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LETTERS
TO THE EDITOR

Forms of LonB Protease from *Archaeoglobus fulgidus* Devoid of the Transmembrane Domain: The Contribution of the Quaternary Structure to the Regulation of Enzyme Proteolytic Activity

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Abstract—Deletion of the transmembrane domain (TM-domain) of *Archaeoglobus fulgidus* LonB protease (*Archaeoglobus fulgidus* (*Af*Lon)) was shown to result in uncontrollable activation of the enzyme proteolytic site and in vivo autolysis yielding a stable and functionally inactive fragment consisting of both α-helical and proteolytic domains (αP). The ΔTM-*Af*Lon-S509A enzyme form, obtained by site-directed mutagenesis of the catalytic Ser residue, is capable of recombination with the αP fragment. The mixed oligomers were shown to be proteolytically active, which indicates a crucial role of subunit interactions in the activation of the *Af*Lon proteolytic site. The thermophilic nature of *Af*Lon protease was found to be due to the special features of the enzyme activity regulation, the structure of ATPase domain, and the quaternary structure.

Key words: Archaeoglobus fulgidus, Lon protease, proteolytic activity, subunit interactions, transmembrane domain

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ATP-dependent Lon proteases (EC 3.4.21.53; MEROPS: clan SJ, S16) and other known enzymes of selective energy-dependent degradation of endocellular proteins (FtsH, ClpAP, ClpXP, HslUV, and 26S proteasome) belong to the superfamily of AAA^+ proteins [1–3]. The Lon family proteases, the active sites of which are represented by the catalytic diad Ser-Lys [4–6], are found in cells of organisms of three basic evolutionary domains, with LonA enzyme subfamily functioning in bacteria and eukaryotes and LonB subfamily, in archaebacteria [7–9]. The representatives of the subfamilies differ in the structure of conserved fragments of the sequences that include catalytic residues and the common structural organization [7]. The endogenous substrates of Lon proteases are the regulatory and also the damaged and alien proteins, whose hydrolysis proceeds by processive mechanism (i.e., without formation of high-molecular intermediates) [10, 11]. Unlike the LonA proteases, classical subjects of studying the energy-dependent proteolysis, LonB proteases practically have not investigated up to now.

A typical representative LonB proteases, the enzyme from *Archaeoglobus fulgidus* (*Af*Lon protease), is an oligomeric thermostable (t_{opt} 70°C) membrane-bound peptide hydrolase, the subunit of which (621 aa., 68.2 kDa) consist of ATPase (A) and proteolytic (P) domains (Fig. 1). The A domain of *Af*Lon is an AAA^+ module specific for AAA^+ proteins. It comprises a nucleotide-binding and α-helical domains (α/β- and α domains, respectively), with an insertion transmembrane (TM) domain being between the consensus Walker's motifs. The catalytic residues of the proteolytic site of the enzyme are Ser509 and Lys552 [6]. *Af*Lon protease carries out the ATP-dependent processive hydrolysis of protein substrates [6, 12].

Deletions were placed in the structure of *lonAf* gene cloned into the vector $pET24a^{(+)}$ to study the structural and functional importance of TM domain of LonB pro-

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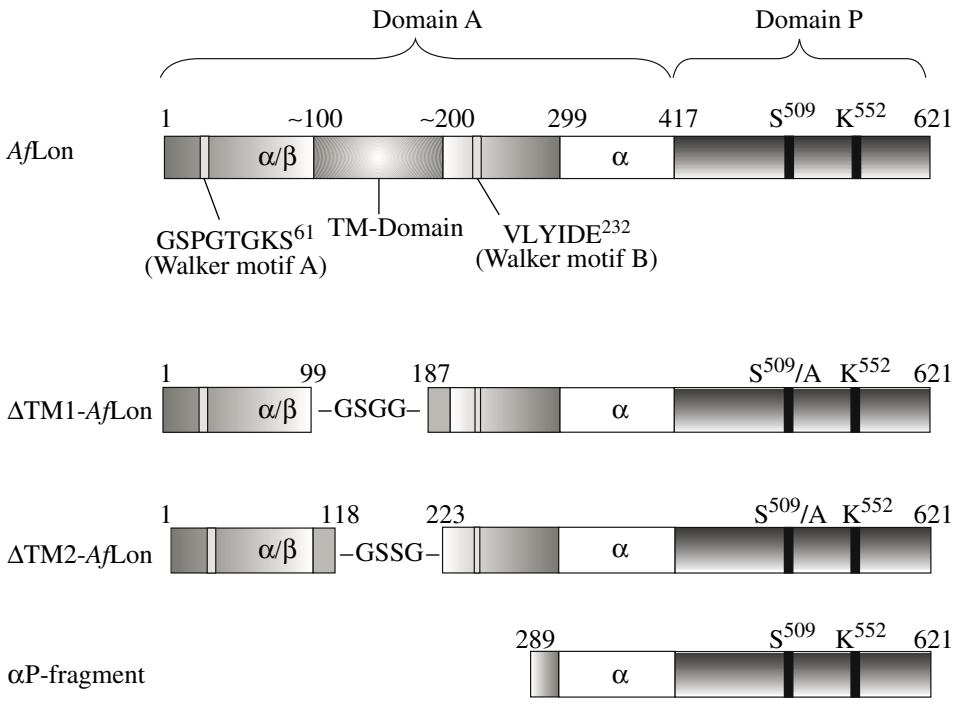

Fig. 1. The structure of*Af*Lon protease and its modified forms.

teases. These led to the elimination of the sequence area presumably corresponding to the TM domain of the enzyme. Without X-ray information, the strict determination of the boundaries of TM domain is impossible, and, therefore, we planned the obtaining of two forms of *Af*Lon protease with the deletion of 100–186 (ΔTM1-*Af*Lon) and 119–222 (ΔTM2-*Af*Lon) fragments of sequence replaced by mobile glycine-serine inserts (Fig. 1). Both deletions led to the removal of the predicted hydrophobic site of the transmembrane domain (121–152, http://smart.embl-heidelberg.de/).

The resulting constructs on the basis of pET24a$^{(+)}$-*lonAf* were checked by sequencing of the structural part of the gene and expressed in the *E. coli* Rosetta (DE3) pLysS like the full-size gene *lonAf* [12]. An unexpected result (Fig. 2) was obtained: the expression of both ΔTM1-*lonAf* and ΔTM2-*lonAf* irrespective of temperature conditions of the induction (37 or 25°C) led to the formation of the same protein with molecular mass ~36 kDa instead of expected protein with M ~ 60 kDa. An analysis of *N*-terminal sequence of the 36-kDa proteins allowed its identification as a fragment of *Af*Lon protease corresponding to combination of α- and P domains of the enzyme (*Af*Lon-(289–621), an αP fragment in Fig. 1).

The main cause of ΔTM(1, 2)-*Af*Lon degradation was the autolysis of the changed forms of enzyme, which was proved by the introduction into the genes ΔTM(1, 2)-*lonAf* of a mutation resulting in the replacement of catalytic of residue Ser509 by Ala and inactivation of proteolytic site in *Af*Lon. One can see in Fig. 2 that the mutant ΔTM forms (ΔTM(1, 2)-*Af*Lon-S509A) that possess neither proteolytic nor ATPase activity are completely stable and can be considered as the models of initial active ΔTM(1, 2)-*Af*Lon forms.

Modified forms of *Af*Lon protease (Fig. 1) were obtained in homogeneous state and analyzed by *N*-terminal sequencing and mass spectrometry. According to the data of a gel-filtration on a HiLoad 16/60 Superdex column (200 pg, 4°C), the αP fragment exists in solution as a monomer, and both forms of ΔTM-*Af*Lon-S509A (ΔTM1 and ΔTM2) are irregular mixtures of associates (from 2 to 8 subunits) and do not show any tendency to association or dissociation at rechromatography.

All the attempts to find out an activity of αP fragment with intact proteolytic site in hydrolysis reactions of protein (β-casein) or peptide (melittin) substrates at various temperatures and incubation times were unsuccessful. This indicates the necessity of participation of additional elements of structure of enzyme in the activation of its proteolytic site. One could think that the localization of αP in a single polypeptide chain with nucleotide-binding domain determines the activated state of P domain in ΔTM-*Af*Lon forms.

At the same time, such a state could also result from the oligomeric organization of ΔTM-*Af*Lon. This circumstance prompted us to study the properties of mixed oligomers formed by mutants ΔTM-*Af*Lon-S509A and the αP fragment. The results of testing proteolytic activity proved to be identical for both ΔTM-forms.

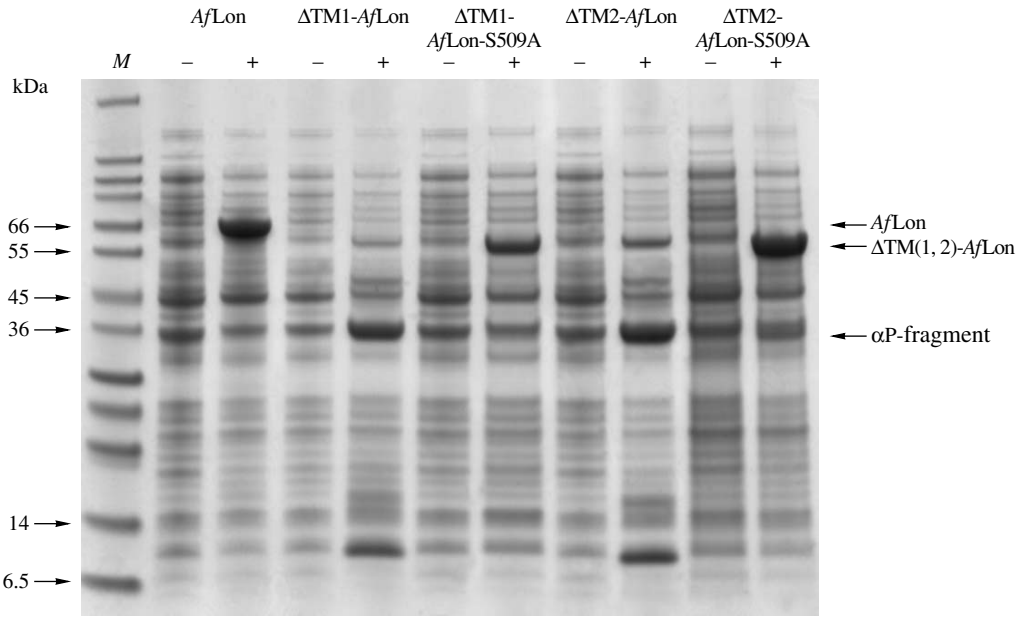

Fig. 2. Expression of A*f*Lon protease and its modified forms in cells of *E. coli* Rosetta (DE3)pLysS strain. The culture was induced with 1 mM isopropyl β-*D*-thiogalactopyranoside at A_{600} 0.5–0.7 for 3 h at 25°C; (-/+), in the absence or in the presence of inducer. Samples of cell lyzates were analyzed in 12% PAG-SDS. *M*, protein markers.

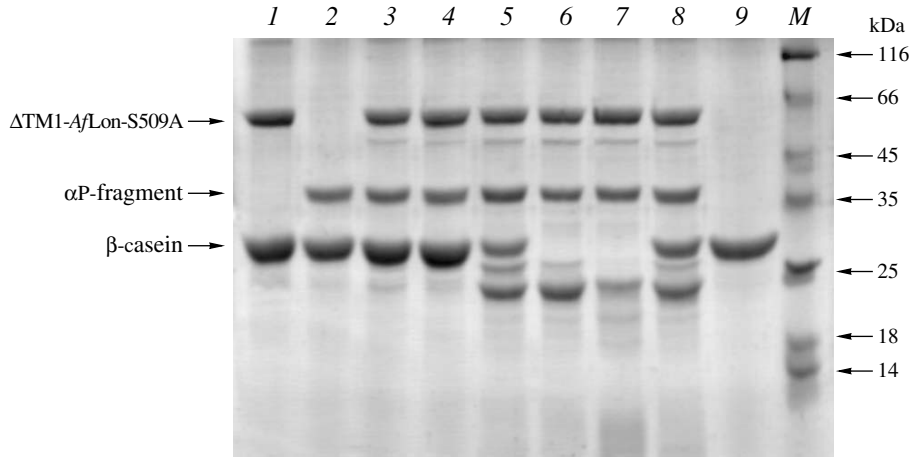

Fig. 3. Proteolytic activity in the system of components αP/ΔTM1-*Af*Lon-S509A (lanes *3*–*8*). Concentrations of proteins: (*1, 3-8*) ΔTM1-*Af*Lon-S509A, 0.5 mg/ml; (*2*–*8*) αP 0.15 mg/ml; (*1*–*9*) β-casein, 1 mg/ml. Effectors: (*4, 7* and *8*) 20 mM $MgCl_2$, (*5* and *7*) 5 mM ATP, (*6* and *8*) 5 mM ADP; *M*, protein markers. Conditions of reaction: 0.1 M bis-Tris-propane buffer, 60°C, 2 h.

As shown by the example of ΔTM1-*Af*Lon-S509A (Fig. 3), the proteolytic activity of αP in the presence of the mutant is restored only upon the introduction in the reaction of ATP or ADP, which indicates the preservation in the deletion forms of ability to bind nucleotides. A distinctive feature of the revealed activity is the nonprocessivity of hydrolysis of protein substrate realized in the magnesium-independent manner. Since the αP fragment does not contain a nucleotide-binding domain and proteolytic site in deletion ΔTM-*Af*Lon-S509A forms is inactivated, it is possible to conclude that the observed activity is a consequence of the recombination of the corresponding forms of *Af*Lon protease and serves as a proof of the key contribution of intersubunit interactions in the activation of proteolytic site of the enzyme P domain.

All the results suggests that (1) the removal of the area of the sequence corresponding to OI domain of *Af*Lon protease leads to the uncontrollable activation of proteolytic site and autolysis of enzyme in vivo; (2) the thermophylicity of *Af*Lon protease is due to the special features of regulation of the enzyme activity, structure

of its ATPase domain, and quaternary structure; and (3) the oligomeric organization of *Af*Lon protease and ATP hydrolysis are necessary prerequisites of realization of the processive degradation of a protein substrate.

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REFERENCES

1. Neuwald, A.F., Aravind, L., Spouge, J.L., and Koonin, E.V., *Genome Res.,* 1999, vol. 9, pp. 27–43.
2. Wickner, S., Maurizi, M.R., and Gottesman, S., *Science*, 1999, vol. 286, pp. 1888–1893.
3. Sauer, R.T., Bolon, D.N., Burton, B.M., Burton, R.E., Flynn, J.M., Grant, R.A., Hersch, G.L., Joshi, S.A., Kenniston, J.A., Levchenko, I., Neher, S.B., Oakes, E.S., Siddiqui, S.M., Wah, D.A., and Baker, T.A., *Cell,* 2004, vol. 119, pp. 9–18.
4. Rotanova, T.V., Melnikov, E.E., and Tsirulnikov, K.V., *Bioorg. Khim.*, 2003, vol. 29, pp. 97–99; *Rus. J. Bioorg. Chem.*, 2003, vol. 29, pp. 85–87.
5. Botos, I., Melnikov, E.E., Cherry, S., Tropea, J.E., Khalatova, A.G., Rasulova, F., Dauter, Z., Maurizi, M.R., Rotanova, T.V., Wlodawer, A., and Gustchina, A., *J. Biol. Chem.*, 2004, vol. 279, pp. 8140–8148.
6. Botos, I., Melnikov, E.E., Cherry, S., Kozlov, S., Makhovskaya, O.V., Tropea, J.E., Gustchina, A., Rotanova, T.V., and Wlodawer, A., *J. Mol. Biol.,* 2005, vol. 351, pp. 144–157.
7. Rotanova, T.V., Melnikov, E.E., Khalatova, A.G., Makhovskaya, O.V., Botos, I., Wlodawer, A., and Gustchina, A., *Eur. J. Biochem.*, 2004, vol. 271, pp. 4865–4871.
8. Lupas, A.N. and Martin, J., *Curr. Opin. Struct. Biol.*, 2002, vol. 12, pp. 746–753.
9. Iyer, L.M., Leipe, D.D., Koonin, E.V., and Aravind, L., *J. Struct. Biol.*, 2004, vol. 146, pp. 11–31.
10. Goldberg, A.L., Moerschell, R.P., Chung, C.H., and Maurizi, M.R., *Methods Enzymol.,* 1994, vol. 244, pp. 350–375.
11. Gottesman, S., *Annu. Rev. Genet.*, 1996, vol. 30, pp. 465–506.
12. Makhovskaya, O.V., Mel'nikov, E.E., and Rotanova, T.V., *Structural Organization and in vitro Functions of ATP-dependent LonB Proteinase from Arshaeoglobus fulgidus, Abstracts and Papers, Int. Conf. Contemporary Problems of Physiology and Biochemistry of Aquatic Organisms, Petrozavodsk, 2004,* Nemova N.N., Ed. Petrozavodsk, 2005, pp. 131–138.