SNAREpins Are Functionally Resistant to Disruption by NSF and αSNAP

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Abstract. SNARE (SNAP [soluble NSF {N-ethylmaleimide–sensitive fusion protein} attachment protein] receptor) proteins are required for many fusion processes, and recent studies of isolated SNARE proteins reveal that they are inherently capable of fusing lipid bilayers. Cis-SNARE complexes (formed when vesicle SNAREs [v-SNAREs] and target membrane SNAREs [t-SNAREs] combine in the same membrane) are disrupted by the action of the abundant cytoplasmic ATPase NSF, which is necessary to maintain a supply of uncombined v- and t-SNAREs for fusion in cells. Fusion is mediated by these same SNARE proteins, forming trans-SNARE complexes between membranes. This raises an important question: why doesn't NSF disrupt these SNARE complexes as well, preventing fusion from occurring at all? Here, we report several lines of evidence that demonstrate that SNAREpins (trans-SNARE complexes) are in fact functionally resistant to NSF, and they become so at the moment they form and commit to fusion. This elegant design allows fusion to proceed locally in the face of an overall environment that massively favors SNARE disruption.

Key words: membrane fusion • SNARE • NSF • αSNAP • liposomes

Introduction

Soluble N-ethylmaleimide–sensitive fusion protein (NSF)[1] attachment protein (SNAP) receptors (SNAREs) are required for many fusion processes (Söllner et al., 1993b; Hong, 1998; Bock and Scheller, 1999; Gerst, 1999; Pelham, 1999). Recent studies of isolated SNARE proteins imply a central role in the act of bilayer fusion. Isolated recombinant neuronal/exocytic vesicle and target membrane SNAREs (v- and t-SNAREs, respectively) reconstituted into separate phospholipid bilayer vesicles spontaneously pair up to form SNARE complexes that link the two bilayers (Weber et al., 1998). Assembly of such SNAREpins (or trans-SNARE complexes) at 0–4°C leads to a metastable docked state from which fusion occurs very slowly, if at all. When warmed to 37°C, a round of fusion is triggered that is complete within a few minutes, as measured by lipid mixing (Weber et al., 1998; Parlati et al., 1999) or content mixing (Nickel et al., 1999). Docking is inhibited by soluble v-SNARE cytoplasmic domain (which binds to unassembled t-SNAREs) or by botulinum neurotoxin D (BoNT D), which cleaves the cytoplasmic domain of the integral membrane protein vesicle-associated membrane protein 2 (VAMP2), also called synaptobrevin 2, the primary neuronal/exocytic v-SNARE, when it is free but not when it is assembled (Hayashi et al., 1994; Weber et al., 1998). Fusion from the docked state is resistant to both treatments.

Docking is the slowest step in the process of fusion by isolated SNAREs (Weber et al., 1998) and is limited by accessibility to the neuronal t-SNARE (Parlati et al., 1999), which consists of a heterodimer of the integral membrane protein syntaxin1 and the multiply palmitoylated synaptosome-associated protein of 25 kD (SNAP-25) (Söllner et al., 1993b; Hayashi et al., 1994; Pevsner et al., 1994). The t-SNARE apparently maintains a closed conformation that

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[1]*Abbreviations used in this paper:* BoNT D, botulinum neurotoxin D; NSF, N-ethylmaleimide–sensitive fusion protein; SNAP, soluble NSF attachment protein; SNAP-25, synaptosome-associated protein of 25 kD; SNARE, SNAP receptor; t-SNARE, target membrane SNARE; VAMP, vesicle-associated membrane protein; v-SNARE, vesicle SNARE.

involves the globular NH_2-terminal domain of syntaxin (Nicholson et al., 1998; Dulubova et al., 1999; Fiebig et al., 1999), which is not involved in the helical bundle that stabilizes the SNARE complex (Poirier et al., 1998b; Sutton et al., 1998). When this regulatory domain is removed, the overall rate of fusion increases dramatically (Parlati et al., 1999) and is sufficient to account for the overall rate of exocytosis in neuroendocrine cells that rely on the same species of SNARE proteins (Chen et al., 1999). Presumably, tethering of vesicles at target membranes and/or regulatory proteins (Kooy et al., 1992; Waters et al., 1992; Nakamura et al., 1995, 1997; TerBush and Novick, 1995; Halachmi and Lev, 1996; TerBush et al., 1996; Barlowe, 1997; Novick and Zerial, 1997; Wang et al., 1997; Grindstaff et al., 1998; Orci et al., 1998; Sacher et al., 1998; Barr, 1999; Gonzalez and Scheller, 1999; Kjer-Nielsen et al., 1999; Munro and Nichols, 1999; Waters and Pfeffer, 1999) present in cells facilitates the switching of t-SNAREs from their basal closed state to a more reactive open state.

Altogether, this evidence supports the conclusion that SNARE proteins are the minimal machinery for intracellular membrane fusion and that their pairing as SNAREpins between bilayers is at the crux of the biophysical mechanism of membrane fusion (Weber et al., 1998). This conclusion has been confirmed by biochemical studies in permeabilized neuroendocrine cells (Chen et al., 1999) and is in concordance with genetic studies using *Drosophila* (Littleton et al., 1998). The topology of the SNARE complex, with both membrane anchors emerging at the same end of a rod based on a parallel arrangement of four α-helices (Hanson et al., 1997; Lin and Scheller, 1997; Hohl et al., 1998; Poirier et al., 1998b; Sutton et al., 1998), the exceptional thermal stability of SNARE complexes (Hayashi et al., 1994; Fasshauer et al., 1998; Poirier et al., 1998a), and the striking overall similarity of the structure of the core of a SNARE complex to the structures of the cores of enveloped viral fusion proteins (Skehel and Wiley, 1998) enhance the inherent physical plausibility of this conclusion.

After fusion, v- and t-SNAREs that had paired between separate membranes now emerge from the same membrane as cis-SNARE complexes. They must be separated to allow future rounds of fusion (Mayer et al., 1996; Nichols and Pelham, 1998), and this is accomplished by the cytoplasmic proteins NSF and SNAP, whose action is needed in many fusion processes (Block et al., 1988; Malhotra et al., 1988; Wilson et al., 1989; Clary et al., 1990; Kaiser and Schekman, 1990; Griff et al., 1992; Söllner et al., 1993a; Whiteheart et al., 1993, 1994). NSF is an ATPase that couples the energy made available from ATP hydrolysis to the disruption of SNARE complexes (Söllner et al., 1993a). Three SNAP proteins coat the rod of the SNARE complex (Hayashi et al., 1995; Hohl et al., 1998), an NSF hexamer binds at the membrane-distal end (Hohl et al., 1998), and a conformational switch between ATP and ADP states of the hexamer provides the driving force for disruption of the SNARE complex (Hanson et al., 1997).

It has been reported that NSF by itself, or with SNAP protein, is fusogenic (Otter-Nilsson et al., 1999). However, this activity is unlikely to be physiologically relevant, as it is only observed with lipid mixtures that are not representative of biological membranes, and which form metastable liposomes whose coalescence is also triggered by unrelated proteins (such as glycolytic enzymes) and even magnesium ion (Brügger et al., 2000).

Implicit in the ability of NSF to disrupt SNARE complexes is a conundrum that must be resolved: since SNARE complexes are effectively disrupted after fusion by NSF, what would prevent NSF from disrupting them before fusion, preventing fusion from occurring at all? In other words, how can SNARE-dependent fusion occur in a cytoplasm containing NSF, SNAP, and ATP? A simple way to resolve this apparent paradox would follow if cis-SNARE complexes were good substrates for NSF disruption, whereas trans-SNARE complexes (SNAREpins) were poor substrates for NSF.

That this may be the case is indicated indirectly by the results of Ungermann et al. (1998), who find that fusion of yeast vacuoles, which requires SNARE pairing, is nonetheless largely resistant to NSF. Whereas most of the trans-SNARE complexes studied by Ungermann et al. (1998) were indeed disrupted by NSF, a distinct subpopulation resisted even vast excesses of NSF, implying two distinct populations of trans-SNARE complexes, one sensitive and the other resistant to disruption by NSF and αSNAP. Alternatively, as proposed by Ungermann et al. (1998), membrane fusion could require transient assembly of NSF-sensitive trans-SNARE complexes, which somehow signals the activation of an unknown pathway that results in bilayer fusion; the latter, rather than SNARE complexes, would constitute the underlying biophysical principle of fusion. Of course, this interpretation ignores the fact that isolated SNAREs can fuse bilayers.

To differentiate these hypotheses, and to address the more general conundrum noted above, we have tested whether SNAREpins mediating bilayer fusion are functionally resistant to NSF by taking advantage of the reconstituted system in which SNAREs are the only proteins present.

Materials and Methods

Protein Expression and Purification

Full-length mouse VAMP2 with a COOH-terminal his_6-tag was expressed in *E. coli* from plasmid pTW2 and purified as described (Weber et al., 1998). The t-SNARE complex between mouse his_6–SNAP-25 and rat syntaxin1A was expressed and purified as follows:

A 100-ml preculture of BL21(DE3) cells transformed with plasmid pTW34 (Parlati et al., 1999) was grown overnight at 37°C in Luria-Bertani (LB) medium containing 0.5% (wt/vol) glucose and 50 μg/ml kanamycin. This preculture was used to inoculate a 12-l containing the same medium. After overnight growth at 37°C, this culture was used to seed 300 l of LB medium containing 50 μg/ml kanamycin. The cells were grown until they reached a density of 0.8 A_{260} and were then induced with isopropylthio-β-D-galactoside (IPTG) (0.2 mM final concentration). After 1 h at 37°C, an additional 15 g of kanamycin sulfate was added and the incubation continued for an additional 3.5 h. After centrifugation, the cell paste was frozen in liquid nitrogen in three aliquots.

One aliquot of this cell paste (~900 g) was resuspended in 2 l of breaking buffer (25 mM Hepes/KOH, pH 7.40, 100 mM KCl, 10% [wt/vol] glycerol and 10 mM β-mercaptoethanol). After addition of 30 ml 200 mM PMSF in ethanol and 500 ml 20% (wt/vol) Triton X-100, the cells were disrupted by one passage through an Emulsiflex C5 cell disrupter (Avestin) at >10,000 psi. Cell debris was then removed by centrifugation in a GS3 rotor for 30 min at 8,000 rpm. The supernatant was additionally clarified by centrifugation in a Ti45 rotor for 45 min at 35,000 rpm. To this lysate, 30 ml packed Ni-NTA agarose (Qiagen) that has been washed first in breaking buffer containing 1% (wt/vol) Triton X-100 and 500 mM imida-

zole (pH adjusted to pH 7.5 with acetic acid) and then equilibrated with breaking buffer containing 1% (wt/vol) Triton X-100 was added. After mixing gently overnight at 4°C on an orbital shaker, the slurry was poured into a column and successively washed with 300 ml of (a) breaking buffer containing 1% (wt/vol) TX-100, (b) breaking buffer containing 1% (wt/vol) n-octyl-β-D-glucopyranoside, and (c) breaking buffer containing 50 mM imidazole and 1% (w/v) n-octyl-β-D-glucopyranoside. Finally, the t-SNARE complex was eluted with a linear gradient (~250 ml) to breaking buffer containing 500 mM imidazole and 1% (wt/vol) n-octyl-β-D-glucopyranoside. All fractions containing significant amounts of t-SNARE complex were pooled. In an effort to remove heat-shock proteins, 1 mM in $MgCl_2$ and 100 mg ATP-agarose (Sigma) was added to the pooled fractions. After overnight incubation at 4°C on a turning wheel, the beads were removed by filtration and the t-SNARE complex frozen in 550 μl aliquots that were stored at −80°C. The protein concentration was determined according to Schaffner and Weissmann (1973) using a bovine IgG as a standard, and was found to be 1.19 mg/ml.

The complex between his_6–SNAP-25 and a thrombin-cleavable version of syntaxin1A was expressed in *E. coli* from the polycistronic plasmid pTW69 purified by Ni-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Parlati et al., 1999). All membrane proteins and protein complexes were purified in 1% (wt/vol) n-octyl-β-D-glucopyranoside. His_6-NSF-myc (Söllner et al., 1993a) and his_6-αSNAP (Whiteheart et al., 1993) were expressed in *E. coli* and purified as described in Whiteheart et al. (1994) and Whiteheart et al. (1993), respectively, with the following modifications: the final gel filtration step in the purification of NSF was omitted and NSF was dialyzed against 25 mM Hepes/KOH, pH 7.5, 150 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 0.5 mM ATP, 15% glycerol, and αSNAP was dialyzed against 25 mM Hepes/KOH, pH 7.8, 100 mM KCl, 1 mM DTT, 10% glycerol. The expression clone for his_6–BoNT D light chain was a kind gift of Dr. Heiner Niemann (Medizinische Hochschale Hannover, Department of Biochemistry, Hannover, Germany) and the protein was expressed and purified as described (Glenn and Burgoyne, 1996). The cytosolic domain of VAMP2 (amino acids 1–94) was expressed in *E. coli* from plasmid pET-rVAMP2CD and purified as described (Weber et al., 1998).

Protein Reconstitution into Liposomes and Thrombin Cleavage of t-SNARE Liposomes

VAMP2 (2.85 mg/ml) and t-SNARE complexes (wild-type, 1.2 mg/ml; thrombin-cleavable, 2.35 mg/ml) were reconstituted into proteoliposomes by dilution and dialysis followed by a Nycodenz density gradient as described in detail previously (Weber et al., 1998). Where indicated, the NH_2-terminal domain of syntaxin was removed by thrombin cleavage as described previously (Parlati et al., 1999).

Fusion Assay and Data Analysis

Fusion reactions and data analysis were performed exactly as described previously (Parlati et al., 1999; Weber et al., 1998). In brief, standard fusion reactions (Weber et al., 1998) contained 45 μl t-SNARE liposomes and 5 μl v-SNARE liposomes and were performed in 96-well microtiter plates at 37°C. Fusion was followed by continuously measuring the increase in NBD-fluorescence at 538 nm (Excitation 460 nm). At the end of the 2-h incubation, the reactions were stopped by adding 10 μl 2.5% (wt/vol) dodecyl-maltoside. The raw NBD-fluorescence was then converted first into percent fluorescence (Weber et al., 1998) and then into rounds of fusion (Parlati et al., 1999) using the following equation: $y = 0.49666 \cdot e^{(0.036031 \cdot x)} - (0.50597 \cdot e^{(-0.053946 \cdot x)})$, where y represents rounds of fusion, and x percent fluorescence. The initial 4 min of each curve was removed to eliminate the decrease in NBD fluorescence due to the change in temperature.

Fusion Reactions Containing αSNAP and NSF

For fusion reactions containing NSF and αSNAP, the following premixes were made Mg-mix: 12 μl 1 M creatine phosphate (Roche), 12 μl 10 mg/ml creatine kinase (Roche), 3 μl 200 mM ATP (Roche), pH 7.0, with NaOH, 4 μl 200 mM $MgCl_2$, 3.5 μl his_6-αSNAP (11.9 mg/ml), and 11.5 μl his_6-NSF-myc (2.2 mg/ml). EDTA-mix was the same as Mg-mix, except that 4 μl 200 mM EDTA, pH 8.0, with NaOH, was substituted for the $MgCl_2$. Where indicated, fusion reactions (or t-SNARE liposomes in Fig. 6) received either 5 μl Mg-mix, EDTA-mix, or buffer (25 mM Hepes/KOH, pH 7.4, 100 mM KCl, 10% [wt/vol] glycerol, 1 mM DTT). Where indicated, 1 μl his_6–BoNT D light chain (2.8 mg/ml) or 1 μl soluble VAMP (5.9 mg/ml) was added. For the fusion reactions for Fig. 1, c and d, his_6-NSF-myc was omitted from the premixes. 4 μl of the respective premix was used for a fusion reaction containing 5 μl v-SNARE donor liposomes and 45 μl t-SNARE acceptor liposomes, respectively. His_6-NSF-myc was added to the final concentrations indicated and the balance, up to a total volume of 60 μl, was made up with reconstitution buffer.

Results

Docked Vesicles Are Functionally Resistant to NSF and αSNAP

v-SNARE vesicles (formed from recombinant VAMP2 and phospholipids) were incubated with t-SNARE vesicles (containing syntaxin1A and SNAP-25B) at 4°C for 18 h. These conditions allow SNAREpin formation, as demonstrated by acquisition of BoNT D resistance, but not fusion (Weber et al., 1998). Incubation was then continued at 37°C for up to 2 h in the absence or presence of NSF and αSNAP and either Mg-ATP or EDTA-ATP. Fusion was followed using a well-defined lipid-mixing assay (Struck et al., 1981) as described previously (Weber et al., 1998), and converted into rounds of fusion of v-SNARE vesicles as described (Parlati et al., 1999). The kinetics of fusion were virtually indistinguishable whether the incubation at 37°C was performed in the presence of protein-free buffer (Fig. 1 a, open diamonds), or NSF and αSNAP with either Mg-ATP (Fig. 1 a, closed circles) or EDTA-ATP (Fig. 1 a, open circles). Magnesium is required for ATP hydrolysis and SNARE complex disruption by NSF (Söllner et al., 1993a; Whiteheart et al., 1994; Hayashi et al., 1995).

In contrast, when the 4°C preincubation was omitted, that is, when SNAREpins were not allowed to form before the addition of NSF, αSNAP, and Mg-ATP, the initial rate of fusion (Fig. 1 b, closed circles) was markedly inhibited. This inhibition clearly demonstrates that the added NSF and αSNAP are functionally active and in sufficient amounts relative to the amount of SNAREs present. Interestingly, over the timecourse of the incubation at 37°C, the initial inhibition of the fusion reaction disappears with what appears to be a half-time of 15–20 min (Fig. 1 b), and eventually the same rate (slope) of fusion is achieved with or without αSNAP, NSF, and Mg-ATP. This is not due to a loss of function of either αSNAP or NSF, nor is it due to exhaustion of hydrolysable ATP or creatine phosphate, as adding more αSNAP, NSF, ATP, and ATP-regenerating system after 30 min of incubation at 37°C had no additional effect on the rate of fusion (data not shown). In contrast, these results indicate that functional SNAREpins can form even in the presence of NSF and αSNAP. The initial inhibition is likely caused by an NSF-induced disruption of the t-SNARE complex (see below). Fusion kinetics were unaffected by NSF and αSNAP when ATP hydrolysis was prevented by the addition of EDTA (Fig. 1 b, open circles). The molar ratio of NSF to αSNAP to t-SNAREs in these experiments was ~1.2:3.1:1.

NSF is expected to act catalytically. However, to assure that NSF was not limiting we titrated the amount of his_6-NSF-myc. The addition of up to 220 μg/ml his_6-NSF-myc (4.4 times higher concentrations than in standard experiments such as in Fig. 1, a and b) had only a marginal effect on both initial rate and final extent of fusion (Fig. 1 c) when v- and t-SNARE liposomes were allowed to dock

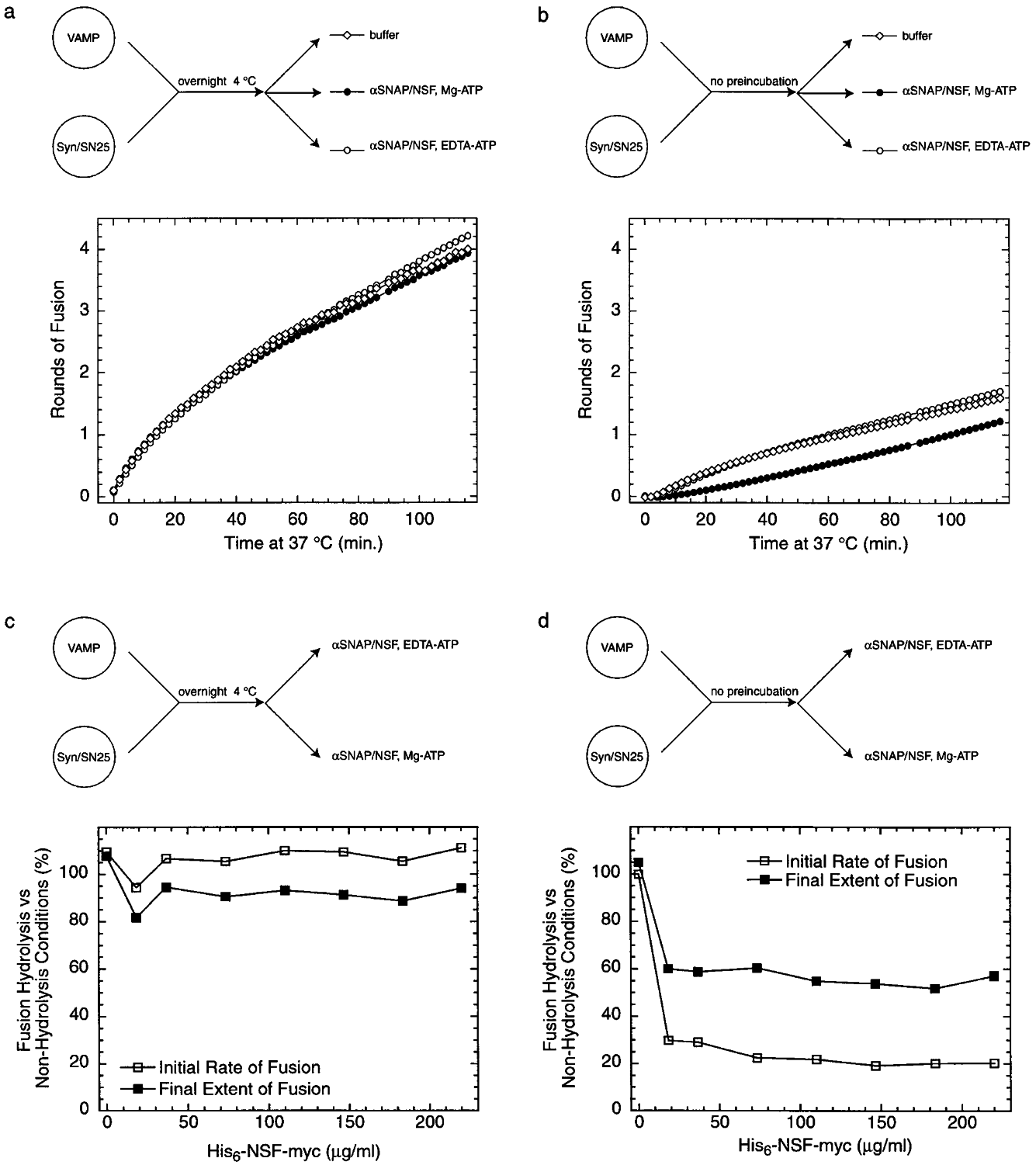

Figure 1. (a) Docked v-t-SNARE vesicles, accumulated during a preincubation at 4°C, conditions under which vesicles do not fuse, are resistant to a subsequent challenge with αSNAP and NSF under fusion conditions. Standard amounts of v- and t-SNARE vesicles were incubated overnight at 4°C. Incubation was then continued at 37°C in the presence of buffer (open diamonds), αSNAP, NSF, and Mg-ATP (closed circles) or αSNAP, NSF, and EDTA-ATP (open circles). Fusion was followed by a probe-dilution assay (Struck et al., 1981; Weber et al., 1998) and converted into rounds of fusion as described previously (Parlati et al., 1999). Data from three independent experiments demonstrated that addition of αSNAP and NSF did not significantly influence fusion. In presence of Mg-ATP, the initial rate of fusion (rounds of fusion at 20 min) was 94.1 ± 6.0% (SD) and the overall extent of fusion (rounds of fusion at 120 min) was 94.0 ± 7.9% (SD), respectively, when compared with buffer control; in presence of EDTA-ATP, the corresponding figures were 93.5 ± 3.9% (SD) and 103.7 ± 7.9% (SD), respectively. (b) Fusion of v- and t-SNARE vesicles without previous preincubation shows a lag phase in the presence αSNAP and NSF. v- and t-SNARE vesicles were incubated at 37°C in the presence of buffer (open diamonds), αSNAP, NSF, and Mg-ATP (closed circles) or αSNAP, NSF, and EDTA-ATP (open circles). Fusion was followed and normalized as

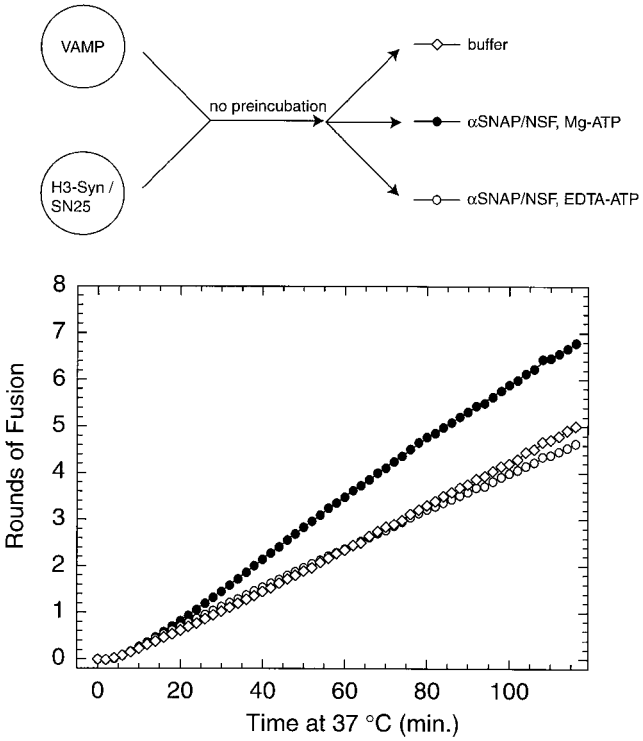

Figure 2. αSNAP and NSF stimulate fusion between v- and t-SNARE vesicles lacking the NH_2-terminal domain of syntaxin. The NH_2 terminus of syntaxin was removed by thrombin treatment of t-SNARE liposomes containing a thrombin-cleavable version of syntaxin as described (Parlati et al., 1999). Standard amounts of v- and t-SNARE vesicles lacking the NH_2-terminal domain of syntaxin were incubated at 37°C in the presence of buffer (open diamonds), αSNAP, NSF, and Mg-ATP (closed circles), or αSNAP, NSF, and EDTA-ATP (open circles). Fusion was followed and normalized as described in Fig. 1 a.

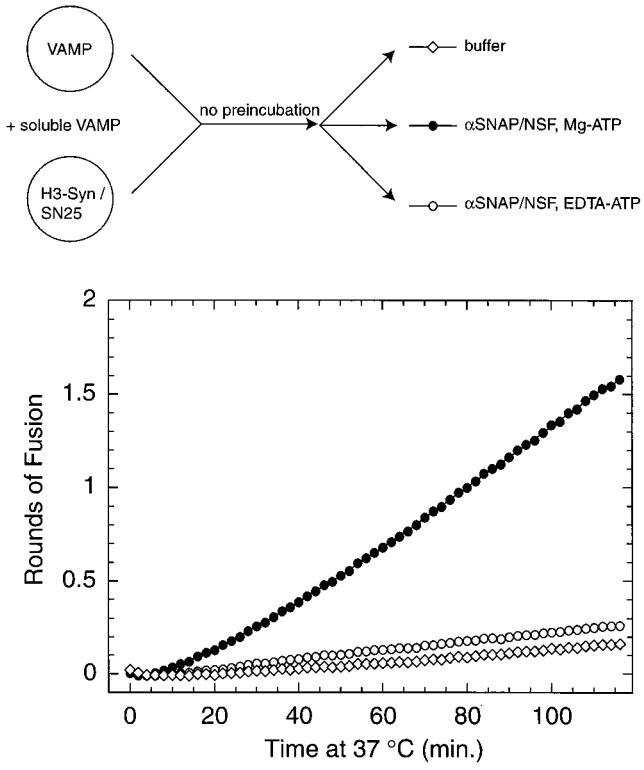

Figure 3. αSNAP and NSF can disrupt bona fide cis-SNARE complexes. The NH_2 terminus of syntaxin was removed by thrombin treatment of t-SNARE liposomes containing a thrombin-cleavable version of syntaxin as described (Parlati et al., 1999). v- and t-SNARE vesicles lacking the NH_2-terminal domain of syntaxin (Parlati et al., 1999) were incubated at 37°C in the presence of cytosolic domain VAMP and in the presence of buffer (open diamonds), αSNAP, NSF, and Mg-ATP (closed circles), or αSNAP, NSF, and EDTA-ATP (open circles). Fusion was followed and normalized as described in Fig. 1 a.

overnight at 4°C. When this preincubation was omitted, addition of very low amounts of NSF (2.7 times lower concentrations than in standard experiments such as in Fig. 1, a and b) led to a considerable reduction of both initial rate and final extent of fusion (Fig. 1 d) consistent with a catalytic role of NSF.

NSF and αSNAP Stimulate Fusion in the Absence of the Regulatory NH_2-Terminal Domain of Syntaxin

When v-SNARE vesicles are incubated at 37°C (without a preincubation at 4°C) with t-SNARE liposomes that lack the NH_2-terminal domain of syntaxin (H3-syntaxin), the initial rate of fusion is virtually identical whether buffer, NSF, αSNAP, and Mg-ATP, or NSF, αSNAP, and EDTA-ATP are added to the reaction (Fig. 2). However, after ~15 min, the rate of fusion of the sample containing NSF, αSNAP, and Mg-ATP markedly increases. This increase begins after about one round of fusion. Since SNAREpins form more rapidly in the absence of the NH_2-terminal domain (Parlati et al., 1999), SNAREpin assembly should be favored relative to SNAREpin disassembly. The disassem-

described in a. Data from three independent experiments demonstrated that the results were highly reproducible. In presence of Mg-ATP, the initial rate and overall efficiency of fusion compared with the buffer control (as defined in a) were 21.8 ± 4.7% (SD) and 66.8 ± 9.0% (SD), respectively; in presence of EDTA-ATP, the corresponding figures were 94.1 ± 7.8% (SD) and 106.4 ± 4.6% (SD), respectively. (c) SNAREpins are functionally resistant to high amounts of NSF. v- and t-SNARE vesicles were incubated overnight (19.5 h) at 4°C. αSNAP and the indicated amounts of NSF were added and the incubation continued at 37°C in presence of either Mg-ATP or EDTA-ATP. Fusion was followed and normalized as described in a. As a measure for the initial rate, the fusion after 20 min under ATP hydrolysis conditions (i.e., in the presence of Mg-ATP) is expressed in percent of fusion under nonhydrolysis conditions (i.e., in the presence of EDTA-ATP) and plotted versus NSF concentration (open squares). As a measure of the overall extent of fusion, fusion at 120 min in the presence of Mg-ATP is expressed in percent of fusion in the presence of EDTA-ATP and plotted versus NSF concentration (closed squares). (d) SNAREpins can form even in the presence of high amounts of NSF. v- and t-SNARE vesicles were incubated at 37°C in the presence of αSNAP, and the indicated amounts of NSF and Mg-ATP or EDTA-ATP. Fusion was followed and normalized as described in a. Further analysis was performed as described in c. Open squares indicate initial rate of fusion, and closed squares indicate overall extent of fusion.

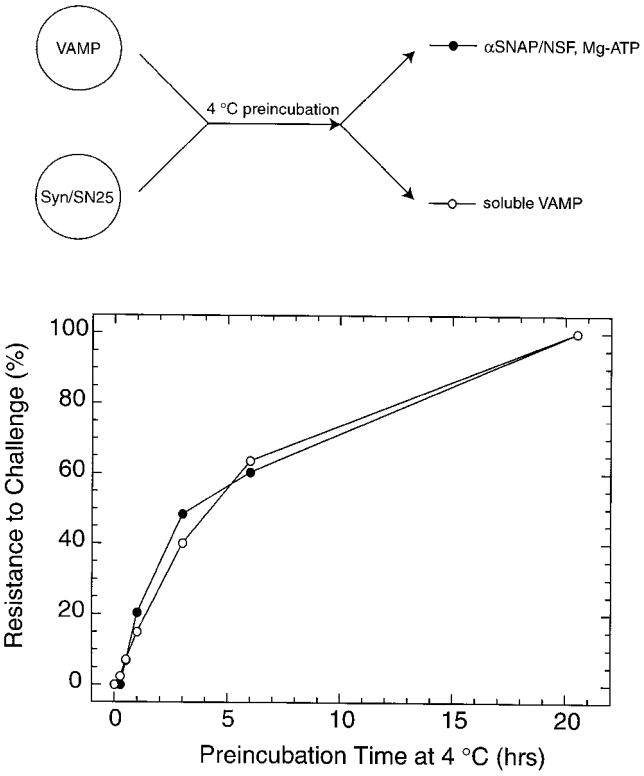

Figure 4. The development of resistance to challenge with αSNAP and NSF shows the same kinetics as development of resistance to challenge with soluble VAMP, i.e., SNAREpin formation. Standard amounts of v- and t-SNARE vesicles were incubated for various times at 4°C. Incubation was then continued at 37°C in the presence of buffer, αSNAP, NSF, and Mg-ATP or soluble VAMP. Fusion was followed and normalized as described in Fig. 1 a. The ratio of rounds of fusion at 20 min (representing a measure for initial rates) between buffer and αSNAP and NSF (closed circles) or buffer and soluble VAMP (open circles) was then determined. Resistance was then normalized by setting the initial resistance in the absence of any preincubation to 0% and the resistance after overnight preincubation to 100%. Percent resistance was plotted as a function of preincubation time at 4°C.

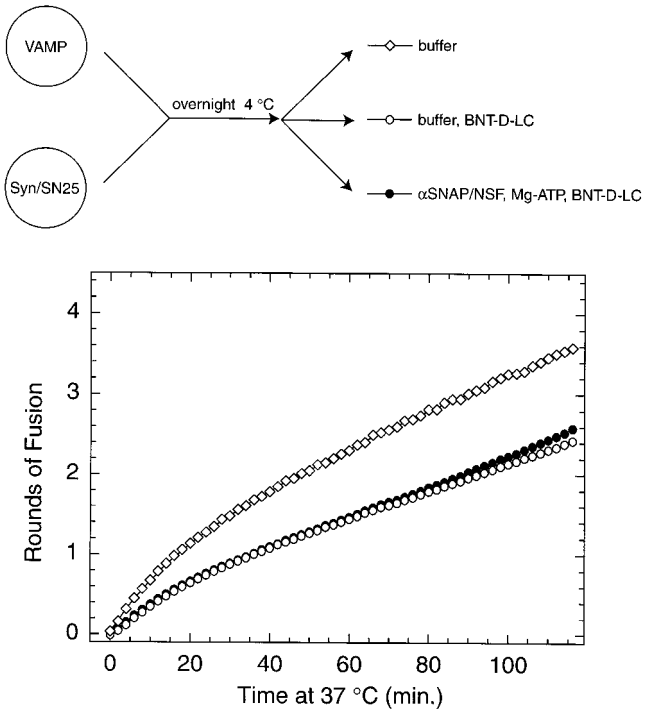

Figure 5. SNAREpins are resistant to a challenge with αSNAP and NSF and BoNT D light chain. Standard amounts of v- and t-SNARE vesicles were incubated overnight at 4°C. The incubation was then continued at 37°C in the presence of buffer (open diamonds), BoNT D–light chain (open circles) or BoNT D–light chain and αSNAP, NSF, and Mg-ATP (closed circles). Fusion was followed and normalized as described in Fig. 1 a.

bly of cis-SNARE complexes formed upon fusion will regenerate free SNAREs that then can engage in another round of fusion.

NSF and αSNAP Can Disrupt Bona Fide cis-SNARE Complexes

The experiments shown in Fig. 1, c and d, strongly indicated that we used sufficient amounts of NSF and αSNAP under our standard conditions (e.g., Fig. 1 a). To provide additional evidence for this, we performed the experiment shown in Fig. 3. Here, v-SNARE vesicles and t-SNARE liposomes that lack the NH_2-terminal domain of syntaxin (H3-syntaxin) were incubated in the presence of sufficient cytosolic domain of VAMP to almost completely inhibit fusion (Fig. 3, open diamonds). The addition of NSF, αSNAP, and Mg-ATP reversed this inhibition (Fig. 3, closed circles) in a magnesium-dependent fashion (Fig. 3, open circles).

For the inhibition of fusion to be reversed under these conditions, cis-SNARE complexes (consisting of VAMP cytoplasmic domain and transmembrane-bound t-SNAREs) must have been disrupted, resulting in the release of the cytoplasmic domain of VAMP from the t-SNARE complex and allowing subsequent SNAREpin formation. At the same time, these SNAREpins must have been functionally resistant to the action of NSF and αSNAP to have allowed fusion to proceed.

Development of Resistance to NSF Parallels SNAREpin Formation

Fusion by SNAREpins, preformed at 4°C, is resistant to challenge with the cytosolic domain of the v-SNARE VAMP (Weber et al., 1998). If the resistance of SNARE-dependent fusion to NSF is due to resistance of SNAREpins to NSF, the timecourse of acquisition of resistance to VAMP and NSF should be similar. To test this, we preincubated v- and t-SNARE vesicles together for variable times at 4°C. At time 0, either cytosolic domain VAMP or NSF, αSNAP, and Mg-ATP were added and incubations were continued at 37°C for 20 min to measure the initial rate of fusion. The ratio of fusion in the presence of inhibitor (i.e., soluble VAMP or NSF) to that in their absence was then determined as a function of time of preincubation. These results were then normalized to a scale obtained by setting the ratio without preincubation to 0% and the ratio after 20 h preincubation to 100%. Fig. 4 shows resistance to in-

hibitor on this scale versus preincubation time. The timecourse for obtaining resistance to the cytosolic domain of VAMP (Fig. 4, open circles) and to NSF, αSNAP, and Mg-ATP (Fig. 4, closed circles) are very similar and develop over a timecourse of several hours.

SNAREpins Are a Very Poor Substrate of NSF and αSNAP

The above results imply that SNAREpins are not an effective substrate for disruption by NSF and αSNAP activity. To further confirm this, we took advantage of BoNT D, which proteolytically cleaves the v-SNARE VAMP in its free form. When assembled in a v-SNARE–t-SNARE complex, however, VAMP is not a substrate for BoNT D (Hayashi et al., 1994). Cleavage of VAMP by BoNT D completely inhibits SNAREpin-mediated fusion when v-SNARE liposomes are preincubated with BoNT D (Weber et al., 1998). In contrast, when SNAREpins are allowed to form by preincubating v- and t-SNARE liposomes extensively at 4°C, subsequent fusion at 37°C is now substantially resistant to BoNT D (Fig. 5, open circles) (Weber et al., 1998).

If SNAREpins were significantly disassembled by NSF and αSNAP, one would predict that VAMP should now become accessible for BoNT D, which in turn would significantly reduce the extent of fusion. This is clearly not the case. v- and t-SNARE liposomes were preincubated at 4°C to allow SNAREpin formation, and αSNAP, NSF, and Mg-ATP were added together with BoNT D. BoNT D had the same effect on subsequent fusion whether NSF and SNAP were absent or present as shown in Fig. 5 (compare open and closed circles). This data confirms that SNAREpins are a poor substrate of NSF and αSNAP.

t-SNARE Liposomes Are Sensitive to the Action of NSF and αSNAP

Why do NSF and αSNAP inhibit fusion reactions when v- and t-SNARE vesicles have not been preincubated (Fig. 1 b)? It has been shown that NSF and αSNAP can induce a conformational change in syntaxin alone (Hanson et al., 1995) and that the t-SNARE complex can be disassembled by NSF and αSNAP as well (data not shown) (Hayashi et al., 1995; Kee et al., 1995; Ungermann and Wickner, 1998). Consequently, it is likely that disrupting free t-SNAREs accounts for the inhibition of fusion by NSF and αSNAP in the initial phase of Fig. 1 b. To test this prediction, we preincubated t-SNARE liposomes (in the absence of v-SNARE liposomes) with NSF and αSNAP and Mg-ATP for 15 min at 37°C. These conditions render SNAP-25 accessible for cleavage by BoNT E, indicative of t-SNARE complex disruption (data not shown). Then the magnesium was chelated by addition of EDTA to stop NSF action, v-SNARE liposomes were added, and fusion followed at 37°C. When we compared the extent of fusion of this reaction to controls in which the t-SNARE liposomes were preincubated in the presence of NSF and αSNAP but with EDTA-ATP or in simple buffer, we observed a significant reduction in both the rate and extent of fusion (compare closed circles to open circles and open diamonds in Fig. 6). Evidently, NSF, αSNAP, and Mg-ATP are able to disrupt t-SNARE complexes, lowering the rate of fusion.

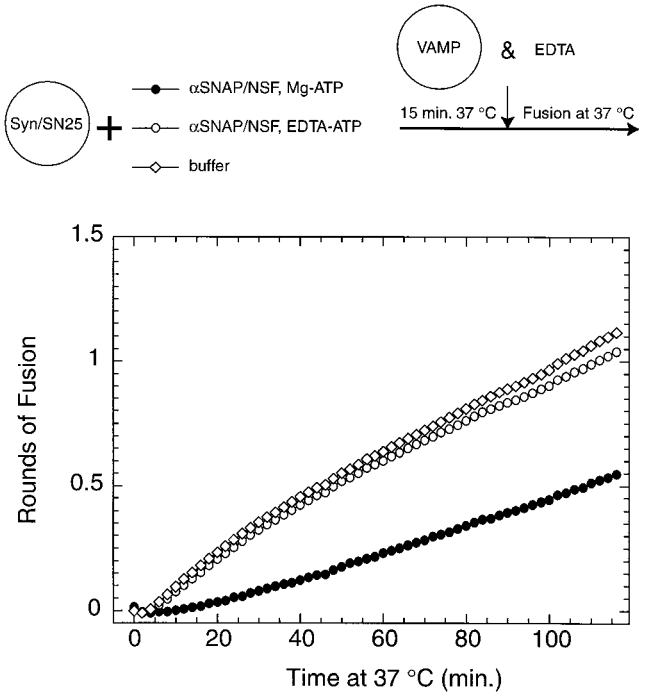

Figure 6. Preincubation of t-SNARE vesicles with αSNAP and NSF inhibits fusion of these vesicles with v-SNARE vesicles. Standard amounts of t-SNARE vesicles were preincubated for 15 min at 37°C in the presence of buffer (open diamonds), αSNAP, NSF, and Mg-ATP (closed circles), or αSNAP, NSF, and EDTA-ATP (open circles). After addition of EDTA (1 μl 200 mM, pH 8.0) and standard amounts of v-SNARE vesicles, the incubation at 37°C was continued and fusion was followed and normalized as described in Fig. 1 a.

These results also help to explain why the initial fusion rate of reactions containing the full-length syntaxin is reduced by NSF (Fig. 1 b), in contrast to reactions containing a syntaxin construct that lacks the NH_2-terminal regulatory domain (Fig. 2). It is likely that NSF and αSNAP can disrupt cis-SNARE complexes formed after an initial round of fusion both in the presence or absence of the NH_2-terminal domain of syntaxin. In principle, this should lead to an increased rate of fusion after the first round under either condition. However, because the NH_2-terminal domain of syntaxin inhibits SNAREpin formation (Parlati et al., 1999), free t-SNARE complexes would be much more abundant in reactions containing full-length syntaxin (Fig. 1 b) than in the absence of the NH_2-terminal regulatory domain (Fig. 2). These free t-SNARE complexes can be disrupted by NSF and αSNAP, leading to the observed inhibition of fusion in the presence of the NH_2-terminal domain of syntaxin (Fig. 1b).

Discussion

The experiments presented here document that fusion relying on SNAREpins is not adversely affected by NSF, even when stoichiometric amounts of NSF and SNAP are added, together with excess Mg-ATP, provided that SNAREpins have already assembled. Before assembly, the NSF system inhibits fusion by acting on free t-SNAREs.

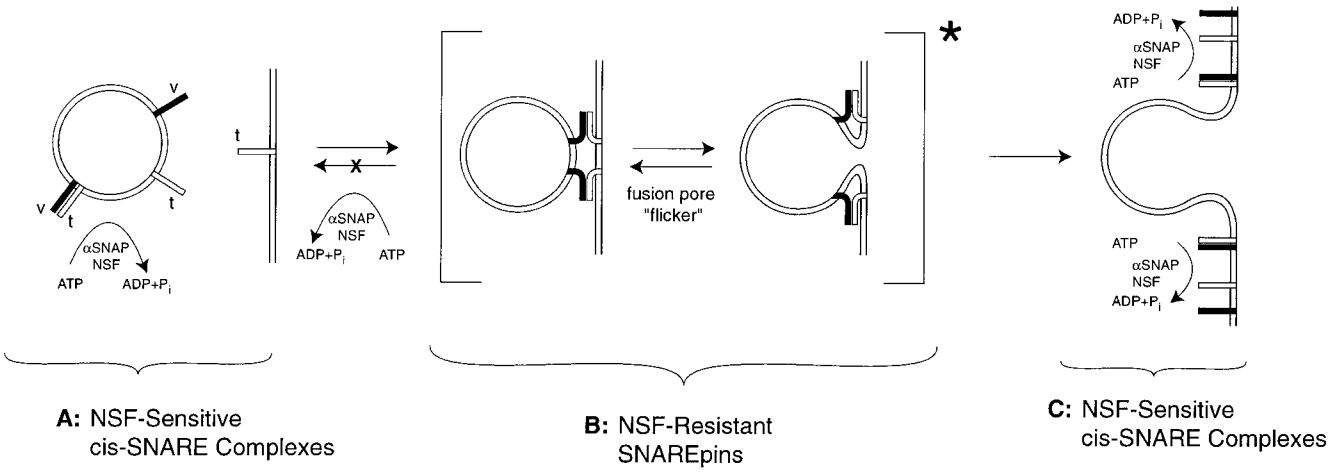

Figure 7. Proposed life cycle of SNAREs. SNARE complexes residing in the same membrane (cis-SNARE complexes) are disrupted by αSNAP and NSF. The energy needed for this process is provided by NSF-catalyzed hydrolysis of ATP. SNAREpins (complexes between vesicles, also called trans-SNARE complexes), on the other hand, are resistant to the action of αSNAP and NSF. It is at this stage that the so-called flickering of the fusion pore (a rapid opening and closing of the fusion pore) might occur. Once the fusion pore is fully dilated, cis-SNARE complexes are formed that are, again, sensitive to αSNAP and NSF. The disruption of cis-SNARE complexes regenerates the SNAREs for a new round of docking and fusion. ATP hydrolysis by NSF supplies the necessary energy input at this point. The resistance of SNAREpins to the action of αSNAP and NSF makes the formation of these SNAREpins, and hence, docking of vesicles, an essentially irreversible step. This confers vectoriality to this process pushing the process to completion, i.e., full fusion.

But even this inhibition (really a delay) in fusion is fully but gradually reversed as t-SNAREs likely reassemble (from separated syntaxin and SNAP-25) (Söllner et al., 1993a) and become resistant to NSF. This happens when they capture v-SNAREs, as shown by the simultaneous acquisition of resistance to NSF, inhibition by cytoplasmic domain of VAMP, and cleavage by a botulinum toxin.

What could account for this functional resistance of trans-SNARE complexes engaged in bilayer fusion (SNAREpins) to disruption by NSF, when virtually the same (cis) complex is sensitive under the same prevailing conditions? Any number of nonexclusive mechanisms can be envisioned, including the following: one possibility is that SNAREpins could be intrinsically resistant to NSF because they assemble into a higher-order assembly, such as a ring. Another possibility is that partially zipped-up trans-SNARE complexes, a proposed intermediate in fusion (Katz et al., 1998; Sutton et al., 1998; Fiebig et al., 1999; Hua and Charlton, 1999; Xu et al., 1999), may not bind SNAPs (Hohl et al., 1998) and therefore not bind NSF. A third possibility is that SNAREpins resist NSF due to a steric exclusion mechanism. SNAREpins, as an intrinsic part of the fusion mechanism, pin the two bilayers closely together (the diameter of the SNARE complex rod is ~2 nm; Hanson et al., 1997; Hohl et al., 1998; Sutton et al., 1998). If sufficiently rigid, this interface would exclude NSF (diameter 13–15 nm) (Whiteheart and Kubalek, 1995; Hanson et al., 1997; Fleming et al., 1998; Hohl et al., 1998). A fourth and different class of possibility is that NSF in fact disrupts SNAREpins, but they rapidly reassemble (before vesicles can dissociate) due to a high local concentration maintained by multivalent docking of vesicles; in other words, the SNAREpins would be functionally but not structurally resistant. However, this fourth possibility seems relatively unlikely, since botulinum toxin would then be expected to cleave the proposed transiently liberated v-SNAREs and thereby inhibit fusion in the presence of NSF, SNAP, and Mg-ATP, and it did not have this effect.

We use the term functional resistance to connote that it remains to be established whether NSF resistance is due to a structural or functional mechanism. Whatever the precise mechanism turns out to be, it is now clear that a mechanism exists that allows SNARE-dependent fusion to occur in a cytoplasm rich in NSF, providing a satisfying explanation of the result of Ungermann et al. (1998) that excess NSF does not inhibit fusion of isolated vacuoles. It also explains why a fraction of the trans-SNARE complexes remain despite the addition of vast excess of NSF; these would be the very complexes mediating lipid bilayer fusion. The trans-SNARE complexes that are sensitive to NSF in Ungermann et al. (1998) are evidently not important for fusion, maybe due to incorrect inter-membrane topology. Alternatively, these complexes might not have attained the conformation committed to fusion. Two structurally distinct populations of trans-SNARE complexes might also help explain similar observations in other systems (Coorssen et al., 1998; Tahara et al., 1998).

The finding that SNAREpins are functionally resistant to NSF adds a deeper appreciation of the role of NSF and SNAPs in intracellular membrane fusion (Fig. 7). The sensitivity of cis-SNARE complexes to NSF assures the availability of uncombined SNAREs before fusion (Fig. 7 A; mainly applies to homotypic fusion), and after fusion assures that v-SNAREs are separated from t-SNAREs (Fig. 7 C) to permit their return to donor membranes (applies to heterotypic fusion). In between (Fig. 7 B), SNAREpins become resistant to NSF. It is this elegant design that allows fusion to proceed in the face of a macro-environment that massively favors SNARE complex disruption.

Because SNAREpins are functionally resistant to disruption by NSF and αSNAP, SNAREpin formation (Fig.

7, A to B) is an essentially irreversible step, making the overall process (Fig. 7, A to C) vectorial. In addition, it is also possible, but entirely speculative, that NSF action after fusion (Fig. 7 C) further helps imparting vectoriality to the fusion mechanism. It is known that the formation of a fusion pore (Fig. 7 B, right panel) is a reversible event; it can either disappear (usually transiently, in a process called flicker [Lindau and Almers, 1995]) or it can open irreversibly (transition from right half of Fig. 7 B to Fig. 7 C). Rapid consumption of cis-SNARE complexes in Fig. 7 C but not the SNAREpins in Fig. 7 B would apparently increase the overall rate of fusion pore opening and decrease the rate of flicker, and therefore increase the overall flux through the fusion process.

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