Structure of the catalytic domain of the human mitochondrial Lon protease: Proposed relation of oligomer formation and activity

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Abstract: ATP-dependent proteases are crucial for cellular homeostasis. By degrading short-lived regulatory proteins, they play an important role in the control of many cellular pathways and, through the degradation of abnormally misfolded proteins, protect the cell from a buildup of aggregates. Disruption or dysregulation of mammalian mitochondrial Lon protease leads to severe changes in the cell, linked with carcinogenesis, apoptosis, and necrosis. Here we present the structure of the proteolytic domain of human mitochondrial Lon at 2 Å resolution. The fold resembles those of the three previously determined Lon proteolytic domains from *Escherichia coli*, *Methanococcus jannaschii*, and *Archaeoglobus fulgidus*. There are six protomers in the asymmetric unit, four arranged as two dimers. The intersubunit interactions within the two dimers are similar to those between adjacent subunits of the hexameric ring of *E. coli* Lon, suggesting that the human Lon proteolytic domain also forms hexamers. The active site contains a 3_{10} helix attached to the N-terminal end of α -helix 2, which leads to the insertion of Asp852 into the active site, as seen in *M. jannaschii*. Structural considerations make it likely that this conformation is proteolytically inactive. When comparing the intersubunit interactions of human with those of *E. coli* Lon taken with biochemical data leads us to propose a mechanism relating the formation of Lon oligomers with a conformational shift in the active site region coupled to a movement of a loop in the oligomer interface, converting the proteolytically inactive form seen here to the active one in the *E. coli* hexamer.

Keywords: ATP-dependent protease; Lon protease; catalytic dyad; mitochondria; oligomerization and activity

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Introduction

ATP-dependent proteases are important for cellular homeostasis, present in all organisms, and degrade abnormal and denatured proteins as well as short-lived regulatory ones.¹ There are a number of ATP-dependent protease families, of which one is the Lon proteases. Indeed the *Escherichia coli* enzyme (*EcLon*) was the first ATP-dependent protease to be discovered² and probably remains the most studied to date. Members of the Lon family are ubiquitous in bacteria, archaea, and eukaryotes, where they are found in chloroplasts,³ mitochondria,⁴ and peroxisomes.⁵ In mitochondria, metabolic activity generates high levels of free radicals which then oxidize proteins leading to their aggregation; this is believed to be a significant cause of aging⁶ and neurodegenerative disease.⁷ Many mitochondrial proteins are synthesized in the cytosol and transported to the mitochondria, where they are assembled into large complexes. Misassembled, nonassembled, and oxidatively damaged proteins must be recognized and degraded, otherwise they can be toxic to the cell. The human mitochondrial Lon protease (*hLon*) has been shown to degrade oxidized proteins including aconitase.⁸ *hLon* has also been shown to bind to mitochondrial DNA in a region overlapping the mitochondrial DNA (mtDNA) promoter and to GC rich sequences along the mtDNA.^{9,10,11} *hLon* is believed to be involved in mtDNA replication, translation, or repair because it has been shown to immunoprecipitate with both mitochondrial twinkle helicase and the mtDNA polymerase γ catalytic subunit A (pol γ A).¹² The mitochondrial Lon proteases are associated with mitochondrial nucleoid proteins such as mtSSB and the recently identified PDIP38.¹³ Lon has been shown to be present in mitochondrial nucleoids.¹⁴ A detailed knowledge of the Lon 3D structure is required for a full understanding of these processes.

There are two Lon families: LonA and LonB. The polypeptide chain of the LonA family members consists of an N-terminal domain, a central ATPase (AAA⁺) domain and a proteolytic domain (henceforth termed LonP) at the C-terminus. In contrast, the LonB family lacks the N-terminal domain and shows differences in the residues conserved in the AAA⁺ domain and the proteolytic domain.¹⁵ *hLon* belongs to the LonA family. The N-terminal domain is thought to be involved in the selective binding of substrates as well as in the oligomeric assembly of the protease, as shown for the *Brevibacillus thermoruber* enzyme.¹⁶ The X-ray structure of the N-terminal domain of *EcLon* has been reported¹⁷ and shown to be similar to that of BPP1347 (PDB code: [1zbo](#)), a member of the cluster of orthologous group (COG) 2802 of proteins of unknown function.

The ATPase domain belongs to the well-characterized AAA⁺ superfamily of proteins.¹⁸ It is com-

posed of two subdomains, a β/α -domain upstream of an α -domain. Many structures of AAA⁺ superfamily proteins have been reported, including those of the α -domains, of the Lon proteases from *E. coli*¹⁹ and *B. subtilis* (PDB code [1x37](#)). In Lon, the role of this domain appears to be the unfolding of substrate proteins before their cleavage by the proteolytic domain.²⁰ The ATPase domain has also been proposed to be involved in DNA binding from studies of the *B. thermoruber* enzyme.¹⁶

The C-terminal proteolytic domain contains a Ser-Lys dyad at the active site. Studies in yeast have shown that the ATPase domain and proteolytic domain, even when expressed separately, are able to interact within the mitochondrion and can fully replace intact Lon.²¹ There appear to be an allosteric interaction between the two domains, and the proteolytic domain is inactive in protein degradation when the AAA⁺ cassette is not present. However, the proteolytic domain of *EcLon* appears to hydrolyze small peptides²² without AAA⁺ cassette participation. Crystal structures of the Lon proteolytic domain from a eubacterium and two archaea have been determined and all have similar overall folds.

The structure from the eubacterium *E. coli* (*EcLon*; PDB code [1rre](#)) was the first to be determined, it had a unique fold and crystallized as a noncrystallographic hexamer.²³ The catalytic serine had been mutated to alanine (S679A). The active site was open to the surface suggesting it was accessible to substrates. This was followed by the structures from two archaea *Methanocaldococcus jannaschii*²⁴ and *Archaeoglobus fulgidus*,²⁵ henceforth *MjLonP* and *AfLonP* with PDB codes [1xhk](#) and [1z0b](#), respectively. The *MjLonP* active site again revealed a Ser-Lys dyad. The structure appeared to form a dimer in the asymmetric unit but this was reported not to be of biological significance. An unexpected feature was that the N-terminal region of the helix carrying the catalytic serine was a 3_{10} helix and not a β -strand as in *EcLonP*, bringing a new residue into the active site, Asp547, which made a salt bridge with the catalytic lysine.

For *AfLonP*, the structures of several mutants were determined, some but not all of which contained a noncrystallographic hexamer similar to that in *EcLonP*, and some of which were monomers. In all the *AfLonP* structures, the catalytic serine pointed away from the lysine and the N-terminal region of the helix carrying the serine was a β -strand. Mutation of the glutamate, equivalent to the aspartate in the *MjLonP* active site, showed no change in ATP-independent activity, but did show a decrease in ATP-dependent protease activity, and was taken to indicate that this aspartate was not directly involved in catalysis.

Structures of a number of other Ser-Lys dyad proteases have been determined.^{26,27} In particular,

Figure 1. The *hLonP* protomer. A: Stereo ribbon diagram color ramped from N- to C-terminus. B: In the same orientation as (A), the structures of *E. coli* (coral), *M. janaschii* (blue), and *A. fulgidus* (yellow) LonP represented as worms superimposed on that of *hLonP* (green), confirming their high similarity. The main differences are in the loops and at the N-terminus of $\alpha 2$. Ser855 and Lys898 composing the catalytic dyad are shown as spheres for the human enzyme. This Figure and Figures 3 and 4 were created with CCP4mg.³¹

the structures of two VP4 proteases from the Birnaviruses (PDB codes: [2pnl](#) and [2gef](#))^{28,29} share the same overall topology as the LonP domains, and indeed they were initially identified as noncanonical Lon proteases.³⁰ These are monomeric single domain proteins expressed as part of a longer viral polypeptide containing several preproteins. The role of the VP4 protease is to produce the mature forms of the individual proteins. One structure ([2gef](#)) contains a well-defined Ser-Lys dyad. In the second ([2pnl](#)), the lysine was mutated to alanine allowing the formation of a covalent intermediate providing direct evidence for the mechanism involving nucleophilic attack by the serine. The active sites of [2gef](#) and [2pnl](#) superimpose closely, and the mutation of lysine to alanine has less impact on the conformation.

Here we present the crystal structure of the proteolytic domain of human mitochondrial Lon protease (*hLonP*) at 2 Å resolutions, the first structural data for an eukaryotic Lon. The active site is blocked by an aspartate as seen in *MjLon* despite the sequence in

this region being almost identical to that of *EcLon*. On the basis of the *hLonP* structure, together with those of homologous LonPs and our biochemical data regarding oligomerization state and activity, we propose a mechanism by which hexamer formation is coupled to a conformational transition at the active site which converts the inactive conformation seen here to one resembling that seen in *EcLon*.

Results

The *hLonP* protomer

The crystal structure of *hLonP* was solved by molecular replacement using *EcLon* (PDB: [1rre](#)) as a search model and was refined at 2.0 Å spacing. It is curious to note that, similar to the *EcLon* and *AjLon* structures, problems were encountered with twinning and the assignment of the space group.

The proteolytic domain used for crystallogenesi contained residues 741–959, but due to presumed degradation of the protein in the crystallization

Figure 2. Sequence alignment of the Lon proteolytic domains for which the structure is known. The secondary structure at the top corresponds to the human orthologue, whereas that at the bottom corresponds to *EcLonP*. Invariant positions are shaded and conserved positions are boxed. Figure created using CLUSTAL W³² and ESPrpt.³³

drop, the protein in the crystal corresponds to residues 754–959, confirmed by N-terminal sequencing. There are six protomers in the asymmetric unit with essentially identical folds as shown by the average pairwise rms difference in C_α positions between chains of 0.44 Å. The protomer structure has nine β -strands and seven α -helices [Fig. 1(A)]. The biggest differences between the protomers lie in the loops connecting the secondary structural elements with the largest deviations for residues 785–797. Up to six residues at the N-termini (754–759) and 11 at the C-termini (949–959) of all six chains are missing in the electron density maps and are assumed to be disordered. In addition, a number of residues in surface loops are missing in each protomer, with a maximum of 13 residues in chain B.

The overall fold is similar to that of other known LonP domains [Fig. 1(B)], and therefore, only differences between the present structure and *EcLonP* will be described. The sequences of the four LonP domains for which the structure is known are aligned in Figure 2. To aid comparison, the numbering scheme for the secondary structural elements of *EcLonP* will be used throughout. As in *EcLonP*, strands β 1– β 4 form a mixed parallel/antiparallel β -sheet. β 1 and β 2 are connected by a type I β -turn, but there was no density for the loop connecting β 2– β 3 (785–797) and it is presumed to be disordered. Following β 3 is helix α 1 and then the short α 1a (a hydrogen-bond turn in *EcLonP*). After this short helix comes β 4 but β 5 is replaced in

hLonP by an extension to α 2. This extension begins as a hydrogen-bonded turn in a close to helical conformation (residues 850–852), becomes a 3_{10} helix (residues 853–856), and from Gly857 α 2 continues as a normal α -helix. This extension causes a substantial conformational change in the region of the active site and is of considerable significance for our proposal regarding LonP activity. Following α 2, there is a short β -bridge in *EcLonP*, which is absent from *hLonP*. The second subdomain comprises a parallel β -sheet (β 6, β 9, and β 10) flanked by α 3, α 4, α 5, and α 6. This subdomain shows no significant changes from *EcLonP* with one exception: α 4 was a 3_{10} helix in *EcLonP* but is a normal α -helix here.

hLonP dimers

Analysis of the surface contacts with PISA³⁴ suggests that four *hLonP* protomers form two equivalent dimers, whereas the other two are monomers. Unfortunately, the Δ^iG values calculated by PISA fall within the uncertainty limit of the method making it difficult to distinguish reliably using buried surface area alone whether A:B and C:D form dimers with E and F forming monomers, or if A:E and C:F are the dimers with B and D as monomers. However, this can be clarified by comparison with other Lon sequences and 3D structures.

In the hypothetical A:E and C:F dimers, the two protomers are related by approximate twofold rotational symmetry, with interactions primarily between their α 1 helices from which residues

interact with their counterparts in the second subunit. A multiple sequence alignment of 60 LonA sequences from SWISSPROT shows that although this particular region is generally well conserved, a number of key residues are not, notably Arg815 and Lys888. Furthermore, it is difficult to imagine how this type of dimer would give rise to the type of hexamer seen in electron micrographs of full-length Lon, nor does it relate to the interface of the hexamers seen in the *EcLonP* and *A/LonP* crystals. This interaction can be presumed to be an artefact of crystal packing and is unlikely to be of physiological significance.

In marked contrast, the A:B/C:D dimer interface is similar to that formed between adjacent subunits of the *EcLonP* and *A/LonP* hexamers. $\alpha 1$ packs against $\beta 3$ in the adjacent subunit [Fig. 3(A)] and the loop between $\beta 7$ and $\beta 8$ makes intersubunit contacts with $\beta 2$. The interactions are mainly hydrophilic and 1520 Å² of accessible surface area are buried, similar to the value in *EcLonP*. However, unlike *EcLonP*, the loop following $\beta 4$ does not contribute any intermolecular contacts, a result of interference from the N-terminus. Met756 in the N-terminus itself packs between Phe779 of the same subunit and the aliphatic portion of Lys888 of the adjacent one. Additionally, Tyr757 forms a hydrogen bond with Thr886 of the adjacent subunit, further stabilizing the position of the N-terminal loop. We conclude that the biologically significant dimers are the protomer pairs A:B and C:D.

The active site

LonA proteins are serine proteases, with a Ser-Lys dyad in which a lysine assists the catalytic serine in proteolytic cleavage.¹⁵ A third catalytic residue, a serine or a threonine, is present in some but not all such proteases.^{26,27,35} The *hLonP* active site has an underlying similarity to that of *EcLonP*, with the positions of several key residues in and around the active site (Pro854, Ser855, Ala856, Gly857, Thr880, and Lys898) being closely conserved (equivalent C α atoms within 0.2 Å). In *hLonP*, the catalytic Ser855 on $\alpha 2$ is hydrogen-bonded to Lys898 in $\alpha 3$ to form the Ser-Lys dyad. Thr880, the third residue, is hydrogen-bonded to Lys898 and possibly Ser855. There is a water molecule (Water3) bound to the Thr880 hydroxyl group. Lys898 is also H-bonded to Gly893.

The 3_{10} helix at the N-terminal end of $\alpha 2$ brings an aspartate residue, Asp852, into the active site where its OD2 atom forms a hydrogen bond with Lys898 [Fig. 4(A)] and is only 3.2 Å from Ser855. In order for the lysine to work as a general base and abstract a proton from the nucleophilic serine, it must be in the deprotonated form. One contribution to lowering the pK_a of the lysine is to place it in a hydrophobic environment. The interaction with

Asp852 must affect the pK_a of Lys898 and prevent its function as a general base. Perhaps more importantly, Asp852 and in addition Trp770 are placed so as to block access to the active site. Thus, it is most unlikely that the present conformation represents a catalytically active form of the *hLonP* domain.

The X-ray data and coordinates have been deposited in the PDB with the accession code [2x36](#).

Oligomerization and activity assays

To understand the relationship between oligomerization and activity of the *hLon* protease and to support the hypothesis of the relationship between oligomerization and activity (below), two constructs of *hLon* were prepared, corresponding to the full-length enzyme and the proteolytic domain (including residues 762–960). For each of them, the oligomeric state and peptidase activities were examined (Table I). The full-length construct formed a hexamer in solution and showed clear peptidase activity in the presence of ATP and also in the presence of nonhydrolyzable analogue AMP-PNP. In contrast, the protease domain on its own was monomeric in solution and possessed no peptidase activity.

Discussion

The structure of the human mitochondrial Lon protease proteolytic domain confirms it has an overall fold similar to the four known LonP structures in the PDB with an overall rmsd of 2.2 Å for the multiple superposition of 162 C α atoms calculated using SSM.³⁶ The major differences are in the loops connecting the various secondary structural elements. First, there is a significant difference at the N-terminus of $\alpha 2$, which affects the active site. Second, the loop between $\beta 2$ and $\beta 3$ is rather long and disordered in *hLonP* and *A/LonP*. It has been proposed²⁵ that this loop might play a functional role in interacting with the ATPase domain. An interaction between these two domains was proposed earlier from yeast two-hybrid studies.²⁰ Third, as in *A/LonP*, the N-terminal tail of *hLonP* points toward the active site surface rather than the putative AAA⁺ interface.

Superposition of the *hLonP* structure on those of the two Birnaviral VP4 proteases (PDB codes [2pnl](#) and [2gef](#)) shows that while the core of the domain is conserved, there are major deletions and insertions as could be expected from the very low level of sequence similarity. The VP4 structures will not be further discussed, but what is highly relevant to LonP is that the catalytic dyads of the two VP4 and three of the four LonP (the exception is *A/Lon*) structures domains superimpose very closely [Fig. 4(B)]. Because for one of the VP4 structures the serine is trapped as a covalent intermediate, this provides compelling evidence that the two residues

Figure 3. The subunit–subunit interface in LonP and relation between oligomerization and activation. A: Stereo view of the *hLonP* dimer interface, formed by chains (A) (gray) and (B) (blue) shown as ribbons. $\alpha 1$ of one chain packs against β -sheet 1 of the adjacent subunit. Side chains (cylinders with carbons in green) are shown for those residues involved in direct intersubunit contacts together with the H-bonds between them. The loop emerging from $\beta 4$ (black) does not make any contacts with the adjacent subunit. Residues in the active site are depicted as cyan cylinders. B: Stereo view of adjacent subunits from the *E. coli* LonP hexamer colored coral and yellow. The side chains are shown as cylinders (green) for those residues involved in direct intersubunit contacts. The extended loop (residues 669–674)—caused by the unfolding of the beginning of $\alpha 2$ in *hLon*—is in red, and makes additional contacts with the adjacent subunit. Residues in the active site are depicted as cyan cylinders. C: Structural differences between the monomeric/dimeric inactive state (represented by *hLon*) and the proposed hexameric active state (represented by *EcLon*). The surfaces of two adjacent subunits of the *EcLonP* hexamer are shown in blue and white: the extensive surface buried in this interface is evident. For *EcLonP*, the following features are shown. (i) The extended loop residues 667–681 (including a length of β -strand) at the N-terminal end of $\alpha 2$ (red ribbon). This makes a substantial contribution to the interface. (ii) The side chains (red cylinders) of two key residues Asp676 and Trp603 (equivalent to Asp852 and Trp770 in *hLonP*). For *hLonP* are shown: (i) the catalytic residues Ser855, Lys898, and Thr880 (cylinders colored by atom type), (ii) the N-terminal segment of *hLonP* $\alpha 2$ as a black ribbon, (iii) the side chains of the two residues as black cylinders (Asp852 and Trp770) which block the active site, and (iv) the chain in the neighborhood of the catalytic serine as a pale green ribbon. The *hLonP* N-terminal segment of $\alpha 2$ with the helical fold places Asp852 directly in the active site with Trp770 physically blocking access, making the monomeric/dimeric form inactive. Thus, in *EcLonP*, the N-terminal section of $\alpha 2$ (which is helical in *hLon*) is unwound so as to contact the adjacent subunit. This movement carries Asp676 (equivalent to Asp852 in human) away from the active site. The loop is H-bonded to the main chain of Trp603 whose side chain is also pulled away from the active site.

Figure 4. A: Active site of the *hLon* proteolytic domain with the $2F_o - F_c$ electron density contoured at 1σ . The hydrogen bond network between the catalytic nucleophile Ser855, the general base Lys898 and Thr880 is shown. Asp852 can be seen to prevent access of substrate by H-bonding to Lys898. B: Superposition of the region around the active site of the *hLonP* domain (green) with those of the VP4 proteases from the infectious pancreatic necrosis virus (2pnl, coral) and the blotched snakehead virus (2gef, blue). The positions of the two residues of the catalytic dyad and the associated threonine are closely similar in the three structures.

making up the dyad are in an appropriate orientation to function in proteolysis.

Comparison with other LonP structures

To assist in the following discussion, some salient points and key features in and around the active site of the four LonP structures are shown in Table

II. In summary, we will argue that the closely similar *hLonP* and *MjLonP* structures represent a closed inactive monomeric (or dimeric) form of the domain, whereas *EcLonP* is an open active hexameric form. *AfLonP* presents something of an enigma, and the structure of *AfLonP* cannot be reconciled with our proposals for the other three structures, as in none the structures of its various mutants do the serine and lysine form the expected dyad, the serine pointing away in the opposite direction.

As can be seen from Table II, the *hLonP* and *MjLonP* active sites are very similar to one another, and will be discussed first. Superposing the two structures brings the active site residues within 0.2 Å with highly conserved hydrogen-bonding interactions. In both structures, there is a 3_{10} helix at the N-terminal end of $\alpha 2$ which brings an extra residue, Asp852, into the active site where it forms hydrogen bonds with Lys898 [Fig. 4(A)]. It was previously suggested for *MjLon* that this aspartate made up a pseudo-catalytic triad²⁴; however, mutation of the equivalent glutamate (E506A) in *AfLon* does not abolish the ATP-dependent catalytic activity and has no effect on the ATP-independent activity, making it appear that the aspartate does not participate directly in catalysis.²⁵ Furthermore, this helix blocks one end of the shallow groove seen in *EcLonP* that is postulated to participate in substrate binding. These considerations lead us to conclude that the conformation seen in *hLonP* and *MjLonP* represents an inactive state which may however represent an important mechanism for the proper regulation of the enzyme.

Although the sequence identity between the proteins in the area of the active site approaches 100%, the active site of the *E. coli* enzyme shows significant differences in 3D structure from those of *hLonP* and *MjLonP*. When compared with *EcLonP*, the loop connecting $\beta 1$ and $\beta 2$ in *hLonP* has shifted by 4.0 Å away from the active site and Trp770 has rotated so that the edge of the indole ring is now over the active site, putting this loop into a conformation similar to that seen in *MjLon* and *AfLon* and rather far from the equivalent residues in *EcLonP*. In *EcLonP*, the 3_{10} helix at the N-terminal end of $\alpha 2$, which allows the aspartate to form hydrogen bonds with the catalytic Lys898, is transformed into $\beta 5$ which causes the equivalent aspartate to move well away from the active site. This opens up the active site

Table I. Oligomerization and Activity of Full-Length *hLon* and of the Proteolytic Domain Alone

Construct	Degree of oligomerization						Peptidase relative activity (%)	
	Superose 12 gel filtration			Glutaraldehyde crosslinking			With ATP	With AMP-PNP
	Hexamer	Mixture	Monomer	Hexamer	Mixture	Monomer		
<i>hLon</i> full length	98%	1%	1%	>99%	–	<1%	100	130
Proteolytic domain	<1%	–	>99%	<1%	–	>99%	<0.1	–

Table II. Comparison of Key Properties and Nature of the Active Sites of the Four Known LonP Structures

	<i>h</i> LonP	<i>Mj</i> LonP	<i>Ec</i> LonP	<i>Af</i> LonP
PDB ID		1xhk	1rre	1z0w and so forth
LonA/B	A	B	A	B
Kingdom	Eukaryote	Archaea	Eubacteria	Archaea
Mutant			S to A	Some mutants
Oligomer	Monomer/dimer	Monomer (dimer not biological)	Hexamer	Monomers and hexamers
Active site 3_{10} or β strand	3_{10}	3_{10}	β strand	β strand in all
Extended loop contact to adjacent subunit in hexamer	Cannot, as 3_{10}	Cannot, as 3_{10}	Yes	Yes, but different from <i>Ec</i> LonP
Ser-Lys Dyad	Yes	Yes	Yes, mutant, modelled	No
Asp blocking Active Site	Yes	Yes	No	No
Lysine	Forms dyad, but buried by Asp	Forms dyad, but buried by Asp	Forms dyad	Accessible but no dyad
Active site	Closed form	Closed form, inactive	Open form, no Asp and Trp	No dyad, serine points away
Proposed activity based on conformation	Inactive	Inactive	Active with modeled serine	Inactive, serine in wrong place to form dyad

making the catalytic serine accessible to substrates and presumably removing the constraint on the pK_a of the lysine. $\alpha 1$, in contrast, has moved about 2 Å away from the active site in *h*LonP, possibly as a result of loss of the interaction between the helix dipole and the equivalent Asp676 in *Ec*LonP.

Thus, there are two quite distinct conformations in and around the active site, a closed form seen in *h*LonP and *Mj*LonP where the active site is blocked by an aspartate, and an open form where the aspartate has moved away. In addition in the *h*Lon monomer, Trp770 is positioned so as to block access to the active site. We propose that the former represents an inactive and the latter an active form. The stability of the two forms appears to be related to the oligomerization state of the domain.

Oligomerization and activity

The oligomerization state of Lon proteases *in vivo* remains somewhat uncertain. *Af*LonP and *Ec*LonP are hexameric in the crystal,^{23,25} whereas transmission electron microscopy suggests that yeast Lon is heptameric.³⁷ In *h*LonP, the two dimers in the crystal formed by chains A-B and chains C-D are closely similar to one another. In addition, they superpose well on two adjacent subunits of the *E. coli* hexamers, and it is possible to generate a model of a *h*LonP hexamer from three dimers. However, some key interactions in the interface are missing and conformational changes in the *h*LonP protomer would be required to convert the α -closed conformation seen here into the β -open conformation seen in the *E. coli* structure.

The balance between the interactions lost and gained between the two conformations appears to be delicate. We propose that both the conformations are likely to exist in the cell. It appears that the conformations seen in *h*LonP and *Mj*LonP both correspond to that of an inactive monomeric form of the protein. In the *Ec*LonP hexamer, there is a conformation in which the loop joining $\beta 4$ – $\alpha 2$ has formed a β -strand ($\beta 5$ in *E. coli*) and also interacts with the neighboring subunit. In contrast, in the structures containing monomers, *Mj*LonP and *h*LonP, there is a helix in the active site with an acidic residue forming hydrogen bonds or a salt bridge with the active site residues.

Our observations allow us to postulate a possible mechanism for the events following the oligomerization of hexameric Lon. In solution, it seems reasonable to suppose that the LonP monomers adopt the closed (α -helical conformation) seen in the present structure, based on the presence of this conformation in *Mj*Lon and in all protomers in *h*Lon regardless of their association state. Following proper association into a hexameric ring, a conformational change is triggered, converting the *h*LonP-type conformation into the *Ec*LonP form: the N-

terminal 3_{10} helical extension to $\alpha 2$ unwinds, bringing Asp852 into contact with the N-terminus of $\alpha 1$, causing it to move ~ 2 Å closer to the active site and closing the cavity, we observed in the present structure [Fig. 3(C)]. The unwound helix, in turn, forms $\beta 5$, whereas the loop connecting $\beta 5$ with $\beta 4$ is stabilized through interactions between Lys851 and the main-chain carbonyl atoms of Val844, Glu846, Thr849, and Gly804. This loop then interacts with the N-terminal region of $\alpha 1$ from the adjacent subunit. Because the unwinding of the $\alpha 2$ extension leads to both a shift of $\alpha 1$ in the same subunit and interactions between the newly formed loop and $\alpha 1$ of an adjacent subunit, it seems that this conformational change must be cooperative and may well require the formation of the hexamer. The formation of dimers in the *hLonP* crystals is insufficient to flip the molecule into the open form. Assuming the conformation of the active site seen in *hLonP* to be proteolytically inactive, this cooperative change would account for the observation that the presence of the hexamer is necessary for peptidase activity (below). Furthermore, this provides a mechanism by which the proteolytic domain might be regulated to prevent uncontrolled proteolysis, activity being dependent on the formation of hexamers through cooperative interactions involving both the AAA⁺ and the protololytic domains.

Although it has been determined that Glu506, the counterpart of Asp852 in *A/LonP*, is not necessary for proteolytic activity,²⁵ it has been shown the equivalent residue is required for proteolytic activity in *EcLon*, where it interacts with the N-terminal part of $\alpha 1$, which contributes to the intersubunit contacts making up the hexamer. This led to the conclusion that the importance of this residue lay in its structural role in maintaining the correct overall fold.¹⁵ Although, in *hLonP*, in the absence of this particular interaction, $\alpha 1$ has moved away from the active site by about 2 Å and that the *Lon* hexamer has not formed, we do observe the presence of two dimers which appear to be components of a proposed *hLonP* hexamer.

The *A/LonP* hexamer shows neither the *E. coli* nor the human conformation. When comparing the *A/LonP* hexamer with *hLonP* and *EcLonP*, however, shows that it appears to be almost an intermediate form between the two. As in *EcLonP*, the loop between $\beta 4$ and $\alpha 2$ has unwound and interacts with the neighboring subunit (albeit in a somewhat different manner). Similarly, it has formed part of $\beta 5$. However, as in *hLon*, its N-terminal tail has been pushed into the cleft between the two domains, possibly disrupting the interface and preventing proper interaction. By preventing proper unfolding of this loop, this insertion may give rise to the unique conformation seen in the *A/LonP* active site. We cannot see how this conformation could be catalytically active given the absence of the serine-lysine dyad.

For this mechanism to be correct, the formation of the hexamer must be necessary for proteolytic activity to occur. This correlates nicely with the biochemical data where the full-length *hLon* is hexameric in solution and shows high peptidase activity both with ATP and with AMP-PNP (nonhydrolyzable analogue), in contrast to the protease domain alone which is a monomer in solution and shows no peptidase activity (Table I).

Although N-terminal degradation was observed in the crystallization solution, this is unlikely to be a result of proteolytic activity by *Lon* itself because the same pattern of degradation is seen when the S855A *hLonP* mutant is overexpressed and purified (data not shown). Furthermore, we tested *hLonP* samples of several concentrations for protease and peptidase activity under several possible crystallization conditions, and in all these cases, the cleavage pattern observed was the same. The addition of the inhibitors MG-132 and lactacystine, which completely block the peptidase activity of the full-length *Lon*, did not suppress this cleavage. Consequently, it is very unlikely that *LonP*, in any oligomeric state, could be responsible for the degradation that we see here.

An additional objection to this mechanism is raised by the mutant *Lon* expressed by the *E. coli lonR9/capR9* gene.³⁸ This mutant is able to form hexamers both with itself and mixed oligomers with *EcLon* wt,³⁹ but it is proteolytically inactive. The only difference between *EcLon* and *LonR9* seems to be an E614K point mutation. Glu614 is located in the subunit interface in the *EcLonP* structure as is Glu781, its equivalent in *hLonP*. In the *EcLonP* structure, Glu614 forms several H-bonds with both the main-chain atoms of Arg710 and Leu709 and with the side chain of Arg710 of the neighboring subunit. Furthermore, this residue is in Van der Waals contact with Pro669, which is part of the active site loop that unfolds to convert the inactive to the active conformation. Therefore, replacing Glu614 with Lys would be expected somewhat to disrupt the hexameric interface, but because this residue is toward the edge of the interface rather than the middle, it would probably not completely abolish it. However, because of its location immediately below the active site loop in the active conformation, it could also plausibly prevent the loop from properly unfolding from the inactive to the active form, in much the same way as the N-terminus of the *A/LonP* structure appears to prevent the correct active site conformation from forming. In other words, instead of preventing the hexamer from forming, this particular substitution should prevent *LonR9* from forming the proteolytically active conformation seen in *EcLonP*; consequently, a crystal structure of the proteolytic domain of *LonR9* or of the mutant E614K ought to show the inactive conformation seen here or, possibly, a conformation similar to that seen in *A/LonP*.

Materials and Methods

Purification

hLonP domain for crystallization (residues 741–959). *hLonP* domain for crystallization (residues 741–959) was overproduced and purified from *E. coli* Rosetta2 cells (Novagen) transformed with the ProEx plasmid containing the *hLonP* domain with a 6 × His tag. Cells were grown in 3% bactotryptone, 2% yeast extract, 1% MOPS pH 7.5 at 37°C until the O.D. 600 nm reached 0.5. Overexpression of recombinant protein was induced by addition of IPTG to 1 mM and further growth of the cultures at 37°C for 90 min. Cells were lysed in 25 mM HEPES pH 8; 150 mM NaCl; 10% glycerol, 10 mM imidazole (Buffer A), and loaded onto a Ni²⁺-NTA affinity column. The column was washed with Buffer A containing 60 mM imidazole, and the tagged protein was eluted in Buffer A plus 250 mM imidazole. The peak containing the proteolytic domain was loaded onto an S200 gel filtration column equilibrated with 25 mM HEPES pH 8, 150 mM NaCl, and 10% glycerol. Fractions containing *hLonP* were dialyzed against 25 mM HEPES pH 8, 150 mM NaCl and concentrated to 15 mg/mL.

Purification of constructs for activity and oligomerization assay. Full-length human Lon and the *hLonP* were overproduced in and purified from the Rosetta *E. coli* strain (Novagen) transformed by plasmids bearing the *hLon* full length lacking its predicted mitochondrial targeting sequence and appropriate truncated form of *hLon*,⁴⁰ all with a 6 × His tag at the N-terminus. The cells were harvested and suspended in buffer A (20 mM HEPES pH 8.0, 150 mM NaCl, 20% glycerol) and sonicated on ice. The lysate was centrifuged at 200,000g at 4°C for 10 min, and the supernatant was loaded onto a Ni²⁺-NTA column (Qiagen). The column was washed thrice with buffer A and five times with buffer A containing 40 mM imidazole. Full-length *hLon* and *hLonP* bound to the column were eluted in three steps with buffer A containing 0.1, 0.2, or 0.3M imidazole.

Multimer formation and stability determination by gel filtration and crosslinking

Gel filtration. Following purification, 6 × His-tagged Lon proteins were loaded in a volume of 200 µL onto a Superose 12 column (Pharmacia) equilibrated with 20 mM HEPES pH 8.0, 150 mM NaCl and 10% glycerol at 4°C. Standards used for size calibration were Blue dextran: 2000 kDa, apoferritin: 440 kDa, BSA: 66 kDa, albumin from hen egg white: 45 kDa, catalase: 232 kDa, aldolase: 158 kDa, carbonic anhydrase: 29 kDa, and cytochrome *c* from bovine heart: 13 kDa.

Chemical crosslinking. Crosslinking experiments were carried out as previously described.^{12,21} The two constructs (20 µg isolated on Ni²⁺-NTA) were cross-

linked using 0.1% glutaraldehyde (Sigma) for 30 min on ice. Crosslinking was stopped by adding reducing sample buffer containing 1M urea and heating at 95°C. Visualization was done on a 3.3% SDS-PAGE stained with Coomassie Brilliant Blue.

Peptidase activity

Peptidase activity was assayed by the fluorescence method⁴¹ using Glu-Ala-Ala-Phe-MNA (GAAP-MNA) as a substrate in a reaction mixture containing 2 µg protease or *hLonP* domain, 50 mM Tris pH 7.9, 5 mM MgCl₂, 0.3 mM fluorogenic peptide, 1 mM ATP or appropriate nonhydrolysable analogue at 37°C for 30 min. Fluorescence was measured on FilterFluorimetrica (Model LS-2B, Perkin-Elmer, Angli-co) at excitation 340 nm and emission 410 nm.

Protein concentration

Protein concentration was estimate by BCA protein assay kit (Pierce Biotechnology) and verified by 10–12% SDS-PAGE and Coomassie staining.

Crystallization, structure solution, and refinement

Crystal screens PACT, HAMPTON I & II and INDEX were set up with protein at a concentration of 15 mg mL⁻¹, using a Mosquito nanoliter-dispensing robot. Promising crystals were obtained following optimization of initial hits in condition F7 of the PACT screen, 0.1M Bis-Tris propanol pH 6.5, 0.25M sodium acetate, and 28% PEG 3350 with 5% glycerol. N-terminal sequencing from a dissolved crystal showed that the crystals contained residues 754–959 of *hLonP* implying that a 13 residue N-terminal truncation had occurred during crystallogenesis.

Data were collected at beamline ID14-4 at the European Synchrotron Radiation Facility (ESRF, Grenoble). Data were integrated using MOSFLM⁴² and scaled and reduced using the CCP4 suite of programs 6.1.0.⁴³ Because of problems associated with twinning and anisotropic diffraction of the crystals, several data sets were collected, of which two were used in the final analysis: one extending to 2.4 Å resolution and the second to 2 Å (Table III). Identification of the correct space group proved to be something of a challenge. Both data sets were originally indexed and integrated in P2₁2₁2, the orthorhombic space group indicated by POINTLESS.⁴⁴ However, as described below, both were finally shown to be in space group P2₁. The value of the β angle was very close to 90° and indeed the value was just over 90° for the 2.4-Å data and just below for the 2-Å crystal. The data merged reasonably well in the orthorhombic space group, with the merging *R* factor being only a little higher when data were merged around the *a** and *c** axes than around *b**.

The structure was first solved in P2₁2₁2 using the 2.0 Å dataset by molecular replacement with

Table III. Data Collection and Refinement Statistics for the hLon Proteolytic Domain

	2.4 Å Data	2 Å Data
Data collection		
Space group	P2 ₁	P2 ₁
Cell dimensions		
a, b, c (Å)	68.24, 82.96, 105.29	69.80, 83.75, 105.49
α, β, γ (°)	90, 89.97, 90	90, 90.05, 90
Resolution (Å)	2.39 (2.52–2.39)	2 (2.11–2)
R_{sym} or R_{merge}	0.096 (0.566)	0.063 (0.73)
$I/\sigma(I)$	14.4 (3.2)	10.9 (1.7)
Completeness (%)	98 (96.7)	99.4 (99.9)
Redundancy	5.5 (5.2)	3.6 (3.7)
Refinement		
Resolution (Å)	2.39	2
No. reflections	42,707	77,079
$R_{\text{work}}/R_{\text{free}}$ (%)	20.85/26.16	19.36/23.23
No. amino acid residues	1098	1100
No. atoms		
Protein	8296	8373
Water	155	330
B -factors (Å ²)		
Protein	47.0	50.5
Water	44.9	51.6
R.m.s. deviations		
Bond lengths (Å)	0.009 (0.022)	0.027 (0.022)
Bond angles (°)	1.147 (1.970)	2.095 (1.968)
Ramachandran plot (%)		
Most favored	91.9	94.5
Additionally allowed	7.9	5.5
Generously allowed	0.2	0
Disallowed	0	0

The numbers in brackets refer to the outer resolution shell. A single crystal was used for each data set. The following residues are disordered or omitted from the final model: chain A: 756–953, loop from 788–797; chain B: 756–948, loop from 784–798; chain C: 755–953, loop from 788–796; chain D: 760–948, loop from 785–797; chain E: 758–948, loop from 786–797; chain F: 759–948, loop from 785–797. For the data collection, the numbers in parentheses correspond to the outer shell of reflections. For the refinement deviations, the values in parentheses are the target values.

MOLREP using the EcLon proteolytic domain (chain A of pdb entry [1lre](#)) as the search model. This identified three independent protomers in the orthorhombic asymmetric unit. Manual corrections to the model were made using COOT⁴⁵ and refinement was carried out with REFMAC.⁴⁶ The resulting structure had an R_{free} of 32% and satisfactory electron density for two of the three chains, but very poor density for the third.

This model was then placed in the cell for the 2.4 Å data and this structure refined, again in P2₁2₁2. The refinement again halted at an R_{free} around 30%. At this stage, the data were reinvestigated and reintegrated in the lower symmetry (and correct) P2₁ space group. In addition, the statistics clearly indicated that the data were twinned. Identification of the correct space group proved to be easier with the 2.4 Å crystals than for that at higher resolution. Refinement then continued with six independent molecules in the asymmetric unit using the recently implemented twinning mode and led to a much improved values for R and R_{free} of 20.8% and 26.1%, respectively, and substantially improved density for the previously poorly ordered third proto-

mer and its pseudo-symmetric mate. The twinning fraction was 0.68.

The 2.0 Å data were reprocessed in the correct P2₁ cell, and the model refined against these data to an R and R_{free} of 19.36% and 23.23%, respectively, again with clear evidence for twinning. The details of data processing and quality of both models are presented in Table III. The data set (2.0 Å) had a twinning fraction of 0.77.

Summary

In summary, we propose that the monomeric form of LonP seen in the present structure and in MjLonP represent inactive conformations of the domain, preventing uncontrolled proteolysis. Formation of a catalytically active form requires the formation of the hexamer accompanied by a conformational change leading to a proteolytically active form similar to that seen in *E. coli*. This hypothesis is supported by our biochemical data.

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