

Polyphosphate, cyclic AMP, guanosine tetraphosphate, and c-di-GMP reduce in vitro Lon activity

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Lon protease is conserved from bacteria to humans and regulates cellular processes by degrading different classes of proteins including antitoxins, transcriptional activators, unfolded proteins, and free ribosomal proteins. Since we found that Lon has several putative cyclic diguanylate (c-di-GMP) binding sites and since Lon binds polyphosphate (polyP) and lipid polysaccharide, we hypothesized that Lon has an affinity for phosphate-based molecules that might regulate its activity. Hence we tested the effect of polyP, cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), guanosine tetraphosphate (ppGpp), c-di-GMP, and GMP on the ability of Lon to degrade α -casein. Inhibition of in vitro Lon activity occurred for polyP, cAMP, ppGpp, and c-di-GMP. We also demonstrated by HPLC that Lon is able to bind c-di-GMP. Therefore, four cell signals were found to regulate the activity of Lon protease.

Introduction

Lon is an 87 kDa ATP-dependent protease¹ that was first purified in *Escherichia coli*,² and it belongs to the AAA⁺ superfamily of ATPases that are associated with diverse cellular activities.³ Lon is divided into two subfamilies, Lon A and Lon B, and this division is based primarily on the characteristics of their catalytic sites.⁴ The catalytic domain of Lon assembles into hexameric rings, suggesting that Lon has a hexameric structure.⁵ In each subunit of this homooligomeric enzyme, there are three functional domains: the N-terminal domain which is involved in substrate recognition and binding, the central ATPase domain, and the C-terminal domain which contains the proteolytic active site.⁶ Lon degradation generates few free residues as it functions as an endo-protease, cleaving substrates into peptides of 5–20 amino acids.⁷

Lon is a global regulator which controls several regulatory proteins including HU, a major DNA binding protein;⁸ SulA, a cell division inhibitor;⁹ RcsA, a positive regulator for capsule synthesis;¹⁰ and several antitoxins (reviewed in Gerdes and Maisonneuve¹¹). Lon also functions as a negative regulator of the type III protein secretion in *Pseudomonas syringae*¹² and degrades naturally unstable proteins as well as misfolded proteins.¹³

In order to ensure normal cellular processes, Lon must be maintained at appropriate levels. In *E. coli* as well as *Streptomyces lividans*, production of Lon from a heterologous promoter results in toxicity.¹⁴ Protein substrates stimulate the intrinsic ATPase

activity of Lon, and this stimulation is unaffected by mutational inactivation of the proteolytic site (reviewed in Suzuki, et al.⁷). Protein sequences rich in aromatic residues are recognized by Lon; these sequences are likely hidden in the hydrophobic cores of proteins but accessible in unfolded polypeptides.¹⁵ The recognition of these signals results in nanomolar binding, leading to subsequent protein degradation.¹⁵ Although Lon is ATP-dependent, Lon has also been shown to respond to other nucleoside triphosphates including GTP, UTP, and CTP, in the hydrolysis of casein, though with less activity than observed with ATP.¹⁶

Several compounds such as chloromethyl ketones, fluorophosphates, and sulfonyl fluorides inhibit Lon activity (reviewed in Rotanova, et al.¹⁷); however, for several of these inhibitors, 50% inhibition of Lon activity generally requires millimolar concentrations of inhibitor.¹ The bacteriophage T4 proteolysis inhibition protein also inhibits Lon protease.¹⁸ Recently, lipopolysaccharide (LPS) was demonstrated to inhibit the peptidase, protease and ATPase activities of Lon.¹⁹ Similar levels of inhibition were observed with mono-phosphoryl and di-phosphoryl lipid, as well as with detoxified LPS and LPS from *Salmonella minnesota* R595; hence, the phosphate groups in the lipid A domain may be responsible for this inhibitory effect, rather than the O-acyl chain or O antigen polysaccharide. Lon also co-precipitated with LPS using an anti-Lon antibody, thus demonstrating direct binding.¹⁹ Furthermore, the evaluation of a series of commercially available peptide-based inhibitors identified

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LALQNEPSGMQVVTAK

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Figure 1. Lon contains c-di-GMP binding motifs. The four RXXXR c-di-GMP-binding motifs which are highly conserved in PilZ-domain containing proteins are shown in yellow. The IGsxxG c-di-GMP-binding motif of BdcA is shown in gray.

the peptidyl boronate MG262 as most potent for inhibiting Lon activity and required binding, but not hydrolysis, of ATP.²⁰

Lon protease forms a complex with polyP.²¹ Proteins degraded by this polyP-Lon complex include free ribosomal proteins; hence, it has been speculated that adaptation to nutritional downshift is mediated in part by action of polyP in directing the degradation of ribosomal proteins.²² The polyP binding site of Lon is localized in the ATPase domain, and therefore competes with DNA for binding to Lon, completely inhibiting the Lon-DNA complex in the presence of equimolar amount of polyP, suggesting that the proteolytic and DNA-binding activities of Lon are likely controlled by polyP.²³ It has been reported that casein hydrolysis by Lon protease is not however affected by polyP.²² Also, both polyP-Lon and DNA-Lon complexes still demonstrate ATPase activity, which has led to the hypothesis that DNA and polyP binding to the DNA-binding domain are dynamic rather than static.²³ Lon also binds double-stranded and single-stranded DNA, and the rate of protein degradation is increased by different DNA species, although this effect was not dependent on any sequence specificity.²⁴ DNA also stimulates the ATPase activity of Lon, and can occur in the absence of a proteolytic substrate.²⁵

Unexpectedly, we found that the sequence of Lon contains several c-di-GMP putative binding motifs (Fig. 1); there are four RxxxR motifs and one IGsxxG motif (Fig. 1). c-di-GMP is an ubiquitous signal that controls many biological processes including motility, biofilm formation, virulence, and cellular morphology.²⁶ The IGsxxG motif is found in the c-di-GMP binding protein BdcA,²⁷ and in PilZ proteins, the c-di-GMP motifs are RxxxR and (D/N)xSxxG.²⁸ These PilZ-domain-containing proteins bind c-di-GMP with variable affinities (sub- μ M to μ M), and it has been speculated that receptors with different or degenerate c-di-GMP binding motifs may exist other than the canonical PilZ domain.²⁹ Furthermore, high affinity binding of c-di-GMP by diguanylate cyclases requires an RXXD motif positioned in close proximity to the active site³⁰; this motif is also conserved in PelD, a degenerate diguanylate cyclase receptor that regulates exopolysaccharide production and binds c-di-GMP³¹ in *P. aeruginosa*. There is additional evidence suggesting that Lon may bind c-di-GMP in that *P. aeruginosa* Lon, which is 84% similar to *E. coli* Lon,³² binds a c-di-GMP analog.³³ Therefore, we explored

the possibility that *E. coli* Lon may bind several cell signals that include phosphate (Fig. 2) and that they may affect its activity.

Results and Discussion

cGMP, cAMP, ppGpp, and polyP inhibit Lon degradation of α -casein

We used α -casein as the reference substrate for Lon due to its high activity on this substrate. Without the addition of the nucleotides or polyP, about 90% of the α -casein was degraded by Lon under these conditions (Fig. 3).

Given that Lon contains five putative c-di-GMP binding sites, we investigated the impact of c-di-GMP binding on Lon activity along with the impact of the similar phosphate-containing cell signals: polyP, cAMP, cGMP, and ppGpp. GMP was also tested as a control. Degradation of α -casein by Lon was nearly completely inhibited by polyP, cAMP, and ppGpp at 850 μ M (Fig. 3). c-di-GMP inhibited Lon activity less (by about 50%). As expected, the Lon degradation products were substantially reduced with polyP, cAMP, and ppGpp (Fig. 3). Similar Lon inhibition was seen at 50 μ M and 450 μ M for polyP, and 450 μ M for c-di-GMP. Since cGMP and GMP did not inhibit the degradation of α -casein, inhibition was not based solely on the presence of a phosphate or guanosine.

The ability of polyP, ppGpp, cAMP, and c-di-GMP to inhibit Lon activity has several physiological implications. PolyP accumulation frequently occurs in response to amino acid starvation; its concentration increases from 700 to 11 000 μ M in response to amino acid starvation³⁶ and can reach 20 000 μ M in the stationary phase.³⁷ This accumulation is frequently accompanied by increased levels of stringent factors including ppGpp and guanosine pentaphosphate (pppGpp). The steady-state concentration of ppGpp in *Escherichia coli* is 25 μ M, although the minimum concentration required to observe penicillin tolerance by amino acid-deprived *E. coli* is 621 μ M.³⁸ Since nutrition stress increases both polyP and ppGpp, we would expect reduced Lon activity under physiological conditions. However, since polyP promotes the degradation of ribosomal proteins,²² the effect of polyP on Lon activity is probably substrate specific.

The secondary messenger cAMP is used by bacteria to control a variety of processes including motility, virulence, and the utilization of a variety of sugars as well as to control sensor proteins such as the cAMP receptor protein and fumarate nitrate reductase.³⁹ The levels of cAMP under starvation conditions are 20 μ M in *E. coli*⁴⁰; hence, cAMP likely has no effect on Lon activity under physiological conditions.

The concentration of the secondary message c-di-GMP is 0.10 μ M in *E. coli*⁴¹ and 50 μ M in *P. aeruginosa*.⁴² Hence, it is unlikely that c-di-GMP should affect Lon activity under physiological conditions unless another factor is involved.

c-di-GMP binds Lon

Since Lon has several putative binding sites, we used HPLC to determine if Lon binds c-di-GMP. We found that Lon binds c-di-GMP since after incubation with c-di-GMP; the peak area was decreased by 50% (Fig. 4).

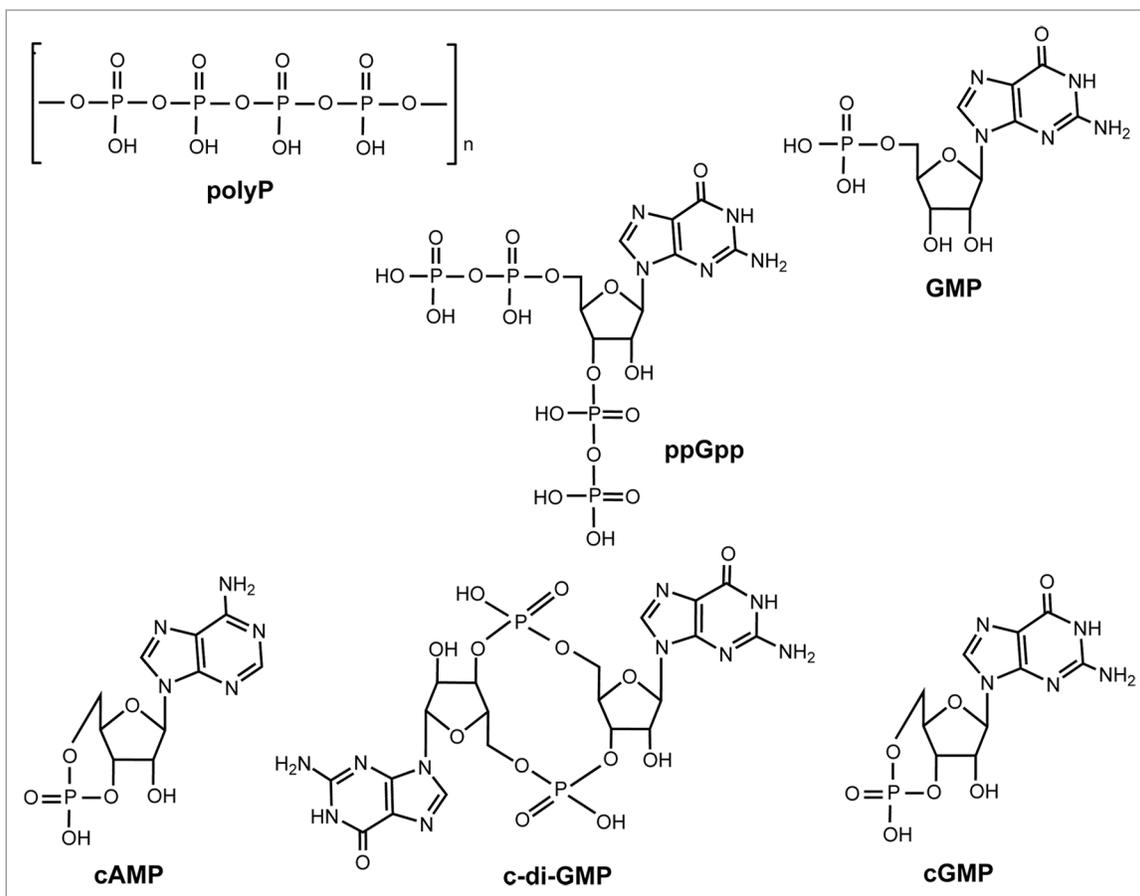


Figure 2. Structures of the compounds tested (polyP, ppGpp, GMP, cAMP, c-di-GMP, and cGMP).

Increasingly, it is becoming evident that Lon regulation occurs through multiple factors. It is also clear that the phosphate groups of different cofactors can interact with Lon and inhibit its activity, as has been demonstrated with Lipid A.¹⁹ The phosphate-rich molecule cardiolipin (phospholipid) selectively binds Lon and inhibits its ATPase and protease functions,⁴³ further highlighting a central role for phosphate-containing molecules in the regulation of Lon activity. Since cGMP and GMP had no effect on the activity of Lon, we can infer that the presence of phosphates is not in itself sufficient for the regulation of Lon. It has been shown that the polyP and DNA-binding sites of Lon are localized in the ATPase domain; hence, polyP competes with DNA for binding to Lon.²³ We speculate that the orientation of phosphates on the small molecules and their ability to interact with the ATPase domain is most critical to the regulation of Lon. This ability to interact with the ATPase domain likely explains why despite the similar orientation of the phosphates on cGMP and cAMP, inhibition is not observed with cGMP but is seen with cAMP. The presence of the carbonyl group of cGMP, likely prevents the orientation necessary for interaction with the ATPase domain. It is noteworthy that with the exception of cAMP, phosphate-based inhibitory small molecules (ppGpp, polyP, and c-di-GMP) contain multiple phosphate groups (Fig. 2). Cardiolipin and Lipid A also contain multiple

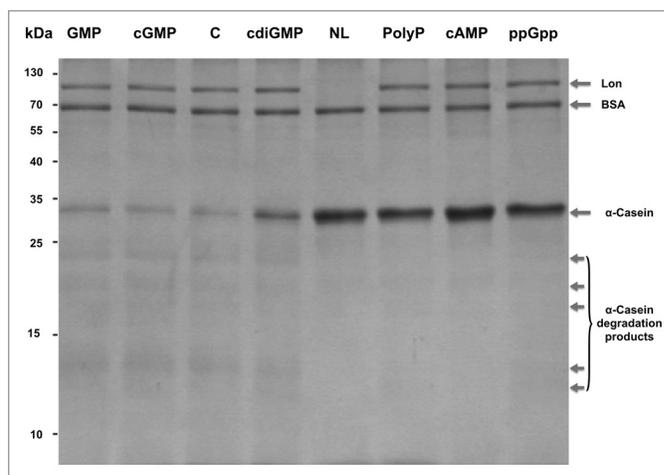


Figure 3. Phosphate molecules inhibit Lon's activity. Casein (6.68 μM) was incubated with Lon (0.19 μM) with 850 μM of GMP, cGMP, c-di-GMP, polyP, cAMP, or ppGpp. Lane C: no phosphate molecule with casein and Lon. Lane NL: no Lon with casein. Samples were incubated for 3 h at 37 °C. BSA (0.68 μM) was added to each reaction.

phosphate groups. Taken together, our findings demonstrate that Lon can be inhibited with phosphate-containing small molecules. Since the levels of ppGpp, polyP, cAMP, and c-di-GMP can

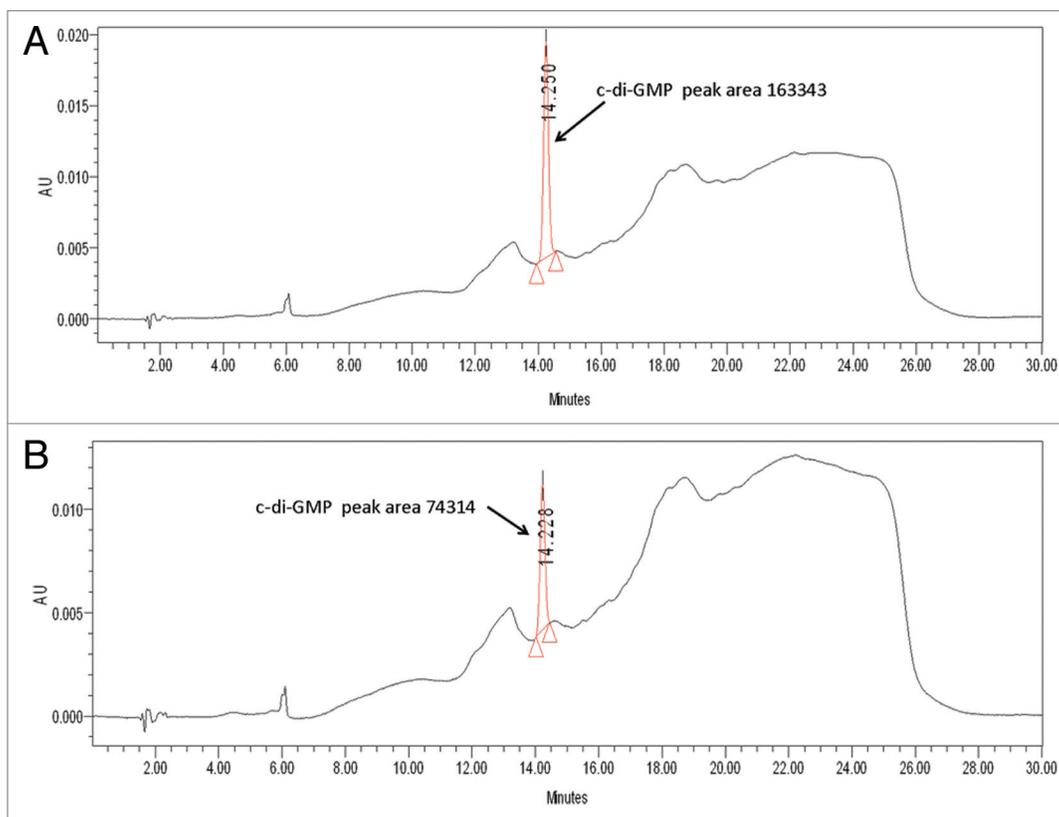


Figure 4. Lon binds c-di-GMP. (A) HPLC chromatogram and spectral index of buffer with 30 μ M of c-di-GMP. (B) HPLC chromatogram and spectral index of Lon incubated with 30 μ M of c-di-GMP.

fluctuate based on environmental factors, we speculate that the inhibitory effect of these nucleotides and polyP might afford the bacteria a way to quickly affect many cellular processes, thus allowing for increased survival and adaptation.

Materials and Methods

In vitro proteolysis assay

The proteolysis reaction was performed based on the method of Kubik et al (2012).³⁴ In brief, 20 μ L reaction volumes contained 0.19 μ M Lon and 6.68 μ M α -casein (Sigma-Aldrich, St. Louis, MO) in reaction buffer (40 mM HEPES-KOH pH 7.6, 25 mM Tris-HCl pH 7.6, 4% w/v sucrose, 4 mM dithiothreitol, 80 μ g/ml BSA, 11 mM magnesium acetate, and 4 mM ATP). cGMP (CalBioChem), GMP (Acros Organics), c-di-GMP (BioLog), ppGpp (TriLink BioTechnologies), cAMP (Sigma-Aldrich, St Louis), and polyP (Kerafast) were added at 850 μ M. Samples were incubated for 3 h at 37 $^{\circ}$ C, the reaction was stopped by the addition of 4 \times Laemmli buffer, and the reaction products were analyzed by SDS-PAGE (gel 15%) with Coomassie Brilliant Blue staining.

HPLC c-di-GMP binding assays

Binding studies were done according to the method of Ma et al.²⁷ In brief, purified His-tagged Lon (30 μ M) was incubated with 30 μ M c-di-GMP for 0.5 h. Since His-tagged Lon is 89 kDa

while c-di-GMP is 0.69 kDa, we used a 10-kDa protein filter unit (EMD Millipore) to separate free and bound c-di-GMP. Samples were run on an HPLC (Waters 2996 Photodiode with 717 plus autosampler) with running buffer A (100 mM KH_2PO_4 , 4 mM tetrabutyl ammonium hydrogen sulfate) and B (75% buffer A, 25% methanol). Peaks were spiked with 5 μ M c-di-GMP in order to verify the correct peak.

Expression and purification of Lon protease

Lon was produced from *E. coli* BW25113/pCA24N-lon by diluting overnight cultures to a turbidity at 600 nm of 0.05 in LB medium and growing to a turbidity of 1.0 at 37 $^{\circ}$ C. Cultures were induced with 1 mM IPTG for 12 h at 25 $^{\circ}$ C to induce expression of 6 \times His-tagged protein.³⁵ Cell lysates were centrifuged and the supernatant was loaded on a His Trap FF Column (GE Healthcare) and the protein purified by AKTA Explorer FPLC (Amersham Biosciences). The activity of Lon produced using this method was found to be the same as that produced at 25 $^{\circ}$ C.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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