Unraveling the Mechanism of the Vesicle Transport ATPase NSF, the N-Ethylmaleimide-sensitive Factor

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The transport of cargo in eukaryotic cells is mediated by the movement of membranous vesicles that pinch off from one membrane and fuse with another. An essential part of this process is the interaction between SNARE1 (soluble NSF attachment protein receptors) proteins from the vesiicle (v-SNARE) and target (t-SNARE) membranes. The resulting SNARE complexes are parallel four-helix coiled-coil structures with melting temperatures between 75 °C and 90 °C, and it is likely that, at least in part, the free energy of SNARE complex formation drives bilayer fusion (1). Regulating the assembly and disassembly of SNARE complexes is thus an important aspect of vesicular transport.

The hexameric ATPase N-ethylmaleimide-sensitive factor (NSF) uses energy from ATP hydrolysis to dissociate SNARE complexes after membrane fusion, allowing the individual SNARE proteins to be recycled for subsequent rounds of fusion (1). NSF binds to and dissociates SNARE complexes only in the presence of the adapter protein, α-SNAP (soluble NSF attachment protein). α-SNAP interacts directly with the SNARE complex and with ATP-bound NSF to form the so-called “20 S particle” (2, 3). In the 20 S particle, α-SNAP stimulates the ATPase activity of NSF, leading to SNARE complex disassembly (Fig. 1) (4, 5). Specific v- and t-SNAREs are associated with each intercompartmental transport step, but NSF and α-SNAP are general cytosolic factors that can disassemble the SNARE complexes from most, if not all, intracellular transport steps (1).

The NSF protomer contains three domains: an N-terminal domain, NSF-N (residues 1–205), responsible for interaction with the α-SNAP-SNARE complex and two homologous ATP-binding domains, NSF-D1 (residues 206–488) and NSF-D2 (residues 489–744) (3). NSF-D1 is an active ATPase that provides the driving force for SNARE complex disassembly (6, 7). NSF-D1 must bind ATP to interact with the α-SNAP-SNARE complex. NSF-D2 is responsible for maintaining NSF as a hexamer (6). It has higher affinity for ATP than NSF-D1 (8) but has no significant ATPase activity. Nucleotide binding by NSF-D2 is, however, important for hexamerization.

The sequences of NSF-D1 and NSF-D2 place NSF in the AAA (ATPases associated with various cellular activities (9)) superfamily (10). AAA proteins, which contain at least one copy of a conserved ~230-amino acid cassette, are involved in a wide variety of cellular roles, including membrane fusion, proteosome regulation, transcription, organelle biogenesis, and microtubule transport and regulation (10, 11). Despite this functional diversity, the ability to assemble or disassemble multisubunit macromolecular complexes, or to fold or unfold polypeptides, appears to be common to the family.

Here, we focus on recent advances that are helping provide a basis for understanding the physical mechanisms that underlie the role of NSF in SNARE complex disruption.

Structural Studies

Crystallographic and electron microscopy studies of NSF, the 20 S complex, as well as other AAA proteins have begun to provide insight into how NSF interacts with the other components of the 20 S complex and responds to changes in bound nucleotide. Electron micrographs of NSF reveal a double ring hexameric structure ~15 nm in diameter and ~12 nm in height (12). The double ring structure is retained by a construct (NSF-D1D2) lacking the NSF-N domain, but the ring height and diameter of NSF-D1D2 are ~2 nm smaller than that of full-length NSF. Similar quaternary arrangements are seen in other AAA proteins (12).

The 20 S complex and α-SNAP-SNARE complexes have also been imaged in the electron microscope (12, 13). α-SNAP appears to form a sleeve around the SNARE complex, binding lengthwise along the rod-like SNARE coiled-coil (13). The 20 S complex resembles a spark plug, with NSF-D1 and NSF-D2 visible as two rings at one end (Fig. 2A). The α-SNAP-SNARE complex sits on the face of the NSF-D1 ring opposite the side that faces NSF-D2. The NSF-D1 hexamer appears flatter and wider than NSF-D2. α-SNAP and the SNARE complex interact in an antiparallel manner. This places the membrane-distal, N terminus of the SNARE complex close to the C terminus of α-SNAP, which then contacts the N-terminal region of NSF (12, 13).

Crystal structures have been determined for NSF-N (14, 15) and its yeast equivalent Sec18p-N (16). The structures of the corresponding domain in p97 (p97-N), a homologue of NSF that functions in SNARE-independent membrane trafficking steps (17), and VAT (VAT-N), an archael homologue of p97, are also known (18). NSF-N and its homologues contain two subdomains: an N-terminal β-barrel and a C-terminal α/β roll. There are significant differences in the structures of the intersubdomain linkers in NSF-N, Sec18p-N, p97-N, and VAT-N, but the relative orientations of the two subdomains are virtually identical (14–18). This, together with the demonstration that NSF-N melts with a single sigmoidal transition (15), makes it likely that the two subdomains remain locked together to form a single structural unit.

NSF binds to the α-SNAP-SNARE complex only in the presence of ATP. When Sec18p is pretreated with the nonhydrolyzable ATP analogue AMP-PNP, it binds to a cation exchange column, whereas under conditions in which hydrolysis may take place, it does not (16). This suggests that a basic surface is exposed during the conformational change(s) that occur upon ATP binding. The groove formed by the intersubdomain interface in NSF-N is rich in basic and hydrophobic residues and is of appropriate size and shape to accommodate an α-helical peptide (15). This region was suggested as a potential site for interaction with the highly negatively charged C-terminal helix of α-SNAP (Fig. 2B) (15, 19). Consistent with this, mutation of a conserved arginine residue (Arg65) near the groove ablates NSF binding to α-SNAP-SNARE complexes while retaining full ATPase activity. Furthermore, hydrophobic residues near the base of the groove have been proposed to interact (16) with...

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a leucine residue required for stimulation of NSF ATPase activity (19), present at the C terminus of α-SNAP.

The crystal structure of NSF-D2 (20, 21) consists of an N-terminal nucleotide-binding subdomain and a C-terminal helical subdomain. The NSF-D2 hexamer is formed by packing of the wedge-shaped nucleotide-binding subdomains, with the nucleotide-binding sites located in the interface between protomers. The C-terminal helical subdomains are located at the apices of the hexamer. The structures of both subdomains show similarities to the δ clamp-loading subunit of Escherichia coli DNA polymerase III (polⅢ) (22), the protease-associated AAA chaperone, HslU (23, 24), and the D1 ATP-binding domain of p97/VCP (p97-D1) (17). Like NSF-D2, both p97-D1 and HslU form hexamers.

The structure of a fragment of p97 containing the p97-N and p97-D1 domains (p97-ND1) shows that the p97-N domains are located at the periphery of the p97-D1 hexamer (Fig. 2C) (17). Contacts between p97-N and p97-D1 are made with both subdomains of p97-D1 and the N-D1 interdomain linker (Fig. 2D) (17). The relative orientation of the p97-N and p97-D1 domains agrees well with that observed in EM images of VAT (25) and p97 (17, 26). Curiously this orientation looks more like EM images of the ATP-saturated than the ADP-bound state of NSF (12), but ADP is bound in the p97-ND1 crystal structure. However, in the p97-ND1 structure, the cleft between the subdomains in p97-N, which includes the surface equivalent to the putative α-SNAP binding surface in NSF-N, is blocked by the ND1 linker on the top side of the domain. If this arrangement were true for NSF, the binding cleft would only be accessible on the side opposite the α-SNAP-SNARE complex observed in images of the 20 S complex (Fig. 2, A and C). This orientation would not be compatible with binding of the α-SNAP-SNARE complex and may explain why the ADP state of NSF is unable to bind α-SNAP-SNARE complexes (8).

EM images suggest that the NSF-N domains move with respect to NSF-D1 (12). A conserved glycine residue, present at the C terminus of the N-D1 linker in NSF (and related AAA proteins), may act as a pivot point for rigid body movements between the domains (17). Structurally this glycine residue is in close proximity to the nucleotide-binding site in both NSF-D2 and p97-D1 and could be sensitive to the state of the bound nucleotide. Another glycine residue, found near the N terminus of the p97 N-D1 linker, may contribute to hinge movement (17). However, this residue is not conserved, even among p97 orthologues, and the structures of NSF-N and p97-N diverge in this region.3

**Conformational Responses to Nucleotide State**

NSF is capable of undergoing large conformational changes upon ATP binding and hydrolysis, most notably with NSF-N domains changing their orientations relative to the rest of the NSF hexamer (12). In doing so, it converts the energy stored in ATP into mechanical work needed to disassemble the α-SNAP-SNARE complex.

NSF is a hexamer in solution, and oligomerization appears to be intimately linked with both ATPase activity and ligand binding (3, 7, 11). A construct of NSF containing only the NSF-N and NSF-D1 domains (NSF-ND1) is monomeric, has reduced ATPase activity and affinity for ligand when compared with full-length NSF (6), and is unable to dissociate SNARE complexes. However, when attached to NSF-D2, NSF-D1 forms a hexameric ring, and NSF-D1D2 has full-length NSF (6). Like NSF, some other active AAA ATPase modules are linked to an oligomerization domain (e.g. FtsH) without which activity is either significantly impaired or lost completely, whereas others (e.g. Vps4p, katanin) exist in equilibrium between monomer and oligomer (11). The stimulation of ATPase activity upon ligand binding and interactions with ligand only when bound to ATP are also common in the AAA family (reviewed in Ref. 11). Oligomerization, ligand binding, conformational change, and ATP hydrolysis therefore appear to cooperate to convert ATP-bound energy to useful work in NSF and other AAA proteins. How are these properties coupled to one another? Although there remains a paucity of direct information on NSF itself, lessons can be learned from recent studies of other members of the AAA family.

AAA proteins contain phosphate-binding P-loop (Walker A) and metal ion-binding DEXX box (Walker B) nucleotide-binding sequences. AAA proteins contain two additional conserved regions, termed sensor-1 and sensor-2 (10, 22), that lie near the nucleotide-binding site. Sensor-1 and sensor-2 are positioned to detect a change in the nucleotide state and to transduce that signal to more remote regions of the protein (10, 20, 22). The structures of two AAA domains that contain consensus sensor-1 (p97-D1 (17)) and sensor-2 (HslU (23, 24)) sequences now provide direct structural information for the sensor regions. However, it remains unclear what role nucleotide binding and hydrolysis play in the respective biological activities of p97-D1 and HslU.

The sensor-1 motif, which overlaps the "second region of homology" (9), contains a highly conserved "sensing" residue (Asn/Ser/ Thr) and a C-terminal proline residue (10). A mutation of Thr394 (the residue N-terminal to the sensing Asn) to proline results in a form of Sec18p that binds α-SNAP but is unable to stimulate ATP hydrolysis (27). The sensor-1 region extends through a short helix to a loop that is in close proximity to the nucleotide-binding site of an adjacent protomer in the hexamer. In most AAA proteins, this loop contains a highly conserved arginine residue (present in NSF-D1 but not NSF-D2). By analogy with G-proteins, this arginine residue has been proposed to be an "Arg finger" that can enhance the rate of hydrolysis of the nucleotide bound to an adjacent protomer (10, 28). In the p97-D1 structure, this arginine residue (Arg365) forms a salt bridge with Glu305 in the DEXX motif of the adjacent protomer. The side chain of p97-Arg365, also conserved among AAA proteins, is in close proximity to the β-phosphatate in the adjacent protomer. Either of these arginine residues could serve as a signal to the adjacent protomer upon nucleotide hydrolysis or release, providing a plausible mechanism for stimulation and cooperativity between hexamer subunits. Mutation of either of the equivalent residues in NSF-D1 (Arg385 or Arg386) prevents 20 S complex disassembly and does not affect either the basal or stimulated rates of hydrolysis. Mutation of the sensing Asn, the putative Arg finger, and other residues in close proximity results in the loss of protease activity in FtsHII (28).

As these mutants remain able to bind ATP, this phenotype has been attributed to the loss either of ATP hydrolysis or the coupling of hydrolysis to protease activity (28). In the HslUV chaperone-protease complex, the sensor-1 region of HslU is in direct contact with HslV (24) and is positioned to transmit changes in nucleotide state directly to its protease partner, HslV.

The sensor-2 motif, located at the N terminus of the third helix in the C-terminal subdomain (α8 in NSF-D2) is in contact with the P-loop. This region has been proposed to be the primary site for transmitting conformational change from the nucleotide-binding site to the C-terminal subdomain (20, 22). The sensor-2 motif in HslU contacts both the P-loop and HslV in the HslUV complex (24) and is positioned to transmit changes upon nucleotide hydrolysis to both the C-terminal subdomain of HslU and HslV. In this light, it is interesting to note that in ClpA, ClpX, and Lon, which are also protease-associated AAA proteins, the C-terminal subdomains

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3 See supplementary information associated with Ref. 17.
Fig. 2. A, negative stain image of the 20 S complex adapted from Ref. 13. Ribbon diagrams of the components of the 20 S complex are shown approximately to scale. The crystal structure of NSF-D1 is not known, and it is modeled by the structure of NSF-D2. NSF-N domains are shown as the trimer observed in Ref. 14. Only three NSF-N domains are shown for simplicity. Only one copy of α-SNAP, based on the structure of Sec1p, is shown for simplicity (there may be up to three copies present in the 20 S complex) (41). The SNARE complex is represented by the structure of a minimal, proteolytically defined complex (44). B, ribbon diagram depicting the structure of the NSF-N domain. The N-terminal subdomain is labeled NC and colored light gray. The C-terminal subdomain is labeled NC and colored dark gray. N- and C-term of the hexamer are labeled. Top and side views of the p97-ND1 hexamer (17). The subdomains of p97-N and p97-D1 are shown in different colors (p97-N N-terminal subdomain (blue), p97-N C-terminal subdomain (gray), p97-D1 N-terminal subdomain (brown), p97-D1 C-terminal subdomain (black)). ADF is shown as white space-filling atoms. The only access to the site equivalent to the putative α-SNAP binding site in NSF-N is indicated. C, ribbon diagram of the p97-ND1 monomer. Subdomains are colored as in B. Regions involved in nucleotide binding and potentially transmitting conformational change (N-D1 linker (pink), P-loop (black), DEEX motif (green), sensor-1 (red), sensor-2 (dark brown)) are highlighted. This figure was drawn using the programs BOBSCRIPT (42) and RASTER3D (43).

Recent electron microscopy data on p97 has presented additional possibilities for how changes in nucleotide state may be coupled to conformational change in AAA proteins (17, 26). In one model, derived from a three-dimensional EM reconstruction, the p97-D1 and p97-D2 hexamers are proposed to pack together in a tail-tail arrangement (17). Movements of the C-terminal subdomains (5–15°) occur about a pivot point in the intersubdomain linker. In the different crystal forms, the packing within the AAA hexamer changes significantly, and in some cases results in a break in the 6-fold symmetry of the hexamer. The changes in packing are probably due to relative movements of the N-terminal and C-terminal subdomains in protomers with different nucleotide occupancy (23). The observed differences may reflect crystal packing constraints rather than genuine conformational states but serve to illustrate that the hexameric interfaces may be sufficiently plastic to change during the catalytic cycle.

Recent electron microscopy data on HslU in the presence of different ATP analogues and in the absence of nucleotide (23, 24). When these structures are superimposed using their nucleotide-binding subdomains, a significant degree of flexibility is observed (23). Rigid body movements of the C-terminal subdomains (5–15°) occur about a pivot point in the intersubdomain linker. In the different crystal forms, the packing within the AAA hexamer changes significantly, and in some cases results in a break in the 6-fold symmetry of the hexamer. The changes in packing are probably due to relative movements of the N-terminal and C-terminal subdomains in protomers with different nucleotide occupancy (23). The observed differences may reflect crystal packing constraints rather than genuine conformational states but serve to illustrate that the hexameric interfaces may be sufficiently plastic to change during the catalytic cycle.

Mechanisms of SNARE Complex Disassembly

However the SNARE complex is disassembled, force must be generated to separate the components. The physical mechanism by which NSF uses α-SNAP to disassemble SNARE complexes is not known, but the superhelical nature of the SNARE complex coiled-coil suggests that any mechanism of disassembly involves rotation of some kind. Physically this can only be achieved if part of at least one helix remains stationary while the remaining helices move, and two obvious possibilities can be considered. The first is that the membrane-anchored C terminus of the SNARE complex remains stationary while NSF and α-SNAP unravel the N-terminal end. This mechanism is unlikely, as NSF can dissociate SNARE complexes formed only from the cytoplasmic portions of the SNARES, i.e. with no membrane anchors (32). Furthermore, the kinetics of 20 S complex disassembly are independent of whether the SNARE complex is in solution or located on membranes (33). The second possibility is that the N terminus of one or more of the components of the SNARE complex is attached directly to NSF, and movement of α-SNAPs bound to the remainder of the SNAREs relative to that anchor point unwinds the SNARE complex. This model requires the presence of a direct contact between components of the SNARE complex and NSF. NSF-α-SNAP can disassemble a core SNARE complex containing only the SNARE motifs of VAMP-2, syntaxin 1a, and SNAP-25 (32). The crystal structure of this core complex showed that the N termini of the syntaxin 1a and SNAP-25 N-
terminal helices extend beyond the rest of the coiled-coil (34). These regions would then be the most likely points of contact with NSF. Although a direct interaction between SNAREs and NSF has never been detected, there is no reason to assume that it cannot take place in the context of the 20 S complex. This mechanism would operate equally well whether the SNAREs were attached to a membrane or in solution.

A further possibility for disassembly of the SNARE complex is that removal of part or all of one of the four helices in the coiled-coil would result in the spontaneous collapse of the remaining complex. In the case of the neuronal SNARE complex, the VAMP-2 SNAP-25 SNAP motif is unstructured in solution in the absence of other SNARE proteins (36), an NSF-induced conformational change imparted solely on syntaxin 1a may initiate melting of the VAMP and SNAP-25 helices, resulting in disassembly of the SNAP complex.

One of the crystal forms of NSF-N contained three protomers that were related by a pure 3-fold rotation axis (14). The dimensions of this trimeric NSF-N assembly were surprisingly concordant with those of the NSF-D2 hexamer and the “collar” region of the 20 S complex seen in electron micrographs (13). This, along with the proposed stoichiometry of 1 NSF hexamer:3 α-SNAP motif (33, 37) in the 20 S complex led to the suggestion that the trimer may represent a functional assembly. This presents the possibility that NSF works by using alternate sets of SNAP-25 SNAP motifs to apply torsion to the SNAP complex and unwind it (14). Alternatively, NSF may use the alternate sets of NSF-N domains to pull one end of α-SNAP toward the outside of the NSF hexamer and unravel the SNAP coiled-coil.

The observation of the central pore in NSF has led to speculation about its possible functional role (21, 34), particularly when considered in the context of other AAA proteins. The protease-associated AAA chaperones feed unfolded proteins through the central pore. The syntaxin family of t-SNAREs has a chemical, and biophysical studies of NSF and related proteins should provide answers to these questions over the next few years."

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REFERENCES


Minireview: Molecular Mechanism of NSF

proteins from different transport stages? Ongoing structural, biochemical, and biophysical studies of NSF and related proteins should provide answers to these questions over the next few years. Acknowledgments—We thank Xiaodong Zhang and Paul Freemont for providing the coordinates of p97-ND1.
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