N-Ethylmaleimide-sensitive Fusion Protein Contains High and Low Affinity ATP-binding Sites That Are Functionally Distinct*  

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Elena A. Matveeva, Ping He, and Sidney W. Whiteheart‡  

From the Department of Biochemistry, Chandler Medical Center, University of Kentucky College of Medicine, Lexington, Kentucky 40536-0084

N-Ethylmaleimide-sensitive factor (NSF) has been shown to be involved in numerous intracellular membrane fusion events of both the regulated and constitutive secretory pathways. Sequence analysis indicates that the NSF subunit contains two nucleotide-binding sites, both with the classical Walker A and B motifs. In this report, we examine the nucleotide binding properties of NSF. The homotrimer contains three high affinity ATP-binding sites with $K_d = 30-40$ nM for ATP and $K_d = 2 \mu M$ for ADP. This class of binding sites did not bind AMP, adenine, or GTP. A second class of lower affinity nucleotide binding sites with a $K_d = 15-20 \mu M$ was also detected. Using various mutant forms of NSF, the high affinity nucleotide-binding sites were localized to the D2 domains and the low affinity sites were localized to the D1 domains. Functionally it is these lower affinity sites in D1 that are crucial for NSF activity. Nucleotide concentration greatly affected the ability of NSF to interact with $\alpha$-SNAP-SNARE (soluble NSF attachment protein-SNARE receptor) complex, suggesting that only when the D1 domain ATP-binding sites are occupied does NSF bind to the $\alpha$-SNAP-SNARE complex.

The N-ethylmaleimide-sensitive factor (NSF) was originally purified based on its ability to restore intercisternal transport activity to Golgi membranes that had been previously treated with the alkylating agent N-ethylmaleimide (NEM) (1, 2). The cDNA encoding this protein was subsequently cloned (3) and shown to have significant homology to a previously identified yeast gene, Sec18 (4). Cells carrying a temperature-sensitive mutation in Sec18, when incubated at restrictive temperature, were unable to secrete proteins and exhibited an intracellular buildup of small vesicles (5–7). When mammalian Golgi membranes were treated with NEM, there was also an accumulation of vesicles that appeared to be proximal to larger cisternal membranes (8). This data demonstrate that NSF/Sec18p is essential for the productive consumption of transport vesicles. Genetic and biochemical experiments clearly show that NSF/Sec18p is used in numerous intercompartmental transport steps of both the constitutive (2, 7, 9–17) and regulated secretory pathways (18–21). It would appear that the NSF/Sec18p is required for most heterotypic membrane fusion events in the cell, with one possible exception (22), and also some of the homotypic membrane fusion events (23, 24).

Many reports have established the importance of NSF in vesicular trafficking processes, yet its mechanism of action has remained elusive. Original data suggested that NSF was required for membrane fusion, since the NEM-accumulated vesicles seemed to be docked to larger membranes which could be construed as target membranes (8). This hypothesis was strengthened by the demonstration that NSF participates in the formation of a 20 S complex composed of peripheral membrane proteins (soluble NSF attachment proteins, SNAPs) and integral membrane proteins (SNAP receptors, SNAREs), which are localized to separate aspects of a membrane fusion junction, i.e. the vesicle (v-SNAREs) and the target membrane (t-SNAREs) (25). NSF, through its ability to hydrolyze ATP, disassembles this 20 S complex in a process that was proposed to be consistent with membrane fusion (26, 27). However, this proposal has recently come into question, based on kinetic experiments using a number of in vitro systems (7, 17–19, 28–31). These data indicate that NSF functions prior to membrane fusion. This early role is most directly shown in the yeast vacuole fusion assay, in which NSF appears to function at a step that can occur even prior to mixing of the two membrane fusion partners. NSF seems to act on the two fusion partners separately by “priming” their SNARE proteins for the subsequent interactions that are involved in membrane docking and fusion (30, 31). In this manner, NSF could be thought to act as a molecular chaperone (32), using ATP hydrolysis to modulate the structure of the SNARE proteins, much like HSP-90 appears to act on steroid receptors (33). Recently it has also been shown that 20 S complexes are present on vesicle membranes, suggesting that the v- and t-SNAREs can bind each other in the same membrane (34, 35). From this data, it seems possible that the NSF-mediated disassembly may in fact be needed to disengage these complexes formed in the same membrane so that they may bind to the SNAREs on opposing membranes.

NSF in dilute solution appears to exist as a homotrimer, yet it has the ability to form homohexamers, as indicated by electron microscopic studies (36–38). Sequence and limited proteolysis analysis indicate that NSF subunits are made up of three domains, an amino-terminal N domain followed by two Walker-type ATP binding domains, D1 and D2 (3, 39). Each domain has been shown to play a distinct role in the overall function of the NSF oligomer. The N domain is required for binding to the SNAP-SNARE complex and thus formation of the 20 S complex (40). Mutational analysis shows that it is the ability of the D1 domain to bind and hydrolyze nucleotide that is critical for transport activity (28, 38, 40). In fact it appears that it is an ATPase-driven interaction between the N and D1 domains that is critical for 20 S complex disassembly (40). This is consistent with structural studies which indicate that when NSF is in the
ATP form, the N domains extend radially from a central core, and these domains are contracted in the ADP form (36). The D2 domain, while apparently not required for its nucleotide binding or hydrolysis capacity, is essential to oligomerization and appears to contribute to the formation of the central core of NSF (36, 38, 40).

To further our understanding of NSF and its properties we have examined the nucleotide binding characteristics of NSF. In this study, we demonstrate that each NSF oligomer contains two distinct classes of ATP-binding sites, low and high affinity, which can be distinguished based on their contribution to NSF structure and function.

EXPERIMENTAL PROCEDURES

Materials—ATP and GTP were obtained from Boehringer Mannheim, and ADP, AMP, and adenine were from Sigma. [alpha-32P]ATP and [gamma-32P]ATP, 800 and >3000 Ci/mmol, were from ICN (Costa Mesa, CA). Pure nitrocellulose transfer and immobilization membranes (0.45 μm for measurement of ATP binding and 0.2 μm for immunoblotting) were purchased from Schleicher & Schuell. Horseradish peroxidase conjugated to anti-immunoglobulin secondary antibodies was from Sigma, and SuperSignal chemiluminescent substrate was purchased from Pierce. Glutathione insolubilized on cross-linked 4% beaded agarose was from Pierce. All chemicals were of reagent grade.

Wild-type NSF and NSF domain rearrangement mutants, α-SNAP and GST-syntxin 1 (cytoplasmic domain), were produced as recombinant proteins in Escherichia coli and purified as described previously (40). Protein concentrations were measured according to the method of Bradford (41) using ovalbumin from Sigma as a standard.

Measurement of ATP Binding—Binding of [alpha-32P]ATP and [gamma-32P]ATP to NSF and to the NSF mutants was determined by the filtration method with a slight modification (42). Briefly, the reaction mixture (20 μl) containing 50 mM HEPES/NaOH (pH 7.8), 150 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol, the indicated concentration of radioactive adenine nucleotide ([alpha-32P]ATP or [gamma-32P]ATP, 500 and >3000 Ci/mmol) and 1.2 nmol of protein was incubated for 10 min at room temperature and filtered through a nitrocellulose membrane (BA-85, 0.45 μm, Schleicher & Schuell). The membrane was washed five times with 1.5 ml of washing buffer containing 10 mM Tris-HCl, pH 7.8, 120 mM NaCl, 2 mM β-mercaptoethanol. The radioactivity retained in the membrane was counted in 3 ml of liquid scintillation mixture (Econo Safe, Research Products International Corp., Mount Prospect, IL) using a Tri-Carb 2100TR (Packard Instrument Co.) liquid scintillation counter. For the competition analysis, radioactive ATP was incubated with NSF or NSF mutants prior to the addition of the indicated concentrations of nonradioactive ATP, ADP, AMP, adenine, and GTP.

Photoaffinity Labeling—Direct photoaffinity labeling of proteins with [alpha-32P]ATP was carried out according to Antonoff (43). Protein samples were irradiated by a UV source with peak emission at 254 nm for 1 h at ~80 °C. After labeling, the proteins were precipitated with trichloracetic acid and analyzed by SDS-polyacrylamide gel electrophoresis. The dried gel was exposed directly on X-Omat film (Eastman Kodak Co.), and the film was developed using a medical film processor Konica QX-70 (Picker, Cleveland, OH).

NSF Binding to the α-SNAP-Syntxin 1 Complex—Soluble protein extracts from Escherichia coli cells expressing the GST-syntxin 1a (cytoplasmic domain) recombinant protein were incubated with pre-washed glutathione-agarose beads at 4 °C with rotation 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 seven times in the same buffer, and then equal volumes of the beads were aliquoted into the reaction tubes. Binding reactions were performed in a final volume of 200 μl containing 10 μg of NSF or mutant in binding buffer (20 mM HEPES/KOH, pH 7.4, 250 mM imidazole, 150 mM potassium acetate, 5 mM EGTA, 1% (w/v) glycerol, 1% (w/v) Triton X-100, and 40% (w/v) ovalbumin) with the indicated nucleotide and divalent cation (see Fig. 6). After 2 h of incubation at 4 °C, the beads were washed five times in binding buffer without ovalbumin. The bound NSF was eluted with SDS-polyacrylamide gel electrophoresis sample buffer and analyzed by Western blotting using the mouse monoclonal 6E6 antibody (59).

Gel Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was performed under reducing conditions on 12.5% slab gels in a discontinuous buffer system according to Laemmli (44). Western blotting onto nitrocellulose was performed according to standard techniques (40) using 5% powdered milk in Tris-buffered saline and horseradish peroxidase conjugated to anti-immunoglobulin secondary antibodies. SuperSignal substrate was used to visualize the immunodecorated antigens using X-Omat x-ray film.

RESULTS

NSF Contains Three Specific High Affinity ATP-binding Sites—Nucleotide binding experiments were performed with recombinant NSF protein using the nitrocellulose filter binding assay. As can be seen in Fig. 1, the binding of radiolabeled ATP increases with increasing concentration, and saturable binding is achieved at approximately 100 nM ATP. These data were subjected to Scatchard analysis (Fig. 1, inset), and a \( K_d \) for ATP binding of 30–40 nM was calculated. (alpha-32P]ATP and (gamma-32P]ATP showed similar binding characteristics, suggesting that true ATP binding was being measured in these experiments with no significant nucleotide hydrolysis. From these data and other saturation curves, 3.3 high affinity ATP-binding sites were detected per NSF trimer. This ratio of 3 mol of ATP/mol of NSF was reproducible when fresh preparations were analyzed but was less so after the proteins had been stored for extended periods at 4 °C.

Competition experiments were used to examine the nucleotide binding specificity of the NSF trimer. As shown in Fig. 2A, only ATP and ADP could effectively compete with \([^{32}P]ATP\) for binding. AMP and adenine were unable to compete for ATP binding even when used at 100-fold excess. GTP showed a slight degree of competition at the highest concentrations, which may reflect the importance of multiple phosphates to the nucleotide binding properties of NSF. In these experiments NSF was prebound with \([^{32}P]ATP\), and then increasing amounts of the indicated unlabeled nucleotides were added to evaluate their ability to displace the bound \([^{32}P]ATP\). The amount of \([^{32}P]ATP\) still bound after incubation was measured and the data was used as another indication of the dissociation constant. The resulting \( K_d \) value calculated for ATP (30–40 nM) was in good agreement to the values calculated from the Scatchard analysis in Fig. 1. Interestingly, the \( K_d \) for ADP was about 2 μM, suggesting that these sites can bind ATP and ADP but with an 100-fold difference in affinity.

To further demonstrate that NSF in our preparations was indeed binding ATP, photoaffinity radiolabeling of NSF was performed (Fig. 2B). In the complete reaction (inset) NSF NSF was covalently modified by bound \([^{32}P]ATP\) after irradiation. This labeling was specific, since it was not present when a 100-fold molar excess of unlabeled ATP was added to the reac-
The analysis above indicates that NSF contains three specific high affinity ATP-binding sites, yet its amino acid sequence suggests that six sites are possible per trimer. Domain rearrangement and ATP-binding site mutants were examined in binding assays to determine which of the two ATP binding domains, D1 or D2, contains the high affinity ATP-binding sites (Fig. 3). Mutant proteins that lacked the D2 domain (ND1) or had the critical lysine residue of the Walker A motif of the D2 mutated (D2K-A) and were unable to bind ATP when the concentration in the binding reaction was held at 0.25 μM (Fig. 3A). Wild type NSF or mutants that contain the D2 domain (ND2, D1D2, and D2) were all able to bind [32P]ATP. In each mutant the stoichiometry of binding was approximately three ATPs per NSF or mutant trimer. These data show that the high affinity ATP-binding site of NSF is in the D2 domain. The photoaffinity labeling of domain rearrangement mutants by [32P]ATP at same concentration as the binding experiment confirms the previous results. Only proteins containing functional D2 domains are labeled. The ND1 mutant and D2K-A mutant, which lack functional D2 domains, were not labeled under these conditions (Fig. 3B).

**NSF Contains Three Lower Affinity Nucleotide-binding Sites**—Previous reports have demonstrated the ability of the D1 domain to bind and hydrolyze ATP. This activity is critical for NSF function in the vesicular transport process; however, this domain (D1) does not appear to contain high affinity ATP-binding sites. The concentrations of [32P]ATP used in the nucleotide binding experiments of Fig. 1 were extended to determine whether NSF possessed a second, lower affinity class of nucleotide-binding sites. As can be seen in Fig. 4A, two plateaus were present in this titration. The first (saturating at 100 nM) corresponds to the high affinity sites described in Fig. 1.
ATP. The reactions with 1.2 nM NSF were incubated in a volume of 20 μl for 10 min and the amount of [32P]ATP bound to NSF was determined as described in Fig. 1. The inset shows a Scatchard plot of the data points of only the second plateau region of the curve. B, characterization of the specificity of the low affinity sites. Specificity was determined by the ability of unlabeled ATP and ADP to displace the [32P]ATP prebound to NSF. The binding reactions were as before with the inclusion of the indicated concentration of unlabeled nucleotide. The concentration of [32P]ATP was 30 μM.

The data points from the second plateau were subjected to Scatchard analysis and a $K_d$ of 15–20 μM was calculated. A Hill plot analysis of the two classes of ATP-binding sites indicated that there is no cooperativity and that both classes of sites appear to be independent. Competition experiments done for these high affinity sites show that the binding for ATP and ADP are quite similar in affinity (Fig. 4B). The $K_d$ values estimated from this experiment were 40 μM for ATP and 140 μM for ADP, which is in good agreement with the Scatchard analysis above (Fig. 4A).

To determine which domain accounts for this class of lower affinity nucleotide-binding sites, the different domain rearrangement mutants were analyzed for ATP binding at the two different ATP concentrations, 0.5 μM and 50 μM (Fig. 5). At the lower ATP concentration only the high affinity sites would be detected, and at the high concentration both sites would contribute as shown for wild type NSF (NSF). Mutants lacking a functional D2 ATP-binding site (ND1 and D2K-A) only showed binding at the high [32P]ATP concentration. Mutants lacking a functional D1 domain binding site (D1K-A and D2) showed binding at the low concentration, but it did not increase at the higher [32P]ATP concentrations. These data indicate that the low affinity site is in the D1 domain, whereas the high affinity site is in the D2 domain.

**Fig. 4. Detection of low affinity ATP-binding sites.** A, the titration experiment of Fig. 1 was extended by isotopic dilution of the [32P]ATP. The reactions with 1.2 nM NSF were incubated in a volume of 20 μl for 10 min and the amount of [32P]ATP bound to NSF was determined as described in Fig. 1. The inset shows a Scatchard plot of the data points of only the second plateau region of the curve. B, characterization of the specificity of the low affinity sites. Specificity was determined by the ability of unlabeled ATP and ADP to displace the [32P]ATP prebound to NSF. The binding reactions were as before with the inclusion of the indicated concentration of unlabeled nucleotide. The concentration of [32P]ATP was 30 μM.

**Fig. 5. Relative [32P]ATP binding to NSF mutants at low and high nucleotide concentrations.** NSF mutants as described in Fig. 3 were incubated with either 0.5 or 50 μM [32P]ATP. The bound nucleotide was determined by filter binding assay as described in Fig. 1. The data were presented as moles of ATP bound per mole of protein. All of the mutants tested have been shown to be trimeric (molecular mass 255 kDa) except ND1, which is monomeric.
In this report, we demonstrate that NSF has two classes of ATP-binding sites that are distinct in their nucleotide binding properties and functional characteristics. One class has a high affinity for ATP ($K_d$ of 30–40 nM) with a 100-fold lower affinity for ADP. These sites are in the D2 domain, but they have been shown not to be catalytically required for NSF activity (38). The second class, found in the D1 domain, has a 1000-fold lower affinity for ATP with a $K_d$ of 15–20 µM. Mutational studies indicate that it is this D1 ATP-binding site that is catalytically important for NSF activity (38). The binding of ATP by the D1 domains is crucial for NSF to interact with other components of the membrane fusion machinery. Specifically, NSF binds to the SNAP-SNARE complex only at nucleotide concentrations which would lead to occupancy of the lower affinity sites. At lower concentrations of ATP, when only the D2 sites are occupied, NSF is incompetent to bind to the SNAP-SNARE complex. This demonstrates that the two distinct aspects of NSF function, 20 S complex assembly and vesicular transport activity, are controlled by the dynamics of the nucleotide bound to the D1 domain.

**Potential Role of the D1 Domain ATP-binding Sites**—Previous reports have indicated the catalytic importance of the D1 domain to NSF activity. Studies done with point mutations in the Walker A and B motifs have shown that the D1 domain accounts for the majority of the ATPase activity of the NSF trimer. Additionally, these studies demonstrated that the ATP binding and hydrolytic activity of this domain are critical for NSF function in the vesicular transport processes (38). From the previous studies with mutant NSF proteins (40) and the studies with wild type protein presented here, it is clear that nucleotide binding to the D1 domain is required for NSF to interact with the other elements of the membrane fusion apparatus. Binding of ATP to the D1 domain appears to induce a conformational change in NSF so that it is able to make all of the protein-protein interactions required for formation of the 20 S complex. This is supported by recent structural data that show a radial extension of the N domains when NSF is in the ATP-bound form (36). This splaying of N domains is essential for NSF binding to the SNAP-SNARE complex (36, 40). Mechanistically, this indicates that only the activated form of NSF, charged with ATP, can participate in formation of the 20 S fusion complex. The ADP-bound form of NSF is incompetent for SNAP-SNARE binding (Fig. 6A).

**Potential Role of D2 Domain ATP-binding Sites**—The D2 ATP-binding sites show a high affinity and selectivity for ATP, but when assayed as an isolated trimeric domain, D2 has very low ATPase activity (40). The fact that the D2 domains bind ATP approximately 100-fold higher than ADP is consistent with other nucleotide binding proteins that lack ATPase activity (45). Unlike the D1 domain, the amino acid sequence of the D2 ATP-binding site is solely distantly related to the 240 amino acid consensus ATP-binding sites of the ATPases associated with a variety of cellular activities (AAA family); therefore the D2 domain may not have the ATPase activity or the associated force-generating capacity predicted for the other AAA protein (46). Aside from its role in multimerization of NSF, it has been difficult to ascribe a function to this domain. Mutations in the ATP-binding site of D2 fail to have a significant phenotype, although the resulting proteins do not appear to be equivalent to wild type NSF in the intra-Golgi transport assay (38). At present, it is hypothesized that the D2 domain plays a structural role in NSF function similar to the proposed role of the α subunit of the $F_1F_0$ ATPase (47). Like the $F_1F_0$ α subunit, the D2 domain has a high affinity ATP-binding site that might be required for the folding and stability of the oligomer (36).
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