Dissecting the N-Ethylmaleimide-sensitive Factor

REQUIRED ELEMENTS OF THE N AND D1 DOMAINS

Chunxia Zhao, Elena A. Matveeva, Qiansheng Ren, and Sidney W. Whiteheart

From the Department of Molecular and Cellular Biochemistry, University of Kentucky Medical Center, Lexington, Kentucky 40536-0509

N-Ethylmaleimide-sensitive factor (NSF) is a homo-hexameric member of the AAA+ (ATPases associated with various cellular activities plus) family. It plays an essential role in most intracellular membrane trafficking through its binding to and disassembly of soluble NSF attachment protein (SNAP) receptor (SNARE) complexes. Each NSF protomer contains an N-terminal domain (NSF-N) and two AAA domains, a catalytic NSF-D1 and a structural NSF-D2. This study presents detailed mutagenesis analyses of NSF-N and NSF-D1, dissecting their roles in ATP hydrolysis, SNAP-SNARE binding, and complex disassembly. Our results show that a positively charged surface on NSF-N, bounded by Arg67 and Lys105, and the conserved residues in the central pore of NSF-D1 (Tyr296 and Gly298) are involved in SNAP-SNARE binding but not basal ATP hydrolysis. Mutagenesis of Sensor 1 (Thr231-Arg375), Sensor 2 (Glu440-Glu445), and Arginine Fingers (Arg385 and Arg388) in NSF-D1 shows that each region plays a discrete role. Sensor 1 is important for basal ATPase activity and nucleotide binding. Sensor 2 plays a role in ATP- and SNAP-dependent SNARE complex binding and disassembly but does so in cis and not through inter-protomer interactions. Arginine Fingers are important for SNAP-SNARE complex-stimulated ATPase activity and complex disassembly. Mutants at these residues have a dominant-negative phenotype in cells, suggesting that Arginine Fingers function in trans via inter-protomer interactions. Taken together, these data establish functional roles for many of the structural elements of the N domain and of the D1 ATP-binding site of NSF.

N-Ethylmaleimide-sensitive factor (NSF) [2] is required for most membrane fusion events in a cell [1, 2]. Conditional mutations in the yeast orthologue and the neuro-specific form in fly (Sec18p and comatoose, respectively) disrupt constitutive secretion [3] or synaptic transmission [4], respectively, and overexpression of a dominant-negative mutant in mammalian cells is cytotoxic [5]. NSF is thought to disassemble SNAP receptor (SNARE) complexes so that they can be recycled for subsequent membrane fusion events. SNAREs are the minimal machinery for membrane fusion [6]. They form a four-helix SNARE complex that spans the apposing membranes and mediates fusion [7]. After fusion, NSF uses the energy from ATP hydrolysis to disassemble spent SNARE complexes for recycling. NSF binds to the SNARE complexes by interacting with an adaptor protein called soluble NSF attachment protein (α-SNAP) [8]. Previous studies [9–12] have suggested that three α-SNAPS coat the length of the SNAP bundle and position NSF at the membrane-distal end of the SNARE complex. This stimulates the ATPase activity of NSF and the subsequent hydrolysis-dependent conformational changes somehow drive unwinding of the SNARE complex. Despite this general outline, little is known about the structural changes that NSF undergoes during its ATP hydrolysis cycle and how the chemical energy is converted into the mechanical energy required for SNARE recycling.

NSF is a member of the AAA+ (ATPases associated with various cellular activities plus) family. Each protomer of the homo-hexamer contains one N-terminal domain (NSF-N), followed by two conserved, Walker-type, nucleotide-binding domains (termed NSF-D1 and NSF-D2). NSF-N contains a double α-β barrel [13, 14] and is required for SNAP-SNARE binding [15]. Positively charged residues on its surface are proposed to interact with the negatively charged C terminus of α-SNAP [8, 16]; however, it is not clear how these residues contribute to SNAP-SNARE complex binding. NSF-D1 accounts for the majority of the ATP hydrolysis; NSF-D2 is required for oligomerization [15]. Each nucleotide-binding domain is divided into two subdomains as follows: a β-sheet core at the base of the nucleotide-binding pocket, and an α-helical domain that partially caps the pocket (Fig. 1A) [12]. In the hexamer, the nucleotide-binding pocket is located at the interface between adjacent protomers. Several conserved motifs contribute to the nucleotide-binding pocket, including the classical Walker A and B motifs (Fig. 1). The conserved lysines in Walker A boxes are crucial for ATP binding [17]. The aspartates in the DEXX sequences of Walker B boxes are thought to coordinate Mg2+ ions, which are required for ATP hydrolysis. The glutamates are proposed to activate water during the hydrolysis reaction. The second region of homology is another sequence motif that is highly conserved in AAA+ proteins [12]. Sensor 1 is at the N terminus of the second region of homology and often contains a threonine/asparagine pair [18]. At its C terminus are two arginine residues termed Arginine Fingers [19]. These residues are thought to be critical for nucleotide hydrolysis similar to Arginine Fingers of GTPase-activating
Dissecting the N-Ethylmaleimide-sensitive Factor

proteins (19). The α-helical subdomain of the nucleotide-binding domains contains a motif, called Sensor 2, which is composed of residues that are positioned adjacent to the ATP-binding site. This motif often contains a conserved arginine (e.g. GAR in ClpA, ClpB (20), and Hsp104 (21) and DLR in Cdc6p (22)) that interacts with the γ-phosphate of ATP. This is absent in NSF-D1 (ELE) and is replaced with a lysine in NSF-D2 (GIK). The importance of Sensor 1 and Sensor 2 for ATP turnover and protein function has been evaluated in a number of AAA⁺ proteins (23), but only limited data are available for their importance in NSF (24).

The six protomers of NSF form two stacked homo-hexameric rings (9). Based on the structure of the NSF-D2, there is a central pore that could be contiguous with a predicted pore in NSF-D1 (11, 25, 26). This pore is thought to be important for substrate binding and processing in other AAA⁺ proteins (12). Three conserved residues YVG (aromatic, hydrophobic, and glycine) are involved in substrate engagement in ClpB and p97 (27, 28). Currently, it is unclear whether the pore region of NSF-D1 is catalytically important to NSF function; however, the pore could be an important element for NSF-mediated SNARE disassembly.

To better understand how NSF binds to and disassembles SNARE complexes, it is necessary to investigate the roles of NSF-N and of the conserved motifs in NSF-D1. Studies of NSF have focused on Walker A and B motifs, showing that they are required for ATP binding and hydrolysis, respectively. In this study, we examine the roles of NSF-N, and Sensor 1, Sensor 2, Arginine Fingers, and the central pore motifs of NSF-D1. Using site-directed mutagenesis, we begin to assign specific functions to the various subdomains of NSF by monitoring four key activities as follows: SNAP-SNARE binding, complex disassembly, basal ATPase activity, and SNAP-SNARE-stimulated ATPase activity. We also test the dominant-negative effects of these mutants in vivo to assess whether mutant protomers can inactivate a hexamer. Our results reveal that NSF-N and the pore of NSF-D1 are important for SNAP-SNARE binding. Also, we show that Sensor 1 and Sensor 2 motifs are involved in ATP binding and hydrolysis, respectively. Finally, our data show that Walker B motif and Arginine Fingers are critical for ATP hydrolysis by NSF hexamers and are likely to facilitate communication between protomers.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids encoding His₆-NSF and His₆-α-SNAP in the pQE9 expression vector were described previously (17, 29). The cytoplasmic domain of human VAMP-8 (amino acids 1-73) was cloned into the BamHI and HindIII restriction sites in the pGEX-KG vector, resulting in a construct with N-terminal glutathione S-transferase (GST) tag. Human SNAP-23 (amino acids 1-211) and the cytoplasmic domain of human syntaxin 2 (amino acids 1-251) were cloned into the BamHI/HindIII and NdeI/Xhol restriction sites, respectively, in the pRSFDuet-1 vector (EMD Bioscience, Madison, WI). This generated SNAP-23 with an N-terminal His₆ tag and syntaxin 2 with a C-terminal S tag. Green fluorescence protein (GFP)-tagged wild-type NSF and the ATP hydrolysis-deficient E329Q mutant (NSF-GFP and E329Q-GFP) in pcDNA4/TO vector were kindly provided by Dr. Phyllis I. Hanson (Washington University, St. Louis, MO) and used for mammalian expression of NSF.

Mutagenesis and Protein Expression—Site-specific mutants were created with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using wild-type His₆-NSF or NSF-GFP as templates. All of the mutations were confirmed by dideoxynucleotide sequencing.

His₆-NSF, His₆-NSF mutants, and His₆-α-SNAP were expressed in the Escherichia coli strain, Rosetta DE3 pLacI, and purified according to published methods (17, 29). SNARE complexes containing GST-VAMP-8 and His₆-SNAP-23-syntaxin 2 were assembled in the E.coli strain, Rosetta DE3, and initial purification was on glutathione-agarose (Sigma). Eluents were dialyzed into 20 mM HEPES, pH 7.4, 200 mM KCl, 10% glycerol, 20 mM imidazole, and 2 mM β-mercaptoethanol and further purified on Ni²⁺-nitritoltriacetic acid-agarose (Sigma).

For mammalian expression, constructs containing wild-type NSF or mutants with C-terminal GFP fusion were transiently transfected into HeLa cells with FuGENE 6 (Roche Applied Science) according to the manufacturer's recommendations. Assays were performed 48 h post-transfection for viability measurements or as indicated for immunofluorescence microscopy. HeLa cells were maintained in Dulbecco's modified Eagle's medium (high glucose) containing 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.292 mg/ml glutamine at 37 °C and 5% CO₂.

SNARE Complex Binding and Disassembly Assay—The procedure was modified from methods described previously (16). SNARE complexes (GST-VAMP-8-His₆-SNAP-23-syntaxin 2), His₆-NSF or mutants, and His₆-α-SNAP (1:1:3 molar ratio) were incubated for 30 min at 4 °C in binding buffer (50 mM HEPES, pH 7.4, 100 mM KCl, 10% glycerol, and 1% Triton X-100, and 5% glycerol with 0.5 mM AMP-PNP (Roche Applied Science) and 2 mM EDTA). Glutathione-agarose beads (30 μl) were then added and incubated for 2 h at 4 °C with continuous mixing. The bound protein complexes were then recovered by brief centrifugation, washed, eluted with SDS-PAGE sample buffer, and then analyzed by Western blotting for His₆ tags with India-His-HRP (Pierce). The reactions lacking α-SNAP were used to determine nonspecific background binding.

For SNAP-SNARE disassembly assays, the binding reactions were performed with 0.5 mM ATP and 2 mM EDTA. The bound complexes were incubated in 0.5 ml of binding buffer containing 5 mM ATP and 10 mM MgCl₂ for 30 min at 4 °C. The bound proteins were eluted with SDS-PAGE sample buffer, and the released proteins were recovered with trichloroacetic acid precipitation. The bound and released proteins were analyzed by Western blotting for His₆ tags with India-His-HRP (Pierce) and quantified by densitometry.

ATPase Activity Assay of NSF—Basal ATPase activity was measured as described previously with minor modifications (15). Basal ATPase assays were carried out using 5 μg of NSF or mutants in ATPase assay buffer (25 mM Tris-HCl, pH 7.4, 100 mM KCl, 2 mM MgCl₂, 0.5 mM 1,4-dithiothreitol, 1% glycerol, 5 mM ATP) supplemented with 10 μCi of [α-³²P]ATP (MP Biomedicals, Santa Ana, CA). After 1 h at 25 °C, the reactions
were stopped by spotting samples (2 μl of each reaction) onto a polyethyleneimine thin layer plate (Selecto Scientific, Suwanee, GA). AMP, ADP, and ATP were separated by ascending chromatography in 0.7 m LiCl and 1 m acetic acid. The radiolabel in each spot was quantified by using a Typhoon 9400 Imager (Amersham Biosciences), and ATPase activity was calculated by determining the percent of [α-32P]ADP produced relative to the total nucleotide present. To measure the SNAP-SNARE-stimulated ATPase activity, SNAP-SNARE complexes were assembled for 2 h at 4 ℃ and then incubated with NSF or mutants. The [α-32P]ADP produced was quantified as above.

The fold increase in ATPase activity was calculated by taking the ratio of ADP produced with and without SNAP-SNARE complexes. In all cases, the ATPase activities of NSF and mutants were sensitive to N-ethylmaleimide (NEM) (Sigma) as reported previously (15), and the reactions were done in duplicate.

Viability Assay—HeLa cells were washed once with Dulbecco’s modified Eagle’s medium (low glucose). Propidium iodide (0.5 ml) (Invitrogen) was added for 30 min at 37 ℃. The cells were washed twice with Dulbecco’s modified Eagle’s medium (low glucose), and the stained cells were viewed using an Eclipse TS100 inverted microscope (Nikon, Japan). The percentage of cell death among transfecteds was calculated by counting the dead cells (red) with green signal (NSF- or mutant-GFP-positive) divided by total GFP-positive cells (n > 200). Transfection efficiency was determined by dividing the number of GFP-positive cells by the total number of cells in a given field.

Immunofluorescence Microscopy—HeLa cells were seeded into sterile glass coverslips in 6-well plates and transfected for 15 or 27 h. Cells were washed once with phosphate-buffered saline (PBS), fixed with 3.7% (v/v) formaldehyde for 15 min at 25 ℃, then quenched with 50 mM NH4Cl for 5 min. Cells were rinsed with 10% FBS/PBS and permeabilized with 0.1% Triton X-100 for 5 min at 25 ℃. Rabbit anti-Giantin IgG (Covance, Princeton, NJ) (1:1000 dilution) in 10% FBS/PBS was then added and incubated for 1 h at 25 ℃. After washing with 10% FBS/PBS, cells were incubated with Texas Red-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) (1:200 dilution) in 10% FBS/PBS for 1 h at 25 ℃. Coverslips were mounted with VECTASHIELD® with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) and examined with an E-600 epifluorescence microscope (Nikon, Japan). To determine the effects on Golgi morphology, the area of the Giantin-positive Golgi was measured and normalized to the area of the 4′,6-diamidino-2-phenylindole-positive nuclei. For this, image analysis was done using Image-Pro Plus 5.0 software (Media Cybernetics, Bethesda).

Immunoprecipitation Assay—HeLa cells were solubilized on ice for 30 min with lysis buffer as described (5). The lysate was cleared by centrifugation. Rabbit anti-GFP polyclonal antibodies (Invitrogen) or rabbit preimmune sera were preincubated with protein G-Sepharose Fast Flow (Amersham Biosciences) for 2 h at 4 ℃ and then incubated with the clarified lysates for another 2 h at 4 ℃. The beads were recovered and washed three times with lysis buffer. The bound proteins were then eluted with SDS-PAGE sample buffer, boiled, and analyzed by Western blotting. The anti-NSF monoclonal antibody, 2E5 (30), was used to detect both endogenous NSF and exogenous NSF-GFP or mutants. The immuno-decorated proteins were quantified by densitometry, and the ratios of NSF-GFP to NSF were calculated.

**RESULTS**

Rationale for Site-directed Mutagenesis of NSF-D1—Previous data suggest that NSF-D1 is the major catalytic domain for NSF (17). Therefore, defining the elements in NSF-D1 that affect ATPase activity should help to elucidate the molecular mechanism of the NSF protein. We introduced mutations into the conserved motifs that were predicted to be adjacent to the nucleotide-binding pocket. Based on the structure of NSF-D2, Sensor 1 and Sensor 2 contribute several residues to the nucleotide-binding pocket (Fig. 1) (25, 26). Sensor 1 in NSF-D1 contains three conserved amino acids (Thr373, Asn374, and Arg375) (light blue in Fig. 1B) that are proximal to the γ-phosphate of ATP. Since there are no conserved amino acids in Sensor 2 of NSF-D1, three corresponding residues (Glu440, Leu441, and Glu442) (dark green in Fig. 1B) were chosen for mutagenesis because they are predicted, by sequence alignments, to be most proximal to the ATP-binding site. Two conserved arginines (Arg385 and Arg388), called Arginine Fingers (dark blue in Fig. 1B) (19), are proposed to polarize the γ-phosphate in trans to facilitate hydrolysis. One early hypothesis posited that the N terminus of the SNARE complex contacts the central pore of NSF during complex disassembly (1). To address this point, single or double mutations of two conserved residues in NSF-D1 (Tyr296 and Gly298) were tested (black in Fig. 1A). All residues discussed above were mutated to either alanines or alternatively charged residues by site-directed mutagenesis. In all cases, the resulting recombinant mutant proteins were hexameric (data not shown) as determined using previously described sizing chromatography (17).

Basal ATPase Activity of NSF—Given the focus on the conserved motifs in NSF-D1, the first assay examined the effects of the mutations on basal ATPase activity. ATPase activity was determined in the presence or absence of NEM; NEM insensitive activity was considered background and subtracted. Under the conditions used, wild-type NSF produced ADP at a rate of 0.64 μmol/mg/h, which is comparable with the published values (16, 31).

Mutations in Sensor 1 showed the greatest negative effects on basal ATPase activity (Fig. 2). Mutation of the first conserved threonine (Thr373) to alanine reduced the activity by ~50%. This is consistent with a previous report that mutation of the corresponding residue in Sec18p (T394P) eliminates ATPase activity (24). A more dramatic effect was observed when the second conserved amino acid, Asn374 was mutated. The asparagine to alanine mutant (N374A) showed an 85% decrease in ATPase activity; the N374D mutation caused a 60% decrease. These effects on basal ATPase activities are consistent with the known importance of Sensor 1 in ATP binding and/or hydrolysis in other AAA+ proteins (12, 23, 32, 33).
Mutations in other regions showed variable but less remarkable effects on basal ATPase activity (Fig. 2).

**SNAP-dependent SNARE Binding**—A critical aspect of NSF function is its ability to bind SNARE complexes via its adaptor protein, α-SNAP. This activity also requires that NSF be in its ATP-bound state (15). To assay for this activity, equal amounts of wild-type or mutant NSF were preincubated with SNARE complexes, with or without α-SNAP, in the presence of a non-hydrolyzable ATP analogue (AMP-PNP). The resulting complexes were recovered with glutathione-agarose beads, and the extent of bound NSF or mutants was determined by Western blotting.

Most of the mutations in Sensor 1 negatively affected binding (Fig. 3). The T373A and N374A mutants were unable to bind above background; the N374D mutant retained only ~20% of binding activity compared with wild-type NSF. This is perhaps caused by the inability of the mutants to bind nucleotide, which is consistent with their lack of ATPase activity (Fig. 2). Interestingly, binding of the R375A mutant was partially reduced, but when the arginine was mutated to glutamate, binding was ablated. The variants in Sensor 2 had mixed effects on SNAP-SNARE binding. The E440R mutation severely affected binding although the E440A mutation was without effect. The L441A mutation partially affected binding, and the mutations at Glu442 either enhanced or had a partial effect on binding. Mutations of Arginine Fingers (R385A and R388A) displayed only ~30% of wild-type binding activity.

In some AAA⁺ proteins, the presence of a conserved YVG motif, in one of the loops that make up the central pore, is essential for substrate binding and/or translocation (12). In NSF, only NSF-D1 contains this conserved motif. To determine its role in SNAP-SNARE binding, mutations of Tyr²⁹⁶ and Gly²⁹⁸ (Y296A/Y296F, G298A, or double mutations Y296A/G298A and Y296F/G298A) were generated. The basal ATPase activities of these mutants were similar to that of wild-type NSF (Fig. 2); however, SNAP-SNARE binding was reduced in all the mutants (Fig. 3). This suggests that this region of NSF-D1 may be important for protein substrate binding (27, 28, 34).

**SNAP-SNARE-stimulated ATP Hydrolysis**—Mutants that showed approximately normal levels of basal ATPase activity and partial SNAP-SNARE binding, e.g. those in Sensor 2 and of Arginine Fingers, were analyzed further to determine whether they were defective in other aspects of NSF function. Two assays were used to investigate the effects of the mutations on
SNAP-SNARE complex disassembly and SNAP-SNARE-stimulated ATPase activity. In Fig. 4, NSF or mutants were prebound to SNAP-SNARE complexes under conditions where ATP hydrolysis was limited (ATP/EDTA). For wild-type NSF, greater than 60% of the initially bound protein was released upon return to hydrolytic conditions. The Sensor 1 mutant R375A was unable to release into the supernatant. The E440A and L441A mutants, in Sensor 2, were also not released into the supernatant, although a little of the E442R mutant was detected. The E442A mutant behaved like the wild-type protein. Both Arginine Finger mutants (R385A and R388A) showed a similar defect in release. Mutations of Tyr296 had mixed effects; the Y296A mutant was defective in release, but the Y296F mutants showed close to wild-type activity. No protein was observed in the supernatant when the G298A mutant was tested. It should be noted that this assay measured the release as a percentage of prebound NSF or mutants and that assay conditions were set so that even poorly binding mutants could be analyzed for their release activity.

![Figure 3](image-url) **FIGURE 3.** Binding of NSF-D1 domain mutants to SNAP-SNARE complexes. A, recombinant SNARE complexes (GST-VAMP-8-His6-SNAP-23-syntaxin 2) were generated as described under “Experimental Procedures.” His6-NSF or mutants and SNARE complexes were mixed in the presence or absence of His6-α-SNAP under nonhydrolyzable conditions (0.5 mM AMP-PNP and 2 mM EDTA). The bound proteins were collected with glutathione-agarose beads, eluted with SDS-PAGE sample buffer, and analyzed by Western blotting using India-His-HRP. The Western blots were quantified by densitometry (B). The binding reaction without α-SNAP served as a background control and was subtracted during calculation. Values have been normalized to that of the wild-type (WT) protein. These data are representative of two to three individual experiment, and the error bars represent the range.

![Figure 4](image-url) **FIGURE 4.** ATPase-dependent release of NSF and NSF-D1 domain mutants from SNAP-SNARE complexes. A, recombinant SNARE complexes (GST-VAMP-8-His6-SNAP-23-syntaxin 2); His6-NSF, or mutants and His6-α-SNAP were incubated in the presence of 0.5 mM ATP and 2 mM EDTA. The bound proteins were recovered with glutathione-agarose beads, and the buffer containing 5 mM ATP and 10 mM MgCl₂ was added. The reactions were incubated, and the proteins released into the supernatant were precipitated with trichloroacetic acid. The bound (p) and released (s) proteins were analyzed by Western blotting as in Fig. 3. The Western blots were quantified by densitometry (B). ATP-dependent NSF release was calculated as follows: released (s) protein divided by total protein (s/(p + s)). Values have been normalized to that of the wild-type (WT) protein. These data are representative of two to three individual experiments, and the error bars represent the range.
Dissecting the N-Ethylmaleimide-sensitive Factor

We next assayed the SNAP-SNARE-stimulated ATPase activities of wild-type and mutant NSF. SNAP-SNARE complexes were assembled and then incubated with NSF or mutants in the presence of [α-32P]ATP. The ADP produced was quantified as in Fig. 2. Fold increases in ATPase activity were calculated by dividing the SNAP-SNARE-stimulated ATPase activity by basal ATPase activity of NSF or mutants. All data are shown with the NEM-treated background controls subtracted. WT, wild type.

Motifs Critical for NSF Function in Vivo—Previous in vitro studies showed that all six protomers must have functional SNAP-SNARE complexes led to an ~18-fold increase in ATPase activity for wild-type NSF (Fig. 5). No ADP was generated when SNAP-SNARE complexes were incubated alone (data not shown). Mutations (E442A and Y296F) that had no effect on NSF release (Fig. 4) showed similar stimulation of ATPase activities (Fig. 5). None of the mutations with impaired release (Fig. 4) showed an increased ATPase activity in the presence of SNAP-SNARE complexes (Fig. 5). Mutations of Sensor 2 and of Arginine Fingers affected SNAP-SNARE-stimulated ATPase activities, which most likely accounts for their negative effects on NSF-SNAP-SNARE complex disassembly.

Important Residues of NSF-N—NSF-N is composed of two subdomains, N$_{A}$ and N$_{B}$, joined by a linker or hinge region (Fig. 8A). The surface of NSF-N has several positively charged amino acids at the apex of the cleft formed by N$_{A}$ and N$_{B}$, which could play a role in binding to the negatively charged C terminus of α-SNAP (13, 14). To characterize this surface, five positively charged residues (Arg$_{10}$, Lys$_{68}$, Lys$_{104}$, Lys$_{105}$, and Lys$_{143}$) and three acidic residues (Asp$_{84}$, Glu$_{15}$, and Asp$_{129}$) were mutated (Table 1 and Fig. 8). Two residues (Tyr$_{83}$ and Cys$_{91}$) (Table 1 and Fig. 8) in the hinge region at the base of the cleft were also mutated because this region has been shown to be a substrate-binding site for the related AAA$^{+}$ protein, p97 (35–37), and these residues have been reported to be the sites of post-translational modification of NSF (38, 39). The SNAP-SNARE binding and basal ATPase activities were measured for most of the mutants, and the results were summarized in Table 1. As expected, none of the mutations had a drastic effect on the
ATPase activity of NSF, although the D14R mutant did show an increase. Mutations of the positively charged residues (Arg10, Arg67, Lys68, Lys104, Lys105, and Lys143) either abolished or severely affected binding to SNAP/SNARE complexes. The E15A, E15R, D142A, and D142R mutants were also defective in binding. The D14A and D14R mutants showed close to wild-type binding levels. Mutation of the residues (Tyr83 and Cys91) in the hinge region showed mixed effects with Y83E having the greatest negative effect and Y83F having only a partial effect. These results suggest that the surface located on the top of interface made by Nα and Nβ is important for SNAP/SNARE complex binding (Fig. 8).

**DISCUSSION**

In this study, we have used site-directed mutagenesis and a battery of assays to understand the mechanism of the general secretory protein NSF. For its cellular function, NSF must bind to SNAP/SNARE complexes and use ATP hydrolysis to disassemble them. The conformational changes in NSF induced by ATP binding and hydrolysis are critical for its function. The data presented here show that the specific elements of NSF-N and NSF-D1 domains are required for ATP hydrolysis, for SNAP-SNARE complex binding and disassembly, and for activity in vivo (summarized in Table 2). Each element of the nucleotide-binding site of NSF-D1, Sensor 1, Sensor 2, Arginine Fingers, as well as the central pore plays a key role. The charged surface formed by the cleft between the subdomains of NSF-N plays a role in SNAP-SNARE binding. These findings may be applicable to other AAA+ proteins that use ATP binding/hydrolysis and adaptor proteins or accessory domains to carry out their cellular functions.

**Sensor 1 and 2**—The crystal structures of the processivity clamp loader γ-complex RuvB or its eukaryotic homologue RuvBL1 have a threonine or asparagine residue in Sensor 1 that forms a hydrogen bond with the γ-phosphate of ATP (33, 40, 41). This may allow discrimination between bound ATP and ADP. A similar arrangement is found in other AAA+ proteins.
Mutagenesis of DnaA has shown a role for Sensor 1 in high affinity nucleotide binding (32). Sensor 2 is also proposed to allow discrimination between the nucleotide-bound and unbound states. A conserved arginine in Sensor 2 of some AAA \(^+\) proteins forms a salt bridge with the \(\beta\)-phosphate of ATP and ADP (33, 40, 41). Mutation of Arg\(^{826}\) in Sensor 2 of Hsp104 equally weakens its affinity for ATP and ADP (21). However, the roles of Sensor 1 and Sensor 2 may extend further than just passive nucleotide-sensing. Several studies demonstrate that the two Sensors are involved in catalysis but not nucleotide binding (44–50).

In the structure of NSF-D2, Ser\(^{655}\) (which corresponds to one of the conserved asparagines in Sensor 1 of NSF-D1) is involved in a hydrogen bond network that positions a water molecule as a potential nucleophile for ATP hydrolysis (26). Although NSF-D2 has limited ATPase activity (17), such an orientation for an asparagine in NSF-D1 suggests that Sensor 1 could have a catalytic role in NSF. In our study, the T373A, N374A, and N374D mutations in NSF-D1 diminished the intrinsic ATPase activity of NSF, consistent with a role of Sensor 1 in nucleotide binding and/or hydrolysis. The mutations also abolished ATP-dependent binding to SNAP-SNARE complexes (Fig. 2 and Fig. 3). This phenotype is similar to that of a mutant in the Walker A motif (K266A), which is defective in nucleotide binding (17). It is thought that K266A does not bind SNAP-SNARE complexes because it cannot attain the ATP-dependent conformation required. Based on the comparison with K266A, the phenotypes of the T373A, N374A, and N374D mutants are consistent with these residues being required more for ATP binding than hydrolysis. Mutations of Arg\(^{375}\) had little effect on basal ATPase activity (Fig. 2), but a negative charge at that position (R375E) did affect SNAP-SNARE complex binding (Fig. 3). Removal of the positive charge (R375A) eliminated ATPase-driven complex disassembly (Fig. 4). These phenotypes suggest that Arg\(^{375}\) could play a role in mediating the nucleotide-dependent conformational changes required for both SNARE

**FIGURE 7.** Mixed hexamer formation in HeLa cells. Forty eight hours post-transfection, immunoprecipitations from cellular extracts were carried out using rabbit anti-GFP antibody. Rabbit preimmune sera were used as a negative control. Samples immunoprecipitated with GFP antibodies were blotted with monoclonal antibody (2E5) for NSF. The endogenous NSF and exogenous NSF-GFP are indicated with arrows.

**FIGURE 8.** Potential SNAP-binding sites on NSF-N. A, crystal structure of the N domain of NSF. N\(_A\) is in light blue, and N\(_B\) is in light purple, and the hinge region is in white. The groove 3 is shown in dark blue. Residues mutated are indicated. Red amino acids are positions where mutations are inhibitory (no binding or less than 30% of wild-type NSF binding); those in yellow are partially inhibitory (30–70% of wild-type NSF binding); and those in green have little effect (more than 70% of wild-type NSF binding). B, top view of the image shows the \(\alpha\)-SNAP-binding surface, which corresponds to 90° clockwise rotation of the image displayed in A. The images are based on Protein Data Bank code 1QDN and were generated using Swiss Protein Data Bank viewer and rendered with Pov-Ray.
complex binding and disassembly. The results presented here suggest that Sensor 1 in NSF-D1 is important for nucleotide binding and inducing a SNAP-SNARE-binding competent state. However, it should be noted that mutation of the first threonine in Sensor 1 (T394P) of Sec18p-D1 has a phenotype similar to that of NSF E329Q, which can bind but not hydrolyze ATP (24).

By contrast, most of the Sensor 2 mutations had only limited effects on basal ATPase activity, suggesting that Sensor 2 plays only a limited role in nucleotide binding (Fig. 2). The E440R (but not E440A), L441A, and the E442R (but not E442A) mutations did affect SNAP-dependent SNARE complex binding (Fig. 3). Of the mutations tested, only the E442A mutant retained wild-type activity in the ATPase-dependent release assay (Fig. 4) and the SNAP-SNARE-stimulated ATPase assay (Fig. 5). Given the differential effects of the E440R and E440A mutations, it would appear that the presence of a positive charge at that position is detrimental to ATP-dependent, SNAP-SNARE complex binding, but the presence of a negative charge is not essential. Comparison of E442A and E442R suggests that a positive charge negatively affects SNAP-SNARE complex-stimulated ATPase activity. Taken together it appears that the elements of Sensor 2 are not essential for nucleotide binding or basal hydrolysis (Fig. 2), but they do play a role either in how the ATPase of the NSF is stimulated by the SNAP-SNARE complex or in how ATP hydrolysis is coupled to complex disassembly. The fact that mixed hexamers, which include Sensor 2 mutants, did not have the dominant-negative effects in vivo (Fig. 6, B and C, and Table 2), indicates that Sensor 2 may act in cis and is not involved in inter-protomer communication during ATP hydrolysis.

Arginine Fingers—Arginine Fingers were first noted in the structure of the Ras/Ras-guanosine, triphosphatase-activating protein (Ras-GAP) complex (19). The positively charged guanidinium group of Arg289 interacts with the fluoride of the GDP-AlF4 complex in the nucleotide-binding site, and the main chain carbonyl oxygen forms a hydrogen bond with a glutamine residue (Gln61) of the switch II region of Ras. This arginine is thought to participate in catalysis by stabilizing the transition state during GTP hydrolysis. A similar interaction was found in p97-D1, where Arg359 from a neighboring protomer interacts with the γ-phosphate of ATP, and Arg622 forms a salt bridge with Glu305 in the Walker B motif on an adjacent subunit (51). The significance of Arginine Fingers in the AAA⁺ family was first noted for FtsH; Arg312 and Arg315 are crucial for ATP hydrolysis and protease activity (52). Subsequently, the importance of Arginine Fingers has been shown for many AAA⁺ proteins (42, 44, 50, 53–57). The role of Arginine Fingers in NSF has been unclear given our previous report that mutations of Arg345 or Arg388 did not affect basal or stimulated ATPase activity (16). These mutants were re-examined in this study. Consistent with previous studies (16), basal ATPase activities

### Table 1

| ATPase and SNAP-SNARE binding activity of NSF-N mutants | Basal ATPase | SNAP-SNARE binding |
|--------------------------------------------------------|--------------|---------------------|
|                                                         | %            | %                   |
| **NSF**                                                | 100 ± 15     | 100 ± 8             |
| R10A                                                   | 100          | 0                   |
| R10E                                                   | 100 ± 1      | 0                   |
| D14A                                                   | 85 ± 5       | 85 ± 5              |
| D14R                                                   | 70 ± 7       | 25 ± 5              |
| E15A                                                   | 100 ± 3      | 0                   |
| E15R                                                   | 100 ± 3      | 0                   |
| R67A                                                   | 110 ± 1      | 0                   |
| R67E                                                   | 36 ± 5       | 36 ± 5              |
| K68E                                                   | 100 ± 1      | 17 ± 2              |
| S73R                                                   | 100 ± 1      | 50 ± 5              |
| Q76R                                                   | 100 ± 3      | 80 ± 5              |
| **N₁₅**                                               |              |                     |
| K104A                                                  | ND           | 0                   |
| K104E                                                  | ND           | 0                   |
| K105A                                                  | ND           | 0                   |
| K105E                                                  | ND           | 0                   |
| K104/K105A                                             | 100 ± 3      | 0                   |
| D142A                                                  | ND           | 20 ± 5              |
| D142R                                                  | 30 ± 8       | 30 ± 8              |
| K143A                                                  | 100 ± 1      | 0                   |
| K143E                                                  | 100 ± 4      | 0                   |
| **Hinge**                                              |              |                     |
| Y83E                                                   | ND           | 0                   |
| Y83F                                                   | 110 ± 1      | 43 ± 4              |
| C91A                                                   | ND           | 83 ± 6              |

*SNAP-SNARE complex binding and basal ATPase activity of wild-type NSF were set as 100%. The values for the mutants were normalized to the wild-type NSF.

### Table 2

| Summary of NSF mutant phenotypes |
|----------------------------------|
| NSF and mutants formed hexamers analyzed by gel filtration chromatography and mixed hexamers in HeLa cells (shown by +). |

| Hexameric | Mixed hexamer | SNAP-SNARE binding | SNAP-SNARE disassembly | Basal ATPase | Stimulated ATPase | Toxicity |
|-----------|---------------|---------------------|-------------------------|--------------|-------------------|----------|
|           |               | %                   | %                       | %            | %                 |          |
| **NSF N₁₅** |               |                     |                         |              |                   |          |
| R67A (N₁₅) | +             | +                   | 100 ± 8                 | 100 ± 20     | 100 ± 15          | 100      |          |
| Y83F (hinge) | +             | +                   | 43 ± 4                  | 91 ± 18      | 110 ± 1           | ND       |          |
| **NSF D1 mutants** |               |                     |                         |              |                   |          |
| E329Q (Walker B) | +            | +                   | 100 ± 4 (15)            | 0            | 30 ± 5 (17)       | 0        | +        |
| R385A (Arg fingers) | +           | +                   | 34 ± 5                  | 0            | 65 ± 25           | 0        | +        |
| E440A (sensor 2) | +            | +                   | 93 ± 16                 | 0            | 85 ± 15           | 0        | –        |
| L441A (sensor 2) | +            | +                   | 36 ± 4                  | 0            | 85 ± 7            | 0        | +/-      |
| Y296F (pore) | +            | +                   | 36 ± 1                  | 94 ± 20      | 70 ± 6            | 100      | –        |
| G298A (pore) | +            | +                   | 22 ± 4                  | ND           | 100 ± 1           | ND       | +/-      |
| Y296F/G298A (pore) | +          | +                   | 84 ± 4                  | ND           | 100 ± 20          | ND       | +/-      |

*Chaperone activity (SNAP-SNARE complex binding and disassembly) and ATPase activity (basal and stimulated ATPase) of wild-type NSF were set as 100%. Toxicity to HeLa cells reflects the ability of the NSF mutants to cause cell death and to disrupt Golgi morphology (+, cell death and Golgi disrupted; +/-, no cell death but Golgi ribbons were swollen; –, no effect). ND means not determined.
Dissecting the N-Ethylmaleimide-sensitive Factor

were close to that of wild-type NSF for both R385A and R388A (Fig. 2) suggesting that the arginines are not required for basal hydrolysis. The R385A and R388A mutations did affect SNAP-SNARE complex binding (Fig. 3), but both mutations eliminated ATPase-dependent release (Fig. 4) and blunted SNAP-SNARE complex-stimulated ATPase activity in vitro (Fig. 5). The latter was a deviation from our previous report (16), perhaps because of our improved preparations of SNARE complexes. These data indicate that Arginine Fingers play a role in inducing a SNAP-SNARE-binding competent state and in mediating ATP hydrolysis when NSF is engaged with SNAP-SNARE complexes. Given their proposed position in NSF-D1 (Fig. 1B), Arginine Fingers are likely to function in trans on the adjacent protomer. Consistently, when expressed in HeLa cells, the R385A mutant had a dominant-negative effect as did the Walker B motif mutant E329Q (Fig. 6). Of the mutants tested, only E329Q and R385A caused appreciable cytotoxicity and alterations in Golgi complex morphology.

Inter-protomer Interactions—AAA + family proteins generally form ring-shaped hexamers, and ATP binding appears to be important for oligomerization of some family members, e.g. yeast Vps4, mammalian SKD1/VPS4B, and bacterial ClpB (42, 55, 56, 58). ATP binding could induce conformational changes that promote oligomerization. Alternatively, oligomerization could bring the residues from neighboring protomers in close contact to the bound nucleotide thus promoting hydrolysis and/or stabilizing the oligomer (these are not mutually exclusive). Arginine Fingers from a neighboring protomer are an example of these types of residues. They can serve as trans-acting elements and directly or indirectly interact with the γ-phosphate. Mutations in Arginine Fingers abolish hexamerization in ClpB (56) and decrease cooperative helicase activity in MCM (50). In p97, the R362E mutation partially disrupts the hexamer, and R359E/R362E double mutants are predominantly monomers (59). A hexamerization defect was not observed in the NSF-D1 mutants reported here. NSF-D2 is required for oligomerization (15) but has no conserved Arginine Fingers nor does it catalyze significant ATP hydrolysis.

Only the E329Q and R385A mutants were toxic when expressed in HeLa cells (Fig. 6C). Given that both mutants did form mixed hexamers (Fig. 7), it seems plausible that inclusion of a mutant protomer “poisons” the hexamer, thus leading to cytotoxicity. This would imply some concerted mechanism to perturb the residues from neighboring protomers in close contact to the bound nucleotide thus promoting hydrolysis, or trans on the adjacent protomer. Consistently, when expressed in HeLa cells, the R385A mutant had a dominant-negative effect as did the Walker B motif mutant E329Q (Fig. 6). Of the mutants tested, only E329Q and R385A caused appreciable cytotoxicity and alterations in Golgi complex morphology.

In vivo expression of Y83F caused no adverse effect on the HeLa cells, a result that differs from previous reports (38). This deviation is hard to explain but may be rooted in the different cell types used. However, the lack of effect when the R67A and Y83F mutants were expressed in HeLa cells is in good agreement with the conclusion that NSF hexamers do not need a full complement of functional N domains for activity (15). This conclusion is also consistent with the “3-in, 3-out” model for NSF-N function, where it is proposed only three N domain-containing protomers are needed to interact with SNAP-SNARE complexes (13).

Central Pore—Several AAA + proteins, (e.g. Hsp104, ClpB, ClpA, ClpX, FtsH, HslUV, and Lon) share a common threading mechanism for interacting with protein substrates (12, 67). The conserved YVG motif forms a hydrophobic patch, which sits above the pore in the hexamer and is responsible for substrate binding and/or translocation into the pore. The central pores in NSF or p97 have been less studied because it is thought that their narrow diameter makes a threading-through mechanism unlikely (12, 68, 69). However, it is possible that the central pore plays other roles. Mutagenesis studies of p97 and VAT suggest that the hydrophobic patch has a similar function to that of the AAA + proteases. The aromatic residue is thought to be
involved in substrate binding and unfolding (28, 49, 70). Single particle analysis of the NSF-SNAP-SNARE complex suggests NSF is arranged as a double-ring structure with a central hole. In NSF-D2, this hole tapers from 3–5 nm at the top (which is proposed to face NSF-D1) to 1.8 nm at the bottom (10, 11). The free SNAP-SNARE complex is 7–8 nm in diameter and 14–15 nm in length. In the NSF-SNAP-SNARE complex, NSF-D1 ring diameter increases (from 12 to 13.5 nm) and the SNAP-SNARE complex shortens (13 nm) (9–11, 25, 26). This change in the dimensions of the components is the only evidence that indicates that the SNAP-SNARE complex rod could partially insert into the NSF-D1 pore. Our studies show that the hydrophobic patch (Tyr296 to Gly298) in one of the loops that protrudes into the NSF-D1 pore may mediate an interaction with the SNAP-SNARE complexes. The mutants in this motif (Y296A, Y296F, G298A, Y296A, Y296F, and G298A) affected SNAP-SNARE binding, although to different extents (Fig. 3). The Y296A (but not Y296F) and the G298A mutants were defective on SNAP assays, we dissected the roles of specific elements of NSF. We characterized most of the important motifs in the catalytic NSF-D1. Combined with our previously published studies of Walker A and Walker B motifs, we have a clearer view of NSF function. The NSF-SNAP-SNARE binding and disassembly process can be divided into four steps as follows: 1) ATP binding; 2) SNAP-SNARE binding; 3) stimulated ATP hydrolysis; and 4) SNAP-SNARE complex disassembly. Walker A and Sensor 1 motifs work in step 1; NSF-N and the central pore region of NSF-D1. We also characterized most of the important motifs in the catalytic NSF-D1. Combined with our previously published studies of Walker A and Walker B motifs, we have a clearer view of NSF function. The NSF-SNAP-SNARE binding and disassembly process can be divided into four steps as follows: 1) ATP binding; 2) SNAP-SNARE binding; 3) stimulated ATP hydrolysis; and 4) SNAP-SNARE complex disassembly. Walker A and Sensor 1 motifs work in step 1; NSF-N and the central pore work in step 2; Sensor 2, Walker B, central pore, and Arginine Finger motifs work in step 3 and/or step 4. The data presented here will be useful in designing future experiments because it describes NSF mutants that are defective at specific steps in the NSF reaction sequence. These mutants will be valuable for future studies that attempt to correlate conformational changes in NSF with specific stages of its reaction cycle.

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Dissecting the N-Ethylmaleimide-sensitive Factor