Each Domain of the N-Ethylmaleimide-sensitive Fusion Protein Contributes to Its Transport Activity*

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N-Ethylmaleimide-sensitive fusion protein (NSF) has been shown to be involved in numerous intracellular transport events. In an effort to understand the basic mechanism of NSF in vesicle-target membrane fusion events, we have examined the role that each of its three domains play in how NSF interacts with the SNAP-SNARE complex. Mutagenesis of the first ATP-binding domain (D1, amino acids 206-477) demonstrates that nucleotide binding by this domain is required for 20 S particle assembly. A second mutation, which permits ATP binding but not hydrolysis, yields a protein that can form 20 S particle but fails to mediate its disassembly. Similar mutations of the second ATP-binding domain (D2, amino acids 478-744) result in trimeric molecules that behave like wild type NSF. Domain rearrangement mutants were used to further probe the functional role of each domain. The amino-terminal domain (N, amino acids 1-205) is absolutely required for binding of NSF to the SNAP-SNARE complex, because the truncated mutant, D1D2, is unable to form 20 S particle. When tested as an isolated recombinant protein, the N domain is not sufficient for binding to the SNAP-SNARE complex, but when adjacent to the D1 domain or in a trimeric molecule, the N domain does mediate binding to the SNAP-SNARE complex. Monomeric N-D1 and trimeric N-D2 could both participate in particle formation. Only the N-D1 mutant was able to facilitate MgATP-dependent release from the SNAP-SNARE complex. These data demonstrate that NSF binding to the SNAP-SNARE complex is mediated by the N domain and that both ATP binding and hydrolysis by the D1 domain are essential for 20 S particle dynamics. The intramolecular interactions outlined suggest a mechanism by which NSF may use ATP hydrolysis to facilitate the vesicle fusion process.

Several groups have now shown a requirement for the N-ethylmaleimide-sensitive fusion protein (NSF/Sec18p) in numerous intracellular fusion events of both regulated and constitutive secretion (reviewed in Ref. 1). Kinetic analysis, using the intra-Golgi transport assay, shows that NSF/Sec18p acts at a late stage in the transport process. N-Ethylmaleimide inhibition of intra-Golgi transport leads to an accumulation of uncoated vesicles that appear to be consumed upon the addition of pure NSF (2). Studies with the temperature-sensitive mutant alleles, sec18-1 or sec18-2, show that under restrictive conditions there is also a build-up of 50 nm vesicles (3). These results demonstrate that NSF/Sec18p is required for vesicle consumption and suggest that it is involved at or near the actual vesicle-target membrane fusion step. Recent experiments have pointed to a role for NSF in earlier stages, such as vesicle formation or priming (4, 5). However, these data conflict with other experiments (6, 7) that show that NSF/Sec18p is not required for production of active transport vesicles in vitro. Although its precise function remains to be elucidated, it is clear that NSF is a general transport factor that plays a central role in many (though perhaps not all; Ref. 8) of the heterotypic fusion events in the cell.

To explain heterotypic fusion events, Rothman and colleagues proposed the SNAP Receptor (SNARE) hypothesis (9), in which the specificity of vesicle-target membrane docking is mediated by the matching of a t-SNARE from the target membrane with its cognate v-SNARE in the vesicle membrane, thereby forming a docking or 7 S complex. This complex then provides binding sites for the soluble NSF attachment proteins (SNAPs), which are absolutely required to mediate the correct positioning of NSF (10-13). This last step serves to complete the formation of the so-called 20 S fusion particle. At least part of the energy for membrane fusion is thought to be provided by the hydrolysis of ATP by NSF, because mutant forms of NSF that are unable to hydrolyze ATP also fail to complete the vesicular transport process (14). To describe the molecular basis of vesicle-target membrane fusion, it becomes critical to understand how NSF interacts with the other elements of the fusion machinery (SNAPs, SNAREs, etc.) and how it might use the energy from ATP hydrolysis to facilitate membrane fusion.

The subunit of the homotrimeric NSF can be divided into three domains: an amino-terminal (N, amino acids 1-205) and two ATP-binding domains (D1, amino acids 206-477, and D2, amino acids 478-744) (15, 16). Initially delineated by sequence analysis, these domains probably represent discrete structural entities because they are released by limited proteolysis of the intact molecule (16). Mutations in the ATP-binding site of domain D1 (binding mutants Lys266 to Ala (D1K-A), Glu or Met or hydrolysis mutant Glu to Gln (D1E-Q)) eliminate intra-Golgi transport activity and cause a 70-80% decrease in ATPase activity relative to wild type NSF (14, 17). These mutant proteins inhibit intra-Golgi transport in a competitive fashion that as demonstrated in this manuscript (see Fig. 1), is most likely due to their ability to form 20 S fusion particle but not mediate MgATP-dependent particle disassembly. The D2 domain is required for trimerization, but its ability to bind
Function of NSF Domains

29183

(mutant Lys549 to Ala (D2K-A), Gln, or Met) or hydrolyze (mutant Asp604 to Glu (D2D-Q)) ATP does not seem to be specifically required for intra-Golgi transport (14, 17). The ATP hydrolytic activity of this domain makes only a small contribution to the overall ATPase activity of NSF (30–40%) (14). The amino-terminal domain has been proposed to exert some control over the ATPase activity of NSF because antibodies directed against it cause a 2-fold increase in hydrolytic activity (17). A similar increase in ATPase activity was observed when NSF was bound to SNAPs that had been immobilized on a plastic surface (18). We present data demonstrating that the N domain of NSF is required for interaction with the rest of the 20 S particle components. Deletion of this domain results in a trimeric molecule (D1D2) with ATPase activity but no ability to bind to the SNAP-SNARE complex. It has been suggested that each of the three domains of NSF has a distinct contribution to the overall activity of the NSF trimer. In this manuscript we propose a role for the N domain in NSF binding to the SNAP-SNARE complex and demonstrate the importance of nucleotide binding and hydrolysis by the D1 domain to 20 S particle dynamics. Further dissection of the role of these domains will undoubtedly shed new light on the cellular function of NSF and may elucidate new aspects of the heterotypic fusion process.

EXPERIMENTAL PROCEDURES

Materials—The monoclonal antibodies, 9E10 (ATCC CRL 1729; Ref. 19) and J66 (17) were prepared from tissue culture supernatants. Anti-amino-terminal domain antibodies were produced in rabbits after immunization with the recombinant N domain and were used as crude sera. HPC1 (20) was prepared from ascites and was a generous gift of Drs. James Rothman and Thomas Söllner. Antibodies for immunoprecipitation were covalently coupled to beads by initially binding the relevant recombinant protein G-Sepharose (Pharmacia Biotech Inc.) followed by cross-linking with dimethyl pimelimidate (Pierce) (21). α-SNAP was purified by NINTA-agarose (QIAGEN Inc., Chatsworth, CA) affinity chromatography as described previously (22). Bovine brain extract was prepared as described (9) and dialyzed against 25 mM Tris/HCl, pH 7.8, 50 mM KCl, 1 mM 1,4-dithiothreitol, and 1% Triton X-100 before use. In vitro translation of [35S]-α-SNAP was performed as described previously, and the radiolabeled product was partially purified by ammonium sulfate precipitation (23). The protein concentration of the bovine brain extract preparation was determined by the bicinchoninic acid assay (Pierce) using bovine serum albumin as standard; all other protein concentrations were determined using the Bio-Rad protein assay reagent and ovalbumin as a standard. All chemicals were of reagent grade.

Production and Purification of NSF Mutants—The point mutation and domain rearrangement mutants of NSF were constructed as described previously (14), cloned into pQE-9 (QIAGEN), and expressed in E. coli after induction with 1 mM isopropyl-thio-β-galactoside (Boehringer Mannheim). For the experiments in Fig. 1, all mutants were constructed with a carboxyl-terminal myc epitope that allows immunoprecipitation of the recombinant protein by the 9E10 antibody (19). This epitope has been shown to have no effect on the activity of the wild type NSF molecule (24). For purification of the recombinant proteins, E. coli cells were disrupted using a French press, insoluble material was removed by centrifugation, and the His-tagged proteins were purified by NINTA-agarose (QIAGEN) affinity chromatography as described (22). Additional purification for ATPase studies or for characterization of the oligomeric state of the mutant proteins was performed by gel exclusion chromatography on Superose 6 (Pharmacia; 1.5 × 45 cm, 0.5 ml/min) in 10 mM HEPES/NaOH, pH 7.0, 300 mM NaCl, 2 mM β-mercaptoethanol, 0.5 mM ATP, 0.5 mM MgCl2, and 5% glycerol. SDS-PAGE was used to stabilize trimeric NSF more effectively than previously used buffers (25).

20 S Particle Formation and Disassembly Assays—Two basic types of assays were used to measure 20 S particle formation and disassembly. In the first assay, based on Wilson et al. (12), particle formation was assayed by the association of [35S]-α-SNAP with wild type or mutant NSF in a SNARE-dependent fashion. For this assay, particle formation was measured by the incorporation of the 9E10 antibody coupled to beads. The complexes were collected on glass fiber filters, and the [35S]-α-SNAP bound was quantified by liquid scintillation counting. In a permutation of this assay, excess unlabelled α-SNAP was used, and the resulting complexes were isolated by immunoprecipitation. The beads were then incubated in buffer containing either 5 mM ATP or ATPγS and 5 mM MgCl2 for 30 min on ice. The release of complex components into the supernatant was determined by Western blotting using the anti-syntaxin antibody (HPC1). For the second type of assay, based on the work of Söllner et al. (20), complexes were formed with α-SNAP, SNAREs from bovine brain extract, and NSF (or mutant form) then immunoprecipitated using the anti-syntaxin antibody HPC1. For these experiments, ATPγS or ATP was added during the formation phase of the reaction. After immunoprecipitation, the proteins co-immunoprecipitated by HPC1 were eluted by 0.2 M glycine, pH 2.7, and 1% Triton X-100 and analyzed by Western blotting. Each type of reaction (final volume, 500 μl) was carried out in 20 mM HEPES/KOH, pH 7.0, 100 mM KCl, 1 mM Triton X-100, 1 mM 1,4-dithiothreitol, 1% polyethylene glycol, and 1% glycerol with ATPγS, ATP, or MgCl2 as noted in the legend. Particle formation was allowed to occur on ice for 45 min. The reactions were centrifuged (8 min at 14,000 × g), and the supernatants were incubated with the relevant antibody conjugate for 2 h with gentle rotation. In experiments done with HPC1, >70% of the available syntaxin protein was immunoprecipitated; likewise, >90% of the myc-tagged proteins were precipitated with the 9E10 antibodies coupled to beads. The amounts of all mutant proteins were equalized based on protein assays and by Coomassie blue staining after gel electrophoresis. Films were developed by Enhanced Chemiluminescence (Amersham Corp.), and all exposures were matched relative to a wild type NSF control, which was included in each experiment. Shown in Fig. 3B is the immunodetection of each NSF mutant. To ensure detection of all mutants, multiple antibody probes were used due to the differences in the epitopes recognized by the various antibodies (see Fig. 3B). Several of the recombinant proteins were partially proteolized during preparation (see Fig. 3B). The data in Fig. 3A represent only the full-length (as calculated from their sequence) proteins.

Miscellaneous Assays of NSF Activity—ATPase assays were performed as described previously (16) using [γ-32P]ATP in a buffer containing 25 mM Tris/HCl, pH 9.0, 100 mM KCl, 0.5 mM 1,4-dithiothreitol, 1 mM MgCl2, 10% glycerol, 1 mM ATP, and 10 μl of [α-32P]ATP (DuPont NEN). At given time points, aliquots (2 μl) of each reaction were spotted on a polyethyleneimine thin layer plate (j.T. Baker Inc., Phillipsburg, N.J.) and chromatographed on 0.7 mm LiCl and 1 M acetic acid. The spots corresponding to an ADP standard were excised and quantified by liquid scintillation counting. In no assays was there a significant amount of AMP formed. Intra-Golgi transport assays in a final volume of 25 μl were performed as described previously (25). Briefly, Golgi membranes were prepared from wild type Chinese hamster ovary cells and mutant 15B Chinese hamster ovary cells (lacking GlcNAc transferase I) that had been previously infected with vesicular stomatitis virus. Transport of a viral marker protein from the Golgi complex (donor) to the wild type Golgi complex (acceptor) was monitored by incorporation of [3H]GlcNAc transferase I into the vesicular stomatitis virus G glycoprotein. Soluble components for transport activity were provided by a cytosolic fraction prepared from wild type Chinese hamster ovary cells. To measure NSF activity in transport, membranes were treated with 1 mM N-ethylmaleimide for 15 min on ice prior to use in the standard transport assay (26).

RESULTS

Role of the ATP-binding Sites of NSF in 20 S Particle Formation and Disassembly—The basic configuration of the SNARE assay of Wilson et al. (12) was used to determine whether the ATP-binding domain mutants of NSF could participate in 20 S particle formation and subsequent MgATP-mediated particle disassembly. In the assay represented in Fig. 1A, the ability of an NSF mutant to form 20 S particle was determined by measuring the amount of [35S]-α-SNAP associated with immunoprecipitated NSF myc (or mutant) in a SNARE-dependent fashion. For wild type NSF, particle formation is maintained by the addition of the nonhydrolyzable ATPγS but is eliminated by MgATP in keeping with the role that ATP hydrolysis is reported to play in particle disassembly (12). In a more sensitive permutation of this assay (Fig. 1B), 20 S particle containing NSF (or mutant) was first formed under saturating conditions (excess unlabelled α-SNAP and SNAPs), immunoprecipitated with anti-myc antibodies, and then incu-
Function of NSF Domains

Bated with either ATPγS/Mg or MgATP to initiate particle disassembly. Syntaxin 1 released from the particle was measured by Western blotting and compared with the total syntaxin bound in the particle.

For both assay configurations (Fig. 1. A and B), neither ATP-binding site mutation (Lys-Ala or Asp-Gln; see Fig. 2) in the D2 domain had any effect on 20 S particle formation or the ATP hydrolysis-mediated dissolution of the complex. Binding of radiolabeled α-SNAP (Fig. 1A) and syntaxin (Fig. 1B, Total) to the two mutants, (D2K-A and D2D-Q), was essentially identical to the binding by wild type NSF and the addition of MgATP-mediated release of particle components (Fig. 1B, ATP). The D1D2 mutant that lacks the N domain failed to support 20 S particle formation. D1D2 was unable to bind either the radiolabeled SNAP (Fig. 1A) or syntaxin (Fig. 1B, Total; also see Fig. 2A). Despite its trimeric nature and ATPase activity (Table I), the D1D2 mutant was not active in intra-Golgi transport (14), probably because it cannot bind to the SNAP-SNARE complex. The ATP-binding mutant D1K-A does not show any 20 S particle formation activity as measured by the SNAP-dependent association of [35S]α-SNAP with mutant or wild type NSF. Mutant or wild type NSF (15 μg) with the carboxyl-terminal myc epitope were incubated with radiolabeled α-SNAP in either the presence or the absence of bovine brain extract (120 μg), which was used as a source of SNARE proteins (SNAREs). Either 0.5 mM ATP (ATP) or ATPγS (ATPγS) was added to the reactions, and all were maintained in 5 mM MgCl₂. Anti-myc antibody coupled to protein G-Superose beads was then added, and the immunoprecipitated complexes were collected on glass fiber filters and quantified by scintillation counting. The error bars represent the range of two separate experiments. Abbreviations for the NSF mutants are explained in the text and in Fig. 2. D1E-Q/D2D-Q participate in 20 S particle formation as measured by the [35S]α-SNAP binding assay (Fig. 1B). When binding was measured in a more sensitive assay under saturating conditions, (Fig. 1B, Total) it appeared that the D1K-A mutant was approximately 10% as efficient at 20 S particle formation as wild type NSF. In the intra-Golgi transport assay, this mutant displayed no transport activity but, interestingly, could inhibit transport when added at high concentrations (IC₅₀ = 157 nM) (14, 17). This mutant showed <10% of the inhibitory activity of the ATP hydrolysis mutants D1E-Q (IC₅₀ = 94 nM) or the double mutant D1E-Q/D2D-Q (IC₅₀ = 13.3 nM) (14). These data suggest that binding of a nucleotide by the D1 domain is an important element of the overall binding of the NSF trimer to the SNAP-SNARE complex. The ATP hydrolysis mutants D1E-Q and D1E-Q/D2D-Q participate in 20 S particle formation as does wild type NSF (Fig. 1. A and B, Total). However, when MgATP is added, the particle formed does not disassemble (Fig. 1. A and B, ATP). Because both mutants are inhibitory to intra-Golgi transport (14) but can form 20 S particle, it seems likely that the MgATP-mediated disassembly step by the NSF trimer is a required intermediate in the vesicular transport process and that the ATPase activity of the D1 domain is crucial to that step.

Differential Role of NSF Domains in 20 S Particle Formation—From the data of Fig. 1, it is clear that the N domain is an important element in NSF binding to the SNAP-SNARE complex. To determine what structural elements of the NSF trimer are important for 20 S complex formation, we examined a series of truncation and domain rearrangement mutants (Fig. 2) using an assay similar to the one employed by Söllner et al. (13). In this assay, mutant proteins were incubated with excess α-SNAP and a detergent-solubilized preparation of bovine brain membrane proteins that is enriched for neuronal SNAREs (syntaxins, synaptobrevins, and SNAP-25; Ref. 9). The resulting 20 S complexes were immunoprecipitated using anti-syntaxin antibody (HPC1), and the amount of NSF or mutant bound was determined by Western blotting. In each case, only SNAP-dependent binding of the NSF or mutant protein was considered significant. The lack of NSF or mutant bound to the immunoprecipitable complex after MgATP addition is indicative of particle disassembly.

As in Fig. 1, the D1D2 mutant does not exhibit SNAP-dependent binding to the 7 S complex, suggesting that the N domain is a critical element of the interactions of NSF with other components of the 20 S particle (Fig. 3A). Surprisingly,
the isolated N domain also failed to show SNAP-dependent binding with the 7 S complex (Fig. 3A). This could simply be due to improper folding of the recombinant domain. However, the His6-tagged protein is very stably expressed in E. coli and migrates as a discrete peak of 28 kDa on gelfiltration chromatography (data not shown). The fact that the isolated, monomeric N domain does not bind to the SNAPzSNARE complex could reflect the need for multiple contact points between NSF and the complex. A single N domain might be incapable of interacting with enough of the requisite binding sites. For the wild type NSF, these contacts could be formed from the cooperative binding of more than one N domain in the context of the trimer or could result from the recognition of more than one NSF domain, i.e. N and D1.

To try to address these possibilities, two mutant forms of NSF were employed, N-D1 and N-D2. N-D1 is monomeric (molecular mass = 580 kDa as determined by gel exclusion chromatography) and has some ATPase activity (18% of wild type; Table I). The low level of ATPase activity is perhaps not surprising because another oligomeric member (p97) of the family of ATPases associated with a variety of cellular activities also has lower ATPase activity when monomeric (27). N-D2 is trim- eric (molecular mass = 186 kDa) but has no measurable ATPase activity (Table I). The N-D1 truncate mutant binds to the 7 S complex in a SNAP-dependent fashion with a low (<10%) binding efficiency compared with wild type NSF. With the addition of MgATP, the bound N-D1 protein releases from the complex in much the same way full-length NSF does (Fig. 3A), but this truncated N-D1 possesses no intra-Golgi transport activity even at very high concentrations (678 nM, data not shown). The N-D2 mutant also binds in a SNAP-dependent fashion, again at much lower affinity, but the bound protein does not release from the complex upon MgATP addition. This same effect is also seen for the ND2D1 mutant (trimeric with ATPase activity (14)), which binds inefficiently and does not release after MgATP addition (Fig. 3). From these data we conclude that it is not simply the presence of the N domain that is important for interaction with the SNAP-SNARE complex but that it is the context in which the N domain is presented. Isolated N domains fail to interact with the SNAP-SNARE complex, unless they are either adjacent to a D1 domain, as would be the case for the wild type NSF and the N-D1 mutant, or presented as a multimer, as would be the case for the wild type NSF or the N-D2 and ND2D1 mutants. These two concepts are not mutually exclusive but are most likely synergistic because none of the mutants bind with the same efficiency as the wild type protein.

| Mutant | ATPasea | ATPase + NEMb |
|--------|---------|--------------|
| Wild type | 4.27 ± 0.40 | 0.36 ± 0.06 |
| N-D1 | 0.77 ± 0.04 | 0.34 ± 0.04 |
| N-D2 | NDc | ND |
| D1D2 | 2.43 ± 0.13 | 0.08 ± 0.03 |

a NSF or mutant concentrations in the assays were 40 μg/ml. The rates of hydrolysis were calculated from time course experiments using 0, 15, 30, and 60 min time points.
b N-Ethylmaleimide (NEM) was added at the start of the assay at a final concentration of 10 mM (10-fold over the free sulphydryl groups in the buffer). c ND indicates that ATPase activity was not detectable.
Function of NSF Domains

The fact that the binding studies (Figs. 1 and 3) were predictive of these inhibition experiments further supports the biological relevance of the binding data.

Multiple Contact Sites Are Needed for Active NSF Trimmers—

The results of earlier experiments (14) suggested that all three of the subunits in the NSF trimer need to be active for the trimer to be functional. In light of the fact that the D1D2 mutant is inactive in the intra-Golgi transport assay (14), it might be expected that mixed NSF/D1D2 trimers containing one or two D1D2 subunits might be inactive as well. However, the mixed trimeric molecules could have some Golgi transport activity because they would possess at least one N domain and the full complement of D1 domains, which as discussed above are required for association with the SNAP-SNARE complex. To test this, His6-tagged D1D2 myc was co-expressed in E. coli together with an untagged form of wild type NSF. The resulting trimers were isolated from E. coli extracts by polyethylene glycol precipitation and fractionated by NiNTA-agarose chromatography using an imidazole gradient, and NSF activity was determined for each fraction. Fig. 5 represents the results of such an experiment with NSF/D1D2 mixed trimers. Any trimers that are bound by the NiNTA agarose contain at least one of the truncated subunits. Two peaks of transport activity are present in the elution profile. The peak early in the profile represents wild type NSF trimers that do not interact with the affinity resin. Because E. coli extracts have an inhibitory effect on Golgi transport, the measurable NSF activity in the first peak is slightly offset from the bulk of the excluded proteins. The second peak, eluted by the imidazole gradient, represents the trimers that bind to the NiNTA resin and, therefore, are a mixture of wild type and D1D2 subunits. This collection of trimers would be made up of molecules possessing two, one, or no N domains. From this experiment we can conclude that for a functional NSF trimer, three N domains are not essential but at least one is required when it is presented in the right context.

2 S. W. Whiteheart, unpublished observation.

**Fig. 3.** A, SNAP-dependent binding of mutant or wild type NSF to the SNARE complex. Mutant or wild type NSF (15 μg) was incubated with bovine brain extract (200 μg) in the presence or the absence (−SNAP) of α-SNAP (5 μg) in the presence of 0.5 mM ATPγS (ATPγS) or ATP (ATP). The resulting complexes were then precipitated using anti-syntaxin antibody coupled to protein G-Superose. The bound proteins were eluted with 0.2 M glycine and were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting with anti-α-SNP (Bound) or anti-syntaxin (Syntaxin) antibodies. Unbound mutant or wild type NSF in the washes (Unbound) was also concentrated and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. The results presented are representative of at least two separate experiments. Abbreviations for the NSF mutants are explained in the text and in Fig. 2, B, Immunodetection of NSF rearrangement mutants. Equal amounts of wild type and mutant NSF were blotted onto nitrocellulose and immunodecorated with either the monoclonal 6E6 antibody (6E6) or the polyclonal anti-N domain antibody (anti-N). The bands were visualized with the appropriate secondary antibody-horseadish peroxidase conjugates using Enhanced Chemiluminescence. The apparent molecular mass of each full-length protein was: wild type (wt), 82.2 kDa; ND2, 57.4 kDa; D1D2, 63.9 kDa; ND1, 61.6 kDa; N, 25 kDa; and ND2D1, 85.3 kDa.

**Fig. 4.** N-D2 and ND2D1 inhibit the Golgi transport activity but only at high concentration. The indicated amounts of each of the proteins were added to a standard (25 μl) intra-Golgi transport assay using wild type Chinese hamster ovary cytosol as a source of soluble transport factors. Interstitial Golgi transport of marker protein from the mutant Golgi complex (donor) to the wild type Golgi complex (acceptor) was measured by the incorporation of [3H]GlcNAc into the vesicular stomatitis virus G glycoprotein. The control 100% value was 5613 dpm with a background of 15 dpm. Abbreviations for the NSF mutants are explained in the text.
with the D1 domain. The position and shape of the second activity peak, relative to the protein profile, indicates that trimers eluting earlier in the imidazole gradient (containing fewer His6-tagged D1D2 myc subunits) account for the bulk of transport activity. This would suggest that trimers containing two N domains are the active species, but further fractionation will be required to determine this conclusively. These findings are consistent with the concept that multiple contact sites are required for NSF to interact with the other components of the 20 S fusion particle.

**DISCUSSION**

In this manuscript we attempt to further understand the functional features of NSF by examining which domains of the molecule are required for which protein-protein interactions in the 20 S fusion complex. To this end, we have demonstrated that interactions of SNAPzSNARE complex with NSF are primarily through the N domains of the trimer. Consistent with this is the observation that the mutation leading to the sec18–1 temperature-sensitive allele is present in a region (ClaI fragment Ile60–Ile177) corresponding to the N domain Sec18p (28). This same mutant allele exhibits synthetic lethality when combined with the sec17–1 mutant, which is the yeast equivalent of α-SNAP (3).

A secondary interaction with the D1 domain of NSF also appears to play a role in 20 S particle formation, but only when D1 is adjacent to the N domain. ATP binding by the D1 may be an important element for NSF to attain the conformation needed for binding to 20 S particle, although this is not completely clear because other replacements of lysine 266 do not exhibit exactly the same inhibitory behavior (17). One might expect that the D1K-A mutant would have similar particle binding properties to the wild type protein because both elements for binding, trimerization and adjacency to the D1 domain, are present in the molecule. The fact that D1K-A mutant can bind only weakly (like N-D2 and ND2D1), suggests that there may be a nucleotide-induced conformational change in D1 that affects the N domain. We speculate that binding of NSF to the SNAP-SNARE complex may be promoted when an appropriate nucleotide (ATP) is bound to the D1 domain. The mutant proteins (especially the N-D1) described in this manuscript should aid in dissecting the role of nucleotide binding to the D1 domain and its relationship to NSF binding to the SNAP-SNARE complex.

The amino acid sequences around the ATP-binding sites in the D1 and D2 domains are characteristic of the family of ATPases associated with a variety of cellular activities (29). Like NSF, these proteins use ATP hydrolysis to carry out their distinctive cellular functions, and mutations of their ATP-binding sites (especially in the domains most homologous to D1) appear to affect the function of the proteins in ways similar to that shown for NSF (discussed in Ref. 14). These similarities between the various ATPases associated with a variety of cellular activities suggest that a common mechanism might be used by all of these proteins to carry out their varied cellular functions. In this manuscript, we demonstrate that the ATPase activity of the D1 domain is directly required for fusion complex disassembly and that the N domain is required for NSF localization. This concept that each domain has a distinct contribution to the overall function of NSF may prove to be a useful paradigm for the study of other ATPases associated with a variety of cellular activities.

The major role of the D2 domain is trimer formation, which appears to be essential for NSF activity (26). The isolated domain, expressed as a recombinant protein in E. coli, is trimeric. Earlier studies showed that ATP binding and hydrolysis by this domain are not required for intra-Golgi transport (14, 17) and, as shown in this manuscript, these properties are also not required for 20 S particle dynamics. The N-D2 and

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3 S. W. Whiteheart and B. Boggess, unpublished observation.
ND2D1 mutants bind to the SNAP-SNARE complex because the N domains are presented as a trimer, but they cannot release once bound because they lack the adjacent D1 domain. These data suggest that even when the D2 domain is placed adjacent to the N domain in a chimeric molecule, it cannot mimic the conformational effects that D1 exerts on the N domain.

In summary, the data presented here suggest roles for each of the three domains of NSF. The N domain is required for SNAP-SNARE complex binding but must be either adjacent to the D1 domain or in a trimeric configuration. These two structural features are most likely synergistic, because neither alone is sufficient to promote the binding efficiency seen for wild type NSF. The D1 domain must be able to hydrolyze ATP to disassemble the 20S particle, and therefore this step is a required intermediate in the transport process. Consistent with this observation is the recent discovery that the temperature-sensitive, paralytic, mutant point mutation (Gly274 to Glu) in the D1 domain in a trimeric configuration. These two structural domains are presented as a trimer, but they cannot mimic the conformational effects that D1 exerts on the N domain.

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