The isolated proteolytic domain of *Escherichia coli* ATP-dependent protease Lon exhibits the peptidase activity

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Abstract Selective protein degradation is an energy-dependent process performed by high-molecular-weight proteases. The activity of proteolytic components of these enzymes is coupled to the ATPase activity of their regulatory subunits or domains. Here, we obtained the proteolytic domain of *Escherichia coli* protease Lon by cloning the corresponding fragment of the *lon* gene in pGEX-KG, expression of the hybrid protein, and isolation of the proteolytic domain after hydrolysis of the hybrid protein with thrombin. The isolated proteolytic domain exhibited almost no activity toward protein substrates (casein) but hydrolyzed peptide substrates (melittin), thereby confirming the importance of the ATPase component for protein hydrolysis. Protease Lon and its proteolytic domain differed in the efficiency and specificity of melittin hydrolysis.

Key words: ATP-dependent proteolysis; Protease Lon; Proteolytic domain; Activity; *Escherichia coli*

1. Introduction

Selective protein degradation is one of the most strictly regulated intracellular processes. As a rule, most defective polypeptides and some short-lived regulatory proteins are hydrolyzed by high-molecular-weight multimeric proteases. In *Escherichia coli* cells, at least four different proteases participate in protein breakdown: Lon [1–5], Clp [3,6–8], HslVU (ClpQY) [9–11], and FtsH (HflB) [8,12]. In all the cases, protein degradation is coupled to ATP hydrolysis. In Clp and HslVU proteases, different subunits are responsible for proteolytic and ATPase activities. These enzymes are heterooligomers composed of proteolytic (ClpP, HslV) and regulatory ATPase (ClpA, HslU) subunits [7,10,13]. The presence of ATPase subunits increases dramatically the activity of the proteolytic components of the enzymes. Thus, it was found that the endoproteolytic activity of individual ClpP protease (21.5 kDa) comprises only 1% of the activity of the ClpAP complex [4], although ClpP retains the capacity to hydrolyze certain peptide substrates [14]. The HslV subunit (19 kDa) exhibits little or even no peptidase activity but is considerably activated after association with HslU [10].

Lon and FtsH proteases differ from Clp and HslVU in the fact that in their molecules, the proteolytic and ATPase sites are localized within the same polypeptide chain, while these enzymes function as homooligomers. Protease Lon is a tetramer, whose subunit consists of three functional domains – N-terminal, ATPase, and proteolytic (further referred to as N, A, and P domains, respectively) [15–17] (Fig. 1). Domain-domain interactions play an essential role in coupling between the proteolytic and ATPase activities and are considered to account for the high selectivity of Lon toward protein substrates. Earlier, we demonstrated that the proteolytic and ATPase domains tightly interact with each other in enzyme function [18]. Studies of the truncated protease Lon composed of the A and P domains only revealed the importance of the N domain for both enzymatic activities of the protease [17]. Also, some amino acid residues involved in coupling between the ATPase and proteolytic activities were identified [18,19]. In view of this, it seemed interesting to elucidate whether or not the isolated proteolytic domain of protease Lon exhibits the activity in the absence of the ATPase and N-terminal domains.

Here, we demonstrated that the isolated proteolytic domain of protease Lon has the peptidase activity but is almost inactive toward protein substrates.

2. Materials and methods

2.1. Bacterial strains and plasmid constructs

The *E. coli* strain MH-1 was used for transformation with plasmid constructs and expression of recombinant proteins.

DNA fragments encoding the full-size and truncated *lon* genes were amplified by polymerase chain reaction with *Taq* DNA polymerase using the earlier constructed plasmid pBR327lon [15] as a template.

The primers

5' AAA AAC CCG GGA ATG AAT CCT GAG CGT TCT GAA C 3' (I)
*Sma*I

5' AA AAA GGA TCC CAT ATC GAA ATT AAC GGC GAT AAC 3' (II)
*Bam*HI

5' AA AAA GAA TTC *TCA CTA* TTT TGC AGT CAC AAC C 3' (III)
*Eco*RI *Stop*

were designed to introduce *Sma*I and *Eco*RI restriction sites at the 5′- and 3′-ends of the full-size *lon* gene (primers I and III) and *Bam*HI and *Eco*RI restriction sites at the 5′- and 3′-ends of its truncated variant (primers II and III).

Cloning of the obtained fragments in the plasmid vector pGEX-KG yielded the recombinant plasmids pGEX-NAP and pGEX-P coding for the hybrid proteins GST-LonNAP and GST-LonP, respectively.

2.2. Expression and isolation of the recombinant proteins

Expression and isolation of the obtained recombinant proteins and their digestion with thrombin were performed as described by Rasulova et al. [17]. *E. coli* cells MH-1 bearing the constructs pGEX-NAP or pGEX-P were grown in 200 ml of LB medium containing 100 μg/ml ampicillin at 37°C to OD_{600} 0.4–0.5. Isopropyl-β-thiogalactopyranoside (IPTG) was added to a concentration of 0.5 mM, and the cells were incubated for another 3 h. The cells were then harvested by centrifugation, resuspended in 5 ml of cold 50 mM Tris-HCl (pH

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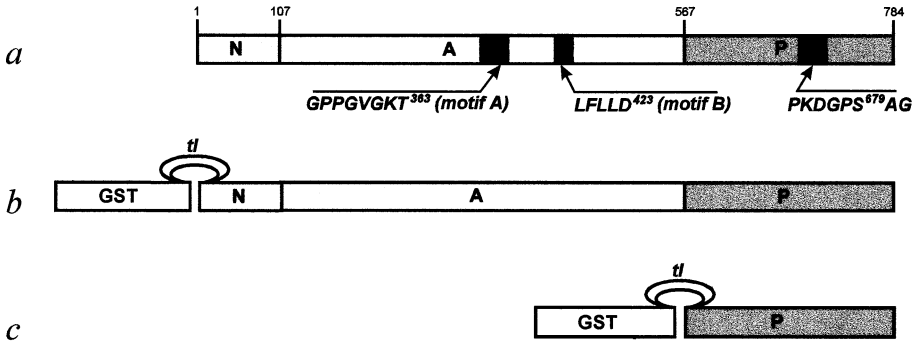

Fig. 1. Structure of *E. coli* protease Lon (a) and hybrid proteins (b) GST-LonNAP and (c) GST-LonP used for obtaining full-size protease Lon (LonNAP) and its proteolytic domain (LonP). N, A, and P, N-terminal, ATPase, and proteolytic domains, respectively; GST, glutathione-*S*-transferase; tl, thrombin-sensitive linker. Conserved Walker's motifs A and B of the ATPase domain and conserved fragment containing the catalytically active Ser^{679} residue are shown.

7.3) containing 10% glycerol (buffer A), disintegrated by sonication, and centrifuged at 40 000×*g* for 30 min at 4°C.

The cell-free extract was applied at a flow rate of 3 ml/h to a glutathione-agarose column (1.0×3 cm) equilibrated with buffer A. The column was washed from nonbound proteins, and the hybrid proteins were eluted with buffer A containing 5 mM reduced glutathione. To remove glutathione, the enzyme preparations were dialyzed against buffer A.

GST-LonNAP and GST-LonP were hydrolyzed with thrombin in 1.5 ml of the reaction mixture containing 0.5 mg of the hybrid protein and 6 μl of thrombin (330 U/ml) in buffer A at 25°C for 2 h. The hydrolysate was applied to a glutathione-agarose column as described above. The target proteins (LonNAP or LonP) did not bind to the sorbent and were eluted with the breakthrough.

Protein was determined by Bradford's method [20]. Protein fractions were analyzed by SDS-PAGE by Laemmli's method [21].

The proteolytic activity of the enzymes was determined from hydrolysis of [^{14}C]acetyl-α-casein [16]. The peptidase activity was assayed by hydrolysis of melittin. The hydrolysis products were separated by HPLC on a Nucleosil C18 column (Machery-Nagel, Germany) in a linear gradient of acetonitrile concentration (0–80%) in 0.1% trifluoroacetic acid [22]. The ATPase activity was determined as described by Bencini et al. [23] with modifications [19].

The molecular masses of LonNAP and LonP were determined by FPLC on a Superose-12 column (Pharmacia, Sweden) in 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl.

3. Results and discussion

3.1. Isolation of the full-size protease Lon and its proteolytic domain

To obtain full-size protease Lon (LonNAP) and its proteolytic domain (LonP, residues 567–784), the corresponding gene fragments were amplified by PCR and cloned in the plasmid vector pGEX-KG [24], which allows hybrid proteins composed of glutathione-S-transferase (GST) and a target protein to be synthesized (Fig. 1). The hybrid proteins GST-LonNAP and GST-LonP, in which the target protein was bound to GST via the thrombin-sensitive linker Leu-Val-Pro-Arg-Gly-Ser-Pro-Gly, were expressed in *E. coli* cells MH-1 with the yields of about 30% of total cell protein (Fig. 2). The hybrid proteins were isolated as described by Rasulova et al. [17] by glutathione-agarose affinity chromatography.

Hydrolysis of the hybrid proteins with thrombin yielded the target proteins LonNAP and LonP bearing additional peptides Gly-Ser-Pro-Gly or Gly-Ser, respectively, at their N-termini. The structure of the obtained proteins was verified by N-terminal sequencing. The purity of the protein fractions was approximately 90% (Fig. 3). The proteolytic and ATPase activities of LonNAP did not differ from those of the native enzyme.

Gel filtration on Superose 12 revealed that LonP was eluted as a protein with a molecular mass of approximately 25 kDa (data not shown). Therefore, the proteolytic domain of protease Lon does not undergo oligomerization, which is characteristic of proteolytic components of ClpAP and HslVU proteases [4,11].

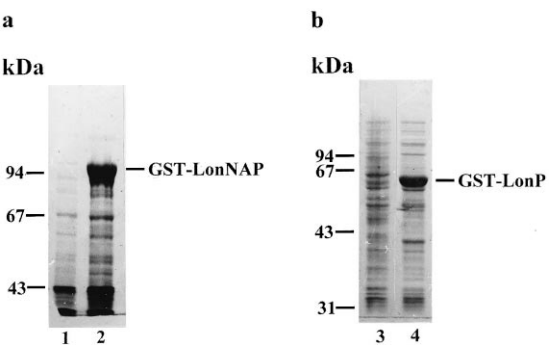

Fig. 2. Expression of hybrid proteins. Analysis by SDS-PAGE in (a) 10% and (b) 15% gel. Lanes 1 and 3, cell lysates before induction; lanes 2 and 4, cell lysates after induction with IPTG. Positions of GST-LonNAP and GST-LonP are indicated.

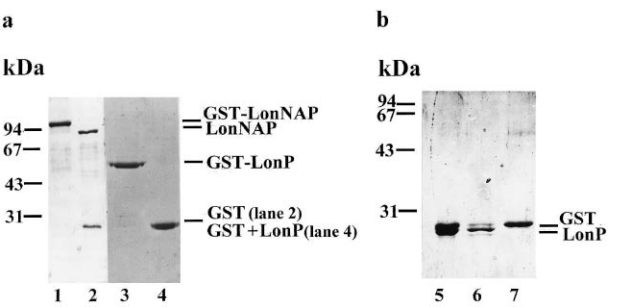

Fig. 3. Thrombin cleavage of the purified hybrid proteins. Analysis by SDS-PAGE in (a) 10% and (b) 18% gel. Lane 1, GST-LonNAP after affinity purification on glutathione-agarose; lane 2, LonNAP and GST after thrombin cleavage of GST-LonNAP; lane 3, affinity-purified GST-LonP; lanes 4 and 5, GST and LonP after GST-LonP cleavage with thrombin; lane 6, LonP after rechromatography of the hydrolysate on glutathione-agarose; lane 7, GST eluted from glutathione-agarose with 5 mM reduced glutathione.

Table 1
Activity of LonP, the hybrid protein GST-LonP, and LonNAP in degrading peptide and protein substrates

Enzyme	Substrate degradation rate (μmol substrate/h·μmol enzyme)	
	α-casein	melittin
LonP	< 0.05	5.0
GST-LonP	< 0.25	10.1
LonNAP (+ATP)	5	33.8
LonNAP (−ATP)	0	9.1

3.2. Catalytic activity of the protease Lon proteolytic domain

The catalytic activity of the isolated LonP toward both protein (casein) and peptide substrates was compared to those of the full-size Lon and the hybrid protein GST-LonP. To assay the peptidase activity, we used melittin, the 26-amino acid peptide from the bee venom (GIGAVLKVLTTGLPALISWIKRLRQQ-NH_2), which had been suggested for testing the specificity of proteases [25,26].

It was found (Table 1) that the activity of the isolated proteolytic domain toward casein was extremely low. The efficiency of casein hydrolysis with LonP comprised ~1% of that with the full-size protease Lon. However, LonP hydrolyzed melittin with approximately the same efficiency as LonNAP in the absence of ATP (in the presence of ATP, the rate of melittin hydrolysis by LonNAP increased 3 to 4 times) (Table 1). The catalytic parameters of the hybrid protein GST-LonP virtually did not differ from those of LonP. Therefore, elimination of the N-terminal and ATPase domains in the Lon protease molecule resulted in the loss of capacity for hydrolysis of protein substrates. However, the isolated proteolytic domain retains the activity toward peptide substrates, thereby resembling proteolytic subunits of ClpAP and HslVU proteases [7,14]. The addition of ATP did not affect the activity of LonP. It should be also noted that both LonP and LonNAP cleaved several peptide bonds in melittin. However, LonP preferentially hydrolyzed the Thr^{10}-Thr^{11} bond, whereas the full-size enzyme hydrolyzed Val^{8}-Leu^{9} bond (as determined by N-terminal sequencing). Hence, the full-size protease Lon and its proteolytic domain exhibit different specificity in melittin hydrolysis.

These results confirm once again the determining role of ATPase components of the complex proteolytic enzymes in the regulation of their proteolytic activity and interaction with protein substrates.

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