The Energy Utilized in Protein Breakdown by the ATP-dependent Protease (La) from Escherichia coli*

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A crucial enzyme in the pathway for protein degradation in Escherichia coli is protease La, an ATP-hydrolyzing protease encoded by the lon gene. This enzyme degrades various proteins to small polypeptides containing 10–20 amino acid residues. To learn more about its energy requirement, we determined the number of ATP molecules hydrolyzed by the purified protease for each peptide bond cleaved. The enzyme hydrolyzed about 1.5 ATP per peptide bond for each amino acid group generated with casein, bovine serum albumin, glucagon, or guanidinated casein as substrates, even though these proteins differ up to 20-fold in size and 3–4-fold in rates of hydrolysis of peptide bonds. Similar values for the stoichiometry (from 1.9 to 2.4) were obtained using fluorescamine or 2,4,6-trinitrobenzene sulfonic acid to estimate the appearance of new amino groups. These values appeared lower at 1 mM than at 10 mM Mg++. The coupling between ATP and peptide bond hydrolysis appeared very tight. However, when the protease was assayed under suboptimal conditions (e.g. at lower pH or with ADP present), many more ATP molecules (from 5 to 12) were consumed per peptide bond cleaved. Our data would indicate that the early steps in protein degradation consume almost as much energy (2 ATPs for each cleavage) as does the formation of peptide bonds during protein synthesis.

Protein degradation in bacterial and animal cells requires metabolic energy (1–5). In Escherichia coli, the energy requirement for the degradation of abnormal polypeptides and certain normal polypeptides (3–5) seems to result from the involvement of protease La, the lon gene product (6–9). This endoprotease (6–10) represents a new type of proteolytic enzyme in which ATP hydrolysis is essential for the cleavage of peptide bonds in proteins (6, 8, 10). A very similar enzyme has been isolated from the matrix of rat liver mitochondria (11), and ATP-dependent proteases appear to exist in the cytosol of mammalian cells, such as mouse erythroleukemia cells (12) and perhaps in reticulocytes (13). Protease La is a 450,000-Da multimer composed of four identical subunits and has both ATPase and proteolytic activities (8, 10). This enzyme appears to catalyze the initial rate-limiting steps in the degradation of certain normal proteins (14, 15) and highly abnormal polypeptides (3, 16, 17). For example, lon mutants, which encode a defective protease (7–10), or htpr mutants, which reduce the content of protease La (18, 19), decrease the cell's capacity to degrade abnormal proteins (16–18, 20). Moreover, lon mutants are defective in the same initial endoproteolytic cleavage that is blocked by ATP depletion (3).

With the purified protease, the cleavage of peptide bonds and the hydrolysis of ATP appear to be closely linked functions (8, 10, 21). Inhibition of the ATPase activity by non-metabolizable ATP analogs or by vanadate (6, 8, 9) causes a proportionate reduction in protein breakdown, while inhibition of proteolytic activity with diisopropyl fluorophosphate reduces its ATPase activity (8). Probably the strongest evidence for a tight coupling between these activities is that protein substrates stimulate ATPase activity 2–4-fold (8), while proteins that are not hydrolyzed have little or no effect.

This energy requirement for peptide bond cleavage cannot be explained by thermodynamic considerations, since the hydrolysis of peptide bonds is an exergonic process and since other proteases show no such energy requirement. Therefore, ATP hydrolysis by this enzyme must serve some kinetic or regulatory function, but neither protease La nor its substrates become phosphorylated or adenylylated (22) during ATP-dependent proteolysis.

New insights into the mechanism of this ATP dependence have been obtained recently from studies with fluorogenic tetrapeptide substrates (22, 24). Although these small peptides are degraded in an ATP-dependent reaction, this process does not require hydrolysis of the nucleotide. In other words, with small peptides, the binding of a nucleotide to the enzyme is sufficient for peptide bond cleavage, while ATP hydrolysis is required for the breakdown of large proteins to acid-soluble fragments. These observations and related ones (23, 24) led us to propose that degradation of proteins is a multistep process in which 1) AT binding is initially necessary to activate the proteolytic site; 2) peptide bond cleavage can then occur; 3) subsequently ATP hydrolysis to ADP takes place, which should terminate protein cleavage until new ATP molecules are bound. For large protein substrates, this reaction cycle seems to occur repeatedly until the protein is converted to oligopeptides. In this mechanism, ATP and peptide hydrolysis occur sequentially in an ordered cyclical process; however, the precise role of the ATP breakdown and the energetic costs of this process remain unclear.

The present studies were undertaken to define the number of ATP molecules actually consumed by protease La in breakdown of proteins of different size. In order to construct a detailed enzymatic mechanism, it is essential to know both the number of ATP molecules cleaved for each peptide bond hydrolyzed and whether coupling between these two processes varies with different substrates and under different experimental conditions. The stoichiometry of protease La is also of appreciable physiological interest since it is unclear how much energy is consumed by the cell in protein breakdown.
Materials and Methods

Protease La was purified, as described by Waxman and Goldberg (8), from E. coli carrying the lon gene on plasmid pMC40. ATP, ADP, O-methylisourea, fluorescamine, leucine, EDTA, and a-casein were obtained from Sigma. BSA 1 was obtained from Miles Pentex Laboratories. HEPES and TNBS were obtained from Research Organics, Inc., Cleveland. Crystalline glucagon was a gift from Dr. R. Chance of Lilly and guanidinylnated casein from Dr. David Chin (Harvard Medical School).

The assay mixture for protease La contained 1-4 μg of protease La, 1 mM ATP, 10 mM Mg++, 50 mM HEPES (pH 7.9), and 10 μg of the protein substrate in a total volume of 200 μl. The assay tubes were incubated at 37 °C for varying periods of time during which the rates of proteolysis were linear (Fig. 1). To test if the stoichiometry for ATP-dependent proteolysis depended on the substrate degraded, we compared the degradation of a-casein, a-casein in which free amino groups were blocked by guanidination with O-methylisourea (25), glucagon, and BSA denatured by reduction and carboxymidomethylation (26).

The ATPase activity was assayed at the same time in the same buffer, although generally ATP was present at 0.5 mM to reduce the blank values in the determination of inorganic phosphate. However, similar data were obtained with ATP at 0.5 or 1 mM as expected, since both concentrations far exceed the Km of the enzyme (20-30 μM) (22). After incubation at 37 °C for varying periods of time, the reaction was stopped by the addition of 100 μl of 1% sodium dodecyl sulfate, and the P, released was estimated, as described by Ames (27). All determinations were made while ATP and protein breakdown proceeded at a linear rate (Fig. 1).

Cleavage of peptide bonds was assayed in most experiments by measuring the appearance of new amino groups with fluorescamine (28). The enzyme reaction was stopped by immersing the tubes in an ice bath, and then 1.2 ml of borate buffer (pH 9.2) was added to each tube followed by 200 μl of fluorescamine dissolved in acetone (0.3 mg/ml). The fluorescence was measured using an excitation wavelength of 395 nm and emission wavelength of 475 nm, and the amino groups generated were estimated using leucine as a standard.

In the experiment in Fig. 3, the amino groups generated from guanidinated a-casein by protease La were estimated with TNBS using a modification of the procedure of Fields (29). The proteolytic reaction was stopped by the addition of 190 μl of 0.1 M NaH2PO4 containing 0.1 M EDTA (pH 9.5). Ten μl of 1.1 M TNBS were then added to each tube, and the tubes were vortexed thoroughly. After 15 min at room temperature, 600 μl of 0.2 M NaH2PO4 containing 1.5 mM Na2SO4 was added to each tube to stop the trinitrophenylation reaction. The absorbance at 420 nm was measured, and the amino groups generated were estimated using leucine as a standard.

Protein concentrations were determined by the method of Bradford (30). To obtain the best estimate of the slopes of graphs relating ATP and protein hydrolysis, data from different experiments were combined (e.g. in Fig. 2) and analyzed using a linear regression program. The correlation coefficients (R) obtained by this method of analysis are also shown.

Results

The assays of protein breakdown by protease La that were employed previously involved the measurement of acid-soluble material generated from radioactive proteins (6-10). However, this approach cannot yield definitive data about the number of peptide bonds cleaved, since many such cleavages do not yield acid-soluble products, and also the acid-soluble peptides may be further degraded by this enzyme. Therefore, we used fluorescamine (28, 31) to detect new amino groups generated during the hydrolysis of peptide bonds in proteins. With this assay, proteolytic activity of the pure enzyme is linear for at least 60 min (Fig. 1). This assay was used in most experiments to determine the relative rates of peptide bond and ATP hydrolysis and to test whether this ratio varies with different protein substrates and under conditions that alter the rate of proteolysis.

The abbreviations used are: BSA, bovine serum albumin; TNBS, 2,4,6-trinitrobenzene sulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

1 The time course of ATP hydrolysis and of the generation of new amino groups from denatured BSA and for the generation of inorganic phosphate from ATP by protease La. The ATPase and protease assays were carried out at 37 °C for 15-60 min, as described under "Materials and Methods," using denatured BSA as substrate. The data reported are based on triplicate assays for amino groups and duplicate assays for the ATPase. Similar results were obtained in multiple experiments with different preparations of the protease. ATP was present in large excess of its Km in the ATPase (0.5 mM) and protease (1 mM) assays.

Fig. 1. The time course of the generation of new amino groups from denatured BSA and for the generation of inorganic phosphate from ATP by protease La. The ATPase and protease assays were carried out at 37 °C for 15-60 min, as described under "Materials and Methods," using denatured BSA as substrate. The data reported are based on triplicate assays for amino groups and duplicate assays for the ATPase. Similar results were obtained in multiple experiments with different preparations of the protease. ATP was present in large excess of its Km in the ATPase (0.5 mM) and protease (1 mM) assays.

Fig. 2. The relationship between ATP and peptide bond hydrolysis by protease La with three different substrates: denatured BSA, casein, and glucagon. The assays were carried out at 37 °C for 5-60 min where both the reactions were linear (Fig. 1). Each set of symbols refers to a single experiment, in which proteolysis and ATPase activity were determined at several times (as in Fig. 1) and which used different enzyme preparations and concentrations. The difference between the basal and the protein-stimulated rates of ATP hydrolysis was used to calculate the stoichiometry. Certain preparations of protease La can degrade glucagon in the absence of ATP at a low rate (23) (10-30% of the ATP-stimulated rate). However, in the presence of 1 mM ATP, the enzyme is saturated with the nucleotide (37), and, therefore, the ATP-independent process was ignored in these measurements. The numbers shown are the best values for the slopes and the correlation coefficient (R) for these data determined by a linear regression program.

The time course of ATP hydrolysis and of the generation of new amino groups from denatured BSA (M, 67,000) are compared in Fig. 1. The enzyme has a basal rate of ATP hydrolysis (10, 21) which was stimulated 2-fold by the addition of denatured BSA. In this experiment (Fig. 1), protein-stimulated ATP hydrolysis averaged 72.2 nmol of P/min/mg of protein. In accord with previous studies, in which the hydrolysis of proteins to acid-soluble material was measured (6-10, 22), there was no detectable degradation of BSA unless ATP and Mg++ were present in the reaction mixture. In the presence of ATP, the rate of degradation of denatured BSA was 29 nmol of amino groups/min/mg of enzyme, and the Kcat value or the turnover number (32) was 13.2 mol of amino groups/min/mol of enzyme. As shown in Fig. 2, the production
of new amino groups was directly proportional to the BSA-induced ATP hydrolysis. Furthermore, this plot, which combines data from several different experiments, shows very little scatter as evidenced by an overall correlation coefficient of 0.98. With several different preparations of protease La, the average slope was 2.4, indicating that between 2 and 3 molecules of ATP were hydrolyzed per peptide bond cleaved.

In order to test if the energy requirement for peptide bond hydrolysis was similar with proteins of different sizes, the degradation of casein (M, 23,000) and glucagon (M, 3,550) was also studied. With casein as the substrate, the specific activity equaled 39 nmol of amino groups/min/mg of enzyme, and the $K_{cat}$ (32) was 17.0 mol of amino groups/min/mol of enzyme. It is interesting that the rate of peptide bond hydrolysis by protease La was similar with casein and denatured BSA, even though the generation of acid-soluble material was previously found to be much faster with $[^3H-\text{CH}_3]$casein than with $[^3H-\text{CH}_3]$-BSA (8, 22). Presumably, many of the radioactive peptides generated from BSA are insoluble in acid. During casein hydrolysis, 109.7 nmol of ATP were cleaved/min/mg of protein. Thus, in the breakdown of casein, 2.6 ATP molecules were hydrolyzed per peptide bond cleaved, and again there was very little scatter in the data. This value is indistinguishable from that obtained with BSA.

Glucagon is degraded at a 3-4-fold faster rate than either BSA or casein (23). Upon incubation with glucagon and ATP, protease La generated 118.2 nmol of amino groups/min/mg of enzyme, and the $K_{cat}$ value was 54 mol of amino groups/min/mol of enzyme. Therefore, it was of special interest to examine the ATP requirement for its breakdown. The average number of ATP molecules hydrolyzed per peptide bond cleaved in glucagon was 2.3, which is very similar to the value obtained with the other substrates. This agreement is noteworthy, since glucagon breakdown can be stimulated either by ATP or by nonhydrolyzable ATP analogs (23), and glucagon (unlike BSA or casein) is cleaved at only 2 or 3 places by protease La. Nevertheless, addition of glucagon promotes the ATPase activity of protease La, and the amount of ATP hydrolyzed is proportional to the number of peptide bonds cleaved (Fig. 2). Thus, with either polypeptides or proteins as substrates, the ATP consumed during peptide bond hydrolysis seems to be identical.

The data in Fig. 2 indicate that on average about 2 molecules of ATP are hydrolyzed per peptide bond cleaved independent of the size or the amino acid composition of the substrate (since similar values were obtained with glucagon, denatured BSA, and $\alpha$-casein). In order to investigate the number of cleavages actually made in different substrates, the enzyme was incubated with several different polypeptides until no further peptide bonds were cleaved. The number of peptides generated was then determined; an average size of the products was then calculated. In a typical experiment (Table I), glucagon was cut about 2-3 times, yielding oligopeptides with an average size of about 10 residues, while BSA yielded oligopeptides containing on the average 19-20 residues (Table I). Analysis of the products of these reactions by gel filtration on Sephadex G-25 also indicated that these products were small (i.e. in the included volume). Together, these results (Table I, Fig. 2) indicate that the ATP requirement per peptide bond cleaved is constant (between 2 and 3), and the size of the products generated is roughly similar regardless of the length of the polypeptide being degraded. These findings would argue against possible mechanisms for ATP-dependent proteolysis, in which large amounts of ATP are utilized for certain cleavages (e.g. rate-limiting ones) while many peptide bonds are hydrolyzed in an energy-independent reaction.

One potential complication in these calculations is that the quantum fluorescence yield with fluorescamine can vary up to 2-3-fold with different amino acid sequences in peptides (31). Because the stoichiometry observed with three different protein substrates was similar, the possible errors due to differences in the reactivity of new end groups are probably not significant. Nevertheless, to confirm these findings, we tried other reagents reported to react specifically with primary amino groups. Ninhydrin (33) and o-phthalaldehyde both gave much larger variations than fluorescamine in the reactivity of different peptides and amino acids (34).

To confirm the results obtained by the fluorescamine method, we quantitated the amino groups released from a protein substrate using TNBS which reacts specifically with primary amino groups. This reaction goes to completion rapidly (35), and the various trinitrophenylated amino acids and peptides have very similar extinction coefficients (35, 36). In addition, to enhance the specificity and sensitivity of the assay, the amino groups of lysine residues in the protein substrate were blocked by guanidination (25). As shown in Fig. 3, in several experiments the rates of hydrolysis of guanidinated casein measured with TNBS were comparable to proteolytic rates obtained with fluorescamine on other proteins (Fig. 2). Furthermore, the ratio of ATP molecules hydrolyzed per peptide bond cleaved was 2.3 (Fig. 3) which is indistinguishable from the ratios obtained above with other substrates.

### Table I

| Substrate          | Number of residues | Mol of peptides generated per mol of substrate | Mean number of residues in peptides produced |
|--------------------|--------------------|-----------------------------------------------|---------------------------------------------|
| Glucagon           | 29                 | 2.8                                           | 10                                          |
| Parathyroid hormone| 84                 | 3.7                                           | 23                                          |
| $\beta$-Globin     | 146                | 9.1                                           | 16                                          |
| $\alpha$-casein    | 139                | 13.6                                          | 15                                          |
| Denatured BSA      | 585                | 30.0                                          | 19.5                                        |

* Leucine equivalent.

**Stoichiometry under Suboptimal Conditions**—These various findings were obtained under conditions which allow maximal rates of proteolysis (pH 7.9, 10 mM MgCl$_2$). To learn more about the coupling between the two processes, we tested whether ATP consumption increased or decreased under conditions where proteolytic rates were slower. When protease La was assayed at pH 7.5, which is optimal for the ATPase activity, but not for the proteolytic function (22), the ratio of ATPs hydrolyzed to peptide bonds cleaved was 6.6 for denatured BSA (Table II) and 4.0 for casein. Thus, the proteolytic process consumes 2-3 times more energy at pH 7.5 than at 7.9, largely because the substrate-activated ATPase activity was 2-3-fold greater at pH 7.5 (while the protein-independent ATPase decreased 40-50%).

A similar effect on stoichiometry was observed when the
reaction was carried out in the presence of ADP. This end product of protease La can markedly inhibit proteolysis (22). In the presence of 0.5 mM ADP, the ratio of ATP consumed per peptide bond hydrolyzed increased from 2.6 to 3.5 (Table II). When assayed at pH 7.5, the addition of 0.5 mM ADP increased this ratio from 5.6 to 9.0, and with 0.1 mM ADP present, it was 13.0. Under the latter conditions, the ADP caused a much greater inhibition of proteolysis than of ATP hydrolysis (Table II); therefore, the energy cost of protein breakdown increased 5-6-fold. Although the addition of ADP and the lower pH partially uncouple ATP hydrolysis from proteolysis, these processes were still directly proportional to one another, and the graphs of amino groups generated versus inorganic phosphate production still showed very little scatter

$$R = 0.98.$$ Thus, the ATPase activity still appeared essential for protein breakdown.

Protease La requires Mg$$^{2+}$$ for both proteolytic and ATPase activities (8, 10, 23). In addition to promoting ATP binding (37), Mg$$^{2+}$$ probably also serves as a cofactor for proteolysis (22), since the Mg$$^{2+}$$ concentration that allows maximal proteolytic activity (10 mM) far exceeds the Mg$$^{2+}$$ concentration (0.1 mM) required for maximal ATP binding. At lower concentrations of Mg$$^{2+}$$ (1 mM), both casein degradation and ATP hydrolysis were slower (20 and 30%, respectively, of the rates measured with 10 mM Mg$$^{2+}$$). The ratio of ATP molecules hydrolyzed per peptide bond generally was lower than at 10 mM Mg$$^{2+}$$. For example, in one preparation, this ratio decreased from 3.1 to 1.9 with casein as the substrate and from 2.7 to 2.1 with denatured BSA. Thus, at more physiological levels of Mg$$^{2+}$$ (1 mM), where proteolysis proceeds more slowly (22), the coupling of the two processes appeared tighter and the stoichiometric ratio more closely approached 2.0.

### DISCUSSION

These various observations together demonstrate that about 2 molecules of ATP are hydrolyzed each time a peptide bond is cleaved by protease La. This figure seems to be in accord with our related studies (37) on nucleotide binding to La. This enzyme has two binding sites for ATP with very high affinities ($$K_D < 1 \mu M$$) and two low affinity binding sites ($$K_D = 20-30 \mu M$$). Nucleotide binding to the two high affinity sites activates the enzyme and allows cleavage of peptide bonds, but maximal rates of proteolysis (as in Figs. 1-3) require the binding of 2 additional ATPs to the low affinity sites (37). We have proposed a cyclical multistep mechanism (28, 24, 38) in which ATP binding first occurs, followed by peptide bond cleavage and then ATP hydrolysis. Thus, these two reactions seem to occur sequentially, but the constant stoichiometry observed here with different enzyme preparations and substrates indicates a strict coordination between these steps.

Nevertheless, the coupling between these processes can be altered in vitro (Table II); for example, in the presence of ADP, or at high Mg$$^{2+}$$ concentrations, or at a suboptimal pH, the ATPase activity is less efficiently coupled to the proteolytic activity. These treatments seem to affect the stoichiometry by distinct mechanisms; for example, ADP differentially decreases the proteolytic activity, while incubation at pH 7.5 primarily stimulates ATP hydrolysis. Therefore, when ADP was present at pH 7.5 (Table II), the amount of ATP utilized per peptide bond was especially large (13.0). However, even under these conditions, ATP hydrolysis was still necessary for proteolysis and proportional to the number of peptide bonds cleaved.

It is of particular significance that the measured stoichiometry is indistinguishable with three substrates, which differ from 3- to 20-fold in chain length. Although protease La cleaves glucagon at only 2 or 3 places, it cuts casein at about 13 points and cuts denatured BSA at about 30 sites (Table I). Therefore, in a given period, when functioning at its maximal rate (as in Fig. 2), this enzyme can attack and degrade completely about 15-20 times more molecules of glucagon than molecules of casein. Yet the energy costs per peptide bond were similar. These findings suggest that under these conditions, the association of protease La with a substrate and the initial cleavages are not the rate-limiting steps that determine how many substrate molecules are attacked; instead, polypeptide length appears to be an important deter-

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* A. S. Menon and A. L. Goldberg, submitted for publication.
degradation of these conjugates to ubiquitin and also for the subsequent function, as well as in mitochondria and chloroplasts proteolytic, as well as in mitochondria and chloroplasts, yielding oligopeptides and small polypeptides which resemble that of protease La. A similar ATP-dependent process is utilized for every nucleotide polymerized in DNA replication. The metabolic costs involved in the breakdown of short-lived proteins by protease La may appear surprisingly large, but these values by themselves probably overestimate the energy costs of complete protein digestion, since other enzymes also must contribute to this process in vivo. Protease La makes a number of endoproteolytic cleavages (3, 24) in proteins, yielding oligopeptides and small polypeptides which in turn are rapidly degraded to amino acids by other proteases and peptidases that do not appear to require ATP (24, 42–45).

It will be interesting to determine if the energy costs for protein breakdown in other cells, both eukaryotic and prokaryotic, as well as in mitochondria and chloroplasts (11, 46) resemble that of protease La. A similar ATP-dependent protease has been isolated from the closely related bacterium, Salmonella typhimurium (22), and the stoichiometry for its function is similar to that reported here (data not shown). ATP-dependent proteases have also been isolated from mitochondria (10) and the cytosol of murine erythroleukemia cells (12). The energy costs of proteolysis may even be greater in the cytosolic ATP-dependent pathway in reticulocytes, where ATP hydrolysis is required both for conjugation of protein substrates to ubiquitin and also for the subsequent degradation of these conjugates (2, 13, 47–51). In any case, the considerable investment of energy made by the bacteria in protein breakdown argues strongly that this process is indeed of appreciable physiological importance. Presumably, this tight coupling of protein and ATP hydrolysis and the resulting large expenditures of energy have evolved to provide tight control and specificity to this process, which if excessive or nonselective, would be lethal to the organism (38, 52).

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