Crystal structure of Lon protease: molecular architecture of gated entry to a sequestered degradation chamber

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Lon proteases are distributed in all kingdoms of life and are required for survival of cells under stress. Lon is a tandem fusion of an AAA+ molecular chaperone and a protease with a serine-lysine catalytic dyad. We report the 2.0-Å resolution crystal structure of *Thermococcus onnurineus* NA1 Lon (TonLon). The structure is a three-tiered hexagonal cylinder with a large sequestered chamber accessible through an axial channel. Conserved loops extending from the AAA+ domain combine with an insertion domain containing the membrane anchor to form an apical domain that serves as a gate governing substrate access to an internal unfolding and degradation chamber. Alternating AAA+ domains are in tight- and weak-binding nucleotide states with different domain orientations and intersubunit contacts, reflecting intramolecular dynamics during ATP-driven protein unfolding and translocation. The bowl-shaped proteolytic chamber is contiguous with the chaperone chamber allowing internalized proteins direct access to the proteolytic sites without further gating restrictions.

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Introduction

ATP-dependent proteases have important functions in cellular and metabolic regulation as well as in protein quality control (Goldberg and John, 1976; Hershko and Ciechanover, 1992; Gottesman, 2003; Sauer *et al.*, 2004; Maupin-Furlow *et al.*, 2005). ATP-dependent proteases are bifunctional enzymes consisting of protein unfolding chaperones coupled to one of several families of broad specificity proteases. Proteasomes, HslUV, and Clp proteases are assembled from separate chaperone and protease components, and, to avoid unregulated damage to cellular proteins, the proteases are assembled into rings of six or seven subunits stacked face to face with the active sites inside and almost totally inaccessible to proteins in the surrounding medium (Lowe *et al.*, 1995; Bochtler *et al.*, 1997; Groll *et al.*, 1997; Wang *et al.*, 1997; Baumeister *et al.*, 1998). The chaperone components recognize specific features in protein substrates and catalyse ATP-dependent unfolding and translocation of the substrate to the proteolytic chamber through designed access channels (Gottesman *et al.*, 1998; Weber-Ban *et al.*, 1999; Kim *et al.*, 2000; Singh *et al.*, 2000; Flynn *et al.*, 2003; Martin *et al.*, 2008). Regulated gating of protein translocation occurs within the chaperone rings and at a constricted entrance to the proteolytic chamber.

The Lon and FtsH families of ATP-dependent proteases function in the same way, but the chaperone and protease components are encoded in tandem in a single subunit. As with other ATP-dependent protease families, the functional enzyme is formed by homotropic association of the ATPase and protease domains into six- or seven-membered rings (Stahlberg *et al.*, 1999; Park *et al.*, 2006). The crystal structure of hexameric FtsH (Bieniossek *et al.*, 2006; Suno *et al.*, 2006) reveals a sequestered degradation chamber encapsulated by the chaperone and protease rings; however, crystal structures of isolated domains and small fragments of Lon (Im *et al.*, 2004; Botos *et al.*, 2004a,b; Li *et al.*, 2005) produced a speculative model in which the proteolytic sites of the hexamer were exposed and accessible from the medium, suggesting that Lon might not have a sequestered degradation chamber or that, to form such a chamber, two hexamers would need to interact to enclose the active sites. Our study establishes that the active sites in Lon hexamers are oriented away from the medium and are sequestered within an aqueous chamber.

Orthologues of Lon are divided into two subgroups (Rotanova *et al.*, 2006): A type (A-Lons), which have a large multi-lobed N-terminal domain together with the ATPase and protease domains, and B type (B-Lons), which lack an N domain, but have a membrane-anchoring region emerging from the ATPase domain. B-Lons are found in Archaea, in which they are the lone membrane-anchored ATP-dependent protease. As Archaea lack FtsH and the Clp proteases, a

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B-Lon together with a cytosolic PAN/proteasome complex carry out all the ATP-dependent proteolysis in those cells. The soluble A-Lons are found in all bacteria and in eukaryotic cell organelles, such as mitochondria and peroxisomes, and are needed for recovery from various stress conditions (Mizusawa and Gottesman, 1983; Suzuki *et al*, 1994; Ngo and Davies, 2007). Lon proteases degrade multiple regulatory and physiological targets including the cell division inhibitor, SulA, in enteric bacteria (Mizusawa and Gottesman, 1983) and the steroid acute regulatory protein in human mitochondria (Granot *et al*, 2007), and they have been identified as virulence factors in pathogenic bacteria (Ingmer and Brondsted, 2009). Deficiencies in mammalian Lon, which targets oxidatively damaged proteins, are correlated with premature ageing (Ngo and Davies, 2007). Here, we report the first structure of an intact, assembled Lon protease. *TonLon* has an enclosed degradation chamber that is contiguous with the chamber formed by the chaperone domains. Two conserved axial loops combine with a unique insertion domain, which also contains the membrane anchor, to form a gated portal through which substrates enter the protein-processing chamber and are then exposed to the proteolytic sites. We propose that this structure serves as a model for all Lon proteases.

Results

Crystal structure of the *TonLon* hexamer

TonLon is a 635-residue protein belonging to the B-Lon family. For crystallization and biochemical studies, we generated a soluble form of *TonLon* by deleting the putative membrane-anchoring region (residues 134–170). The membrane anchor is found within a larger domain of ~110 residues inserted after the Walker-A helix in the AAA+ domain. We refer to this inserted domain as Ins1 (Supplementary Figure S1). The purified protein was enzymatically active for peptide cleavage and both ATP-dependent

and -independent protein degradation (Supplementary Figure S2B; Supplementary Table I). To avoid self-degrading activity during crystallization, we introduced two point mutations (Ser523Ala and Lys566Ala) that abolished proteolytic activity, but had little effect on ATPase activity (Supplementary Table I). Hereafter, references to the *TonLon* structure apply to the mutant with both the deletion and the point mutations.

Crystals of *TonLon* were grown in the presence of ATP (An *et al*, 2010). The crystals were of a hexagonal space group in which a molecular symmetry axis coincided with the crystallographic six-fold screw axis (Figure 1A). The asymmetric unit contained two monomers, one with a tightly bound ADP (T-monomer) and one with a loosely bound ADP (L-monomer), indicating that the *TonLon* hexamer in the crystals is a trimer of dimers. The ADP was most likely generated by hydrolysis of ATP during crystallization. Three such T-L heterodimers make up the hexamer (Figure 1B). The hexamer has overall dimensions of ~110 Å in height and ~120 Å at its widest. The subunits are integrated in a parallel to form a three-layered container with the protease ring covered by the AAA+ ring, which is overlaid with a domain composed of Ins1 and two smaller insertion domains, Ins2 and Ins3, that emerge from the AAA+ domain (Figures 1A and 2). The topography of the inserts corresponds to those found in clade 3 of AAA+ proteins (Iyer *et al*, 2004). In the native enzyme, Ins1 contains the two tandem transmembrane helices. In the structure, the residues flanking the deletion appear at the apex of an α - β turn located on the apical surface near the outer edge of the hexamer (Figure 1A), placing the anchors in a position to tether *TonLon* with the apical surface facing the cell membrane.

Gated access to the internal chambers of *TonLon*

A channel passes all the way through the molecule along the symmetry axis. From the narrow entrance on the apical surface, the channel widens towards the middle, forming

Figure 1 Overall structure of *TonLon*. (A) The hexameric structure of *TonLon*. The hexamer is shown with five subunits in surface representation and one monomer as a ribbon diagram. The molecule is oriented with the protease domain (P) (blue and lavender ribbons) at the bottom, the AAA+ domain (A) (bright and light green ribbons) in the middle, and the apical insertion domain (I) (orange and magenta ribbons) at the top. The orange ribbon depicts Insert 1, to which the membrane anchor (missing in our structure) is attached at the top. MA is the putative membrane-anchoring region. ADP is represented as a stick figure bound between the α/β (bright green) and α (light green) subdomains, and lavender balls indicate the positions of the protease catalytic residues. The other five monomers are in parallel alignment with T- and L-monomers in yellow and magenta tints, respectively. (B) Surface representation showing the top view of the hexamer, which has pseudo-six-fold symmetry. ADPs are shown in red. In subsequent figures, 'T' (yellow) and 'L' (cyan) represent T- and L-monomers, respectively.

Figure 2 Domain structure of *TonL*on. **(A)** Ribbon diagram showing the ATPase domain of a T-monomer. The α/β - and the α -helical subdomains are coloured in green and metallic green, respectively. Ins1, Ins2, and Ins3, which branch out of the α/β subdomain, are coloured in orange, magenta, and pink, respectively. MA is the putative membrane-anchoring region. Dots represent disordered regions in the final model. All residue numbers correspond to the sequence position in the full-length protein. The arginine finger (R311) is shown in stick. Important residues in the Walker-A (D245 and E246), Walker-B (K73), sensor-1 (N297), and sensor-2 (R379) motifs are shown in sticks and labelled. **(B)** Ribbon diagram showing the protease domain of T-monomers. The S-subdomain and the K subdomain, with catalytic K566, are labelled and are coloured in light blue and dark blue, respectively. A red ball indicates conserved glycine residue (G441 in *TonL*on and G596 in *EcL*on). The linker helix between the ATPase and protease domains is coloured yellow. Catalytic residues, S523 and K566, are shown as balls and labelled.

Figure 3 Clipped view of *TonL*on. **(A)** Ribbon drawing of a vertical section through the centre of the hexamer. Joining the AAA + (I + A) and protease (P) domains forms a broad chamber with wide channels leading from the portal and to the exit pore. The portal opening is determined by two loops, F216 (red), in the centre of the aromatic-hydrophobic loop, and M275 (green), in the centre of the pre-sensor-1 β hairpin, are displaced downwards on the vertical axis in the T-monomers. **(B)** Surface rendering of a similar vertical slab. Red arrows point to the substrate-binding grooves in the protease layer, which are accessible to proteins from the chaperone portion of the chamber. **(C)** Top view of the hexamer in a ribbon drawing. Red and green spheres indicate C α atoms of F216 and M275, respectively. These residues are closer to the vertical axis in the T-monomers.

Figure 4 Sequence alignment between α/β subdomains of *TonLon* and *EcLon*. The secondary structure assignments correspond to *TonLon*. A red arrow indicates the position of Ins1 (residues 85–189), which is shown in full in Supplementary Figure S1. Red and black open boxes frame Ins2 and Ins3, respectively. The Walker-A, Walker-B, and sensor-1 motifs are framed by blue, green, and yellow boxes, respectively. The arginine finger (R311) is shaded in pink. Asterisks indicate identical residues between *TonLon* and *EcLon*. The aromatic-hydrophobic loop motif of *EcLon* (Martin *et al*, 2008) is in bold letters.

a cylindrical chamber, and becomes narrower again as it passes through the last portion of the protease ring (Figure 3A and B). By analogy with other ATP-dependent proteases, the channel opening in the apical domain likely serves as a gateway for substrate entry and will be referred to as the portal. The portal is formed by flexible loops contributed by Ins2 and Ins3.

Phe216 in the centre of Ins2 has a disordered side chain and frames the circumference of the portal (Figure 3A and C). Ins2 corresponds to the aromatic-hydrophobic loop of *EcLon*, which has a tyrosine residue in the central position, but otherwise shows no other sequence similarity to the loop in *TonLon* (Figure 4). The aromatic-hydrophobic loop, commonly observed in the AAA+ protein family, has been proposed to have a critical function in driving substrate unfolding and translocation, with the aromatic residue interacting with the protein substrate and enabling movement of the loop to impart an unfolding or translocating force (Martin *et al*, 2008). Consistent with this function, a Phe216Ala mutant of *TonLon* lost almost all of the ATP-dependent proteolytic activity against α casein and the aromatic peptide, Glt-AAF-MNA (Supplementary Figure 2A and B). *EcLon* recognizes sequences rich in aromatic residues (Gur and Sauer, 2008), and the presence of the aromatic residue at the entrance of the portal in *TonLon* might enable it to interact with similar sequence motifs in its substrates. *TonLon* degrades several known substrates of *EcLon*, including UmuD, Arc-SulA, and Lambda N-GFP fusion, in an ATP-dependent manner (Supplementary Figure 2C); however, a detailed analysis of the substrate specificity has not been conducted.

The narrowest constriction in the portal is defined by Ins3 with Met275 in the centre position (Figure 3C). The proteolytic activity of an Met275Ala mutant is comparable with that of wild-type *TonLon* (Supplementary Table I), indicating that Met275 is not required for substrate interaction. How this loop participates in substrate gating and whether specific residues in the loop interact with substrates remain to be determined. There is virtually no constriction of the chamber between the chaperone domain and the protease active sites, in contrast to the multiple-component ATP-dependent proteases, such as ClpP and the proteasomes, in which a narrow channel into the proteolytic chamber restricts access to one or two polypeptide chains in extended conformations. In *TonLon*, substrate polypeptides that have passed through the portal into the chaperone chamber would have direct access to the proteolytic active sites.

Nucleotide-dependent asymmetry within the *TonLon* hexamer leads to oscillations in the portal

Unequal binding of ADP to the alternating T- and L-monomers correlates with a dramatic difference in the orientations of the AAA+ domains relative to their respective protease domains and the molecular symmetry axis (Figure 5). In contrast, the protease domains are arranged with close to perfect six-fold symmetry, with only slight ($1\text{--}2^\circ$) differences in the rotational alignment of alternating domains, and the small α -helical subdomains display a similar pseudo-six-fold symmetry. The α/β subdomains of the T- and L-monomers are shifted and rotated $8\text{--}9^\circ$ relative to each other (Figure 5). Superposition of the protease domains of the T- and L-monomers reveals that rotation occurs as a rigid body movement about a point near Thr323 in strand 13, one of the pair of β strands connecting the α/β and the α subdomains (Figure 5). As a result, the entire α/β subdomain and apical insertion domain of the T-monomer move in towards the molecular symmetry axis and down towards the protease domains, constricting the opening of the portal. The minimum width of the portal is determined by the positions of the Ins1 and Ins2 loops extending from the T-monomers. The diameter of the circle circumscribing the α carbons of Phe216 in the T-monomers is $\sim 13\text{ \AA}$, whereas it expands to $\sim 28\text{ \AA}$ in the L-monomers (Figure 3C), which are rotated up and away from the molecular symmetry axis. Similarly, in the plane of the α carbons of Met275, the portal width is $\sim 8.5\text{ \AA}$ in the T-monomers, but grows to $\sim 17\text{ \AA}$ in the L-monomers (Figure 3C). Transitions between the T and L states upon nucleotide exchange would cause the diameter and shape of the portal to fluctuate and importantly would bring the individual axial loops into contact with different parts of the substrate, influencing protein binding and release and the resultant structural perturbations imparted on the bound protein. Another consequence of the nucleotide-dependent change in position of the α/β subdomain is that the Ins2 and Ins3 loops are displaced by $12\text{--}15\text{ \AA}$ down the axis of the channel (Figure 3A). Such movement along the axis would provide a mechanism for active translocation of bound proteins into the channel and towards the proteolytic sites.

Examination of the crystal packing reveals that interactions among symmetry-related molecules in the crystals are made between AAA+ domains of T-monomers and protease domains of L-monomers, which allows the possibility that the characteristic conformation of T-monomers could be influenced by packing interactions with the robust

Figure 5 C α -tracing of superimposed T- and L-monomers. A T-monomer is superimposed onto an L-monomer in the hexamer, which is represented by a transparent surface. The protease domains were optimally aligned to emphasize the relative change in position of the AAA + domain in the T-monomer, which is displaced down and in towards the molecular symmetry axis. The boxed region on the top right is the close-up view of the ATP-binding sites in the two monomers. The boxed region on the bottom right shows the pivot point near T323 about which the α/β subdomain rotates in the transition between the T and L states.

protease domains. Remarkably, the crystal structure of the isolated AAA + domain with a tightly bound ADP (unpublished data), which was used for the structure determination of *TonLon* (see 'Materials and methods'), is virtually identical to that of T-monomers with RMSD of 0.6 Å for all atoms. The identical conformation of the two AAA + domains under different crystal packing environments strongly suggests that the observed conformation of T-monomers is not a crystallographic artefact.

Structural basis of tight and weak binding of ADP

ADP is bound at the interface between the α/β and α subdomains and is positioned to interact with highly conserved residues that constitute functional motifs in the majority of AAA + proteins. The large α/β subdomain (residues 1–321) with its central five-stranded parallel β sheet sandwiched by α -helices has three highly conserved motifs: Walker A, which interacts with the β and γ phosphates of ATP; Walker B, which activates a water molecule that attacks the β – γ phosphodiester bond of ATP; and sensor-1, which communicates with the γ phosphate and is used to discriminate between ATP and ADP (Figures 2A and 4). The α/β subdomain also has an arginine-finger residue (Arg311), which is required for activation of ATPase activity upon assembly. These residues are positioned to interact with the tightly bound ADP in three of the subunits (Supplementary Figure S4). Mutations in Walker-A or Walker-B residues or in the arginine-finger residue caused a drastic decrease in ATPase activity and ATP-dependent proteolytic activity (Supplementary Table I). The smaller mostly α -helical subdomain (residues 322–412) contains the sensor-2 motif, Arg379 (Figure 4), which is also positioned to interact with bound nucleotide. The sensor-2 motif also serves to help discriminate among nucleotide states.

The ADP situated at the subdomain interface in the T-monomer (T-ATPase domain) (Figure 2A) is tightly bound

and shows strong electron density (Supplementary Figure S3). In contrast, the electron density of ADP in the L-ATPase domain is weak (Supplementary Figure S4), and the B factor of ADP is much higher there (48.9 Å^2) than in the T-ATPase domain (22.1 Å^2), implying lower occupancy and/or weaker binding. Earlier studies with *EcLon* had suggested the existence of high- and low-affinity states for the nucleotide-binding sites in Lon oligomers (Menon and Goldberg, 1987a; Vineyard *et al*, 2005; 2006). To confirm that ATP and ADP bind unequally to different subunits of *TonLon* in solution, we measured binding of ATP γ S and ADP by isothermal titration calorimetry. ATP γ S bound tightly to *TonLon* ($K_d \sim 2 \mu\text{M}$) and the stoichiometry of binding was 3.2 ± 0.04 per hexamer, and ADP bound even tighter ($K_d < 1 \mu\text{M}$) with a stoichiometry of 2.7 ± 0.03 (Supplementary Figure S2D). To evaluate the binding of ATP under assay conditions, we measured the ATP concentration dependence of peptide cleavage by *TonLon*. The ATP titration curve for activation of peptidase activity was sigmoidal followed by a very steep slope, which when fit to the Hill's equation gave a Hill's coefficient of 3.4 (Supplementary Figure S2E). These data suggest that unequal binding of ADP to adjacent subunits observed in the crystal reflects an intrinsic property of *TonLon*.

In the subunits with tightly bound ADP, the base is stacked between Met75 in the α/β subdomain and Leu378 in the α -helical subdomain, whereas N1, N3, and NH2 at C-6 make hydrogen-bonding interactions and the 2'-OH of ribose interacts with Glu342 at the entrance of the pocket (Supplementary Figure S2). The diphosphate, nestled between the N-terminal end of helix-4 and the Walker-A motif, makes multiple-polar interactions with the Walker-B and sensor-1 motifs in the α/β subdomain and the sensor-2 motif in the α -helical subdomain. Notably, the only interaction of ADP with a neighbouring L-monomer (L-ATPase domain) is the contact between the 3'-OH of the ribose and

Figure 6 A close-up view of the TL interface. (A) At the interface between AAA+ domains, a modelled ATP is in stick representation and veiled by a transparent surface. A curved red arrow represents modelled rotation of the side chain of R311 upon ATP binding. Important residues in the Walker-B, sensor-1, and sensor-2 motifs are represented as sticks. Dotted lines point out interatomic distances <3 Å indicative of bonding. (B) The regions surrounding the linker helices of T- and L-monomers (yellow and cyan ribbons, respectively) are shown. Purple sticks represent residues from the adjacent L-monomer that come within 3 Å of the T-monomer linker. Residues within the protease domain that are displaced towards the T-monomer linker of the same subunit are shown as sticks. Linker residues whose side chains adopt different orientations in the two monomers are labelled green.

Tyr315 (Figure 5; Supplementary Figure S4). When the T- and L-monomers are superimposed, the rotation of the α/β subdomain is seen to produce a shift of ~ 3 Å in helix-4 including the Walker-A motif (Figure 5). The change in position of Met75 in helix-4 disturbs its interactions with the adenine ring, and the accompanying change in position of the ADP leads to the loss of the interactions between the ribose and Tyr315 of the adjacent subunit and between the α phosphate and the sensor-2 residue, Arg379. Although ADP is more exposed to the medium in the L site, it appears that a conformational change would be required to allow dissociation of ADP from the T site, consistent with experiments showing that protein substrate binding is required to dislodge tightly bound ADP in *EcLon* protease (Menon and Goldberg, 1987b).

The docking of alternating T-ATPase and L-ATPase domains (Figure 3A) produces two different interfaces (Figure 1B). We refer to the interface with a T site and tightly bound ADP as the LT interface, and the one with an L site and weakly bound ADP as the TL interface. At the LT interface, 3240 Å^2 of solvent-accessible area is buried between the ATPase domains, and slightly less, 2730 Å^2 , is buried at the TL interface, suggesting that the TL interface is looser and more mobile. The differences in the interfaces would be expected to also affect the relative ATPase activity at the respective sites as a consequence of the altered position of an arginine-finger residue, Arg311 (Figure 2A), which is brought closer to or further from the nucleotide in the adjacent active site by a rotation in helix-13 (Figure 6A). Arginine-finger residues are common in oligomeric AAA+ proteins. They are brought into the active site of adjacent subunits upon assembly and activate ATPase activity by stabilizing negative charge accumulating at the γ phosphate in the transition state (Ogura *et al*, 2004). A rotation of the side chain of Arg311 brings it into position to interact with the γ phosphate of the ATP in the L-ATPase site, as we found by modelling ATP into the site (Figure 6A). Mutating Arg311 to alanine led to a significant impairment of *TonLon* protease and ATPase activity (Supplementary Table I), which supports the conclusion that this residue acts as an arginine finger.

Structure in the protease domain: formation of an enclosed proteolytic chamber

TonLon and *EcLon* differ in the linker between the protease and ATPase domains, which is a 16-residue helix in *TonLon* and an 11-residue coil in *EcLon* (Figures 2B and 4). A U turn of 180° after helix-18 (Supplementary Figure S4) orients the protease domain of *TonLon* so that the catalytic site opens towards the ATPase domain. The N-terminal portions of several other B-Lon protease domains also loop back towards the AAA+ domain in the manner of *TonLon* (Im *et al*, 2004; Botos *et al*, 2005). Given the high degree of homology between the A-Lon and B-Lon families within the AAA+ and protease domains, we think it is likely that the orientation of the ATPase and protease domains observed for *TonLon* is universally adopted by Lon proteases, despite a previous speculative model in which the protease domain was oriented in the opposite direction (Botos *et al*, 2004b). A recent structure of LonA from *Bacillus subtilis*, which shows the AAA+ and protease domains oriented in the manner shown here for *TonLon* (Duman and Lowe, 2010), lends support to our proposal.

The protease domain in *TonLon* has an overall structure very similar to those of the isolated Lon protease domains from *Escherichia coli* (Botos *et al*, 2004b), *Methanococcus jannaschii* (Im *et al*, 2004), and *Archaeoglobus fulgidus* (Botos *et al*, 2005). The domain is composed of the S subdomain (residues 441–538) and the K subdomain (residues 539–635) (Figure 2B), which harbour the catalytic residues, Ser523 and Lys566, respectively. The catalytic dyad extends into a hydrophobic canyon that sits at the interface between the S and K subdomains (Figure 3B). The side chains of Ser523 and Lys566 (both mutated to alanine) are oriented towards each other and align well with the catalytic residues in the crystal structures of the *EcLon* and *MjLon* protease domains (Supplementary Figure S6). Thr548, a universally conserved residue, is juxtaposed to the catalytic dyad and overlaps precisely with the equivalent threonine residue in all structures determined to date. In our holoenzyme structure, neither Asp522 nor Glu520 is oriented towards the active site cavity (Supplementary Figure S5),

and neither would be in a position to make hydrogen bond contact with the ϵ amino of catalytic Lys566. Thus, it appears unlikely that either of these residues is part of the catalytic machinery in B-Lons as has been proposed (Im *et al*, 2004).

Viewed from the position of the linker, the protease domain is shaped like a bowl with the exit pore at the bottom. The axially positioned exit pore is formed by six repeats of a long loop between strands 17 and 18 with the disordered side chain of Glu470 protruding into the centre (Figure 3A and B; Supplementary Figure S7). The width of the exit pore is ~ 13 Å, sufficient to allow the passage of unstructured cleavage products. A wide ledge runs around the circumference of the bowl about half-way down. The six active sites lie along the circumferential ring ~ 28 Å from the centre, and adjacent sites are separated by ~ 32 Å (Figure 3B; Supplementary Figure S6). The hydrophobic canyons with the catalytic residues face towards the ATPase domain. The ATPase ring forms a lid over the protease domain producing a large hollow chamber, so that both unfolded or partially folded substrate proteins that have entered the ATPase chamber will easily encounter the active sites. Large protein substrates and most peptides would only have access through the axial channel in the AAA+ domain, but there are small rectangular openings roughly 10 by 13 Å near the linker in each subunit through which small peptides or nucleotides might pass. There is also some evidence of dynamic movement in the protease domain, which could influence catalytic activity as well as the accessibility of the active sites. The backbone atoms of the individual protease domains overlay with an RMSD of < 1.0 Å; however, as a result of the shift in the linker in response to nucleotide binding and release, the domains connected to the L ATPases are rotated slightly relative to those of the T-ATPase subunits causing a displacement of ~ 1.5 Å in the main chain along the periphery. Mutations in Glu614, His665, and His667 of *EcLon* (Glu460, His509, and Gln511 in *TonLon*, which are extensively involved in interdomain contacts of the protease layer) decrease ATPase and protease activity of *EcLon* (Oh *et al*, 1998; Starkova *et al*, 1998), pointing to the importance of the conformational communication between the protease and ATPase domains in coupling the two activities

Discussion

TonLon as a model for all Lon proteases

The structure of *TonLon* presented here confirms that the proteolytic sites are located within a gated chamber that is accessible to proteins only through restrictive axial channels. To what extent does *TonLon* provide a model for A-Lons such as *EcLon*? The protease domains (Supplementary Figures S5 and S6) as well as the α -helical subdomains of *TonLon* and *EcLon* are homologous in their tertiary and quaternary structures. Sequence alignment confirms that all major functional motifs are well matched (Figure 4; Supplementary Figure S1), and sequence-based secondary structure analysis predicts that *EcLon* has the insertions corresponding to Ins2 and Ins3 of *TonLon*. Consequently, except for Ins1, a strategic insertion needed to allow anchoring of *TonLon* to the cell membrane, the α/β subdomains of *TonLon* and *EcLon* are likely to exhibit close structural similarity.

A notable difference between *EcLon* and *TonLon* is the linker between the ATPase and protease domains, which

is a helix in *TonLon* and an extended coil in *EcLon* (Supplementary Figure S1). In a previous model of *EcLon* (Botos *et al*, 2004a, b), the protease ring was proposed to take the opposite orientation with the active sites directed away from the ATPase and exposed to the medium. However, we note that, in the crystal of the *EcLon* protease domain, the N-terminal portion of the main chain adopts a hook-like conformation reminiscent of the U turn that brings the chain back towards the AAA+ domain in *TonLon* (Supplementary Figure S5). Gly441 of *TonLon*, which is part of the U turn (Figure 2B), is absolutely conserved in Lon proteases, and the orthologous Gly596 contributes to the hook in *EcLon* (Supplementary Figure S5). When the α -subdomain of *EcLon* is superimposed onto that of *TonLon*, the gap between the end of the terminal helix (Gln582) and the glycine in the hook (Gly596) is ~ 40 Å. No portions of the structure obstruct the region between Gln582 and Gly596, and the 13 intervening residues are predicted by the PHDsec programme (Rost *et al*, 2004) to adopt an extended coil. Thus, the gap in *EcLon* could easily be bridged with the domains in this orientation. We conclude that LonAs and LonBs share a hexameric framework constructed from homologous ATPase and protease domains, and that the closed degradation chamber and the basic mechanism of substrate-processing encrypted in this hexameric architecture are common to both families.

A 'bowl and lid' design for sequestered proteolytic chambers in single-polypeptide ATP-dependent proteases

The architecture of *TonLon* is reminiscent of that of FtsH, which is also a single-polypeptide ATP-dependent protease (Bieniossek *et al*, 2006), and differs in several respects from the multi-component proteases such as Clp and the proteasomes. In *TonLon* and FtsH, there are only six active sites per holoenzyme and they are located in a bowl-like chamber that is covered with the hexameric assembly of ATPase domains that serve as a concave lid, producing a large protein-processing chamber in which protein unfolding and degradation can apparently occur simultaneously. The architectural design explains several properties reported for Lon proteases. The enclosed degradation chamber is consistent with biochemical data showing that the majority of products of degradation by Lon are small peptides (Maurizi, 1987; Nishii *et al*, 2005). The contiguous chaperone and degradation chambers would allow Lon to make initial cuts at multiple sites within protein substrates and even to cleave native forms of some proteins (Ondrovicova *et al*, 2005). The latter activity suggests that the apical gate can open sufficiently to allow at least small domains to enter without unfolding. Lon protease from *Thermococcus kodakaraensis* KOD1 can degrade unfolded proteins in the absence of ATP (Fukui *et al*, 2002). One possibility is that, in the absence of tightly bound nucleotide, all six α/β domains are rotated up and out in the manner of the L subunits, which would generate an axial channel sufficient to allow unfolded and perhaps even small folded proteins to pass through.

In all ATP-dependent proteases, substrate access is controlled at the apical surface of AAA+ domains through axial loops whose positions are changed in response to rigid domain movements as nucleotides bind and are hydrolysed and released from the AAA+ domains. Multi-component

proteases such as ClpXP, ClpAP, HslUV (ClpYQ), and 26 S proteasomes, which function as dynamic complexes of chaperone and proteolytic components, also control substrate access at the entrance to the protease. The sequestered proteolytic chambers are constructed by joining two heptamers (or hexamers in the case of ClpQ) face to face so that the active sites are inside. Access to the chamber is through axial channels, which are gated and regulated by interaction with the chaperone. As the AAA+ components of multi-component ATP-dependent proteases also function independently to remodel protein structures and can release proteins that will refold to the native state, gating at the point of entry to the protease might provide a final checkpoint for deciding between degradative and refolding fates for specific proteins. The use of a single gate may reflect that Lon selectively targets unfolded proteins. In fact, several of the physiological substrates of *EcLon*, λ N-protein and the cell division inhibitor, SulaA, have structurally unstable regions (Gur and Sauer, 2008). Such proteins might not be capable of refolding if released, and a one-pass gating mechanism would assure their efficient disposal.

Substrate-processing loops are manipulated by rigid body movement of the α/β subdomain

The location of Ins2 and Ins3 on the apical surface forming a cover over the axial channel places them in position to engage substrates, control access to the interior, and actively participate in unfolding and translocation of bound proteins as has been proposed for similar structures in other AAA+ proteins (Martin *et al*, 2008). The inserts jut out from the α/β subdomain at an unusually sharp angle and thus extend from the surface towards the membrane to which *TonLon* is anchored *in vivo*, possibly to allow them to survey exposed regions of membrane proteins and to facilitate the capture of appropriate substrates. Phe216, located in an aromatic-hydrophobic loop of Ins2, and Met275, in the pre-sensor-1 β -hairpin loop, move in and down during the transition from the L to the T state, whereas the corresponding residues move up in the other three subunits. The narrowing of a region of the portal would serve to grip substrates at specific locations and the downwards motion could translocate them into the interior. When the protein is too large to pass through the narrow region of the portal, the vectorial movement of Ins2 during the ATP-powered L $\rightarrow$ T transition would tend to tug proteins against the portal, generating counteractive forces that would lead to unfolding (Sauer *et al*, 2004; Zhang *et al*, 2009). Therefore, the L $\rightarrow$ T transition is likely to be the mechanism underlying both translocation and unfolding of substrates in *TonLon*.

The buried ADP in the T site is consistent with studies with *EcLon* showing that dissociation of ADP generated *in situ* is slow and requires allosteric activation induced by protein substrate binding (Menon and Goldberg, 1987a). Our direct binding studies indicate that ADP binds to *TonLon* with a $K_d < 1 \mu\text{M}$ (Supplementary Figure 2D). The ATPase activity of *TonLon* is enhanced approximately three-fold in the presence of an unfolded protein substrate (Supplementary Table I), which is also consistent with the studies of *EcLon* showing that protein substrates accelerate the rate-limiting release of ADP during the hydrolysis cycle. ADP/ATP exchange should occur in the L domain, in which the ADP is more exposed and exhibits a high B factor (Figure 3A).

If the L- and T-monomers are taken to represent conformational states before ATP binding and after ATP hydrolysis, respectively, it is reasonable to propose that each monomer in *TonLon* repeatedly interconverts between T- and L-conformations in concert with ATP hydrolysis cycles. A modelling study in which an L-monomer is replaced with a T-monomer shows a significant clash between α/β subdomains from adjacent T-monomers (Supplementary Figure S8), indicating that adjacent monomers cannot maintain the T-conformation simultaneously. When an L-monomer adjacent to a T-monomer undergoes an ATP-powered L $\rightarrow$ T transition, high-affinity binding of ATP to the L state might impel a T $\rightarrow$ L transition of the T-monomer to relieve the clash. We propose that the L $\rightarrow$ T transition powered by ATP and substrate binding in an L-monomer is coupled to T $\rightarrow$ L transition in an adjacent T-monomer. Owing to the clash between adjacent T-monomers, no more than three non-adjacent monomers may work in concert in the hexamer. Studies with several AAA+ proteins now point to a general mechanism in which ATP hydrolysis occurs at different times in different subunits, in either a random or alternating manner, and is facilitated allosterically by nucleotide and substrate binding to other subunits within the ring (Martin *et al*, 2005, 2007; Augustin *et al*, 2009). The crystal structure presented here will serve as a model to study similar details of how Lon proteases capture and degrade their substrates and how those substrates are recognized as appropriate targets by this important component of cellular protein quality control.

The limited differences in conformations between the T and L subunits suggest that Lon might undergo a smaller range of motion than observed recently for ClpX (Glynn *et al*, 2009) and FtsH (Bieniossek *et al*, 2006, 2009), in which the α -subdomain undergoes a displacement by as much as 80°, but is in line with motions proposed for HslU (Wang, 2004). ATP binding to one or more subunits could induce a conformation different from that observed with ADP and thus add additional asymmetry within the ring. A fuller understanding of the conformational dynamics and range of motion will require structural determination of multiple nucleotide states.

Materials and methods

Purification and crystallization

Details of expression, purification, and crystallization of *TonLon* have been described elsewhere (An *et al*, 2010). Protein concentrations were measured by absorbance using either experimentally determined absorption coefficients or absorption coefficients calculated from the aromatic amino-acid content using the Pepstats programme (<http://helixweb.nih.gov/emboss/html/pepstats.html>). Molar concentrations of *TonLon* refer to units of hexamers.

Structure determination and refinement

It was extremely difficult to obtain *TonLon* crystals of diffraction quality (An *et al*, 2010), which prohibited our using MIR or MAD methods to get phase information. As an alternative, we determined to solve the structure of *TonLon* using molecular replacement. As no high-resolution structural data was available for the AAA+ domain of Lon proteases, we first solved the structure of the AAA+ domain of *TonLon*, which we obtained by limited proteolysis. The purified full-length *TonLon* was treated with trypsin with 1:100 (w/w) ratio for 30 min and the AAA+ domain was then separated by gel filtration. The AAA+ domain was crystallized in a precipitant solution containing 12% polyethylene glycol 4000, 0.2 M potassium chloride, 0.01 M magnesium acetate, and 0.05 M tri-sodium citrate dehydrate pH 4.5. The crystals belong to space group P1 with unit cell parameters $a = 40.83$, $b = 61.00$, $c = 76.32$,

Table 1 Refinement statistics

Space group	P63
Resolution (Å)	30–2.0
Completeness (>0 s) (%)	99.9 (99.9) ^a
R/R_{free} (%) ^b	20.6/23.5
Number of reflections in working set/test set	103966/5516
Average B factor (Å ²)	30.1
Number of protein atoms	8680
Number of water molecules	835
Number of ADPs	2
Number of PEG 400	2
RMSD bonds (Å)	0.008
RMSD angles (deg)	1.4
Ramachandran plot	
Residues in most favourable regions (%)	92.6
Residues in additional allowed regions (%)	7.2
Residues in generously allowed regions (%)	0.2

^aValue for the highest shell (2.03–2.0 Å) is given in parentheses.

^b $R = \sum |F_o - F_c| / \sum F_o$, where F_o and F_c are the observed protein structure factors and calculated protein structure factor from the atomic model, respectively. R_{free} was calculated with 5% of the reflections.

$\alpha = 74.36$, $\beta = 86.48$, and $\gamma = 83.89$. To solve the structure of the isolated AAA + domain by the MAD method, the seleno-methionine substituted AAA + domain was prepared as above and crystallized. MAD data sets at 2.0-Å resolution were collected at three wavelengths (peak, 0.97909; edge, 0.97934; remote, 0.96411) (Supplementary Table II). Selenium positions were located and phases were calculated using the programme SOLVE. The subsequent solvent flattening by RESOLVE gave rise to an interpretable map, based on which the *de novo* model building was easily completed. The initial model containing ADP molecules was subjected to several rounds of refinement and manual refitting, giving rise to a model with R and R_{free} values to 25.99 and 30.18%, respectively. At this stage, we stopped the refinement without incorporating water molecules into the model, because water molecules were not required to use the model for the subsequent molecular replacement determination of *TonLon*.

The structure of *TonLon* was determined by molecular replacement with MolRep in the CCP4 programme suite. Structures of the isolated AAA + domain of *TonLon* and the *EcLon* protease domain (Botos *et al*, 2004b) were separately used as search models. The position of the protease domain was first determined and then fixed in the subsequent search for the position of the isolated AAA + domain. Refinement was performed with the maximum likelihood algorithm implemented in the CNS programme (Table I). Molecular graphics manipulations and calculations of solvent-accessible surface area were performed with QUANTA (Molecular Simulations Inc., San Diego, CA). In the final model, a T-monomer contains residues 18–130, 179–216, and 221–635 and an L-monomer contains residues 20–128, 178–216, and 222–634. The Ramachandran plot indicates 92.9% of non-glycine and non-proline residues are in the most favoured regions, and no residues are in the disallowed regions in the final model.

In vitro protein degradation

*TonLon*ΔTM with wild-type catalytic residues in the protease domain was used for *in vitro* degradation assays. Proteolytic activity against unfolded proteins was assayed with fluorescein isothiocyanate casein (FITC casein) and with α casein (both from Sigma). The assay buffer consisted of 50 mM Tris-HCl, pH 8.0, and 10 mM MgCl₂, with or without 1 mM ATP. *TonLon*ΔTM, the F216A mutant, or the M275A mutant (50 nM hexamer) was incubated in 200 μ l of assay buffer at 70°C with 2 μ M substrate. The fluorescence increase upon degradation of FITC casein was monitored using a fluorescence spectrophotometer with excitation at 490 nm and emission at 525 nm. For α -casein degradation, aliquots were removed at timed intervals and mixed with SDS sample buffer at 95°C. After SDS-PAGE, the α casein remaining was detected by staining with Coomassie blue.

Activity against folded substrates was assayed at 37°C with 0.2 μ M *TonLon* in the above buffer solution with and without 1.5 mM ATP. The solution also contained 50 mM creatine phosphate and 0.3 U/ml of phosphocreatine kinase to regenerate ATP and minimize the accumulation of ADP. No degradation was observed with the regenerating system alone. N-GFP is a fusion of the phage λ N-protein with GFP-mut3.1 and was kindly provided by Fatima Rasulova, LCB, NCI, NIH (unpublished data). *E. coli* UmuD was provided by Roger Woodgate (NGI, NICHD, NIH). Arc-SulA is a fusion of the Arc repressor protein with the C-terminal 11 amino acids of SulA and was provided by David McKay (Stanford University School of Medicine). N-GFP, UmuD, and Arc-SulA were present in the assay solution at 4.0, 10, and ~2 μ M, respectively. Aliquots were quenched into SDS sample buffer and the proteins were separated by SDS-PAGE. The remaining intact protein was detected by staining with Coomassie blue or, in the case of Arc-SulA, by silver staining.

Peptidase activity

Stock solutions of glutaryl-Ala-Ala-Phe-methoxynaphthyl amide (Glt-AAF-MNA) (Sigma) in DMSO were diluted to 0.3 mM in 50 mM Tris/HCl buffer, pH 8.0, with 10 mM MgCl₂, with or without 1 mM ATP. After incubation at 70°C with 12.5 nM *TonLon* hexamer for various times, the reaction was quenched by dilution into 2% SDS and the increase in fluorescence was measured in an Aminco Bowman spectrofluorometer with excitation at 335 nm and emission at 410. Continuous assays of peptidase were conducted using a peptide with the following sequence: aminobenzoic acid-Ala-Phe-His-Met-Ala-Leu-nitrotyrosine-Pro-Val (Fluo-FV) (Li *et al*, 2010). The fluorescence of the donor, nitrotyrosine, is reduced by the presence of the acceptor, aminobenzoic acid. Increased fluorescence emission at 415 nm upon cleavage of the peptide was monitored with excitation at 320 nm. Typical assay mixtures contained 50 mM creatine phosphate, 0.3 U/ml of phosphocreatine kinase as an ATP regenerating system, 0.25 μ M (100 μ g/ml) of *TonLon* hexamer, and 4 μ M of Fluo-FV. Assays were conducted at 37°C with variable concentrations of ATP.

ATPase activity

To measure ATPase activity, *TonLon* (2 μ g) was incubated with 1 mM ATP and 10 mM MgCl₂ in 100 μ l of 50 mM Tris-HCl buffer (pH 8.0) at 37°C for 15 min. Inorganic phosphate released was determined from the increase in absorbance at 660 nm after addition of ammonium molybdate and malachite green. Stimulation of the ATPase activity of *TonLon* by α casein was investigated at a casein concentration of 0.3 mg/ml.

Nucleotide-binding assays

Binding of ATP γ S and ADP to *TonLon* was measured by isothermal titration calorimetry at 25°C using a VP-ITC microcalorimeter (Microcal). A solution of 1.2 ml of 50 mM Tris/HCl, pH 8.0, containing 10 mM MgCl₂ and 5 μ M hexamer *TonLon* was titrated with ATP γ S or ADP dissolved in the same buffer adjusted to 10.2 mM MgCl₂. ATP γ S or ADP (0.2 mM) was added in nine aliquots of 2.5 μ l each followed by 17 aliquots of 14 μ l each.

Addendum in revision

During the time this manuscript was under revision, a report appeared online with a 3.4 Å structure of *B. subtilis* LonA with ADP bound (Duman and Lowe, 2010). The subunit structure is consistent with the orientation of the protease active sites with respect to the AAA + domain that we observe in the *TonLon* hexamer and confirms our proposal that this orientation is maintained in the Lon A family as well.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Bethesda, MD. The atomic coordinates have been deposited in the Protein Data Bank (accession code 3K1J).

Conflict of interest

The authors declare that they have no conflict of interest.

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