Protease II from Escherichia coli

PURIFICATION AND CHARACTERIZATION

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MICHELE PACAUD AND CATHERINE RICHAUD

From the Laboratoire de Chimie des Proteines, Institut de Recherches Scientifiques sur le Cancer, Villejuif, France and the Institut de Microbiologie, Universite de Paris, F-91405 Orsay, France

We have previously demonstrated the existence of two types of endopeptidase in Escherichia coli. A purification procedure is described for one of these, designated protease II. It has been purified about 13,500-fold with a recovery of 24%. The isolated enzyme appears homogeneous by electrophoresis and gel filtration. Its molecular weight is estimated by three different methods to be about 58,000. Its optimal pH is around 8. Protease II activity is unaffected by chelating agents and sulfhydryl reagents. Amidase and proteolytic activities are stimulated by calcium ion, which decreases the enzyme stability.

Like pancreatic trypsin, this endopeptidase catalyses the hydrolysis of α-amino-substituted lysine and arginine esters. It appears distinct from the previously isolated protease I, which is a chymotrypsin-like enzyme. The apparent Michaelis constant for hydrolysis of N-benzoyl-L-arginine ethyl ester is $4.7 \times 10^{-4}$ M. The esterase activity is inhibited by diisopropylphosphorofluoridate ($K_{i(app)} = 2.7 \times 10^{-3}$ M) and tosyl lysine chloromethyl ketone ($K_{i(app)} = 1.8 \times 10^{-5}$ M), indicating that serine and histidine residues may be present in the active site. However, protease II is insensitive to phenylmethanesulfonyl fluoride and several natural trypsin inhibitors. Its amidase and esterase activities are competitively inhibited by free arginine and aromatic amidines.

The proteolytic activity measured on azocasein is very low. In contrast to trypsin, protease II is without effect on native β-galactosidase. It easily degrades aspartokinase I and III. Nevertheless both enzymes are resistant to proteolysis in the presence of their respective allosteric effectors. These results provide further evidence that such differences in protease susceptibility can be related to the conformational state of the substrate. The possible implication of structural changes in the mechanism of preferential proteolysis in vivo, is discussed.

The intracellular breakdown of proteins to the amino acid level probably involves the participation of proteases and peptidases. Despite early reports suggesting the absence of protein degradation in log phase cells (1-3), it is now well established that a pool of labile proteins (2 to 7% of the total bacterial proteins) is hydrolyzed rapidly in growing and starved cells (4-6). An additional protein degradation occurs (20 to 40% of the total proteins), at a slow rate, under certain conditions of starvation in some strains of Escherichia coli (7). In addition, several studies have shown that the incomplete polypeptides produced after a deletion in the gene coding for lac repressor (8) or by nonsense mutation in the structural gene of β-galactosidase (9, 10) are selectively degraded. Experiments of Pine (11) and Goldberg (12) also indicate a general system for the degradation of abnormal proteins in growing cells. Mutants of E. coli with a defect in the degradation of β-galactosidase nonsense fragments have been isolated (13), but the biological significance of this phenomenon is unclear.

During the past 10 years, considerable information has been obtained about general protein degradation in mammalian and bacterial cells. However, few studies have been directed toward the characterization of intracellular proteolytic enzymes despite their importance for understanding the regulation of enzyme levels. Prouty and Goldberg who studied the effects of protease inhibitors on protein breakdown in E. coli have suggested that starving cells contain a serine protease, probably similar to trypsin (14).

We have previously reported the presence of two endopeptidases (I and II) in soluble extracts from E. coli growing cells. These enzymes hydrolyze the N-acylphenylalanine amides, and N-acylarginine amides, respectively; they therefore can be classified in the group of chymotrypsin and trypsin-like enzymes. Protease I has already been isolated and characterized (15). While we began to isolate the second protease, Richaud et al. (16), studying the stability of pure aspartokinase III, were intrigued by the appearance of new molecular species from the native enzyme, under controlled conditions. These modifications have been attributed to proteolysis in preparation, in which an esterase activity similar to that of the second endopeptidase was found. A comparative study of the behaviors of aspartokinase III and of this proteolytic enzyme suggested that the two enzymes could be co-purified. This has
been subsequently confirmed. The present paper reports a purification procedure of the trypsin-like enzyme, which is referred to as protease II. Some physicochemical and kinetic properties of the purified protein are presented, including in vitro proteolysis of three enzymes isolated from E. coli.

The two endopeptidases of E. coli differ by numerous properties. Assays of cellular localization suggest that protease I is a periplasmic protein, while protease II is a cytoplasmic one. This different localization supports the hypothesis of a distinct physiological function for each.

MATERIALS AND METHODS

Bovine trypsin (twice crystallized), chromatographically purified β-galactosidase from Escherichia coli, and other enzymes were purchased from Worthington. Aspartokinase I was a kind gift from Dr. Michel Veron (Institut Pasteur, Paris); Kunitz inhibitor (Iniprol) was from Choay Laboratoire (France). N-Benzoyl-L-arginine ethyl ester, N-benzyloxyl-L-tyrosine ethyl ester, and proteins for molecular weight determinations were obtained from Schwarz/Mann. Exopeptidase substrates and L-phenylalanine-L-arginine-2-naphthylamide were supplied by Bachem, Inc. Amino acids and all N-acetylamiino acid-p-nitrophenyl esters were products of Cyclo Chemical. Benzoyl-DL-arginine-p-nitroanilide, amidines, and phenylmethanesulfonyl fluoride were purchased from Sigma. Tosyl-L-phenylalanine chloromethyl ketone, tosyl-L-lysine chloromethyl ketone, and disopropylphosphorofluoridate were from Serva (West Germany). Dioxane and dimethylformamide (spectral grade) were from Carlo Erba (Italy). All other materials were obtained from commercial sources. Deionized water was used for both the preparation of solutions and for the rinsing of glassware.

Bacterial Cells—E. coli strain B (ATCC 11303) was purchased from Miles Laboratories (England). The cells were grown on a yeast extract/glucose/salt medium with aeration. They were harvested at late log phase, and kept frozen at -30°.

Cellular Localization—E. coli K12 cells cultivated in aerated minimal medium with 2% glycerol as carbon source were used. Osmotic shock was applied under different conditions to growing and starving cells, following the procedure of Nosell and Hoppel (17). β-Galactosidase and alkaline phosphatase were chosen as reference enzymes.

Hydrolysis of Synthetic Substrates—Activity on N-benzoyl-D-arginine-p-nitroanilide was measured at 410 nm in 100 mM Tris-HCl buffer, pH 8, as described by Erlanger et al. (18). A molar absorption difference of 8900 M⁻¹ cm⁻¹ was used in all calculations. The hydrolysis of N-benzyloxyl-L-tyrosine ethyl ester was determined in the same buffer at 255 nm by the method of Kedzdy and Lorand (19) using a molar absorption difference of 808 M⁻¹ cm⁻¹. For these two standard assays units of enzyme activity are defined as nanomoles of product liberated per min.

Activities upon the N-acylamino acid-p-nitrophenyl esters were performed at 410 nm in 100 mM potassium phosphate buffer, pH 7.5 (20). The hydrolysis of N-benzoyl-L-tyrosine ethyl ester was followed at 255 nm (21), and that of N-acetyl phenylalanine β-naphthyl ester at 328 nm (15). Carboxypeptidase B activity was tested according to the method of Folk et al. (22). Activities on N-acetamidoc 2 naphthyl amides were measured at 335 nm. Initial velocities were calculated from the zero time slopes of recorded optical density variations versus time plots. The absorbance scale used ranged from 0 to 0.1. All assays were done at 25° in a Beckman Acta III spectrophotometer equipped with a thermostated cell compartment.

Proteolytic Assays—Azocasein hydrolysis was measured by the method described by Leighton et al. (23) with minor modifications. Protease II was incubated for 6 hours at 37°. The β-galactosidase proteolysis was tested at pH 7.6 in the following buffer: 20 mM Tris, 10 mM EDTA, 10 mM NaCl, and 10 mM 2-mercaptoethanol (24).

Enzyme Purification

Aspartokinase I homoserine dehydrogenase I was performed under experimental conditions described by Veron et al. (25). Protease II substrate ratios were approximated 0.1 and 0.5% (w/w). Aspartokinase III degradation was determined in purified preparations of this enzyme mixed with varying amounts of protease II (0.1 to 0.5%, w/w). Incubations were carried out in the absence of lysisin in Tris-glucose buffer (10 mM Tris/75 mM glucose, pH 8.6) as previously described by Richard et al. (18). All reactions were followed by measuring enzyme activities and by electrophoresis in polyacrylamide-agarose gels with sodium dodecyl sulfate.

Other Enzyme Activities—Alkaline phosphatase was assayed according to Torriani (26), β-galactosidase according to Ullmann et al. (27), and alcohol dehydrogenase was determined by the method of Valee and Hoch (28). The activity of aspartokinase III was estimated by the hydroxamate-ferric chloride method (29). For aspartokinase I-α-ketoisocaproate dehydrogenase I, aspartokinase activity was determined by a coupled assay using pyruvate kinase and lactate dehydrogenase (30) and dehydrogenase activity was tested in the forward direction as described by Patte et al. (31). Irreversible Competitive Inhibitions—A stock solution of 100 mM DFP in 2-propanol (kept at -20°) and a fresh solution of 10 mM TLCK in 1 m HCl were suitably diluted before each experiment. The enzyme was incubated at 37° in the presence of a constant concentration of inhibitor: 10⁻⁴ M for DFP and 2 x 10⁻⁴ M for TLCK. The reaction mixtures were composed of 100 m sodium phosphate buffer, pH 7 (80 μl), inhibitor solution (10 μl), and enzyme solution at 90 μg/ml (10 μl). The remaining activities in each assay were determined after a given time, by addition of 0.9 ml of Bz-Arg-ONa at different concentrations. Hydrolysis was immediately monitored at 235 nm. Incubation times were 20 min with 10 mM DFP.

Protein Concentration—Proteins were assayed throughout the purification by a modified biuret technique (32) and the amount of purified enzyme was estimated by the procedure of Lowry et al. (33). Both methods had been calibrated with a bovine plasma albumin solution, prepared by weight. For proteins the absorption coefficient of which is known, concentrations were calculated from optical density.

Trypsin—Various dilutions of trypsin were prepared from a fresh solution of this protein (5 mg/ml in 1 m HCl). Concentrations were spectrophotometrically determined at 280 nm using E₀ cm⁻¹ m⁻¹ = 1.43.

Electrophoresis and Esterase Activity—Electrophoresis in composite gels of acrylamide/agarose was carried out by the procedure of Uriel (34). The gels (2 mm thick) were 7% acrylamide, 0.1% bisacylamide, and 0.8% agarose, with a running Tris glycine buffer, pH 8.7. Esterase activity on benzoyl-L-arginine ethyl ester was revealed in the gels by the colorimetric method of Fujimoto et al. (35) with minor modifications. Proteins were stained with Coomassie brilliant blue.

Electrophoreses with sodium dodecyl sulfate were carried out on acrylamide/agarose gels according to the method of Weber and Osborn (36). For molecular weight estimations, the following proteins were used as standards: bovine serum albumin, M₈ = 67,000; ovalbumin, M₈ = 45,000; chymotrypsinogen, M₈ = 25,000; and myoglobin, M₈ = 17,800.

Determination of Molecular Weight by Gel Filtration—Descending gel filtration (37) on Sephadex G-100 was performed as follows: the column (96 x 2.4 cm) was equilibrated and eluted in appropriate buffer, with a flow rate of 12 ml/hour. Absorbance was determined in a spectrophotometer at 280 nm or at 412 nm (cytochrome c). Enzyme activity was assayed by coupling method II. Cultures of protease II. The logarithm of the molecular weight was correlated to the elution volume of each protein. Protein standards were: yeast glucose-6-phosphate dehydrogenase, M₈ = 108,000; bovine serum albumin, ovalbumin, and peptin, M₈ = 36,000; and cytochrome c, M₈ = 12,400.

Sucrose Density Gradient Centrifugation—Sedimentation coefficients were measured according to the method of Martin and Ames (38). The purified enzyme and yeast alcohol dehydrogenase (30 μl) were layered on the top of a sucrose gradient (4.4 ml) 5 to 20%, in 100 mM Tris buffer, pH 8, containing 1 m KCl. Centrifugation was performed for 14 hours at 4°, at 50,000 rpm, in the rotor 50 of a Spinco L3 centrifuge.

RESULTS

Enzyme Purification

Unless otherwise stated, all operations were performed at 4°.

Preparation of Cell Extracts

About 600 g of frozen cells were allowed to thaw overnight in

1The abbreviations used are: DFP, diisopropylphosphorofluoridate; Bz-Arg-ONa, N-benzyloxyl-L-arginine-p-nitroanilide; Bz-Arg-OEt, N-benzyloxyl-L-arginine ethyl ester; TLCK, tosyl-L-lysine chloromethyl ketone.
the cold room at 4°C. Cells were suspended in 800 ml of 20 mM potassium phosphate buffer, pH 7.2, containing 2 mM magnesium Titrplex, 150 mM KCl, and 2 mg of deoxyribonuclease. The whole suspension was homogenized by stirring, and bacteria were broken in a French press, by 40-ml fractions. The insoluble material then was removed by centrifugation at 17,000 × g for 40 min. To the supernatant streptomycin sulfate (2% w/v) was added slowly with mechanical stirring. After standing overnight the supernatant fluid was collected by centrifugation at 17,000 × g for 2 hours and was designated as "crude extract."

**Step 1: First Ammonium Sulfate Precipitation**—To the cooled crude extract (50 to 60 mg of protein/ml) solid ammonium sulfate was added to 50% saturation, with stirring over a period of 20 min. After 1 hour of stirring at room temperature, the suspension was centrifuged for 30 min at 12,000 × g. The precipitate was collected and dissolved in 20 mM potassium phosphate buffer, pH 7.2, containing 150 mM KCl. This solution then was centrifuged for 2 hours at 78,000 × g and the clear supernatant was dialyzed for 16 hours against three changes of 5 liters of 20 mM potassium phosphate buffer, pH 7.10, containing 280 mM KCl. Enzyme was eluted in a relatively sharp peak by increasing KCl concentration stepwise to 580 mM KCl. The active fractions were pooled and concentrated by ultrafiltration through a Diaflo UM-2 membrane. Enzyme was dialedyzed against 100 mM Tris/HCl buffer, pH 7.8, and stored at -80°C.

The purification results are summarized in Table I. The yield of enzyme activity was 24%. Starting with 600 g of cells we have obtained 50 g of protein, from which 0.88 mg of enzyme was prepared with a 10,500-fold purification. This high value proves that the enzyme exists in very low concentration within the cells (about 7.5 mg/100 g of soluble proteins).

The purified enzyme showed an absorption maximum at 278 to 280 nm and a minimum at 250 nm. No significant absorbance was detected above 320 nm. The ratio A₂₈₀nm/A₂₅₀nm was 1.33.

![Fig. 1. Elution pattern on Sephadex G-100. Proteins obtained from hydroxylapatite were filtered through the column.](http://www.jbc.org/)

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>50.000 mg</td>
<td>59,000 units</td>
<td>1.04 units/mg protein</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>35,000 mg</td>
<td>53,800 units</td>
<td>1.53 units/mg protein</td>
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<tr>
<td>35%</td>
<td>12,236 mg</td>
<td>45,353 units</td>
<td>3.70 units/mg protein</td>
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<tr>
<td>DEAE-Sephadex</td>
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<td></td>
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<tr>
<td>(pH 7.1)</td>
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<td>41,300 units</td>
<td>5.85 units/mg protein</td>
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<tr>
<td>(pH 7.4)</td>
<td>109.2 mg</td>
<td>24,792 units</td>
<td>181 units/mg protein</td>
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<tr>
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<td>16,500 units</td>
<td>2,500 units/mg protein</td>
<td>2,403</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>0.88 mg</td>
<td>12,420 units</td>
<td>14,100 units/mg protein</td>
<td>13,557</td>
</tr>
</tbody>
</table>

Data refer to 600 g of wet cells. Hydrolytic activity on Bz-Arg-ONAn was measured as described under "Materials and Methods." A unit is defined as the quantity of enzyme which liberates 1 nmol of p-nitroanilide/min.
Criteria of Homogeneity

*Escherichia coli* protease II was routinely eluted from Sephadex G-100 with constant specific activity. Rechromatography of peak fractions on Sephadex G-100 resulted in a single peak with constant specific activity, identical with the applied material. From analytical gel electrophoresis only a single band with no minor contaminants was detected by protein staining which was also active on Bz-Arg-OEt (Fig. 2). When 25 μg of enzyme were analyzed by electrophoresis in sodium dodecyl sulfate, a single component was found, again indicative of homogeneity.

Molecular Weight Estimation

By sucrose gradient centrifugation a sedimentation coefficient of 4 S was obtained using yeast alcohol dehydrogenase ($M_r = 150,000$) as a reference. The molecular weight calculated was 59,000.

From the analytical gel filtration of the purified enzyme (150 μg/ml), using the empirical calibration method of Andrews (37), several values depending on the ionic strength of eluting buffer used, were obtained. In 10 mM Tris/HCl buffer, pH 8, containing 100 mM KCl, a value of 110,000 was found. In 100 mM Tris/HCl buffer, pH 8, containing various concentrations of KCl, the values estimated were: 78,000 in 0.1 M KCl, 67,000 in 0.5 M KCl, and 58,000 in 1 M KCl (Fig. 3). Each measure was made with a ±5000 precision.

Electrophoresis in sodium dodecyl sulfate acrylamide gels gave an average value of 58,000 (with a ±4000 precision) (Fig. 4). The relative mobility of the protein was unchanged by addition of iodoacetate in the incubation medium. These results suggest that the enzyme is composed of a single subunit of molecular weight of about 58,000, which is able to form dimers at low ionic strength.

Stability

Maximum stability was obtained at -80^°C in dilute solutions at protein concentrations no less than 0.3 mg/ml within a pH range of 7.8. Nevertheless, after 3 months the enzyme gradually lost activity, presumably due to irreversible denaturation. Repeated freezing and thawing did not affect the esterase activity. Although calcium ion protects many proteolytic enzymes, it caused a marked decrease in stability of protease II. For example, when the enzyme was stored (at 150 μg/ml in Tris/HCl buffer, pH 7.6) for 5 weeks at -80^°C either without or with 25 and 50 mM calcium chloride, the remaining activities were, respectively: 88, 50, and 32%. The effect of calcium was still more drastic in more dilute protein solutions.

Influence of Several Agents on Enzyme Activity

*pH*

The pH dependence for protease II was determined in potassium phosphate (pH 5 to 7.5) and Tris/HCl (pH 7.5 to 9.5) buffers. Amidase activity on N-benzoyl-D-arginine-p-nitroanilide was minimal at pH 5.5, rose sharply to the optimum at pH 7.5, and remained on a plateau until pH 9. The optimal proteolytic activity on azocasein ranged from pH 7.6 to 8.9.
Effective in the absence of urea. This suggests that a free -SH group does not participate in enzymatic catalysis and that the native enzyme has at least one disulfide bridge.

Ionic Strength

In 100 mM Tris/HCl buffer, pH 8, the amidase activity was unchanged when the final ionic strength was increased from 0.05 to 1 by the addition of appropriate amounts of NaCl.

Metal Chelators and Sulfhydryl Reagents

No decrease in activity was obtained by incubation in either EDTA or a-phenanthroline for 9 hours, indicating there is no metal ion requirement. The enzyme was unaffected by sulfhydryl reagents (see Table II), but 70% of the amidase activity was lost when the reduced protease was treated with iodoacetate in the absence of urea. This suggests that a free -SH group does not participate in enzymatic catalysis and that the native enzyme has at least one disulfide bridge.

Bivalents Cations

The effects of some bivalent cations are given in Table II. Whereas Zn²⁺, Fe²⁺, Hg²⁺, and Co²⁺ decreased the activity below the value of the control, Ca²⁺ slightly increased it. From determinations of the initial rate of Bz-Arg-ONAn hydrolysis above the value of the control, Ca²⁺ slightly increased it. From determinations of the initial rate of Bz-Arg-ONAn hydrolysis above 25 mM.

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Substrate Specificity and Kinetic Parameters

Synthetic substrates of trypsin, N-benzoyl-b-L-arginine-p-nitroanilide, N-benzoyl-L-arginine ethyl ester, N-carbobenzoxy-L-lysine-p-nitrophenylester, and L-phenylalanine-L-arginine-2-naphthylamide were all hydrolyzed, exhibiting amidasic and esterolytic properties of the enzyme. Molar specific activities of protease II and bovine trypsin upon the first two substrates above and azocasein were determined and compared. Results in Table III indicate that Bz-Arg-ONAn was hydrolyzed, respectively, 5 and 30 times faster by protease II than by the pancreatic enzyme, whereas protease II was approximately a thousand times less active upon azocasein. The apparent $K_m$ values for Bz-Arg-ONAn and Bz-Arg-ONAn using protease II were measured with and without calcium from substrate concentrations ranging from 0.5 to 5.10⁻⁴ M. From data in Table IV, it can be seen that Ca²⁺ increased $K_m$ (app) of Bz-Arg-ONAn and slightly increased $K_m$ (app) of Bz Arg OEt. These results suggest that calcium ion is an activator of the amidase activity, but a weak inhibitor of the esterase activity.

No carboxypeptidase B activity would be measured on hippuryl-L-arginine, and the two aminopeptidase substrates, L-arginine-2-naphthylamide and L-lysine-2-naphthylamide, were not significantly hydrolyzed, which means that it is a carboxyendopeptidase. Protease II showed no detectable activity against chymotryptic substrates: N-acetyl-L-tyrosine ethyl ester, N-benzoyl-L-tyrosine ethyl ester, N-benzoylcarbonyl-L-tyrosine-nitrophenyl ester, and N-benzoylcarbonyl-L-tryptophan-p-nitrophenyl ester. Among the following substrates, N-benzoylcarbonyl-glycine-p-nitrophenyl ester, N-benzoylcarbonyl-L-leucine-p-nitrophenyl ester, N-benzoylcarbonyl-L-valine-p-nitrophenyl ester, N-benzoylcarbonyl-L-alanine-p-nitrophenyl ester, N-benzoylcarbonyl-L-proline-p-nitrophenyl ester, N-benzoylcarbonyl-L-glutamine-p-nitrophenyl ester, and N-benzoylcarbonyl-L-methionine-p-nitrophenyl ester, only N-benzoylcarbonyl-glycine-p-nitrophenyl ester was weakly hydrolyzed. As trypsin is able to cleave N-acetyl-glycine-p-nitrophenyl ester in a nonspecific fashion (39), protease II and the pancreatic enzyme probably have a similar specificity toward small substrates.

Inhibition Studies and Kinetics

The effects of protease inhibitors are presented in Table V. Protease II was inhibited only by DFP and TLCK and the esterase activity was not restored after extensive dialysis. Thus as in the case of trypsin, DFP and TLCK are irreversible inhibitors of protease II. However, esterase and amidase activities of the Escherichia coli protease were unaffected by prolonged incubation at pH 8 in a 40-fold molar excess of each of the following trypsin inhibitors: ovomucoid, Kunitz (pancreatic), and soybean.

The enzyme inhibitor dissociation constants $K_i$ (app) were determined for DFP and TLCK according to the method of Lineweaver and Burk (40). Initial velocities were measured at concentrations of Bz Arg OEt varying from 0.6 to 2 x 10⁻⁴ M.
L-arginine, n-arginine, pH 8, at three constant concentrations of Bz-Arg-ONAn: 5 x 10^{-5} M. Initial velocities were measured in Tris/HCl buffer; inhibition constants were measured according to the method of Dixon (41). Initial activity was recovered after dialysis. Therefore, the inhibition was competitive. The K_i values obtained were 1.8 x 10^{-5} M for TLCK and 2.3 x 10^{-2} M for DFP.

Benzamidine and p-aminobenzamidine which are known to be highly effective competitive inhibitors of trypsin also inhibited protease II. The inhibition was time-independent and initial activity was recovered after dialysis. Therefore, these two compounds are reversible inhibitors of the enzyme. Inhibition constants were measured according to the method of Dixon (41). Initial velocities were measured in Tris/HCl buffer, pH 8, at three constant concentrations of Bz-Arg-ONAn: 5 x 10^{-4} M, 5 x 10^{-4} M, and 2 x 10^{-4} M, with inhibitor concentrations ranging from 0.5 to 5 mM. Plots of 1/1 versus either benzamidine or p-aminobenzamidine concentrations intersected at I = -K_i and cut the vertical axis at different points, indicating the competitive nature of the inhibition.

The inhibitory effect of several amino acids (L-histidine, L-arginine, D-arginine, and L-lysine) was assayed on the initial rate of Bz-Arg-OEt hydrolysis at pH 8. Only L-arginine inhibited protease II activity. The inhibition was of the competitive type. Hydrolysis of ester derivatives of either lysine or arginine was inhibited to a similar extent. Unlike protease II, bovine trypsin was unaltered by preliminary incubation in L-arginine solutions.

All kinetic constants of protease II are listed in Table VI and compared with those of trypsin. From these data it can be deduced that TLCK is a more potent inhibitor of protease II than of trypsin. In contrast, K_i values of Bz-Arg-OEt and K_i values of amidines are approximately 100 times larger for protease II than for the pancreatic enzyme.

Table III

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Protease II</th>
<th>Trypsin</th>
<th>Molar activity ratio, protease II to trypsin</th>
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<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mole</td>
<td>mg</td>
</tr>
<tr>
<td>Bz-Arg-OEt</td>
<td>97.5 x 10^{-4} M</td>
<td>5655 M</td>
<td>47 x 10^{-4} M</td>
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<tr>
<td>Bz-Arg-ONAn</td>
<td>14.1 x 10^{-4} M</td>
<td>817.1 M</td>
<td>1.136 x 10^{-4} M</td>
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<tr>
<td>Azocasein</td>
<td>0.23 unit</td>
<td>13.94 x 10^{4} units</td>
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Table IV

Kinetic parameters of protease II in Tris/HCl buffer, pH 8, with or without calcium ion

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>K_i (app) x 10^{-8} M</th>
<th>V_{max} (amol/min/mg)</th>
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</thead>
<tbody>
<tr>
<td>Bz-Arg-OEt</td>
<td>4.76 x 10^{-8} M</td>
<td>148.3 x 10^3</td>
</tr>
<tr>
<td>Bz-Arg-ONAn</td>
<td>5.55 x 10^{-8} M</td>
<td>157.6 x 10^2</td>
</tr>
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</table>

*Kinetic constants determined without Ca^{2+} addition.

Table V

Effect of synthetic inhibitors

Each compound was added to samples of protease II diluted in 0.1 M Tris/HCl buffer, pH 8. The mixture, after standing at 25°C for 1 hour, was assayed for the esterase activity by the standard method. TPCK, L-1-tosylamido-2-phenylethylchloromethyl ketone; PMSF, phenylmethylsulfonyl fluoride.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Relative activity</th>
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<tr>
<td>TPCK</td>
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<tr>
<td>PMSF</td>
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<td>TLCK</td>
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<tr>
<td>DFP</td>
<td>54</td>
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Table VI

Comparison of apparent kinetic constants for Escherichia coli protease II and bovine trypsin

<table>
<thead>
<tr>
<th>Inhibitor or substrate</th>
<th>Protease II</th>
<th>Trypsin</th>
<th>References</th>
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<tbody>
<tr>
<td>TLCK</td>
<td>1.8 x 10^{-4} M</td>
<td>2.1 x 10^{-4} M</td>
<td>42</td>
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<tr>
<td>DFP</td>
<td>2.7 x 10^{-4} M</td>
<td>3.7 x 10^{-4} M</td>
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<tr>
<td>p-Aminobenzamidine</td>
<td>8 x 10^{-4} M</td>
<td>8.2 x 10^{-4} M</td>
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<tr>
<td>Benzamidine</td>
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<tr>
<td>L-Arginine</td>
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<tr>
<td>Bz-Arg-ONAn</td>
<td>5 x 10^{-4} M</td>
<td>6.5 x 10^{-4} M</td>
<td>(Measured)</td>
</tr>
<tr>
<td>Bz-Arg-OEt</td>
<td>4.7 x 10^{-4} M</td>
<td>5 x 10^{-4} M</td>
<td>44</td>
</tr>
</tbody>
</table>

*Kinetic data for protease II have been determined under experimental conditions similar to those used for trypsin, except for the systematic absence of Ca^{2+}.

Assays for Proteolytic Activity

As protease II action upon denatured casein was less extensive than on azocasein, the proteolytic activity was ascertained against three purified proteins from E. coli: \( \beta \)-galactosidase, aspartokinase I-homoserine dehydrogenase I, and aspartokinase III. Aspartokinase I is involved in the regulation of biosynthesis of threonine and is inhibited by threonine (45); aspartokinase III is implicated in the regulation of the lysine biosynthetic pathway, and is under allosteric control by lysine (29). At low ionic strength and in the absence of their allosteric ligands, aspartokinase I and III appear to be in a dissociated state (16, 25).

\( \beta \)-Galactosidase did not appear altered by the endopeptidase. The electrophoretic pattern and activity of the native enzyme were not modified by incubation with 2% of protease II (w/w) at 28°C for 48 hours. When purified preparations of aspartokinase III containing 0.1 to 1% of protease II (calculated from the esterase activity) were incubated at low ionic strength in Tris/glycine buffer, pH 8.6, kinase activity disappeared completely within 24 hours (16). Conversion of the native

Plots of 1/V against 1/S intersected on the 1/V axis, indicating that the inhibition was competitive. The K_i (app) values obtained were 1.8 x 10^{-5} M for TLCK and 2.3 x 10^{-2} M for DFP.

Calculations for molar specific activities were based on molecular weight of 23,800 for trypsin and 58,000 for protease II. All assays were done in Tris/HCl buffer, pH 8. Amidase and esterase activities, determined without calcium, are given as moles of substrate hydrolyzed per min at 55°C. For azocasein hydrolysis, 1 unit is arbitrarily defined as an absorbance change of the optical density of 1 unit at 420 nm/hour/ml, at 37°C.
The enzyme purified by the procedure described here appears homogeneous by gel filtration and electrophoresis. Further purity tests have been limited by the small amount of enzyme that is presently available.

The apparent molecular weight is estimated to be about 58,000 by sucrose gradient centrifugation and electrophoresis in sodium dodecyl sulfate acrylamide gels. However several values are obtained from Sephadex G-100 filtration. Gel filtration data suggest that the $M_r = 58,000$ species, in dilute salt solutions at pH 8, exists in equilibrium with another species, presumably a dimer with a molecular weight near 110,000.

E. coli protease II is neither activated by 2-mercaptoethanol nor inhibited by sulphydryl reagents. Although chelating agents are without effect, calcium ion increases amidase and proteolytic activities, but not esterase activity; it also decreases the stability of the enzyme. This ion stimulates the activity of trypsin and causes a conformational change of the molecule (48), which becomes more compact and less susceptible to autolysis. Our observations suggest that protease II is structurally less rigid in the presence of Ca$^{2+}$ and presumably more sensitive to autolysis. Amino acid ester and amide substrates bind to trypsin in a different fashion (49), but as the catalytic mechanism of their hydrolysis by protease II is still unknown, the discrepant effect of calcium of each of them remains unexplained.

The enzymatic activity is unaltered by natural trypsin inhibitors from soybean, egg white, and pancreas. Identical observations have been reported for thrombin (50), a blood-
clotting enzyme. Nevertheless both enzymes have a specificity similar to that of trypsin, as cleavage of the simple substrates occurs at the carbonyl bonds of arginine and lysine residues. Proteolytic enzymes generally show a narrower specificity toward natural inhibitors, than upon the low molecular weight inhibitors or substrates. The specificity of protease II towards polypeptide chains is under investigation.

The esterase activity is irreversibly lost in the presence of an excess of DFP, although it is unaffected by phenylmethylsulfonyl fluoride. The same results were obtained with protease I (15). Phenylmethylsulfonyl fluoride is a larger molecule than DFP, and as with acetylcholinesterase (51), the essential serine residue reacting with DFP may be supposed unavailable to phenylmethylsulfonyl fluoride. Thus protease II belongs to the group of bacterial serine proteases. As with pancreatic trypsin (52) the inhibition of protease II by TLCK, and the large increase of activity between pH 6 and 7, strongly indicate that a histidine residue is involved in the catalytic site, beside the serine residue. Benzamidine and p-aminobenzamidine competitively inhibit the amidase activity with inhibition constants about 100 times higher than for trypsin. It has been demonstrated that amidines competitively inhibit trypsin by steric hindrance of substrate accessibility to the hydrophobic specificity pocket (43) and also to the active site itself (53). Thus the two proteases would appear to have a certain degree of structural homology in the area of the active site.

While protease I does not hydrolyse heat-denatured casein as measured by Kunitz's method, protease II is weakly active on this substrate. β-Galactosidase is not attacked by prolonged incubation with 2% of protease II (w/w). Surprisingly, assays of proteolysis upon aspartokinase I-homoserine dehydrogenase I and aspartokinase III show that the two enzymes are cleaved in an effective way in the absence of their allosteric effectors with a protease to enzyme ratio equal to 0.1%/w/w. The digestion of aspartokinase I appears to result in a consistently limited proteolysis, because the $M_{r} = 33,000$ polypeptide is degraded by trypsin, but not by protease II. The present studies confirm the earlier suggestion (15) that two types of esterase activities are the manifestation of two individual endopeptidases (I and II). Besides the hydrolysis of natural and synthetic substrates, protease II differs from protease I in regard to its molecular weight, isolectric point, stability, sensitivity to trypsin inhibitors, and cellular localization. However, they can be both included in the neutral protease group and are not excreted in the culture medium by exponentially grown or starved cells. Other trypsin-like enzymes have been isolated from several bacterial species, but all were extracellular.

In a previous report on the study of the effects of protease inhibitors in E. coli, Rossman et al. (54) have described some properties of a trypsin-like enzyme probably similar to protease II. Prouty and Goldberg have found that protein degradation is decreased by trypsin inhibitors only in starving cells (14). From this observation, the authors have proposed the existence of separate proteases for different growth states. However, electropherograms of the crude extract from cells in exponential growth or starvation reveal two similar active bands for protease I and II, respectively. By contrast, protease II activity increases in glucose-starved cells. Such results show that the proteolytic enzymes present in starved cells are present in exponential growth, but are subjected to different mechanisms of regulation.

In spite of the very low proteolytic activity of protease II in vitro, enhancement of this enzyme activity in glucose-starved cells suggests that it could be implicated in "general protein turnover." Preliminary results (not shown) indicate that E. coli also contains an arginine and lysine aminopeptidase activity, evidenced by the fact that, using cell-free extracts, the two exopeptidase substrates: L-arginine-2-naphthylamide and L-lysine-2-naphthylamide are cleaved to a similar extent. By comparison of hydrolytic activities of crude extracts upon L-arginine-2-naphthylamide, and upon the endopeptidase substrate L-phenylalanine-L-arginine-2-naphthylamide (measured by the amount of 2-naphthylamide liberated) it was concluded that exopeptidase activity is 5-fold larger than the endopeptidase activity (protease II). This aminopeptidase is of interest because it could possibly play an important role in the breakdown of proteins sensitive to protease II in vivo. Moreover exopeptidases may attack the peptides produced by nonsense mutants (55, 56). Among the peptidases present in E. coli two have been well characterized, one cleaves NH$_2$-terminal proline residues (57), while the other is specific for β-aspartyl peptides (58). According to Sussman and Gilvarg, the peptidases split oligopeptides released by protease cleavage and the regulation of proteolysis occurs primarily at the level of protease activity (59). Work is in progress to elucidate the mechanism of regulation of both proteases I and II. The presence of cytoplasmic peptidases raises the problem of how the preferential proteolysis is achieved.

In vitro degradation of aspartokinases I and III by protease II only occurs in the absence of their respective effectors, suggesting that the two allosteric enzymes must first dissociate to become susceptible to proteolytic attack. The dissociation-mediated proteolysis has already been well documented for aspartokinase I (46). It is known that binding of substrate and effector can also stabilize other enzymes against proteolytic attack, both in vivo and in vitro. Tryptophan pyrrolase of liver is protected by tryptophan (60), while pyridoxal enzymes are only degraded in the presence of pyridoxal phosphate (61). Structural features susceptible to determine the rate of protein cleavage in vitro have been minutely investigated by Naslin et al. (62). These authors have found that yeast cytochrome b$_5$ is 10 times more sensitive to trypsin in the dissociated state than the native tetrameric enzyme. A wide variety of oligomeric enzymes are able to associate and dissociate in the presence of ligand. Some of them, which have been listed by Levitski and Koshland (63), exhibit such behavior in the range of the physiological concentration of enzyme and of substrate; aspartokinase I (30) and aspartokinase III (64) can be placed in this category. In addition a proteolyzed form of aspartokinase III has been detected from E. coli cells in stationary phase (65). Plausibly the effector-induced change in structure of polymeric enzymes (whether it does or does not lead to dissociation) could play an important role in controlling the rate of degradation of individual proteins in vivo, even in bacterial cells. This hypothesis is indirectly strengthened by the observation that proteins usually stable during exponential growth, become very susceptible to in vivo proteolysis when their structure is altered by some mutations (8, 9).

The present findings lead to the hypothesis that the enhancement of protein degradation in starved bacteria might be due to both an increase of protease activity and to effector-induced changes in protein conformation which result in a greater susceptibility to enzymatic digestion.

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