentrations of NSF were added, an additional in-
crease in fluorescence anisotropy was observed (data not
shown), which probably signifies the formation of the 20S com-
plex. When we finally triggered disassembly with MgCl$_2$, the
reaction proceeded efficiently as can be seen by a gradual
decrease of fluorescence anisotropy (Fig. 1d). The rate of disas-
sembly is comparable to the one observed in the FRET mea-
surements, underlining the validity of both methods. In the fol-
lowing, the buffering conditions were optimized, eventually
leading to a standard composition for disassembly buffer, which includes K glu/K acetate at concentrations of 120 and 20 mm
respecitively, as well as 2 mm ATP, 5 mm MgCl$_2$, and 50 mm
HEPES, pH 7.4.

α-SNAP Efficiency Is Low in Solution—To determine the op-
timal concentrations of NSF, ATP, and α-SNAP under the con-
ditions used in the fluorescence assays, we monitored disas-
sembly kinetics, varying the concentration of one of these
factors at a time. As shown in supplemental Fig. S1, the ATP
and NSF requirements were in line with earlier studies (39).

Also in line with earlier findings (20, 21), micromolar
amounts of α-SNAP were needed for efficient disassembly. As
outlined above, an advantage of the fluorescence anisotropy
approach is that it can also serve as a monitor of α-SNAP bind-
ing, although the small change in anisotropy renders it difficult
to quantify the exact amount of bound molecules. Nevertheless
an optimal increase in fluorescence anisotropy was observed
only at higher α-SNAP concentrations (Fig. 2a), suggesting that
α-SNAP binds with only moderate affinity to the soluble
SNARE complex. Keeping in mind that the stoichiometry of
20S complexes has been reported to be 6:3:1 (NSF:α-SNAP:
SNARE complex) and that NSF does not interact with α-SNAP
in solution (14), the need for such high α-SNAP amounts to
saturate SNARE complex binding is not likely to be
physiological.

In principle, the low α-SNAP efficiency could be inherent to
the recombinant α-SNAP, possibly due to invisible degradation
or a lack of putative posttranslational modifications. Neverthe-
less, we have recently shown that our recombinant α-SNAP
disassembles SNARE complexes on sheets of PC12-cell mem-
branes efficiently when applied in a concentration of less than
100 nM (26). The apparently high α-SNAP affinity in the sheet
experiments strongly suggested that a factor that is missing in
the experiments using the soluble SNARE complex but present
on membrane sheets is responsible for the different α-SNAP
efficiency.

SNARE Complex Incorporation into Liposomes via Their
Transmembrane Domains Potentiates α-SNAP Efficiency—
Considering that the disassembly reaction in the cell is most
likely to occur only after vesicle fusion is complete and all three
SNAREs are located on one membrane, it was tempting to spec-
ulate that the membrane is of some importance during the dis-
assembly reaction. To test this hypothesis, measurements were
carried out using SNARE complexes incorporated into lip-
osomes. Indeed, α-SNAP turned out to be more potent in disas-
sembling SNARE complexes on liposomes than in solution.
This is illustrated in Fig. 3a, where decreasing amounts of
α-SNAP were used. As little as 30 nM α-SNAP promoted disas-
sembly, 45 nM sufficed to disassemble all complexes and 120 nM
saturated the assay, which is very similar to the requirements
observed in the sheet assay (26). A direct comparison of
α-SNAP efficiency on liposomes and in solution (Fig. 3b) fur-
thermore illustrates that the improvement on liposomes is
quite substantial: In solution, roughly 20 times more α-SNAP is
required to reach comparable kinetics as on liposomes. The
α-SNAP dependence on liposomes was identical regardless of
which SNARE transmembrane domain (synaptobrevin or syn-
taxin) was used for complex incorporation. This renders it
unlikely that the presence of the membrane anchors caused the increased α-SNAP efficiency.

At this point, we did not know whether the actual anchorage of the complex to the membrane is a prerequisite for the markedly improved α-SNAP performance or whether the mere presence of the hydrophobic transmembrane domains or lipids leads to an increased α-SNAP efficiency. To resolve this question, we treated liposome-incorporated SNARE complexes with detergent prior to disassembly to dissolve the liposomes, carefully staying below concentrations that would be harmful for the disassembly machinery. Indeed, stepwise dissolution of liposomes by application of detergent correlated with a stepwise reduction of the fraction of SNARE complexes that were quickly disassembled. This indicates that the SNARE complexes indeed need to be anchored to the membrane to serve as high affinity targets of α-SNAP (see supplemental Fig. S2).

**Design of α-SNAP Mutants Lacking the Putative Lipid-interacting Domain** — α-SNAP is an amphiphilic protein that has been reported to bind even to plastic surfaces (40, 41). Furthermore, α-SNAP was shown to bind lipids independently of SNAREs (42). Alongside these observations, the data collected so far made it tempting to speculate that a direct interaction between α-SNAP and the membrane lipids is responsible for the increased α-SNAP efficacy on liposomes. If this were true, the lipid binding property might possibly be mapped to a certain region of α-SNAP. No crystal structure has been solved for α-SNAP so far, but the structures of the α-SNAP isoform (43) and of the yeast homolog Sec17 are known (44). Based on interaction studies using various point-mutated α-SNAPs, a model of α-SNAP bound to the SNARE complex has been proposed by Marz et al. (20). According to this model, mostly basic residues on the conserved ridge of α-SNAP form a diagonal band across...
that interacts with a complementary stretch on the SNARE complex bundle. Within the 32 very N-terminal residues of the α-SNAP homology model, an arm-like structure points away from the complex. This arm consists of a loop of mostly hydrophobic amino acids (residues 27–32) that are highly conserved in different SNAPs, as can be gleaned from a sequence alignment shown in Fig. 4a. If this region were the interaction site of α-SNAP with the membrane as suggested by the depiction in Fig. 4b, its deletion should interfere with membrane binding and hence the higher α-SNAP efficiency on membranes. We chose to design two mutants: One large-scale deletion (deleting residues 1–32) mutant designated α-SNAPdel, and a subtler one, where the mutations were confined to only the loop region. In detail, we changed two

FIGURE 2. Micromolar amounts of α-SNAP are required for efficient disassembly of the soluble SNARE complex. a, binding of α-SNAP to the purified labeled SNARE complex (SybC28TR:SNAP25:SyxH3, 75 nM) leads to increase of fluorescence anisotropy in the absence of NSF. The signal changes upon binding of increasing amounts of α-SNAP are shown as multiples of the anisotropy prior to addition of α-SNAP. Mean values of anisotropy change are plotted against the α-SNAP concentration. Adding NSF in the absence of MgCl₂ leads to a further increase in anisotropy. The disassembly reaction was then started by the addition of 5 mM MgCl₂. A typical sequence of fluorescence changes is shown in the inset (1.5 μM α-SNAP was added). b, subsequently, the disassembly reaction was followed by a decrease in fluorescence anisotropy. To compensate for the differences in the starting value of fluorescence anisotropy resulting from the different amounts of bound α-SNAP, the reactions were normalized. For disassembly, 10 nM NSF and 2 mM ATP were added, and the reaction was started by addition of 5 mM MgCl₂. Note that an optimal binding and reaction rate was reached only at about 1.25 μM α-SNAP.

FIGURE 3. Incorporating the SNARE complexes into liposomes renders α-SNAP more efficient. a, disassembly kinetics of the liposomal SNARE complex (SybTMRC28TR:SNAP25:SyxH3, 35 nM) using different amounts of α-SNAP. The reaction in the presence of 5 nM NSF and 2 mM ATP was started by adding 5 mM MgCl₂. Note that 120 nM α-SNAP sufficed to promote fast disassembly of membrane-inserted SNARE complexes. b, to disassemble soluble SNARE complexes (SybC28TR:SNAP25:SyxH3) at approximately similar speed as observed for liposomal SNARE complexes (SybTMRC28TR:SNAP25:SyxH3), more than 20 times more α-SNAP had to be added.
conserved phenylalanines (residues 27 and 28) for the polar amino acid serine (\(H9251\)-SNAPF27S,F28S).

Both Mutated \(H9251\)-SNAPs Have Lost the Lipid-mediated Efficiency Boost—We found that both mutants were able to disassemble soluble SNARE complexes. \(H9251\)-SNAPF27S,F28S turned out to promote disassembly of soluble SNARE complexes as efficiently as wild-type \(H9251\)-SNAP (Fig. 5a), whereas \(H9251\)-SNAPdel was somewhat less efficient. The latter was not surprising, because a deletion of residues 1–28 had been reported to hamper SNAP efficiency before (25). We therefore chose to concentrate on the less severe \(H9251\)-SNAP mutant, \(H9251\)-SNAPF27S,F28S. We directly compared its disassembly kinetics in solution and on liposomes of \(H9251\)-SNAPF27S,F28S and of \(H9251\)-SNAPwt, using the fluorescence anisotropy set-up. Knowing that \(H9251\)-SNAPwt efficiency increases 20-fold on liposomes, we employed \(H11011\) 20-fold less of the respective \(H9251\)-SNAP (60 nM) for the experiments on liposomes, leaving everything else as in solution. Remarkably, whereas 60 nM \(H9251\)-SNAPwt efficiently promoted disassembly of membrane-inserted SNARE complexes, the same amount of \(H9251\)-SNAP itself did not support disassembly on liposomes at all (Fig. 5b). Increasing the concentration of \(H9251\)-SNAPF27S,F28S led to a gradual increase of disassembly speed on liposomes. When we also tested the \(H9251\)-SNAPdel mutant, notwithstanding its reduced overall \(H9251\)-SNAP efficiency, we found that the membrane-mediated \(H9251\)-SNAP-potentiation was also abolished (supplemental Fig. S3). Together, these findings suggest that the arm-like structure at the N-terminal tip of \(H9251\)-SNAP might serve as a hitherto unknown membrane attachment site.

The Membrane Boost Is Conserved for Other SNAP Proteins—We next asked whether the membrane boost is conserved for other homologs of the disassembly machinery. To answer this question, we focused on the mammalian brain-specific SNAP isoform \(H9251\)-SNAP and the SNAP homolog of yeast, Sec17 (13, 45).

FIGURE 4. A hydrophobic loop in the N-terminal region of SNAP might serve as membrane attachment site. a, structure-based sequence alignment of the N-terminal portion of SNAP proteins from different organisms indicates that the hydrophobic loop between the first two helices is conserved. At the top, boxes indicate the first two helices and the connecting loop from the Sec17 crystal structure. The yeast Sec17 (SaCe_Sec17, Saccharomycyes cerevisiae, gi 6319421) was aligned with several fungal and animal SNAP homologs: LoLe_Sec17, Lodderomyces elongisporus, gi 14926407; CaAl_Sec17, Candida albicans, gi 68475136; CaGl_Sec17, Candida glabrata, gi 50287489; CiIn_\(H9251\)-SNAP, Ciona intestinalis, gi 198424864; DrMe_\(H9251\)-SNAP, Drosophila melanogaster, gi 17737681; TrAd_\(H9251\)-SNAP, Trichoplax adhaerens, gi 196014845; StPu_\(H9251\)-SNAP, Strongylocentrotus purpuratus, gi 72082731; BrFl_\(H9251\)-SNAP, Branchiostoma floridae, gi 219425561; and BoTa_\(H9251\)-SNAP, Bos taurus, gi 423236; BoTa_\(H9251\)-SNAP, Bos taurus, gi 423230. Arrowheads indicate the highly conserved aromatic residues that were mutated to serines. b, schematic drawing of the crystal structures of Sec17 from Baker’s yeast (PDB code 1QQE, S. cerevisiae) (56) and of the membrane-embedded neuronal SNAP complex including its transmembrane regions (Ref. 57, PDB codes 3HD7 and 3HD9). This illustration indicates that the loop between the first two helices of Sec17 might touch the membrane when the protein is bound to the SNAP bundle. Note that the illustration is largely based on the model of the \(H9251\)-SNAP: SNARE complex interaction given in Marz et al. (20).

\(H9251\)-SNAP Contains a Membrane Attachment Site

\(H9251\)-SNAP contains a membrane attachment site that is conserved in other SNAP homologs.
Because of the high sequence similarity of β-SNAP and α-SNAP, we were not surprised to find that β-SNAP behaved very similar to α-SNAP with respect to promoting disassembly. Like α-SNAP, β-SNAP was also more efficient in disassembling SNARE complexes in membranes (supplemental Fig. S4).

To investigate the activity of the yeast SNAP Sec17, we first established that the yeast enzymes could substitute for their mammalian counterparts. Strikingly, both the yeast and the mammalian disassembly machinery were able to disassemble the neuronal SNARE complex as well as the yeast complex (data not shown), although the neuronal disassembly machinery was clearly more efficient, possibly because of the higher purity and stability of these proteins. We then compared Sec17 function on liposomes and in solution (Fig. 6). Here, the Sec17-concentration capable of mediating disassembly on liposomes did not successfully disassemble soluble SNARE complexes. It can thus be concluded that, like α-SNAP, Sec17 efficacy is higher when the target complexes are incorporated into membranes. Similarly, the higher efficiency of SNAPS on membranes was not restricted to disassembly of the neuronal SNARE complex target, but was also observed for the disassembly of membrane-inserted yeast SNARE complexes (data not shown).

Complexin 1 Interferes with Disassembly of SNARE Complexes—The SNARE complex-interacting protein complexin has been reported to displace α-SNAP from SNARE complexes (46), thereby inhibiting the disassembly reaction. Meanwhile its crystal structure when bound to the SNARE complex has been solved (47, 48), revealing that complexin binds to a groove of the four-helix bundle formed by the helices of syntaxin 1a and synaptobrevin 2. In fact, it is easy to envision that bound complexin can interfere with the activity of the disassembly machinery, especially considering that complexin binds with very high affinity (49). Gel-based experiments, however, did not corroborate the inhibitory role of complexin (49). As such experiments only have a limited resolution and are mainly suited to detect strong effects, we re-investigated the influence of complexin on SNARE disassembly in vitro using our fluorescence-based assays. To this end, disassembly was carried out as usual except that complexin was added to the solution prior to the reaction trigger. We found that, in solution, 370 nM Cpx1 (370 nM) were able to inhibit SNARE disassembly strongly at an α-SNAP concentration of 1.1 μM in the presence of 3 nM NSF, whereas no disassembly was observed when 60 nM α-SNAP was employed.

FIGURE 5. On liposomes, the mutant α-SNAPF27S,F28S does not show the increased efficiency seen for wild-type α-SNAP. α, α-SNAPwt and α-SNAPF27S,F28S support disassembly of soluble SNARE complexes with comparable efficiency. Disassembly of ~80 nM labeled SNARE complex (Syb28TR:SNAP25:SynH3) was monitored by fluorescence anisotropy. Both α-SNAP variants were employed at 1.1 μM in the presence of 3 nM NSF. b, α-SNAPwt supported disassembly of liposomal SNARE complexes (SybTMRC28TR:SNAP25:SynH3) at 60 nM concentration, whereas no disassembly was observed when 60 nM α-SNAPF27S,F28S were employed.

FIGURE 6. The yeast SNAP homolog Sec17 shows membrane dependence as well. Roughly 700 nM Sec17 and 1.5 nM NSF were able to disassemble 40 nM of membrane-inserted SNARE complexes (SybTMRC28TR:SNAP25:SynH3). The same amount of Sec17 did not promote disassembly of 40 nM SNARE complex in solution (SybC28TR:SNAP25:SynH3). The reactions were started by the addition of 5 mM MgCl2 (arrow). Note that even though the non-cognate combination of yeast Sec17 and mammalian NSF disassemble neuronal SNARE complexes less efficiently than the combination of Sec17 and Sec18, we chose to perform this experiment with this combination, because Sec18 was very fragile and tended to lose its activity rapidly.
were added (Fig. 7b). If one assumes that the affinity of Cpx1 does not vary drastically between liposomal and soluble complexes, this finding corroborates that α-SNAP binds with higher affinity to membrane-bound SNARE complexes.

**DISCUSSION**

The key function of the ATPase NSF is the disassembly of tight cis-SNARE complexes that arise form the fusion of a transport vesicle with its target membrane. NSF does not bind directly to the SNARE complex, but requires the adaptor α-SNAP. According to the current model, three copies of α-SNAP bind to the rod-like SNARE complex along its length (14). Although the approximate architecture of the entire 20S particle has been established for about a decade (19, 50), progress in understanding the disassembly mechanism, i.e. how NSF and α-SNAP take apart the tight SNARE bundle, has been surprisingly slow. Furthermore, it has remained unclear how much of the cofactor α-SNAP is required for NSF-driven SNARE complex disassembly. It seemed that nm amounts of α-SNAP were sufficient to saturate SNARE complex binding in some studies, whereas others reported an EC50 as high as 5 μM α-SNAP. As the studies used different methods, no clear parameter causing such differences was identifiable between the various studies.

One reason for these conflicting results might be because the disassembly factors are biochemically rather difficult to handle and so building up a reliable enzymatic assay with high-time resolution is challenging. A major step toward such an assay has been achieved recently by using a FRET assay based on GFP-variants of the SNARE proteins. Here, we have taken this approach a step forward by specifically labeling the SNARE proteins with fluorescent dyes. This approach avoided the bulky GFP moiety, allowing us to use a variety of different SNARE constructs, even the ones containing their transmembrane domains. In turn, the high time-resolution and flexibility of our assay allowed us to establish a protocol for a consistent preparation of highly active disassembly enzymes. We noticed that some of the proteins we used were more difficult to maintain in an active state. For example, due to ongoing degradation of β-SNAP during purification, the efficiencies of α-SNAP and β-SNAP could not be compared in an absolute manner. A degradation product of β-SNAP appears to be capable of inhibiting the disassembly reaction, which might explain why this homologue has been claimed to have a different role from α-SNAP in earlier studies (51, 52), although the proteins show 83% sequence identity (53). Despite the stability problem, and in agreement with another study (54), we were able to show that both proteins behave in a very similar manner during disassembly.

It soon became evident that when we used the soluble SNARE complex, the α-SNAP requirements agree with those studies reporting a low α-SNAP affinity: μM amounts of α-SNAP were needed to achieve optimal disassembly and to saturate SNARE complex binding. However, when we incorporated the SNARE complex into lipid membranes, much lower α-SNAP concentrations (~100 nm at most) were sufficient. This is comparable to the α-SNAP concentration recently found to disassemble SNARE complexes efficiently in “ex vivo” membrane sheets. Our results strongly suggest that α-SNAP is capable of interacting directly with membrane lipids, increasing its efficiency.

It is conceivable that the additional membrane attachment site serves to stabilize the interaction between α-SNAP and the SNARE rod, thereby substantially improving the efficiency of the disassembly process. We found similar activity increases for the α-SNAP homologs β-SNAP and Sec17, suggesting that SNAP cofactors in general are able to interact with the membrane. It had been proposed early on that SNAP cofactors might primarily recognize the overall shape of the four-helix bundle of

**FIGURE 7.** The SNARE complex-interacting protein complexin 1 interferes more efficiently with disassembly in solution than on membranes. a, in solution, substoichiometric amounts of Cpx1 reduced α-SNAP efficiency during disassembly. Soluble, FRET-active complexes (SybC28TR-SNAP25130TR-SyxH3, ~75 nm) were disassembled by 4.5 nm NSF with the help of 1.1 μM α-SNAP in the presence or absence of 370 nm Cpx1. b, for the disassembly of membrane-inserted SNARE complexes, ~20 times less α-SNAP was needed. Accordingly, 45 nm α-SNAP was added to disassemble ~75 nm liposomal SNARE complex (SybTM28TR-SNAP25130TR-SyxH3). Addition of 370 nm Cpx 1 inhibited the reaction on membranes as well, but the inhibition could be overcome effortlessly when an additional 450 nm α-SNAP was added (indicated by an arrow). Note that in this reaction, less than half the amount of α-SNAP is present compared with the reaction shown in panel a. This corroborates the view that affinity of α-SNAP to the SNARE bundle is augmented in the presence of the membrane.
the SNARE complex, because the disassembly enzymes must be able to attack all the different types of SNARE complexes within the cell. Our results show that the affinity of SNAP to the core four-helix bundle, be it the neuronal SNARE complex or the yeast secretory complex, is only moderate. This suggests that the additional membrane attachment site is necessary to improve the overall affinity. The membrane might also function as a SNAP collector, providing a first SNAP binding site and thereby increasing its local concentration on the membrane, facilitating binding to cis-SNARE complexes. Alternatively, the membrane interaction might induce a conformational change in SNAP that strengthens its binding to the SNARE complex.

In our assay, complexin was able to inhibit disassembly of cis-SNARE complexes. The fact that this inhibition can be overcome by an increase in the α-SNAP concentration suggests that both proteins compete for binding to the SNARE complex. Because of the increased efficiency of α-SNAP on membranes, much less α-SNAP was needed to overcome complexin inhibition on liposomes than in solution. From this, we conclude that the affinity of complexin to the SNAP bundle is not increased by the presence of membranes in the same way as the affinity of α-SNAP. This is in line with the literature, where the affinity of complexin to the SNAP bundle has been reported to be even lower on membranes (55) than in solution (49). One could imagine that this helps to secure binding of SNAPs and complexin at the relevant stage of the SNARE cycle: SNAREs in cis-configuration might offer a high affinity binding site for SNAPs, whereas complexin might favor SNAREs in trans-configuration.

Our results demonstrate that NSF-catalyzed disassembly of SNARE complexes needs to be studied in the context of the lipid bilayer. Only when the high affinity SNAP binding site is established can stoichiometric amounts of α-SNAP be used. This is important, because unnaturally high α-SNAP requirements may occlude bottlenecks of the reaction or regulatory mechanisms. Certainly, this should also help to decode the protein-protein interactions during disassembly. For example, even though three α-SNAPs may bind to one SNARE complex in purified 20S complexes, as determined by quantitative amino acid analysis (14), it is not known whether three α-SNAPs actually have to be bound for functional disassembly.

Based on a multitude of mutations on the surface of α-SNAP, an α-SNAP:SNARE complex binding model has been generated. This model is based on shape and charge complementary between the surface of α-SNAP and the SNARE complex (20). In this model, the N-terminal region of α-SNAP points outwards. Remarkably, the C-terminal globular bundle domain of α-SNAP had to be taken out of the model in order to avoid a clash between the α-SNAP and the SNARE complex. Therefore, the authors suggested that the C-terminal domain of α-SNAP might bind upon binding to the SNARE complex (20). Of course, such a scenario cannot be ruled out by our findings. Yet if one assumes, that the conformation of α-SNAP does not change drastically upon binding, binding of α-SNAP alongside the SNARE bundle would position the N-terminal region of α-SNAP more toward the lipid bilayer (Fig. 4a). The N-terminal region consists of a twisted sheet of nine α-helices. The first two helices are connected by an extended loop that sticks out of the twisted sheet. The loop contains mostly hydrophobic amino acids (residues 27–32), suggesting that it accommodates the membrane attachment site. Indeed, the presence of the membrane did not longer improve the disassembly reaction when we deleted the entire putative membrane anchor region (aa 1–32) or, more subtly, when the two highly conserved phenylalanine residues (F275, F285) on the extended loop in α-SNAP were mutated. These phenylalanines appear to be perfectly suited to sink into the lipid bilayer, where they can anchor the protein. It should be noted that the mutant efficiency in solution was comparable to that of wild-type α-SNAP, indicating that despite their fundamental importance for lipid binding, the two phenylalanine residues are not involved in SNAP-SNARE or SNAP-NSF interaction. Interestingly, the homologous loop is shorter in γ-SNAP, a distantly related isoform, and adopts a different local conformation (43). Nevertheless, the loop in γ-SNAP also contains exposed aromatic residues, Phe-23 and Trp-26, which might be able to interact with a membrane. Taken together, these findings strongly suggest that the hook-like structure is essential for a protein-lipid interaction that potentiates the α-SNAP efficiency during SNARE complex disassembly. Hence, the disassembly machinery seems perfectly adapted to attack membrane-inserted SNAP25 bundles.

Acknowledgments—We thank Alexander Stein, Ursel Ries, and Wolfgang Berning-Koch for protein preparations and Thorsten Lang and Dana Bar-On for fruitful discussions.

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α-SNAP Contains a Membrane Attachment Site

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A conserved membrane attachment site in α-SNAP facilitates NSF-driven SNARE complex disassembly

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Suppl. Fig. 1: NSF and ATP requirements of the SNARE complex disassembly reaction.

The disassembly of purified SNARE complexes containing synaptobrevin labeled at position 28 with Texas Red (Syb\textsuperscript{C2STR}: SNAP25: SyxH3) was monitored by fluorescence anisotropy in solution.

a) Increasing amounts of the disassembly enzyme NSF were added to 130 nM labeled SNARE complex in the presence of excess α-SNAP (3.6 μM) and ATP (2 mM). As little as 1 nM of NSF was sufficient for optimal performance, and NSF activity was in a linear range up to a concentration of 16 nM of enzyme with speeds of disassembly of ~15 pmoles of SNARE complex per mg NSF per minute as calculated by the respective half-times. Ongoing reassembly at later points during the reaction caused by the increasing concentrations of free SNAREs suggests that these rates are probably underestimated.

b) Increasing amounts of ATP were used for disassembly of 75 nM labeled SNARE complex in the presence of 10 nM NSF and 1.25 μM α-SNAP. Each reaction was started by the addition of 5 mM MgCl\textsubscript{2} (arrow). These ATP and NSF-requirements are in line with earlier findings (Matveeva et al. 1997, JBC 272: 26413-18.)
α-SNAP contains a membrane attachment site

**Suppl. Fig. 2: Membrane anchorage of SNARE complexes is a prerequisite for higher efficiency of α-SNAP.**

Liposomes were dissolved to determine whether the actual anchorage of the SNARE complex to a membrane (rather than the mere presence of lipids or the transmembrane domains) is a prerequisite for the requirement of relatively low α-SNAP concentrations for efficient disassembly. Increasing amounts of the detergent n-dodecyl-β-D-maltoside (DDM) were used to dissolve the liposomes. Solubilization of the liposomes was monitored by FRET between NBD-PE and rhodamine-PE.

a) Due to its low critical micelle concentration, relatively low concentrations of DDM, which did not interfere with the disassembly reaction, were necessary to dissolve the liposomes. To monitor disassembly a FRET-active SNARE complex (SybTMR Alexa594: SNAP25130OG: SyxH3) was incorporated into liposome membranes.

b) Increasing amounts of DDM were added to the mix in order to dissolve increasing fractions of liposomes before the disassembly reaction was started by the addition of 120 nM α-SNAP (indicated by an arrow), which was enough to disassemble membrane-bound SNAREs swiftly. After 280 s, an additional 1.5 μM α-SNAP was added to the reaction (indicated by an arrow) to disassemble the
remaining complexes quickly. In the absence of detergent, the increase in α-SNAP concentration led to only a marginal acceleration of the reaction. At relatively low concentrations of DDM (e.g. 125 nM DDM), the second addition of α-SNAP also led to only a minor acceleration of the disassembly reaction. At high DDM concentrations (e.g. 375 & 500 nM DDM), a drastic acceleration was observed. As can be seen in a), these higher DDM concentrations disrupted a relatively large fraction of liposomes and thus solubilized more SNARE complexes, whereas only a minor fraction of liposomes was affected by the presence of 125 nM DDM. Together, this suggests that low concentrations of α-SNAP can only support efficient disassembly of membrane-bound SNARE complexes. The increase in α-SNAP concentration after 280 s then allowed for efficient disassembly of SNARE complexes that were solubilized by the detergent.

Suppl. Fig. 3: The deletion mutant α-SNAPdel does not support more efficient disassembly on liposomes.

Approximately 70 nM of FRET-active SNARE complexes (SybC28TR : SNAP25130OG; SyxH3 for reaction in solution; SybTMRAlexa594 : SNAP25130OG; SyxH3 for reaction on liposome membranes) were disassembled in solution (black line) or incorporated into liposome membranes via the transmembrane domain of synaptobrevin (blue line). At the beginning, 1.5 µM of α-SNAPdel was added before the reactions were started by the addition of 5 mM MgCl₂ (arrow). At the indicated times (arrows), an additional 1.5 µM of α-SNAPdel was added twice to the ongoing reactions. For comparison, the fluorescence signal changes of both reactions were normalized.
Suppl. Fig. 4: β-SNAP exhibits membrane dependence as well.

Approximately 70 nM of FRET-active SNARE complexes were disassembled in solution (Syb\textsuperscript{C28TR} : SNAP25\textsuperscript{130OG} : SyxH3) (a) or incorporated into liposome membranes via the transmembrane domain of synaptobrevin (SybTMR\textsuperscript{Alexa594} : SNAP25\textsuperscript{130OG} : SyxH3) (b) in the presence of 6 nM NSF. In solution, higher concentrations of the adaptors α-SNAP (0.6 μM) or β-SNAP (3.4 μM) were necessary compared to the reaction on liposome membranes (45 nM or 160 nM, respectively), demonstrating that both adaptor molecules support NSF-driven disassembly much more efficiently when the SNARE complex is inserted in membranes. Note that in both reactions, higher concentrations of β-SNAP than α-SNAP were needed. Each reaction was started by the addition of 5 mM MgCl\textsubscript{2} (arrow).
A Conserved Membrane Attachment Site in α-SNAP Facilitates N
-Ethylmaleimide-sensitive Factor (NSF)-driven SNARE Complex Disassembly
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J. Biol. Chem. 2009, 284:31817-31826.
doi: 10.1074/jbc.M109.045286 originally published online September 17, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M109.045286

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