Structure
Article

Local and Global Mobility in the ClpA AAA+ Chaperone Detected by Cryo-Electron Microscopy: Functional Connotations

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SUMMARY

The ClpA chaperone combines with the ClpP peptidase to perform targeted proteolysis in the bacterial cytoplasm. ClpA monomer has an N-terminal substrate-binding domain and two AAA+ ATPase domains (D1 and D2). ClpA hexamers stack axially on ClpP heptamers to form the symmetry-mismatched protease. We used cryo-electron microscopy to visualize the ClpA-ATPγS hexamer, in the context of ClpAP complexes. Two segments lining the axial channel show anomalously low density, indicating that these motifs, which have been implicated in substrate translocation, are mobile. We infer that ATP hydrolysis is accompanied by substantial structural changes in the D2 but not the D1 tier. The entire N domain is rendered invisible by large-scale fluctuations. When deletions of 10 and 15 residues were introduced into the linker, N domain mobility was reduced but not eliminated and changes were observed in enzymatic activities. Based on these observations, we present a pseudo-atomic model of ClpAP holoenzyme, a dynamic proteolytic nanomachine.

INTRODUCTION

Protein degradation, a vital function shared by all kingdoms of life, serves in the programmed removal of regulatory factors and to maintain the integrity of the cellular proteome by eliminating defective proteins (Wickner et al., 1999). In the prokaryotic cytoplasm, proteolysis is performed by several related protein complexes, members of the Clp family: ClpAP, ClpXP, ClpYQ (HslUV), Lon, and the membrane-anchored FtsH. Each complex has a distinct range of substrate specificities (Gottesman, 2003). Similar complexes are found in archaea and in the mitochondria and chloroplasts of eukaryotes. They share two fundamental properties. First, as they operate intracellularly, their activity has to be stringently controlled in order to avoid damaging unintended targets. Second, they are processive; once degradation is initiated on a given substrate, it continues until the entire polypeptide chain has been reduced to peptides of seven to eight residues (Choi and Licht, 2005). These functional requirements are met by combining two oligomeric components: a peptidase whose active sites are sequestered inside the complex, thus having access only to cleavage sites that are specifically presented to them; and an unfoldase that recognizes *bona fide* substrates, unfolds them, and feeds them into the peptidase for degradation (Baker and Sauer, 2006; Striebel et al., 2009).

The unfoldases belong to the AAA+ family of mechanoenzymes (Ammelburg et al., 2006; Erzberger and Berger, 2006; Hanson and Whiteheart, 2005; Snider and Houry, 2008). ClpA, a type II enzyme with two AAA+ modules, and ClpX, a type I enzyme with a single AAA+ module, form hexameric rings in the active state; both partner the ClpP peptidase, which consists of two apposing heptameric rings. As the two components mount coaxially, there is a 6:7 symmetry mismatch at their interface (Beuron et al., 1998; Kessel et al., 1995). ClpY/HslU is a hexameric type I unfoldase that pairs with a double hexamer of the peptidase ClpQ/HslV, hence, no symmetry mismatch in this system (Sousa et al., 2000). In Lon, the ATPase and the peptidase are colinear segments of the same polypeptide chain so that there is necessarily no symmetry mismatch in these oligomers (Rotanova et al., 2006).

In terms of overall architecture and other key properties, these proteases resemble the proteasome, a complex that degrades proteins tagged with polyubiquitin chains in the cytoplasm and nucleus of eukaryotic cells, thus playing key roles in numerous processes (Goldberg, 2003). The proteasome also features a 6:7 pseudosymmetry mismatch between its ATPase ring and the peptidase. However, the elaborate subunit composition of its unfoldase component—the 19S regulatory complex—complicates analysis. For this reason, the Clp enzymes whose unfoldases are relatively simple homomeric rings afford valuable model systems for the proteasome.

In the present study, we have focused on the structure of the ClpA unfoldase of *E. coli* (Katayama et al., 1988) in the context of the fully assembled protease. Its subunit has three domains, an α-helical N domain connected by a flexible linker to the D1 and D2 domains that have canonical AAA+ folds (Guo et al., 2002). In the hexamer, D1 and D2 form two stacked ring-like tiers (Beuron et al., 1998; Ishikawa et al., 2004). The D2 tier engages ClpP by means of flexible loops containing a conserved IGL motif (Kim et al., 2001). The N domains undergo large-scale

fluctuations about positions distal to the D1 tier (Ishikawa et al., 2004), on whose surface substrates such as RepA initially bind, and are then unfolded and translocated axially into the degradation chamber of ClpP (Ishikawa et al., 2001; Reid et al., 2001; Weber-Ban et al., 1999). Substrate binding does not suppress N domain mobility (Ishikawa et al., 2004). Loops lining the axial channel of ClpA were identified by crosslinking and mutagenesis experiments as important for this process (Hinnerwisch et al., 2005). Of these, the so-called diaphragm loop in the D2 tier, which contains a conserved GYVG motif, has been proposed to promote substrate translocation by adopting different conformations, depending on the protein's nucleotide state, alternating between an "up" position closer to D1 and a "down" position closer to ClpP (Bohon et al., 2008; Farbman et al., 2008; Hinnerwisch et al., 2005).

The symmetry mismatch and the property that ClpA can bind to one or both ends of ClpP have thwarted attempts to obtain crystal structures for ClpAP. Several structures have been determined for ClpP, all of which are very similar except for the conformation of the N-terminal 17 residues (Yu and Houry, 2007), which are envisaged to be dynamic loops (Bewley et al., 2006; Gribun et al., 2005; Szyk and Maurizi, 2006; Effantin et al., 2010). A crystal structure has also been determined for the ClpA monomer in the ADP-bound state (Guo et al., 2002). Here, we have analyzed ClpAP complexes by cryo-electron microscopy (cryo-EM) and image reconstruction to ~12 Å resolution, and by fitting in known crystal structures, we derived a pseudo-atomic model for the ATP-containing ClpA hexamer. In comparison with a model of the ADP-containing hexamer (Guo et al., 2002), we infer that ATP hydrolysis is accompanied by substantial (20°) rigid-body rotations of the D2 domains, relative to a stable platform of tightly coupled D1 domains. From their diminished density in the cryo-EM density map, the "diaphragm" loops that line the axial channel in the D2 tier are seen to be mobile, a property consistent with an imputed role in substrate translocation. Even greater mobility is exhibited by the distally protruding N domains, and this mobility is reduced but not eliminated by a 15 residue deletion in the D1/N domain linker. Overall, this analysis reveals novel aspects of mobility of likely functional significance in a complex already known to be highly dynamic.

RESULTS

Cryo-EM Reconstruction of ClpP-Bound ClpA Hexamers

At ~500 kDa, ClpA is a relatively small protein complex for cryo-EM analysis and its hollow nature (Beuron et al., 1998) gives it an approximately donut-like appearance when projected in any direction. This property raises the risk of misidentifying axial views as side views and vice versa, particularly for micrographs recorded close to focus. To avoid this difficulty, we analyzed ClpA complexed with ClpP, because side views of ClpAP are readily identifiable as such (Figure 1) and they surely present side views of ClpA (Kessel et al., 1995). With these data, the range of orientations to be searched for each particle when calculating a reconstruction is limited to the angular setting around its symmetry axis and, eventually, a small out-of-plane tilt. Moreover, a complete set of side views affords a full sampling of the 3D Fourier transform and thus a reconstruction that is not compromised by missing data (Roseman et al., 1996; Ortega et al., 2005).

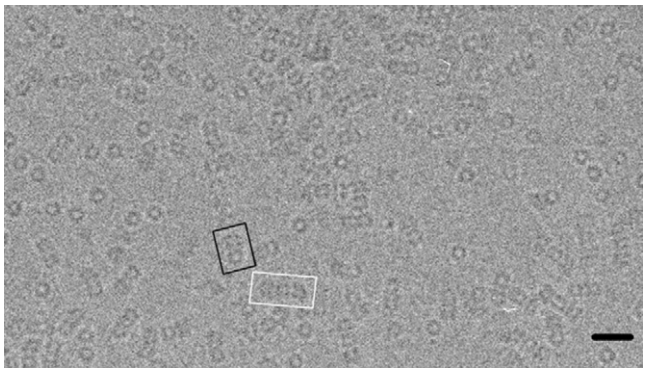

Figure 1. A Field of ClpAP Complexes Visualized by Cryo-EM
Examples of 1:1 and 2:1 complexes are highlighted in black and white, respectively. Scale bar, 300 Å.

ClpA was assembled into hexamers by adding ATPγS, and then mixed with ClpP at a 1:1 molar ratio of ClpA hexamers to ClpP tetradecamers. This condition favors the formation of 1:1 complexes, while some 2:1 complexes are also produced (Figure 1). Cryomicrographs were recorded as focal pairs and the ClpA-containing portions were masked out from side views of 1:1 and 2:1 complexes. These data were combined in the ensuing analysis. In brief, the reconstruction was performed by projection matching, assuming six-fold symmetry. Two quite different starting models (see Experimental Procedures) were used to confirm that the final reconstruction was not biased by the choice of initial template. According to the FSC criterion, the reconstruction, which included 6000 focal pairs, has a resolution of 12.5 Å (see Figure S1 available online).

The density map depicts a three ring structure (Figure 2A). The bottom ring represents ~50% of a ClpP heptamer. This part of ClpP (~70 kDa), which was included in the excised images to ensure that the experimental volume would contain all of ClpA, is too small to subvert angular determination of the ClpA hexamers. As ClpP is heptameric and six-fold symmetry was applied, the representation of this ring is almost perfectly cylindrical. Of the other two rings, the one in contact with ClpP comprises the D2 domains of ClpA and the distal ring its D1 domains. They have the same dimensions and a generally similar appearance as in the reconstruction of Ishikawa et al. (2004); i.e., the D2 ring has a slightly greater diameter than D1 (145 Å versus 130 Å). However, the side ports that were seen between the D1 and D2 tiers when the latter reconstruction was surface rendered are much smaller. With the possible exception of a small density at the axial channel entrance on the apical surface of D1 (Figure 2C, red arrow), the six 16 kDa N domains are invisible, reflecting their high mobility (Ishikawa et al., 2004). This density apart, ClpA has a continuous axial channel that widens into large internal cavities between the D1 and the D2 tiers, and at the interface between D2 and ClpP (the "vestibule") (Figure 2B). However, when the axial region is viewed in grayscale sections (Figures 2C–2E), the channel appears considerably narrower, as some lower but nevertheless significant density is visible (Figure 2C, top red oval). The diameter of