Short Communication

Three-Dimensional Reconstruction of the S885A Mutant of Human Mitochondrial Lon Protease

(transmission electron microscopy / 3D reconstruction / AAA+ protease / human mitochondrial protein)

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Abstract. The Lon protein is a protease belonging to the superfamily of ATPases associated with diverse cellular activities (AAA+). Its main function is the control of protein quality and the maintenance of proteostasis by degradation of misfolded and damaged proteins, which occur in response to numerous stress conditions. It also participates in the regulation of levels of transcription factors that control stress conditions. It also participates in the regulation of many cellular processes, which occur in response to numerous stress conditions (Suzuki et al., 1994; Bulteau et al., 2006). Furthermore, it has been shown to regulate levels of short-lived regulatory proteins involved in many cellular processes (Vieux et al., 2013; Ambro et al., 2014). It also participates in the regulation of many transcription factors and it was shown to play a role in the aging mechanism and replication of mitochondrial DNA as well (Bota et al., 2002; Ngo et al., 2013).

In humans, the altered Lon expression is related to several severe diseases such as lateral sclerosis, myopathy or epilepsy (Venkatesh et al., 2012), and recently mtLon was identified as a potential anti-lymphoma tar-

Received June 27, 2014. Accepted July 21, 2014

This work was supported by the grants: OPVK CZ.1.07/2.3.00/30.0030 from the Ministry of Education, Youth and Sports of the Czech Republic, No. CZ.2.16/3.1.00/24010 from the OPPK programme of the European Fund for Regional Development, the Czech Science Foundation P302/12/G157 and 13-32339P, Prvouk/1LF/1 and UNCE204022 from Charles University in Prague, and by project “BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University” (CZ.1.05/1.1.00/02.0109) from the European Regional Development Fund. V. P., L. A., J. B. and E. K. were supported by the Slovak Research and Development Agency (Grant APVV-0123-10) and by the Slovak Grant Agency (Grant VEGA 2/0113/14).

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get (Bernstein et al., 2012). Despite the fact that Lon protease has been studied in several organisms, neither its structure nor its oligomeric state are known in human cells. Biochemical studies indicated that six or seven protein subunits might assemble into a ring-shaped complex with internal cavity (Ondrovicova et al., 2005; Ambro et al., 2014). Each Lon subunit of the E. coli type (Rotanová et al., 2004) is composed of three separate domains: (1) a very flexible N-domain, (2) a central AAA+ domain, and (3) a C-terminal (proteolytic) domain, which is in charge of the peptidase activity. The Lon protease structure analysed by electron microscopy in E. coli showed a hexameric ring-like shape, in which the two active domains of the subunits, the proteolytic and the nucleotide binding site, seem to be located on each side of the ring, respectively (Park et al., 2006). Recently, it was shown that under physiological conditions the E. coli Lon was able to form a dodecameric structure, which supports the idea of substrate size exclusion by a gating mechanism to control the substrate size by its assembly state. The dodecameric structure will degrade smaller substrates preferentially (Vieux et al., 2013).

The typical mechanism of the Lon’s action may be described by (1) recognition and binding of the substrate to the complex, (2) unfolding of the substrate, (3) translocation of the unfolded substrate to the degradation chamber, (4) degradation of the substrate. All these steps are ATP-dependent reactions (Lee and Suzuki, 2008; Venkatesh et al., 2012). It was shown that the structure of various AAA+ homologue complexes could undergo conformational changes depending on the physiological conditions and/or the binding of its co-factors during the protein degradation cycles (Rouiller et al., 2002; Lee et al., 2007).

Our aim is to establish the Lon structure through the degradation cycle to better understand its mechanism of action. Therefore, we focused on the study of the hLon S885A mutant that has a point mutation (serine to alanine) located in the proteolytic domain at position 885. The mutation has in consequence disabled the proteolytic function of the LON subunits without disturbing the structure of the oligomeric complex (Ambro et al., 2014). The knowledge we acquired regarding its oligomeric state allows us to go further in the understanding of the regulatory mechanism of Lon.

**Material and Methods**

The recombinant hLon production protocol we used is the same as described in Ambro et al. (2012). Shortly, we purified the recombinant hLon by harvesting cells and resuspending them in a buffer containing 20 mM HEPES, pH 8.0, 150 mM NaCl, 20% (v/v) glycerol, and 10 mM imidazole. Then the cells were sonicated on ice, and the cell lysate was centrifuged for 15 min at 100,000 g and the supernatant was loaded onto a Ni²⁺-nitritotriacetic acid column (Qiagen, Hilden, Germany). The cDNAs encoding the Lon point mutants were produced using QuikChange XL site-directed mutagenesis (Agilent Technologies, Santa Clara, CA). Complementary oligonucleotide primers were used to introduce a single-point mutation, which was verified by DNA sequencing (Macrogen, Seoul, Korea). To purify the sample preparation, gel filtration was performed with a Superose™ 6 10/300 GL column (GE Healthcare, Uppsala, Sweden) using a buffer containing 50 mM Tris/ HCl, pH 8.0, 150 mM NaCl, 10% (v/v) glycerol. The fraction of interest was observed using electron microscopy techniques. Grids were prepared according to the carbon sandwich technique (Valentine et al., 1968; Tischendorf et al., 1974). Five µl of S885A protein suspension were applied between the carbon layer and the mica sheet, the sandwich was bathed in 2% uranyl acetate at pH = 4.2 to be negatively stained, and then fished onto a C-flat™ grid (Protochips, Inc., Raleigh, NC). The images were recorded at a final magnification of 62,000 x with a defocus of 300 nm in Tecnai G2 Sphera 20 microscope (FEI, Hillsboro, OR) equipped with a LaB6 cathode and a 2048×2048 Gatan Ultrascan 1000 CCD camera (Gatan, Inc, Pleasanton, CA) of a pixel size 14 Å. The microscope was operated at the accelerating voltage of 120 kV. Data image analysis and 3D reconstruction was done in EMAN2 (Tang et al., 2007).

**Results and Discussion**

Lon has been shown to exist in several oligomeric forms at the same time under physiological conditions (Ambro et al., 2012). In our case, after size exclusion chromatography, the fraction corresponding to the expected size of the hexameric or heptameric complexes in E. coli hLon complex was stabilized by the presence of both Mg²⁺ within the buffer (Rudyak et al., 2001) and ADP bound to the ATPase region. An overview of the sample preparation is shown in Fig.1A. A first dataset of 20,000 particles was collected, some non-convenient structures were easily recognized and discarded from the analysis. The dataset was reduced to a structurally homogeneous set of 4,000 particles entering the three-dimensional refinement. The high number of the rejected particles was also caused by the high flexibility of the N domain and the dynamic properties of the oligomeric complex as well (Ondrovicova et al., 2005). Nevertheless, some particles were structured but about twice the size of a heptameric or hexameric structure, and those were 2D-analysed separately. 2D classification revealed several class averages exhibiting hints of a 6-fold symmetry; one of them is presented in Fig.1B1, and a couple of views identified as side views (Fig.1B2-3). In these side views, extra densities binding the N-terminal domain can be observed (arrows), whose densities however decreased in 3D reconstruction, so that they could be unambiguously recognized and masked. Their presence illustrates the high affinity of the N domains towards substrates. Therefore, we imposed 6-fold symmetry in the three-dimensional refinement and we achieved a structure with a resolution of 19 Å estimated with FSC = 0.5 criterion (Fig.1C). The achieved model of the
hLon mutant is formed as a hexameric ring of 120 Å diameter having 90 Å in height. The structure is in agreement with the early reports of X-ray structures of Lon protease in *Thermococcus onnurineus* (Cha et al., 2010), *Bacillus subtilis* (Duman and Lowe, 2010), and the partially resolved ones in *E. coli* (Li et al., 2010), human (Garcia-Nafria et al., 2010), and the partially resolved ones in *E. coli* (Li et al., 2010). hLon has a diameter of 12 nm, which is close to the one described in *S. cerevisiae* (Stahlberg et al., 1999), but which is smaller than the 16 nm diameter reported in the dodecameric structure of *E. coli* (Vieux et al., 2013). It is important to notice that within the AAA+ family several oligomeric ring-like structures have a diameter of about 14 nm and the height close to 90 Å (Lee et al., 2007). Our result allows us to answer the question about the Lon’s oligomeric state in human. In addition to the fact that hLon is a hexamer we can also assign the positions of the three principal domains to the resolved structure thanks to the high sequence homology with the existing resolved structures (Fig.1C, colour arrows). According to Vieux et al. (2013), the presented single-ring conformation of Lon should be able to degrade large protein substrates. In our preparation, we have also obtained dodecameric class averages in the 2D classification (images not shown). This primary result represents a first step towards deeper understanding of the mechanism of Lon action in humans. We are now working on the establishment of the native structure by the frozen hydrated approach to achieve a resolution improvement and go deeper within the conformational states of each kinetic state.

**References**


