Studies of the ATP-dependent Proteolytic Enzyme, Protease La, from *Escherichia coli*

(Received for publication, April 16, 1981)

Fred S. Larimore, Lloyd Waxman, and Alfred L. Goldberg

From the Harvard Medical School, Department of Physiology, Boston, Massachusetts 02115

The ATP-dependent proteolytic activity, protease La, from *Escherichia coli* has been partially purified, and the role of ATP investigated. ATP (3 mM) stimulated degradation of [methyl-3H]casein and [methyl-3H]caspohemoglobin 6-40-fold but only when Mg<sup>2+</sup> was present. The nucleotide had to be continuously present for this process, since rapid depletion of ATP prevented further proteolysis. A concentration of 250 μM MgATP gave half-maximal activity, but the effects of different concentrations of ATP depended also on the Mg<sup>2+</sup> concentration, and high concentrations of nucleotides inhibited. dATP, CTP, and UTP also stimulated proteolysis but not as well as ATP, while GTP, ADP, AMP, pyrophosphate, and the nonmetabolizable analogs α,β-methylene-ATP, β,γ-methylene-ATP, adenosine 5'-O-(3-thiotriphosphate), and 2',3'-dialdehyde-ATP, had little or no effect. These analogs, as well as AMP and ATP, inhibited the effect of ATP, probably by competition for the same binding sites. Both ATP and ADP appeared to bind to and stabilize the protease (even in the absence of Mg<sup>2+</sup>), since they prevented the rapid loss of activity that occurred at 42 °C.

No protein kinase or protein adenyllylase activity was demonstrable in these preparations. ATP-dependent proteolysis also was not affected by the addition of termination factor rho or ubiquitin, and these preparations did not conjugate ubiquitin to protein substrates. Although certain inhibitors of ATPases (N,N'-dicyclohexylcarbodimide, oligomycin, azide) did not affect casein hydrolysis, others (vanadate, Dio-9, quercetin) markedly inhibited this process. Proteolysis ceased rapidly after addition of vanadate; thus, continued splitting of ATP seems necessary, but a simple relationship between ATPase activity and proteolysis was not clear in these impure preparations. At concentrations above 200 mM, phosphate markedly inhibited the ATP-dependent activity and stimulated proteolysis slightly in the absence of ATP.

These various properties suggest that ATP cleavage is required for proteolysis and distinguish protease La from the recA gene product and the cytoplasmic ATP-stimulated proteases of mammalian cells. for metabolic energy (1). In *Escherichia coli* (1–8) as in mammalian cells (1, 8–10), the rapid degradation of proteins with highly abnormal structures (4, 8, 10), of specific enzymes (9), and of normal proteins during starvation (1, 2, 5, 8) requires the continuous production of ATP. In mammalian cells, this energy requirement was initially thought to be related to lysosome function (1, 11–13). However, ATP is also essential for certain degradative processes, for which there is growing evidence against lysosomal involvement, such as the rapid breakdown of abnormal proteins. Furthermore, bacteria or mammalian erythrocytes do not contain lysosomes, yet show a clear ATP requirement for intracellular protein degradation (2–8, 14).

This energy requirement for proteolysis has been investigated most extensively in *E. coli* (4, 5, 8). Studies with various inhibitors in wild type and mutant cells indicated an essential role for high energy phosphate bonds rather than for the "energy-rich membrane state" (8). Furthermore, in the degradation of abnormal proteins, inhibitors of ATP production seem to block the initial endoproteolytic cleavages of the substrate (5). In recent years, cell-free preparations have been developed from *E. coli* which show ATP-stimulated proteolysis (7, 15–18). In these crude extracts, an ATP stimulation of 60 to 300% was found (15, 18). Our initial attempts to isolate the responsible activity indicated an association of the ATP-stimulated proteolytic system with the cell membrane (16), although a soluble form was also found (18).1 Swamy and Goldberg (18) demonstrated in *E. coli* extracts eight soluble proteases, only one of which is dependent on ATP for activity (17–20). This enzyme, named protease La, appears identical with that found by Voellmy and Goldberg (16) and Chung and Goldberg (19). In addition to this enzyme, the *recA* gene product in *E. coli* can carry out proteolytic cleavages of the λ repressor and the *lex A* gene product in an ATP-dependent process (21–23). Although the *recA* product and the ATP-dependent protease La appear quite distinct (18–20), the present studies investigated whether ATP affects protease La in a manner similar to the *recA* protein.

We have carried out a series of experiments to define more precisely the role of ATP in the proteolytic process and to test certain possible mechanisms, including whether ATP might be hydrolyzed in conjunction with proteolysis, whether phosphorylation or adenylylation of substrates or enzyme may occur, and whether this process involves the transcription termination factor, *rho*, as has been suggested by Simon et al. (24). These questions are also relevant to understanding the role of ATP in protein breakdown in mammalian cells. Ethington and Goldberg and co-workers (6, 10) have described a nonlysosomal ATP-dependent proteolytic system in reticulocytes that seems responsible for the degradation of abnormal and some normal proteins. The role of ATP in this process is

1 K. H. S. Swamy, R. S. Voellmy, and A. L. Goldberg, unpublished observations.
still controversial. Cytoplasmic proteases that are stimulated 2-3-fold by ATP have been isolated from liver and red cells (7, 14, 25). Alternatively, Hershko and colleagues (26-29) have proposed that ATP may not affect a proteolytic enzyme directly, but instead, is required for the conjugation of proteolytic substrates to a small polypeptide, ubiquitin, and that this derivatization is essential to increase their proteolytic susceptibility (26-29). We therefore investigated the possible involvement of ubiquitin in the ATP-dependent proteolytic activity from E. coli.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli K-12, grown to logarithmic phase on Luria Broth, was obtained as a frozen paste from the Grain Processing Corp. (Muscatine, IA). Hexokinase, calf thymus histone type II A, N,N,N′-dicyclohexylcarbodiimide, ATP, ADP, AMP, Trizma base, quercetin, and oligomycin were obtained from Sigma. α,β-Methylene-ATP, β,γ-methylene-ATP, and adenosine 5′-O-(3-thiotriphosphate) were obtained from P-L Biochemicals. Dic was kindly provided by Dr. R. J. Roon (University of Minnesota) and purified termination factor rho by Dr. W. R. McClure (Harvard University). Bovine thymus ubiquitin was a generous gift of Dr. G. Goldstein, Ortho Pharmaceutical. 2,3-Dialdehyde-ATP was prepared according to the procedure of Easterbrook-Smith et al. (30). Sodium vanadate was obtained from Fisher. All other chemicals were of reagent grade.

Protein Substrates—The substrates for protease assays were prepared by methylation of bovine hemoglobin with [3H]formaldehyde (40-49 μcmol, New England Nuclear) and bovine α-casein with [3H]formaldehyde (85 μcmol, New England Nuclear) according to the procedure of Rice and Means (31). Typically, the specific activities obtained were 2-4 × 10^6 cpm/mg for [3H]globulin and 10 × 10^6 cpm/mg for [3H]casein. To increase its proteolytic susceptibility, hemoglobin was extracted from the hemoglobin by use of methylmethylethylene (32).

Protease Assay—Proteolytic activity was determined by the release of 3- or 14C-labeled material soluble in trichloroacetic acid. Assays were carried out on 500-μl aliquots of the pooled fractions from the DEAE-columns that contained maximal ATP-stimulated proteolytic activity (Fig. 1). Typically, the material pooled from DEAE-cellulose contained about 3 mg of protein/ml and from DEAE-Sepharose 2 mg/ml which corresponds to 0.9-1.1 mg and 0.5-0.6 mg of protein added per 500 μl of assay mixture. To the enzyme were added 10 μl of 1 M Tris-HCl (pH 7.5 at 22 °C) and 25 μl of 60 mM ATP or 25 μl of CF buffer. Ten μl of [3H]casein stock or 25 μl of [3H]globulin stock solutions were then added such that the reaction mixture (0.5 ml) contained 50-100 μg/ml of substrate and 1-2 × 10^6 cpm/mg of [3H]casein. The reaction mixture was incubated at 30 °C for 4 h, except where noted, and the reaction was terminated by the addition of 40 μl of 3% bovine serum albumin (Sigma) as a carrier and 60 μl of 100% trichloroacetic acid. After 30 min on ice, the solution was centrifuged, and 150 μl of CF buffer, 1 mM ATP (specific activity, 100 cpm/pmol), and 50 mM Tris-HCl (pH 7.5) were added to the supernatant. After 30 or 90 min at 30 °C, the reaction was stopped by the addition of 50 μl of 10% sodium dodecyl sulfate and boiling for 3 min. The samples were dialyzed against water overnight in the cold to remove salts and then lyophilized.

The samples were dissolved in electrophoresis buffer and electrophoresed on 17% polyacrylamide slab gels in the presence of sodium dodecyl sulfate according to the method of Laemmli (35) as modified by Mcgregor et al. (36). The gels were stained with Coomassie blue and destained by diffusion with 40% methanol and 7.5% acetic acid. The gels were prepared for fluorescent autoradiography by treatment with Enhance (New England Nuclear) according to the manufacturer's directions. The film was exposed for 3 weeks at −70 °C. With this procedure, less than 500 cpm of [3H]casein could be readily seen by eye. Thus, in the adenyllylation assay, 5 pmol of [3H] ATP incorporated into protein could be detected.

Preparation of ATP-dependent Proteolytic Enzyme—Approximately 75 g of E. coli K-12 were suspended in 150 ml of 50 mM Tris-HCl (pH 7.8 at 22 °C), 10 mM MgCl2, and the mixture was stirred to pH 7.5 with the addition of 1 m Tris-HCl. All subsequent steps were performed at 4 °C. The cell suspension was passed through an Aminco French Press at an operating pressure of 14,000 p.s.i. The suspension was centrifuged at 150,000 g for 30 min at 30 °C, and 2 m sodium chloride and 20 μl of protein could be detected in the supernatant. The supernatant was dialyzed against 10 mM Tris-HCl (pH 7.5 at 22 °C) and 5 mM MgCl2 for 2-4 h until its conductivity was less than 0.02 mmo.

The dialyzed crude extract was loaded on a DEAE-cellulose column (2.5 × 30 cm) (Whatman DE52) equilibrated with 10 mM Tris-HCl (pH 7.5 at 22 °C) and 5 mM MgCl2 (TMD buffer) at a flow rate of about 40 ml/h. After extensive washing with TMD buffer (until A280 of the effluent was less than 0.05), a linear gradient of 0-0.2 m NaCl in TMD buffer was run (2.4 liters total volume). Chromatography of the crude extract on DEAE discovered four peaks with proteolytic activity against [3H]casein, one of which was stimulated by ATP and correspondingly to protease La (18, 20). Fractions containing ATP-stimulated proteolytic activity (which generally was eluted between 0.11 and 0.13 m NaCl) were pooled. They were dialyzed against 10 mM Tris-HCl (pH 8.0 at 22 °C), 5 mM MgCl2, and 10 mM 2-mercaptoethanol (TMD buffer) until pH 8.0 and a conductivity of 2.5 mho was reached. This material was then loaded on a DEAE-
The ATP-dependent Protease from E. coli

Preparations of Protease La—In accord with prior findings (18, 20), chromatography of E. coli extracts on DEAE-cellulose resolved the ATP-dependent protease La from six other soluble E. coli enzymes capable of hydrolyzing [3H]casein and [14C]globin. When the most active fractions under this peak were pooled, ATP (3 mM) increased the hydrolysis of [3H]casein or [14C]globin 5–10-fold, depending on the preparation. With enzyme obtained by DEAE-cellulose or Sepharose chromatography (Fig. 1), proteolysis in the presence of ATP occurred at linear rates for up to 120 min, while ATP-independent activity was negligible. In addition, proteolysis was proportional to enzyme concentration over a wide range (Fig. 1) and was proportional to the concentration of [3H]casein or [14C]globin until saturation was observed (Fig. 1). The levels of casein or globin used routinely in the assays (100 μg/ml) exceeded the apparent Km values which generally ranged from 10–40 μg/ml but were occasionally as high as 100 μg/ml with certain preparations of the radioactive substrate or enzyme. In any case, such variations did not alter any of the conclusions presented below, and in all cases, the fraction of substrate hydrolyzed was less than 10%.

When both substrates were present at concentrations of 100 μg/ml, the [3H]casein was degraded to acid-soluble material much more rapidly than [14C]globin. Nevertheless, the relative ATP stimulation with [14C]globin was larger (10–15-fold) than with [3H]casein (5–10-fold), probably because the low amount of ATP-independent activity contaminating protease La was more active against [3H]casein than [14C]globin. Further chromatography on DEAE-Sepharose removed most of this remaining ATP-independent activity (18, 20). After this step, the stimulation by ATP was typically about 40-fold with either substrate (Fig. 2) (both of which gave similar results in the various experiments). Chromatography on either DEAE-cellulose or Sepharose yielded a peak containing 80–90% of the protease La activity put on the column (20). Based on the amount of protein removed by these steps, we estimate that after the DEAE-cellulose step, the enzyme was purified about 20–30-fold over crude lysates, and after DEAE-Sepharose chromatography (Fig. 2) about 60–80-fold. Nevertheless, the ATP-dependent proteolytic activity obtained by either approach behaved similarly in subsequent experiments, and therefore data are shown that were obtained with either preparation.

The ATP Requirement—To test whether ATP is continuously required for proteolysis or whether it only activated this process and then was no longer necessary, the added ATP was rapidly depleted from the assay mixture by the addition of hexokinase and glucose. These agents were added at concentrations sufficient to reduce ATP levels by 96% in 2–3 min, as shown by high pressure liquid chromatography. The hexokinase and glucose together (Fig. 3) caused proteolysis to cease abruptly. By contrast, addition of hexokinase alone had no significant effect, while glucose (18 mM) caused only a minor reduction in proteolysis (for unknown reasons). Thus, the presence of the nucleotide is required throughout the process.

When ATP production in E. coli is inhibited, protein breakdown does not decrease until ATP levels fall to less than 20% the levels normally found in growing cells (about 3 mM) (5, 8), and below this point degradation is blocked almost completely (5, 8). To compare these in vivo findings with the behavior of protease La, casein hydrolysis was measured with widely different ATP concentrations (Fig. 4). Proteolysis fell off dramatically with ATP concentrations below 0.6 mM, as was seen with intact cells (5). An apparent Km for ATP of 200–250 μM in the presence of 7 mM MgCl2 was determined graphically from the data in Fig. 4.

The actual ATP dependence of this process depended upon
ATP, or an inhibitory effect of high Mg$^{2+}$ concentrations on the proteolytic enzyme may all possibly contribute to these complex effects. In any case, data obtained from experiments with widely varying concentrations of magnesium and ATP suggest a Mg$^{2+}$/ATP ratio of 2.5-4 permitted maximal proteolysis.

In the absence of Mg$^{2+}$, no proteolysis was found in the presence of 3 mM ATP. Mn$^{2+}$ and Ca$^{2+}$ could be substituted for Mg$^{2+}$, with Mn$^{2+}$ being slightly more effective, and Ca$^{2+}$ slightly less effective than Mg$^{2+}$ (data not shown). Because similar results were observed when hydrolysis of casein or globin was measured, these effects of metal ions were probably related to interactions with the nucleotides and the proteolytic

To determine if ATP must continually be present for proteolysis, protease La was incubated in the absence (Δ−−Δ) and presence (Δ−−Δ') of 3 mM ATP. At 60 min, the assay mixture was divided into three 4-ml aliquots. To one, 12.5 μg of hexokinase and 75 mM of glucose were added (○--○); to another 12.5 μg of hexokinase (○—○) alone or 75 mM of glucose (▲—▲) alone was added as a control. The incubation was carried out for an additional 60 min. At the points indicated, 0.5-ml aliquots were removed, and the acid-soluble radioactivity was measured. Measurements by high pressure liquid chromatography showed that this concentration of hexokinase and glucose lowered ATP levels by at least 97% within 2-3 min of addition. These data have been corrected for the effects of dilution. The enzyme preparation was obtained from DEAE-cellulose.

The magnesium concentration in a complex fashion. Thus, with 7 mM MgCl$_2$, maximal hydrolysis of casein or globin was observed at 0.6 mM ATP, but above 1 mM this activity decreased sharply. When the MgCl$_2$ concentration was increased to 20 mM, maximal proteolytic activity was observed at ATP concentrations between 1 mM and 5 mM (Fig. 4), and the fall in proteolysis with high ATP levels was less pronounced. However, in the presence of 20 mM MgCl$_2$, maximal casein hydrolysis was significantly less than with 7 mM MgCl$_2$, and with 50 mM Mg$^{2+}$ an even greater fall in maximal proteolytic rate was found (data not shown). The reasons for this fall in activity at high ATP and at high Mg$^{2+}$ concentrations are not clear. An inhibition of proteolysis by excess substrate (ATP-Mg), an inhibition of the effect of ATP-Mg by free

![Graph](https://example.com/graph1.png)

**Fig. 3.** Effect of depletion of ATP on $[^{14}C]$globin hydrolysis. To determine if ATP must continually be present for proteolysis, protease La was incubated in the absence (Δ−−Δ) and presence (Δ−−Δ') of 3 mM ATP. At 60 min, the assay mixture was divided into three 4-ml aliquots. To one, 12.5 μg of hexokinase and 75 mM of glucose were added (○--○); to another 12.5 μg of hexokinase (○—○) alone or 75 mM of glucose (▲—▲) alone was added as a control. The incubation was carried out for an additional 60 min. At the points indicated, 0.5-ml aliquots were removed, and the acid-soluble radioactivity was measured. Measurements by high pressure liquid chromatography showed that this concentration of hexokinase and glucose lowered ATP levels by at least 97% within 2-3 min of addition. These data have been corrected for the effects of dilution. The enzyme preparation was obtained from DEAE-cellulose.

**Fig. 4.** ATP dependence of protease La. ATP at the concentrations indicated was added to the assay mixture either in the presence of 7 mM (○—○) or 20 mM (○—○) MgCl$_2$. A stock of the disodium salt of ATP was neutralized to pH 7.5 before addition to the incubation mixtures. These data were obtained on enzyme preparations from DEAE-cellulose.

**Fig. 5.** Double reciprocal plot of the effects of 2',3'-dialdehyde-ATP, AMP, and ADP on the initial velocity of casein hydrolysis measured in the presence of ATP. Proteolytic activity was measured at several ATP concentrations (○—○), and in the presence of 100 μM 2',3'-dialdehyde-ATP (△—△), 1 mM AMP (Δ−−Δ), and 1 mM ADP (X—X). These data were obtained with preparations from DEAE-Sepharose which showed insignificant activity in the absence of ATP (<4% that with ATP).
The ATP-dependent Protease from E. coli

The ATP-dependent Protease from E. coli

AMP by themselves inhibited proteolysis (Fig. 5), data are presented as substrates.

We studied the effects of other nucleoside mono-, di-, and triphosphates and pyrophosphate (Table I). Deoxyribose-ATP was the most effective of the various compounds tested and maximal proteolysis was about 75% that observed in the presence of ATP. UTP and CTP had small but significant effects, while GTP, ADP, AMP, and pyrophosphate were less than 15% as effective as ATP in stimulating proteolysis (Table I). In addition, ADP (1 mM), AMP (1 mM), and the ATP analog, 2',3'-dialdehyde-ATP (100 µM), were all competitive inhibitors of ATP as found by double reciprocal plots of ATP concentration versus initial velocity of proteolysis in the presence and absence of these compounds (Fig. 5). These plots also gave an apparent K_m for ATP of 250 µM.

Stabilization by Nucleotides—These findings raised the possibility that ATP might interact directly with the protease. Accordingly, ATP was found to stabilize the enzyme at both 4 and 42 °C. Preparations of the enzyme from the DEAE-celulose column lost activity progressively at 4 °C. After 4 days in the absence of ATP, this activity was only 40% of the initial level. However, in the presence of 3 mM ATP, very little change in proteolytic activity was observed. The enzyme was quite unstable at higher temperatures. Upon incubation at 42 °C, proteolytic activity decreased by about 80% in 1 h. When 3 mM ATP was present, no loss in activity occurred (Fig. 5). Lower concentrations of ATP were less effective in preventing inactivation at 42 °C. At 100 µM, which is below the apparent K_m for stimulation of proteolysis (250 µM), very little protection was observed (data not shown). Progressive greater stabilization was obtained with 0.5 and 1.0 mM. Thus, the stabilization of the protease appears to correlate with the ability of ATP to activate this enzyme (Fig. 4).

In analogous experiments, ATP was added to the enzyme at 42 °C in the absence of Mg. Under these conditions, the enzyme was stabilized as well as in the presence of Mg^{2+} (data not shown). The addition of Mg^{2+} by itself had no stabilizing effect under these conditions. Thus, ATP by itself appears to bind to the proteolytic system although the divalent cation is essential for proteolysis.

ATP and the analogs were compared for their ability to stimulate proteolysis, measured as in Table I on similar preparations from the DEAE-Sepharose column.

| Nucleotide (3 mM) | % activity |
|------------------|------------|
| ATP              | 100        |
| ATP-s            | 22         |
| 2',3'-Dialdehyde-ATP | 10       |
| α,β-Methylene-ATP | 11         |
| β,γ-Methylene-ATP | 5          |

TABLE II

Effects of ATP and various ATP analogs (3 mM) on globin-degrading activity

ADP and AMP, which appear to be competitive inhibitors of ATP-dependent proteolysis (Fig. 5), were also found to protect the enzyme from inactivation at 42 °C but less effectively than ATP (Fig. 6). These results strongly suggest that ATP, ADP, and AMP bind directly to the proteolytic enzyme and induce some structural alteration in it (rather than in the substrate).

Effects of Nonmetabolizable ATP Analogs—To determine whether ATP hydrolysis is also required for protein breakdown, we tested the effects of a series of nonmetabolized or poorly metabolized ATP analogs. 2',3'-Dialdehyde-ATP and ATP_5'S (3 mM), which can be slowly cleaved by many ATPases (37, 38), stimulated proteolysis about 20% as well as ATP, while α,β-methylene-ATP and β,γ-methylene-ATP showed less than 10% of the activity seen with ATP (Table II). The β,γ-methylene analog cannot be cleaved at the terminal phosphate by ATPases (33). In the α,β-methylene analog, the methylene group appears to interfere with binding to many enzymes, such that the γ-phosphate is not cleaved as effectively as in ATP (39). The simplest interpretation of these results is that ATP cleavage is essential for proteolysis, in accord with our earlier suggestions (15). These findings with ATP_5'S distinguish protease La from the ATP-stimulated cleavage of the λ repressor by the recA gene product (40). The latter process was stimulated severalfold more effectively by ATP_5'S than by ATP.

The low levels of proteolysis (Table II) observed with the analogs may be explained by residual contamination with ATP. To evaluate this possibility, we determined whether ATP was present in these preparations by high pressure liquid chromatography. No ATP was observed; however, ATP contamination of the analogs (added at 3 mM) of less than 0.3% could have produced this low stimulation of proteolytic activity (Table II and Fig. 4), and such a small amount of ATP (i.e. 10 µM ATP) would not have been detected by this assay.

A possible explanation for the lack of proteolysis with the analogs is that they do not bind to the enzyme. However, all four analogs were found to inhibit the effect of ATP on proteolysis (Table III), and thus probably are competing with ATP.

The abbreviation used is: ATP_5'S, adenosine 5'-O-(3-thiotriphosphate).

Fig. 6. Stabilization of protease La at 42 °C by ATP, ADP, and AMP. Protease La was incubated at 42 °C in the absence of adenine nucleotides (O—O) or in the presence of 3 mM ATP (■—■), 3 mM ADP (△—△), or 3 mM AMP (□—□). At the times indicated, aliquots were put on ice and then all assayed simultaneously with [3H]casein in the presence of 3 mM ATP. Because ADP and AMP by themselves inhibited proteolysis (Fig. 5), data are presented as per cent initial activity. The preparations studied were similar to those used in Fig. 5.

TABLE III

Effects of ATP analogs on globin-degrading activity in the presence of ATP (1 mM)

Enzyme preparations resembled those in Table I.

| Analog (3 mM) | % activity |
|---------------|------------|
| None          | 100        |
| ATP_5'S       | 37         |
| 2',3'-Dialdehyde-ATP | 14       |
| α,β-Methylene-ATP | 64        |
| β,γ-Methylene-ATP | 56        |

The ATP-dependent Protease from E. coli
ATP for binding. Furthermore, 3 mM α,β-methylene-ATP and ATPγS were found to cause some stabilization of the protease at 42 °C (data not shown). Thus, they appear to interact with the enzyme.

Effects of ATPase Inhibitors—To examine further whether hydrolysis of a high energy phosphate bond is essential for proteolysis, the effects of various inhibitors of E. coli ATPases were examined. Three inhibitors of the membrane-bound (Ca²⁺-Mg²⁺)-ATPase, N,N'-dicyclohexylcarbodiimide, sodium azide, and oligomycin, had little or no effect on casein hydrolysis (Table IV). (The slight inhibition with oligomycin is probably not meaningful; these latter observations are complicated by the presence of ethanol as a solvent, which by itself inhibited proteolysis by about 50%.) On the other hand, ATP showed 10% of the proteolytic activity seen in its presence (see Fig. 8).

Additional experiments tested whether high concentrations of phosphate might mimic the effect of vanadate on proteolytic activity. Concentrations less than 25 mM phosphate slightly increased proteolytic activity, but concentrations above 100 mM dramatically inhibited ATP-stimulated proteolysis. In addition, above 200 mM, the proteolytic activity in the absence of ATP increased 2-fold. By contrast, at concentrations above 200 mM, NaCl and KCl inhibited ATP-dependent proteolysis only slightly and did not stimulate protein hydrolysis in the absence of ATP. Perhaps these very high levels of phosphate act like ATP to cause the formation of a

| Inhibitor                  | % activity |
|---------------------------|------------|
| None                      | 100        |
| N,N'-Dicyclohexylcarbodiimide | 92         |
| 50 μM                     | 87         |
| 100 μM                    |            |
| Oligomycin                |            |
| 2.5 mM                    | 78         |
| Sodium azide              |            |
| 5 mM                      | 91         |
| 10 mM                     | 93         |
| Dio-9                     |            |
| 100 μg/ml                 | 29         |
| 500 μg/ml                 | 1          |
| Quercetin                 |            |
| 100 μM                    | 62         |
| 500 μM                    | 9          |
| Sodium vanadate           |            |
| 100 μM                    | 61         |
| 500 μM                    | 11         |

Fig. 7. Effects of vanadate on ATP-dependent proteolytic activity. [³⁵S]Globin hydrolysis was assayed in the presence of 3 mM ATP with (– – –) or without (– – –) 100 μM vanadate. At 60 min, the assay mixture without vanadate was divided into 2 equal portions and water (– – –) or 100 μM vanadate (– – –) were added. At the times indicated, aliquots were removed, and acid-soluble radioactivity was measured. These data were obtained on enzyme preparations from DEAE-cellulose which in the absence of ATP showed 10% of the proteolytic activity seen in its presence (see Fig. 8).

Fig. 8. Effects of different concentrations of vanadate on globin hydrolysis. Assays were conducted in the presence (– – –) or absence (– – –) of 3 mM ATP under standard conditions. These data were obtained on enzyme preparations from DEAE-cellulose.

Vanadate appeared to be a rather specific inhibitor of protease La (Fig. 7). At 50 μM, 50% of the proteolytic activity was lost, and at 500 μM, no proteolysis was observed (Fig. 8). At these concentrations, vanadate did not significantly inhibit any of the seven ATP-independent proteases in E. coli (data not shown). This inhibition of protease La was not reversible. After treatment with 1 mM vanadate, the enzyme was dialyzed extensively. The yellow color characteristic of vanadate was still evident, and the enzyme still showed no activity. Cantley et al. (46) have restored activity to vanadate-treated (Na⁺-K⁺)-ATPase by using norepinephrine to reduce the vanadate ion to the vanadyl ion, a much less effective inhibitor. In our preparations, the vanadyl ion was also less effective, but proteolytic activity was still inhibited in the presence of 2 mM norepinephrine and 1 mM vanadate (data not shown).

Additional experiments tested whether high concentrations of phosphate might mimic the effect of vanadate on proteolytic activity. Concentrations less than 25 mM phosphate slightly increased proteolytic activity, but concentrations above 100 mM dramatically inhibited ATP-stimulated proteolysis. In addition, above 200 mM, the proteolytic activity in the absence of ATP increased 2-fold. By contrast, at concentrations above 200 mM, NaCl and KCl inhibited ATP-dependent proteolysis only slightly and did not stimulate protein hydrolysis in the absence of ATP. Perhaps these very high levels of phosphate act like ATP to cause the formation of a
“phosphoenzyme intermediate” or to induce an altered enzyme conformation which is active in proteolysis.

Appreciable ATPase activity was demonstrable in these preparations but did not co-elute from DEAE-Sepharose with the proteolytic activity (Fig. 2). Therefore, even if an ATPase activity is associated with protease La (see below), in these impure preparations the contaminating ATPases make its study impossible (Fig. 2). Accordingly, no simple correlation was observed between the effects of these inhibitors on proteolysis (Table IV) and the total ATPase activity in preparations from DEAE-Sepharose. For example, azide showed a 30 to 40% inhibition of the total ATPase without affecting proteolysis. Of the three ATPase inhibitors that decreased proteolysis (Table IV, Figs. 7 and 8), Dio-9 had little effect, vanadate showed about 50% inhibition, and quercetin (500 μM) caused about 80% inhibition of ATP hydrolysis.

Possible Mechanisms of the ATP Effects—To explain the energy requirement for proteolysis, Hersksoh and co-workers (26-29) have suggested that in rabbit reticulocytes and other cells, ATP was required for an energy-dependent ligation of the polypeptide ubiquitin to protein substrates to enhance their proteolytic susceptibility. Although ubiquitin seems to be present in E. coli (48), our DEAE-chromatography steps should have removed this polypeptide. Also, experiments with anti-ubiquitin antibody indicated that ubiquitin was immemorably low or not present in our preparations. When ubiquitin was added to assay mixtures at 10 μg/ml, no change in proteolysis occurred in the presence or absence of ATP. Ciechanover et al. (26) reported that ubiquitin became associated with many proteins in reticulocyte extracts, and we have been able to confirm this observation with reticulocyte extracts. However, when we carried out similar experiments (26, 27) with the preparations of protease La, we could not obtain any evidence for such an ATP-stimulated ligation process with casein or E. coli proteins.

Another possibility to account for ATP-stimulated proteolysis is that a protein kinase is required to phosphorylate the casein substrate or the protease prior to degradation. There is growing evidence for the existence of several protein kinases in enteric bacteria (49-51). We therefore assayed for protein kinase activity by incubating [32P]ATP and protease La fractions from the DEAE-cellulose column with casein (1.25 mg/ml), histone (1.25 mg/ml), or no exogenous proteins added. The preparation did not phosphorylate the exogenous substrates. When control, 12.0 pmol of phosphate were incorporated per mg per mg of protein and in the presence of casein (10.5 pmol) and histone (11.0 pmol). Since casein is rapidly hydrolyzed by the protease, phosphorylation of the substrate does not seem essential for proteolysis.

Moreover, no evidence was obtained for phosphorylation of the protease. Although some 32P was precipitated with the protein as discussed above, when these preparations were analyzed by polyacrylamide gel electrophoresis and autoradiography, no phosphorylated proteins were observed. By contrast, it was possible to detect incorporation of 32P into distinct E. coli proteins when crude extracts were incubated with [32P]ATP and treated similarly.

Another possibility is that ATP is essential for the adenylation of the substrate or enzyme, as occurs in the regulation of E. coli glutamine synthetase (52). To determine whether this form of modification might be part of the ATP-stimulated proteolytic mechanism, we tested whether radioactivity from [2,3-3H]ATP might be incorporated into casein or into endogenous proteins. Following the incubation period, the proteins were fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and the results were analyzed by autoradiography. With this approach, which should be able to detect less than several hundred disintegrations/min of 3H (53), we did not observe any incorporation into proteins by visual inspection of the autoradiograms.

Possible Involvement of rho—Certain mutations affecting the transcription termination factor rho have been reported to lead to increased proteolysis (24), and it was suggested that the rho ATPase may have a role in protein degradation (24). To test this possibility, we studied the effects of purified rho on globin hydrolysis. rho itself had no proteolytic activity with globin as a substrate, either in the presence or absence of polycytidylic acid (Table V). Adding rho to the enzyme mixture also had no effect on proteolysis. In addition, polycytidylic acid (6 μg/ml) did not enhance proteolysis although it stimulated the ATPase activity 4-10-fold in the preparations from the DEAE-cellulose column. This polynucleotide did not affect the ATPase in the preparations from DEAE-Sepharose (Fig. 2) which probably no longer contain the rho factor. Thus, no evidence for involvement of this protein was obtained.

### DISCUSSION

The properties of the ATP-dependent proteolytic enzyme, protease La, described here can account for the apparent energy requirement of protein degradation in vivo. For example, in intact E. coli, ATP or some other nucleoside triphosphate is required for degradation of abnormal proteins, but cellular ATP levels must be reduced by 80-90% before rates of proteolysis fall significantly (5, 8). With protease La, ATP was much more effective at stimulating proteolysis than other nucleoside triphosphates (Table I), and approximately physiological levels of ATP (2-3 mM) (5) and Mg2+ (5-10 mM) (54), gave maximal or near maximal rates of protein breakdown. As the ATP concentration was reduced below 0.6 mM, proteolysis decreased abruptly (Fig. 4) in a similar fashion as seen in vivo (5, 8). Changes in protein degradation occur very rapidly when bacteria are depleted of ATP or when ATP production is restored (5). Similarly, protease La activity fell rapidly upon removal of ATP (Fig. 3) or addition of vanadate to block ATP hydrolysis (Fig. 4).

Recent studies have provided further strong evidence that protease La catalyzes the rate-limiting step in protein breakdown in vivo. In lon+ (also called capR+ or deg+) strains, the degradation of abnormal proteins occurs at reduced rates (55, 56), apparently because of a deficiency in their capacity to carry out an ATP-dependent endoproteolytic cleavage (4). Such mutants have recently been shown to have defects in protease La, while E. coli carrying the cloned lon gene contain

### Table V

| Condition                      | % activity |
|-------------------------------|------------|
| rho + globin + ATP            | 0          |
| rho + globin + ATP + poly(C)  | 0          |
| Protease La + globin + ATP    | 100        |
| Protease La + globin + ATP + poly(C) | 100 |
| Protease La + globin + ATP + rho | 100 |
| Protease La + globin + ATP + rho + poly(C) | 100 |
greater than normal amounts of this ATP-dependent enzyme (19).

The present findings on protease La can also account for our earlier observations with ATP in crude extracts (15). In the crude preparations, ATP caused only a 2-3-fold stimulation (15, 17), apparently because of the presence of several additional ATP-independent proteases (18, 20). When freshly grown E. coli are lysed by more gentle techniques than were used here on frozen cells (15, 16), a large fraction of the ATP-stimulated activity is associated with the cell membrane. The relationship between the membrane-associated and the soluble activities is not yet clear. Perhaps protease La is associated with the membrane in vivo or possibly there exist more than one ATP-stimulated proteolytic enzyme in E. coli.

Protease La seems to require a Mg$^{2+}$-ATP$^-$ (or Mn$^{2+}$-ATP$^-$) complex rather than free ATP$^-$ (40). Furthermore, the marked ability of several adenine nucleotides and ATP in particular to protect the protease against inactivation at 4 and 42 °C strongly suggests that the Mg$^{2+}$-ATP$^-$ binds directly to the enzyme. Some stabilization was also observed with ATP alone although less than with Mg$^{2+}$-ATP. Interestingly, the ATP concentrations capable of stabilizing the protease at 42 °C correlated with the concentrations that supported proteolysis (Fig. 4). Thus below the K_	ext{m} for ATP did not protect completely the enzyme from thermal denaturation, while at levels above the K_	ext{m} the enzyme is maintained in a heat-stable conformation. The protection of the enzyme at 42 °C by AMP, ADP, α,β-methylene-ATP, and ATP$\gamma$S also suggests that these compounds bind to the enzyme in a similar fashion as ATP (Fig. 6) and induce a conformational change to a more stable state. Nevertheless, the nonmetabolized nucleotides, AMP and ADP, do not support proteolysis, presumably because they cannot be cleaved. Since these compounds inhibit the stimulation of protein breakdown by ATP (Table V) apparently by competition (Fig. 4), cleavage of ATP or proteins is not required for this stabilization.

It appears unlikely that an additional ATP-utilizing enzyme, such as a protein kinase, adenyllylation system, or protein-ligation factor, is present and acts to stabilize the protease. Such models cannot explain how nonfunctional nucleotides can also protect the protease under these circumstances (Fig. 6). The possibility that a protein kinase or adenylase modifies the protease or protease also is unlikely because we were not able to detect any such activity in the preparations. In addition, the model proposed by Ciechanover et al. (26, 27) to explain ATP-dependent proteolysis in reticulocytes does not appear applicable to protease La. Even though ubiquitin has been reported in E. coli and shown to be very similar immunologically to the polypeptide from bovine thymus (48), this protein had no effect on degradation of casein or globin and was not conjugated to them.

These various results together strongly suggest that Mg$^{2+}$-ATP$^-$ binds directly to the proteolytic enzyme. Nucleotide binding, while necessary, is not sufficient for proteolysis, as was shown with the nonmetabolizable ATP analogs which appear to bind (Fig. 5) but do not stimulate proteolysis (Table III). Thus, cleavage of the γ-phosphate seems necessary for this process. The marked inhibition of casein hydrolysis by vanadate, Dio-9, and quercetin further suggest that an ATPase activity is associated with the protease and is essential for its function. Vanadate’s mode of inhibition has been well studied (unlike that of Dio-9 or quercetin). At the active site of kidney (Na$^{+}$-K$^+$)-ATPase (42-46), vanadate serves as a “transition state analog.” It binds very tightly to the site normally occupied by phosphate and effectively prevents phosphate or ATP binding. In contrast, vanadate seems to inhibit myosin ATPase through the slow formation of an enzyme-ADP-vanadate complex (47). The rapidity of vanadate’s inhibition of protease La and the lack of a requirement for prior ADP accumulation suggests that vanadate is also acting as a “transition state analog” (cf. Refs. 42-46).

Although the inhibition by vanadate strongly suggests that ATP hydrolysis is essential for proteolysis, these partially purified preparations contain appreciable ATPase activity unrelated to protease La (Fig. 2), and not surprisingly, no simple correlation between the inhibition of proteolysis (Table IV) and ATPase was obtained in these impure preparations. In recent studies (57-59) with purer preparations (18-20), protease La has been shown to contain an ATPase function. This ATPase is inhibited by vanadate and is required for protein cleavage, in accord with the present findings (4).

An interesting observation in this context was the marked inhibition of casein hydrolysis by high concentrations of phosphate (Fig. 9) and the small but reproducible, stimulation of proteolysis in the absence of ATP (Fig. 9). Such observations may also possibly indicate that ATP hydrolysis is coupled to peptide cleavage, since a large excess of phosphate or ADP would then be expected to inhibit ATP hydrolysis. It is also possible that high phosphate levels cause the formation of a “phosphoenzyme” intermediate, as has been observed for the (Na$^{+}$-K$^+$)-ATPase where high phosphate mimics the effect of ATP (60).

These properties of protease La differentiate it from known proteolytic enzymes including the ATP-stimulated proteolytic systems described recently in bacterial or mammalian cells. In addition to its roles in DNA recombination, the recA gene product cleaves the λ repressor and the lexA gene product in an ATP-dependent fashion (21-23) in the presence of a polynucleotide. Although this protein contains ATPase function (21-23), ATP$\gamma$S stimulates the cleavage of these repressors severalfold more effectively than ATP (40); thus ATP hydrolysis is not required for this process. By contrast, ATP$\gamma$S promoted casein hydrolysis by protease La only 20% as well as ATP (Table IV), and this process occurred in the absence of polynucleotides (Table V). These two enzymes are also distinctly different from the reporter assay described by different genes (18-20).²

Recently, ATP-stimulated alkaline proteases have been purified from reticulocytes (7, 14, 23), rat liver (25), and other tissues (7). After purification, these mammalian endoproteases are stimulated 2-3-fold by ATP; however, nonmetabolized

² L. Waxman, unpublished observations.
ATP analogs and pyrophosphate also cause some stimulation, and vanadate has no effect (17, 14, 25). Although these properties clearly distinguish these cytoplasmic enzymes from protease L, an ATP-dependent protease has been isolated from rat liver mitochondria5 that appears very similar to the E. coli enzyme described here.

The interaction of protease L with ATP and the apparent requirement for nucleotide cleavage are not consistent with the models suggested by Hershko and co-workers (26-29) for ATP's role in degradation of proteins in reticulocytes (10). By analogy to other ATP-dependent enzymes, we suggest two general models to explain the effects of ATP on protease L.

1) The hydrolysis of ATP would switch the enzyme into an active conformation for peptide bond cleavage. This process could involve formation of a "phosphoenzyme intermediate" as occurs for the (Na⁺-K⁺)-ATPase (60). 2) Alternatively, by analogy to a possible mechanism of adenylate cyclase (61), ATP binding to the protease may put it in a "high affinity" state for binding of protein substrates. After peptide cleavage, ATP hydrolysis would allow release of the cleaved polypeptide and return of the enzyme to the low affinity state. In either mechanism, the role of ATP is to regulate the conformation of the protease rather than to modify the substrate.

Acknowledgments—We are grateful to Ms. Robi Levine and Ms. Maureen Rouh for their expert assistance in the preparation of this manuscript. We are also grateful to our colleagues, Drs. Chin Chung and K. H. S. Swamy and to Mr. Timothy Meixsell for their assistance in many of these preparations.

REFERENCES
1. Goldberg, A. L., and St. John, A. C. (1976) Annu. Rev. Biochem. 45, 747-803
2. Mandelstam, J. (1960) Bacteriol. Rev. 24, 289-308
3. Goldberg, A. L. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 422-426
4. Kowit, J. D., and Goldberg, A. L. (1977) J. Biol. Chem. 252, 8360-8367
5. Olsen, K., and Goldberg, A. L. (1978) Biochim. Biophys. Acta 542, 385-398
6. Goldberg, A. L., Kowit, J. D., Ettinger, J. D., and Klemes, Y. (1978) in Protein Turnover and Lysosome Function (Segal, H., and Doele, D. ed.) pp. 171-196, Academic Press, New York
7. Goldberg, A. L., Strom, N. P., and Swamy, K. H. S. (1980) Ciba Found. Symp. 76, 227-261
8. St. John, A. C., and Goldberg, A. L. (1978) J. Biol. Chem. 253, 2765-2771
9. Hershko, A., and Tomkics, G. M. (1971) J. Biol. Chem. 246, 4628-4629
10. Ettinger, J. D., and Goldberg, A. L. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 54-58
11. Hayashi, M., Hiroi, Y., and Natari, Y. (1973) Nature New Biol. 243, 153-166
12. Huisman, W., Bouma, J. M. W., and Gruber, M. (1974) Nature 240, 428-429
13. Brostrom, C. O., and Jeffay, H. (1970) J. Biol. Chem. 245, 600-606
14. Boche, F. S., and Goldberg, A. L. (1980) Fed. Proc. 39, 1682
15. Murakami, K., Voellmy, R., and Goldberg, A. L. (1979) J. Biol. Chem. 254, 8194-8200
16. Voellmy, R. W., and Goldberg, A. L. (1981) Nature 290, 419-421
17. Goldberg, A. L., Voellmy, R., and Swamy, K. H. S. (1979) in Biological Functions of Proteosomes (Holtz, H., and Tschesche, H. ed.) pp. 35-48, Springer-Verlag, Berlin
18. Swamy, K. H. S., and Goldberg, A. L. (1981) Nature 292, 652-654
19. Chung, C. H., and Goldberg, A. L. (1981) J. Biol. Chem. 256, 7525-7528
20. Goldberg, A. L., Swamy, K. H. S., Chung, C. H., and Lasimone, F. S. (1982) Methods Enzymol. 80, in press

5 M. Desautels and A. L. Goldberg, manuscript in preparation.
Studies of the ATP-dependent proteolytic enzyme, protease La, from Escherichia coli.

F S Larimore, L Waxman and A L Goldberg

J. Biol. Chem. 1982, 257:4187-4195.

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/257/8/4187.citation.full.html#ref-list-1