Mechanistic insights into the recycling machine of the SNARE complex

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Evolutionarily conserved SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptors) proteins form a complex that drives membrane fusion in eukaryotes. The ATPase NSF (N-ethylmaleimide sensitive factor), together with SNAPs (soluble NSF attachment protein), disassembles the SNARE complex into its protein components, making individual SNAREs available for subsequent rounds of fusion. Here we report structures of ATP– and ADP–bound NSF, and the NSF/SNAP/SNARE (20S) supercomplex determined by single-particle electron cryomicroscopy at near-atomic to sub-nanometre resolution without imposing symmetry. Large, potentially force-generating, conformational differences exist between ATP– and ADP–bound NSF. The 20S supercomplex exhibits broken symmetry, transitioning from six-fold symmetry of the NSF ATPase domains to pseudo four-fold symmetry of the SNARE complex. SNAPs interact with the SNARE complex with an opposite structural twist, suggesting an unwinding mechanism. The interfaces between NSF, SNAPs, and SNAREs exhibit characteristic electrostatic patterns, suggesting how one NSF/SNAP species can act on many different SNARE complexes.

Membrane fusion is essential for many physiological processes in eukaryotic cells, including protein and membrane trafficking, hormone secretion, and neurotransmission. The evolutionarily conserved SNARE proteins have a key role in these processes. Specific combinations of SNARE proteins are located on opposite membranes. Upon zippering into a highly stable four-helix bundle—the SNARE complex—they provide the energy for membrane fusion. The combinations of SNARE proteins depend on the source of vesicles and the identity of target membranes, but other factors also contribute to the specificity of the membrane targeting. To maintain the pool of individual SNARE proteins, the ATPase NSF, together with SNAPs, disassembles post-fusion and non-productive SNARE complexes into individual protein components using the energy from ATP hydrolysis.

NSF was the first protein found to play a key role in eukaryotic trafficking. It is a member of AAA+ (ATPases associated with diverse cellular activities) superfamly of ATPases, and it forms a homomeric hexamer with a molecular weight of ~500 kDa, with each protomer consisting of an amino-terminal domain (termed N) and two ATPase domains (termed D1 and D2) (Fig. 1a). The D1 domains are responsible for most of the ATPase activity of NSF, whereas the D2 domains are primarily responsible for hexamerization. The N domains are involved in SNAP and possibly SNARE binding. Prior to ATP hydrolysis, the NSF, SNAP, and SNARE complex form the so-called 20S supercomplex.

Individual components of 20S supercomplex have been structurally characterized, including the crystal structures of several SNARE complexes, SNAPs, and the D2 and N domains of NSF. Structural studies of full-length NSF and the 20S supercomplex have also been carried out using quick-freeze/deep-etch, negative-staining electron microscopy, and electron cryomicroscopy (cryo-EM). However, due to the low resolution limits of these studies, the detailed molecular architecture of the 20S supercomplex is unknown and critical questions remain to be answered such as how the adaptor protein SNAP recognizes SNARE complexes, how many SNAPs are involved; how one NSF/SNAP species disassembles many different SNARE complexes in a promiscuous fashion; as well as the question of what is the molecular mechanism of disassembly.

Here we present the structures of full-length NSF in two different nucleotide states (ATP- and ADP-bound, at 4.2 Å and 7.6 Å resolution, respectively), and structures of two different 20S supercomplexes involving different SNARE substrates determined by single particle cryo-EM, ranging from 7.6 to 8.4 Å resolution. The cryo-EM structures reveal large conformational differences of NSF between ATP- and ADP-bound states, and upon binding to SNAPs and SNAREs. We confirmed by site-directed mutagenesis that the molecular interfaces between SNAPs, SNAREs, and NSF play important roles in disassembly, and propose that recognition at these interfaces is based on characteristic electrostatic patterns. Based on these new insights we speculate about the molecular mechanisms of NSF-mediated SNARE complex disassembly.

ATP– and ADP–bound NSF structures

We developed a new purification scheme to address the heterogeneity of NSF samples caused by mixtures of nucleotide states. In essence, hexameric NSF was monomerized by completely removing the bound nucleotides through size-exclusion chromatography (SEC) (Methods and Extended Data Fig. 1a, b). The resulting NSF protomers could be reassembled into hexamers in the presence of the desired nucleotide. The reassembled ATP- and ADP-bound NSF hexamers were studied by single particle cryo-EM; EDTA was included to prevent hydrolysis. Our reassembled NSF hexamers are functionally active (Extended Data Fig. 1g and Methods).

The reconstruction of ATP-bound NSF is shown in Fig. 1b, c and Extended Data Fig. 2. The reconstruction has an estimated overall resolution 27 of 4.2 Å after masking out flexible N domains (Extended Data Fig. 2e and Extended Data Table 1). All D2 domains, and five out of six D1 domains were well resolved in the final three-dimensional (3D) density map (Fig. 1b and Extended Data Figs 2d and 3a–c). Consistent with the estimated resolution, we observed grooves in α-helices, β-strands...
The 3D reconstruction of ADP-bound NSF is shown in Fig. 1d, e. The overall estimated resolution is 7.6 Å (Extended Data Fig. 4 and Extended Data Table 1), with well-resolved tubular densities for α-helices (Fig. 1d). To obtain an atomic model of ADP-bound NSF, we docked our cryo-EM structure of the D1 domain protomer (obtained from ATP-bound NSF) and the crystal structure of the D2 domain hexamer into the corresponding cryo-EM densities, followed by refinement. To complete the models, the crystal structure of NSF N domain was docked into the corresponding densities (Methods).

The cryo-EM data sets of the NSF particles used in this study were of sufficient quality to determine and refine 3D reconstructions to high resolution without imposing any symmetry, which turned out to be critical for resolving the NSF N domains and asymmetries in the ATPase rings (Extended Data Fig. 5, see Supplementary Information for a detailed discussion).

**Asymmetric features of ATP- and ADP-bound NSF**

Both the ATP- and ADP-bound structures of NSF are organized into three layers: two rings consisting of six D2 domains and six D1 domains, respectively, and a layer of six (four) N domains for ATP (ADP)-bound NSF (Figs 1c, e and 2a, b). For ADP-bound NSF, the remaining two N domains are flipped along the sides of the ATPase rings with well resolved densities compared to the N domains atop the D1 ring, leaving little doubt as regards the identity of these two densities (Fig. 1e and Extended Data Figs 4c, e and 7c).

For ATP-bound NSF, the D2 ring is planar and approximately sixfold symmetric. The D1 ring is reminiscent of a right-handed ‘split washer’, with each chain stepping up about 5 Å as manifested by the relative positions of the α2 helix in the D1 domains (Fig. 2c and Extended Data Fig. 6a). Chain F (purple) is an exception, which does not step up relative to chain E (blue), but instead slightly steps down towards chain A (red); there is a large step down from chain F to chain A. The D1 domain of chain F was not as well resolved in the density map as the other D1 domains (Fig. 1b), indicating its potential flexibility (Supplementary Video 1). However, the density for this domain is clear enough to indicate that the α subdomain has a different position relative to the α/β subdomain, compared to the other five D1 domains that can all be

**Figure 1 | 3D density maps of ATP- and ADP-bound NSF.**

a. Domain diagram of the NSF protomer. b. Different views of the sharpened map (6.5Å) of ATP-bound NSF filtered to a resolution of 4.2 Å with each domain colour-coded to match the domain diagram in panel a. A single chain of NSF (protomer) is coloured in gold to help with visualization. The density of one D1 domain (subsequently referred to as the D1 domain in chain F) is not well resolved (see black arrow). c. Different views of the unsharpened map (1.8Å) of ATP-bound NSF showing the positions of the N domains. d. Different views of the sharpened map (6.5Å) of ADP-bound NSF filtered to a resolution of 7.6 Å with each domain colour-coded to match the domain diagram in panel a. A single chain of NSF (protomer) is coloured in gold to help with visualization. e. Different views of unsharpened map (1.3 Å) of ADP-bound NSF showing the positions of the N domains. The gap in the D1 ring is indicated by a black arrow.

Within β-sheets, and backbone zigzags corresponding to ~3.8 Å Cx distances, along with densities of some aromatic side chains (Extended Data Fig. 3a, b). The crystal structure of the ATP-bound NSF D2 hexamer was readily docked into the corresponding cryo-EM density, followed by refinement. The densities of the D1 domains were of sufficient quality to build and refine a de novo atomic model of the D1 domain with bound ATPs. The D1 domain is a typical AAA + module with two subdomains (α and α/β) and motifs that are generally found in ATPases (Extended Data Fig. 3d, e). The α2 helix of the D1 domain is bent (Extended Data Fig. 3d), a distinctive feature compared to the straight α2 helices found in the D2 domain of NSF, as well as in both D1 and D2 domains of the closest related relative, the AAA + ATPase valosin-containing protein (VCP/p97).}

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![Image of NSF proteins](https://example.com/nsf_proteins.png)

**Figure 2 | Structures of ATP- and ADP-bound NSF.**

a. Side-view of ATP-bound NSF. b. Side-view of ADP-bound NSF. The six protomer chains are rainbow coloured anticlockwise based on the relative positions of the D1 domains to the D2 ring in the ATP-bound NSF model; the chain with the closest distance between D1 and D2 domains is named chain A (red). Nucleotides are shown as grey surfaces. See Methods for generation and refinement of the atomic models. c. A schematic diagram showing the topology of ATPase rings of ATP- and ADP-bound NSF, respectively. D1 rings are coloured according to the models shown in panels a and b.
well superposed (Extended Data Fig. 6c). Densities for ATP molecules are clearly visible in the nucleotide-binding pockets of the D1 domains of chains A through E, and in all D2 domains (Extended Data Fig. 3f, g); however, there is no clear density in the nucleotide-binding pocket of the D1 domain of chain F.

For ADP-bound NSF, the D2 ring slightly deviates from a near perfect six-fold symmetric conformation, producing a small gap (Extended Data Fig. 7a). The D1 ring is more expanded and planar compared to ATP-bound NSF (Extended Data Figs 6b and 7b), with a large opening between chains A and F, which coincides with the small gap in the D2 ring, that is, it forms an open ‘flat washer’ (Fig. 2c). The structures of all six D1 domains can be well superposed, but adopt different orientations relative to the D2 domains (Extended Data Fig. 6b, d).

When superposing the α/β subdomains of all the D1 domains of both ATP- and ADP-bound NSF (except for the flexible chain F in ATP-bound NSF), the α7 helix in the α subdomain is translated between the ATP and ADP-bound states (Extended Data Fig. 6e). The nucleotides are likely absent in the D1 ring of ADP-bound NSF as the conformational changes would not favour binding of nucleotides because of possible clashes between the nucleotide and the translated α7 helix (Extended Data Fig. 6e). This conformational change of the α subdomain is correlated with the large difference of the D1 ring between the ATP- and ADP-bound states. The ATP loaded D1 ring is more compact, with a total interface area of 5938 Å² compared to that of 3746 Å² in the ADP-bound state, resembling a spring-like transition from a ‘loaded’ split-washer state to a ‘relaxed’ open-flat-washer state (Extended Data Fig. 7b and Supplementary Video 2). This transition is further correlated with outward rotations of the D1 domains of chains A and B and the changes in their N domains (Extended Data Figs 6b and 7b, c). For a detailed comparison of our NSF structures with other members of the AAA+ family, see the Supplementary Information.

**Structures of the 20S supercomplex**

We prepared 20S supercomplex consisting of AMPPNP-bound hexameric NSF, αSNAP, and neuronal SNARE complex that is composed of syntaxin-1A, synaptobrevin-2/VAMP-2 (vesicle-associated membrane protein 2), and SNAP-25 (synaptosomal-associated protein 25) (Methods and Extended Data Fig. 1c–f). We used a truncated neuronal SNARE complex (green shaded fragments in Fig. 3a), identical to the one of which the high-resolution structure had been determined; it was chosen because it remains monomeric in solution even at high concentration, and is sufficient for reversible assembly and disassembly (Extended Data Fig. 1g). The 3D classification of the single particle cryo-EM data of the 20S supercomplex produced four different reconstructions that each represents an asymmetric molecular state of 20S, referred to as states I, II, IIIa, and IIIb (see Methods). The corresponding refined density maps of the 20S supercomplex (without symmetry) have an overall resolution ranging from 7.6 to 8.4 Å after gold-standard refinement by RELION (Extended Data Fig. 8f)27,31.

The structures of the four states of the 20S supercomplex have the same overall architecture, so we discuss state I as a representative of all states in the following; detailed differences between the states are discussed later. The 20S supercomplex resembles a tower with different domains organized into layers (Fig. 3b). At the base of the tower (in the orientation shown in Fig. 3b, middle panel) are the D2 and D1 ATPase rings of NSF, and at the top of the tower is a ‘spire’, made up of four αSNAP molecules and one SNARE complex, surrounded by the six N domains of NSF. The 20S supercomplex is a striking example of broken symmetry: The approximate six-fold symmetry at the base of the complex is progressively violated in the D1 and N domains, allowing the complex to transit from six-fold symmetry to a pseudo four-fold symmetry at the top (Fig. 3b and Extended Data Fig. 8e).

The four α-helix bundle of the SNARE complex at the centre of the spire is clearly visible along with its characteristic twisted left-handed grooves, although the chemical identity of each polypeptide chain cannot be uniquely assigned at the available resolution (Fig. 3b and Extended Data Fig. 6c).

**ATPase rings are tightened upon SNAP/SNARE binding**

When comparing the ATPase domains of the AMPPNP-bound 20S supercomplex to those of ATP-bound NSF, there are similar overall features, along with some important differences. The overall root-mean-square-deviation (r.m.s.d.) of the main chain atoms of D1 and D2 ATPase rings is 4 Å, based on a superposition of the D1 ring. The D2 ring of 20S supercomplex is approximately six-fold symmetric, whereas the D1 ring has a split-washer-like arrangement, similar to that of ATP-bound NSF (Extended Data Fig. 7d). Overall, the ATPase rings of the 20S supercomplex adopt a tighter conformation than NSF alone (Extended Data Fig. 7d).
Four states of the 20S supercomplex

While the D1/D2 ATPase rings of NSF are very similar among the four states, the αSNAP–SNARE spire and the N domains differ (Fig. 4a). The four states were grouped into three observed patterns (I, II, and III) based on the mode of interaction between αSNAP molecules and N domains (Fig. 4b). Considering that each αSNAP can interact with either one or two nearby N domains, one expects a total of nine theoretical patterns taking into account the split-washer asymmetry of the D1 ring (see Supplementary Discussion). However, only three patterns consistently emerged in the 3D classification. One explanation for this phenomenon is that the position of the αSNAP–SNARE spire is not random, which might favour certain patterns over the others. Indeed, the centre of the spire—the SNARE complex—is always located close to chains E and F, which are at the raised edge of the D1 split washer (arrows in Fig. 4b), suggesting possible interactions between the pore loops (YVG motif)\(^7\) of D1 domains and the SNARE complex. For pattern III, two subclasses were refined separately, resulting in states IIIa and IIIb. The main difference between the two states involves the relative position of the spires, not the pattern of αSNAP and N domain interactions (Supplementary Video 5). Thus, the 20S supercomplex exhibits four major states, which are mainly characterized by the patterns of the N domains and the position of the αSNAP–SNARE spire, whereas the conformations of the spire and base themselves do not differ much.

Multi-modal interactions between N domains and αSNAP

The structures of the four states of 20S reveal eight instances of αSNAP molecules that are interacting with two N domains of NSF, and another eight instances of αSNAP molecules that are interacting with one N domain (Fig. 4). When superposing αSNAP molecules separately based on the 1:2 and 1:1 binding scenarios, two distinct N-domain binding sites on the surface of the C-terminal region of αSNAP appear in the case of 1:2 binding, whereas in the case of 1:1 binding, the N domains bind somewhere between these two sites (Fig. 5a). The electrostatic potential surface of the C-terminal region of αSNAP is quite negative, and both distinct binding sites are located in this negatively charged area (Fig. 5b). The N domains of NSF interact with either of the two binding sites on αSNAP via the same positively charged area (Fig. 5c). The two interfaces involve five positively charged residues of the N domain of NSF and eight negatively charged residues of the C-terminal region of αSNAP (Fig. 5d).

Previous mutagenesis studies suggested that certain positively charged residues of the N domains are important for αSNAP and SNARE binding\(^3\), and that the C-terminal region of αSNAP is important for 20S

**Figure 4 | Top views of the four states of 20S supercomplex.** The identified four states were aligned with respect to the D1 rings. a. Sharpened maps (state I: 4.7σ, state II: 4.5σ, state III: 4.2σ, state IIIb: 4.0σ). b. Schematic drawings to help with visualization. The N domains and αSNAP molecules are shown as spheres and squares, respectively. The D1 rings are rainbow coloured using the same scheme as in Fig. 2, with black arrows indicating the split between chain A and chain F. The D2 rings are omitted for clarity. Each N domain is labelled with its corresponding chain identifier. The numbers of particles that contributed to the reconstruction of each state are listed.
We infer that the C terminus of the SNARE complex is facing away from NSF since it would normally continue into the transmembrane \(\alpha\)-helices of the SNAREs syntaxin-1A and synaptobrevin-2 (transmembrane domains were not included in the constructs used). This inference is consistent with the orientations of \(\alpha\)SNAPs, the hydrophobic loops of which are pointing away from NSF for possible membrane association\(^36\) (Fig. 6a). Moreover, our structures provide a molecular explanation for the previous finding that the trans-SNARE complex before membrane fusion is resistant to NSF disassembly\(^37\): the \(\alpha\)SNAP–SNARE subcomplex would not be able to form since the trans-SNARE complex is probably in a half-zippered state\(^38\).

The electrostatic potential surface of the SNARE complex has a highly conserved pattern with negative charges at the centre (Fig. 6b)\(^39\). The interacting surface of \(\alpha\)SNAP has two extruding positively charged residues (K122, K163) close to the central ionic layer (zero layer)\(^39,40\) of the SNARE complex, and another two close to the C-terminal region of \(\alpha\)SNAP (K203, R239) (Fig. 6c, d). Previous mutagenesis studies suggested the importance of K122, K163, and K203 (ref. 41). To further test the functional importance of these possible interactions, we mutated these two groups of residues, as well as a conserved conserved negative patch close to the N terminus of \(\alpha\)SNAP (E39, E40, E43, D80), and performed the SNARE complex disassembly assay. Remarkably, the K122E/K163E double mutant was completely inactive and the K203E/R239E double mutant showed impaired kinetics (Fig. 6e, f). The negative-patch quadruple mutant E38A/E40A/E43A/D80A affected kinetics to a lesser degree, with slightly decreased initial reaction rates; the presence of a membrane may make this interaction between SNAPs and SNAREs more important\(^36\).

**Variable \(\alpha\)SNAP stoichiometry**

To investigate how NSF disassembles a different SNARE complex, we prepared a SNARE complex consisting of VAMP-7 (vesicle-associated membrane protein 7), syntaxin-1A and SNAP-25 (referred to as the V7-SNARE complex); this complex includes the N-terminal Habc domain of syntaxin-1A and the N-terminal Longin domain of VAMP-7 (Fig. 7a). We assembled the 20S supercomplex with NSF and \(\alpha\)SNAP (referred to as the V7-20S supercomplex, Extended Data Fig. 9a–c). This complex is functionally active since the V7-SNARE complex is disassembled upon ATP hydrolysis\(^39\). We determined a cryo-EM reconstruction to an estimated resolution of 8.0 Å without imposing any symmetry (Extended Data Fig. 9d–f). Only two \(\alpha\)SNAP molecules bound to the rather inclined SNARE complex bundle (Fig. 7b, c). Note that the two N-terminal domains of the V7-SNARE complex and two of the six N domains of NSF were not visible. Although the spire is not as well resolved as in the 20S supercomplex (as indicated by the lack of separation of the four \(\alpha\)-helices of the V7-SNARE complex), the cryo-EM reconstruction of V7-20S revealed that NSF can use fewer \(\alpha\)SNAP molecules and readjust the N domains when binding to different SNARE complexes. The supercoil axis of the truncated neuronal SNARE complex in the 20S structures is approximately perpendicular to the plane of ATPase rings, whereas in the V7-20S structure the V7-SNARE complex is angled at 76 degrees relative to the plane of ATPase rings (Fig. 7b, d). Despite these differences, the mode (for example, right-handed twist) by which \(\alpha\)SNAP interacts with SNARE complex and the location of the SNARE complex atop the D1 ATPase ring in V7-20S are similar to those of 20S (compare Fig. 3c and Fig. 7c).

To further confirm the stoichiometry of the \(\alpha\)SNAP–SNARE subcomplexes in solution, we conducted composition-gradient multi-angle light scattering (CG-MALS) experiments using the two different SNARE complexes mixed with \(\alpha\)SNAP in a composition gradient (Extended Data Fig. 10a, b). The CG-MALS data analysis revealed that \(\alpha\)SNAP binds to the truncated neuronal SNARE complex at a maximum ratio of 4:1, whereas it binds to V7-SNARE complex at a maximum ratio of 2:1 (Extended Data Fig. 10c, d). In solution, multiple species of the \(\alpha\)SNAP–SNARE subcomplex are in equilibrium, but the cryo-EM structures of
Figure 7| 3D density map and structure of V7-20S supercomplex.

a. Domain diagram of the V7-SNARE complex. Transmembrane domains (grey) were not included in the complex. The two N-terminal domains of VAMP-7 and syntaxin-1A are highlighted by red boxes. b. Different views of the 3D density map (4.5σ) coloured similarly to Fig. 3b. The angle between the long axis of V7-SNARE complex and the plane of ATPase rings is approximately 76 degrees as shown in the inset. c. Corresponding views of the atomic model fit to the density map of V7-20S. Note that each of the two αSNAP molecules interacts with two NSF N domains. d. An illustration of the top view similar to Fig. 4b. The location of the N termini of the V7-SNARE α-helix bundle is indicated by a green dotted circle.

Figure 8| Model of NSF-mediated SNARE complex disassembly. The model consists of four stages (a–d). The model refers to the neuronal SNARE complex (consisting of synaptobrevin-2 (Syb2), syntaxin-1A (Stx1A), and SNAP-25) and αSNAPs, but the model is also applicable to other SNARE complexes, along with a different number of SNAP molecules as observed in both 20S and V7-20S suggest that NSF catches the saturated complex in both cases (Extended Data Fig. 10e, f).

NSF mediated SNARE complex disassembly

Based on our cryo-EM structures, we propose a working model of NSF mediated SNARE complex disassembly (Fig. 8). Starting with the cis-SNARE complex (that is, with both transmembrane domains in the same membrane), the first step (Fig. 8a, b) is the binding of SNAP molecules. Our cryo-EM structures suggest that depending on the particular component proteins of the SNARE complex, up to four SNAP molecules are present. A stoichiometry higher than 4:1 is unlikely, due to packing considerations. Dozens of SNARE proteins exist in an eukaryote, but there are only a few SNAP and very few NSF species. We propose that one NSF/SNAP species can disassemble all SNARE complexes (including yeast SNAREs) using shape and characteristic electrostatic pattern recognition of SNARE complexes by SNAPs, rather than specific ‘lock into key’ interactions (Fig. 6).

The second step of our model (Fig. 8b, c) is the binding of NSF, that is, the formation of 20S supercomplex. Upon binding to the SNAP-SNARE subcomplex, which acts like a fastener, both NSF ATPase rings are tightened, akin to a loaded spring (Extended Data Fig. 7d–f and Supplementary Video 4). The N domains are immobilized due to the interactions with SNAP molecules; characteristic electrostatic patterns may also play a role in these interactions. The opposing twists of SNAP molecules and the SNARE four α-helix bundle in both the 20S and V7-20S supercomplexes (Fig. 6a), along with the existence of four distinct molecular states (Fig. 4), suggests that the 20S supercomplex exerts a torque to unwind or loosen the SNARE complex while switching between the four states. This step requires ATP hydrolysis to initiate the movement of the NSF N domains, and subsequent force transmission via SNAPs. A second possibility is that the four states represent independent binding modes: each would apply a force on its own upon binding to the SNAP-SNARE subcomplex to unwind or loosen the SNARE complex.

The final step of our model (Fig. 8c, d) is the hydrolysis of multiple ATP molecules. We observed large conformational differences of NSF between the ATP- and ADP-bound states (Fig. 1c, e and Extended Data Fig. 10).

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Author Contributions M.Z. and Z.-Q. D.C. expressed and purified all protein samples; M.Z. and S.W. collected cryo-EM data; S.W. processed cryo-EM data; M.Z. built and refined the atomic models based on cryo-EM density maps; M.Z. performed the disassembly assay; S.V. performed the CG-MAoS experiment; M.Z., S.W., Y.C., and A.T.B. designed experiments, analyzed data and wrote the manuscript.

Author Information 3D cryo-EM density maps of ATP- and ADP-bound NSF, and the 20S supercomplex have been deposited in the Electron Microscopy Data Bank (EMDB), with accession codes EMDB-6204 (NSF-ATP), EMDB-6205 (NSF-ADP), EMDB-6206 (20S-ATP state I), EMDB-6207 (20S-ATP state II), EMDB-6208 (20S-state III) and EMDB-6209 (7V–20S). The coordinates of atomic models of ATP- and ADP-bound NSF, the 20S supercomplex have been deposited in the Protein Data Bank (PDB) with accession codes 3M3A, 3J94, 3J95, 3J96, 3J97, 3J98 and 3J99.

Supplementary Information is available in the online version of the paper.
**METHODS**

**Protein expression and purification.** Chinese hamster NSF with a tobacco etch virus (TEV) protease cleavable N-terminal His-tag was expressed from pPROEX-1 vector in *E. coli* BL21(DE3)-RIL cells (Agilent Technologies) at 25 °C overnight using autoinducing LB medium. After collecting the cells by centrifugation, they were resuspended in lysis buffer (50 mM TrisCl, pH 8.0, 300 mM NaCl, 50 mM imidazole, and 0.5 mM TCEP), and were subjected to sonication and centrifugation. The cleared lysate was loaded onto a HisTrap column (GE Healthcare), and washed with lysis buffer. NSF was eluted using elution buffer (lysis buffer supplemented with 350 mM imidazole). The fresh elution was pooled, concentrated, and supplemented with TEV protease, and incubated overnight at 4 °C. Tag-cleaved NSF was run through a HiTrap column to remove the TEV protease and the cleaved tags. Note that our method differs from that previously reported, which used apyrase to monomerize NSF. Monomerized NSF was frozen and stored at −80 °C for future use. To reassemble the hexameric NSF in a specific nucleotide state, for example, ATP or ADP, monomerized NSF was dialyzed at 4 °C overnight in reassembly buffer (50 mM TrisCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM nucleotide, 1 mM DTT, and 10% glycerol). The concentrated dialyzed NSF was loaded onto a Superdex 200 16/60 column that was pre-equilibrated with SEC buffer (50 mM TrisCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM ATP, 1 mM DTT, and 10% glycerol). Fractions containing hexameric NSF was separated from aggregated NSF eluted from the void volume. Concentrated hexameric NSF was loaded onto a Superdex 200 16/60 column that was pre-equilibrated with monomerization buffer (50 mM sodium phosphate, pH 8.0, 150 mM NaCl, 0.5 mM TCEP) in the absence of Mg2+, for example.

**SNARE complex.** However, the first anion exchange chromatography step for the NSF, 20S and V7-20S). The same vitrification conditions were applied to all samples (ATP- and ADP-bound NSF, 20S and V7-20S).

**Cryo-EM data collection.** Grids were transferred to TF30 Polara equipped with a field emission source and operated at 300 kV. Images were recorded on a Gatan K2 Summit direct electron detector operated in super-resolution counting mode following the established dose fractionation data acquisition protocol. Images were recorded at a nominal magnification of 31000×, corresponding to a calibrated super resolution pixel size of 0.61 Å on the specimen. The dose rate on the detector was set to be −8.2 counts (corresponding to −10.9 electrons) per physical pixel per second. At this setting, the coincidence loss is about 11.5% and the total loss, including the loss due to imperfect detector quantum efficiency, is about 25%. The total exposure time was 6 s, leading to a total accumulated dose of 44 e⁻ per particle. Image processing. Super-resolution counting images were 2 × 2 binned by Fourier cropping for motion correction, resulting in a pixel size of 1.22 Å. Motion corrected frames were summed to a single micrograph for subsequent processing. Defocus values were determined for each micrograph using CTFIND3. A semi-automated procedure similar to a previous work was used to pick particles. Briefly, for each data set, ~2,000 particles were manually picked to calculate 2D class averages. Uniform class averages were selected as templates for automated particle picking. Picked particles were visually inspected. Obviously falsely picked particles were removed. 2D classification was performed using RELION and SPIDER. Initial 3D models were generated using the common lines method. 3D classification and gold-standard refinement were performed using RELION. The initial model from the common lines method was low-pass filtered to 60 Å and used as the starting model for the initial auto-refinement, which generated a consensus model. This consensus model was again filtered to 60 Å and used for 3D classification. We used prior knowledge of NSF to distinguish ‘good’ classes from ‘bad’ classes: 3D class averages that showed incorrect features of NSF were considered as bad, including the wrong numbers of apparent D1 and/or D2 domains or a seriously deteriorated D2 ring. All good class averages had the correct number (six) of all the domains and well-defined D2 ring density.

For 20S good 3D class averages showed the correct number of all the domains, and well-defined densities of the αSNAP- SNAP spire and D2 ATPase ring. Particles...
in distinct good 3D classes were refined separately, yielding four final reconstructions (Fig. 4a). The four final reconstructions belong to three patterns (I, II, and III) based on the mode of interactions between nSNAP and N domains. When we tested different settings of the 3D classification procedure, other patterns were either rarely populated or showed deteriorated features in some domains, preventing refinement of the pattern to a reasonable resolution. Therefore, these other patterns may represent minor populations if they actually exist. Note that when we sub-classified pattern I or II, we also observed multiple states, but the differences between these states were smaller than the differences between the rigid body model minimization was carried out for the zβ and zβ subdomains of each ATPass domain (six D1 and six D2) separately using COOT.

Densities for ADP were clearly visible for four nucleotide-binding sites of the D2 domains (except those in chains A and F). The resulting model was refined using PHENIX as described above for the ATP-bound structure. The model was completed by docking the N domains into the unsharpened map, without fitting and further refinement; the quality of these densities was good enough to determine the approximate positions of the N domains.

For 20S supercomplex structure, the model of ATP-bound NSF containing the D1 and D2 domains was docked into each of the four sharpened maps corresponding to the four states rather than the rigid body model minimization was carried out for the zβ and zβ subdomains of each ATPass domain (six D1 and six D2) separately using COOT.
any self-association and a dual-component ‘crossover’ gradient to assess the interaction between nSNAP and each SNARE complex (Extended Data Fig. 10a). For each composition, the Calypso system prepared an aliquot of protein solution, injected it into the detectors and stopped the flow for 60–240 s to allow the reaction to come to equilibrium within the MALS detector flow cell. Equilibrium light scattering and concentration data were fit to an appropriate association model using the CALYPSO software (Wyatt).

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Extended Data Figure 1 | Purification of recombinant NSF bound to specific nucleotide and 20S supercomplex. a, Schematic diagram of the purification steps of NSF. Chromatography columns and buffer conditions are provided. b, Size-exclusion chromatograms corresponding to the coloured steps in panel a. Major peaks are labelled. c, A scheme showing the purification steps of the 20S supercomplex. d, Size-exclusion chromatogram of the 20S supercomplex. Major peaks are labelled. e, SDS–PAGE gel of fractions collected in panel d. The samples were not boiled. f, SDS–PAGE gel of the same fractions as in panel e. The samples were boiled. g, SDS–PAGE-based SNARE disassembly assay of the truncated neuronal SNARE complex. This complex is stable in SDS without boiling. Disassembly by NSF/αSNAP is observed as a function of time.
Extended Data Figure 2 | 3D reconstruction of ATP-bound NSF by single-particle cryo-EM. a, A representative electron micrograph (out of 1,150 micrographs) of ATP-bound NSF particles in vitreous ice. b, Selected 2D class averages (6 out of 50). c, Plots of the angular distribution of particle projections. The radius of the sphere at each projection direction is proportional to the number of particle images assigned to it. Two alternative views are shown, with either the z axis or the y axis pointing out towards the viewer. Two corresponding re-projection images of the final density map are shown under the plots. d, Selected slice views of the final reconstruction. The slice numbers are indicated. e, FSC curves for the 3D density map after RELION post-processing. The resolution is estimated to be 4.2 Å by the gold-standard refinement criterion, as indicated by the red arrow. The FSC curve between the refined atomic model and the 3D density map is shown in blue. f, FSC curves for cross-validation. Black, model versus summed map (full data set); green, model versus half map 1 (not used for test refinement); orange, model versus half map 2 (not used for test refinement). See Methods for details. g, 3D density map coloured according to the local resolution as estimated by ResMap.
Extended Data Figure 3 | Representative densities from the cryo-EM reconstructions of ATP-bound NSF and the 20S supercomplex. a–g, ATP-bound NSF. Density maps were sharpened by XMIPP using a B-factor of $-123\,\text{Å}^2$. a, Representative densities (black mesh, 7.8σ) for an α-helix and a β-strand of the D1 domain with the refined model (coloured sticks) superposed. b, Representative density (black mesh, 7.0σ) of the β-sheet of the α/β subdomain of D1 (chain C) with the refined model (yellow Cα ribbon) superposed. c, Density (black mesh, 7.0σ) and model (yellow Cα ribbon) for the D1 and D2 domains of chain B. Note that the linker between the two domains is well resolved. d, De novo model (yellow cartoon) of the D1 domain built from the cryo-EM density map (black mesh, 7.0σ). The arrangement of the subdomains and nucleotide is illustrated in the inset. The pore loop (YVG motif) and two α-helices: α2 from the α/β subdomain, and α7 from the α subdomain are highlighted in the red dotted boxes. e, Density (black mesh, 6.5σ) and model (yellow Cα ribbon) of the ATP binding pocket of the D1 domain (chain C). Motifs that are typical for AAA+ ATPases are indicated. f, Superposition of the crystal structure of the ATP-bound D2 domain with Mg$^{2+}$ (ref. 19, PDB accession code: 1NSF, coloured sticks and balls), and the cryo-EM density map (black mesh, 7.6σ) of ATP-bound NSF (density of chain C). The crystal structure was docked into the density as a rigid body without any refinement. Note that no Mg$^{2+}$ was present in the samples for cryo-EM studies, but the ATP molecule and the protein coordinates from the crystal structure match the cryo-EM density well. g, Nucleotide-binding sites of the D1 domains from ATP-bound NSF. The density (translucent surface, chains A–E: 8.2σ, chain F: 5.0σ) of each D1 domain is shown together with the built model in ribbon representation. The nucleotide-binding pockets are highlighted by dotted circles. Five out of the six D1 domains show clear density for ATP. h–l, Representative densities (translucent surface, 4.7σ) from the reconstruction for state I of the 20S supercomplex with the model (cartoon) superposed. All densities are representative except for the N domain in panel j, which represents the better-resolved half of the N domain densities (12 out of 24 cases).
Extended Data Figure 4 | 3D reconstruction of ADP-bound NSF by single-particle cryo-EM. a, A representative electron micrograph (out of 840 micrographs) of ADP-bound NSF particles in vitreous ice. b, Selected 2D class averages (6 out of 30). c, Selected focused 2D class averages (5 out of 10). The first image shows the focused classification mask, which locates the flipped N domains. d, Plots of angular distribution of particle projections. The radius of the sphere at each projection direction is proportional to the number of particle images assigned to it. Two alternative views are shown, with either the z axis or the y axis pointing out towards the viewer. Two corresponding re-projection images of the final density map are shown under the plots. e, Selected slice views of the final reconstruction. Slice numbers are indicated. f, FSC curve for the 3D density map after RELION post-processing. The resolution is estimated to be 7.6 Å by the gold-standard refinement criterion. g, 3D density map coloured according to the local resolution as estimated by ResMap.
Extended Data Figure 5 | Detrimental effect of imposing C6 symmetry on the reconstruction of ADP-bound NSF and C3 symmetry on the reconstruction of the 20S supercomplex. a, For the NSF maps, in order to visualize densities of the N domains, an unsharpened map is displayed (translucent surface, C1: 1.2σ, C6: 0.6σ) together with the sharpened map using no symmetry (C1) or C6 symmetry during reconstruction (coloured surface, C1: 5.9σ, C6: 7.0σ). For the reconstruction that uses C6 symmetry, symmetric densities for the N domains at top and side positions appear in the unsharpened map, however, these densities cannot be matched to the crystal structure of the N domain. Likewise, the D1 domains appear compressed and cannot be fit well using the structure of the D1 domain that we obtained by asymmetric reconstruction. b, Reconstruction of state I of the 20S supercomplex without symmetry (C1) or with C3 symmetry. Density maps are shown in coloured surfaces similar to Fig. 3 (C1: 4.7σ, C3: 4.9σ). The C3 averaging causes the D1 domains to display alternating up and down positions. The density for the SNARE complex is a featureless rod without the characteristic left-handed twist of the four α-helix bundle. Densities for only three SNAPs emerge, but without any interpretable features (for example, there are no grooves between helices), preventing a match with the crystal structure of the known homologue of αSNAP, Sec17. The N domain densities are weak and none of them exhibit the expected kidney shape.
Extended Data Figure 6 | Comparison of AAA+ ATPase domains from ATP- and ADP-bound NSF structures. a, Unrolling of the ATPase domains of ATP-bound NSF. Two orthogonal views are shown. Individual chains are aligned based on the D2 domains (white) to show the split-washer arrangement of the D1 domains. b, Unrolling of the ATPase domains of ADP-bound NSF. Individual chains are aligned as in panel a. Dotted boxes in panels a and b highlight the α2 helices of the D1 domains in order to help with visualization of the relative positions. The six protomer chains are rainbow coloured as in Fig. 2. c, Superposition of the six D1 domains of ATP-bound NSF based on the α/β subdomains (white). The relative positions of α7 helices from the α subdomains are illustrated in the inset. d, Corresponding superposition of the ADP-bound NSF D1 domains. e, Superposition of the five D1 domains (without chain F) of ATP-bound NSF (grey), and six D1 domains of ADP-bound NSF (white) based on the α/β subdomains. The α7 helices from the α subdomains are highlighted by red dotted boxes. The relative translation of the α7 helices between the ATP-bound state and the ADP-bound state is shown in the inset.
Extended Data Figure 7 | Comparison of ATP- and ADP-bound NSF structures, and ATPase domains of ATP-bound NSF and 20S supercomplex. 

a–c, Surface representations of the D2, D1 and N domains of ATP- and ADP-bound NSF (looking down from the N-terminal side of the NSF hexamer). The maximum diameters of the D2 and D1 rings, and the interface areas (calculated by PISA®) between ATPase domains are indicated. Each protomer chain is coloured as in Fig. 2. The D1 ring is also shown in panel c and coloured white to help with visualization. 
d–f, The ATPase domains of the structure of the 20S supercomplex (state I) were superposed on the ATP-bound NSF using the D1 ring as the reference for the fit. Six protomer chains from ATP-bound NSF are rainbow coloured as in Fig. 2. The ATPase domains of the 20S supercomplex are colored in white and grey. Note that the density of chain F in the reconstruction of ATP-bound NSF alone is poorly resolved (Fig. 1b), whereas in the 20S reconstruction it is well defined, although the overall resolution of the 20S reconstruction is lower. 

d, Side views. e, Top view of the D2 rings. Each individual D2 domain is labelled. Percentages of interface area change (from NSF to 20S) between the D2 domains are provided in the figure. The interface areas between the D2 domains are similar in the NSF and 20S structures, except for a significant increase (12%) between chains D and E for 20S compared to NSF alone. 
f, Top view of the D1 rings. Each D1 domain is labelled, with the split between chains A and F indicated by a black arrow. The translation of the \( \alpha \) helix in \( \alpha \) subdomain of chain A is illustrated in the inset. Percentages of interface area change (from NSF to 20S) between the D1 domains are shown. Three interfaces stay the same; the one between chains A and B decreases, whereas those between chains E and F, and chains F and A increase significantly.
Extended Data Figure 8 | 3D reconstruction of 20S supercomplex by single-particle cryo-EM. a, A representative electron micrograph (out of 610 micrographs) of the 20S supercomplex in its original purification buffer recorded using the TF20 microscope and the TVIPS TemCam-F816 CMOS camera. The inset shows selected 2D class averages (5 out of 50). b, A representative electron micrograph (out of 2,459 micrographs) of 20S supercomplex in the buffer containing additional 0.05% Nonidet P-40 recorded using the TF30 Polara microscope and the Gatan K2 Summit detector. c-g, Results from this imaging condition. c, Selected 2D class averages (6 out of 50). d, Plots of angular distribution of particle projections. The radius of the sphere at each projection direction is proportional to the number of particle images assigned to it. Two alternative views are shown, with either the z axis or the y axis pointing out towards the viewer. Two corresponding re-projection images of the final density map are shown under the plots. e, Selected slice views of the final reconstruction. Slice numbers are indicated. Slices from different layers are framed in different colours: SNAREs and αSNAPs: yellow, N domains: pink, D1 ring: blue, and D2 ring: purple. f, FSC curves for the 3D density maps of the four states after RELION post-processing. The estimated resolution ranges from 7.6 Å to 8.4 Å as estimated by the gold-standard refinement criterion. g, 3D density map coloured using local resolution estimated by ResMap. The right panel shows a cut-through view of the interior of the map. c-e and g are results from a subclass representing state I.
Extended Data Figure 9 | Purification and 3D reconstruction of V7-20S supercomplex. 

a, Size-exclusion chromatogram of the V7-20S supercomplex. Major peaks are labelled. Only fraction 10 was concentrated and used for single-particle cryo-EM. 
b, SDS–PAGE gel of fractions collected in panel a. The samples were not boiled. 
c, SDS–PAGE gel of the same fractions as in panel b. The samples were boiled. 
d, A representative electron micrograph (out of 993 micrographs) of the V7-20S supercomplex. 
e, Selected 2D class averages (6 out of 50). 
f, FSC curve for the 3D density map after RELION post-processing. The estimated resolution is 8.0 Å as estimated by the gold-standard refinement criterion.
Extended Data Figure 10 | CG-MALS characterization of αSNAP–SNARE subcomplex. a, Concentration gradient setup for the experiment that measures the binding between αSNAP and truncated neuronal SNARE complex. b, Measured molar mass for different components. Note that there were two independent runs for αSNAP over the specified concentration ranges. c, Measured molecular mass of αSNAP–SNARE (truncated) subcomplex converted from light scattering over the concentration gradient. The experimental data are represented by blue dots. Simulated curves with different αSNAP:SNARE (truncated) stoichiometry are shown. The best fit is 4:1. d, Measured molecular mass of the αSNAP-V7-SNARE subcomplex calculated from light scattering over the concentration gradient. The experimental data are represented by blue dots. Simulated curves with different αSNAP-V7-SNARE stoichiometry are shown. The best fit is 2:1. e, Calculated mole fractions of different αSNAP–SNARE (truncated) species over the concentration gradient based on 4:1 stoichiometry. f, Calculated mole fractions of different αSNAP–V7-SNARE species over the concentration gradient based on 2:1 stoichiometry.
## Extended Data Table 1 | 3D reconstructions of NSF and 20S supercomplex by single particle cryo-EM

| NSF (ATP) | NSF (ADP) | 20S | V7-20S |
|-----------|-----------|-----|--------|
| Electron microscope | TF30 Polara | TF30 Polara | TF30 Polara | TF30 Polara |
| Acceleration voltage (kV) | 300 | 300 | 300 | 300 |
| Defocus range (µm) | -1.8 - 2.8 | -1.8 - 2.8 | -1.8 - 2.8 | -1.8 - 2.8 |
| Electron dose (e/Å²) | 44 (26.4) | 44 | 44 | 44 |
| Pixel size (Å) | 2.4312 | 2.4312 | 2.4312 | 2.4312 |
| Particles processed | 89,289 | 30,848 | 116,082 | 65,126 |
| Resolution of unmasked map (Å) | 6.4 | 9.2 | 8.6 | 8.6 |
| Resolution of masked map (Å)† | 4.2 | 7.6 | 7.6 | 7.6 |
| Map sharpening B-factor (Å²)‡ | -129 | -479 | -428 | -428 |

* The accumulated dose of the first 18 frames.
† Resolution of the masked map is estimated from masking-effect-corrected FSC curves.
‡ B-factors automatically determined by RELION.
## Extended Data Table 2 | Statistics of model refinement

| Model composition | NSF (ATP) | NSF (ADP) | 20S supercomplex |
|-------------------|-----------|-----------|------------------|
| Total atoms       | 21,712    | 21,407    | 41,435           |
| Peptide chains    | 6         | 6         | 14               |
| Protein residues  | 2,906     | 2,887     | 5,431            |

| Refinement        |           |           |                  |
|-------------------|-----------|-----------|------------------|
| Unit cell (P1)    |           |           |                  |
| $a = b = c$ (Å)   | 311.2     | 311.2     | 311.2            |
| $\alpha = \beta = \gamma$ (°) | 90     | 90     | 90               |
| Resolution (Å)    | 4.2       | 7.6       | state I 7.6      |
|                   |           |           | state II 7.8     |
|                   |           |           | state IIIa 8.4   |
|                   |           |           | state IIIb 8.2   |

| R.m.s. deviations |           |           |                  |
|-------------------|-----------|-----------|------------------|
| Bond lengths (Å)  | 0.008     | 0.011     | 0.009            |
| Bond angels (°)   | 1.762     | 1.976     | 1.596            |

| Ramachandran plot |           |           |                  |
|-------------------|-----------|-----------|------------------|
| Favored (%)       | 93.0      | 92.8      | 89.6             |
| Outliers (%)      | 2.0       | 2.2       | 2.3              |

| MolProbity        |           |           |                  |
|-------------------|-----------|-----------|------------------|
| Overall score     | 2.62      | 2.54      | 2.54             |
| Rotamer outliers (%) | 1.8   | 0.8       | 0.9              |

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