Effects of Inorganic Polyphosphate on the Proteolytic and DNA-binding Activities of Lon in *Escherichia coli*

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Lon belongs to a unique group of proteases that bind to DNA and is involved in the regulation of several important cellular functions, including adaptation to nutritional downshift. Previously, we revealed that inorganic polyphosphate (polyP) increases in *Escherichia coli* in response to amino acid starvation and that it stimulates the degradation of free ribosomal proteins by Lon. In this work, we examined the effects of polyP on the proteolytic and DNA-binding activities of Lon. An order-of-magnitude experiment suggested that polyP first binds to Lon, which stimulates Lon-mediated degradation of ribosomal proteins. A polyP-binding assay using Lon deletion mutants showed that the polyP-binding site of Lon is localized in the ATPase domain. Because the same ATPase domain also contains the DNA-binding site, polyP can compete with DNA for binding to Lon. In fact, an equimolar amount of polyP almost completely inhibited DNA-Lon complex formation, suggesting that Lon binds to polyP with a higher affinity than it binds to DNA. Collectively, our results showed that polyP may control the cellular activity of Lon not only as a protease but also as a DNA-binding protein.

Inorganic polyphosphate (polyP) is a linear polymer of many hundreds of orthophosphate residues linked together by ATP-like high energy phosphoanhydride bonds. This polymer naturally occurs in microbes, fungi, plants, and animals (1). In *Escherichia coli*, the level of polyP is very low in the exponential phase, but it increases up to 1000-fold in response to amino acid starvation and during the onset of the stationary phase (2, 3).

We demonstrated previously that polyP forms a complex with the ATP-dependent Lon protease, an enzyme that degrades intracellular proteins during nutritional downshift (4). However, whether polyP first binds to Lon or to the ribosomal proteins has not been established.

Lon is a highly conserved enzyme present in Archaea and eubacteria as well as in the mitochondria of eukaryotes. The Lon monomer includes an N terminus of unknown function, a central ATPase domain containing a typical ATP-binding motif, and a C-terminal proteolytic domain with a catalytically active residue at Ser-679 (5–8). The N-terminal region also contains a “basic-acid-basic” region (9). A sensor and substrate discrimination domain has been identified between the ATPase and protease domains (10). Purified sensor and substrate discrimination domains from known Lon substrates (10), suggesting that it plays a role in substrate recognition. Although *E. coli* Lon is thought to be a homo-tetramer or -octamer, recent structural analysis of the proteolytic domain of Lon revealed that it assembles into hexameric rings (11).

Lon contributes to the regulation of several important cellular functions, including radiation resistance, cell division, filamentation, capsular polysaccharide production, lysogeny of certain bacteriophages (reviewed in Refs. 12–14), and adaptation to nutritional downshift (4). *E. coli* Lon is known to bind non-specifically to DNA (15). However, human Lon binds specifically to a single-stranded GT-rich DNA sequence of human mitochondrial DNA (16). In addition, Fu et al. (17) showed that the *E. coli* Lon binds to a TG-rich site (pets) of the human immunodeficiency virus type-2 enhancer. Whether *E. coli* Lon has a specific binding site on the *E. coli* chromosome is unclear.

It has been suggested that the basic-acid-basic domain is involved in DNA binding (5). Another report speculated that the protease domain is involved in DNA binding, because the amino acid sequence of this domain is similar to that of the DNA-binding protein LexA (18). In both cases, the DNA-binding sites of Lon were predicted from its amino acid sequence. Here, our experimental data suggested that both the polyP- and DNA-binding sites of Lon are localized in the ATPase domain. This explains why polyP competes with DNA for binding to Lon. Finally, based on these findings, we discuss the regulatory effects of polyP on the proteolytic and DNA-binding activities of Lon.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—Lon protease fused with maltose-binding protein (MBP-Lon) was expressed in *E. coli* (pMal-Lon) and purified using amylose resin as described by Sonezaki et al. (19). MBP-Lon was further purified by Hi Trap Q anion exchange chromatography (Amersham Biosciences). MBP-Lon was eluted from the Hi Trap Q column with a linear gradient of 0–1 M NaCl in buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 20% glycerol). Lon was purified after the cleavage of proteolytic removal of MBP as described previously (19). The proteolytically inactive mutant, Lon-S679A, was also expressed as a fusion with MBP (a gift from Dr. S. Sonezaki (Toto Ltd., Kitakyushu, Japan)). MBP-SulA was purified as described previously (20).

Ribosomal proteins were purified from isolated ribosomes as follows. Ribosomes were isolated from *E. coli* MG1655 by ultracentrifugation (21). Ribosomal proteins were extracted by incubating the purified...
ribozones for 20 h at 4°C with 4.5 mM LiCl and 6 mM β-mercaptoethanol (22). After precipitating the ribosomal RNAs by centrifugation (5000 × g, 5 min), the supernatant containing ribosomal proteins was dialyzed against buffer B (50 mM ammonium acetate, pH 5.6, 10% glycerol, and 0.15 mM NaCl). The supernatant was applied to a carboxymethyl cation exchange column (PerSeptive Biosystems, Framingham, MA), and ribosomal proteins were eluted with a linear gradient of 150 mM NaCl in buffer B over 15-column volumes. Some ribosomal proteins were further purified by gel filtration chromatography with Superdex 75 (Amersham Biosciences) in buffer A containing 150 mM NaCl.

Degradation of Ribosomal Proteins by Lon—Ribosomal proteins (5 μg) and Lon (1.5 μg) were incubated in a buffer containing 25 mM Tris-HCl, pH 7.8, 4 mM ATP, and 5 mM MgCl2 in the presence and absence of 1.4 μM polyP, as a polymer at 37°C and then separated by 12.5% SDS-PAGE. PolyP- and DNA-binding Assays—Deletion mutants of Lon fused to MBP were expressed in E. coli DH5α on 2% YT medium (16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl/liter) (23) with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were collected by centrifugation and suspended in SDS sample buffer (23). After boiling for 5 min, samples were separated by 12.5% SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes. The membranes were washed twice with an incubation buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM MgCl2, and 0.1% Triton X-100) and incubated for 12 h at 37°C in the same buffer containing 1% bovine serum albumin. PolyP- and DNA-binding activity of Lon was assessed as described above except using 32P-labeled DNA instead of polyP. The radioactivity remaining on the filter was measured by scintillation counting.

Sequencing of DNA Extracted from DNA-Lon Complex—Protolytically inactive Lon fused with MBP was expressed in E. coli harboring pMal-Lon(S679A). After the E. coli cells were disrupted, a lysate containing MBP-Lon-DNA complexes was applied to the immobilized amyllose column. The column was washed with five volumes of 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.25% Tween 20 and then washed with five volumes of the same buffer containing 200 mM NaCl. After free DNA was completely eluted from the column, MBP-Lon was eluted with the same buffer containing 10 mM maltose. The fraction containing MBP-Lon was extracted twice with the same volume of phenol-chloroform, after which DNA was precipitated with ethanol. The DNA was dissolved with water, inserted into the Smal site of pUC119, and then used to transform E. coli. Inserted DNAs obtained from 21 individual recombinants were sequenced (23).

Gel-shift Assay—LeuC DNA (0.5 pmol) and Lon (S679A) (1 μg) were incubated for 5 min at room temperature in the presence or absence of polyP. The reactions were then mixed with loading dye and separated by 1% agarose gel electrophoresis (23). After staining with ethidium bromide, DNA was visualized under UV light. Double-stranded pets DNA (GATCCAGCTATACTTGGTCAGGGCGAATTCTAACTA) (17) was used to transform E. coli cells, which were disrupted, and total DNA was isolated. DNA sequences of these primers are shown in Table I.

TABLE I

| Primer   | DNA sequence                                      |
|----------|---------------------------------------------------|
| F1       | ATGAACTCTCAGGCGGCTTCGA                           |
| F138     | ACGGCAGGTGGAAGGCTCAT                             |
| F181     | CAGTGTCTCCTGAGATGCTC                             |
| F302     | GACAGATCCGCAAGGCTCAT                             |
| F320     | GATGCCAGCATTTGCTT                               |
| F472     | TGGACCTCCCCATGATACCTCC                            |
| F618     | GTGCCGGATGGGGAACGAAACTG                         |
| R302     | CCACGAACTTCAGGCGGCTCTCCTCAT                     |
| R437     | AAGGAACTCTTCCAGGCGGCTCTCCAT                     |
| R616     | ACAGGAATCTTCCATGATACCTCC                        |
| R713     | ACAGGAATCTTCCATGATACCTCC                        |
| R755     | CGTGAATCTCCAGGTCATATTGTCAGGCA                    |
| R784     | ACAGGAATCTTCCATGATACCTCC                        |

RESULTS

PolyP Affects the Substrate Specificity of Lon—In the presence of polyP, Lon degrades most LiCl-extracted ribosomal proteins (4). We purified several ribosomal proteins of the large subunit (L1, L3, L6, L9, L13, L15, L17, L18, and L24) and subjected them to Lon-mediated degradation in the presence or absence of polyP. Lon degraded L1, L3, L6, and L24 in the presence but not in the absence of polyP (Fig. 1A). PolyP also stimulated the degradation of purified L9, L13, and L17 but not
L15 and L18 (data not shown). Lon is known to be responsible for the rapid turnover of regulatory proteins, including SulA, a cell division inhibitor produced in response to DNA damage. Therefore MBP-SulA was subjected to degradation by Lon in the presence and absence of polyP. In contrast to the ribosomal proteins tested, the degradation of the MBP-SulA protein was slightly inhibited in the presence of 1.4 \( \mu \text{M} \) polyP (Fig. 1B).

Therefore, our results showed that polyP does not always stimulate Lon-mediated proteolysis and that polyP affects the substrate specificity of Lon.

**PolyP-Lon Complex Formation Is Important for the Stimulation of Ribosomal Protein Degradation**—Most ribosomal proteins bind to polyP and are degraded by Lon in the presence of polyP (4). The MBP-SulA protein does not bind to polyP, indicating that polyP binding is necessary for the polyP stimulation of Lon-mediated proteolysis. However, not all polyP-binding proteins are degraded by Lon in the presence of polyP. For example, polyP kinase, L15, and L18 can bind to polyP, but they are not degraded by Lon in the presence of polyP. Therefore, it is likely that polyP binding is necessary but not sufficient for the polyP stimulation of Lon-mediated proteolysis.

Lon also binds polyP (4). Does polyP first bind to Lon and then the polyP-Lon complex degrades the ribosomal proteins? Alternatively, does polyP bind to ribosomal proteins and then Lon recognizes the polyP-ribosomal protein complex for degradation? In the latter case, polyP may function as a molecular tag, much like ubiquitin in eukaryotic cells (reviewed in Refs. 24–26), to identify ribosomal proteins for degradation by proteases. To answer these questions, we examined the effect of the order of addition on L24 cleavage (Fig. 2). We found that...
Lon efficiently degraded L24 when Lon and polyP were mixed together prior to the addition of L24, whereas there was little degradation of L24 when L24 was first mixed with polyP before the addition of Lon (Fig. 2). This indicated that polyP-Lon complex formation is important for the stimulation of protein degradation and that polyP does not act as a tag for Lon-mediated degradation.

PolyP- and DNA-binding Sites of Lon Are Localized within the ATPase Domain—Lon consists of basic-acid-basic, ATPase, sensor and substrate discrimination, and proteolytic domains (Fig. 3A). To determine the polyP-binding site of Lon, we generated proteins containing deletion mutants of Lon fused to the C terminus of MBP. These proteins were expressed and transferred to a nitrocellulose membrane (Fig. 3B). PolyP blotting was performed using [32P]polyP (Fig. 3B). The results of this experiment showed that the polyP-binding site is localized on Lon between amino acids 272 and 437. Because Lon 320–784 also bound to polyP, the polyP-binding site is presumably restricted to the region between amino acids 320 and 437. Therefore, polyP binding appears to be mediated by the ATPase domain.

We examined this further using purified Lon deletion mutants. The fusion proteins were purified by amylose chromatography and then used in a polyP-binding assay (Fig. 3C). We found that the binding activity of the purified Lon 272–437 was almost same as that of intact Lon. This supports the idea that the polyP-binding site is localized in the ATPase domain of Lon.

Although Lon was originally identified as a DNA-binding protein (5, 15), the location of the DNA-binding site has not been determined. We therefore performed a DNA-binding assay using the purified Lon deletion mutants. We used 32P-labeled E. coli leuC DNA as a probe because, as shown below, a leuC DNA fragment was co-purified with MBP-Lon. Fig. 3C shows that the DNA-binding site also appears to be localized in the ATPase domain of Lon.

Lon Binds to polyP More Strongly than to DNA—Because the ATPase domain contains both polyP- and DNA-binding sites, polyP may compete with DNA for the binding to Lon. To examine this possibility, we determined the effect of polyP on the DNA-Lon complex using a gel-shift assay (Fig. 4). DNA (leuC) formed a complex with Lon, but in the presence of an equimolar amount of polyP, formation of the DNA-Lon complex was almost completely inhibited. This suggested that polyP binds to Lon more strongly than to DNA. To further confirm that the DNA-Lon complex is inhibited in the presence of polyP, we examined the effect of polyP on MBP-Lon binding to a DNA-cellulose column. MBP-Lon was not eluted from the column by a buffer alone, but was eluted when the buffer contained polyP (Fig. 5). This result suggested that polyP can bind to Lon at the same site as DNA (Fig. 5).

During the course of the studies using amylose chromatography, we observed that purified MBP-Lon contains bound DNA. This bound DNA was lost when the MBP-Lon was further purified using an anion-exchange column. Interestingly, when the E. coli cells were subjected to nutritional downshift, which increases polyP level as much as 1000-fold (2, 3), we found a significant amount of polyP associated with the amylose resin-purified MBP-Lon (data not shown). Together, our results showed that although Lon can form a complex with DNA in vitro and in vivo, Lon forms a complex with polyP when it is present.

Lon Binds Nonspecifically to E. coli Chromosomal DNA—Although Fu et al. (17) showed that the E. coli Lon binds to a TG-rich pets site of the human immunodeficiency virus type2 enhancer, it is unclear whether Lon binds to specific sites on the E. coli genome. To examine this in greater detail, we cloned and sequenced the DNA fragments that were co-purified with the amylose resin-purified Lon. Twenty-one DNA clones were randomly selected for analysis. Their DNA sequences corresponded to 382–1009-bp fragments containing b0899, b2324, ftsJ, leuC, lon, lpdX, malE, rhsE, secD, yacK, yafA, ybbC, yecA, yeyB, yhfK, ygcU, and ymfE, and 496–679-bp fragments containing the intercistronic regions between b2973 and b2972, cynS and cynX, rplM and rpsL, and yecS and yecC (Table II). There was no significant similarity among the sequences of
these DNA fragments. Thus, in agreement with Charette et al. (15), it appears that Lon binds nonspecifically to *E. coli* chromosomal DNA.

We also examined the relative affinities of *leuC* and the pets DNA. We used synthesized 36-bp TG-rich pets DNA as a competitor in the *leuC* DNA-binding experiment. The *leuC* DNA-Lon complex was still observed in the presence of a 100-fold molar excess of the pets DNA (data not shown), suggesting that the pets DNA has lower affinity toward Lon than the *leuC* DNA.

**DISCUSSION**

PolyP Regulates the Proteolytic Activity of Lon by Forming a polyP-Lon Complex—Regulatory proteolysis is generally quite specific, and the features of proteins that target them for degradation are well known in eukaryotic cells. Specifically, conjugation of proteins with ubiquitin (ubiquitination) targets them to the proteasome and is essential for the degradation of many proteins in eukaryotic cells (24–26). In bacteria, the SsrA-mediated tagging and degradation system provides a unique mechanism for the destruction of abnormal proteins produced by inappropriate translation termination (27). Therefore, we suspected that polyP acts as a tag to direct Lon-mediated protein degradation. However, our current order of addition experiments suggested that polyP first binds to Lon rather than to the targeted ribosomal proteins, suggesting that polyP does not function as a tag for Lon-mediated degradation.

We recently found that based on gel chromatography, the polyP-Lon complex is very large (>2000 kDa). Furthermore, dynamic light scattering measurements indicated a molecular mass of ~8750 kDa (data not shown). Because one molecule polyP binds to approximately four molecules of Lon (4), it might be considered that the polyP-Lon complex is a multiple of the Lon hexamer. Additional studies must be performed to more precisely determine the structure of the polyP-Lon complex.

PolyP Affects the DNA-binding Activity of Lon—Analysis of polyP and DNA binding by Lon mutants indicated that the polyP- and DNA-binding sites of Lon are localized in the ATPase domain. Also, polyP bound to Lon more strongly than DNA, which allows polyP to displace the Lon-associated DNA in *vitro* as well as in *vivo*. The ATPase domain is thought to be involved in the important chaperon activity (10), and DNA has been reported to stimulate the ATPase activity of Lon (15). However, we have shown previously that polyP slightly inhibits the ATPase activity of Lon in the absence of substrates (4). It is a bit puzzling that both of the polyP-Lon and DNA-Lon complexes show ATPase activities if polyP and DNA persistently cover the ATPase domain. It might be possible that polyP and DNA binding to the ATPase domain are dynamic rather than static.

Regulatory Effects of polyP on the Proteolytic and DNA-binding Activities of Lon—Goff and Goldberg (28) reported that overproduction of Lon causes the proteolysis of normal proteins in cells and is lethal to the host cells. We also observed that *E. coli* growth stopped immediately when Lon was overproduced (data not shown). Excess Lon is unfavorable to cells under normal conditions. Sonezaki et al. (29) speculated that Lon is normally localized in the multiprotein-DNA complex (nucleoid), which might restrict its ability to access and degrade proteins under normal conditions. They also showed that MBP-Lon was dissociated from DNA in the presence of denatured but not native bovine serum albumin and that treatment of MBP-Lon at 47 °C reduced its DNA-binding ability but does not affect its proteolytic activity. These findings suggested that exposure to heat shock causes a release of Lon from DNA allowing it to degrade denatured proteins. Similarly our data suggested that when the levels of polyP increase in response to nutritional downshift, the polyP-Lon complex is released from DNA whereupon it can access and degrade free ribosomal proteins.

It is also possible that Lon binds to DNA at a site adjacent to a regulatory protein-binding site, thus leading to enhanced degradation of the regulatory protein. In agreement with this model, some early studies have suggested that Lon controls the level of mRNA transcription from the *E. coli* gal operon (30). In this case, polyP may modulate the function of Lon in transcriptional regulation. However, like Charette et al. (15), we suspected that *E. coli* Lon is a DNA-binding protein with low specificity. Further investigations are required to clarify the role of Lon in transcriptional regulation.

It remains unclear whether the main role of the DNA-binding ability of Lon is to prevent it from accessing and degrading substrate proteins or to mediate enhanced degradation of regulatory proteins. In any event, the drastic change in intracellular polyP levels that occurs during nutritional downshift can affect the DNA-binding ability of Lon and its regulation of cellular functions.

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