LIMITED PROTEOLYSIS OF *Escherichia coli* ATP-DEPENDENT PROTEASE LON

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***E. coli* ATP-dependent protease Lon is a homooligomeric proteolytic complex, where each subunit consists of three functional domains: the C-terminal proteolytic, the central displaying the ATPase activity, and the N-terminal, the function of which is still unclear. To study the properties of the isolated functional domains of the native enzyme we employed the experimental conditions for limited digestion of the protease Lon by the *St. aureus* protease V8 that provided obtaining a low number of large fragments roughly corresponding to its functional domains. There were also characterised limited proteolysis of the protease Lon and its mutant forms in the presence or absence of adenine or guanine nucleotides. The character of the nucleotide was shown to play a key role for interacting with nucleotide-binding site of protease Lon. This suggests that ATP or ADP binding causes the enzyme conformation changes, when the ATPase domain transforms from the most vulnerable part of the molecule into a spatially inaccessible one, evidently hidden inside a globular or an oligomeric structure.**

Protein selective proteolytic degradation appears to be rather significant in homeostasis maintaining and metabolism regulation in the cell. Along with short-lived regulatory proteins, the polypeptide chains with a disrupted or changed structures are selectively hydrolysed. Such defects might arise from inaccuracy during protein biosynthesis, chemical or physical damage. Oligomeric ATP-dependent proteolytic complexes essentially contribute into the selective digestion of such proteins.

Five different ATP-dependent proteases, viz. Lon (La), ClpAP (Ti), ClpXP, FtsH (HflB) and HslVU (ClpQY) were found in the *E. 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 (Mr. 87304 Da) [4]. A monomer of protease Lon is supposed to consists of three functional domains [5, 6]: the N-terminal, with its still unknown function, the central ATPase covering so-called A and B Walker's motives (ATP-binding fragment GPPGVGKT and region KVGCKNPLFLLD responsible for coordination of Mg^{2+}) [7]; the C-terminal proteolytic domain containing a catalytically active residue Ser679 [8].

Although the enzymatic properties of protease Lon are thoroughly described, its structural features as well as the coupling mechanism of ATAase and proteolytic activities are still obscure. The present studies are aimed at obtaining the enzyme isolated domains for their further structure-functional investigation. To tackle this problem successfully, we tried to find appropriate conditions for limited proteolysis of protease Lon and its mutant forms. The influence was studied of adenine and guanine nucleotides on the fragmentation of the protease Lon under the found conditions of limited proteolysis.

Methods of Research

To obtain protease Lon, the strain BL21 *E. coli* cells containing no gene *lon*, were transformed by a plasmid vector pLON involving a full gene *lon* with its own regulatory elements [9]. Biosynthesis of protease Lon mutant forms containing amino acid substitutions Ser679Ala and Lys362Ala was performed in the *E. coli* cells BL21, converted by plasmid vectors pLON/SA and pLON/KA, respectively. The plasmids were kindly granted by Prof. R. Glockshuber, the Institute of Molecular Biology and Biophysics, ETH (Zürich, Switzerland).

These cells carrying native or mutant genes for protease Lon were grown in the LB medium containing 50 μg/ml of ampicillin. After incubation for one night at 30°C and for 2h at 42°C isolation was carried out by the Zehnbauer modified method [10]. *E. coli* cells were precipitated by centrifuging, resuspended in the 0.1 M potassium-phosphate buffer, pH 6.8, 1 mM DTT, 5% glycerol (buffer A), then subjected to destruction with lysozyme and by ultrasound. Ultrasound cell destruction was done by desintegrator UZDN-4A (22 Hz, three 40 sec-cycles, with a 30 sec-cycle pause). The lysate was centrifuged at 100 000 g for 1.5 h. The enzyme isolation at all its stages was conducted at 4°C.

Free cellular extract was separated by chromatography on phosphocellulose P-11 (Whatman, UK) in the buffer A (column 1.5×10 cm) at the flow rate 0.25 ml/min. The column was washed in the buffer A (150 ml) and eluted by the 0.5 M potassium-phosphate buffer, pH 6.8, 1 mM DTT, 5% glycerol (100 ml). The collected fractions containing protease Lon was chromatographed on the column (1.5×10 cm) with DEAE-EMD-Fractogel (Merck, Germany) and equilibrated by 50 mM Tris-HCl buffer, pH 8.0, 1 mM DTT, 5% glycerol (buffer B). The elution ran with the rate 0.25 ml/min in the

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NaCl linear gradient (0–1.0 M) in the buffer B (100 ml). The enzyme was purified up to 98%.

SDS-Electrophoresis was used for controlling the isolation in the 15% PAG according to the Laemmli method [11].

Applying BSA as a standard, the protein concentration was measured according to the Bradford method [12].

Use was made of the procedure described for isolating native as well as mutant forms of protease Lon: Lon/S679A and Lon/K362A.

The proteolytic activity of the protease Lon and its mutant forms were measured by the level of the β-casein hydrolysis and controlled by SDS-electrophoresis in the 15% PAG, as well as by hydrolysis of a low molecular weight substrate—thiobenzyl ether of N-succinyltripeptide (Suc-Phe-Leu-Phe-SBzl) [13].

The ATPase activity was established by the amount of inorganic phosphate released upon the ATP hydrolysis. The phosphate was spectrophotometrically detected by the reaction with the ammonium molybdate and zinc acetate [14].

Limited proteolysis of protease Lon and its mutant forms were carried out using protease V8 from *St. aureus*. The hydrolysis ran at the enzyme-substrate ratio 1 : 50, at 30 °C for 18 h in the buffer solution containing 50 mM Tris-HCl buffer, 1 mM DTT, 5% glycerol (pH 8.0). The concentration of the nucleotides added (ATP, ADP, AMP, GTP, GDP, GMP, and nonhydrolysed ATP analogues: AMPPNP, AMPPCP, AMPCPP) made up 4 mM, as to the Mg^{2+} ions—15 mM. SDS-Electrophoresis was used for controlling limited proteolysis in the 15% PAG.

To determine the amino acid sequence of the protease Lon fragments, electroblotting was employed to transfer them onto the membrane of immobilon-P. Electroblotting was performed in 0.025 M $NaHCO_3$ buffer solution (pH 9.0) containing 20% methanol and 0.1% SDS, for 2h at 15 °C and 400 mA. The membrane was washed with methanol, the protein bands were detected by staining with 0.1% water solution of amidoblack 10B. The amino acid sequences of the above fragments were established by Applied Biosystems gas phase protein sequencer 470A (USA); PTH-derivatives of the amino acids were identified by Applied Biosystems PTH-analyzer 120A (USA).

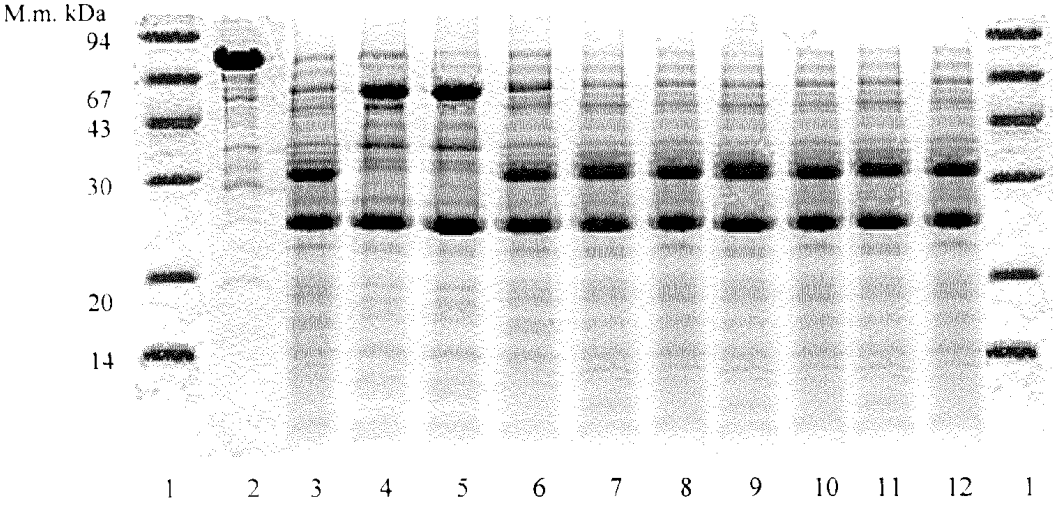

Fig. 1. Electrophoresis of limited proteolysis products of protease Lon from *E. coli* (2) in the absence (3) and in the presence of adenine and guanine nucleotides (4 mM): ATP (4), ADP (5), AMP (6), GTP (7), GDP (8), GMP (9), AMPPNP (10), AMPPCP (11), AMPCPP (12); (1)—protein SDS-PAGE standards.

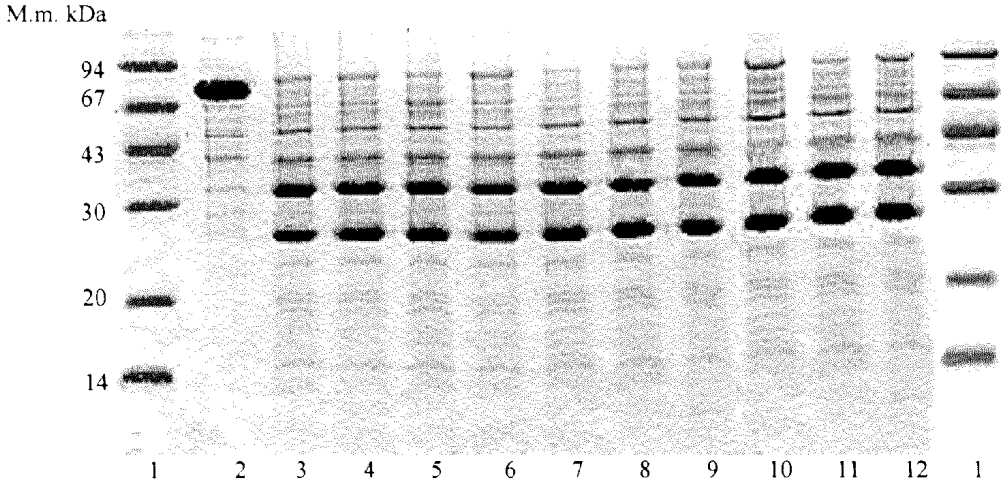

Fig. 2. Electrophoresis of limited proteolysis products of Lon/KA mutant form (2) in the absence and (3) in the presence of adenine and guanine nucleotides (4 mM): ATP (4), ADP (5), AMP (6), GTP (7), GDP (8), GMP (9), AMPPNP (10), AMPPCP (11), AMPCPP (12); (1)—protein SDS-PAGE standards.

Results and Discussion

The *E. coli* ATP-dependent protease Lon is a homooligomeric energy-dependent proteolytic complex, with the subunits each consisting of three functional domains: the C-terminal proteolytic, the central displaying the ATPase activity, and the N-terminal, with the function still unclear. With the aim in view to cleave the enzyme polypeptide chain within the interdomain segments and to obtain the fragments, which might resemble the functional domains of the protease Lon, the method of limited proteolysis was of much help here.

To tackle the problem, the proteolytic enzymes and appropriate conditions were assorted, so that the formation of stable large fragments could be observed. For the research there were used such enzymes as trypsin, chymotrypsin, thermolysin, duodenase, and protease V8 from *St. aureus* in different enzyme-substrate ratios and under different conditions of hydrolysis (t °C, time, pH). The positive results were achieved when using 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 protease Lon contains 69 –Glu–X– bonds, distributed rather evenly polypeptide chain , most of them are not hydrolysed during limited proteolysis (enzyme–substrate ratio 1 : 50, 18 h, 30 °C, pH 8.0). Evidently, spatially accessible peptide bonds within interdomain segments are mainly hydrolysed.

The N-terminal fragment (Arg5-Glu240, 25 kDa) and the C-terminal one (Val487-Lys784, 33 kDa), including a proteolytic domain, resulted from limited proteolysis of the native protease Lon in the nucleotide absence. A series of studies was performed in the presence of ATP, ADP, AMP, GTP, GDP, GMP, and nonhydrolysed ATP analogues: AMPPNP, AMPPCP, AMPCPP as well as of the Mg^{2+} ions. An influence of these nucleotides on the fragmentation character of the native protease Lon and its mutant forms was studied. It turned out that limited proteolysis of the enzyme only in the presence of ATP or ADP led to the formation of a large polypeptide chain fragment (Leu241-Lys784, 63 kDa) containing both the ATPase and proteolytic domains. The N-terminal fragment (25 kDa) identical with that formed upon the limited proteolysis in the nucleotide absence was also obtained. Limited proteolysis in the presence of AMP, nonhydrolysed ATP analogues as well as guanine nucleotides produced the fragments similar to those formed in the nucleotide absence (Fig. 1). Interestingly, the Mg^{2+} ions present in the above cases did not affect proteolysis. Addition of AMP, nonhydrolysed ATP analogues or guanine nucleotides obviously gave no effect on the protease Lon fragmentation.

Thus it becomes clear that the presence of ATP (or ADP) induces changes in the enzyme spatial structure. The results obtained echoed well the assumption that ATP is an allosteric effector on the functioning of the ATP-dependent protease Lon. Consequently, the nucleotide itself appears to be significant during interaction with the nucleotide-binding site of protease Lon. ATP or ADP affects much the enzyme conformation changes, when the ATPase domain transforms from the most vulnerable part of the molecule into a spatially inaccessible one, evidently, hidden inside a globular or an oligomeric structure.

The results obtained for the enzyme Lon/S679A mutant form were identical, viz. the replacement of catalytically active Ser679 by Ala did not entail essential changes in the enzyme conformation.

Limited proteolysis of the enzyme Lon/K362A mutant form resulted in the fragments with Mr. 25 kDa and 33 kDa both in the nucleotide presence or absence (Fig. 2); this confirms an importance of Lys362 in protease Lon binding to nucleotides. As expected the replacement of Lys362 by Ala is not accompanied by conformational changes of protease Lon in response to ATP and ADP addition.

References

1. Gottesman S. (1996) *Regulation of Gene Expression in Escherichia coli*, ed. by E.C.C. Lin and A. Simon Lynch, RG Landers Co., 503–519.
2. Goldberg A.L., Swamy K., Chung C., and Larimore F. (1981) *Meth. Enzymol.,* **80,** 680–702.
3. Goldberg A.L., Morschell R.P., Chung C.H., and Maurizi M.R. (1994) *Meth. Enzymol.*, **244,** 350–375.
4. Chin D.T., Goff S.A., Webster T., Smith T., and Goldberg A.L. (1988) *J. Biol. Chem.*, **263,** No. 24, 11718–11728.
5. Maurizi M.R. (1992) *Experientia,* **48,** 178–201.
6. Rotanova T.V., Kotova S.A., Amerik A.Yu., Lykov I.P., Ginodman L.M., and Antonov V.K. (1994) *Russian J. of Bioorg. Chem.*, **20,** 114–125.
7. Yoshida M. and Amano T. (1995) *FEBS Letters,* **359,** No. 1, 1–5.
8. Amerik A.Yu., Antonov V.K., Gorbalenya A.E., Kotova S.A., Rotanova T.V., and Shimbarevich E.V. (1991) *FEBS Letters,* **287,** No. 1–2, 211–214.
9. Fischer H. and Glockshuber R. (1993) *J. Biol. Chem.*, **30,** 22502–22507.
10. Zehnbauer B.A., Foley E.C., Henderson G.W., and Marcovitz A. (1981) *Proc. Natl. Acad. Sci. USA*, **78,** No. 4, 2043–2047.
11. Laemmli U.K. (1970) *Nature*, **227,** 680–685.
12. Bradford M.M. (1976) *Anal. Biochem.*, **73**, 248–254.
13. Fischer H. and Glockshuber R. (1994) *FEBS Letters*, **356,** 101–103.
14. Melnikov E.E., Tsirulnikov K.B., Ginodman L.M., and Rotanova T.V. (1998) *Russian J. of Bioorg. Chem.*, **24,** 293–299.