N-Ethylmaleimide-sensitive Fusion Protein: A Trimeric ATPase Whose Hydrolysis of ATP Is Required for Membrane Fusion

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Abstract. The NEM-sensitive fusion protein, NSF, together with SNAPs (soluble NSF attachment proteins) and the SNAREs (SNAP receptors), is thought to be generally used for the fusion of transport vesicles to their target membranes. NSF is a homotrimer whose polypeptide subunits are made up of three distinct domains: an amino-terminal domain (N) and two homologous ATP-binding domains (D1 and D2). Mutants of NSF were produced in which either the order or composition of the three domains were altered. These mutants could not support intra-Golgi transport, but they indicated that the D2 domain was required for trimerization of the NSF subunits. Mutations of the first ATP-binding site that affected either the binding (K266A) or hydrolysis (E329Q) of ATP completely eliminated NSF activity. The hydrolysis mutant was an effective, reversible inhibitor of Golgi transport with an IC_{50} of 125 ng/50 μl assay. Mutants in the second ATP-binding site (binding, K549A; hydrolysis, D604Q) had either 14 or 42% the specific activity of the wild-type protein, respectively. Using coexpression of an inactive mutant with wild-type subunits, it was possible to produce a recombinant form of trimeric NSF that contained a mixture of subunits. The mixed NSF trimers were inactive, even when only one mutant subunit was present, suggesting that NSF action requires each of the three subunits in a concerted mechanism. These studies demonstrate that the ability of the D1 domain to hydrolyze ATP is required for NSF activity and, therefore is required for membrane fusion. The D2 domain is required for trimerization, but its ability to hydrolyze ATP is not absolutely required for NSF function.

INTERCISTERNAL Golgi transport is a multistep process (36). Initially, transport vesicles carrying cargo proteins bud from their compartment of origin. Both the forming buds and resulting vesicles are coated with a seven-protein complex known as coatomer (29, 30, 32, 51), and the small molecular weight GTP-binding protein, ADP ribosylation factor (40). The coated vesicles move to their target compartment, they are uncoated in a process that requires GTP hydrolysis by ADP ribosylation factor (47), and dock with the target membrane. Finally, the vesicles fuse with and release their cargo into their target compartment. Vesicle-target membrane fusion is inhibited by the alkylating agent *N*-ethylmaleimide (NEM)[1] (2, 16, 25), a property that was exploited to purify the NEM-sensitive component of the transport process from CHO cell cytosol (5). The protein thus purified was named NEM-sensitive fusion protein (NSF) because of its apparent role in membrane fusion (25). Kinetic studies indicate that NSF acts late in transport, and morphometric experiments show that NEM treatment of Golgi membranes causes a buildup of uncoated transport vesicles that are consumed after the addition of the pure NSF protein (25). NSF is also required in several intracellular fusion events such as ER-to-Golgi transport (3), endosome-endosome fusion (10), and transcytotic vesicles-to-plasma membrane transport (45). The NSF homologue in yeast, SEC18p (55), is involved in several transport events such as ER-to-Golgi and Golgi-to-plasma membrane transport (17). These data taken together suggest that NSF is a general component of the intracellular fusion machinery.

NSF, in its active form, is a homooligomeric protein consisting of 76-kD subunits as determined by SDS-PAGE (5). The cDNA encoding NSF shows that each subunit contains two consensus ATP-binding sites (55) consisting of a Walker A box motif (50) and a permutation of the Walker B motif known as the DEAD box (24). These two ATP-binding sites are homologous to those of a family of ATP-binding proteins (22). These proteins contain either one or two conserved

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1. *Abbreviations used in this paper*: NEM, *N*-ethylmaleimide; NSF, NEM-sensitive fusion protein; NTA, nitrolotriacetic acid; SNAPs, soluble NSF attachment proteins; SNAREs, SNAP receptors; VSV-G, vesicular stomatitis virus.

ATP-binding sites and they have a wide variety of cellular functions. Like other members of this family, NSF possesses an ATPase activity with a high pH optimum, pH 9.0 (33, 46). Sequence analysis of NSF suggested that it could be separated into three distinct domains: an amino terminal (N) followed by the two homologous ATP-binding (D1, D2) domains (55). Limited proteolysis showed that the predicted domain boundaries do indeed correspond to structural elements of the NSF molecule (46).

NSF is a soluble cytosolic protein that binds transiently to membranes in an ATP-dependent fashion (53). This association is mediated by a set of proteins called soluble NSF attachment proteins (SNAPs) (8). Two of the forms, alpha and gamma, are expressed in all tissues and have been immunolocalized to several subcellular compartments (54), suggesting that they too are general fusion components. NSF and SNAPs stably associate with integral-membrane protein receptors, SNAP receptors (SNAREs), when ATP hydrolysis is prevented (57). This property allowed the purification of a detergent-soluble particle made up of SNAREs from both vesicle and target membranes, as well as stoichiometric amounts of SNAPs and NSF (42). The particle assembles when ATP hydrolysis is prevented (ATPγS or EDTA/ATP) and disassembles under hydrolytic conditions (42, 57). Since NSF is the only protein in the complex that binds ATP, it is likely that the assembly/disassembly cycle of this 20S docking/fusion complex is governed by ATP hydrolysis by NSF.

In this manuscript, we present the first data directly demonstrating the critical role of ATP hydrolysis by NSF in membrane fusion. We also demonstrate that NSF is a homotrimer. Each subunit is made up of three distinct domains that appear to be responsible for different aspects of NSF function in the fusion event. The first ATP-binding site is absolutely required for fusion, and the second domain is needed for trimerization. Additionally, it is demonstrated that all three subunits must be active to form an active NSF trimer. This observation suggests the NSF may use a concerted mechanism to facilitate membrane fusion.

Materials and Methods

Production of Domain Switch Mutants

To produce mutant proteins in which the domain structure of NSF was changed, PCR primers were designed to produce DNA fragments encoding the isolated NSF domains with BamHI and XhoI cloning sites for insertion into pKS II$^+$ Bluescript (Stratagene, La Jolla, CA). The PCR primers were constructed to include restriction sites that would maintain the reading frame after blunt end ligation. DNA fragments encoding the isolated domains can be combined by blunt-end ligation in any order or combination to create the desired form of NSF. The ligation joints were checked by dideoxy sequencing using Sequenase (United States Biochemical Corp., Cleveland, OH), and the constructs were cloned into the BamHI/SalI sites of the pQE-9 expression vector (Qiagen, Chatsworth, CA).

Production of ATP-binding Site Mutants

Point mutations to change the residues of the ATP-binding sites were created by the overlapping PCR technique (18). Briefly, two complementary PCR primers, each of which contains the desired mutation, are used with appropriate flanking primers to generate two short PCR products that contain the mutation in their overlapping regions. These PCR products were then denatured, annealed to each other, and a "full-length" product that contains an internal mutation was amplified using the flanking primers. New mutagenesis primers were made for each mutation, but the same outer primers were used for all constructs of the same domain. The outer primers were chosen to make use of endogenous restriction sites in the NSF DNA (EcoNI/ClaI for the domain 1 ATP-binding site and BstEII/Bsu36I for the domain 2 ATP-binding site). Once a piece of the coding region was mutated by PCR and confirmed by dideoxy sequencing, it was cloned into an NSF expression construct. After generating the single-site mutations, a double mutant in which both ATP-binding sites are changed was made by simple restriction digestion and ligation. NSF constructs were cloned into the BamHI/SalI site of the pQE-9 plasmid, allowing high levels of expression of the NSF mutants with a hexahistidine tag at the amino terminus. The plasmids were transformed into XL-1 Blue *Escherichia coli* (Stratagene), and colonies were selected based on resistance to ampicillin and tetracycline, as well as their ability to express the NSF construct.

Production of Mixed NSF Oligomers

To produce the mixed oligomers of NSF, it was necessary to construct an expression plasmid that could be used in the same cells with pQE-9. This expression vector was generated by replacing the promotor, operator, and ribosome-binding sites of the plasmid pMON5839 (11, 28) with those of the plasmid pQE-3 (Qiagen). Initially, the pMON5839 was modified by deleting its unique BamHI site by digestion with BamHI, filling in the sticky ends with DNA polymerase I (Klenow fragment; Boehringer Mannheim Biochemicals, Indianapolis, IN), and religation. This modified plasmid was cut with BglII, filled in to blunt ends with DNA polymerase I, and then cut with SalI to delete its promotor, operator, and ribosome-binding sites. The analogous region of pQE-3 (Qiagen) was excised by digestion of the plasmid with XhoI, filling in to blunt ends with DNA polymerase I, and SalI digestion. This promotor, operator, and ribosome-binding region of pQE-3 was then ligated into the blunt/SalI sites of the modified pMON5839. The final construct, called pMONQE, contains the kanamycin resistance gene from the pMON5839 plasmid and the cloning sites of the pQE-3 plasmid but with no His_6-tag. The wild-type NSF construct was cloned into this plasmid, cotransformed with the K266A NSFmyc construct in pQE-9 (Qiagen), and cotransformants were selected by resistance to ampicillin, tetracycline, and kanamycin. These cotransformants expressed approximately equal amounts of NSF subunits from each plasmid (data not shown). This coexpression was used to generate NSF oligomers that contain two different types of subunits that could be detected with anti-NSF (6E6) and anti-myc epitope (9E10) monoclonal antibodies.

Purification of NSF and NSF Mutants

A purification scheme for wild-type and mutant forms of NSF, based on published procedures for the purification of His_6-tagged proteins and NSF, was used (54). Cells expressing the appropriate construct were grown to a density of A_{600} = 0.8 in Super medium (25 g bacto-tryptone, 15 g yeast extract, and 5 g NaCl/liter with 100 μg/ml ampicillin and 50 μg/ml tetracycline) and induced for 4 h with 1 mM isopropyl-thio-β-D-galactoside. The cells were harvested by centrifugation, and they were washed in breaking buffer (100 mM Hepes/KOH, pH 7.0, 500 mM KCl, 5 mM ATP, 5 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 2 mM PMSF, 10 μg/ml leupeptin, and 1 μM pepstatin). The pellet was resuspended in 1/60 of the original volume in Breaking buffer and disrupted by French press. The extract was clarified by centrifugation at 250,000 g for 1 h, and the supernatant was loaded onto a Ni^{2+}nitrilotriacetic acid (NTA) agarose (19) (Qiagen) column (5 ml) that was equilibrated in 20 mM Hepes/KOH, pH 7.0, 200 mM KCl, 2 mM 2-mercaptoethanol, 0.5 mM ATP, 1 mM $MgCl_2$, and 10% glycerol (buffer A). After loading, the column was washed with three column volumes of buffer A, three column volumes of buffer A + 20 mM imidazole, and was then eluted with an eight-column volume gradient of buffer A from 20 to 500 mM imidazole. NSF elutes at an imidazole concentration of ~200 mM and in most cases is >80% pure. Additional purification (>95%) was achieved by gel filtration chromatography on a Superose 6 Fast Flow (Pharmacia Diagnostics Inc., Farifield, NJ) column (1.6 × 50 cm) in 50 mM Hepes/KOH, pH 7.8, 100 mM KCl, 2 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 0.5 mM ATP, and 5% glycerol.

In Vitro intercisternal Golgi transport assays

NSF activity was measured as outlined in Block et al. (5). Briefly, the endogenous NSF from donor and acceptor Golgi membranes was inactivated by treatment for 15 min on ice with 1 mM NEM and was quenched with 2 mM DTT. These membranes were then used together with heat-inactivated CHO cells cytosol (that lacks NSF activity) in a standard transport assay incubated for 1.5 h at 37°C. The reaction was stopped, and the

^{3}H-glycosylated vesicular stomatitis virus (VSV-G) protein transported from the donor to acceptor compartment was recovered as described (1) and quantitated by liquid scintillation counting. To determine the specific activity of some of the mutant forms, a time course experiment in which aliquots of the transport assay were taken at different times was used. The rate of transport was calculated based on counts per minute of [^{3}H]*N*-acetyl-glucosamin-VSV-G produced per minute after the initial 10-min lag.

ATPase Assays

NSF and the mutant forms were tested for ATPase activity using the technique described in Tagaya et al. (46). [α^{32}P]ATP (3,000 Ci/mmol) was purchased from Du Pont NEN (Wilmington, DE). Briefly, wild-type or mutant NSF (1 μg/20 μl) was incubated in buffer (0.77 mM ATP, 8 μCi [α^{32}P]ATP; 25 mM Tris/HCl, pH 9.0, 100 mM KCl, 0.5 mM DTT, 0.65 mM mercaptoethanol, 1 mM $MgCl_2$, 10% glycerol) for 1.5 h at 37°C. 1 μl of the reaction was then spotted in duplicate onto cellulose polyethyleneimine thin-layer chromatography plates (MN 300; Brinkmann Instruments, Inc., Westbury, NY) and developed in 0.7 M LiCl, 1 M acetic acid. After exposure of the thin layer plates to x-ray film, the [α^{32}P]ADP spots were identified relative to standards, cut out of the plate, and counted by liquid scintillation. The amount of [α^{32}P]ADP produced was compared for samples that had been either treated or not with 4.95 mM NEM (threefold excess over sulfhydryl groups). NEM treatment has been shown to completely abolish the ATPase activity of NSF. NEM treatment was used in these assays to distinguish the NSF-specific ATPase activity from that of contaminating phosphatases.

Velocity Sedimentation on Glycerol Gradients

Wild-type and mutant NSF were analyzed by velocity sedimentation on glycerol gradients using a modification of the method described in Wilson et al. (57). 50 μg of protein were loaded onto a 5.0-ml linear glycerol gradient (10–35%) in 20 mM Hepes/KOH, pH 7.0, 100 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, and 0.5 mM ATP. The gradients were then subjected to centrifugation for 14 h at 4°C at a speed of 29,000 rpm in a rotor (SW-55; Beckman Instruments, Inc., Spinco Division, Palo Alto, CA). The gradients were harvested (at 330 μl/min) from the bottom into 15 fractions, and aliquots were analyzed by SDS-PAGE. The velocity sedimentation standards were bovine serum albumin (4.6 S), catalase (11.4 S), and α2-macroglobulin (19.6 S).

Sedimentation Analysis of NSF

Sedimentation analysis was performed in an analytical ultracentrifuge (model E; Beckman Instruments, Inc., Palo Alto, CA) equipped with a high-intensity light source and a ultraviolet scanning system. Double-sector cells (12 mm path length) with sapphire windows were used in an AnG rotor. To detect possible concentration-dependent dissociation, the meniscus depletion technique (58) was applied using an initial concentration of 0.3 and 1.7 mg/ml for NSF and NSFmyc, respectively. The temperature was kept at 2°C and 12°C, and the scanning wavelengths were 230 and 280 nm, respectively.

s values were determined at 24,000 rpm, plotting ln *r* vs time, and correcting for 20°C and water viscosity. Sedimentation equilibria at 8,000 rpm were evaluated from ln *c* vs r^2 plots, making use of computer programs developed by A. Minten, National Institutes of Health (Bethesda, MD), and G. Boehm (Institut für Biophysik und Physikalische Biochemie, Regensburg, Germany). The partial specific volume was calculated from the amino acid composition.

Cross-linking of the NSF Oligomer

NSF (0.36 mg/ml) was cross-linked with various concentrations (0.1–10 mM) bis(sulfosuccinimidyl) suberate (BS^3; Pierce Chemical Co., Rockford, IL) in 20 mM Hepes/KOH, pH 7.4, 200 mM KCl, 0.14 mM ATP, 0.28 mM DTT, and 3% glycerol for 30 min on ice. The reactions were quenched with 100 mM glycine, pH 7.4, and the cross-linked species were separated by SDS-PAGE on a 5% acrylamide gel.

Miscellaneous Techniques

Protein concentrations were determined using the Bio Rad protein assay reagent (Bio Rad Laboratories, Richmond, CA) and ovalbumin as a standard. Western immunoblotting was visualized using horseradish peroxidase-coupled anti-mouse immunoglobulin secondary antibodies (Bio Rad Laboratories) and enhanced chemiluminescence reagents (Amersham Corp., Arlington Heights, IL). SDS-PAGE gels were prepared and run as described (23) and stained with Coomassie brilliant blue R-250.

Results

Rationale of Mutant Construction

The sequence of the NSF subunits contains several features of interest: specifically, two consensus ATP-binding sites (centered around residues 266 and 549) and an apparent tridomain structure consisting of an amino-terminal domain (1-206) and the two homologous ATP-binding domains (207-487, 488-744). The ATP-binding site sequences have been found in other proteins and used as a criterion for inclusion into a family of cellular ATPases found in a number of organisms (22). The predicted domain structure was demonstrated by the fact that the individual domains of NSF can be generated by limited proteolysis (46). To understand the role of these domains in the function and structure of NSF, we constructed a series of rearrangement mutants in which the order and composition of the domains in the NSF subunit were changed (see Materials and Methods and Fig. 1). Specifically, we were interested in what elements of the NSF subunit were involved in oligomer formation and what was required for its function in Golgi transport.

ATP hydrolysis by NSF has been proposed to play a central role in the fusion of vesicles with their target membranes. To directly determine whether ATP hydrolysis by NSF is required for intercisternal Golgi transport and to delineate the relative roles of the two ATP binding domains, site-directed mutagenesis was used to change the conserved lysine residues (266, 549) in the G/A-X-X-X-X-G-K-T/S sequences of the two Walker A boxes (50) and acidic amino acids (E329 or D604) in the DEAD box elements of the Walker B boxes (24). The conserved lysine residues (part of the P-loop [37]) have been reported to be involved in nucleotide binding in a number of ATP- and GTP-binding proteins, and their mutation is expected to yield an NSF subunit with reduced ATP-binding capacity. These lysine residues were changed to alanines since that switch has been calculated to cause the least structural perturbation to the polypeptide chain (6). The DEAD box motif was originally seen in RNA helicases, which use ATP hydrolysis to unwind RNA for translation (38). The first two acidic amino acids are thought to bind the Mg^{2+} ion that coordinates the phosphates of ATP for hydrolysis (31). Replacement of either of these residues with a positively charged amino acid eliminates ATP hydrolysis without affecting nucleotide binding in RNA helicases (35). This series of DEAD box mutants is more likely to retain the ability to assume any change in tertiary structure that might be induced by ATP binding than would the lysine to alanine mutants discussed above.

Characterization of Domain Rearrangement Mutants of NSF

Plasmids encoding the domain rearrangement mutants were constructed by blunt end ligation of the separate domain-encoding DNA fragments as outlined in Materials and Methods. The ligation joints were sequenced to ensure that the correct reading frame was maintained. These constructs were then cloned into pQE-9, and His_6-tagged proteins were produced in *E. coli* and purified by Ni^{2+}NTA agarose

chromatography. Compared to the wild-type NSF construct (Fig. 1 *A*), some of the rearrangement mutants suffered from increased proteolysis during their preparation, especially the mutants in which a domain was repeated, eg., N-D1-D1 and N-D2-D2. This may reflect a lack of stability of the mutant proteins in the *E. coli* cell.

Initially, the mutant forms of NSF were analyzed by velocity sedimentation to determine whether they contained the molecular information needed to form homooligomers. Because of the small size of the gradients, the accuracy of the size estimates is limited, but as is shown in Fig. 1 *B*, oligomeric wild-type NSF can be easily separated from monomeric mutant forms. From Table I, it is clear that all of the mutant forms that lacked the D2 domain were incapable of forming proper oligomeric structures. The N-D1-D1 and N-D1 migrated only a short distance on the velocity gradient with an apparent size of 6 S. Mutants that contained the D2 domain migrated with an *s* value similar to wild-type NSF. These data suggest that the second ATP-binding domain (D2) of NSF is required for the oligomerization of the NSF subunits. The prominent proteolysis fragment (~50 kD) present in the N-D2-D2 preparation, despite containing the 2C8 epitope (see below), did not migrate as an oligomeric species. These mutant proteins were next tested in an NSF-dependent intra-Golgi transport assay. While the wild-type NSF saturated the assay at 5 ng per 50 μl assay, none of the mutants had any transport activity using up to 40 ng per assay (see Table I). This would indicate that the rearrangement mutants have less than one eighth of the activity of the wild-type protein, and they are considered inactive in intra-Golgi transport.

Transport Activity of ATP-binding Site Mutants of NSF

Point mutations of amino acids in the ATP-binding sites of NSF were made by PCR mutagenesis and were sequenced to insure that only the desired mutation was made. The mutant proteins were expressed in *E. coli* and were purified by Ni^{2+}NTA agarose chromatography, followed by sizing chromatography on Superose 6 (see Fig. 2 *A*). The elution position on Superose 6 (~300 kD) insured that all of the mutants were oligomeric. The proteolysis fragments in the D604Q preparation appeared after storage of the pure protein and were not initially present after purification.

Mutant proteins were assayed in an NSF-dependent, intra-Golgi transport assay to assess the effect that the point mutations in the two ATP-binding sites had on NSF activity (Fig. 2, *B* and *C*). Mutations made in the first ATP-binding site (D1) led to a completely inactive form of NSF. Two distinct mutations, K266A and E329Q, expected to affect ATP binding and hydrolysis, respectively, resulted in the complete inactivation of the NSF produced. While wild-type NSF and

Table I. Summary of the NSF Domain Rearrangement Mutants

Mutant	Oligomeric	s Value*	Activity‡
Wild-type	+	14.1	100%
N-D2-D1	+	12.9	0%
N-D1-D1	−	6	0%
N-D2-D2	+	12.9	0%
N-D1	−	6	0%
D1-D2	+	12.9	0%

Construction of the different NSF configurations is discussed in Materials and Methods. The mutant proteins were expressed in *E. coli* and purified by Ni^{2+}NTA agarose chromatography.

* The purified proteins were analyzed by velocity sedimentation on a glycerol gradient and compared to standard proteins to determine their s values (see Fig. 2 *b*). *s* values are given in Svedberg units, S.

‡ The purified proteins were tested for NSF activity by titration into a standard transport assay (50 μl assay) using NEM-treated membranes and heat-inactivated CHO cell cytosol (see Materials and Methods).

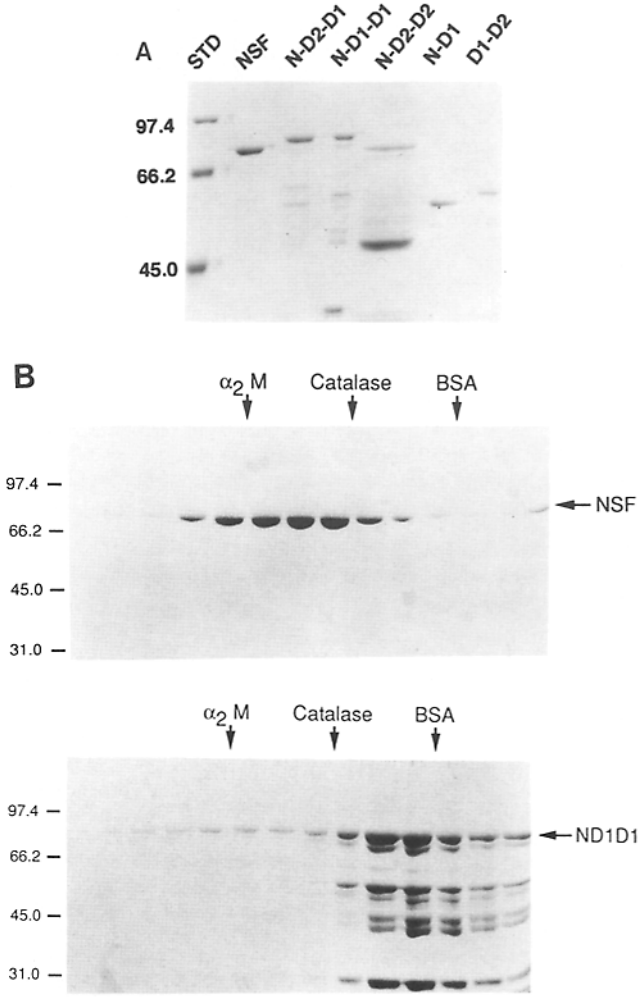

Figure 1. Characterization of the NSF domain rearrangement mutants. Domain rearrangement mutant constructs were produced and expressed in *E. coli* as described in Materials and Methods. The proteins were purified by affinity chromatography on Ni^{2+}NTA agarose. Approximately equal amounts of NSF or the mutants were separated by SDS-PAGE and stained with Coomassie brilliant blue (*A*). Wild-type NSF and the N-D1-D1 mutant were analyzed by velocity sedimentations as described. Gradients were loaded on the top (*right side of the panel*) and subjected to centrifugation as described (*B*). The gradients were fractionated from the bottom (*left side of panel*), and aliquots of each fraction was analyzed by SDS-PAGE on an 8% acrylamide gel and stained with Coomassie brilliant blue. The sedimentation standards are α_2-macroglobulin, 19.6 S; catalase, 11.4 S; and bovine serum albumin, 4.6 S. The molecular mass standards are phosphorylase B, 97.4 kD; bovine serum albumin, 66.2 kD; and ovalbumin, 45 kD.

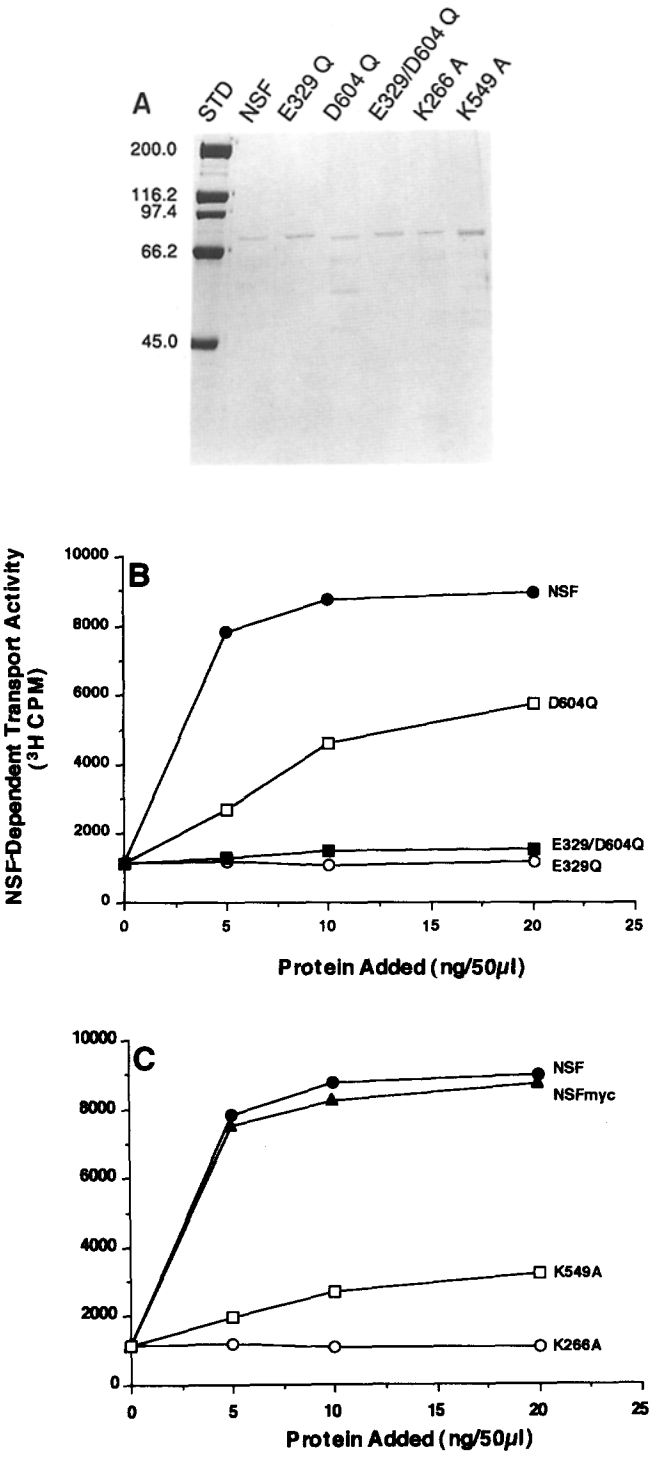

Figure 2. Characterization of the ATP binding site mutants of NSF. Point mutations in both ATP binding sites of NSF were made as described in Materials and Methods. The proteins were purified by Ni^{2+}NTA affinity chromatography and gel exclusion chromatography on Superose 6. Approximately equal amounts of NSF or mutant proteins were analyzed by SDS-PAGE on an 8% acrylamide gel and Coomassie brilliant blue staining (*A*). The molecular mass standards were myosin, 200 kD; β-galactosidase, 116.2 kD; phosphorylase B, 97.4 kD; bovine serum albumin, 66.2 kD; and ovalbumin, 45 kD. The mutants were then assayed for NSF activity in a standard transport assay (50 μl) using NEM-treated membranes and heat-inactivated CHO cell cytosol (*B* and *C*) (see Materials and Methods). Wild-type NSF and NSF with the COOH-terminal myc epitope (NSF and NSFmyc) were used as controls, and the mutants are denoted by the amino acid changed (as in D604) and what it was changed to (e.g., Q).

NSFmyc saturated the transport assay at 5 ng/50 μl (authentic CHO cell NSF saturates at 2.6 ng per 50-μl assay [5]), the two D1 mutants showed no transport activity when used up to 20 ng/50 μl. Since the mutations were made in two discrete regions of the ATP-binding site, the inactivation that they cause is more likely a result of the effect on ATP binding/hydrolysis rather than conformational alterations. These results demonstrate that the ability of D1 to hydrolyze ATP is required for the function of NSF.

Mutations in the second ATP-binding site resulted in an attenuated form of NSF that could support intra-Golgi transport albeit less effectively than wild-type protein (Fig. 2, *B* and *C*). The two mutations K549A and D604Q yielded forms of NSF that apparently saturate the transport assay at a concentration four times higher than wild-type NSF, and they supported only a fraction of the total transport (36% for K549A, 64% for D604Q). The specific activity of transport for these two mutants was determined by measuring the rate of NSF-dependent transport vs incubation time, and it was 14% (for K549A) and 42% (for D604Q) that of the wild-type NSF. Because of the presence of proteolysis fragments in the D604Q preparation, the specific activity measured could be an underestimate. The relative difference in specific activity between the binding (K549A) and hydrolysis (D604Q) mutants was consistent in two different preparations of the mutant proteins (data not shown). Perhaps, the ATP binding to D2 may be needed to induce an appropriate conformation in the NSF molecule. These results demonstrate that the ability of the D2 domain to hydrolyze ATP is not absolutely required for NSF function. Whatever the role of this D2 domain in NSF function, its ability to at least bind ATP appears to be critical. As expected, the double hydrolysis mutant in which both E329 and D604 were changed to glutamine had no NSF activity.

ATPase Activity of ATP-binding Site Mutants

The ATPase activity of NSF has two characteristics: it is maximally active at pH 9.0, and it is completely eliminated by treatment of the protein with *N*-ethylmaleimide (46). These properties were exploited in measuring the ATPase activity of the ATP-binding site mutants. Assays were performed at pH 9.0, and the ATPase activity was expressed as NEM sensitive, to distinguish it from contaminating phosphatases that might be present in the various preparations of mutant NSF.

Mutations in either domain led to an overall decrease in the NEM-sensitive ATPase activity of the mutant proteins compared to wild-type (see Fig. 3). Mutation in the D1 domain had the greatest effect, causing a 69 and 82% decrease in ATPase activity for the E329Q and K266A mutations, respectively. Mutations in the D2 domain were not as effective at eliminating ATPase activity (28% decrease for D604Q and 41% decrease for K549A), suggesting that the D1 domain accounts for the major portion of the NEM-

sensitive ATPase activity of NSF. The double E/D-Q mutant completely lacked ATPase activity. The one rearrangement mutant tested (N-D2-D1) had 77% of wild-type ATPase activity. Since this mutant was inactive in intra-Golgi transport, it is clear that correct positioning of the domains, as well as ATPase activity, are required for NSF activity.

Inhibition of Intra-Golgi Transport by NSF Mutants

Mutants that were inactive in the transport assay were tested to determine whether they could effectively inhibit intra-Golgi transport in a reversible fashion. Fig. 4 *A* shows the results of an experiment in which the mutant NSF were titrated into a transport assay. The amount of endogenous NSF in a standard transport assay is estimated to be <5 ng per 50-μl assay. The E329Q and the E329/D604Q mutants inhibited transport with IC_{50}'s of 125 and 170 ng per 50-μl assay. The K266A mutant also inhibited the transport assay but required 2 μg per 50-μl assay to achieve 50% inhibition (data not shown). The inhibitory action of these mutants were completely reversed by the addition of 2 μg of wild-type NSF (Fig. 4 *B* and data not shown). These data indicate that the mutant NSF's can compete with endogenous NSF for binding to appropriate sites on the Golgi membranes. The domain rearrangement mutants were also tested and inhibited only to ~30% when 800 ng were used per 50-μl assay. The only efficient, reversible inhibitors of intra-cisternal Golgi transport were the E329Q and the E329/D604Q mutants.

Characterization of Anti-NSF Monoclonal Antibodies

Monoclonal antibodies (2C8, 2E5, and 6E6), produced by Tagaya et al. (46) have been shown to be inhibitory in the intra-Golgi transport assay. One of these antibodies, 2C8, stimulates the ATPase activity of NSF by twofold (46). The

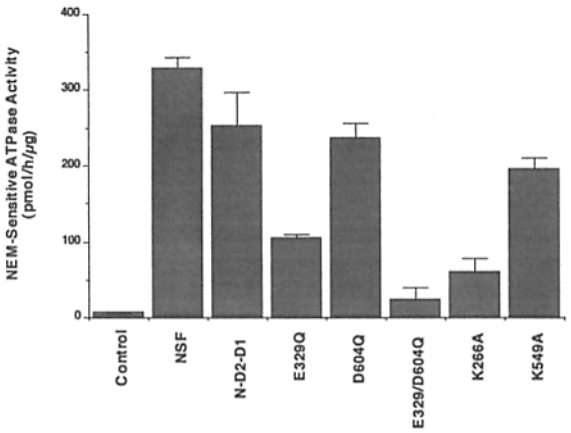

Figure 3. ATP-binding site mutants of NSF lack ATPase activity. Mutant NSF proteins were assayed for ATPase activity as described in Materials and Methods. The NSF specific ATP hydrolysis is presented as picomoles of ADP produced per hour per micrograms of protein. The NSF specific hydrolysis was determined by subtracting the NEM resistant ATPase activity from the total ATP hydrolysis observed. The proteins assayed were the wild-type NSF, the rearrangement mutant, N-D2-D1, and mutants in the ATP-binding site as listed in Fig. 3. Each assay was done in duplicate and the data represents the average and the error bars represent the range.

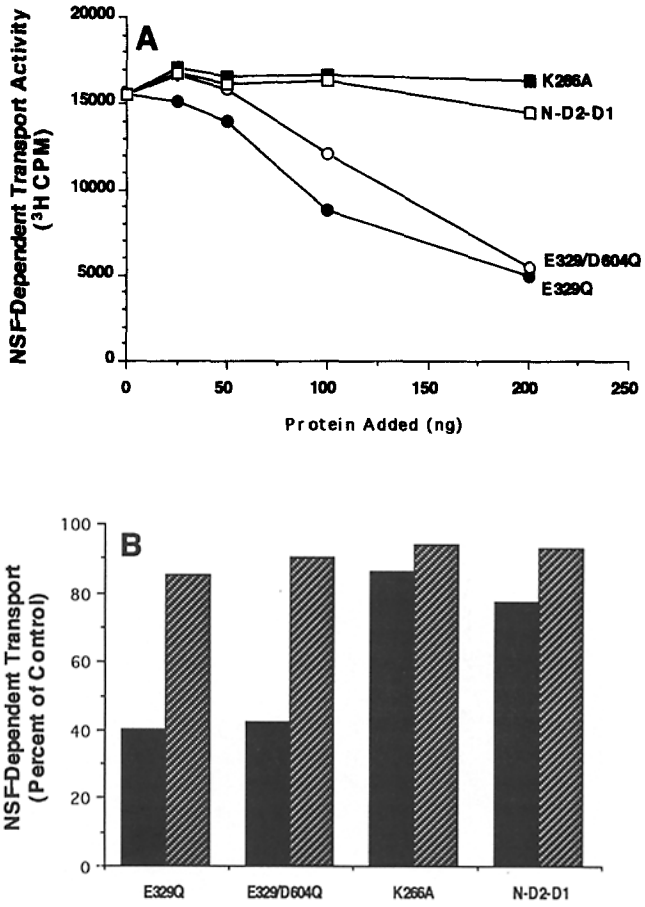

Figure 4. Inhibitory effect of ATP binding site mutants of NSF on intra-Golgi transport. The effect of the ATP binding site mutants and rearrangement mutants of NSF on intra-Golgi transport was determined by adding increasing amounts of the NSF mutants into a standard transport assay using untreated membranes and heat-inactivated CHO cytosol (*A*). The standard intra-Golgi transport assay was inhibited by adding 200 ng of mutant NSF (*B, solid bars*) and the inhibition was reversed by the addition of 2 μg of wild-type NSF (*slashed bars*). The intra-Golgi transport assays were performed as described in Balch et al. (1), and the data represents the average of duplicates.

domain rearrangement mutants were used to determine what region(s) of the NSF molecule is recognized by these monoclonal antibodies. Epitope mapping was accomplished using a series of Western blots in which the same amount of each rearrangement mutant was blotted onto nitrocellulose and probed with equal dilutions of the monoclonal antibodies. Reactivity was judged by the intensity of immunodecoration as detected by the enhanced chemiluminescence method. Two of the antibodies, 2E5 and 6E6, reacted strongly with constructs in which the D1-D2 domain boundary was maintained (wild-type NSF and D1-D2). Additionally, these antibodies had some minor cross-reactivity with recombinant SEC18p. The third antibody, 2C8, reacted with all proteins containing the N domain (wild-type NSF, N-D1-D1, N-D2-D2, N-D2-D1, and N-D1) and did not react with SEC18p (or D1-D2). From this data it is apparent that the 2E5 and 6E6 antibodies recognize an epitope made up of elements from

both the D1 and D2 domains, whereas the 2C8 antibody reacts with the N domain.

Oligomeric Nature of NSF and Its Functional Relevance

Initially, NSF was suggested to be a homotetramer using approximate but standard methods. This estimation was based on a sedimentation constant of 12S and a calculated Stokes radius of 63 Å (57). The sedimentation constant was determined by comparison relative to known standards in a sucrose gradient (as was done in Table I), and the Stokes radius was approximately determined from sizing chromatography on Superose 12. Since it is important to be certain of the oligomeric state of NSF, an independent and more precise series of experiments was undertaken. Chemical cross-linking, used to determine the oligomeric state of several protein complexes (43), was applied to NSF. Fig. 5 *A* shows the results of an experiment in which NSF was incubated with the cross-linker bis(sulfsuccinimidyl) suberate (BS^3). As the concentration of cross-linker is increased, it is possible to see the accumulation of dimer (2N) and trimer (3N), but no tetramer was detected. The apparent molecular masses of the dimer and trimer were 152 and 210 kD, respectively. This data supports the conclusion that NSF is a trimer.

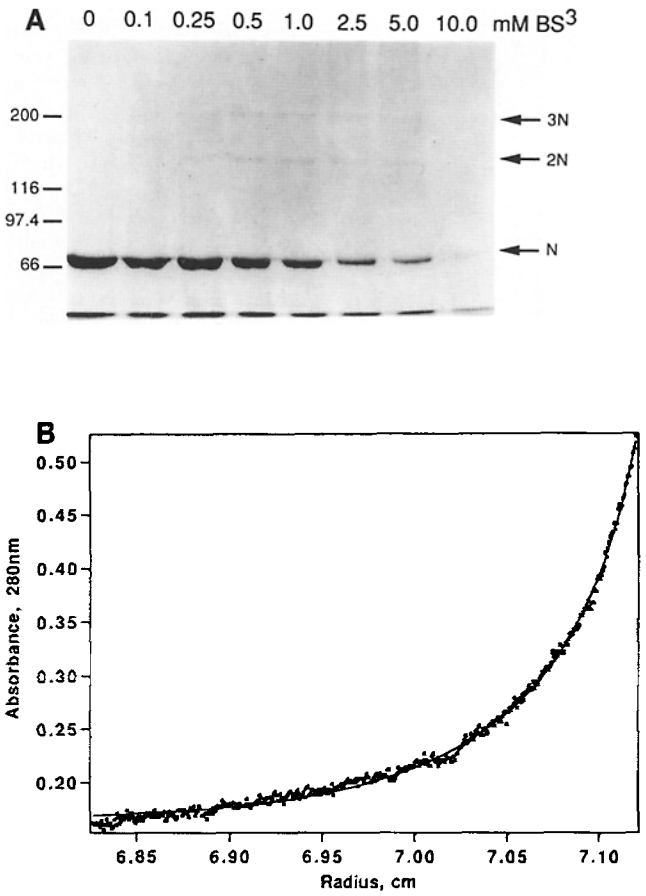

Figure 5. Oligomeric nature of NSF. (*A*) Wild-type NSF was incubated with the indicated concentrations of the crosslinker, bis(sulfosuccinimidyl) suberate (BS^3), quenched with glycine, and the products were separated by SDS-PAGE on a 5% acrylamide gel and stained with Coomassie brilliant blue. Products corresponding to dimers (2N) and trimers (3N) of NSF were readily seen; however, no tetramer was detected. The molecular mass standards were myosin, 200 kD; β-galactosidase, 116.2 kD; phosphorylase B, 97.4 kD; and bovine serum albumin, 66.2 kD (*B*) Sedimentation analysis of NSFmyc in 50 mM Hepes/NaOH, pH 7.8, 100 mM NaCl, 2 mM mercaptoethanol, 1 mM $MgCl_2$, 0.5 mM ATP, and 1% (vol/vol) glycerol. Meniscus depletion sedimentation equilibrium was determined at 8,000 rpm with an initial protein concentration of 1.7 mg/ml, 12°C, scanning at 280 nm. Assuming homogeneity of the sample, the data points fit (correlation coefficient >0.9998) the curve predicted for a complex of 255,282 D (*solid line*), i.e., a trimer of 85,094 D NSF subunits.

Analytical centrifugation of NSF was used to precisely determine the sedimentation coefficient and the molecular mass of the oligomer in solution. NSFmyc migrates as a single band with a sedimentation coefficient, $s_{20,w}$ = 12.8 ± 0.3S, in accordance with a globular protein of 250–300 kD mass. No tendency to form high molecular weight aggregates was detected. The sedimentation equilibrium at 8,000 rpm confirms the above estimate. The molecular mass of the recombinant His_6-NSFmyc determined by this method is 255 ± 5 kD (correlation coefficient = 0.9998), and it is in perfect agreement with a trimer using the calculated subunit molecular mass of 85,094 D. Fig. 5 *B* illustrates the fit of the experimental data points (taken from the scanning profile at 280 nm) with the theoretical curve for the trimeric complex (molecular mass = 255,282 D). A similar result was obtained when NSF was analyzed (data not shown). This sample was subjected to freezing before ultracentrifugation and gave a clear indication for dissociation of the trimer. The mass average for the major component was 218,600–232,700 D, and a minor component with a minimum molecular mass of 80 ± 10 kD. These data indicate that in solution, NSF is clearly a trimer.

Although NSF must be an oligomer to be active in intra-Golgi transport (5), it is unknown if all of the subunits need to be functional for the protein to attain maximal activity. To address this question, mixed NSF trimers were produced in *E. coli* by coexpressing a K266A mutant with a His_6 at the amino terminus and a myc epitope tag at the carboxy terminus together with a wild-type NSF. The mixed NSF trimer could then be separated by Ni^{2+}NTA agarose chromatography into pools that contained no K266A subunits (trimers that did not adhere to the resin) and those that contain at least one K266A mutant subunit. Using monoclonal antibodies that recognized the myc epitope (56), it is possible to show that in the initial pool of NSF (runthrough) does not contain any of the mutant subunit (Fig. 6, *A* and *B*). Trimers with a higher percentage of wild-type subunits eluted in the initial stages of the imidazole gradient, and those that were predominantly mutant (His_6/myc) eluted in the later portion of the gradient. When assayed for NSF activity, only the pool made up of all wild-type subunits had any activity (Fig. 6 *C*). Thus, even the NSF trimers that elute early in the imidazole gradient and contain only one mutant subunit are not active. These data demonstrate that the coexpressed subunits associate randomly in *E. coli*, and that for trimeric NSF to be active, all of the subunits must be functional.

Discussion

The data presented in this paper directly demonstrate that the ATPase activity of NSF is absolutely required for it to facili-

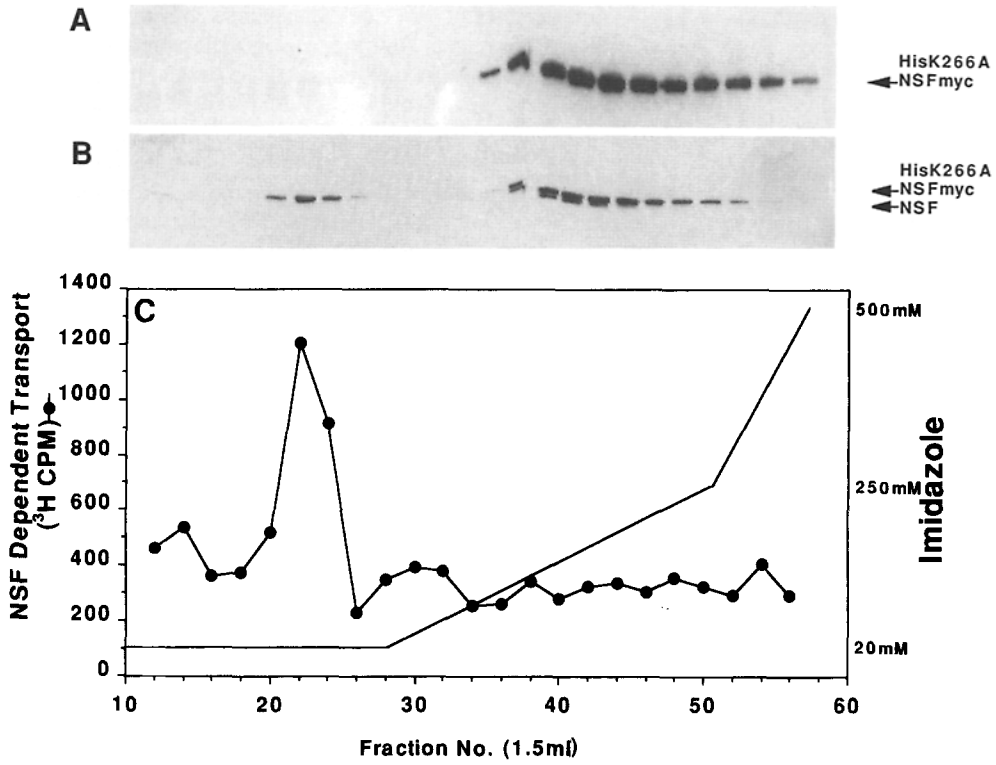

Figure 6. Effect of mutant subunits on the activity of the NSF trimer. NSF trimers were produced in *E. coli* such that they contained a random mixture of wild-type NSF subunits (non-His_6 tagged) and K266A mutant subunits that contain His_6 at the amino terminus and a myc epitope at the carboxy terminus (see Materials and Methods). The mixed trimers were then separated by affinity chromatography on Ni^{2+}NTA agarose using a gradient of imidazole from 20–500 mM (as denoted in *C*). Aliquots (2 μl of 1 ml) of each gradient fraction were initially analyzed by Western blotting with antibodies that would recognize only the mutant subunit (*A*) (9E10, which reacts with the myc epitope of the HisK266Amyc subunit) and all forms of NSF subunits (*B*) (6E6, see text). The mutant subunit with the two recombinant tags has a slightly higher molecular weight by SDS-PAGE. Aliquots (0.1 μl of 1 ml) were then assayed in a standard NSF dependent intra-Golgi transport assay (50 μl) using NEM-treated membranes and heat-inactivated CHO cell cytosol (see Materials and Methods). A background of 1,000 cpm was subtracted from each data point.

tate membrane fusion as measured by intra-Golgi transport. While this has been proposed (25, 42, 57), the data presented here is the first demonstration that NSF used ATP hydrolysis to mediate the fusion of vesicle and target membrane bilayers. We also present data arguing that each of the three domains of the NSF subunit has a distinct role in NSF function. Using site-directed mutagenesis to change two distinct regions of the ATP-binding sites, the relative importance of each of the two ATP-binding sites was demonstrated. The ability of the first ATP-binding site to hydrolyze ATP is absolutely required for NSF to be functional. This domain also accounts for the majority of the NEM-sensitive ATPase activity of NSF. The second ATP-binding domain is required for trimer formation, but its ATPase activity does not appear to be essential for NSF activity. Mutants of this domain, though able to support intra-Golgi transport, do so at a reduced level. The amino-terminal domain is not required for trimer formation, yet its deletion yields an inactive NSF trimer. This domain is recognized by the 2C8 antibody, which stimulates the ATPase activity of NSF (46), suggesting that the N domain may play some regulatory role.

The impact of the oligomeric state of NSF would greatly affect hypotheses regarding how NSF might facilitate membrane fusion. NSF was originally reported to be a tetramer based on approximate, hydrodynamic measurements (5), yet cross-linking experiments could only detect a trimer of NSF subunits. To resolve this important point, more detailed measurements were made by analytical ultracentrifugation. The new data clearly show that NSF is a trimer, and they further demonstrate that each of the three subunits must be functional for NSF to be active. The implication is that the NSF subunits must be oriented in a parallel fashion, since that is the only way to arrange a globular trimeric protein, and that the mechanism of fusion must involve a concerted or unified action of all of three subunits according to a mechanism that is asymmetric relative to the junction between vesicle and target membrane.

NSF is a member of a family of ATP-binding proteins delineated by a conserved 185–amino acid region encompassing the ATP-binding sites (22). This family contains proteins of widely divergent function, but with some structural and mechanistic similarities. Where known, the proteins are predominantly oligomeric (NSF, trimer; p97, hexamer [34]; mei-1, oligomer [7]; S4, heterooligomer [12]) and contain ATPase activities with high pH optima (pH 9.0, NSF [46] and p97[33]). Each family member has one well-conserved ATP-binding domain (D1 or NSF), and the family can be subdivided based on the presence of a second, less well-conserved ATP-binding domain (14). Members that are involved in transcription (SUG1 [44], TBP-1 [39], MSS1 [26]), proteolysis (S4 [12]), septation (FstH [48]), and meiosis (mei-1 [7]) contain a single ATP-binding domain. The family members that have two domains are thought to be involved in peroxisome biogenesis (PAS1p [14], PAY4p [27], PAS8p [49]), cell division (SAVp [9], VCP [21], p97 [33], CDC48p [15], cm20a12p [52]) and secretion (NSF [55], SEC18p [13]).

Several mutations of the well-conserved ATP-binding domain (D1 in NSF) have been made for many of the proteins with the same result: inactivation of the protein. When the

conserved lysine (K744 equivalent to K266 in NSF) was mutated to glutamic acid, the resulting PAS1p was unable to rescue a PAS1 deletion in yeast (20). When the other conserved lysine (K467 equivalent to K549 in NSF) was changed to glutamine, the mutant PAS1p was able to rescue the deletion, suggesting that, like NSF, this ATP-binding site does not have to hydrolyze ATP to maintain an active PAS1p (20). Mutations of the ATP-binding site of mei-1 of *C. elegans* are dominant negative inhibitors of meiotic spindle formation (7). These examples, together with the data presented in this paper, demonstrate that the ability of this conserved ATP-binding region (D1 in NSF) to hydrolyze ATP is essential to the function of the ATP-binding protein family. A likely possibility would be that these proteins use this conserved domain as an energy transducer to convert the chemical energy of ATP hydrolysis into some physical force needed to carry out their cellular function. By extension of this idea, the other domains might be responsible for the specificity of each protein's interactions in the cell.

With the identification of the SNAREs and the realization that they may control the specificity of transport vesicle-to-target membrane docking, it is now possible to make more meaningful speculations as to the role of NSF in facilitating membrane fusion. The SNARE hypothesis holds that the vesicle SNARE binding to its cognate target membrane SNARE is the basis for core specificity of vesicle targeting (4, 41, 42). The resulting docking complex is the site of the binding of SNAPs (α and γ), which then act to mediate the binding of NSF and the formation of the 20 S fusion particle (57). Presumably, once bound, NSF uses its ability to hydrolyze ATP in some way to help initiate the fusion process. In vitro experiments using detergent-solubilized components have shown that the disassembly of the 20 S particle is triggered by ATP hydrolysis, apparently by NSF (25, 41, 42). In one model of membrane fusion, this disassociation of the solubilized 20 S particle has been interpreted to be a manifestation of membrane fusion. Data presented in this manuscript support this idea: ATP hydrolysis by the D1 domain is absolutely required for intra-Golgi transport and, therefore, membrane fusion. Since this domain accounts for the majority of NSF's ATPase, it is not hard to imagine that it accounts for the ATP hydrolysis that drives the disassembly of the 20 S particle. The E329Q mutant is an efficient inhibitor of transport, suggesting that this mutant would incorporate into the 20 S fusion particle and, because of its inability to hydrolyze ATP, fail to progress through fusion, thus locking the 20 S particle into an inactive conformation. This model would hold that the ATPase activity of D1, which is essential to transport, is in fact the energy source for membrane fusion as manifested by 20 S particle disassembly.

What could be the role of the D2 domain? Although ATP hydrolysis by D2 is not necessary for a round of fusion, it is still possible that ATP hydrolysis by D2 may be needed for recycling NSF after fusion. In this model, the D1 domain would be responsible for driving membrane fusion, and the D2 domain would be involved in disassembly/recycling of the fusion machine. This model is also supported by the data presented in this manuscript. The fact that the mutations of the D2 domain are able to sustain a fraction of the total transport suggests that these mutants may be able to only facilitate a single fusion event per NSF molecule.

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