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Nucleotide-dependent conformational changes in the *N*-Ethylmaleimide Sensitive Factor (NSF) and their potential role in SNARE complex disassembly

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ABSTRACT

Homohexameric, *N*-Ethylmaleimide Sensitive Factor (NSF) disassembles Soluble NSF Attachment Protein Receptor (SNARE) complexes after membrane fusion, an essential step in vesicular trafficking. NSF contains three domains (NSF-N, NSF-D1, and NSF-D2), each contributing to activity. We combined electron microscopic (EM) analysis, analytical ultracentrifugation (AU) and functional mutagenesis to visualize NSF's ATPase cycle. 3D density maps show that NSF-D2 remains stable, whereas NSF-N undergoes large conformational changes. NSF-Ns splay out perpendicular to the ADP-bound hexamer and twist upwards upon ATP binding, producing a more compact structure. These conformations were confirmed by hydrodynamic, AU measurements: NSF-ATP sediments faster with a lower frictional ratio (f/f_0). Hydrodynamic analyses of NSF mutants, with specific functional defects, define the structures underlying these conformational changes. Mapping mutations onto our 3D models allows interpretation of the domain movement and suggests a mechanism for NSF binding to and disassembly of SNARE complexes.

1. Introduction

The *N*-Ethylmaleimide Sensitive Factor (NSF), first purified in 1988 (Block et al., 1988), has been shown to be an essential enzyme required for all membrane trafficking events in a cell. This homohexameric ATPase binds to Soluble NSF Attachment Protein (SNAP)-Receptor (SNARE) complexes and mediates the recycling of spent SNARE complexes for subsequent rounds of membrane fusion (Zhao et al., 2011). As with other ATPases associated with diverse cellular activities (AAA) proteins, NSF couples the hydrolysis of ATP to the conformational modulation of a protein complex, in this case the SNARE complex (Hanson and Whiteheart, 2005). NSF protomers are composed of three domains: NSF-N (1–205); and two AAA domains (NSF-D1, 206–477; NSF-D2, 478–744) (Tagaya et al., 1993). Each uniquely contributes to overall activity. NSF-N is required for SNAP–SNARE binding and positively charged residues at the top of the interface between the N_A and N_B subdomains are important (Nagiec et al., 1995; Zhao et al., 2009). NSF-D1 and -D2 contain the ~230 amino acid motif that is the hallmark of the AAA family. NSF-D1 catalyzes ATP hydrolysis, which is required for SNARE complex disassembly (Whiteheart et al., 1994; Nagiec et al., 1995). Conserved elements of the AAA motif in NSF-D1 (*i.e.* Walker A and B box, Arginine Fingers, Sensor 2) have been shown, by mutagenesis analysis, to be critical for NSF function (Zhao et al., 2009). NSF-D2 is not catalytic, but is required for hexamerization.

NSF binds to the SNARE complex *via* an adaptor protein called α-Soluble NSF Attachment Protein (α-SNAP) (Clary et al., 1990). Three α-SNAPs mediate binding of one NSF hexamer to the SNARE complex (Wimmer et al., 2001). Electron microscopy studies and crystallographic data indicate that SNAP binds along the length of the four-helical, coiled-coils of the SNARE complex in an antiparallel manner, such that its C-terminus is opposite to the SNARE's transmembrane domains (Hanson et al., 1997; Hohl et al., 1998; Furst et al., 2003). Consistently, mutagenesis suggests that the C-terminus of SNAP is important for NSF binding and the penultimate leucine is required to stimulate NSF's ATPase activity and thus SNAP–SNARE complex disassembly (Barnard et al., 1996, 1997; Marz et al., 2003). Under non-hydrolysis conditions, the complex of NSF/SNAP/SNARE resembles a "sparkplug" with the NSF hexamer at the wide end and the transmembrane domains at the other (Hohl et al., 1998; Furst et al., 2003). Based on this

Abbreviations: AAA, ATPase associated with diverse cellular activities; AU, analytical ultracentrifugation; EM, electron microscopy; NSF, *N*-Ethylmaleimide Sensitive Factor; PDB, Protein Data Base; SNAP, Soluble NSF Attachment Protein; SNARE, SNAP Receptor; VCP, Valosin Containing Protein.

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arrangement, one could envision a mechanism by which NSF untwists the SNARE complex to mediate its disassembly.

Full-length NSF has not been crystallized but structures of NSF-N (May et al., 1999; Yu et al., 1999) and NSF-D2 (Lenzen et al., 1998; Yu et al., 1998) are available, as are several EM analyses of NSF (Hanson et al., 1997; Furst et al., 2003) and the NSF–SNAP–SNARE complex (Hohl et al., 1998; Furst et al., 2003). Despite these structural data, it is not clear how NSF mediates SNAP–SNARE complex disassembly. To advance this endeavor, we have characterized the conformational changes of NSF primed to bind the SNAP–SNARE complex and of NSF at the end of the ATPase cycle using single particle analysis and analytical ultracentrifugation of NSF in the ATP- and ADP-bound states, respectively. We have also characterized the conformations of two NSF mutants that are deficient at specific steps in the SNARE disassembly process. Based on these data, we begin to gain insight into how NSF disassembles SNARE complexes.

2. Materials and methods

2.1. Plasmids, mutagenesis, and protein expression

His_6-NSF-myc (1–744) from Chinese Hamster Ovary (CHO) cells was cloned into pQE9 (Qiagen, Valencia, CA). His_6-NSF-D1D2-myc fragment (206–744), and His_6-NSF-D2 fragment (488–744) were inserted into the pQE9 vector. The NSF-L441A and NSF-R67A mutants were created with the QuickChange, site-directed mutagenesis kit (Stratagene, La Jolla, CA) using wild-type His_6-NSF-myc as template. The mutations were confirmed by dideoxy nucleotide sequencing by Davis Sequencing (Davis, CA). NSF wild-type (NSF-WT) or mutants were expressed in the *Escherichia coli* strain, Rosetta DE3 pLacI, and purified according to the published methods (Zhao et al., 2009). The purified proteins were finally dissolved in Buffer A (50 mM HEPES/KOH, pH 7.4, 300 mM KCl, 1 mM $MgCl_2$, 2 mM β-mecaptoethanol, 5% glycerol) containing 0.5 mM ATP and stored at −80 °C. The purity of the proteins was examined on a 12.5% SDS–PAGE gel and stained by Coomassie Blue R-250. NSF or mutants were exchanged to different nucleotide states by Size Exclusion Chromatography (SEC) on a column (0.7 × 30 cm) of Sephadex G-50 (Sigma, St. Louis, MO) at 200 µl/min.

2.2. Electron microscopy and single particle analysis

The samples, in their respective nucleotide-bound states, were incubated with an additional 2 mM AMP-PNP or 2 mM ADP with 2 mM $MgCl_2$ at 0 °C for 30 min, reflecting a 50- to 100-fold excess over the K_d for the two ATP binding nucleotide sites (NSF-D1 and -D2; Matveeva et al., 1997). This ensures that at least 95% of NSF's binding sites are occupied by the indicated nucleotide. All samples were diluted to ~100 µg/ml in 50 mM HEPES/KOH, 50 mM KCl, pH 7.4, immediately prior to application on freshly glow-discharged carbon coated C-flat grids and preserved with a 1% solution of uranyl formate (Ohi et al., 2004). Two Tecnai F20 Twin transmission electron microscopes were used to acquire all datasets. The microscopes were operating at 120 kV, using a dose of 25 $e^-/Å^2$ (ADP, ATP and D1D2), and a nominal underfocus ranging from 0.2 to 2.0 µm. 858 (ATP), 909 (ADP), and 448 (D1D2) images were recorded at a nominal magnification of 62,000× (ATP and ADP) and 50,000× (D1D2) at pixel sizes of 0.137 nm and 0.226 nm, respectively, at the specimen level (Supplementary Fig. 1). Images were recorded with a Tietz F415 4 k × 4 k pixel CCD camera utilizing the Leginon software (Suloway et al., 2005). Experimental data were processed by the Appion software package (Lander et al., 2009). The defoci were estimated using ctffind3 (ATP/ADP) (Mindell and Grigorieff, 2003) and ACE2 (D1D2). The phases were flipped on the whole micrograph. Particles were automatically selected (Roseman, 2004) and extracted at a box size of 160 pixels and binned by a factor of 2 for the final reconstruction. The stacks contained: 113,259 (ATP), 66,846 (ADP) and 57,004 (D1D2) particles. Symmetry-free initial models were created for each dataset independently using Imagic-5 on reference-free aligned 2D class averages (van Heel et al., 1996). To exclude model bias all datasets were also refined against all other starting models, resulting in no change to the final models. A variety of symmetry groups (C1, C3 and C6) were tested during image processing and the resulting maps were in agreement with the final model presented here. Since a C6 point group symmetry was also apparent in reference-free class averages generated by the Xmipp tools: clustering 2D reference-free alignment, and the reference-free maximum likelihood alignment procedure (Scheres et al., 2005a,b; Lander et al., 2009; Sorzano et al., 2010), a C6-pointgroup symmetry was applied to the final refinement cycles. Only particles contributing to the best class averages, as judged by eye, were retained for processing. To further eliminate particles that were not fully assembled, aggregated, or just noise, 3D maximum likelihood, implemented in Xmipp, was applied (Scheres et al., 2008). For each dataset, three models were generated and those that showed clearly distinguishable features, such as the top or bottom view, hexameric shape, or the double stacked rings side views, were used to select the contributing single particles into a new stack for further refinement using standard iterative projection matching in Xmipp and Imagic-5 (van Heel et al., 1996; Sorzano et al., 2004). For the final refinement round only the best 10–40% from each dataset were used (AMP-PNP: 12,978, ADP: 14,192, D1D2: 20,104). This level of particle inclusion is consistent with the percent hexamer measured by AU for each of the NSF preparations (see Table 1). The angular increment started at 14° and was reduced by 2° every second iteration until the models stabilized at 2°. Resolution was assessed by calculating the Fourier Shell Correlation (FSC) at a cutoff of 0.5, which provided values between 16 and 20 Å resolution (Supplementary Fig. 2).

To map amino acids of interest, the D1 domain of NSF was modeled using the I-Tasser webserver (Zhang 2007; Roy and Kucukural, 2010). It generated 5 homology models of the NSF monomer with high confidence values. The best homology model (C-score: −0.06, TM-score 0.71 ± 0.12) was used together with the D2 and N domains of the available X-ray structures of NSF (Lenzen et al., 1998; May et al., 1999). Automatic docking of individual domains was performed using the Chimera software package (Pettersen et al., 2004). In the first step, the available crystal structure of the individual D2 domain was automatically positioned into the density map using the "fit in map" and "search" functions. Five additional copies were then automatically generated according to the 6-fold symmetry. In the second step, the homology model was placed using the same function. The "search" function allows the user to manually scroll through different docking options. Since the highest correlation for this domain was at the same position as the D2 domain, the next best docking solution was chosen. This corresponded to the expected position of the D1 domain. To verify this fitting, two hexamers were docked using the "fit sequence" function in Chimera to minimize overlap between the individual PDBs. These results were very similar to the positions estimated by the docking of individual domains, supporting the docking solution. In this step, the orientation of the D2 hexamer was also tested. The presented orientation of the D2 domain was docked with a correlation coefficient of 8.78 for AMP-PNP- and 8.76 for the ADP-NSF reconstructions; the same hexamer rotated by 180° had values of 8.26 and 8.16, respectively. These data further support the chosen orientation for the D2 domains. The N domains were placed using the same techniques. Finally, to minimize overlaps the "fit sequence" function was again used respecting