PinA Inhibits ATP Hydrolysis and Energy-dependent Protein Degradation by Lon Protease*

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The bacteriophage T4 PinA protein inhibited degradation of [3H]o-methyl casein by purified Lon protease from Escherichia coli, but inhibition was noncompetitive with respect to casein. PinA did not inhibit cleavage of the fluorogenic peptide, N-glutaryl-alanylalanylphenylalanyl-3-methoxynaphthylamide and, moreover, did not block the ability of protein substrates, such as casein, to activate cleavage of fluorogenic peptides by Lon. Thus, PinA does not block the proteolytic active site or the allosteric protein-binding site on Lon. Inhibition of basal ATPase activity was variable (50–90%), whereas inhibition of protein-activated ATPase activity was usually 80–95%. Inhibition was noncompetitive with respect to ATP. PinA did not block activation of peptide cleavage by nonhydrolyzable analogs of ATP. These data suggest that PinA does not bind at the ATPase active site of Lon and does not interfere with nucleotide binding to the enzyme. PinA inhibited cleavage of the 72-amino acid protein, CcdA, degradation of which requires ATP hydrolysis, but did not inhibit cleavage of the carboxy-terminal 41-amino acid fragment of CcdA, degradation of which does not require ATP hydrolysis. PinA thus appears to interact at a novel regulatory or enzymatic site involved in the coupling between ATP hydrolysis and proteolysis, possibly blocking the enzyme unfolding or remodeling step essential for degradation of high molecular weight protein substrates by Lon.

Lon protease from Escherichia coli is an oligomeric enzyme composed of identical 87-kDa subunits (1, 2). Each Lon monomer contains a proteolytic site and an ATP-binding site. Lon has ATPase activity that is activated by protein binding (3, 4). ATP hydrolysis is essential for degradation of proteins, whereas ATP binding alone is sufficient to activate cleavage of small nonstructured proteins and oligopeptides (5–7). For high molecular weight protein substrates, ATP hydrolysis is rate-limiting, and the rates of proteolysis are directly proportional to the rates of ATP hydrolysis (5).

Protein substrates bind Lon at two sites, the proteolytic active site and an “allosteric site” that probably lies within the ATPase domain. Occupancy of this latter site by potential substrates, such as unfolded polypeptides, activates ATPase activity and enhances cleavage of low molecular weight peptides (5, 8). Yeast Lon mutants lacking proteolytic activity but having an intact ATPase can promote assembly of protein complexes (9), indicating that Lon can catalyze conformational changes in proteins. The allosteric protein-binding site is likely to be the site where specific degradation motifs in proteins are recognized and is part of the catalytic domain for unfolding proteins. The ability to alter protein conformation may allow Lon to partially unfold proteins to give them greater access to the proteolytic active sites (10, 11).

Cells expressing the T4 pinA gene are phenotypically Lon−, suggesting that PinA functions to inhibit the Lon protease in vitro (12). In the preceding paper, we showed that purified PinA forms a complex with Lon protease and inhibits its casein degradation activity in vitro (13). Here, we investigate the mechanism of Lon inhibition by PinA and suggest that PinA interferes with the coupling between ATP hydrolysis and protein degradation, possibly by preventing protein unfolding by Lon.

EXPERIMENTAL PROCEDURES

Purification Procedures and Standard Methods—The purification of PinA and Lon, assay for casein degradation, buffer composition, and methods for SDS-PAGE and gel filtration were described in the preceding paper (13). An ATP-regenerating system consisting of 50 mM creatine phosphate and 20 μg/ml phosphocreatine kinase was used in some assays. Degradation of CcdA and N Protein—A phase N protein was purified and degraded as described previously (14). For degradation, 2 μg of N protein was added to 250 μl of buffer containing 50 mM Tris-HCl, pH 8.0 at 25 °C, 25 mM MgCl₂, 1 mM DTT, and 4 mM ATP; in other assays, 0.5 mM AMP-PNP or deionized H₂O was substituted for ATP. The solutions were incubated for 5 min at 37 °C, and degradation was initiated by the addition of 2 μg of Lon. After 45 min at 37 °C, the reaction was terminated by adding 310 μl of ice-cold 10% trichloroacetic acid. Insoluble protein was collected by centrifugation for 15 min at 14,000 × g in an Eppendorf centrifuge. The pellet was washed twice in 0.5 ml of acetone, air-dried, and dissolved in 10 μl of SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE, stained with Coomassie Blue, and quantitated by scanning on a Pharmacia-LKB UltroScan XL densitometer using GelScan XL software.

Purified CcdA and synthetic CcdA41, consisting of the carboxy-terminal 41 amino acids of CcdA, were degraded by Lon protease as described by Van Melder et al. (7). For quantitation of CcdA and CcdA41 degradation, the reactions were quenched with 4 mM guanidine-HCl and degradation products were analyzed by reverse phase chromatography as described previously (7).

Assays for Peptidase Activity—Cleavage of the fluorogenic tetrapeptide, glutaryl-Ala-Ala-Ala-Phe-MNA, was assayed in a solution containing 50 mM Tris-HCl, pH 8.0 at 25 °C, 10 mM MgCl₂, 1 mM DTT, 4 mM ATP, 50 μM peptide, 1 μg of Lon, and 5 μg of o-casein (6). The 50-μl reaction

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The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; AMPPNP, adenylyl-5′-imidodiphosphate; DTT, dithiothreitol; glutaryl-Ala-Ala-Ala-Phe-MNA, glutaryl-alanyl-alanyl-phenylalanyl-methoxynaphthylamide.

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PinA Blocks Protein-activated ATPase Activity of Lon

RESULTS

Inhibition of ATP-dependent Proteolysis—Casein degradation by Lon was measured in the presence of varying amounts of PinA and casein (Fig. 1). PinA inhibited the $V_{\text{max}}$ for casein degradation by Lon, but the apparent $K_{m}$ for casein (2.6 $\mu$M) was unaffected by the presence of excess PinA. Thus, PinA is not competitive with respect to casein and apparently does not bind in the protein substrate-binding site. The inset to Fig. 1 shows the Dixon plot for PinA inhibition of casein degradation; the calculated $K_i$ for PinA was 4 ± 1 nM.

**PinA Inhibits Substrate-stimulated ATPase Activity of Lon**—The effect of PinA on the ATPase activity of Lon was examined in the presence or absence of protein substrate. The PinA concentration required for inhibition of Lon ATPase activity was similar to that required for inhibition of casein degradation (Fig. 2). In the experiment shown, PinA inhibited about 60% of the basal ATPase activity of Lon, whereas inhibition of substrate-stimulated ATPase activity was much greater (Fig. 2). In different experiments, inhibition of basal activity varied between 50 and 80%, and inhibition of substrate-stimulated ATPase activity was usually greater, about 80–95%. The residual substrate-stimulated activity was similar to uninhibited basal ATPase activity.

To determine if PinA inhibition of ATPase activity was competitive with ATP, assays were performed with a fixed amount of PinA and varying concentrations of ATP. As shown in Table I, increasing the concentration of ATP to 1 mM (20 times the $K_{m}$) did not overcome PinA inhibition. No changes in the ATP concentration dependence for either basal or substrate-stimulated activity were seen in the presence of PinA (data not shown). Thus, PinA lowered the $K_{m}$ for ATP hydrolysis in the presence of Lon but did not appear to affect the affinity of ATP binding to Lon.

**PinA Does Not Inhibit Nucleotide-activated Peptidase Activity of Lon**—Hydrolysis of both peptide and protein substrates occurs at the same active site in Lon (6). Cleavage of glutaryl-Ala-Ala-Phe-MNA is activated by nucleotide binding but does not require ATP hydrolysis. PinA did not inhibit the peptidase activity of Lon on glutaryl-Ala-Ala-Phe-MNA (Table II). PinA also did not inhibit cleavage of oxidized insulin B chain or a synthetic peptide, FAPGHMALVPV, in the presence of AMPNP (data not shown). Thus, PinA does not prevent binding of nucleotides to Lon, nor does it interfere with the allosteric activation of peptidase activity upon nucleotide binding. To test whether inhibition by PinA is mediated through ADP, a potent

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**Table I**

| ATP | Casein | ATP hydrolysis |
|-----|--------|---------------|
|     | −PinA | +PinA         |
| 10  | 0.15   | 0.072         |
| 50  | 0.34   | 0.22          |
| 1000| 1.35   | 0.52          |

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**Fig. 1.** Casein titration of Lon in the presence of varying concentrations of PinA. Purified Lon (2 $\mu$g) was incubated in 0.25 ml of the reaction mixture for 30 min at 37 °C (see “Experimental Procedures”) with the amounts of $[3H]$-methyl casein and PinA indicated. ○, 72 ng of PinA; ■, 36 ng of PinA; ●, 8 ng of PinA; □, 12 ng of PinA; △, 6 ng of PinA; ▼, 3 ng of PinA; ○, 0 ng of PinA. *Inset,* Dixon plots of 1/specific activity versus [PinA]. Specific activity is expressed as milligrams of casein degraded/h/mg of Lon protease. PinA concentrations were varied in the presence of different amounts of the substrate, casein. ○, 9 $\mu$g of casein; ■, 18 $\mu$g of casein; ●, 36 $\mu$g of casein; △, 60 $\mu$g of casein.

**Fig. 2.** Inhibition of ATP hydrolysis by PinA. Lon ATPase activity was measured with and without addition of the indicated concentrations of PinA. Reaction mixtures contained 1 mM ATP, 0.5 $\mu$g of purified Lon, and where indicated, 5 $\mu$g/ml $\alpha$-casein in 50 $\mu$l of reaction mixture (see “Experimental Procedures”). ■, basal ATPase activity; ●, ATPase activity in the presence of casein.

mixture was incubated in the dark at 37 °C for 20 min, after which the reaction was terminated by the addition of 0.2 M sodium borate, pH 9.1, and 2 mM EDTA. Fluorescence was read on a LS-3B Perkin-Elmer fluorometer at an excitation wavelength of 335 nm and an emission wavelength of 415 nm. To examine the effect of nucleotide on peptidase activity, nucleotide was omitted, or 50 $\mu$M AMPNP was substituted for ATP.

**Assays for ATPase Activity**—ATPase activity was measured by the release of $[^{32}P]$orthophosphate from $[^{32}P]$ATP. Reaction mixtures (50 $\mu$l) contained 50 mM Tris-HCl, pH 8.0 at 25 °C, 25 mM MgCl$_2$, 1 mM DTT, and 1 mM $[^{32}P]$ATP (specific activity 2 $\mu$Ci $\mu$mol$^{-1}$) (15). The mixture was incubated at 37 °C for 5 min, and the reaction was initiated by the addition of 1 $\mu$g of Lon. After 20 min, the reaction was terminated by the addition of 200 $\mu$l of an ice-cold stop solution consisting of a 1:1 mixture of 5 mM siliotungstic acid in 1 M H$_2$SO$_4$ and 5% ammonium molybdate in 2 M H$_2$SO$_4$. The phosphomolybdate was extracted with 0.5 ml of solvent composed of a 1:1 mixture of isobutanol in toluene (16), and was centrifuged for 3 min at 14,000 $\times$ g at 4 °C. The organic phase containing $[^{32}P]$orthophosphate (250 $\mu$l) was counted in 10 ml of Scintiverse BD scintillation fluid (Fisher).
inhibitor of both peptide and protein degradation, casein degradation assays were conducted in the presence of an ATP-regenerating system. No difference in the extent of inhibition was observed, indicating that the Lon is not acutely sensitive to inhibition by ADP in the presence of PinA.

Binding of protein substrates, such as casein, to an “allosteric site” on Lon stimulates peptidase activity of Lon (8). This substrate-stimulated peptidase activity was also unaffected by excess PinA (Table II). These results indicate that protein substrate binding at the proteolytic active site and at the allosteric site on Lon is not affected by PinA.

PinA Inhibition of N Protein Degradation—The ability of PinA to inhibit degradation of natural substrates of Lon was examined first with λ N protein, which is degraded by Lon in the presence of ATP (14). N protein degradation also occurs in the presence of nonhydrolyzable ATP analogs but at ≤25% of the rate seen with ATP (10). Fig. 3A shows that PinA inhibited degradation of N protein in the presence of Lon and ATP. In the experiment shown, inhibition was greater than 50%, but a low rate of degradation was seen even in the presence of excess PinA. Incubation of N protein with Lon in the presence of AMPPNP resulted in degradation of ~50% of the N protein in 60 min, and PinA inhibited this degradation to a lesser extent (20–25%) (Fig. 3B). Since N protein degradation is only partially dependent on ATP hydrolysis, these experiments suggested that PinA inhibition is more complete when ATP hydrolysis is required. This conclusion was confirmed by the effects of PinA on degradation of a different physiological Lon substrate, CcdA.

PinA Blocks Degradation Coupled to ATP Hydrolysis—CcdA, a protein made by F factor, is degraded in vitro by Lon only when accompanied by ATP hydrolysis (7). A truncated form of CcdA, CcdA41, lacks stable secondary structure and can be degraded by Lon in the presence of nonhydrolyzable analogs of ATP (7). PinA completely blocks CcdA degradation in the presence of ATP but did not inhibit degradation of CcdA41 in the presence of AMPPNP (Fig. 4). CcdA41 degradation occurs at a similar rate in the presence of ATP or AMPPNP, and PinA was unable to inhibit degradation in either case (Table III). Thus, PinA blocks a step in Lon-dependent degradation that requires ATP hydrolysis, probably energy-dependent conformation rearrangement (or unfolding) of substrate proteins. Since PinA blocks ATP hydrolysis, these data also show that ATP binding produces a similar allosteric activation of Lon activity as is produced by nonhydrolyzable analogs.

**DISCUSSION**

Purified PinA inhibited casein degradation by Lon protease, and inhibition appeared to be noncompetitive with casein. The apparent $K_i$ for PinA (3–4 µM) is consistent with tight binding between the two proteins, as had been suggested by the isola-

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**TABLE II**

| PinA addition | Nucleotide | +Casein | +Casein | +Casein/+casein |
|---------------|------------|---------|---------|----------------|
| None          | ATP        | 4.8     | 11.0    | 1.25           |
| ATP           | AMPPNP     | 2.7     | 4.3     | 1.87           |
| AMPPNP        | + PinA     | 2.8     | 4.0     | 1.70           |

**TABLE III**

| Substrate | Nucleotide | Degradation rate (min⁻¹) |
|-----------|------------|--------------------------|
| CcdA      | ATP        | 2.0                      |
| CcdA      | AMPPNP     | 0.0                      |
| CcdA41    | ATP        | 4.1                      |
| CcdA41    | AMPPNP     | 4.0                      |

Activity is expressed as the number of substrate molecules degraded per min per protease active site, assuming a single active site per subunit of Lon.

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**Fig. 4. Effects of PinA on CcdA and CcdA41 degradation by Lon.** CcdA41 and CcdA were incubated with Lon (2 µg) for 60 min at 37 °C. Degradation was monitored by disappearance of the starting material and appearance of peptide products after reverse phase chromatography (see “Experimental Procedures”). Upper profiles, incubations without PinA; lower profiles, incubations with PinA. A, 10 µg of CcdA41 degraded in the presence of AMPPNP; B, 10 µg of CcdA degraded in the presence of ATP.
or the nonhydrolyzable analog, AMPPNP, was used, PinA does
"freeze" Lon in a conformationally inactive state (3). Since PinA
peptide bond cleavage, and failure to release ADP would
release of ADP allows Lon to continue the catalytic cycle of
the "activated" state.

nucleotides in the presence of PinA, PinA does not appear to
binding is not sufficient to allow degradation of proteins with
enhances peptidase activity of Lon (6). However, nucleotide
ATP analogs, or CTP, induces a conformation change which
distinct from previously described functional sites on Lon.

The lack of inhibition of Lon peptidase activity and the
noncompetitive nature of inhibition of casein degradation indicate
that PinA binds outside of the proteolytic active site on
Lon. Protein substrates bind to Lon and stimulate hydrolysis,
suggesting that there is an allosteric binding site for protein
substrates on Lon (8). PinA did not interfere with the ability of
casein to stimulate peptidase activity (Table II), indicating that
PinA does not block casein binding at the allosteric site, and
thus PinA does not appear to bind, or at least does not
completely occupy, the allosteric protein-binding site on Lon.

PinA does not completely inhibit nucleotide hydrolysis, as evidenced by the 50% basal ATPase activity remaining at concentra-
tions of PinA that block 90–95% of protein degradation (Fig. 2). However, protein-activated ATPase activity was completely blocked by PinA. Inhibition of both basal and
substrate-stimulated ATP hydrolysis is independent of ATP
concentration. Peptidase activity and CcdA41 degradation are
stimulated by nucleotide binding and were unaffected by PinA.
PinA is not competitive with ATP or ATP analogs and does not
bind at the nucleotide site. Thus, PinA binds to a unique site
distinct from previously described functional sites on Lon.

Nucleotide binding and hydrolysis have distinct roles in Lon
activity. Binding of nucleotides, such as ATP, nonhydrolyzable
ATP analogs, or CTP, induces a conformation change which
enhances peptidase activity of Lon (6). However, nucleotide
binding is not sufficient to allow degradation of proteins with
stable secondary or tertiary structure, which requires ATP
hydrolysis (7). Since Lon peptidase activity is stimulated by
nucleotides in the presence of PinA, PinA does not appear to
affect the nucleotide-induced conformational change in Lon to
the "activated" state.

Binding of protein substrates at the allosteric site induces a
conformational change in Lon that releases bound ADP (3). The release of ADP allows Lon to continue the catalytic cycle of
peptide bond cleavage, and failure to release ADP would
"freeze" Lon in a conformationally inactive state (3). Since PinA
did not inhibit peptide or CcdA41 degradation when either ATP
or the nonhydrolyzable analog, AMPPNP, was used, PinA does
not lock Lon in an inactive ADP-bound state.

Recent data suggest that energy-dependent proteases need
ATP hydrolysis to drive enzyme catalyzed conformational changes or unfolding of protein substrates. Yeast Lon protease and
the ATPase domains of Clp proteases have chaperone-like
activities and can remodel protein structures (9, 10, 17). Studies
with E. coli Lon have shown that the presence of stable
secondary structure in a substrate is the determinant of the
requirement for ATP hydrolysis (7). With Clp proteases and the
26 S proteasome, disruption of the secondary structure of
protein substrates is apparently required to allow substrates to get to
the proteolytic active sites, which are located in an interior aqueous cavity accessible through relatively narrow channels. Although the structure of Lon is not known, it is likely that the
active sites of Lon are similarly inaccessible to folded proteins.

PinA specifically blocks the steps in protein degradation that
require ATP hydrolysis. Degradation of CcdA (which requires
unfolding) but not of CcdA41 (which does not require unfolding)
was inhibited, suggesting that PinA inhibits the chaperone activity of Lon. We propose that PinA prevents the unfolding of protein substrates by interfering with ATP hydrolysis and the conformational changes in Lon that accompany ATP hydrolysis. These conformational changes in Lon may alter its interactions with bound proteins, which in turn result in structural changes in the protein substrate.

Our previous data showed that a dimer of PinA binds to a
tetramer of Lon (13). Lon has four potential ATPase sites per
tetramer, and inhibition of all stimulated ATPase activity by a
single PinA dimer implies that Lon subunits act in a cooperative
manner. Since it does not block access to the active sites for
several protein substrates, PinA probably does not bind in the
substrate channel to the active sites. We propose that PinA
binds to a flexible region on Lon that is important in coopera-
tive ATP-driven conformational changes that are coupled to
unfolding of protein substrates. This binding site on Lon may represent a functional site that is occupied by other effectors of
Lon in vivo, and its identification should give additional infor-
mation about physiological regulation of Lon.

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TABLE IV
Comparison of $K_i$ values of protein protease inhibitors

| Enzyme | Substrate | Inhibitor | $K_i$ |
|--------|-----------|-----------|-------|
| Lon    | Casein    | PinA      | $3.6 \times 10^{-9}$ |
| Papain | Z-Phe-Arg-NMeC | Cystatin | $<1.0 \times 10^{-11}$ |
| Trypsin| BAPNA     | Exotin    | $2.2 \times 10^{-8}$ |
| Subtilisin | NA      | SSI       | $1.0 \times 10^{-10}$ |

$a$ Ref. 18.
$b$ Ref. 19.
$^{c}$ Binding constant was determined by direct physical measurement (20).
$d$ N-Carboxbenzoxo-Phe-Arg-7-amido-4-methyl coumarin.
$e$ N-Benzyl-ll-Arg-p-nitroanilide.
$f$ Not applicable.
$g$ SSI, Streptomyces subtilisin inhibitor.