The effects of SNAP/SNARE complexes on the ATPase of NSF

Elena Matveeva, Sidney W. Whiteheart*

Department of Biochemistry, Chandler Medical Center, University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY 40536-0084, USA

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Abstract The ATPase of the *N*-ethylmaleimide sensitive factor (NSF) appears to be central to the events that culminate in vesicle-target membrane fusion. Complexes containing different combinations of NSF, α-SNAP, Vamp-2 (synaptobrevin 2), syntaxin 1, and SNAP-25 were reconstituted and then tested for their effect on the ATPase of NSF. While NSF interacts with all α-SNAP-containing complexes, only the α-SNAP/t-SNARE complex significantly stimulated ATPase activity. This stimulation was dependent on increasing SNAP/t-SNARE complex and was saturable. The apparent stimulation of ATPase activity is due to a 10-fold increase in initial hydrolysis rate. Complex containing both v- and t-SNAREs bound significantly more α-SNAP but did not stimulate the ATPase of NSF.

Key words: *N*-Ethylmaleimide sensitive factor; Soluble NSF attachment protein; SNAP receptor; Membrane fusion; AAA ATPase; Vesicular traffic

1. Introduction

The SNARE hypothesis, as originally stated [1], has given a useful paradigm with which to dissect the processes that lead to transport vesicle-target membrane fusion. A recent adaptation of this model holds that an integral membrane protein from the vesicle (v-SNARE, i.e. Vamp-2 (synaptobrevin 2)) pairs with a heterodimeric complex in the target membrane (t-SNAREs, i.e. syntaxin 1 and SNAP-25) to form a complex (called the 7S complex) which is necessary and sufficient to promote bilayer fusion [2,3]. Other cytosolic, accessory factors such as the *N*-ethylmaleimide sensitive factor (NSF) and its adapters, the soluble NSF attachment proteins (SNAPs) bind to the SNAP receptor (SNARE) complexes and facilitate membrane fusion; however, they exert their effect either before or after the actual fusion event [4,5]. These soluble proteins are essential for the in vitro reconstitution of several vesicular transport processes, yet they are not required for membrane fusion of defined SNARE-containing liposomes [2]. These data would suggest that the soluble proteins such as NSF and SNAPs are not specifically required for membrane fusion but are required for efficient SNARE-mediated fusion through SNARE activation or SNARE recycling.

The critical role of NSF and its ability to hydrolyze ATP is well established [6–8]. NSF is required for a number of vesicular trafficking events and mutations in its D1 ATP-binding site render it inhibitory to transport [6,7]. What has remained elusive is the actual step which NSF catalyzes. Recent evidence shows that NSF may act as a molecular chaperone using ATP hydrolysis to modify the conformation of a complex of SNAP and SNARE proteins [9–13]. It would appear that NSF, by 'unwinding' these complexes, creates a partially stable intermediate that can subsequently participate in vesicle-target membrane fusion. As with many chaperone molecules (i.e. [14]), the ATPase activity of NSF appears to be stimulated by the complex to which it is bound. Binding of α-SNAP to the SNAREs is thought to induce a conformational change in α-SNAP making it competent to interact with NSF. This has been mimicked in vitro by adsorbing α-SNAP to a plastic surface [15–17]. Using this assay, Burgoyne and colleagues have demonstrated that α-SNAP can stimulate the ATPase activity of NSF and that a conserved residue in the SNAP C-terminus is required [18,19]. Since α-SNAP acts as an adapter to correctly position NSF on the SNARE complex, the question arises as to what aspects of the SNARE complexes are required for this α-SNAP-mediated stimulation of NSF's ATPase. In this study, we demonstrate that α-SNAP bound to the t-SNARE complex of syntaxin and SNAP-25 is sufficient to stimulate the initial rate of ATP hydrolysis by NSF. Interestingly, the 7S complex containing α-SNAP/syntaxin/SNAP-25 and Vamp-2 did not significantly stimulate NSF's ATPase activity though it, too, was disassembled.

2. Materials and methods

2.1. Materials

[α-^{32}P]ATP (800 Ci/mmol) was purchased from ICN (Costa Mesa, CA, USA). ATP and ATPγS (adenosine 5′-*O*-(thiotriphosphate)) were obtained from Boehringer Mannheim (Indianapolis, IN, USA). Glutathione immobilized on cross-linked 4% beaded agarose and chemicals for electrophoresis were from Sigma (St. Louis, MO, USA), polyethyleneimine cellulose plates for thin layer chromatography were from Selecto Scientific (Norcross, GA, USA). All chemicals were of reagent grade.

2.2. Production and purification of recombinant proteins

His_6-NSFmyc, His_6-α-SNAP, His_6-SNAP-25, His_6-Vamp-2 (cytosolic domain, 1–94 a.a.) and GST-syntaxin 1 (cytosolic domain 1–265 a.a.) were produced as recombinant proteins in *Escherichia coli* and purified as described previously [8,20]. Protein concentrations were measured using the Bio-Rad protein assay reagent and ovalbumin as a standard.

2.3. 20S particle and SNAP/SNARE complex formation

The complex formation procedure was modified from our previously described method [21]. GST-syntaxin 1 (cytosolic domain) was incubated with preswollen glutathione-agarose beads (100 μg protein per 100 μl beads) at 4°C in phosphate-buffered saline with 0.01% Tween 20, 0.1% β-mercaptoethanol and 2 mM EDTA. After 1 h of incubation, the beads were washed four times in the same buffer, and then equal volumes of the beads were aliquotted into the reaction tubes. Particle formation reactions were performed in a final volume of 500 μl containing 15 μl beads with GST-syntaxin 1 in binding buffer (20 mM HEPES/KOH, pH 7.4, 250 mM imidazole, 150 mM potassium acetate, 5 mM EGTA, 1 mM ATPγS, 5 mM $MgCl_2$, 1% (w/v) glycerol, 1% (w/v) Triton X-100 and 10% (w/v) ovalbumin) and

*Corresponding author. Fax: (1) (606) 323-1037.
E-mail: whitehe@pop.uky.edu

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saturating amounts of α-SNAP, SNAP-25, Vamp-2 and NSFmyc or different combinations of these proteins. After 3 h of incubation at 4°C with rotation, the beads were washed five times in binding buffer without ovalbumin. The bound proteins were eluted with SDS-polyacrylamide gel electrophoresis sample buffer or used for ATPase assay.

2.4. Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed under reducing conditions on 12.5% slab gel in a discontinuous buffer system according to Laemmli [22]. All gels were stained with 0.02% Coomassie Brilliant Blue R-250, 40% methanol and 10% acetic acid, washed and scanned with UMAX scanner (UMAX Data Systems, Taiwan, ROC) and Photoshop 3.0 software (Adobe Systems, San Jose, CA, USA). NIH Image 1.59 was used to estimate the amount of binding proteins by measuring pixel density.

2.5. Assay of NSF activity

ATPase assay was performed as a modification of the one described previously [23] using [α-^{32}P]ATP in a buffer containing 25 mM Tris-HCl, pH 7.6, 100 mM KCl, 0.5 mM 1,4-dithiothreitol, 1 mM $MgCl^2$, 1% glycerol, 1 mM ATP, 10 μCi of [α-^{32}P]ATP, 10 μg NSF and indicated amount of SNAP/SNARE complex. At given time points aliquots (2 μl) of each reaction were spotted on a polyethyleneimine thin layer plate and chromatographed in 0.7 M LiCl and 1 M acetic acid. Plates were dried and then analyzed using a PhosphorImager from Molecular Dynamics (Sunnyvale, CA, USA). ATPase activity was measured by determining the percent of ADP produced relative to the total nucleotide present. In no reactions was there any AMP produced.

3. Results and discussion

To determine the effect of the SNAP/SNARE complexes on the ATPase activity of NSF, it was first necessary to demonstrate that correct complexes can be produced using recombinant proteins. Complexes were constructed on glutathione agarose beads using a syntaxin 1-glutathione-*S*-transferase (GST) fusion protein, His_6-SNAP-25, His_6-α-SNAP, and His_6-Vamp-2. The concentrations of the complex constituents were varied systematically to determine the amounts required for saturation binding, thus insuring that the complexes formed and used were as homogeneous as possible. Fig. 1A shows a Coomassie Blue stained SDS-PAGE gel of the resulting complexes formed from the indicated components. As predicted, α-SNAP binds in all cases that GST-syntaxin 1 is present [24,25]. This binding increases slightly as more components are added to the complex but the most significant increase occurs when NSF is added (Fig. 1B). As expected, α-SNAP has little effect on SNAP-25 binding, yet SNAP-25 binding did lead to a slight increase in α-SNAP binding. As has been shown, SNAP-25 binding is required for the stable binding of Vamp-2 to the GST-syntaxin 1 [26]. NSF binding is dependent on α-SNAP and α-SNAP binding was enhanced by NSF regardless of the other SNAREs present (Fig. 2A). These data demonstrate that the complexes formed with recombinant proteins exhibit the same properties as have been reported by a number of groups and justify their use in the examination of their effect on the ATPase of NSF. It is important to note that no α-SNAP is present in Fig. 1A, lane 1 (see also Fig. 2A, lane 1). This demonstrates that the addition of 10% ovalbumin in the complex formation step effectively inhibits the adherence of α-SNAP to both the agarose beads and the plastic surface of the reaction tube.

NSF binding to the partial and complete SNAP/SNARE complexes appears to be quite similar when α-SNAP is present (Fig. 2A). NSF binds to the α-SNAP/GST-syntaxin 1 complex (lane 2) to approximately the same extent as it does to complexes which also contain SNAP-25 (lane 3) or SNAP-25 and Vamp-2 (lane 4). When α-SNAP is omitted (lane 5), NSF does not bind. When GST-syntaxin 1 is omitted (lane 1), no complexes are recovered on the GSH-agarose beads. These complexes were readily assembled under non-hydrolysis conditions, i.e. ATP-γ-S/EDTA. When ATP/Mg^{2+} was added for 30 min, each NSF containing complex 'disassembled' releasing the non-GST tagged proteins into the supernatant (data not shown). Taken together, these data indicate that these in vitro-formed SNAP/SNARE complexes are competent to form the appropriate interactions required for NSF binding and ATP-dependent disassembly.

To determine the effect of the various complexes on the

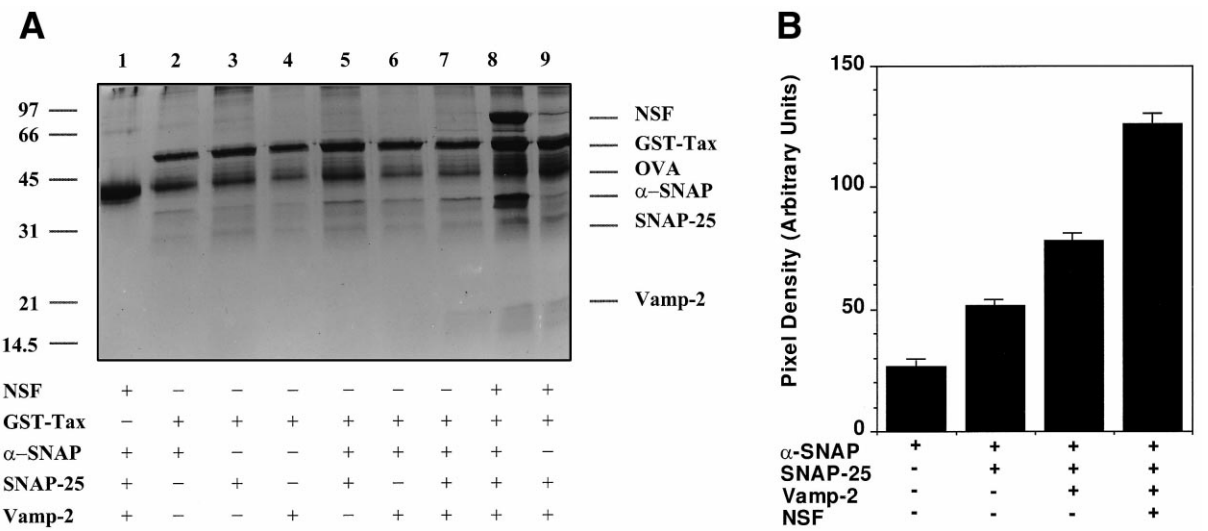

Fig. 1. 20S particle and SNAP/SNARE complex formation. A: A Coomassie Blue stained SDS-PAGE gel of the complexes formed from the indicated combinations of NSF, GST-syntaxin 1 (GST-Tax), α-SNAP, SNAP-25, and Vamp-2 in the presence of ATPγS and EDTA. Saturating amounts of each protein were used and complexes were captured on GSH-agarose beads. B: Analysis of binding of α-SNAP to GST-syntaxin 1 in the presence of different components of 20S particle. Densitometry was used to determine the amount of α-SNAP associated with the beads under each of the conditions in panel A. The protein density for each was normalized to the GST-Tax in the same lane.

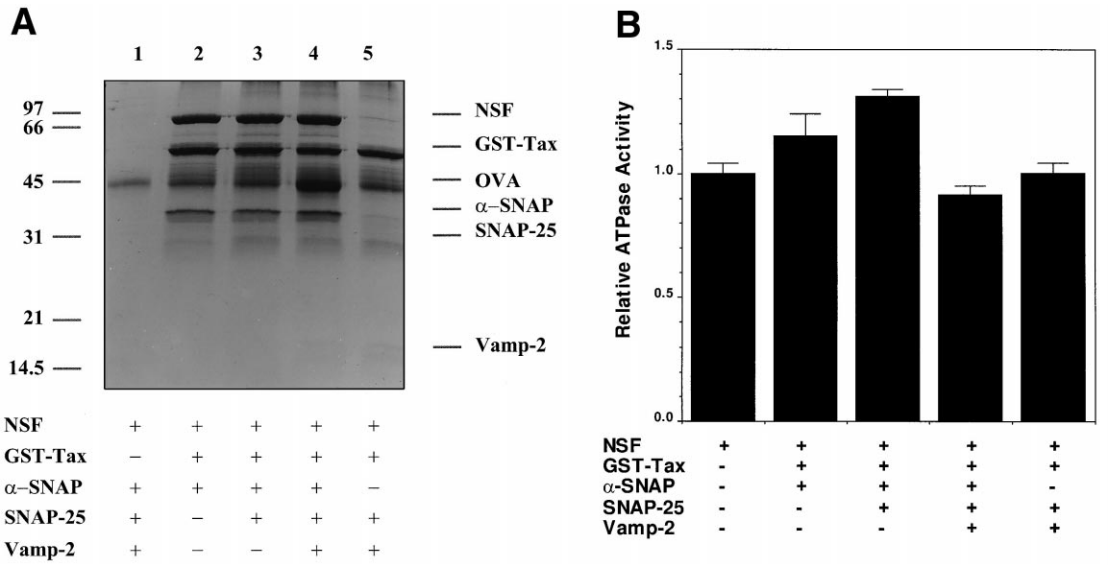

Fig. 2. Effect of SNAP/SNARE complex on the ATPase activity of NSF. A: Complexes were formed from the indicated combinations of NSF, GST-syntaxin 1 (GST-Tax), α-SNAP, SNAP-25, and Vamp-2 in the presence of ATPγS and EDTA and then captured on GSH-agarose beads. As described in Section 2, NSF binding to these complexes was assessed by Coomassie Blue staining. B: The indicated complexes were formed in the absence of NSF, harvested and washed on GSH-agarose beads and then incubated with a constant amount of NSF (10 μg) and [α-^{32}P]ATP. The amount of ADP produced by NSF during 1 h in the presence of GSH-agarose beads without other components was normalized to 1. The data represents the mean of 6 experiments (±S.E.M). In no case did the complexes show nucleotidase activity in the absence of NSF.

ATPase activity of NSF, complexes were first constructed on GSH-agarose beads, washed to remove unbound proteins, and then a constant amount of NSF was added to the complex-bound beads. The amount of ADP produced in 60 min was measured and compared to the amount of remaining ATP. To determine the basal hydrolysis rate of NSF, an equal volume of GSH-agarose beads was added. As shown in Fig. 2B, the SNAP/t-SNARE complex containing α-SNAP, syntaxin, and SNAP-25 stimulated the ATPase activity of NSF by ~35%. Complexes containing syntaxin and α-SNAP only slightly stimulated NSF but the complex containing α-SNAP, syntaxin, SNAP-25, and Vamp-2 showed no significant stimulation of ATPase activity. In no case did the complexes show any detectable nucleotidase activity in the absence of NSF.

From the data presented in Fig. 2B, it would appear that the amount of ADP produced by NSF during the 60-min incubation was increased by the addition of the α-SNAP/t-SNARE complex, yet these experiments represent an endpoint and therefore do not truly address changes in the kinetics of the reaction. Time course experiments were performed to determine the effect of the α-SNAP/t-SNARE complex on the rate of ATP hydrolysis by NSF. As shown in Fig. 3A, increasing amounts of the α-SNAP/t-SNARE complex lead to an increase in the initial rate of ATP hydrolysis by

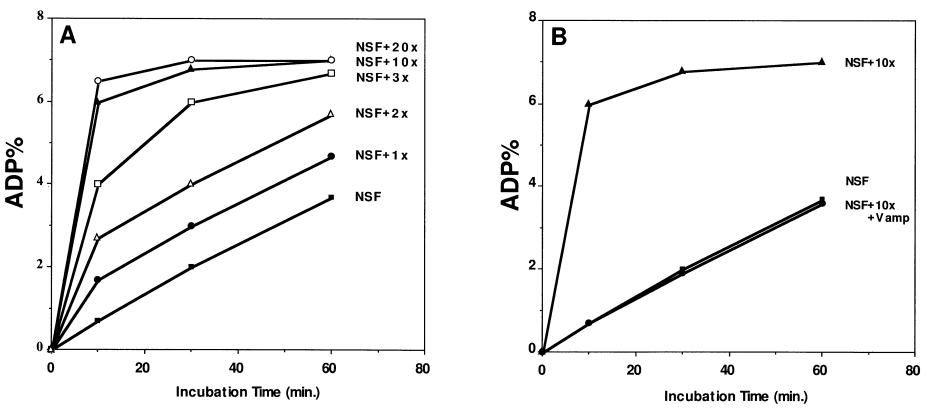

Fig. 3. The effect of the SNAP/t-SNARE complex on the kinetics of ADP production by NSF. A: SNAP/t-SNARE complexes were prepared as described in Section 2 and were defined as 1× and 2×, 3×, 10× and 20× based on the volume of complex-containing beads used. The curves represent a 2-, 3-, 10- and 20-fold excess of SNAP/t-SNARE complex relative to that used in Fig. 2B. For the data presented, the molar ratio of hexameric NSF to SNAP/t-SNARE complex was estimated by densitometry of Coomassie Blue stained gels and was found to vary between 1:7 and 1:140. To maintain the liquid-solid phase ratio, fresh GSH-agarose beads were added to equalize the volume of beads in each sample. B: Comparison of the ADP production by NSF (NSF), NSF in the presence of 10× SNAP/t-SNARE complex (NSF+10×), and NSF in the presence of a complex that contained both v- and t-SNAREs and α-SNAP (NSF+10×+Vamp). In both panels the amount of ADP produced is expressed as a percentage of the total nucleotide present in the reaction. The percent ADP in the original nucleotide preparation before starting the reactions (0–6%) was subtracted as background.

NSF. When the α-SNAP/t-SNARE complex is limiting, the hydrolysis time course is biphasic with an initial phase of higher hydrolysis followed by a return to basal activity. Upon ATP hydrolysis, the SNAP/SNARE complex would disassemble and the isolated syntaxin has been shown to assume a conformation that is incompetent to bind to α-SNAP [25]; therefore the biphasic stimulation curve (Fig. 3A) is consistent with a reaction scheme in which the stimulating complex is consumed during the reaction. As more complex is added to the reaction, the increase in initial ATP hydrolysis becomes more pronounced and ultimately plateaus indicating saturation of the reaction (compare 10× with 20× complex). The plateau shown in the time course in Fig. 3A most likely represents inactivation of NSF rather that product inhibition since the amounts of ADP produced are insufficient (~70 μM) [27] and no significant amounts of ADP-bound NSF were detected at the 60-min time point (data not shown).

Comparison of the initial rates of ATP hydrolysis between NSF alone with the α-SNAP/t-SNARE complex in Fig. 3B shows that the α-SNAP/t-SNARE complex causes a 10-fold increase in the rate of ATP hydrolysis by NSF. Interestingly, in this same experiment, the α-SNAP/v-,t-SNARE complex had no significant effect on the basal ATPase activity of NSF. The fact that the α-SNAP/t-SNARE complex has a greater effect on the activity of NSF is consistent with the observation by Ungermann et al. [13] in which the ATP-dependent release of Sec17p (yeast α-SNAP) from t-SNARE-containing membranes is faster than release from vacuolar membranes that contain both t- and v-SNAREs. While it is clear that NSF can disassemble both SNAP/t-SNARE and SNAP/v-,t-SNARE complexes, from the data presented it seems that disassembly of the former complex could represent a more kinetically favored reaction. Further experimentation will be needed to specifically address this point.

NSF has been proposed to serve as a molecular chaperone [5,9], modulating the conformation of the SNARE proteins so that they can participate in membrane fusion reactions. Two points in the membrane fusion pathway might require such activity: just prior to SNARE engagement (priming), and sometime after bilayer fusion (recycling). The first step would be required for activation of the SNAREs and the second would be required to recycle them. It has been shown that NSF/Sec18p-dependent priming is required for activation of the t-SNAREs since the transport competence of t-SNARE-only vacuoles is greatly inhibited by anti-Sec18p antibodies [12,13]. For priming NSF could activate the syntaxin protein by 'unfolding' it, thereby allowing it to interact with accessory molecules such as LMA-1 [28] or a member of the Sec1/mUNC18 family [29] which serve to stabilize the activated conformation of syntaxin for subsequent interactions. In fact, syntaxin/Munc18 complexes represent a major syntaxin-containing complex in cells unless NSF is attenuated ([30] and Lemons and Whiteheart, unpublished). When NSF is inactivated, a buildup of v-,t-SNARE complexes is observed suggesting the NSF is required to break up these complexes for the recycling of the constituents. Unfortunately, from these studies it is not clear whether all of the syntaxins are associated with a v-SNARE-containing complex or just with a SNAP-25-like t-SNARE partner. While NSF can carry out both types of reactions, t-SNARE and v-,t-SNARE complex disassembly, the data presented in this manuscript suggest that it may be the priming of the t-SNARE that is kinetically favored. Perhaps this differential rate in ATP hydrolysis and, by extension, complex disassembly is related to the proposed roles of the various complexes. Since only the v-,t-SNARE complex is required for membrane fusion, it might be detrimental for that complex to be disassembled too rapidly. Recycling of the SNAREs, however, does not need to be on as rapid a time scale as does the priming of a t-SNARE.

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