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The Effect of Mutations in the Inserted Domain of ATP-Dependent Lon Protease from *E. coli* on the Enzyme Function[1]

A. M. Kudzhaev, A. G. Andrianova, O. V. Serova, V. A. Arkhipova, E. S. Dubovtseva, and T. V. Rotanova[2]

Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, ul. Miklukho-Maklaya, 16/10, Moscow, 117997 Russia

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Abstract—The ATP-dependent protease LonA from *E. coli* (Ec-Lon) belongs to the superfamily of AAA$^+$ proteins and plays a key role in the quality control system of the cell proteome. Ec-Lon functions as a homohexamer and destroys abnormal and defective polypeptides, as well as a number of regulatory proteins, according to a "processive degradation" mechanism. A Ec-Lon subunit includes an ATPase component and a proteolytic component (AAA$^+$ module and P-domain, respectively), as well as a noncatalytic region formed by the *N*-terminal (*N*) domain and an inserted α-helical (HI(CC)) domain; this region is unique for AAA$^+$ proteins. Mutant forms of Ec-Lon were obtained by replacing R164, R192, or Y294 residues localized in the HI(CC) domain, and the properties of these proteins were investigated in order to elucidate the role of the HI(CC) domain in enzyme functioning. The *C*-terminal part of the HI(CC) domain was shown to have an allosteric effect on the efficiency of the functioning of both ATPase and proteolytic sites of the enzyme, while the coiled-coil (CC) fragment of this domain was shown to interact with the protein substrate.

Keywords: AAA$^+$ proteins, ATP-dependent proteolysis, Lon proteases, domain organization, coiled-coil region, site-directed mutagenesis, E. coli

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INTRODUCTION

The family of Lon proteases (EC 3.4.21.53; MEROPS: clan SJ, S16) plays a key role in the functioning of the protein quality control system (QCS), which maintains cellular proteome integrity in all natural kingdoms [1, 2]. The QCS includes molecular chaperones involved in protein remodeling and disaggregation in the cell, as well as selective ATP-dependent peptide hydrolases, Lon proteases among them, that eliminate damaged and mutant proteins from the cells and degrade short-lived regulatory proteins. QCS peptide hydrolases are oligomeric bifunctional enzymes that contain proteolytic components (P-domains or individual subunits) belonging to different classes of peptidases and ATPase components, namely, Hsp100 heat shock proteins that belong to the superfamily of AAA$^+$ proteins (**A**TPases **a**ssociated with diverse cellular **a**ctivities). Clp/Hsp104 chaperones disaggregating the proteins targeted by QCS also belong to the AAA$^+$ protein superfamily [3].

All proteins of the Hsp100 family are formed by ATPase modules (also termed AAA$^+$ modules) and extra domains localized either at the *N*-terminus of the protein (N-domain) or within the AAA$^+$ module (I-domain) [4]. AAA$^+$ proteins usually contain one or two ATPase modules (in case of Class II and Class I proteins, respectively), with each of the modules including two domains, namely, the nucleotide-binding (NB) domain and the α-helical (H) domain. Domains of both types contain specific conserved elements.

Lon proteases are homooligomeric enzymes with AAA$^+$ modules localized in the same polypeptide chain as the P-domains [5]; the latter domains are represented by serine-lysine endopeptidases [6]. The Lon protease pool consists of two subfamilies (A and B); the A subfamily includes cytoplasmic and mitochondrial enzymes from bacteria and eukaryotes, while the B subfamily includes membrane-bound enzymes of Archaea [7]. Both the environment of the catalytic serine and lysine residues and the overall subunit architecture of LonA and LonB proteases are different; for instance, the extra domain of LonA enzymes is *N*-terminal (N),while in LonB it is inserted (I).

The presence of an extended variable *N*-terminal region preceding the AAA$^+$ modules is a characteristic feature of LonA proteases distinguishing them from

Abbreviations: AMPPNP, adenosine 5'-(β, γ-imido) triphosphate, DTDP, 4,4'-dithiodipyridine, Nu, nucleotide; PepTBE, Suc-Phe-Leu-Phe-SBzl; QCS, protein quality control system.

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[2] Corresponding author: phone: +7 (495) 335-42-22, fax: +7 (495) 335-71-03; e-mail: tatyana.rotanova@ibch.ru).

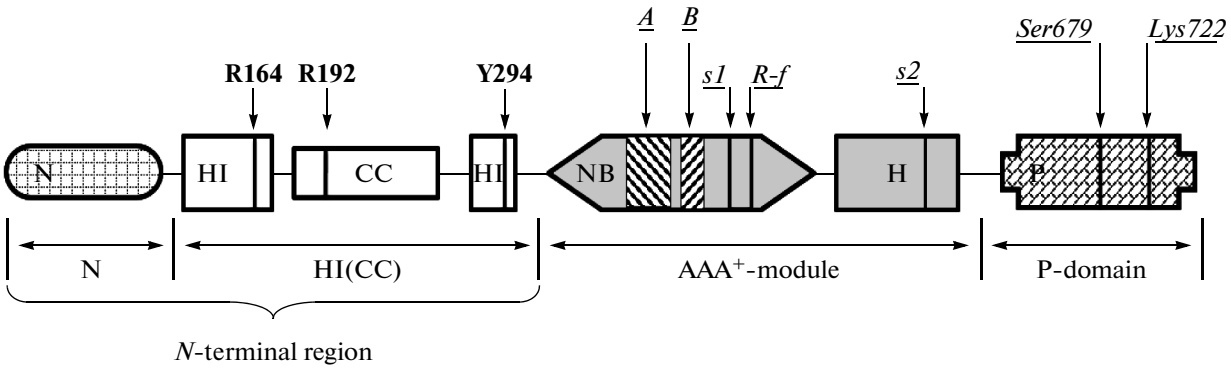

Fig. 1. Domain organization of *E. coli* LonA protease. Domain identifiers: N—*N*-terminal; HI(CC)—α-helical with a coiled-coil fragment; NB—nucleotide binding; H—α-helical; P—proteolytic. Identifiers of conserved elements: *A* and *B*—Walker motifs, *s1* and *s2*—sensor residues, *R-f*—"arginine finger" residue, *Ser679* and *Lys722*—catalytic residues of the proteolytic center. **R164**, **R192**, and **Y294**—residues subjected to mutagenesis.

other AAA+ proteins. We performed a comparative analysis of the primary and secondary structures of LonA proteases from distantly related organisms to demonstrate that the *N*-terminal region of these enzymes consists of two domains, namely, the true *N*-terminal domain (N) and an inserted α-helical domain, HI(CC), which includes a sequence fragment with a specific coiled-coil (CC) conformation and is markedly similar to both the H-domain of the AAA+ module of the Lon proteases and the α-helical domain (H1(M)) of the first AAA+ module of ClpB/Hsp104 chaperones [8, 9]. Thus, the domain organization of LonA proteases can be schematically represented as N–HI(CC)–NB–H–P, with a single nucleotide-binding domain (NB) flanked by two similar α-helical domains (HI(CC) and H) (Fig. 1). Therefore, LonA proteases can be considered unique representatives of the AAA+ protein superfamily presumably belonging to Class I, but devoid of the nucleotide-binding domain of the first AAA+ module. The role of the preserved HI(CC) domain in the functioning of LonA proteases remains uncharacterized.

The aim of the present work was to investigate the effect of substitutions of conserved amino acid residues located in different fragments of the inserted HI(CC) domain of LonA proteases on the functioning and/or structural organization of the enzymes. LonA protease from *Escherichia coli* (Ec-Lon, Fig. 1) was used as an example. The study involved directed mutagenesis of highly conserved residues R192 (CC fragment) and Y294 (*C*-terminal part of the domain) and comparative characterization of the properties of the mutant forms of the enzyme and those of intact Ec-Lon protease and a previously obtained mutant enzyme with a substitution of the R164 residue localized in the *N*-terminal part of the HI(CC) domain.

RESULTS AND DISCUSSION

Inserted HI(CC) domains of LonA proteases include eight α-helices, with four helices (H1–H4) forming the actual HI-domain and four other helices (C1–C4) forming the CC fragment. The C1–C4 fragment is located between the helices H3 and H4, and therefore the secondary structure of the domain corresponds to the following scheme: H1-H2-H3-C1-C2-C3-C4-H4 [9]. The insertion domain is followed by a linker region joining HI(CC) and NB domains; the linker region contains an α-helical fragment (H-link) as well. HI(CC) domains are similar in size (178–190 amino acid residues), and sequence similarity of the domains within the groups of bacterial and eukaryotic enzymes is quite high [9]. Coiled-coil fragments (C1-C4) exhibit higher similarity than full-sized HI(CC) domains, while the "long" C2 helix comprising 55 amino acid residues is the most highly conserved element of the CC fragments (as shown for bacterial LonA proteases, Table 1). The Arg192 residue localized at the beginning of the C2 helix in the CC fragment (conserved in 98% of all enzymes) and the Tyr294 residue localized in the *C*-terminal helix H4 (conserved in 95% of all enzymes) were chosen to assess the role of individual fragments of the HI(CC) domain of Ec-Lon protease (residues 124–301) in the functioning of the enzyme. Point replacement of these residues by alanine was performed, and the properties of the mutant forms Lon-R192A and Lon-Y294A were investigated and compared with the properties of the intact enzyme and the previously obtained mutant Lon-R164A (substitution in helix H3) [10].

Mutants Lon-R192A and Lon-Y294A were derived from the recombinant form of Ec-Lon containing a hexahistidine tag at the *C*-terminus of the protein (C-His-Lon) [10]. The intact enzyme and its mutant forms were isolated by affinity chromatography on Ni-Sepharose and gel filtration on Sephacryl S-400.

Table 1. Similarity between HI(CC) domains (A), linkers connecting to NB domains (B), CC fragments (C), and the helices constituting these domains (H1–H4, C1–C4, and H-link) for a group of 45 bacterial LonA proteases

LonA protease fragment (size, aa; Xaa-Yaa in Ec-Lon)										
A (179 aa; E123–V301)										B (24 aa; P302–Y325)
	Identifier	H1	H2	H3	C (108 aa; M173–M280)				H4	H-link helix
					C1	C2	C3	C4		
Helices	Helix length, aa (Xaa-Yaa in Ec-Lon)	22 (E124-N145)	10 (P149-H158)	11 (P162-H172)	5 (Q181-E185)	55 (V189-E243)	9 (E252-A260)	13 (E266-L278)	15 (A286-Q300)	12 (D311-T322)
	Helix or fragment similarity, %	9.1	0.0	18.2	40.0	52.7	0.0	30.7	33.3	33.3
Entire fragments (A, B, and C)					(C) 31.5					(B) 33.3
		(A) 24.6								

ATPase Activity of Mutant Forms of Ec-Lon Protease

The presence of equimolar amounts of ATP and magnesium ions in the reaction medium and a pH of 8.0–8.2 were previously shown to provide for maximal ATPase activity of the native Ec-Lon protease. Inhibition of ATPase activity of the enzyme was observed under simulated physiological conditions (that is, at elevated concentration of Mg^{2+} ions); the presence of a protein substrate alleviated the inhibition [11].

All three mutant forms of C-His-Lon with residue substitutions in the HI(CC) domain retained the ability to hydrolyze ATP at a 1 : 4 ratio of ATP and Mg^{2+} concentrations in the medium (Fig. 2); however, all mutations caused significant reduction of enzyme activity, which was especially pronounced for the Lon-Y294A mutant (95% loss of activity). ATPase activity of both the native enzyme and its mutant forms increased severalfold in the presence of the protein substrate (Fig. 2). These data show that the HI(CC) domain is not essential for the formation of the ATPase center of Ec-Lon protease, but still has an effect on the functional efficiency of this center.

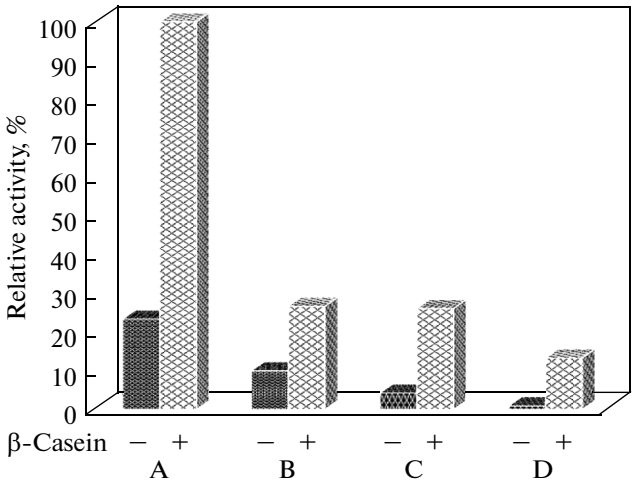

Fig. 2. ATPase activity of the intact C-His-Lon protease (A) and its mutant forms Lon-R164A (B), Lon-R192A (C), and Lon-Y294A (D) in the presence (+) or absence (–) of the protein substrate β-casein. Experimental conditions: 50 mM Tris-HCl-buffer, pH 8.1; 0.15 M NaCl; 37°C; 5 mM ATP; 20 mM $MgCl_2$; 0.5 mg/mL β-casein, and 1 μM enzyme.

Activity of Peptidase Centers of the Mutant Forms of Ec-Lon Protease

The efficiency of the peptidase centers of intact Ec-Lon protease and its mutants was evaluated using the reaction of hydrolysis of the N-protected tripeptide thiobenzyl ester Suc-Phe-Leu-Phe-SBzl (PepTBE) [12].

Both C-His-Lon protease and its mutant forms were capable of cleaving the peptide substrate in the absence of effectors; however, substitutions in the HI(CC) domain led to a marked reduction of activity of the peptidase center, with the rate of PepTBE hydrolysis decreasing by more than an order of magnitude in the case of the mutant Lon-Y294A. The ratio of specific peptidase activities of C-His-Lon, Lon-R164A, Lon-R192A, and Lon-Y294A was approximately 15 : 5 : 7 : 1. Peptidase centers of all forms of the enzyme were activated by magnesium ions (Table 2).

The effects of nucleotides and nucleotide complexes with Mg^{2+} on peptidase properties of C-His-Lon and its mutants varied considerably. ATP and the nonhydrolysable nucleotide AMPPNP, as well as the complexes of these compounds with magnesium ions, had an activating effect on peptidase centers of all forms of Ec-Lon. The activating effect on C-His-Lon and Lon-R164A increased in the series AMPPNP < ATP < AMPPNP-Mg ≈ ATP-Mg, while the mutant forms Lon-R192A and Lon-Y294A were activated to a

Table 2. Influence of effectors on the activity of peptidase centers in C-His-Lon, Lon-R164A, Lon-R192A, and Lon-Y294A*

Effector	C-His-Lon		Lon-R164A		Lon-R192A		Lon-Y294A	
	v	n	v	n	v	n	v	n
No effector	5.88	1	1.71	1	2.88	1	0.39	1
Mg	33.1	5.62	15.1	8.81	14.3	4.95	1.43	3.67
ATP	47.1	8.01	26.4	15.4	19.1	6.64	5.03	12.9
ADP	0.49	0.08	1.06	0.62	0	0	0.09	0.23
AMPPNP**	14.2	2.41	3.30	1.93	21.6	7.49	4.00	10.3
ATP-Mg	63.5	10.8	36.7	21.4	19.9	6.90	5.70	14.6
ADP-Mg	10.1	1.73	4.95	2.89	0.39	0.14	0.24	0.62
AMPPNP-Mg	58.0	9.86	36.4	21.3	19.5	6.78	5.05	12.9

* The values of specific hydrolysis rate for Suc-Phe-Leu-Phe-SBzl (v, $\mu M_S/(\min \mu M_E)$ are shown; μM_S and μM_E— hydrolysed substrate and enzyme concentrations, respectively). n—the degree of inhibition ($n < 1$, gray background) or activation ($n > 1$) of substrate hydrolysis. Experimental conditions: 50 mM Tris-HCl-buffer, pH 8.1; 0.15 M NaCl; 10% DMSO; 0.1 mM PepTBE; 0.2 mM DTDP; 2.5 mM Nu; 20 mM $MgCl_2$; 0.2 mM enzyme; 37°C.

** Adenosine 5'-(β, γ-imido)triphosphate, a nonhydrolyzable analogue of ATP.

similar extent in the presence of all these nucleotide effectors. Free ADP inhibited all forms of Ec-Lon, while a complex of ADP with magnesium ions (ADP-Mg) activated C-His-Lon and Lon-R164A and inhibited the mutant forms Lon-R192A and Lon-Y294A (Table 2).

The results suggest that the HI(CC) domain exerts an allosteric effect (either direct or mediated by the ATPase center of the enzyme) on the peptidase center of Ec-Lon, with the *C*-terminal part of the domain having a greater impact.

Proteolytic Activity of the Mutant Forms of Ec-Lon Protease

Proteolytic activity of C-His-Lon-protease and its mutant forms towards the model protein substrate β-casein was assessed using gel electrophoresis of the reaction products [13].

As shown in Fig. 3, Ec-Lon-protease forms with mutations in the HI(CC) domain did not lose the distinguishing feature of the intact enzyme, namely, the capacity for "processive degradation" of protein substrates (not accompanied by accumulation of large intermediate fragments) concomitantly to ATP hydrolysis (in the presence of ATP and magnesium ions). However, the efficiency of proteolysis varied between the enzyme forms: the activity of Lon-R164A was virtually similar to that of C-His-Lon, while the mutants Lon-R192A and Lon-Y294A had significantly lower activity. The proteolytic activity of Lon-R192A was minimal, although the peptidase activity of this mutant enzyme (both basal and elevated due to the presence of ATP-Mg complex) was considerably higher than that of Lon-Y294A (Table 2). This implies disturbances in binding of casein by the mutant enzyme Lon-R192A, and therefore an important role in the interaction with the protein substrate can be ascribed to the CC region of the HI(CC) domain.

Unlike C-His-Lon and Lon-R164A the mutant enzymes Lon-R192A and Lon-Y294A were not capable of nonprocessive degradation of β-casein in the presence of an AMPPNP-Mg complex; the reaction neither occurred under the conditions described above (Fig. 3) nor during 24 hours of incubation (data not shown). However, fragments of Lon-Y294A were detected among the reaction products in the latter case, this being indicative of the possibility of partial degradation of the mutant enzyme in case of prolonged incubation at 37°C.

Autolytic Capacity of Ec-Lon Protease and Its Mutant Forms

Analysis of the propensity of C-His-Lon and mutant enzymes Lon-R164A, Lon-R192A, and Lon-Y294A for autodegradation revealed that Lon-Y294A was most prone to autolysis (Fig. 4). Slight autolysis of the intact enzyme and the mutant forms of Lon-R164A and Lon-R192A was observed only in the

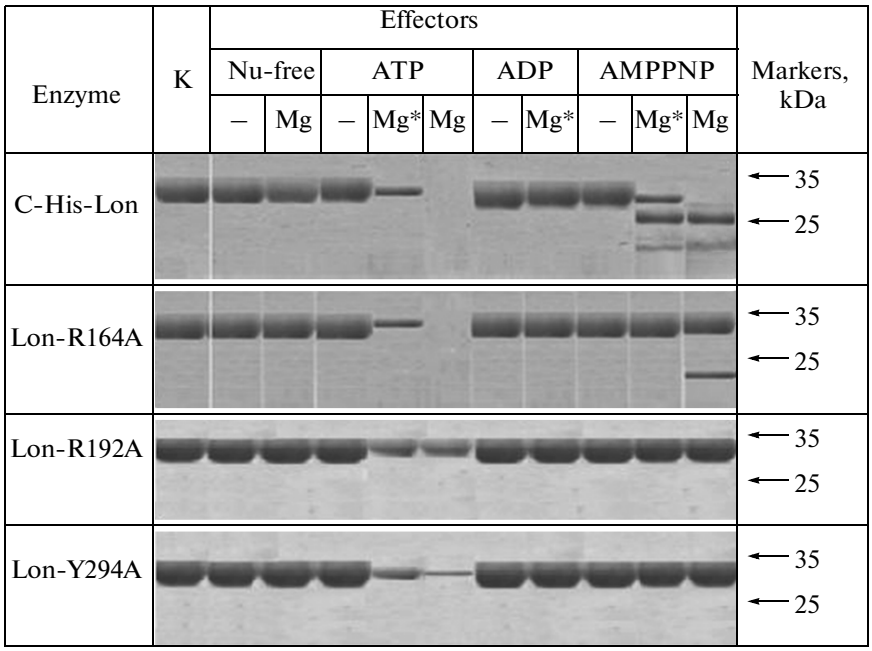

Fig. 3. Hydrolysis of β-casein by C-His-Lon protease and mutant enzymes Lon-R164A, Lon-R192A, and Lon-Y294A in the absence and in the presence of effectors (electrophoresis in 12% PAGE). Experimental conditions: 50 mM Tris-HCl buffer, pH 8.1; 0.15 M NaCl; 37°C; reaction duration: 2 hours or 20 minutes (for lanes marked Mg*); concentrations: 5 mM Nu, 20 mM $MgCl_2$, 0.5 mg/mL β-casein, and 5 μM enzyme. K (control)—β-casein, "_ _ _"—in the absence of Mg^{2+} ions, Mg—in the presence of Mg^{2+} ions.

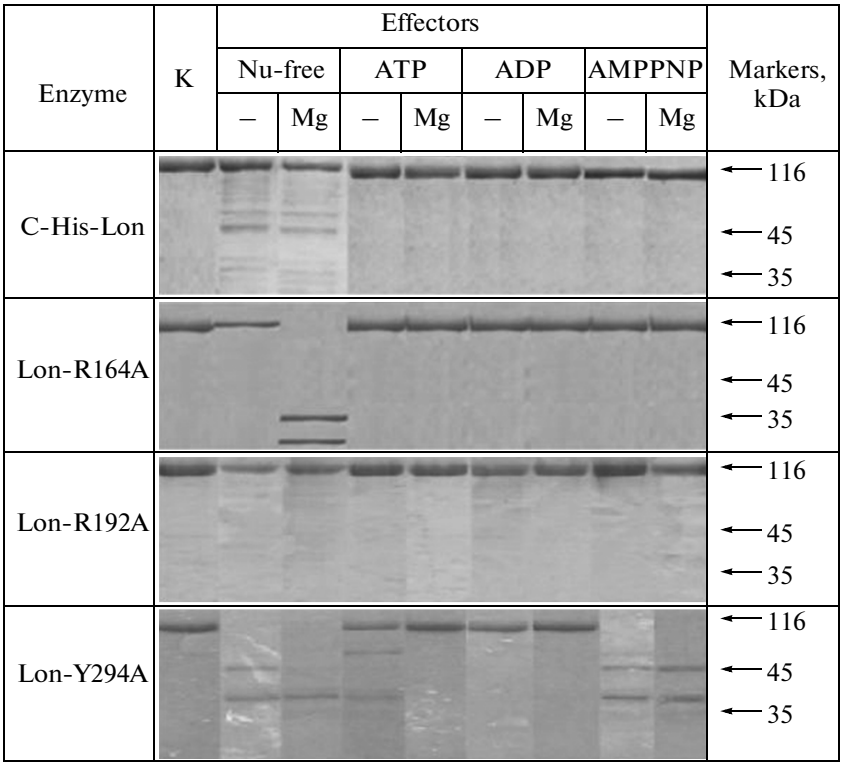

Fig. 4. Autolysis of C-His-Lon-protease and its mutant forms Lon-R164A, Lon-R192A, and Lon-Y294A in the absence and in the presence of effectors. The experimental conditions and designations are the same as in the legend to Fig. 3, except the reaction time (24 h). K (control)—intact enzyme (at zero reaction time).

Table 3. Primers used to produce C-His-Lon-protease and its mutant forms

Ec-Lon modification	Primer	Primer structure (5'-3")	Restriction enzymes
– (C-His-Lon)	f1	AATCCTGAGCGTTCTGAACGC'	
– (C-His-Lon)	Lon_full_C_His (f2)	GGG*CTCGAG*TTTTGCAGTCACAACCTGCATG	XhoI
R164A	Lon_R164A	CAGCAATGGTATCCGCCAGAGCGGCCGGATCGTC-GATGCTATT	
R164A, R192A, Y294A	Lon_HindIII (f3)	CGCAGAAAG*AAGCTT*CAACGG	HindIII
R164A, R192A	Lon_Acc65I (f4)	CGCATTCCAC*GGTACC*TGTAC	Acc65I
R192A	Lon_R192A_XbaI	GAGATGTCCGACGTTAACGAAGC*TCTAGA*ATATCT-GATGGCAATGATGG	XbaI
Y294A	Lon_Y294A_MunI	GAAGCGACCGTAGTGCGTGGTG*CAATTG*ACTGGATG-GTACAGGTACC	MunI
Y294A	Lon_BamHI_rev	GTTCTGCTCT*GGATCC*AGCAC	BamHI

* Restriction enzyme recognition sites are italicized and underlined.

absence of nucleotides (notably, the intensity of LonR164A autolysis increased considerably in the presence of magnesium ions). Nucleotides and Nu-Mg complexes had a stabilizing effect on these forms of Ec-Lon.

The mutant enzyme Lon-Y294A decayed both in the absence of nucleotides and in the presence of free ATP; the degradation was accelerated by AMPPNP and AMPPNP-Mg complex. The loss of the stabilizing effect of nucleotides on Ec-Lon due to mutation of the Y294 residue shows that the *C*-terminal part of the HI(CC) domain is important for the maintenance of the correct enzyme conformation.

CONCLUSION

The data obtained show that the HI(CC) domain of Ec-Lon protease neither plays a role in the formation of ATPase and proteolytic centers of the enzyme nor is involved in the unique phenomenon of processive proteolysis coupled with ATP hydrolysis. However, the HI(CC) domain, especially its *C*-terminal portion, exerts an allosteric effect on the functioning of both active centers of the enzyme, while the CC fragment is involved in the interaction with the protein substrate. Moreover, the *C*-terminal part of the HI(CC) domain is involved in the formation of the correct conformation of the enzyme.

EXPERIMENTAL

Commercial reagents produced by Sigma, Aldrich, and Bio-Rad (United States), Fluka (Switzerland), Boehringer Mannheim (Germany), Pharmacia (Sweden), Difco (England), Panreac (Spain), Fermentas (Lithuania), and Reakhim (Russia) were used in the present work.

Site-directed mutagenesis of C-His-Lon protease was carried out according to the procedure described in [10] using the high-yield vector pET28. The primers used in the present work are shown in Table 3.

Sequencing of cloned DNA and primer synthesis were performed by ZAO EVROGEN (www.evrogen.ru). Restriction and ligation were carried out according to the protocols provided by manufacturers of the enzymes used.

Isolation and purification of C-His-Lon-protease and its mutant forms was performed according to a procedure reported previously [10]. The final purification steps—affinity chromatography on Ni-sepharose and gel filtration—were performed on HiTrap^{TM}FF columns (tandem 2 × 5 mL) from GE Healthcare and Sephacryl S-400 column (volume 120 mL, GE Healthcare), respectively.

Protein concentration was determined according to the procedure of Bradford [14].

Homogeneity of the protein preparations was assessed by electrophoresis as described in [15] using a commercial marker kit (*M*, kDa): β-galactosidase (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase (35.0), restriction endonuclease Bsp98I (25.0), β-lactalbumin (18.4), and lysozyme (14.4).

ATPase activity assays were performed at 37°C in 50 mM Tris-HCl-buffer, pH 8.1, containing 150 mM NaCl, 5 mM ATP, 20 mM $MgCl_2$, and 1 μM enzyme [16]. The enzyme was replaced with buffer for a blank test. Accumulation of inorganic phosphate was monitored, and the initial reaction rate values were calculated using the absorbance values of mixtures contain-

ing 200 μL of the reaction mixture and 600 μL of the reagent (100 mM $Zn(AcO)_2$, 15 mM $(NH_4)_6Mo_7O_{24}$, 1% SDS, pH 4.5–5.0) at a wavelength of 350 nm ($\varepsilon_{350} = 7800\ M^{-1}\ cm^{-1}$).

Thioesterase activity. Hydrolysis of an *N*-substituted tripeptide thiobenzyl ester Suc-Phe-Leu-Phe-SBzl (PepTBE) was followed by spectrophotometry at a wavelength of 324 nm to detect optical absorption of 4-thiopyridone ($\varepsilon_{324} = 16500\ M^{-1}\ cm^{-1}$) formed upon the interaction of the hydrolysis product (benzyl thiolate, $BzlS^-$) with 4,4'-dithiodipyridine (DTDP) [17]. PepTBE hydrolysis was performed at 37°C in 50 mM Tris-HCl buffer, pH 8.1, containing 150 mM NaCl, 10% DMSO, 0.2 mM DTDP, 0.1 mM PepTBE, and 0.2–0.3 μM enzyme. Reaction mixtures used to characterize the influence of effectors were supplemented with Nu (to 2.5 mM) and $MgCl_2$ (to 20 mM).

Proteolytic activity of the enzymes was tested electrophoretically [15]. The reaction was conducted at 37°C in 50 mM Tris-HCl-buffer, pH 8.1, containing 150 mM NaCl, 20 μM β-casein, and 5 μM enzyme, either in the absence or in the presence of 5 mM Nu and 20 mM $MgCl_2$. A 20-μL aliquot of the reaction mixture or the control solution was mixed with 7 μL of lysis buffer (0.2 M Tris-HCl-buffer, pH 8.9, 4% SDS, 20% glycerol, 0.5 mM EDTA, 0.8% bromophenol blue, 3% β-mercaptoethanol), boiled for 10 min, and loaded onto 12% PAAG for electrophoresis.

Autolytic activity of the enzymes was tested electrophoretically [15] under conditions similar to those of the proteolytic activity assay except for the absence of β-casein.

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