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Role of α-Helical Domains in Functioning of ATP-Dependent Lon Protease of *Escherichia coli*

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Abstract—Homooligomeric ATP-dependent LonA proteases are bifunctional enzymes belonging to the superfamily of AAA^+ proteins. Their subunits are formed by five successively connected domains, i.e., *N*-terminal (N), α-helical (HI(CC)), nucleotide-binding (NB), the second α-helical (H), and proteolytic (P) domains. The presence of the inserted HI(CC) domain determines the uniqueness of LonA proteases among the AAA^+ proteins. The role of the α-helical domains in the LonA protease functioning was studied with an example of *E. coli* Lon protease (Ec-Lon). The properties of the intact Ec-Lon and its mutant forms, i.e., Lon-R164A and Lon-R542A bearing the substituted arginine residues at the similar positions in the HI(CC) and H domains, were compared. The H domain was shown to play a crucial role in ATP hydrolysis and enzyme binding to the target protein. The HI(CC) domain is not decisive for the manifestation of the catalytic properties of the enzyme. However, it affects the functioning of Lon ATPase and peptidase sites and is involved in maintaining enzyme stability. The participation of the HI(CC) domain in the formation of three-dimensional structures of LonA proteases and/or their complexes with DNA is suggested.

Keywords: AAA^+ proteins, ATP-dependent Lon protease, domain organization, α-helical domains, site-directed mutagenesis, Escherichia coli

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INTRODUCTION

The family of ATP-dependent Lon proteases (EC 3.4.21.53; MEROPS: clan SJ, S16) is a key component of the quality control system (QCS), which ensures the integrity and functionality of cellular proteins [1–4]. QCS combines molecular chaperones represented mainly by heat shock proteins (Hsp) of different families [5, 6] and a number of selective peptide hydrolases and proteolytic complexes (proteasomes) [7, 8]. Peptide hydrolases of QCS (or AAA^+ proteases) are bifunctional enzymes, which contain proteolytic components relating to different classes of proteases and ATPase components representing the superfamily of chaperones-disaggregases of the Hsp100 or AAA^+ proteins (ATPases associated with different cellular activities [9, 10]). During functioning QCS, the AAA^+ proteases selectively degrade cellular regulatory proteins and release cells from defective, damaged, and mutant proteins.

Homooligomeric Lon proteases belong to the rare representatives of the AAA^+ proteins whose ATPase components consist of nucleotide-binding (NB) and α-helical (H) domains and proteolytic components (P domains), i.e., serine-lysine endopeptidases [11] are sequentially arranged in a single polypeptide chain. The Lon family is divided to subfamilies A and B. The former combines cytoplasmic bacterial enzymes and mitochondrial eukaryotic proteases, and the latter combines membrane-bound Lon proteases of archaea. LonA and LonB proteases differ by the surroundings of the catalytic serine and lysine residues in the P domain and by the general architecture, i.e., LonA contains prolonged *N*-terminal regions and LonB contains transmembrane domains inside the AAA^+ modules [12].

It was shown in our previous papers that the *N*-terminal regions of LonA proteases are formed by two domains, i.e., the *N*-terminal domain itself (N domain) and the inserted α-helical domain containing the region with the **c**oiled-**c**oil (CC) conformation (**h**elical **i**nserted CC-containing domain, HI(CC) domain) [13, 14]. Thus, the domain organization of individual subunits of LonA proteases can be represented by the following scheme: N–HI(CC)–NB–H–P, where the NB–H fragment forms the AAA^+ module.

We found the similarity between LonA proteases and chaperons of the ClpB/Hsp104 family [14]. The subunits of the latter are formed by the *N*-terminal domain and two AAA^+ modules, and the H1 domain of the first AAA^+ module of chaperons includes the

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

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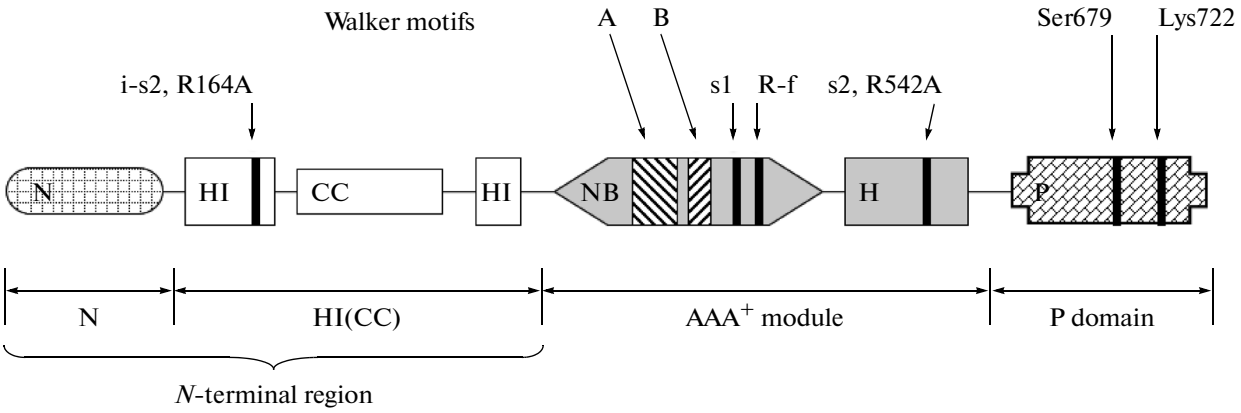

Fig. 1. Domain organization of Lon A protease of *E. coli*. Designations of domains: N, *N*-terminal; HI(CC), α-helical with coiled-coil region; NB, nucleotide-binding; H, α-helical; P, proteolytic. NB and H domains form AAA^+ module. Designations of conserved elements: A and B, Walker motifs; s1 and s2, sensor residues; i-s2, potential sensor residue; R-f, arginine finger. Ser679 and Lys722 are catalytically active residues.

fragment with the CC conformation, the so-called M domain that corresponds to the scheme of N–NB1–H1(M)–NB2–H2. The HI(CC) domain appeared to have a pronounced similarity with the H1(M) domain of chaperons. At the same time, the enzyme structure does not contain the nucleotide-binding domain, the analogue of the NB1 domain of chaperons, and this fact provides the uniqueness of the structure of the LonA proteases among the AAA^+ proteins, which usually contain either one or two full two-domain AAA^+ modules. The role of the inserted HI(CC) domain in the functioning of the LonA proteases and/or in the maintaining of their active structure has not yet been investigated.

The goal of this work was a comparative study of the contribution of two α-helical domains (classical H and inserted HI(CC)) in the manifestation of the enzymatic properties of LonA proteases with an example of the Lon protease from *E. coli* (Ec-Lon).

RESULTS AND DISCUSSION

The α-helical domains (H), which form the ATPase modules along with the nucleotide-binding domains (NB), are the distinctive characteristic of the AAA^+ proteins structure [10, 15, 16]. The classical H domains are formed by four α-helices and include the positively charged consensus sensor-2 residue (s2) located in the *N*-terminal region of the third helix. This residue along with other conserved fragments of the NB domain (the A and B Walker motifs, the sensor-1 residues (s1), and the arginine finger (R-f)) is involved in the formation of the ATPase center of the AAA^+ protein.

The scheme of the domain organization of the Ec-Lon protease subunit (784 aa) is shown in Fig. 1. The H domain of the AAA^+ module contains the residues (491–579) where the role of the sensor residue s2 plays Arg542. The inserted HI(CC) domain is formed by the 124–302 residues; like in the H domain, the arginine residue Arg164 (i-s2, Fig. 1) is localized in the beginning of the third helix of the HI(CC) domain. To reveal the role of the α-helical domains of the Ec-Lon protease in the functioning of the enzyme, the Arg164 and Arg542 residues were substituted by alanine and the properties of the resulting mutant forms were studied in comparison with those of the intact enzyme.

To optimize the scheme of the isolation of the Ec-Lon protease and its mutant forms, we prepared the recombinant form of the enzyme containing the hexahistidine fragment (in octapeptide LEHHHHHH) at the *C*-end of the enzyme (C-His-Lon), as well as the mutants on its basis bearing the substitutions in both the sensor s2 residue and similar i-s2 residue (Lon-R542A and Lon-R164A, respectively). C-His-Lon and the mutants R542A and Lon-R164A are obtained in the preparative amount using affinity chromatography on Ni-Sepharose and gel-filtration on Sephacryl S-400.

Activity of ATPase Centers of Mutant Forms of Ec-Lon Protease

It is known that the native Ec-Lon protease hydrolyzes ATP in the absence of a protein substrate (the basic ATPase activity) in the range of pH 7.0–9.0 (with the maximum at pH 8.0–8.2). The rate of the hydrolysis of ATP is maximal at the equimolar ratio of the nucleotide and magnesium ions. The presence of free Mg^{2+} ions inhibits the ATPase activity of the enzyme, and this effect is eliminated in the presence of the protein substrate [17].

It is shown that the C-His-Lon protease and the Lon-R164A mutant retain the property of the native enzyme to hydrolyze ATP, albeit the ATPase activity of Lon-R164A is markedly reduced (Fig. 2, where the initial rate of the ATP hydrolysis by the C-His-Lon protease in the presence of the protein substrate is

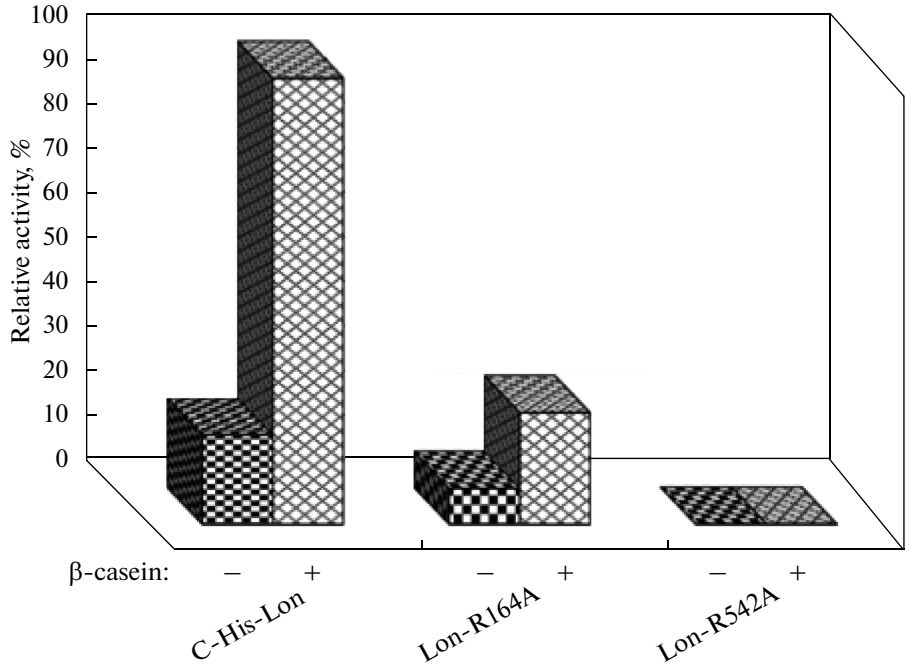

Fig. 2. ATPase activity of intact C-His-Lon protease and its mutant Lon-R164A and Lon-R542A forms. Experimental conditions: 50 mM Tris-HCl buffer, pH 8.1; 0.15 M NaCl; 37°C; 5 mM ATP; 20 mM $MgCl_2$; 1 mg/mL β-casein; 1 μM enzyme.

taken for 100%). At the same time, the Lon-R542A mutant does not have the ATPase activity in the range of pH 7.0–9.0 at any ratio of the nucleotide and magnesium ions and in the presence of either the protein (β-casein) or peptide (melittin) substrate. Thus, it should be stated that Lon-R542A completely loses its ability to hydrolyze ATP.

The results confirm the participation of the s2 sensor residue (R542 in Ec-Lon) in the formation of the ATPase center in the LonA proteases (as in the other representatives of the AAA^+ proteins) and is consistent with the data that the mutant forms of ATP-dependent proteases containing the substitutions of the s2 residues (ClpXP from *E. coli* [18], HslUV from *E. coli* [19], and LonB from *Thermoplasma acidophilum* [20]) lose their ability to hydrolyze ATP. The significant decrease in the ATPase activity of the Lon-R164A mutant in comparison with the intact C-His-Lon protease may be due to a violation of the proper conformation of the enzyme when replacing the i-s2 residue.

Activity of Peptidase Centers of Ec-Lon Protease Mutant Forms

We previously proposed to use thiobenzyl ester of *N*-protected tripeptide Suc-Phe-Leu-Phe-SBzl (PepTBE) as an indicator of the functional efficiency of the peptidase centers of the Ec-Lon protease [21]. The use of this substrate allowed us to evaluate the effect of the mutations on the activity of the enzyme peptidase centers.

The data in Table 1 show that both mutants retain the ability to hydrolyze PepTBE in the absence of effectors. However, the substitution of the i-s2 residue (R164) led to a significant decrease in the activity of the peptidase center, while the replacement of the s2 residue (R542) caused significant activation of the peptidase center of the mutant. The ratio of the specific activities of C-His-Lon, Lon-R164A, and Lon-R542A was approximately 100 : 25 : 200. The Mg^{2+} ions markedly activate all enzymes, and the common inhibitor was only ADP.

Nucleotides (Nu) and their complexes with the magnesium ions (Nu-Mg) similarly influence the activity of the peptidase centers in C-His-Lon and Lon-R164A, i.e., the rate of the hydrolysis of PepTBE with these enzymes in the presence of the effectors increases in the order ADP < without effector < AMPPNP < ADP-Mg < Mg^{2+} < ATP < AMPPNP-Mg ≤ ATP-Mg (Table 1). The maximal efficiency of the peptidase centers in the presence of ATP-Mg indicates that the Lon-R164A mutant retains the property of the coupled functioning of ATPase and peptidase centers, which is typical for the intact enzyme.

Any nucleotides and the Nu-Mg complexes inhibit the action of Lon-R542A. The effectors change the rate of the PepTBE hydrolysis in the following order: ADP = ATP < AMPPNP = ADP-Mg < ATP-Mg < AMPPNP-Mg < without effector < Mg^{2+} (Table 1). These data indicate that despite the loss of the ability to hydrolyze ATP, the ATPase center of the Lon-R542A mutant retains the property to bind nucle-

Table 1. Effect of effectors on the activity of peptidase centers of C-His-Lon, Lon-R164A, Lon-R542A, and mixed oligomer Lon-R542A/Lon-S679A

Effector	C-His-Lon		Lon-R164A		Lon-R542A		Lon-R542A/Lon-S679A	
	v	n	v	n	v	n	v	n
Without effector	7.41	1	1.71	1	14.9	1	21.2	1
Mg	48.6	6.56	15.1	8.81	21.4	1.44	ND	ND
ATP	138	18.7	26.4	15.4	1.91	*0.13*	ND	ND
ADP	1.41	*0.19*	1.06	*0.62*	1.96	*0.13*	ND	ND
AMPPNP*	9.09	1.23	3.30	1.93	4.19	*0.28*	ND	ND
ATP-Mg	163	22.0	36.7	21.4	4.89	*0.33*	46.8	2.21
ADP-Mg	12.0	1.61	4.95	2.89	4.24	*0.28*	5.50	*0.26*
AMPPNP-Mg	158	21.3	36.4	21.3	8.76	*0.59*	53.3	2.54

Here are the values of the specific rate of hydrolysis of Suc-Phe-Leu-Phe-SBzl (v, $\mu M_S/(\min \mu M_E)$, where μM_S and μM_E are concentration of substrate and enzyme, respectively); n is the extent of inhibition ($n < 1$, italic) or activation ($n > 1$, regular) of the substrate hydrolysis; ND, not determined. 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 μM enzyme (in case of individual enzymes) or 0.05 μM Lon-R542A + 0.5 μM Lon-S679A (in case of mixed oligomers); 37°C.

* Nonhydrolyzable analogue of ATP, adenosine-5'-(β, γ-imido)triphosphate.

otides and influence the functioning the peptidase center in intact enzyme, which however, differs considerably from the effects of the ATPase center in the intact enzyme. It was shown for the ClpXP [18] that the mutation of the s2 residue violates the interaction between the ATPase and proteolytic subunits. One can assume that in the case of Ec-Lon protease, the substitution of the R542 residue by alanine leads to the change in the interdomain and/or intersubunit contacts in the enzyme.

The partial recovery of these contacts is observed in mixed oligomers consisting of the Lon-R542A form without the ATPase activity and the previously obtained Lon-S679A form, which is mutant for the catalytic center and not capable of hydrolyzing peptide and protein substrates. The effect of the nucleotide-magnesium complexes on the hydrolysis of PepTBE by the Lon-R542A mutant in the mixed Lon-R542A/Lon-S679A oligomers was similar to the influence of these effectors on the native enzyme. ATP-Mg and AMPPNP-Mg activate the hydrolysis of the thioester substrate with the same efficiency, and ADP-Mg exhibits the inhibitory properties (Table 1). The results can be considered as a confirmation of our previous conclusion on the interactions between the ATPase and peptidase/proteolytic centers of the enzyme localized in different subunits of oligomeric Ec-Lon protease [22].

The comparison of the efficiency of the influence of ATP, ADP, and AMPPNP on the peptidase activity of the C-His-Lon protease and its mutant forms indicates that the side chain of the R542 residue in the intact enzyme is directly or indirectly involved in the interaction with β-γ-phosphate bond in nucleoside triphosphate. The mutation of the R542 residue leads to the fact that AMPPNP but not ATP becomes the most complementary nucleotide to the ATPase center.

Proteolytic Activity of Mutant Forms of Ec-Lon Protease

The proteolytic activity of the C-His-Lon protease and its mutant forms was tested by the efficiency of the degradation of the model protein substrate, i.e., β-casein [22]. The activity of the enzyme in the absence presence of the effectors was evaluated by gel electrophoresis.

According to the presented results (Fig. 3), the C-His-Lon protease and the Lon-R164A mutant are capable of cleaving the protein substrate with a similar efficiency by the processive mechanism (without the accumulation of the intermediate products of large size) under the conditions of ATP hydrolysis (in the presence of ATP and magnesium ions). Moreover, the intact enzyme and the Lon-R164A form nonprocessively cleave β-casein in the presence of the AMPPNP-Mg complex. In this case, however, the properties of the mutant and intact enzymes differ. Against the background of the reduced hydrolysis rate of the protein target, Lon-R164A is able to efficiently cleave the intermediate proteolysis product, i.e., the fragment of 25 kDa, which, on the contrary, is accumulated during the hydrolysis of casein by the C-His-Lon protease (Fig. 3).

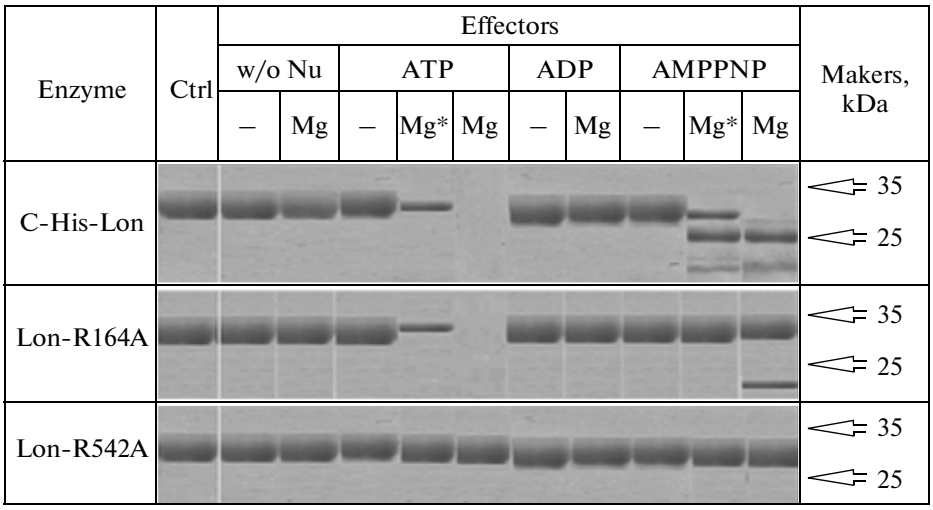

Fig. 3. Hydrolysis of β-casein by C-His-Lon protease and mutants Lon-R164A and Lon-R542A in the absence and presence of effectors (electrophoresis in 12% PAAG). Experimental conditions: 50 mM Tris-HCl buffer, pH 8.1; 0.15 M NaCl; 37°C; reaction time: 2 h or 20 min (lanes designated as Mg*); concentrations: 5 mM Nu; 20 mM $MgCl_2$; 0.5 mg/mL β-casein; 5 μM C-His-Lon and Lon-R164A; 10 μM Lon-R542A. Abbreviations: Nu, nucleotides, Ctrl(control), β-casein; "—", in the absence of Mg^{2+}; Mg, in the presence of Mg^{2+}.

The Lon-R542A mutant does not exhibit the proteolytic activity either under experimental conditions (Fig. 3) or when the incubation time increases to 24 h (data not shown). Thus Lon-R542A completely loses the ability to hydrolyze the protein substrate. Nevertheless, the possibility of the binding of casein to the Lon-R542A mutant requires additional study.

Autolysis of Ec-Lon Protease and its Mutant Forms

The investigation of the proteolytic properties of the C-His-Lon protease and its mutants showed that the prolonged incubation of the reaction mixture can cause the partial destruction of the enzymes. Therefore, we studied the autocatalytic properties of the C-His-Lon protease and the Lon-R164A and Lon-R542A mutants. The slow degradation for C-His-Lon and Lon-R164A is observed solely in the absence of nucleotides, and this process is pronounced in the presence of the magnesium ions (Fig. 4). In this case, the autolysis efficiency of the mutant is significantly higher than that of the intact enzyme.

It was unexpected that the Lon-R542A form can autodegrade, with the autolysis of the mutant being observed in both the absence and presence of the nucleotide effectors (Fig. 4). In general, the low efficiency of the autolysis increases in the following order: ADP-Mg = ATP-Mg = AMPPNP-Mg < ADP < ATP < AMPPNP < Mg^{2+} < without effector. These results show that, in contrast to the effect of the magnesium ions on the peptidase activity of Lon-R542A (see above, Table 1), the Mg^{2+} ions do not increase but rather reduce the extent of the nucleotide action on the proteolytic center during the autolysis of the mutant. Thus, we can conclude that, despite the inability to hydrolyze the target protein, the proteolytic center of the Lon-R542A mutant remains catalytically active, and the results support the intramolecular nature of autolysis.

CONCLUSION

According to the data presented, the H domain of the AAA^+ module of the Ec-Lon protease plays a crucial role in both the implementation of ATP hydrolysis and the binding of the protein substrate to the enzyme. The activity of the peptidase center of the Lon protease depends on the condition of the ATPase center and reaches a maximum when the enzyme (mutant Lon-R542A) loses the ability to hydrolyze ATP. The HI(CC) domain does not have a fundamental value for the manifestation of the catalytic properties of the enzyme; however, it influences the efficiency of the functioning of the ATPase and peptidase centers of the Lon protease and supports the stability of the enzyme. One can assume that the real role of the unique HI(CC) insert is the involvement in the formation of the tertiary and/or quaternary structure of the LonA proteases and, most likely, the formation of complexes with DNA. The choice between these possibilities requires further study.

EXPERIMENTAL

We used reactants from the commercial suppliers: Sigma, Aldrich, Bio-Rad (United Sates), Fluka (Switzerland), Boehringer Mannheim (Germany), Pharmacia (Sweden), Difco (England), Panreac (Spain), Fermentas (Lithuania), and Reachim (Russia).

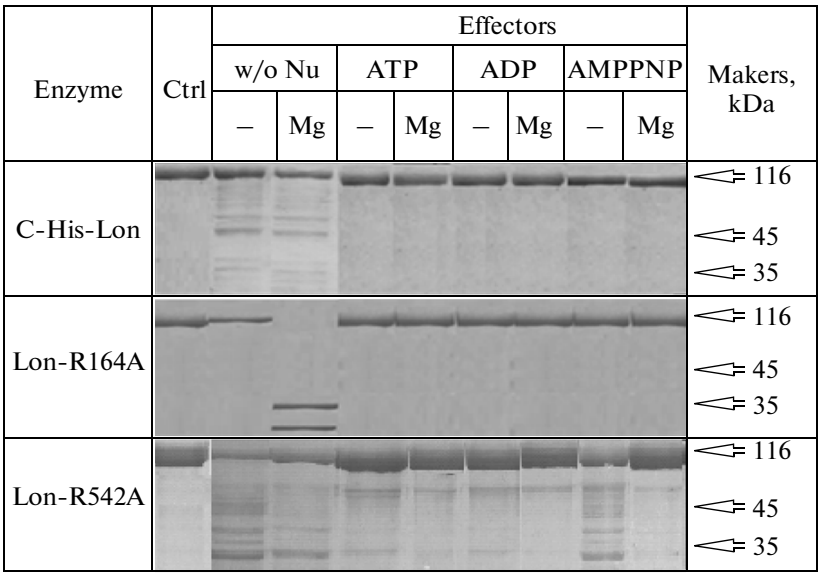

Fig. 4. Autolysis of C-His-Lon protease and its mutants Lon-R164A and Lon-R542A in the absence and presence of effectors. Experimental conditions and designations see in the caption to Fig. 3.

Site-directed mutagenesis. The cloning of the full-sized His-Lon protease into the highly producing pET28 vector was carried out by the standard PCR protocol for amplification of the *lon* gene using DNA of pBRlon as a template [23]. The DNA fragment with a length of 2400 bp was prepared using oligonucleotides f1 and f2 as the forward and reverse primers (Table 2). The target PCR product was treated with *Xho*I restrictase and cloned into the pET-28a(+) vector (Novagen, Cat. No 69864-3), which was pretreated with NcoI, *Pfu* polymerase, and XhoI restrictase. Plasmid pET28-lon DNA was then isolated.

To obtain the mutant of the C-His-Lon protease containing the replacement of Arg164Ala the primers Lon_R164A, f3, and f4 (Table 2) were designed by megaprimer method. The amplification of the gene fragment was performed in two stages using plasmid pET28-lon DNA as a template. Using the Lon_R164A and f4 primers at the first stage, we prepared the PCR fragment, which was used as the primer at the second stage along with the f3 primer. The resulting DNA fragment (~730 bp) was cloned into the pET-28-lon vector using the unique *Hind*III and *Acc*65I restriction sites. The mutation was confirmed by the sequencing of the isolated plasmid DNA (pET28-lon-R164A).

Plasmid DNA (pET28-lon-R542A) was obtained by the same way and used to prepare the mutant of the C-His-Lon protease with the Arg542Ala substitution. For this purpose, we used the f2, f5, and Lon_R542A primers (Table 2). The cloning was carried out using the unique SalI and XhoI restriction sites.

The sequencing of the cloned DNA from several primary clones and the synthesis of the primers were performed in the EVROGEN company (www.evrogen.ru). The restriction and ligation procedures were carried out according to the manufacturer's protocols for the corresponding enzymes.

Isolation and purification of C-His-Lon protease and its mutants. The competent *E. coli* cells of the BL21(DE3) strain were transformed with the corresponding plasmid constructions by the heat shock method [24]. The transformed cells were incubated in the LB culture medium for 1 h at 37°C to induce the gene of the resistance to kanamycin (the selective marker of the pET28a vector and its derivatives). An aliquot of the cell suspension was seeded on the agar medium containing kanamycin and incubated for 12 h. The selected colonies were grown for 12 h at 37°C in small volumes of the LB medium. The resulting culture was transferred to a large volume of medium, containing kanamycin and grown to the value of A_{600} ~ 0.5 ou. IPTG was then added to the concentration of 0.5 mM, and the mixture was incubated for 3 h. The resulting cells were precipitated by centrifugation.

The cellular biomass (2 g) was resuspended in 50 mM Tris-HCl buffer, pH 7.5 (60 mL), incubated with lysozyme (0.4 mg) for 1 h at 4°C, lysed by sonication in an ultrasonic disintegrator (15 kHz, 4 × 1 min) at 0°C and centrifuged for 30 min at 10000 g 4°C.

The chromatography of the cell-free extract was carried out on Ni-Sepharose (HiTrap™FF columns, tandem of 2 × 5 mL, GE Healthcare) in 20 mM Tris-HCl buffer, pH 7.5 containing 0.5 M NaCl and 5 mM imidazole. The bound proteins were eluted in a gradient of imidazole concentrations (5 mM–1 M) in the same buffer. Gel filtration of the C-His-Lon protease and its mutant forms was performed on a Sephacryl S-400 column (120 mL, GE Healthcare) in 20 mM Tris-HCl buffer, pH 7.5 containing 0.15 M NaCl.

Table 2. Structure of oligonucleotide primers for preparation of C-His-Lon protease and its mutant forms Lon-R164A and Lon-R542A

Modification of Ec-Lon	Primer	Structure of the primer (5'-3')*	Restrictase
C-His-Lon	f1	AATCCTGAGCGTTCTGAACGC	
C-His-Lon Lon-R542A	f2	GGG*CTCGAG*TTTTGCAGTCACAACCTGCATG	*Xho*I
Lon-R164A	Lon_R164A	CAGCAATGGTATCCGCCAG**AGC**GGCCGGATCGTCGATGCTATT	
Lon-R164A	f3	CGCAGAAAG*AAGCTT*CAACGG	*Hind*III
Lon-R164A	f4	CGCATTCCAC*GGTACC*TGTAC	*Acc65*I
Lon-R542A	f5	CTGACC*GTCGAC*GATAGCGC	*Sal*I
Lon-R542A	Lon_R542A	CAGTTTGGAGATTTCACGC*TCTAGA*CC**AGC**CACGCCCGCCTCACGGGT	*Xba*I

* Restriction sites are in italic and underlined; mutation sites are in bold.

Standard analytical methods. The protein concentration was evaluated by the Bradford method [25]. The homogeneity of the protein preparations were tested electrophoretically [26] using the commercial marker kit (*M* kDa) containing β-galactosidase (116.0), bovine serum albumin (66.2), ovalbumin (45.0), lactate dehydrogenase (35.0), restrictase Bsp981 (25.0), β-lactalabumin (18.4), and lysozyme (14.4).

ATPase activity was tested by the accumulation of inorganic phosphate during the hydrolysis of ATP [27]. The reaction was carried out at 37°C in the reaction mixture (1.2 mL) containing 50 mM Tris-HCl buffer, pH 8.1, 150 mM NaCl, 5 mM ATP, 20 mM $MgCl_2$, 1 μM enzyme. The enzyme was replaced by the buffer in the control experiment. To evaluate the initial rates of the reactions, aliquots (200 μL) of the reaction and control mixtures were taken at regular intervals and shaken in quartz cuvettes with the reagent (600 μL) consisting of 100 mM $Zn(AcO)_2$, 15 mM $(NH_4)_6Mo_7O_{24}$, and 1% SDS, pH 4.5–5.0. Optical absorption was registered at 350 nm (ε_{350} = 7800 M^{-1} cm^{-1}).

Thioesterase activity. Hydrolysis thiobenzyl ester of *N*-substituted tripeptide Suc-Phe-Leu-Phe-SBzl (PepTBE) was monitored spectrophotometrically by measuring optical absorption of 4-thiopyridine at 324 nm (ε_{324} = 16500 M^{-1} cm^{-1}), which is formed as a result of the interaction of the hydrolysis product with 4,4'-dithiopyridine (DTDP) [28].

The reaction was carried out at 37°C in the reaction mixture (1 mL) containing 50 mM Tris-HCl buffer, pH 8.1, 150 mM NaCl, 10% DMSO, 0.2 mM DTDP, 0.1 mM PepTBE, 2.5 mM Nu, 20 mM $MgCl_2$, and 0.2–0.3 μM enzyme. When the reaction was carried out in the absence of the effectors, they were replaced by the same volumes of the buffer. The thioesterase activity of mixed oligomer Lon-R542A/Lon-S679A was evaluated at the concentration of the mutant Lon-R542A and Lon-S679A forms in the reaction mixture being 0.05 and 0.5 μM, respectively.

Proteolytic activity of the enzymes was evaluated electrophoretically [26]. The reaction was carried out at 37°C in the presence or absence of the effectors in the reaction mixture containing 50 mM Tris-HCl buffer, pH 8.1, 150 mM NaCl, 5 mM Nu, 20 mM $MgCl_2$, 20 μM β-casein, 5 μM enzyme. When the reaction was carried out in the absence of the effectors, they were replaced by the same volumes of the buffer. Aliquots of the reaction or control mixture were added to 4× lysis buffer (7 μL) containing 0.2 M Tris-HCl buffer, pH 8.9, 4% SDS, 20% glycerol, 0.5 mM EDTA, 0.8% bromophenol blue, and 3% β-mercaptoethanol. The reaction mixture was denatured by boiling and loaded on 12% PAAG for electrophoresis.

Autolytic activity of the enzymes was evaluated electrophoretically [26] under the conditions similar to those for the evaluation of the proteolytic activity except the use of β-casein.

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