Isolation and Characterization of the Phage T4 PinA Protein, an Inhibitor of the ATP-dependent Lon Protease of Escherichia coli*

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The bacteriophage T4 PinA protein, expression of which leads to inhibition of protein degradation in Escherichia coli cells, has been purified from cells carrying multiple copies of the pinA gene. PinA is a heat-stable protein with a subunit M₆ of 18,800 and an isoelectric point of 4.6. Under nonnondenaturing conditions on a gel filtration column, PinA migrated in two peaks corresponding to a dimer and a tetramer. Purified PinA inhibited ATP-dependent protein degradation by Lon protease in vitro; it did not inhibit the activity of other E. coli ATP-dependent proteases, ClpAP or ClpYQ. Furthermore, PinA did not inhibit ATP-independent proteolysis in E. coli cell extracts. PinA binds with high affinity to Lon protease (Kᵣ ~ 10 nm for dimer binding), and a complex with ~1 dimer of PinA per tetramer of Lon protease could be isolated by gel filtration. Lon activity was partially restored upon dilution of the PinA-Lon complex to subnanomolar concentrations, indicating that inhibition was reversible and that PinA did not covalently modify Lon protease. PinA was not cleaved by Lon protease, and heating the Lon-PinA complex at 65 °C denatured Lon protease and released active PinA. The properties of PinA in vitro suggest that PinA inhibits its protein degradation in vivo by forming a tight, reversible complex with Lon protease.

Lon protease, the product of the lon gene (1), is one of the major ATP-dependent proteases of Escherichia coli. In vivo, Lon is responsible for the degradation of such specific proteins as SulA (2) ResA (3), and the λ N protein (4), as well as unfolded and abnormal proteins (5). Purified Lon, subunit M₆ 87,000, is an oligomeric protein that has been reported to exist in either tetrameric or octameric form (6, 7). Sequence analysis suggests an oligomeric protein that has been reported to exist in either dimer and a tetramer. Purified PinA inhibited ATP-dependent protein degradation by Lon protease in vitro; it did not inhibit the activity of other E. coli ATP-dependent proteases, ClpAP or ClpYQ. Furthermore, PinA did not inhibit ATP-independent proteolysis in E. coli cell extracts. PinA binds with high affinity to Lon protease (Kᵣ ~ 10 nm for dimer binding), and a complex with ~1 dimer of PinA per tetramer of Lon protease could be isolated by gel filtration. Lon activity was partially restored upon dilution of the PinA-Lon complex to subnanomolar concentrations, indicating that inhibition was reversible and that PinA did not covalently modify Lon protease. PinA was not cleaved by Lon protease, and heating the Lon-PinA complex at 65 °C denatured Lon protease and released active PinA. The properties of PinA in vitro suggest that PinA inhibits its protein degradation in vivo by forming a tight, reversible complex with Lon protease.

Hydrolysis of ATP may provide energy to help unfold protein substrates, giving them greater access to the proteolytic active site and making them more susceptible to cleavage (13, 16, 17). Recent studies of CdcA degradation by Lon in vitro indicated that the absence of stable secondary structure in protein substrates decreased the requirement for ATP hydrolysis (11). Protein substrates bind to two sites on Lon, the proteolytic active site and an allosteric site, which may serve as the site for the protein remodeling function. Occupancy of the allosteric site by substrates such as unfolded polypeptides activates the peptidase activity against small peptides and enhances proteolysis (12).

E. coli cells infected with bacteriophage T4 show reduced proteolysis of abnormal proteins and protein fragments (18). Inhibition of protein degradation requires synthesis of T4 proteins made during the first 10 min after infection at 37 °C (18–20). Clones of T4 genes that lead to inhibition of proteolysis in E. coli cells were obtained by Simon and co-workers (19, 20), and one specific gene, pinA (proteolysis inhibition A), which resulted in inhibition of abnormal protein degradation, was cloned by Skorupski et al. (21). The target of PinA in vivo appears to be the ATP-dependent Lon protease, because E. coli pinA⁺ cells expressing a single copy of the T4 pinA gene are phenotypically Lon− (21).

We have purified the PinA protein and shown that purified PinA binds to Lon protease and inhibits its ATP-dependent protease degradation activity in vitro. In the accompanying paper (22), we demonstrate that PinA exerts its affect by blocking ATP hydrolysis by Lon.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were obtained from commercial sources unless otherwise specified. [H]Formaldehyde was obtained from NEN Life Science Products. Clp protease was purified as described previously (23–25).

Growth of Bacterial Strains—Bacterial strains and plasmids used are described below. Bacteria used to make cell extracts were grown in glucose L broth (31), which contained (per liter) 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 2 g of glucose. Glucose L agar contained glucose L broth with 1.5% (w/v) agar. Ampicillin was used at 50 μg/ml as needed.

Purification of Lon Protease—Lon protease was purified from E. coli SG22030 (lon−) carrying the multicopy plasmid pLon50, which contains lon under its own promoter (5). The purification method has been described (6). Lon was estimated by SDS-PAGE to be 90–95% pure and was free of other proteases.

Overexpression and Preparation of PinA—The pinA gene product was expressed from a plasmid constructed by J. Tomaszewski using the expression vectors of Tabor and Richardson (26). The host strain was E. coli LS101, a derivative of K38 (27) carrying a gale mutation to prevent production of excess capsular polysaccharide in the absence of

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Purification of the PinA Protein—PinA was purified from cells growing at 28 °C with the aid of Novagen’s T7 expression system pET11a. Cells were harvested at 6 h after shaking and transferred to an ice bath. After centrifugation, PinA was solubilized from the cell pellet by adding 10 ml of buffer containing 200 mM PIPES, pH 6.0, and 0.2 M NaCl, 1 mM EDTA. After clarification, the solution was loaded onto a Superose-12 column equilibrated in buffer T, and the elution gradient was linear from 0.2 to 0.6 M NaCl in Buffer T at a flow rate of 1 ml/min. Fractions containing PinA identified by SDS-PAGE were pooled, concentrated, and analyzed by gel filtration on a Superose-12 column packed with Sephacryl Superfine S-200 according to the manufacturer’s instructions. The specific activity of the [3H]methyl a-casein preparation was determined by filtering the buffer through a 0.22-μm filter (Millipore) in aliquots of 20 ml, and aliquots were loaded onto separate MonoQ HR 10/10 columns. Proteins were eluted with a linear gradient of 0.2–0.6 M KCl in buffer T if a flow rate of 1 ml/min. Frac-tions containing PinA identified by SDS-PAGE were pooled, and ammonium sulfate was added to 40% saturation. After centrifugation for 30 min at 30,000 × g, the pellet was suspended in 10–15 ml buffer T with 0.1 M KCl. Insoluble matter was removed by centrifugation, and the resulting supernatant was loaded in aliquots of 2.5 ml onto separate 2.3 × 60-cm TSK250 gel filtration columns equilibrated in buffer T with 0.1 M KCl. Proteins were eluted in the same buffer at a flow rate of 2 ml/min. Purified PinA was stored at 80 °C.

The ammonium sulfate pellet was dissolved in 80 ml of buffer T, and the solution was centrifuged at 20,000 × g for 20 min to remove insoluble material. The supernatant was filtered through a 0.22-μm filter (Millipore) in aliquots of 20 ml, and aliquots were loaded onto separate MonoQ HR 10/10 columns. Proteins were eluted with a linear gradient of 0.2–0.6 M KCl in buffer T if a flow rate of 1 ml/min. Fractions containing PinA identified by SDS-PAGE were pooled, and ammonium sulfate was added to 40% saturation. After centrifugation for 30 min at 30,000 × g, the pellet was suspended in 10–15 ml buffer T with 0.1 M KCl. Insoluble matter was removed by centrifugation, and the resulting supernatant was loaded in aliquots of 2.5 ml onto separate 2.3 × 60-cm TSK250 gel filtration columns equilibrated in buffer T with 0.1 M KCl. Proteins were eluted in the same buffer at a flow rate of 2 ml/min. Purified PinA was stored at 80 °C.

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Biochemical Characterization and Physical Properties—One-dimensional SDS-PAGE gels were run essentially as described by Laemmli (28), using Mini-PROTEAN II Ready Gels (Bio-Rad). Except as noted, 12% gels were used. The molecular weight of PinA under nonnaturating conditions was determined by gel filtration on a Superose-12 column (Pharmacia Biotech Inc.). Proteins were eluted from the Superose-12 column in 20 ml of buffer containing 0.5 M NaCl, 1 mM EDTA, and 10% glycerol (buffer B), and stored at −80 °C.

Amino Acid Analysis—Purified PinA was hydrolyzed in 6 N HC1 at 115 °C for 30 and 60 min. The hydrolysates were washed, dried, and derivatized with phenylisothiocyanate (PTC). PTC-amino acids were separated on a C18 reverse phase column (4.6 mm × 15 cm) using the solvent system described by Bidlingmeyer et al. (30). The cysteine content of PinA protein was determined by performing acid oxidation (31), followed by acid hydrolysis and amino acid analysis. Aromatic amino acids were determined spectrophotometrically by second derivative UV spectroscopy in 6 M guanidine hydrochloride, as described by Levine and Federici (32). Using the amino acid content calculated from the DNA-derived sequence of PinA, the extinction coefficient of the protein was determined from the calculated aromatic amino acid con-

tent of the protein and the measured absorbance of a standard solution of the protein.

NH2-terminal Sequence Determination—To remove salts and buffers prior to sequencing, purified PinA was passed through a HR 10/10 Fast desalting column (Pharmacia) in deionized H2O. Sequencing was performed according to the manufacturer’s directions in Applied Biosystems model 470A Protein Sequencer with a model 120A on-line PTH Amino Acid Analyzer (33) and a model 610A Data Analysis Module and the ABI model 475 Report Generator.

Methyl a-Casein Preparation—a-Casein was radioactively labeled with [3H]formaldehyde by the method of Jentoft and Dearborn (34). Using the specific activity of the [3H]methyl a-casein was approximately 5 Ci/mg.

Assays for ATP-dependent Proteolytic Activity—Assays for proteolytic activity were performed as described previously (9, 23) or as follows. A solution with 9 μg of [3H]methyl a-casein in 250 μl of buffer containing 50 mM Tris-HCl, pH 8.0, 25 mM MgCl2, 1 mM DTT, and 4 mM ATP was incubated for 5 min at 37 °C, and the reaction was initiated by the addition of 0.5–2.0 μg of Lon. Incubation at 37 °C was continued for 15–30 min. The reaction was terminated by the addition of 310 μl of ice-cold 10% trichloroacetic acid and 40 μl of 10 mg/ml bovine serum albumin. Precipitated proteins were separated from trichloroacetic acid-insoluble proteins by centrifugation at 4 °C in an Eppendorf centrifuge at 14,000 × g for 6 min. Radioactivity was determined by liquid scintillation counting using 0.5 ml of supernatant in 10 ml of Scintiverse BD (Fisher) or Aquasol (NEN Life Science Products). Assays were performed in duplicate, and the measured activity had a variance of <5%.

Inhibition of Lon by PinA—PinA was added to standard assays solutions 1–5 min prior to addition of Lon to initiate the assays. Mixing PinA and Lon before adding them to the assay mixtures did not affect the results. The effect of pH on the inhibitory activity of PinA was determined by substituting the following buffers in the assay mixture: 50 mM MES, sodium salt, 10 mM MgCl2, pH 6.0 and 7.0; 50 mM Tris-HCl, 10 mM MgCl2, pH 7.0–9.0; and 50 mM 2-amino-2-methyl-1-propa-noic acid, 10 mM MgCl2, pH 9.0–10.5.

Stoichiometry of the Lon-PinA Complex—A 2.5-ml Sephacyr S-200 (Pharmacia) column was equilibrated at room temperature in buffer B. Lon (20 μg) was loaded onto the column, and 250-μl aliquots of buffer were added at 2 min intervals. Aliquots of 250 μl were collected from the column at each step. The same procedure was used for PinA (20 μg) and for a mixture of 20 μg of Lon and 20 μg of PinA. Nucleotide requirement was determined by equilibrating the column in buffer B containing 50 μM AMPNP, and then chromatographing Lon and PinA. Alternatively, Lon was dissolved in water and used without dilution. The column packed with Sephacyr Superfine S-200 according the manufacturer’s instructions.

Lon (250 μg), PinA (250 μg), and mixtures of the two proteins were also analyzed by gel filtration on a Superose-12 column equilibrated in buffer B. Proteins were eluted at a flow rate of 0.4 ml/min in the same buffer. Protein in the fractions was detected by densitometry with a Hewlett-Packard ScanJet IIc/ADF using Deskscan and Collage software or by capturing a digital image of the gel with an Eagle Eye Frame Integrator and using NIH Image software. Known amounts of Lon or PinA were used as standards. To determine if formation of the PinA-Lon complex was reversible, the complex isolated by gel filtration was diluted into assay solutions of increasing volume (100 μl to 1.6 ml) to reduce the concentrations of PinA and Lon below the apparent Kd, and the increase in Lon activity was measured.

RESULTS

Purification of the PinA Protein—PinA was purified to near homogeneity from extracts of cells in which the protein was overproduced. Previous work had shown that PinA was a component of the cytosol and was not associated with the cell membrane.3 Purified PinA in fractions from the final gel filtration step is shown in Fig. 1A; PinA was estimated to be 95% pure in the best fractions which were used for all experiments described below. PinA had a maximum absorbance at 281 nm and an extinction coefficient of 1.95 (mg/ml)−1 determined by analysis of the second derivative of the UV absorbance spectrum (32).

3 H. J. Kim, unpublished observations.
In the presence of 1 molar excess of PinA, inhibition of Lon was less than 1% of the casein present in the assay solution, and, therefore, inhibition was not due to interaction of PinA with the substrate. PinA apparently binds very rapidly to Lon, since prior incubation of the two proteins together did not increase the degree of inhibition (data not shown). PinA has a very high affinity for Lon under the conditions used for these assays; half-maximal inhibition occurred at about 20 nM PinA subunit or only 10 nM PinA dimer. PinA purified by both procedures described under “Experimental Procedures” had comparable inhibitory properties.

In vivo studies suggested that other ATP-dependent E. coli proteases were not affected by PinA (22). As shown in Table I, PinA did not inhibit casein degradation by the ATP-dependent proteases, ClpAP or ClpYQ (HslUV). Thus, PinA appears to specifically recognize Lon protease.

Stability of PinA—PinA was stable when heated. Table II shows that PinA inhibited Lon to the same extent before and after heating for 10 min at –100 °C. Inhibition of Lon by PinA required the intact PinA protein, because digestion of PinA by trypsin or chymotrypsin inactivated PinA (data not shown). This experiment also demonstrated that Lon inhibition was not due to nonproteinaceous inhibitors contaminating the PinA.

To determine if PinA was cleaved by Lon, PinA was incubated with Lon for 5 min at 37 °C, and the reaction was terminated by boiling the proteins in SDS. The proteins were separated by SDS-PAGE and quantitated by densitometry after staining with Coomassie Blue. As shown in Fig. 3, no loss of PinA was detectable after incubation with Lon. Thus, PinA does not appear to be a substrate for Lon.

Oligomeric Structure of PinA—Freshly purified PinA migrated with an apparent molecular weight of 40,000 on a Superose 12 gel filtration column in the presence of 0.1–0.2 M KCl and thus appears to be a dimer. After storage at –20 °C for more than 1 year, PinA species that appeared by gel filtration to be tetramers (see Fig. 4) and octamers (data not shown) predominated. No noticeable effect on the ability of PinA to inhibit Lon protease accompanied these changes in oligomeric state, and it is possible that the aggregated PinA dissociated to dimers and tetramers at the dilutions used for assays. High salt concentrations (>0.5 M KCl) cause PinA to dissociate into monomers and, when present in assay solutions, decrease the inhibitory effects of PinA (data not shown). These last data suggest either that the monomeric form of PinA does not inhibit Lon or that the interaction between PinA and Lon is disrupted by high ionic strength.

PinA inhibition of Lon was optimal between pH 8 and 9 (data not shown). Above pH 9.5, inhibitory activity decreased considerably, but the decreased inhibition could reflect confor-
PinA, an Inhibitor of Lon Protease

Table I

Specificity of PinA for Lon protease

| Protease                        | Protease activity<sup>a</sup> |
|---------------------------------|-------------------------------|
|                                 | mg casein/h                  |
| **-PinA**                       | Lon                           |
|                                 | 1.4                           |
|                                 | +PinA                         |
|                                 | 0.5                           |
| ClpAP (excess ClpP)             | 21                            |
| ClpAP (excess ClpA)             | 52                            |
| ClpYQ (HslUV)                   | 8.0                           |

Table II

Heat stability of PinA

ATP-dependent casein degradation was assayed with 1 μg of Lon with or without 2 μg of PinA. Where indicated, PinA was heated at 100 °C for 5 min before adding to the assay solution. Results of a single experiment are shown; heat stability was also observed in separate experiments with PinA heated at 5 min at 60, 80, or 100 °C.

| Components in assay | Remaining casein degrading activity<sup>a</sup> |
|---------------------|-----------------------------------------------|
|                     | %                                            |
| Lon alone           | 100                                           |
| Lon + PinA          | 15                                            |
| Lon + heated PinA   | 17                                            |

<sup>a</sup> Activity is expressed as a percent of that observed without inhibitor (~2 mg of casein/h/mg of Lon).

Fig. 3. PinA is not cleaved by Lon protease. PinA and Lon protease were incubated for 5 min at 37 °C in standard assay buffer. The proteins were precipitated, separated by SDS-PAGE, and stained with Coomassie Blue. Lane 1, 10 pmol of Lon; lane 2, 10 pmol of Lon and 10 pmol of PinA; lane 3, 10 pmol of PinA; lane 4, 20 pmol of Lon and 10 pmol of PinA; lane 5, 20 pmol of Lon.

Fig. 4. Isolation of a PinA-Lon complex by gel filtration. A, absorbance profiles following gel filtration on Superose 12. – – –, 250 μg of Lon protease alone; ---, 250 μg of PinA alone; ——, 250 μg each of Lon and PinA. B, activity of Lon protease and PinA-Lon complex after gel filtration. The fractions were assayed for ATP-dependent casein degradation using 20-μl aliquots of the fractions shown. ●, Lon alone; ■, PinA-Lon complex. C, SDS-PAGE profiles of Lon and PinA in fractions from the three Superose 12 columns. The standards used to calibrate the column were thyroglobulin, immunoglobulin G, ovalbumin, myoglobin, and cyanocobalamine.

The proteolytic activity of Lon in fractions containing the isolated complex was assayed and compared with that present in similar fractions when Lon protease was run alone. As shown in Fig. 4B, the casein degrading activity of the PinA-Lon complex was only 5–10% of the activity of Lon protease alone.

The amount of PinA bound to Lon was determined in a separate experiment by mixing the two proteins in a ratio of 5 PinA dimers per subunit of Lon and isolating the complex by gel filtration on a Sephacryl S-200 column. Protein in the fractions corresponding to the complex was quantitated by comparing the intensity of the Coomassie-stained bands to known amounts of Lon or PinA that had been run and stained separately in parallel. Approximately 1–2 dimers of PinA were bound to 1 tetramer of Lon. Increasing the amount of PinA added to Lon did not increase the PinA found complexed to Lon (data not shown).
PinA, an Inhibitor of Lon Protease

TABLE III

Active PinA can be recovered from the PinA-Lon complex

In part A, PinA alone, Lon alone, or a PinA/Lon complex in which Lon was inhibited >90% was spun through a Sephacryl S-200 spin column. Lon in the eluate was assayed for casein degradation before and after heating at 65 °C for 10 min. In part B, untreated Lon protease was assayed before or after addition of the heated eluates from the above spin columns to assay solutions. Each experiment was performed once with duplicate assays.

| Elute added to assay | Lon-dependent casein degradationa % |
|---------------------|-------------------------------------|
| A. Lon in spin column eluates |                                    |
| Lon alone (untreated) | 100                                 |
| Lon-PinA complex (untreated) | <4                                  |
| Lon alone (heated) | <4                                  |
| Lon-PinA complex (heated) | <4                                  |
| B. Fresh Lon treated with heated eluates |                                |
| Lon alone | 100                                 |
| Lon + heated Lon eluate | 96                                  |
| Lon + heated PinA eluateb | 105                                 |
| Lon + heated Lon/PinA complex eluate | 7                                   |

a Untreated Lon activity was about 2 mg of casein degraded/h/mg of Lon.
b PinA is retained in the column and is not present in the eluate.

Release of Active PinA from the Lon-PinA Complex—The release of active PinA from the PinA-Lon complex was demonstrated by taking advantage of the thermal stability of PinA. Aliquots of Lon and the PinA-Lon complex from the Sephacryl spin-column eluates were incubated at 65 °C for 10 min, after which the aliquots were immediately added to reaction mixtures with and without fresh Lon. Table III shows that, after 10 min at 65 °C, Lon alone or in the complex with PinA had no proteolytic activity. Addition of the heated PinA-Lon complex to assay solutions with fresh Lon resulted in inhibition. The inhibition was not due to interference from the heated and presumably denatured Lon, since the addition of heated Lon alone to the reaction mixture had no effect on casein degradation by fresh nondenatured Lon. Thus, no irreversible change in PinA accompanies binding to and inhibition of Lon protease.

Reversibility of Lon Inhibition by PinA—The PinA-Lon complex was isolated by gel filtration on Sephacryl S-200. Eluates were collected and were assayed for ATP-dependent casein degradation in reaction solutions of different volumes. Table IV shows that Lon in the complex isolated from the column was inhibited >90% when assayed at high concentrations of the complex, but dilution of the complex resulted in a progressive increase in the Lon activity. Similar results were obtained without isolation of the complex by gel filtration. PinA and Lon were premixed in a ratio sufficient to cause >90% inhibition of Lon activity when assayed in 100-μl reaction solutions. Dilution of the same mixture into larger volumes for assay resulted in dissociation of the complex and a 40% gain in Lon activity (Table IV). In other experiments, 40–85% of Lon activity was recovered at 8–16-fold dilution of the PinA-Lon complex into assay solutions (data not shown). These results suggest that, although the PinA-Lon complex is quite stable, complex formation is reversible.

DISCUSSION

The bacteriophage T4 PinA protein has been purified from E. coli cells carrying a multicopy plasmid with the pinA gene under control of a T7 promoter. Purified PinA inhibits ATP-dependent casein degradation by Lon protease by more than 90%. PinA shares several properties with other polypeptide protease inhibitors. It is relatively small (M, 20,000), heat-stable, and acidic (pI = 4.6); however, PinA does not inhibit proteases such as trypsin, chymotrypsin, subtilisin, or pepsin but is degraded and inactivated by treatment with these proteases. PinA appears to target Lon protease specifically, and elsewhere (22) we demonstrate that PinA inhibits ATP-dependent protein degradation by Lon protease by blocking the coupling between ATP hydrolysis and peptide bond cleavage.

Inhibition of [3H]methyl α-casein degradation by Lon protease occurs at very low concentrations of PinA, and an apparent Kᵢ of about 5 nM was calculated under standard assay conditions. The low Kᵢ implies that PinA binds tightly to Lon, which was confirmed by showing that it was possible to isolate a complex of Lon and PinA after gel filtration chromatography. The complex was formed in the absence of nucleotide as well as in the presence of the nonhydrolyzable ATP analog, AMPPNP, indicating that tight binding of PinA to Lon does not require any of the energy-driven steps involved in protein degradation.

As expected, when used at concentrations comparable to those in standard assays, the PinA-Lon complex isolated by gel filtration showed little proteolytic activity against [3H]methyl α-casein. However, dilution of the complex resulted in a small increase in proteolytic activity, suggesting that inhibition of Lon by PinA is at least partially reversible. Heating the PinA-Lon complex released PinA from denatured Lon protease. The free PinA was able to bind to Lon and inhibit proteolytic activity. SDS gel analysis of PinA after forming a complex with Lon or released from the complex by heating showed no evidence of cleavage of the PinA.

The classical protease inhibitors from plants and microbial organisms are characterized by either reversible or irreversible mechanisms (37). Reversible inhibitors have a specific peptide bond, which combines with the active site of the target protease and is then cleaved by the protease. Hydrolysis does not proceed to completion, but an equilibrium between intact and cleaved peptide bonds is established.Irreversible inhibitors combine with the protease and are cleaved at a specific peptide bond. However, the acyl intermediate between the inhibitor bond, which combines with the active site of the target protease and is then cleaved by the protease. Hydrolysis does not proceed to completion, but an equilibrium between intact and cleaved peptide bonds is established. Irreversible inhibitors combine with the protease and are cleaved at a specific peptide bond. However, the acyl intermediate between the inhibitor and the protease is not hydrolyzed and the inhibitor remains covalently bound to the protease. The latter inhibitor–enzyme complex resists dissociation by urea and SDS. The results of the thermal inactivation and dilution studies of the PinA-Lon complex suggest that PinA may differ from other reversible inhibitors in that it is not cleaved by the protease.

Skorupski et al. (21) have shown that lon+ cells lysogenic for λ in which the pinA gene has been cloned behave phenotypically like lon mutants. These lysogens produce mucoid colonies, filament in response to DNA damage, permit efficient plaque formation by λ Ots phage at 40 °C, and exhibit reduced levels of

TABLE IV

Reversibility of Lon inhibition by PinA

The PinA-Lon complex was formed, and the Lon was assayed at different dilutions. In part A, the PinA-Lon complex formed with saturating PinA was isolated by gel filtration on Sephacryl S-200 and assayed in increasing reaction volumes to dilute the complex. In part B, PinA was titrated into Lon to produce >90% inhibition in the smallest reaction volume, and identical aliquots of the mixture were assayed in solutions of increasing volume. Results from single experiments are shown.

| Assay volume | Lon activity | Unitsa % |
|--------------|--------------|---------|
| A. PinA/Lon from Sephacryl S-200 |             |        |
| 100          | 0.15         | <8      |
| 200          | 0.22         | 11      |
| 400          | 0.36         | 18      |
| 800          | 0.60         | 30      |
| B. Mixture of PinA and Lon |             |        |
| 100          | 0.22         | 11      |
| 200          | 0.34         | 17      |
| 400          | 0.56         | 28      |
| 800          | 0.96         | 48      |

a A unit of Lon activity is 2 mg of casein degraded/h/mg of Lon.
abnormal protein degradation, all typical of E. coli cells lacking functional Lon protease (17, 38). Expression of pinA has no detectable effect on abnormal protein degradation in E. coli null lon strains (21). Our finding that PinA does not inhibit ClpAP or ClpYQ activity in vitro is consistent with the idea that PinA displays specificity for Lon protease alone.

ATP-dependent proteases tend to be high molecular weight, multimeric enzymes with potential binding sites for regulatory components. Several endogenous inhibitors that bind reversibly to the eukaryotic 20 S and 26 S proteasomes have been described, each of which appears to have a unique mode of action. For example, an inhibitor described by Chu-Ping et al. (39) apparently acts allosterically since it inhibits three distinct peptidase activities of the 20 S proteasome, whereas a ubiquitinated inhibitor from rabbit reticulocytes blocks ATP-dependent degradation of ubiquitinated proteins by the 26 S proteasome but has only partial activity against peptidase activities (40). A multimeric factor, CF2, combines with the 20 S proteasome but has only partial activity against peptidase activities (41). Since CF2 and the 20 S proteasome are both components of the 26 S proteasome, this inhibition probably reflects changes in accessibility of the proteolytic active sites during assembly of the 26 S proteasome, which has ATP-dependent proteolytic activity and has a more stringent specificity, preferentially degrading ubiquitinated proteins. No inhibitors of the E. coli ClpAP or ClpXP proteases have yet been described. The CIII protein of A inhibits the ATP-dependent FtsH (HflB) protease; however, in vivo data suggest that CIII simply acts as a competitive substrate for the protease, and CIII does not inhibit Lon protease in vitro.4 A search of the GenBank and Swiss-Prot data bases (42) revealed no homologies to pinA at either the DNA or amino acid sequence level. PinA, therefore, appears to be a novel protease inhibitor, highly specific for Lon protease. Further characterizations of the effects of PinA on the proteolytic, peptidase and ATPase activities of Lon are described in the accompanying paper (22).

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