Domain structure and ATP-induced conformational changes in *Escherichia coli* protease Lon revealed by limited proteolysis and autolysis

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Abstract ***Escherichia coli*** **protease Lon (La) is an adenosine triphosphate (ATP)-regulated homo-oligomeric proteolytic complex responsible for the recognition and selective degradation of abnormal and unstable proteins. Each subunit of the protease Lon appears to consist of three functional domains: the C-terminal proteolytic containing a serine active site, the central displaying the ATPase activity, and the N-terminal with still obscure function. We have used limited proteolysis to probe the domain structure and nucleotide-induced conformational changes in the enzyme. Limited proteolysis of the native protease Lon generated a low number of stable fragments roughly corresponding to its functional domains. Conformational changes in the wild-type enzyme and its mutant forms in the presence or absence of adenine and guanine nucleotides were investigated by limited proteolysis. The nucleotide character was shown to play a key role for susceptibility of the protease Lon to limited proteolysis, in particular, for resistance of the ATPase functional domain. ATP and adenosine diphosphate displayed a protective effect of the ATPase domain of the enzyme. We suggest that these nucleotides induce conformational changes of the enzyme, transforming the ATPase domain from the most vulnerable part of the molecule into a spatially inaccessible one. Both limited proteolysis and autolysis demonstrate that the most stable part of the protease Lon molecule is its N-terminal region. Obvious resistance of the protease Lon C-terminus to proteolysis indicates that this region of the enzyme molecule including its substrate-binding and proteolytic domains has a well folded structure.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: ATP-dependent protease; Protease Lon; Functional domain; Limited proteolysis; Autolysis; *Escherichia coli*

1. Introduction

Protein selective proteolytic degradation appears to be rather significant in maintaining homeostasis and metabolism regulation in the cell. Along with short-lived regulatory proteins, the polypeptide chains with disrupted or changed structures selectively split. Such defects might arise from inaccuracy during protein biosynthesis, chemical or physical damage. Intracellular protein degradation is responsible for the rapid turnover of specific regulatory proteins and clearing of abnormal proteins from the cytoplasm. Oligomeric adenosine triphosphate (ATP)-dependent proteolytic complexes essentially contribute to the selective proteolysis of proteins.

Five different ATP-dependent proteases, viz. Lon (La), ClpAP (Ti), ClpXP, FtsH (HflB) and HslUV (ClpYQ) were found in the *Escherichia coli* cells [1]. *E. coli* ATP-dependent protease Lon is a serine protease displaying a chymotrypsin-like activity [2,3]. The enzyme is a tetramer formed with identical subunits, each containing 784 amino acid residues (M_r = 87 304 Da) [4]. The monomer of protease Lon is supposed to consist of three functional domains [5,6]: the N-terminal with its still unknown function, the central ATPase containing a typical ATP-binding motif, the so-called Walker A sequence [7], and the C-terminal proteolytic domain with a catalytically active Ser679 residue [8]. Studies of the substrate-induced activation of the *E. coli* protease Lon indicated that the interaction of protein substrates with the enzyme enhanced its ability to hydrolyze ATP and peptide bonds [9,10]. Recently a 'sensor and substrate discrimination' (SSD) domain of the protease Lon was purified and shown to fold as a monomer and recognize known Lon substrates [11]. Although the enzymatic properties of the protease Lon are thoroughly described, its structural features and the mechanism of interaction between functional domains as well as the reasons for the high selectivity of the enzyme are still obscure. The present studies are aimed at probing the domain structure of the enzyme and obtaining the isolated domains for their further structure–functional investigation. To generate large stable fragments of the protease Lon we found appropriate conditions for limited proteolysis of the wild-type enzyme and of its mutant forms. The boundaries of the structural domains and nucleotide-induced conformational changes of the enzyme molecule were investigated by limited proteolysis. The products of the protease Lon autolysis were also assayed.

2. Materials and methods

2.1. Bacterial strains and plasmid constructs

The wild-type protease Lon and its mutant forms were expressed in the *lon*-deficient *E. coli* strain BL21 [12]. The expression plasmid pLON containing the *lon* gene as well as the expression plasmids

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pLON/SA and pLON/KA coding for the mutant forms of the protease Lon were a kind gift from Prof. Rudi Glockshuber, Institute for Molecular Biology and Biophysics, ETH, Zurich, Switzerland. The expression plasmid pLON/SA codes for proteolytically inactive protease Lon containing amino acid substitution Ser679Ala. The expression plasmid pLON/KA codes for the ATPase-inactive protease Lon containing amino acid substitution Lys362Ala. The cells of *E. coli* BL21 harboring the corresponding plasmid (pLON, pLON/SA or pLON/KA) were grown in Luria–Bertani medium containing 50 μg/ml of ampicillin at 37°C. At an optical density at 550 nm of 1.0, isopropyl-1-thio-β-D-galactopyranoside was added to the final concentration of 1 mM, the cells were grown for 4 h.

2.2. Isolation of the protease Lon and its mutant forms

The wild-type protease Lon and its mutant forms were isolated according to the modified Zehnbauer method [13]. The cells of *E. coli* BL21/pLON were harvested by centrifugation (10 000 × *g*, 4°C, 30 min). The cells were resuspended in 50 mM potassium phosphate buffer solution containing 5% glycerol and 1 mM dithiothreitol (DTT) (pH 6.8) and sonificated at 22 kHz. The extract was centrifuged (100 000 × *g*, 4°C, 1.5 h) and a soluble fraction was sequentially applied onto phosphocellulose P-11 (Whatman, UK) and DEAE-EMD-Fractogel (Merck, Germany) columns as described previously [13]. The obtained protease Lon was about 98% pure as estimated from SDS–electrophoresis in 15% polyacrylamide gel (PAG) according to the Laemmli method [14]. The protein concentration was determined by measuring the absorbance at 280 nm ($\varepsilon_{280} = 47\,000$ cm^{-1} M^{-1}) and according to the Bradford method applying bovine serum albumin as standard [15]. Use was made of the procedure described for isolating wild-type and mutant forms of protease Lon: Lon/S679A and Lon/K362A.

2.3. Enzyme assays

The proteolytic activity of the protease Lon and its mutant forms was measured by the level of the β-casein hydrolysis controlled by SDS–electrophoresis in 15% PAG. The peptidase activity was assayed by hydrolysis of melittin with subsequent mass spectrometry of the hydrolysis products and by hydrolysis of a low molecular weight substrate – thiobenzyl ether of *N*-succinyltripeptide (Suc-Phe-Leu-Phe-SBzl) [16]. The ATPase activity was measured at 37°C in the standard buffer as described in [17] by detecting an inorganic phosphate as a complex with the ammonium molybdate and zinc acetate at 350 nm.

2.4. Limited proteolysis

Limited proteolysis of the protease Lon and its mutant forms was carried out using the protease V8 from *Staphylococcus aureus*. The hydrolysis ran at 30°C for 18 h at an enzyme–substrate ratio of 1:50 in a 50 mM Tris–HCl buffer solution containing 1 mM DTT and 5% glycerol (pH 8.0). The concentration of the nucleotides added (ATP, ADP, adenosine monophosphate (AMP), guanosine triphosphate (GTP), guanosine diphosphate (GDP), guanosine monophosphate (GMP) and non-hydrolyzable ATP analogs: AMPPNP, AMPPCP, AMPCPP) made up 4 mM, as to the Mg^{2+} ions − 15 mM. SDS–electrophoresis in 15% PAG was used for controlling limited proteolysis. The gels were stained with Coomassie brilliant blue G-250 solution [14].

2.5. Autolysis of the isolated Lon protease

Autolysis of the protease Lon was performed at room temperature for 3 days in the absence or in the presence of 4 mM ATP, and controlled by SDS–electrophoresis in 15% PAG. The gels were stained with Coomassie brilliant blue G-250 solution [14].

2.6. Electrophoresis and electroblotting

SDS–electrophoresis was carried out in 15% PAG according to the Laemmli method [14] followed by electroblotting onto the Immobilon-P PVDF membrane (Millipore) for 2 h at 15°C and 400 mA in 0.025 M $NaHCO_3$ buffer solution (pH 9.0) containing 20% methanol and 0.1% SDS. The membrane was washed with methanol, the protein bands were detected by staining with 0.1% solution of amido black 10B. The excised membrane bands containing the protein were used for sequencing and hydrolysis of the C-terminal amino acids.

2.7. N-terminal sequencing

The amino acid sequences were determined using the Applied Biosystems Model 491 Procise cLC Protein Sequencing System (USA).

2.8. Hydrolysis of C-terminal amino acids

Hydrolysis of the C-terminal amino acids of the membrane-immobilized proteins was done in two steps using carboxypeptidases A (55 U/mg protein) and B (460 U/mg protein) in mixture and then carboxypeptidase Y (132 U/mg protein) (all carboxypeptidases were from Calbiochem). To prevent non-specific adsorption of the carboxypeptidases, the Immobilon membrane was treated with polyvinyl pyrrolidone PVP-40 (Serva). The membranes were incubated in 100 μl of 0.5% PVP-40 dissolved in 100 mM acetic acid for 30 min at 37°C. The PVP-40 excess was removed by a five-fold washing with water and a two-fold washing with 0.1 M NH_4HCO_3, pH 8.5. The incubation with carboxypeptidases A and B was carried out for 1 h at 37°C at pH 8.5. After supernatant removal the incubation with carboxypeptidase Y was performed for 1 h at 37°C at pH 6.0. The carboxypeptidase A, B, Y supernatants were collected, dried and analyzed by dansylating the amino acid mixture as described in [18].

3. Results and discussion

3.1. Limited proteolysis of the wild-type protease Lon

In this study, we have used limited proteolysis to probe the domain organization of the *E. coli* protease Lon. With the aim of cleaving the enzyme polypeptide chain within the most accessible regions and obtaining the fragments which might correspond to functional domains of the protease Lon, limited proteolysis was of much help. Different proteolytic enzymes and cleavage conditions were assorted so that the formation of large stable fragments of the protease Lon could be observed. To this purpose there were used such enzymes as trypsin, chymotrypsin, thermolysine, duodenase, and protease V8 from *St. aureus* in different enzyme–substrate ratios and under assorted hydrolysis conditions (*T* in °C, time, pH). The best results were achieved with the protease V8 from *St. aureus*, known to be specific to the peptide bonds formed by a glutamic acid carboxyl group (Glu-X). Despite the fact that the protease Lon contains 69 Glu-X bonds, distributed rather evenly along the polypeptide chain, most of them were not hydrolyzed under limited digestion conditions (enzyme–substrate ratio 1:50, 18 h, 30°C, pH 8.0). Two major stable fragments of molecular masses 27 and 33 kDa resulted from limited proteolysis of the wild-type protease Lon in the absence of nucleotides (Fig. 1). The obtained fragments were separated by SDS–electrophoresis and electroblotted onto the Immobilon-P membrane. The N-terminal protein sequencing and analysis of C-terminal amino acids with carboxypeptidases

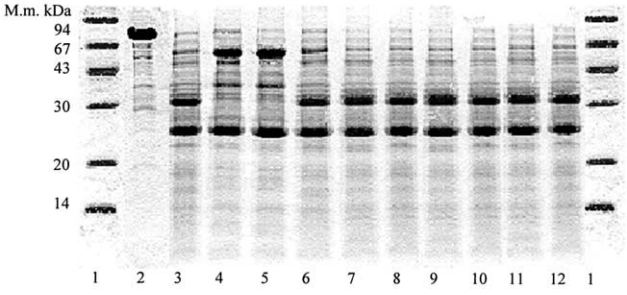

Fig. 1. Limited proteolysis of the *E. coli* protease Lon (2) in the absence (3) or in the presence of 4 mM ATP (4), ADP (5), AMP (6), GTP (7), GDP (8), GMP (9), AMPPNP (10), AMPPCP (11), AMPCPP (12). Analysis by SDS–PAGE in 15% gel; (1), molecular mass standard.

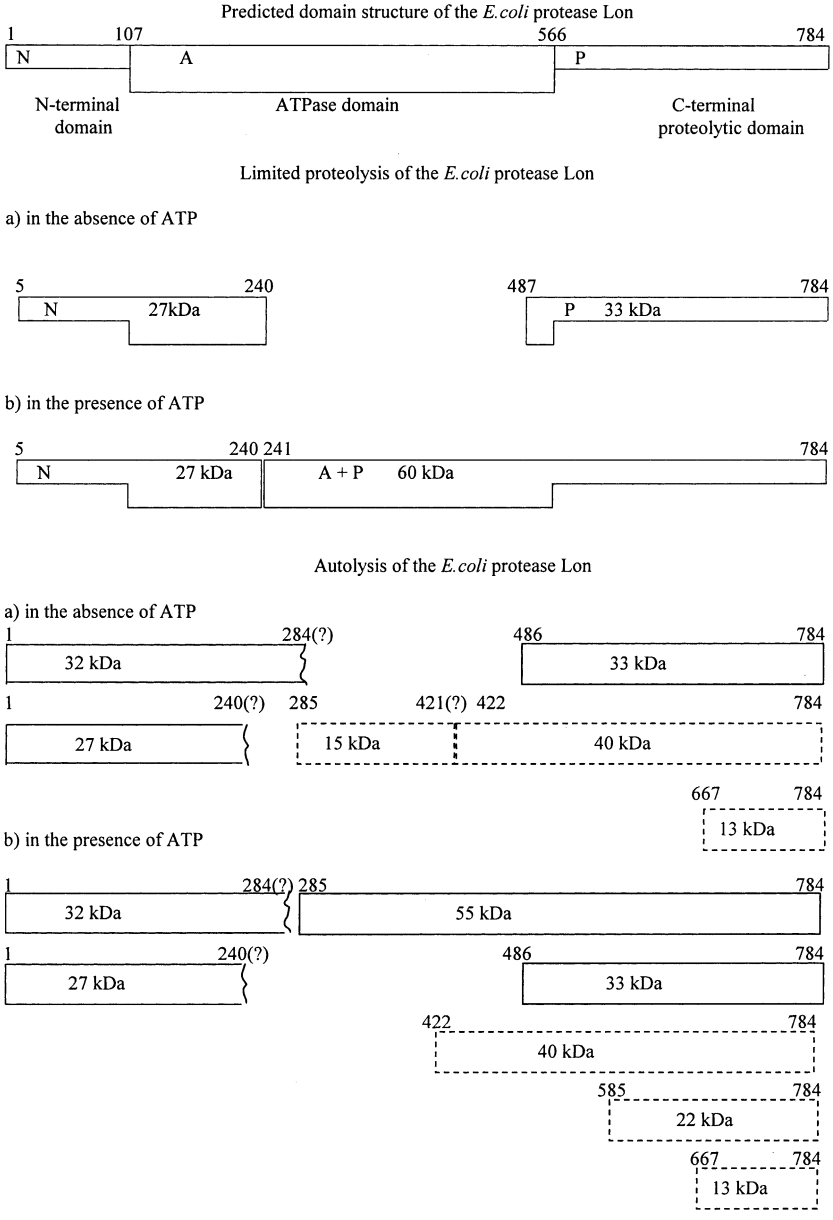

Fig. 2. Fragmentation of the *E. coli* ATP-dependent protease Lon by limited proteolysis and autolysis.

have been performed to clarify the fragments boundaries. A smaller fragment (Arg5–Glu240, 27 kDa) was localized in the N-terminal region of the molecule. The C-terminal fragment (Val487–Lys784, 33 kDa) included the substrate-binding SSD and proteolytic domains of the enzyme (Fig. 2).

3.2. *Nucleotide binding and Mg^{2+} influence on the protease Lon fragmentation*

Our previous studies [19] demonstrated protection of protease Lon from proteolytic digestion under limited proteolysis conditions afforded by ATP binding. These data substantiate the observation that in the presence of ATP the enzyme molecule undergoes conformational changes, reducing its susceptibility to proteolysis. We wanted to know whether such conformational changes were produced by other ligands besides ATP. A series of studies was performed in the presence of ADP, AMP, GTP, GDP, GMP or non-hydrolyzable ATP analogs: AMPPNP, AMPPCP, AMPCPP as well as of the Mg^{2+} ions. The nucleotide influence on fragmentation of the wild-type protease Lon and its mutant forms was studied in parallel. The enzyme-limited proteolysis in the presence of ATP or ADP led to the formation of a large polypeptide chain fragment (Leu241–Lys784, 60 kDa) containing the ATPase and proteolytic domains (Fig. 1). The obtained N-terminal fragment (Arg5–Glu240, 27 kDa) was identical to that formed upon limited proteolysis in the absence of nucleotides (Fig. 2). Limited proteolysis in the presence of AMP, non-hydrolyzable ATP analogs, and guanine nucleotides produced fragments similar to those formed in the absence of nucleotides (Fig. 1). Thus, addition of AMP, non-hydrolyzable ATP analogs or guanine nucleotides had no influence on the protease Lon conformation. Interestingly, the presence or absence of Mg^{2+} did not affect the fragmentation in the above cases (data not shown).

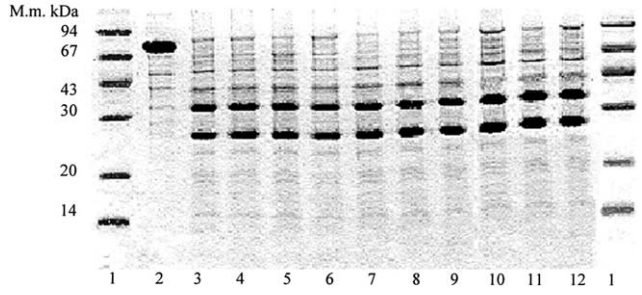

Fig. 3. Limited proteolysis products of the Lon/KA mutant form of the *E. coli* protease Lon (2) in the absence (3) or in the presence of 4 mM ATP (4), ADP (5), AMP (6), GTP (7), GDP (8), GMP (9), AMPPNP (10), AMPPCP (11), AMPCPP (12). Analysis by SDS–PAGE in 15% gel; (1), molecular mass standard.

Thus, it is clear that the presence of ATP or ADP induces conformational changes in the enzyme structure. Nucleotide binding was shown to be crucial for susceptibility of the protease Lon to limited proteolysis, in particular for the resistance of its ATPase functional domain. In the presence of ATP or ADP, the ATPase domain is transformed from the most vulnerable to proteolysis part of the molecule into a spatially inaccessible one, evidently embedded inside the globular or oligomeric structure.

3.3. Limited proteolysis of the mutant forms of the protease Lon

The results obtained with the Lon/S679A mutant form of the enzyme were absolutely identical to those of the wild-type protease Lon. Besides, the fragmentation character in the presence or absence of nucleotides did not differ from that of the wild-type protease Lon either (data not shown). This comparative characterization demonstrated that replacement of catalytically active Ser679 by Ala did not entail essential difference in the enzyme fragmentation by limited proteolysis and did not influence the character of the ATP-induced conformational changes of the enzyme. This accords well with the previous studies [12], suggesting that Ser679 was likely not to be involved in the formation and stabilization of the three-dimensional structure of the protease Lon.

Limited proteolysis of the Lon/K362A mutant form of the protease Lon resulted in fragments of molecular masses 27 and 33 kDa in the presence or absence of nucleotides (Fig. 3); this confirms the importance of Lys362 in the protease Lon binding to nucleotides. As expected, ATP or ADP addition was not accompanied by any changes in the fragmentation character. As Lys362 was supposed to interact directly with the β- or γ-phosphate of ATP [20], the replacement of Lys362 by Ala caused the indifference of the Lon/K362A mutant enzyme to ATP addition not accompanied by any noticeable conformational changes of the molecule.

3.4. Autolysis of the protease Lon

The protease Lon autolysis took place both in the presence and in the absence of ATP. The dominant fragment resulting from the protease Lon autolysis in the presence or absence of ATP had molecular mass 27 kDa (Fig. 4) and turned out to be an N-terminal fragment practically identical to the N-terminal product obtained by limited proteolysis but beginning with the N-terminal amino acid residue of the protease Lon. This fragment displayed a high stability to further cleavage and dominated among other autolysis products. Another N-terminal fragment of molecular mass 32 kDa was obtained after autolysis in the presence or absence of ATP. These data demonstrate that the most stable part of the protease Lon molecule is invariably its N-terminal region. Obvious resistance of the protease Lon N-terminus to proteolysis indicates that this region of the enzyme molecule has a well folded structure.

The C-terminal fragments resulting from the autolysis in the presence and absence of ATP had molecular masses 40, 33, 22 and 13 kDa (Fig. 2), moreover, the latter two were obtained in trace amounts as a result of internal proteolysis of larger intermediate products. The fragment of 22 kDa was observed only in the presence of ATP. The fragment of 33 kDa resulting from the protease Lon autolysis in the presence or absence of ATP corresponded to the C-terminal fragment obtained by limited proteolysis in the absence of ATP. This fragment dominated among other C-terminal autolysis products. The above results demonstrated a conformational stability of the C-terminal part of the protease Lon molecule including its substrate-binding SSD and proteolytic domains.

A protective effect of ATP was demonstrated by autolysis in the presence of the nucleotide that prevented the cleavage of the fragment of molecular mass 55 kDa, containing the ATPase and proteolytic domains, and corresponding to the polypeptide 60 kDa resulting from limited proteolysis in the presence of ATP. The results of the autolysis in the absence of ATP (Fig. 4) showed predominant cleavage of the ATPase domain region. The fragments of 15 kDa (localized in the ATPase region) and 40 kDa resulting from internal proteolysis of the fragment of 55 kDa were obtained in trace amounts.

Together with the results of limited proteolysis of the *E. coli* protease Lon, the obtained data revealed conformational changes in the enzyme molecule in response to the ATP presence. ATP displayed a protective effect on the ATPase region, which turned out to be the most accessible to proteolysis. Mg^{2+} did not affect the enzyme cleavage. Both limited proteolysis and autolysis revealed relative resistance of N- and C-terminal parts of the enzyme molecule. The above results demonstrated a conformational stability of the proteolytic and N-terminal domains of the protease Lon. The information on the domain organization of the protease Lon is essen-

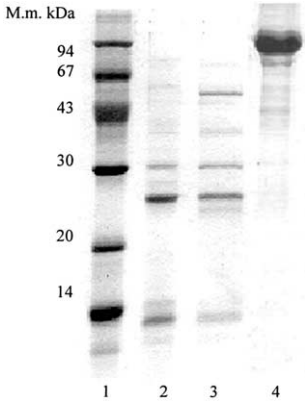

Fig. 4. Autolysis of the *E. coli* protease Lon (4) in the absence (2) or in the presence of 4 mM ATP (3). Analysis by SDS–PAGE in 15% gel; (1), molecular mass standard.

tial for a better understanding of the interactions between the functional parts of ATP-dependent proteases.

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