Cryo electron microscopy structures of Hsp100 proteins: crowbars in or out?1

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Abstract: Independent cryo electron microscopy (cryo-EM) studies of the closely related protein disaggregases ClpB and Hsp104 have resulted in two different models of subunit arrangement in the active hexamer. We compare the EM maps and resulting atomic structure fits, discuss their differences, and relate them to published experimental information in an attempt to discriminate between models. In addition, we present some general assessment criteria for low-resolution cryo-EM maps to offer non-structural biologists tools to evaluate these structures.

Key words: Hsp104, ClpB, cryo EM, protein disaggregation, AAA+.

Introduction

In vivo protein aggregation was thought of as irreversible until members of the Hsp100 protein family were found to mediate recovery of proteins from amorphous inclusion bodies and ordered, prion-like aggregates. This group of chaperones, which include yeast Hsp104, bacterial ClpB, and plant Hsp101, appear unique in their ability to reverse aggregation in cooperation with other chaperones (Glover and Lindquist 1998; Goloubinoff et al. 1999; Mogk et al. 1999; Queitsch et al. 2000). They belong to the AAA+ (ATPases associated with various cellular activities) superfamily of ATPases and the driving force for substrate remodelling is provided by tandem AAA+ ATPase domains, which are preceded by an N-terminal domain (Squires and Squires 1992; Schirmer et al. 1996). A coiled-coil propeller insertion between the ATPase domains is essential for disaggregation activity and is not found in other Hsp100 proteins (Lee et al. 2003; Mogk et al. 2003; Schirmer et al. 2004). The location and role of this insertion in the functional hexamer have been studied by cryo electron microscopy (cryo-EM) of ClpB (Lee et al. 2003; Lee et al. 2007) and Hsp104 (Wendler et al. 2007; Wendler et al. 2009), but the results have been controversial. In the ClpB structures, the coiled coil projects outwards from the hexamer and was proposed to have a crowbar-like action, whereas in the Hsp104 structures the coiled coil is intercalated into the wall of the hexamer. In this review, we will analyse the differences between the studies and guide non-structural biologists through criteria for assessing low-resolution cryo-EM maps.

Why is it so difficult to get good EM maps of AAA+ proteins?

The high mobility of AAA+ oligomers makes structural analysis very difficult. Flexibility limits the resolution obtainable by both X-ray crystallography and EM because molecules in different conformations are averaged together, resulting in a loss of structural detail. Despite the growing number of depositions of intermediate-resolution EM maps to the EM data bank (EMDB; http://www.ebi.ac.uk/pdbe/...
emdb), AAA+ maps showing secondary structure information are lacking. To obtain maps of this quality, a large number of identical single particles (~100,000 without imposed symmetry, 10,000 – 20,000 with 6-fold symmetry; Frank 2001, 2002) have to be averaged into a single cryo-EM reconstruction. However, for Hsp104, we found that increasing the number of particles in the dataset from ~4,000 to ~24,000 failed to significantly improve the resolution of the final, symmetrized, three-dimensional (3D) reconstruction (Wendler et al. 2009).

Considering the dynamic nature of Hsp100 complexes, the most likely reason for the limitation in resolution is conformational heterogeneity. Differences in hexamer conformations arise when subunits are exchanged or when individual subunits or domains adopt different conformations within the ring. Some degree of subunit coordination is expected, since both inter- and intra-subunit communication have been found in Hsp104 and ClpB (Schirmer et al. 2001; Hattendorf and Lindquist 2002; Cashikar et al. 2002; Doyle et al. 2007). Our recent work indicates that nucleotide-bound Hsp104 hexamers adopt asymmetric conformations, suggesting a sequential firing mode in the ring (Wendler et al. 2009). Very large datasets (>100,000 particles) and assortment into different structural classes would...
be necessary to obtain an EM map with secondary structure information. Heterogeneity due to ring instability and particle aggregation poses a serious experimental challenge for cryo-EM work on Hsp100 proteins. ClpB is in rapid subunit exchange (Werbeck et al. 2008) and, like Hsp104, is very sensitive to protein, salt, and nucleotide concentrations (Schirmer et al. 1998, 2001; Schlee et al. 2001). One approach is to stabilize the hexamer with a cross-linking reagent such as glutaraldehyde. However, the effects of cross links on the structures of highly mobile AAA+ assemblies have yet to be investigated.

**Assessment of EM maps**

Unlike more established methods for 3D structure determination, cryo-EM techniques are still developing and are not entirely standardized. Nevertheless, there are some basic criteria for the assessment of cryo-EM structures (reviewed in Saibil 2000, 2007; van Heel et al. 2000; Frank 2002). The raw images are two-dimensional (2D) projections of the 3D structures and the aim of single particle analysis is to determine the positions and orientations of the 2D projections as accurately as possible. An important criterion for internal consistency is the resemblance of raw images or class averages showing characteristic views of the ice-embedded complex to re-projections of the 3D reconstruction. When the particle orientation is determined by cross correlation with a known or model 3D reconstruction as reference, there is a risk of model bias (dependence of the outcome on the reference chosen; Shaikh et al. 2003; Grigorieff 2000). For new structures it is therefore important that an independent initial 3D density map is generated. To obtain an initial map (starting model) without prior assumptions, it is necessary to determine the relative angles between different views of the particle. This can be done either by collecting pairs of images at different, known tilt angles (random conical tilt; Radermacher et al. 1987; Radermacher 1988) or by computationally searching pairs of images to find their relative orientations (common lines or angular reconstitution; Crowther et al. 1970; Van Heel 1987). Heterogeneity greatly complicates this analysis and mixed data sets need to be sorted by statistical methods (Penczek et al. 2006; Scheres et al. 2007; Elad et al. 2008) to produce multiple reconstructions of each dataset grouped into subsets with less variation, representing the most frequently occurring states or assemblies.

An initial judgment about the resolution of single-particle 3D reconstructions can be made from the overall appearance of density. Tubular densities represent α-helices and indicate better than 9–10 Å resolution, whereas smooth surfaces...
characterize domains and subunits with worse than 10 Å resolution. Currently, most AAA+ cryo-EM maps are resolved in the 10–20 Å range, allowing for the fitting of rigid body domains of atomic structures, but not for the analysis of subdomain or secondary structure movement. A general criterion for atomic structure fitting is that the EM map should account for all known subunit domains and that there should not be significant unfilled density or clashes after fitting. In this context, it is important to show cut open views of the fitted maps, so that the fit for internal density becomes visible. The interpretation of low-resolution EM reconstructions by atomic structure fitting usually generates a working hypothesis of the assembly requiring validation by other means.

**Comparison of ClpB and Hsp104 cryo-EM maps**

ClpB and Hsp104 are orthologs and share ~45% overall identity in protein sequence with up to 60% identity in the strongly conserved AAA+ domains. Their functional characteristics have been extensively reviewed (Weibezahn et al. 2004; Bösl et al. 2006; Mogk et al. 2008; Doyle and Wickner 2009). Here we address the discrepancies between the published Hsp104 and ClpB cryo-EM reconstructions.

Each Hsp100 protein has been analysed assuming 6-fold symmetry in 4 different nucleotide states (Fig. 1). The hexameric structure of the sensor 1 mutant protein Hsp104ΔN from *Saccharomyces cerevisiae*, which can bind but not hydrolyse ATP in the C-terminal AAA+ domain, has been determined in the presence of ADP, ATP, and ATPyS (Wendler et al. 2009). In addition, an N-terminal Hsp104 deletion mutant with wild type AAA+ domains has been reconstructed in the presence of ATPyS (Wendler et al. 2007). The structure of *Thermus thermophilus* ClpB has been determined in its apo state and in the presence of AMPPNP and ADP (Lee et al. 2007). Furthermore, the structure of the double Walker B mutant (DWB; E279A/E678A), that can bind but not hydrolyse ATP in both AAA+ domains, was determined in the presence of ATP (Lee et al. 2007). The resolution of the cryo-EM maps is in the range 11–17 Å and none show secondary structural features. The ClpB complexes were stabilized with 0.01% glutaraldehyde, whereas the Hsp104 complexes were not cross linked. The number of images used to determine the 6-fold symmetric reconstructions range between 8200 and 16 200 for ClpB, and between 2000 and 7000 for Hsp104. For the Hsp104 structures, an independent starting model was generated for each assembly by angular reconstitution, whereas the first ClpB–AMPPNP map was used as a starting model for all subsequent structures (Lee et al. 2007). The subunit orientation in the EM maps was based on a comparison between a full length and an N-terminal deletion variant in the case of Hsp104 (Wendler et al. 2007) and on the protrusions that are thought to account for part of the coiled coil in the ClpB reconstructions (Lee et al. 2003).

In all maps, the tandem AAA+ domains feature a double-tier, hexameric complex. The N termini are not visible in the ClpB maps, but are present as a smaller ring on top of the NDB1 (nucleotide binding domain 1) ring in the Hsp104 maps. In different nucleotide states, the ClpB complex appears to differ mainly in the length of the radially extending...
spikes, whereas the relative positions and orientations of the AAA+ domains remain almost identical (Fig. 2A). In contrast, the presence of different nucleotides and particularly ATP binding and hydrolysis in NBD1 produce large domain rotations in Hsp104 (Fig. 2B). Unlike the AAA+ arrangement in the double layer of another tandem AAA+ protein, p97 (Huyton et al. 2003; DeLaBarre and Brunger 2003), which positions NBD1 directly above NBD2, Hsp104 and ClpB cryo-EM structures show similar offsets between the AAA+ domains (Fig. 1; Wendler et al. 2007; Lee et al. 2003).

The ClpB DWB complex should trap ATP in both AAA+ domains and thus is in a state comparable with Hsp104 in the presence of ATPγS (Fig. 3). Intriguingly, the height of the AAA+ double layer and the outer diameter of NBD2 are very similar in both Hsp100 maps. However, a striking difference is observed in the first AAA+ layer, which is narrower and exhibits extended spikes in the ClpB map. When the maps are overlaid, it becomes obvious that the density distribution in the ClpB NDB1 layer is very different from that in Hsp104 ATPγS (Fig. 3A). Adjusting the rendering threshold so that only the core densities of the domains remain visible confirms that the two maps have a similar distribution of density in NBD2, but differ in NBD1 (Fig. 3B). Comparison of the EM maps with the X-ray structure of the p97 hexamer reveals that the diameter of the ring and the distribution of the core densities in NBD2 is not compatible with the p97 hexameric subunit packing (Figs. 3C and 3D). The NBD2 AAA+ domain would be located mainly...

Fig. 5. Visualization of engineered sulfhydryl cross links in ClpB and Hsp104 structures. (A) ClpB X-ray structure (1QVR_A) as described in Lee et al. (2003). NBD1 proximal and distal wings of the coiled-coil propeller domain are indicated. (B) Subunit conformation in the Hsp104 homology structure derived from atomic structure fitting into the Hsp104 cryo-EM map (Wendler et al. 2007). (C) Enlarged view of the NBD1 – coiled-coil interface in the Thermus thermophilus ClpB X-ray structure. Residues that were mutated to cysteine in the study of Lee et al. (2003) are drawn as spheres. The closest approach of the cysteine pairs is given in Angstroms (Å) in the inset. The R355C–E520C crosslinked state has 60% of the wild-type chaperone activity and the other crosslink pairs have 20% of the wild-type activity. (D) Enlarged view of the NBD1 – coiled-coil interface in the Hsp104 homology model. Residues homologous to those described in C are drawn as spheres and distances of the closest atoms of each pair are given in Angstroms in the inset. (E) Enlarged view of the NBD1 – coiled-coil interface in the Escherichia coli ClpB homology model generated with SWISS-MODEL (Schwede et al. 2003). Residues that were mutated to cysteine in the study of Haslberger et al. (2007) are drawn as spheres. The closest approach of the cysteine pairs is given in Angstroms in the inset. (F) Enlarged view of the NBD1 – coiled-coil interface in the HSP104 homology model. Residues homologous to those described in E are drawn as spheres and distances of the closest atoms of each pair are given in Angstroms in the inset.
outside the EM density in both maps if the hexamers are formed with p97-like packing.

**Fitting and biochemical validation of the maps**

Atomic structure fitting has been undertaken for both Hsp100 assemblies (Fig. 4). For ClpB, a model of the hexameric structure was obtained by rigid body fitting of 4 fragments of the crystal structure: the large NBD1 domain, the small NBD1 domain, the coiled-coil domain, and NBD2 (Lee et al. 2007). The homology model of Hsp104 was also fitted as 4 rigid bodies: the N-terminal domain, NBD1, the coiled coil, and NBD2. However, the overall fit for Hsp104 differs considerably from the packing in the ClpB crystal structure, whereas the fit for ClpB is strongly based on the domain arrangement of the p97 hexamer. The current resolution of the maps is insufficient to distinguish between structural differences that arise from species differences and those that arise from varying interpretations or processing of the EM data. It also reduces reliability of atomic structure fitting, so that additional experimental information is required to discriminate between the different structural models.

The placement of the coiled-coil domain is the most striking difference between the fits and has therefore been a target of biochemical validation (Figs. 5A and 5B). Lee and colleagues (Lee et al. 2003) found that immobilizing the coiled coil in *T. thermophilus* ClpB via engineered sulfhydryl cross links between NBD1 and coiled-coil residues results in 40%–80% less reactivation of heat-aggregated glucose-6-phosphate dehydrogenase (Fig. 5C). An even stronger effect was observed when mutating leucine residues at the interface between the NBD1 and coiled-coil domain. Haslberger and colleagues (Haslberger et al. 2007) designed cross links between the coiled coil and NBD1 that confirm the proximity of the domains, but also suggest conformational flexibility in the NBD1-associated helix wing because the relevant residues in the ClpB X-ray structure are too far apart to form the observed disulfide bridges (Fig. 5E). In the Hsp104 structure, the proximity between the coiled coil and NBD1 is maintained (Figs. 5D and 5F), and therefore the choice of cross links does not effectively discriminate between models. The more shielded position of the coiled coil in the Hsp104 model is supported by the observations that the domain is less accessible to several monoclonal antibodies in the hexameric than in the monomeric form (Wendler et al. 2009). Furthermore, it was found that point mutations in the NBD1 distal wing of the coiled coil largely abolish ATP hydrolysis in Hsp104 (Wendler et al. 2007) and thus support a close interaction between this domain and the AAA+ domains as suggested by the Hsp104 ATPyS EM structure. Point mutations in the NBD1 proximal wing of the coiled coil severely affect thermostolerance, disaggregation, and ATPase activity (Lee et al. 2005; Schirmer et al. 2004; Haslberger et al. 2007). In Hsp101, the plant homolog of Hsp104/ClpB, these effects can be suppressed by mutations in the cavity-facing surface of NBD1 (Lee et al. 2005), providing indirect support for an interaction between the coiled-coil domain and NBD1.

Finally, the ClpB reconstructions in the presence of different nucleotides show essentially the same domain arrangement and do not explain how ATP binding and hydrolysis is translated into protein disaggregation activity by Hsp100 oligomers (Fig. 2A). In contrast, the observed domain movements between different Hsp104 states suggest a peristaltic translocation activity in the hexamer (Fig. 2B).

**Conclusions**

Given the resolution of the EM maps and the considerable difference between the domain arrangement in the ClpB crystal structure and the Hsp104 cryo-EM reconstruction, the fitting of atomic structures into the EM map is far from definitive. It has been shown that Hsp100 proteins are flexible and change conformation during the ATP cycle (Haslberger et al. 2007; Wendler et al. 2009). Thus, assuming only one set of rigid atomic coordinates for the entire range of movements accomplished by these molecular machines is not sufficient to solve the structural mysteries underlying protein disaggregation. It will be a challenge to identify the dynamic structural changes in more detail and to combine all available data into a refined mechanistic model.

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**References**


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