FEBS Letters 422 (1998) 218–220 FEBS 19780

# Mutations in the proteolytic domain of *Escherichia coli* protease Lon impair the ATPase activity of the enzyme

Natalie N. Starkova*, Ekaterina P. Koroleva, Lev D. Rumsh, Lev M. Ginodman, Tatyana V. Rotanova

*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya st. 16/10, 117871 Moscow, Russia*

Received 18 December 1997

**Abstract Conserved residues of the proteolytic domain of *Escherichia coli* protease Lon, putative members of the classic catalytic triad (H665, H667, D676, and D743) were identified by comparison of amino acid sequences of Lon proteases. Mutant enzymes containing substitutions D676N, D743N, H665Y, and H667Y were obtained by site-directed mutagenesis. The mutant D743N retained the adenosine triphosphate (ATP)-dependent proteolytic activity, thereby indicating that D743 does not belong to the catalytic site. Simultaneously, the mutants D676N, H665Y, and H667Y lost the capacity for hydrolysis of protein substrates. The ATPase activity of these three mutants was decreased by more than an order of magnitude, which suggests a close spatial location of the ATPase and proteolytic active sites and their tight interaction in the process of protein degradation.**



*Key words:* Adenosine triphosphate-dependent proteolysis; Protease Lon; Active site; Site-directed mutagenesis; *Escherichia coli*

## 1. Introduction

Proteases Lon are the most studied among enzymes performing adenosine triphosphate (ATP)-dependent degradation of proteins in prokaryotes and eukaryotes [1–5]. In *Escherichia coli* cells, protease Lon hydrolyses with a high selectivity abnormal and foreign overexpressed proteins, as well as some short-lived regulatory proteins [3–5].

*E. coli* protease Lon is an oligomeric enzyme composed of four identical subunits [1]. It was suggested that each subunit consists of three functional domains [6,7]: N-terminal, whose function is still unknown; central ATPase domain; and C-terminal proteolytic domain, which includes the early identified catalytically active serine residue S679 [8].

Proteolysis catalyzed by protease Lon is strictly coupled to ATP hydrolysis. Since hydrolysis of a peptide bond does not require energy, the role of ATP hydrolysis in proteolysis remains obscure. ATP was proposed to be involved in promoting conformational changes in the enzyme or substrate unfolding and translocation in the substrate-binding region of protease Lon [5,9]. The enzyme exhibits the ATPase activity in the absence of protein substrates; however, addition of such substrates stimulates ATP hydrolysis.

At present, the mechanism of the ATP hydrolysis coupling to proteolysis and the reasons of the high selectivity of protease Lon are poorly understood. Elucidation of the structure of the proteolytic active site of the enzyme may promote the solution of these problems. This work was designed to reveal by site-directed mutagenesis of putative functionally active amino acid residues of the proteolytic domain of *E. coli* protease Lon involved in the formation of the catalytic site and/or in the coupling of proteolytic and ATPase activities of the enzyme.

## 2. Materials and methods

Transformation of competent cells, DNA digestion with restriction endonucleases, DNA isolation from agarose gel and ligation were performed by standard procedures [10]. Plasmids were isolated using a Wizard™ DNA isolation kit (Promega, USA) as recommended by the manufacturer.

Amino acid sequences were compared using MEGALIGN program of the DNASTAR program package. Mutagenesis was performed by SOE (splicing by overlapping extention) method of PCR mutagenesis [11]. The structures of the PCR products were verified by sequencing by dideoxynucleotide method using Sequenase™ v. 2.0 (USB, USA).

Wild-type and mutant forms of Lon protease were expressed in *E. coli* cells AB1899. The enzymes were isolated as described in [7]. Electrophoresis was performed by Laemmli's method [12].

The proteolytic activity of the isolated enzymes was assayed by measuring the degradation of [$^{14}$C]acetyl-α-casein in the absence and presence of 3 mM ATP [7,13]. The ATPase activity was determined from the amount of released inorganic phosphate as described in [14] with modifications [15].

## 3. Results and discussion

### 3.1. Identification of residues, potential members of the catalytic site of E. coli protease Lon

The search for putative catalytically active residues of *E. coli* protease Lon was based on the suggestion that the catalytic site of the enzyme is similar to those of classic serine proteases [16,17] and includes histidine and aspartic acid residues in addition to the earlier revealed serine residue S679 [8].

It is known that functionally active residues are usually located in highly conserved regions of proteins. We compared amino acid sequences of proteolytic domains of Lon proteases from different organisms (Fig. 1). Despite the fact that the enzymes compared were from evolutionary distant organisms, the homology between their proteolytic domains was found to be considerably high (over 50%). It appeared that these enzymes contain no conserved sequences that are characteristic of other families of serine proteases (chymotrypsin, subtilisin) and include residues of the classic catalytic triad [16,17]. The earlier identified active serine residue S679 of *E. coli* protease Lon was found to be located in the strictly conserved fragment PKDGPS*AG that has not been found in any known serine proteases. Proteolytic domains of protease Lon contain only two conserved histidine and two conserved aspartic acid residues. In *E. coli* protease Lon, they are H665, H667, D676,

*Corresponding author. Fax: (7) (95) 3107007.
E-mail: nr@enzyme.siobc.ras.ru


*PII* S0014-5793(98)00012-X

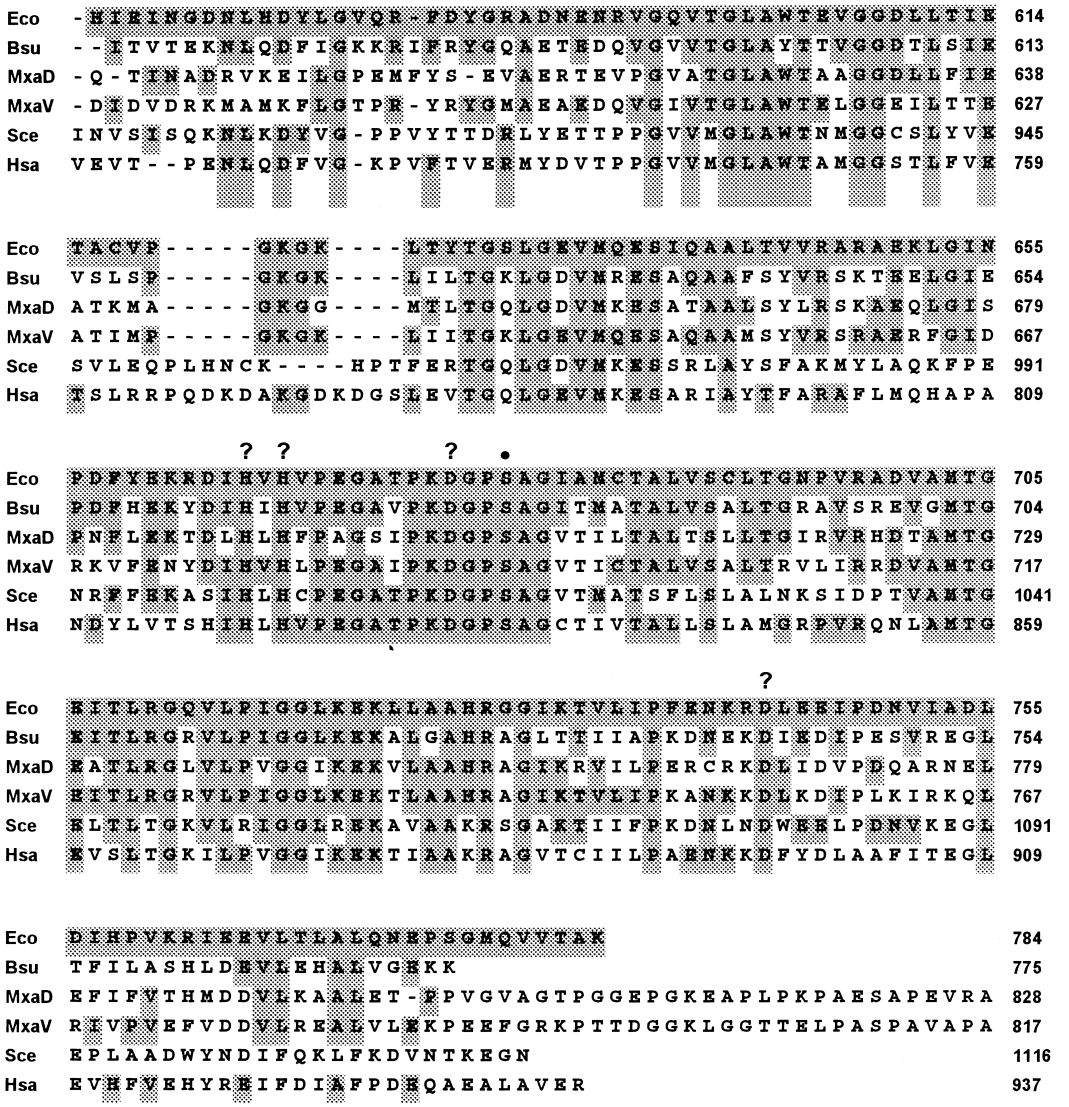


Fig. 1. Comparison of amino acid sequences of proteolytic domains of proteases Lon. Eco, *E. coli* [16]; Bsu, *Bacillus subtilis* [19]; MxaV, *Myxococcus xanthus* V [20]; MxaD, *M. xanthus* D [21,22]; Sce, *Saccharomyces cerevisiae* [23]; Hsa, human brain [24,25]. Identical amino acid residues are dashed. Putative members of the catalytic triad (?) and active serine (●) are indicated.

and D743 (Fig. 1). The first three residues are located in close vicinity to S679 in the highly conserved fragment I664–G681. Such a close location of catalytically active residues has not been noted earlier in classic serine proteases. To elucidate the possible role of the identified residues in enzyme functioning, we subjected them to site-directed mutagenesis.

### 3.2. Site-directed mutagenesis and characterization of mutants

Mutants H665Y, H667Y, D676N, and D743N were obtained by SOE PCR-mutagenesis [11] using the pBRlon plasmid bearing the full-size wild-type *lon* gene under the control of its native regulatory region [6].

The mutants were characterized by the ability of the mutant plasmids to affect the phenotype of *E. coli* cells AB1899. When grown on a mineral medium, these cells display a characteristic phenotype (slimming of bacterial colonies because of overproduction of capsular polysaccharides and formation of typical elongated structures) [2,5]. Transformation of *E. coli* cells AB1899 with the pBRlon plasmid leads to the complete disappearance of the mutant phenotype. We found that transformation of *E. coli* cells AB1899 with the pBRlonD743N plasmid also completely eliminated the mutant phenotype, whereas the plasmids pBRlonH665Y, pBRlonH667Y, and pBRlonD676N failed to restore the normal phenotype. Therefore, in the case of pBRlonD743N, the active enzyme was synthesized in the cells; in other cases, it could be expected that the proteins of mutants H665Y, H667Y, and D676N were inactive.

### 3.3. The catalytic activity of mutant enzymes

The mutant proteins were expressed in *E. coli* AB1899 cells and isolated as described earlier [7]. It appeared that the mutant D743N retained the ATP-dependent proteolytic activity, therefore D743 is not a catalytically active residue. Three other mutants completely lost the capacity for casein hydrolysis (Table 1), which shows that residues D676, H665, and H667 are essential for the proteolytic activity of the enzyme.

Interesting results were obtained when we assayed the ATPase activity of the proteolytically inactive mutants. Mutation of the catalytically active serine did not affect the ATPase

Table 1
The catalytic activity of *E. coli* protease Lon and its mutant forms

| Mutant | Proteolytic activity, % | ATPase activity | | | | | |
|---|---|---|---|---|---|---|---|
| | | In the absence of casein | | | In the presence of 0.3 mg/ml casein | | |
| | | $k_{cat}$, min | $K_M$, mM | $k_{cat}/K_M$, mM/min | $k_{cat}$, min | $K_M$, mM | $k_{cat}/K_M$, mM/min |
| Lon (wild-type) | 100 | 19 | 0.16 | 119 | 48 | 0.18 | 267 |
| S679A | 0 | 19 | 0.17 | 112 | 39 | 0.20 | 195 |
| H665Y | 0 | 2.8 | 0.54 | 5.18 | 5.2 | 0.51 | 10.2 |
| H667Y | 0 | 1.9 | 0.50 | 3.80 | 4.2 | 0.48 | 8.75 |
| D676N | 0 | 4.0 | 0.56 | 7.14 | 4.4 | 0.58 | 7.59 |

The reaction mixture contained 0.3–3 mM ATP, 10 mM $MgCl_2$, 150 mM NaCl, 50 mM Tris-HCl (pH 7.8), 20 μg of the enzyme.

activity of protease Lon (Table 1). This result is consistent with the data obtained earlier [18]. Surprisingly, the ATPase activity ($k_{cat}/K_m$) of the mutant proteins D676N, H665Y, and H667Y was by more than an order of magnitude lower than that of the wild-type enzyme and the S679A mutant (Table 1). It should be noted that such a low ATPase activity could hardly be the reason for elimination of the proteolytic activity, since it was found that certain Lon mutants with substitutions in the ATPase domain, whose ATPase activity was decreased to the same extent, retain the capacity for efficient hydrolysis of casein [15]. The effect of mutations in the proteolytic domain on the ATPase activity may be explained by the close spatial location of the two catalytic sites of the enzyme, and deteriorations in the structure of the proteolytic site may affect the functioning of the ATPase catalytic site. Additionally, we found that the mutants S579A, H665Y, and H667Y retained the capacity for ATPase activity stimulation with casein, which is characteristic of the native protease Lon, whereas D676N lost it (Table 1). This indicates that the residues under study are involved in the interactions between the domains to a different extent.

Despite the apparent importance of the identified residues for the activity of protease Lon, the question whether or not H665, H667, and D743 belong to the catalytic triad still remains open. Recently, it was found that some serine proteases contain the catalytic serine–lysine dyad [19] instead of the classic triad. It may be suggested that the proteolytic site of protease Lon is another example of such a dyad; however, this suggestion needs further investigation.

*Acknowledgements:* We are grateful to Dr. N.S. Bystrov for the synthesis of oligonucleotides. This work was supported in part by the Russian Foundation for Basic Research (project no. 93-04-6201) and the International Science Foundation (project NF8000).

## References

[1] Goldberg, A.L., Swamy, K.H., Chung, C.H. and Larimore, F.S. (1981) Methods Enzymol. 80, 680–702.
[2] Gottesman, S. (1996) Annu. Rev. Genet. 30, 465–506.
[3] Goldberg, A.L., Moerschell, R.P., Chung, C.H. and Maurizi, M.R. (1994) Methods Enzymol. 244, 350–375.
[4] Gottesman, S. and Maurizi, M.R. (1992) Microbiol. Rev. 56, 592–621.
[5] Maurizi, M.R. (1992) Experientia 48, 178–201.
[6] Amerik, A.Yu., Antonov, V.K., Ostroumova, N.I., Rotanova, T.V. and Chistyakova, L.G. (1990) Bioorg. Khim. 16, 869–880 (Russ.).
[7] Rotanova, T.V., Kotova, S.A., Amerik, A.Yu., Lykov, I.P., Ginodman, L.M. and Antonov, V.K. (1994) Bioorg. Khim. 20, 114–125 (Russ.).
[8] Amerik, A.Yu., Antonov, V.K., Gorbalenya, A.E., Kotova, S.A., Rotanova, T.V. and Shimbarevich, E.V. (1991) FEBS Lett. 287, 211–214.
[9] Van Melderen, L., Thi, M.H.D., Lecchi, P., Gottesman, S., Couturier, M. and Maurizi, M.R. (1996) J. Biol. Chem. 271, 27730–27738.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[11] Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K. and Pease, L.R. (1989) Gene 77, 61–68.
[12] Laemmli, U.K. (1970) Nature (London) 227, 680–685.
[13] Chin, T.D., Goff, S.A., Webster, T., Smith, T. and Goldberg, A.L. (1988) J. Biol. Chem. 263, 11718–11728.
[14] Bencini, D.A., Shanley, M.S., Wild, J.R. and O'Donnovan, G.A. (1983) Anal. Biochem. 132, 259–264.
[15] Melnikov, E.E., Tsirulnikov, K.B., Ginodman, L.M. and Rotanova, T.V. (1998) Bioorg. Khim. (in press) (Russ.).
[16] Antonov, V.K. (1993) Chemistry of Proteolysis, Springer-Verlag, Berlin, Heidelberg.
[17] Rawlings, N.D. and Barrett, A.J. (1994) Methods Enzymol. 244, 19–61.
[18] Fischer, H. and Glockshuber, R. (1993) J. Biol. Chem. 268, 22502–22507.
[19] Paetzel, M. and Dalbey, R.E. (1997) Trends Biochem. Sci. 22, 28–31.
[20] Riethdorf, S., Volker, U., Gerth, U., Winkler, A., Engelsmann, S. and Hecker, M. (1994) J. Bacteriol. 21, 6518–6527.
[21] Tojo, N., Inouye, S. and Komano, T. (1993) J. Bacteriol. 175, 2281–2287.
[22] Gill, R.E., Karlok, M. and Benton, D. (1993) J. Bacteriol. 175, 4538–4544.
[23] Tojo, N., Inouye, S. and Komano, T. (1993) J. Bacteriol. 175, 4545–4549.
[24] Van Dyck, L., Pearce, D.A. and Sherman, F. (1994) J. Biol. Chem. 269, 238–242.
[25] Wang, N., Gottesman, S., Willingham, M.C., Gottesman, M.M. and Maurizi, M.R. (1993) Proc. Natl. Acad. Sci. USA 90, 11247–11251.