Purification of a Protease from *Escherichia coli* with Specificity for Oxidized Glutamine Synthetase*

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A soluble *Escherichia coli* protease has been identified and purified to homogeneity. The protease cleaves glutamine synthetase which has been modified by mixed function oxidation; native glutamine synthetase is not a substrate. Using [14C]glutamine synthetase as a substrate (prepared by growing *E. coli* on 14C-labeled amino acids), protease activity was assayed by determining the release of trichloroacetic acid-soluble material. The pure protease cleaves glutamine synthetase near the carboxyl terminal yielding 4,500 and 47,000 M<sub>r</sub> products. The characteristics of this enzyme distinguish it from proteases previously purified from *E. coli*. These characteristics include a molecular weight of 75,000, alkaline pH optimum, lack of inhibition by serine protease inhibitors, and the ability to degrade insulin and casein.

Oxidation of glutamine synthetase and other enzymes can be catalyzed by a variety of mixed function oxidase systems from bacterial and mammalian sources. Mixed function oxidation may be a "signal" or "marker" which consigns a protein for proteolytic degradation. Susceptibility to oxidation is subject to metabolic regulation, thereby providing control of proteolytic turnover. Isolation of a protease specific for modified glutamine synthetase provides the enzymatic basis for the specificity of this scheme.

The turnover of intracellular proteins has been recognized and extensively studied (Schoenheimer, 1942; Goldberg and St. John, 1976; Heräsko and Giechansky, 1982; Pine, 1972). Degradation has been shown to be selective and responsive to the nutritional state of the cell (Maurizi, 1980; Pine, 1972), demonstrating that the process is metabolically regulated. A number of proteases and peptidases have been purified from bacteria (Goldberg et al., 1981). These enzymes have been isolated using radiochemically labeled proteins or synthetic di- or tripeptides as substrates. However, with the exception of the cleavage of the λ repressor by the RecA protease (Roberta et al., 1978), the cellular functions of the proteases are not known. Their role in protein turnover, including the identification of their substrates and how their action is regulated, remains to be demonstrated.

Glutamine synthetase (1-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2) is a key enzyme in nitrogen metabolism. In *Escherichia coli* the enzyme has been shown to be regulated by cumulative feedback inhibition, covalent modification (adenylation), and repression and derepression of its synthesis (Stadtman and Ginsburg, 1974). While normally stable, it is one of the enzymes turned over when cells are starved for nitrogen (Fulks and Stadtman, 1986; Maurizi, 1980), suggesting its intracellular level is also regulated by proteolysis.

Previous studies (Levine et al., 1981) suggested that the turnover of glutamine synthetase occurred in two steps. In the first step, glutamine synthetase is oxidized, resulting in inactivation, loss of at least one histidine, and the generation of one or more carbonyl groups. In the second step, oxidized glutamine synthetase is degraded. It was proposed that the two-step process provided the means to regulate glutamine synthetase degradation with control provided by the modification reaction and specificity by one or more proteases which recognize the modified form. We used a model-inactivating system composed of ascorbate, iron, and oxygen to covalently modify the glutamine synthetase and then looked for a protease that degrades the modified form. We describe here the purification and characterization of a protease from *E. coli* which degrades oxidized but not native glutamine synthetase.

**EXPERIMENTAL PROCEDURES**

**Materials**

Uniformly labeled 14C-L-amino-acid mixture, specific activity 1.84 Ci/mg, and 131I-insulin, specific activity 88.4 mCi/mg, were purchased from ICN Radiochemicals; [14C]methy1 homoglutamic, specific activity 0.23 mCi/mg, and [14C]methy1-α-casein, specific activity 0.82 μCi/mg, were obtained from New England Nuclear; reductively methylated [14H]bovine serum albumin (BSA), specific activity 2.6 μCi/mg, was a gift from Dr. Kelvin Davies, University of Southern California, Los Angeles, CA. DEAE-cellulose DE52 and CM-cellulose CM52 were purchased from Whatman; ammonium sulfate from Schwarz-Mann; phenyl-Sepharose CL-4B and Sephadex G-25M were obtained from Pharmacia; HPLC Bio-Gel Phenyl-5-PW, HPLC Bio-Gel TSK DEAE-6-PW, and Bio-Gel P10 from Bio-Rad; Sigma supplied MES, MOPS, HEPES, CHES, and TAPS buffers, gel filtration standards, porcine trypsin (10,000-13,000 BAE units/mg), synthetic substrates, and all protease inhibitors except aprotinin and leupeptin, which were purchased from Boehringer Mannheim; C<sub>10</sub> Vydac 218TP54 was purchased from Sep/a/ra/tions Group; trifluoroacetic acid from Pierce Chemical Co.; acetoni trile from Burdick and Jackson; Dye-Matrex<sup>TM</sup> gels were prepared by Amicon. Colloidon bag dialysis sacs were from Schleicher and Schuell, and SDS gradient gels were from Sepragel. All other chemicals were of laboratory reagent grade.

Deionized water was obtained by passing distilled water through a water filtration system supplied by HydroService and Supplies, Inc. For use on the HPLC, this water was also passed through a Millex<sup>QM</sup> System (Millipore).

1 The abbreviations used are: BSA, bovine serum albumin; MES, 2-(N-morpholino)ethanesulfonic acid; MOPS, 3-(N-morpholino)-propanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHES, 2-(N-cyclohexylamino)ethanesulfonic acid; TAPS, 3-[tris(Hydroxymethyl)methyl]aminopropanesulfonic acid; SDS, sodium dodecyl sulfate; BAEE, N<sub>3</sub> benzoyl-L-arginine ethyl ester; HPLC, high-performance liquid chromatography.
Buffers Employed

During purification and characterization of the protease the following buffers were used: Buffer A, 25 mM imidazole, 1 mM MnCl₂, 75 mM 3-ketoglutarate, pH 7.2; Buffer B, 50 mM Tris-HCl, pH 7.5; Buffer C, 100 mM sodium phosphate, pH 7.0; Buffer D, 100 mM sodium phosphate, 1 mM ammonium sulfate, pH 7.0; Buffer E, 100 mM sodium phosphate, 0.75 mM ammonium sulfate, pH 7.0; Buffer F, 20 mM Tris-HCl, pH 9.0; Buffer G, 50 mM HEPES, 100 mM KCl, 10 mM MnCl₂, pH 7.2; Buffer H, 50 mM Tris, 100 mM NaCl, pH 7.5; Buffer I, 100 mM sodium phosphate, 8 mM urea, pH 7.0; Buffer J, 10 mM imidazole, 100 mM KCl, 1 mM MnCl₂, pH 7.2. The pH of each buffer was adjusted at room temperature by addition of either HCl or NaOH.

Glutamine Synthetase Assays

Glutamine synthetase specific activity was determined at pH 7.57 by the γ-glutamyl transferase assay, described by Stadtman et al. (1979). The state of adenylylation was determined spectrophotometrically (Ginsburg et al., 1979) and by the γ-glutamyl transferase assay at pH 7.18 and 7.57 in the presence and absence of ADP (Stadtman et al., 1979).

Preparation of 14C-Labeled Glutamine Synthetase

Unadenylated glutamine synthetase was purified from a strain of E. coli K12 (Pgln6/YMC10) which has a plasmid containing the glutamine synthetase structural gene (Bachman et al., 1981). Details of the procedure first summarized by Roseman and Levine (1983) and also successfully applied to the purification of mammalian proteins (Woolfolk et al., 1966) are described here. Cells were grown on 29 mM CaCl₂, 62 μM FeCl₃, 0.63 μM ZnSO₄, 0.64 μM CuSO₄, 0.76 μM CoCl₂, and 53.7 μM Na₂EDTA, supplemented with 0.5% (w/v) glucose, 4 mM α-ketoglutarate, pH 7.2; Buffer B, 50 mM Tris-HCl, pH 7.5; and 10 mM HEPES buffer, pH 8.0.

Glutamine synthetase specific activity varied from 136 to 140 units/mg, electrophoresis under conditions where 1% contamination would have diminished the purity of glutamine synthetase. After precipitation with zinc, the glutamine synthetase was usually less than 1.0, as is the ratio of glutamine synthetase activity to acid-soluble radioactivity from the [14C]glutamine synthetase. The rate of degradation of four batches of the 8-h oxidized substrate varied by only 20%.

Other Modifications of Glutamine Synthetase

Each modified form of glutamine synthetase was prepared from the 14C-labeled unadenylated enzyme as described below. Except for adenylation, the modification reactions were dialyzed into a collodion sac (75,000 cutoff) containing the radioactive glutamine synthetase. After modification, the glutamine synthetase was dialyzed against Buffer G.

Acetylated Glutamine Synthetase

Acetylation with N-acetyl imidazole was carried out according to the procedure of Cimino et al. (1970). The number of tyrosine residues modified, determined by the decrease in absorbance at 278 nm, was 0.5 residues of 17.

Adenylylated Glutamine Synthetase

Glutamine synthetase was adenylylated to an average state of 11.1 with adenylyltransferase by Dr. T. Martensen of this laboratory (Anderson et al., 1979).

Nitrated Glutamine Synthetase

Nitration with tetranitromethane was carried out according to the procedure of Cimino et al. (1970). The number of tyrosine residues modified, determined from the absorbance at 428 nm (Sokolovsky et al., 1966), was 2 of 17 alanin.

Reduction of Glutamine Synthetase with Sodium Borohydride

Reduction with sodium borohydride was accomplished by a procedure modified from that of Rilev and Snell (1968). Either native or oxidized glutamine synthetase was dialyzed against Buffer G at pH 8.0 in a collodion sac (75,000 cutoff). The solution was warmed to 37°C and then brought to 20 mM sodium borohydride by addition of freshly prepared reagent (1 M NaBH₄ in 0.1 N sodium hydroxide). The solution was incubated at 37°C for 30 min and then dialyzed against Buffer G to remove reagents.

Urea Denaturation

Glutamine synthetase was reductively denatured with urea and dithiothreitol and then carboxymethylated with iodoacetic acid (Martensen and Stadtman, 1982). Reagents were removed by dialysis against 10 mM HEPES buffer, pH 8.0.

Protease Assays

Proteolysis was measured by determining release of trichloroacetic acid-soluble radioactivity from the [14C]glutamine synthetase. The reactions were conducted in 0.1-ml volumes (in capped Eppendorf tubes) which contained 50 mM HEPES buffer, 100 mM KCl, 1 mM MnCl₂, pH 7.5, and 4–5 μg of labeled substrate (about 100,000 cpm). Tri replaced HEPES in assays performed at pH 9.0. After incubation at 37°C for 1 h (unless stated otherwise), the reaction was stopped by addition of 0.5 ml of 10% trichloroacetic acid followed by 0.1 ml of bovine serum albumin (5 mg/ml in 0.1 N HCl), and the mixtures were agitation vigorously on a Vortex mixer. After 15 min at 0°C, the reaction mixtures were centrifuged at 10,000 × g for 5 min in a Microfuge. Then, 0.5 ml of supernatant was mixed with 16 ml of Agassol (New England Nuclear) and counted in a Beckman model LS250 liquid scintillation counter. With each set of assays, blanks were included in which the protease was replaced by an equal volume of Buffer H. A unit is defined as the amount of activity which liberates 1 μg of trichloroacetic acid-soluble radioactivity/h.

Proteolysis of [14C]methyl hemoglobin, [14C]methyl-r-casem, and [125I]insulin was measured in similar assays using 4–5 μg (70,000 cpm), 12 μg (10,000 cpm), and 5 μg (90,000 cpm) of each substrate, respectively.

Proteolysis of [3H]bovine serum albumin was measured using 30 μg (400,000 cpm) of substrate. Assays were also carried out in 50 mM Tris buffer, 10 mM MnCl₂, pH 8.1, for comparison with other previously identified proteases (Goldberg et al., 1981). In experiments with radioactive substrates, 0.6 μg of trypsin (6–8 BAEE units) was used. Trypsin activity was measured by hydrolysis of azocasein (Bergmeyer, 1974).
Protein Determination

Protein concentration was determined by the procedure of Bradford (1976) using BSA as a standard. Protein concentration of the protease from purification Step 8 was determined from the integrated absorbance at 210 nm calibrated to BSA. Glutamine synthetase concentration was determined spectrophotometrically (Ginsburg et al., 1970).

Predicted Protein Structure

The rules of Chou and Fasman (1978) were followed to predict secondary structure using the program of Lowe (1986). Hydrophobic plots were constructed according to the method of Laemmli (1970).

SDS-gel electrophoresis in 10% or 20%–20% gradient slab gels was performed according to the method of Laemmli (1970).

High-performance Liquid Chromatography

High-performance liquid chromatography (HPLC) was performed on an IBM LC/9533 ternary gradient liquid chromatograph connected to a Hewlett-Packard 1040A detection system and controlled by an IBM 9000 computer. Chromatograms were taken at 210 nm with a 20-nm bandwidth. The reference wavelength was 550 nm with a 100-nm bandwidth.

Estimation of Molecular Weights

The subunit molecular weight of the protease was estimated by linear regression analysis (method of least squares) from data obtained by SDS-PAGE. Glutamine synthetase was estimated using phosphorhydrolase (92,500), ovalbumin (44,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysosome (14,000) as molecular weight markers.

The molecular weight of the native protease was estimated by linear regression analysis of data obtained by gel filtration. Sephadex G-150 gel filtration was performed on a column (1 × 30 cm) eluted with Buffer F at a flow rate of 10 ml/h. Aldolase (158,000), serum albumin (67,000), ovalbumin (44,000), and cytochrome c (12,500) were used as molecular weight markers. HPLC Bio-Sil gel filtration was performed on a column (7.5 × 500 mm) eluted with Buffer C at a flow rate of 0.5 ml/min using thyroglobulin (670,000), IgG (158,000), ovalbumin (44,000), myoglobin (17,000), and B12 (1,350) as molecular weight markers.

Molecular weights of proteolytic products were estimated by Bio-Gel P10 gel filtration on a column (9.7 × 28 cm) eluted with Buffer I at 1 ml/h using cytochrome c (12,400), protamine (6,500), insulin B chain (3,450), insulin A chain (2,530), and oxidized glutathione (600) as molecular weight markers.

Analysis of Proteolytic Products

The products of glutamine synthetase or insulin B chain digestion were isolated using HPLC reverse phase chromatography. Insulin B chain, 0.25 mg/ml, was incubated with protease under the same conditions as the glutamine synthetase incubation. Reaction mixtures (0.1–0.25 ml) were acidified by addition of an equal volume of 1% trifluoroacetic acid. The resulting solution, at pH 2.3, was applied to a Vydac 218TP54 column (0.46 × 25 cm) equilibrated in an aqueous solution of 0.05% trifluoroacetic acid at 30 °C. The products were eluted with a linear gradient of 0–70% acetonitrile (2% per min) containing 0.05% trifluoroacetic acid at a flow rate of 1 ml/min. Products were detected by absorbance at 210 nm. Ultraviolet spectra of the product peaks were measured during their elution using the HP1040A on-line detector, and peaks were collected for determination of their molecular weight and amino acid composition. In experiments which used radioactive substrates, fractions (0.5 ml) were collected and counted. Amino acid analysis of the products was performed using the o-phthalaldehyde precolumn derivatization method of Jones and Gilligan (1983). The column was a 15-cm C-18 from IBM Instruments, Inc.

Protease Purification

E. coli K12 cells were grown in a 300-liter minimal medium containing 0.5% glucose (w/v) and 4 mM L-glutamine. Cells (1300 g) were harvested in stationary phase, frozen in liquid nitrogen, and stored at −20 °C. Ammonium sulfate is referred to concentration saturation at 0 °C (69.7 g/100 ml). The first three steps in the purification (performed at 4 °C unless stated) were modified from the glutamine synthetase purification procedure of Miller et al. (1974).

Step 1. Homogenization—Three hundred grams of frozen cells suspended in 600 ml of Buffer A were disrupted by passage twice through a French pressure cell at 10,000 psi, and cell debris was removed by centrifugation at 17,000 × g for 90 min.

Step 2. Streptomyein Sulfate Fraction—Nucleic acids were removed by streptomyein sulfate precipitation. After adjusting the pH to 5.85 by slow addition of 1 M acetic acid, 1 g of streptomyein sulfate was added to each 100 ml of crude extract, and the mixture was stirred for 15 min at room temperature. The pH was then readjusted to 5.85 with 1 M NaOH, and the suspension was centrifuged at 17,000 × g for 15 min. The pellet was discarded.

Step 3. Zinc/Magnesium Precipitation—This step removes the endogenous unlabeled glutamine synthetase which might decrease the sensitivity of the radioactive assay. The supernatant was warmed to 25 °C and brought to 50 mM MgCl₂, 1.5 mM ZnSO₄ by addition of 1 M MgCl₂ and 1 M ZnSO₄. After readjusting the pH to 5.85 with 1 M acetic acid, the mixture was stirred for 30 min at room temperature and then centrifuged at 17,000 × g for 60 min. The pellet was discarded.

Step 4. Ammonium Sulfate Fractionation—Solid ammonium sulfate was slowly added to the zinc/magnesium supernatant to 50% saturation, and the mixture was allowed to stir for 60 min. After centrifugation at 10,800 × g for 20 min, the pellet was discarded, and ammonium sulfate was added to the supernatant to 70% saturation. The mixture was allowed to stir an additional 1 h and then centrifuged as before. The supernatant was discarded and the pellet was dissolved in about 50 ml of Buffer B and dialyzed for 16 h against 3 liter volumes of Buffer B. Insoluble proteins were removed by centrifugation at 20,000 × g for 10 min.

Step 5. DEAE-cellulose Chromatography—The fraction obtained in the previous step was subjected to ion-exchange chromatography on a DE52 column (5 × 15 cm) previously equilibrated with Buffer B. The protease activity did not bind to the column in this buffer.

The column was washed with Buffer B at a flow rate of 300 ml/h, and the nonbinding fractions were assayed for protein and protease activity. The active fractions were pooled and adjusted to 100 mM sodium phosphate, 1 M ammonium sulfate, pH 7.0, by addition of 300 mM sodium phosphate, 3 M ammonium sulfate, pH 7.0. The pH was adjusted to 7.9 with 1 M phosphoric acid.

Step 6. Phenyl-Sepharose Hydrophobic Chromatography—This step concentrates the protease preparation and removes a contaminating material that interferes with subsequent HPLC steps. The active fractions from the last step were applied to the phenyl-Sepharose column (4 × 8 cm) previously equilibrated in Buffer D. The column was washed with Buffer D until no protein was detected in the eluent (A₂₈₀ < 0.01). Protease activity was eluted with Buffer C. The active fractions were pooled and concentrated by adding solid ammonium sulfate to 90% saturation and stored overnight at 4 °C. The suspension was centrifuged at 12,000 × g for 20 min.

Step 7. HPLC Bio-Gel Phenyl Hydrophobic Chromatography—This step was performed at 20 °C. The pellet from Step 6 was dissolved in Buffer D at a final protein concentration of 5 mg/ml, filtered through a 0.2-μm Millex GV filter, and applied via a 5-ml injection loop to a Bio-Gel phenyl column (7.5 × 75 mm) equilibrated with Buffer D. After washing the column with 1 ml of Buffer D followed by 0.1 ml of Buffer E, the protease activity was eluted with a linear "reversed" gradient of 750–0 mM ammonium sulfate in Buffer C (50 mM/min). The flow rate was 1 ml/min, and each fraction contained 1 ml. Fractions 29–33 were pooled and buffer exchanged for Buffer F by subjecting 2.5-ml aliquots to gel filtration on Sephadex G-25 columns (PD 10).

Step 8. HPLC Bio-Gel DEAE Ion-exchange Chromatography—The protease preparation from the last step was filtered through a 0.2-μm Millex GV filter and applied via a 5-ml injection loop to a Bio-Gel DEAE column (7.5 × 75 mm) which had been equilibrated in Buffer F. After washing the column with 6 ml of Buffer F, the protease was eluted with a linear gradient of 0–108 mM NaCl in Buffer F (50 mM/min), and the column was then washed with 1 ml NaCl. The flow rate was 1 ml/min, and each fraction contained 1 ml. Fractions 26–27 were pooled and dialyzed against 50 mM HEPES, 100 mM KCl, pH 7.5, and stored at 4 °C.
RESULTS

When crude E. coli extracts were examined for proteolytic activity against native glutamine synthetase, no degradation was detected. In contrast, crude extracts degraded oxidized glutamine synthetase at an initial rate of 4-6 units/mg of extract protein. This result suggested that one or more proteases specifically recognized oxidized glutamine synthetase, and purification of this protease activity was undertaken.

The purification results are summarized in Table I. Starting with a crude extract containing 9.7 g of soluble protein, 5 µg of purified protease was obtained, representing 0.2% of the initial activity. It is estimated that the protease has been purified by a factor of 3,300 with the assumptions that no inhibitors or activators of the protease are present in the enzyme preparations throughout the purification and that no other proteases contribute to the measured activity. If the protease activity measured in crude extracts does reflect the action of other proteases, then the values reported for recovery and -fold purification are underestimated. Fig. 1 shows the pattern obtained when samples from each purification step are subjected to SDS-polyacrylamide gel electrophoresis. One unit of protease was applied to each lane. Lane 7 shows the purified protein following the final DEAE step. This band appears less intense in cruder preparations (lanes 1-6) suggesting these preparations contained other proteases or activators of this protease. Some preparations contained an additional band (Mr, 94,000) whose presence did not correlate with protease activity. Only homogenous preparations were used in the characterization studies reported here.

Specificity

The purified protease is specific for oxidized compared with native glutamine synthetase (Fig. 2). The initial rate of degradation of the oxidized is over 50-fold greater than that of the native enzyme.

Molecular Weight

HPLC gel filtration indicated that the molecular weight of the native protease is 75,000. Similar results were obtained using Sephadex G-150. Electrophoresis in 10% SDS-polyacrylamide gels also gave a value of 75,000 (Fig. 1); thus, the enzyme is monomeric.

Stability

The activity of the protease is stable for 3 months at 4 °C in 50 mM HEPES, 100 mM KCl, pH 7.5, at protein concentrations of about 10-20 µg/ml. The protease can also be stored as an 80% ammonium sulfate slurry with no detectable loss of activity for at least 2 months. The protease loses at least 50% of its activity upon freezing at -20 °C, -70 °C, or in liquid nitrogen. No degradation of the purified protease was detected by SDS-polyacrylamide gel electrophoresis following its incubation at 37 °C for 4 h.

pH Dependence

The pH optimum for degradation of oxidized glutamine synthetase is 8.5-9.0 (Fig. 3). Proteolysis is diminished in CHES compared with Tris buffers at comparable pH values.

Effect of Protease Inhibitors and Metal Ions on Protease Activity

Inhibitors—The effect of various inhibitors and group specific reagents is shown in Table II. Of the serine protease inhibitors tested, only aprotinin exhibits significant inhibition (46%) under conditions which fully inhibit trypsin. The bacterial protease is partially inhibited by the metal chelators EDTA and o-phenanthroline. The thiol reagent ethyleneimine gives 85% inhibition, and this is due to inhibition of the protease rather than to modification of the substrate. This was determined by separately incubating both glutamine synthetase and protease with ethyleneimine, followed by dialysis. The treated glutamine synthetase could be degraded by protease, but the treated protease was no longer capable of degrading glutamine synthetase.

Cations—The effects of divalent cations are given in Table III. Inhibition of activity was seen upon incubation with Cu²⁺, Fe²⁺, and Fe³⁺. Protease activity in the presence of Ba²⁺, Ca²⁺, Mg²⁺, Mn²⁺, and Zn²⁺ was reduced to a lesser extent. Cd²⁺ and Co²⁺ increased protease activity slightly.

Effect of Substrates and Products of Glutamine Synthetase on Proteolysis

Substrates protect native unadenylylated glutamine synthetase from oxidative modification, providing a mechanism for regulation of the reaction (Levine et al., 1981; Levine, 1983b). It was of interest to examine their effect on the proteolysis of oxidized glutamine synthetase. Data presented in Table IV show that the substrates ATP and glutamate and the products ADP and glutamine only slightly protect oxidized glutamine synthetase from degradation.

Degradation of Other Proteins and Synthetic Substrates

As shown in Table V, the protease degrades both casein and insulin to trichloroacetic acid-soluble products, but neither hemoglobin nor BSA. It degrades insulin more rapidly than does trypsin.

A partially purified preparation (Step 5), presumably still containing other proteases, was incubated with synthetic substrates of the serine proteases trypsin, chymotrypsin, and elastase. During a 16-h incubation, 22% of the oxidized [¹⁴C] glutamine synthetase was degraded to trichloroacetic acid-soluble products, but no degradation was seen of the following

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total units</th>
<th>Total mg</th>
<th>Specific activity</th>
<th>Yield %</th>
<th>Purification -fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>40,600</td>
<td>9,760</td>
<td>4.2</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Streptomycin sulfate</td>
<td>24,400</td>
<td>7,300</td>
<td>3.3</td>
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<td>0.8</td>
</tr>
<tr>
<td>Zinc/magnesium supernatant</td>
<td>39,400</td>
<td>6,200</td>
<td>4.7</td>
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<td>1.1</td>
</tr>
<tr>
<td>Ammonium sulfate fraction</td>
<td>24,600</td>
<td>1,770</td>
<td>14</td>
<td>61</td>
<td>3.3</td>
</tr>
<tr>
<td>DEAE-cellulose, pH 7.5</td>
<td>7,770</td>
<td>211</td>
<td>37</td>
<td>19</td>
<td>8.8</td>
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<tr>
<td>Phenyl-Sepharose</td>
<td>1,300</td>
<td>135</td>
<td>10</td>
<td>32</td>
<td>2.4</td>
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<tr>
<td>HPLC phenyl</td>
<td>200</td>
<td>1.32</td>
<td>150</td>
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<td>36</td>
</tr>
<tr>
<td>HPLC DEAE</td>
<td>72</td>
<td>0.005</td>
<td>14,000</td>
<td>0.2</td>
<td>3,300</td>
</tr>
</tbody>
</table>

TABLE I
Purification summary

Samples from each stage of the purification were equilibrated in Buffer H and assayed for protease activity.

E. coli Protease
phenyl pool; were applied to magnesium supernatant; standards. The gel was stained with Coomassie Blue.

Lung protease and modification of the standard assay at pH glutamine synthetase in a total volume of glutamine synthetase. Protease activity was assayed by a minor aliquots were removed and assayed for trichloroacetic acid glutamine synthetase. Adenylylation modifies a single tyrosine on each synthetic substrates tested: N-benzoyl-L-prolyl-L-phenylalaninyl-L-arginine-p-nitroanilide; D-valyl-L-leucyl-L-lysine-p-nitroanilide; L-alanyl-L-alanyl-L-phenylalanine-p-nitroanilide; methoxy succinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-p-nitroanilide. In another experiment, the partially purified preparation did not degrade the collagenase substrates 4-phenylazonbenzoxy carbonyl-L-prolyl-L-leucyl-L-glycyl-L-prolyl-D-arginine nor diazotized collagen (hide powder azure) during a 24-h incubation.

Other Modifications of Glutamine Synthetase

Other modified forms of glutamine synthetase were examined for their susceptibility to proteolysis. The results are summarized in Table VI. Denaturation with urea followed by carboxymethylation produced changes which result in a degradation rate comparable to that of the oxidized form of the substrate. Adenylylation modifies a single tyrosine on each glutamine synthetase subunit with no loss of \( \gamma \)-glutamyl transferase activity (Shapiro et al., 1967). Adenylylation did not render the protein susceptible to the protease. Tyrosine residues other than that which is adenylylated are modified by nitration and acetylation. However, either of these treatments results in conversion of the enzyme to a form with catalytic characteristics similar to that of the adenylylated enzyme (Cimino et al., 1970). (The possible acetylation of lysine residues was not determined.) Acetylation, but not nitration, also allows attack by the protease. Whether acety-

**FIG. 1.** SDS-polyacrylamide gel electrophoresis of protease purification. Dialyzed samples containing 1 unit of protease activity were applied to lanes 1–7: Lane 1, crude extract; Lane 2, zinc/magnesium supernatant; Lane 3, 50–70% ammonium sulfate fraction; Lane 4, DEAE eluent; Lane 5, phenyl-Sepharose pool; Lane 6, HPLC phenyl pool; Lane 7, HPLC DEAE pool; Lane 8, molecular weight standards. The gel was stained with Coomassie Blue.

**FIG. 2.** Specificity of the protease for oxidatively modified glutamine synthetase. Protease activity was assayed by a minor modification of the standard assay at pH 9.0 described under "Experimental Procedures." The reaction mixtures contained 0.15 \( \mu \)g of protease and 60 \( \mu \)g of either oxidized or native \(^4\)C-labeled glutamine synthetase in a total volume of 600 \( \mu \)l. At each time point, 100-\( \mu \)l aliquots were removed and assayed for trichloroacetic acid (TCA)-soluble radioactivity. Results with native (O—O) and oxidized glutamine synthetase (●—●) represent the average of duplicate measurements.

**FIG. 3.** pH dependence for the degradation of oxidized glutamine synthetase. Protease activity was measured in the standard assay with 10 \( \mu \)g of glutamine synthetase, 5 ng of protease, and incubation for 30 min. Buffers were MES (+), HEPES (\( \Phi \)), Tris (\( \Delta \)), TAPS (\( \times \)), and CHES (\( \wedge \)). TCA, trichloroacetic acid.

**TABLE II**

*Effect of protease inhibitors*

The various compounds at the final concentrations indicated were incubated with 0.2 \( \mu \)g of pure protease for 30 min at 25 °C, prior to addition of substrate which was oxidized synthetase for the *E. coli* protease and casein for trypsin. Relative activities refer to controls with vehicle. This was buffer for all except diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride (1-propanol) and pepstatin (dimethyl sulfoxide).

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration</th>
<th>Relative activity</th>
<th>E. coli protease</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
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<td>100</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>0.15</td>
<td>54</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>0.015</td>
<td>89</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tosyl-L-lysine</td>
<td>2.7</td>
<td>120</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>chloromethyl ketone</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>0.047</td>
<td>113</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Disopropyl fluorophosphate</td>
<td>1.0</td>
<td>112</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Leupeptin</td>
<td>1.0</td>
<td>129</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride</td>
<td>1.0</td>
<td>105</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

Other Modifications of Glutamine Synthetase

Other modified forms of glutamine synthetase were examined for their susceptibility to proteolysis. The results are...
TABLE III

Effect of cations on protease activity

Cations were incubated at 50 μM final concentration with 0.2 μg of protease for 20 min at 25 °C, prior to addition of oxidized glutamine synthetase. The reaction was then incubated for 1 h at 37 °C.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Activity</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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</tr>
<tr>
<td>Ba²⁺</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>123</td>
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<tr>
<td>Co²⁺</td>
<td>110</td>
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<tr>
<td>Cu²⁺</td>
<td>15</td>
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<tr>
<td>Fe²⁺</td>
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<tr>
<td>Fe³⁺</td>
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<tr>
<td>Mg²⁺</td>
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</tr>
<tr>
<td>Mn²⁺</td>
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<tr>
<td>Ni²⁺</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>64</td>
<td></td>
</tr>
</tbody>
</table>

TABLE IV

Effect of substrates and products of the reaction catalyzed by glutamine synthetase on protease activity

The compounds were incubated with 4 μg of oxidized glutamine synthetase for 5 min at 25 °C prior to the addition of 0.2 μg of protease. The concentration of glutamate and glutamine was 30 mM, and the concentration of ATP, ADP, and P_i was 1.0 mM.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Glutamate</td>
<td>91</td>
</tr>
<tr>
<td>ATP</td>
<td>76</td>
</tr>
<tr>
<td>Glutamate + ATP</td>
<td>78</td>
</tr>
<tr>
<td>ADP</td>
<td>76</td>
</tr>
<tr>
<td>P_i</td>
<td>94</td>
</tr>
<tr>
<td>ADP + P_i</td>
<td>80</td>
</tr>
<tr>
<td>Glutamine</td>
<td>93</td>
</tr>
</tbody>
</table>

TABLE V

Degradation of hemoglobin, casein, bovine serum albumin, and insulin

Assays contained 0.2 μg of E. coli protease or 0.6 μg of trypsin (6–8 BAEE units) and either [14C]methyl hemoglobin (4–5 μg), [3H]methyl-a-casein (12 μg), 125I-insulin (5 μg), [3H]bovine serum albumin (35 μg), or [14C]-labeled glutamine synthetase (4 μg) in 50 mM HEPES, 100 mM KCl, 0.1 mM MgCl₂, pH 7.5. All reaction mixtures were incubated for 1 h at 37 °C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Trichloroacetic acid soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. coli protease</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>0</td>
</tr>
<tr>
<td>Casein</td>
<td>10</td>
</tr>
<tr>
<td>Insulin</td>
<td>19</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.2</td>
</tr>
<tr>
<td>Oxidized glutamine synthetase</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*ND, not determined.

TABLE VI

Degradation of modified forms of glutamine synthetase

Assays contained 4 μg of [14C]-labeled glutamine synthetase and 0.2 μg of protease in 100 mM HEPES, 100 mM KCl, 0.1 mM MgCl₂, pH 7.5. Reaction mixtures were incubated for 1 h at 37 °C.

<table>
<thead>
<tr>
<th>Form of glutamine synthetase substrate</th>
<th>Glutamine synthetase activity</th>
<th>Protease activity (% trichloroacetic acid soluble)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Adenylylated</td>
<td>96</td>
<td>0.1</td>
</tr>
<tr>
<td>Nitrated</td>
<td>62</td>
<td>0.4</td>
</tr>
<tr>
<td>Acetylated</td>
<td>53</td>
<td>2.4</td>
</tr>
<tr>
<td>Urea denatured</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>Oxidized</td>
<td>0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

TABLE VII

Effect of borohydride treatment of oxidized and native glutamine synthetase on proteolysis

Proteolysis was measured as trichloroacetic acid-soluble cpm as described under "Experimental Procedures." Approximately 0.2 μg of protease was used.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Trichloroacetic acid soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated</td>
</tr>
<tr>
<td>Oxidized glutamine synthetase</td>
<td>3.8</td>
</tr>
<tr>
<td>Native glutamine synthetase</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**FIG. 4.** Proteolysis of insulin B chain. Oxidized insulin B chain (250 μg/100 μl) was incubated with protease (0.1 μg/100 μl) in the standard reaction condition, pH 7.5. At 30 min, aliquots of 400 μl were removed for amino acid analysis of proteolytic products as described under "Experimental Procedures."

Analysis of Digestion Products

Products of Insulin B Chain—Fig. 4 shows the course of insulin B digestion by the pure protease. Degradation of the intact B chain generated six major peaks which were collected for amino acid analysis (Table VIII). A small peak at 12.02 min was not present in sufficient quantities for analysis. Comparison of the amino acid composition of the 6 peptides with the published B chain sequence permitted unequivocal identification of the three cleavage sites (Fig. 5). The fastest rate of cleavage was the Val-Glu bond at residues 12–13. The Tyr-Leu bond at residues 16–17 was attacked at a slightly slower rate. The valine-cysteic acid bond at positions 18–19 was cleaved at a much slower rate.

Products of Proteolysis of Oxidized Glutamine Synthetase—
The protease initially cleaves the oxidized glutamine synthetase at a single site to produce two products, readily separated by HPLC. One of these products (peak at 22.3 min, Fig. 6) has a molecular weight of 47,000 as determined by chromatography on Bio-Gel P-10 and 4,500 by amino acid analysis and sequence data (Table IX). The other product which results from this initial cleavage had a molecular weight of 3,600 as determined by chromatography on reverse phase HPLC. The major product at 21 min had M, of 3600 by gel filtration and 4500 from amino acid analysis.

The presence of tyrosine in the low molecular weight product places the initial cleavage site near the carboxyl terminus. This conclusion follows from examination of the primary sequence (Colombo and Villafranca, 1986). From the amino terminus the first tyrosine occurs at residue 100. Cleavage to include this residue would generate a product with a molecular weight of at least 11,000. However, tyrosines are at positions 465 and 466, the third and fourth residues from the carboxyl terminus. Hence, cleavage of all monomers confirmed that it was the carboxyl-terminal peptide (Table IX).

From this analysis, the proteolytic cleavage appears to occur between Ala430 and Gly431. (The sequence in this region is Glu429, Phe430, Leu431-Lys432-Ala433-Gly434-Gly435-Val436. Phe431-Thr432.) The calculated molecular weight of the peptide is 4,460, which is 8.6% of the molecular weight of the glutamine synthetase monomer. Hence, cleavage of all monomers by this protease would generate a maximum of 8.6% trichloroacetic acid-soluble counts, consistent with the experimental results (Fig. 2).

Products of Other Modified Forms of Glutamine Synthetase

Fig. 6. Initial proteolytic product from oxidized glutamine synthetase. Oxidized glutamine synthetase (25 mg/100 ml) was incubated with protease (0.1 mg/100 ml) at pH 9.0. At 0 and 15 min, aliquots of 20 ml were removed for analysis of proteolytic products by reverse phase HPLC. Initial profile; and 15-min profile. The major product at 21 min had M, of 3600 by gel filtration and 4500 from amino acid analysis.
proteases have been classified as serine proteases based on their inhibition by diisopropyl fluorophosphate or phenylmethylsulfonyl fluoride; however, this protease is not inhibited by these nor by most other serine protease inhibitors. Its molecular weight, estimated by gel filtration and SDS-polyacrylamide gel electrophoresis, is 75,000 in contrast to smaller \( E.\ coli \) proteases 1 (43,000) (Pacaud and Uriel, 1971), II (58,000) (Pacaud and Richaud, 1975), and leader peptidase (39,000) (Zwizinski and Wickner, 1980), and larger enzymes such as protease IV (650,000) of Pacaud (1982) and Do (520,000) and La (350,000) previously studied in Goldberg's laboratory (Goldberg et al., 1981). Protease La, the product of the lon gene, is stimulated by ATP whereas this protease is not. In addition to glutamine synthetase, this protease degrades both insulin and casein, and it, therefore, can be contrasted with the proteases purified by Goldberg and his co-workers (Goldberg et al., 1981) which degrade either iodinated insulin or methylated casein but not both. The major sites of cleavage of insulin B chain by this \( E.\ coli \) protease differ from those of \( E.\ coli \) protease III (Cheng and Zipser, 1979). The cleavage site of the other two \( E.\ coli \) proteases, P, and C, which degrade insulin has not been reported (Goldberg et al., 1981).

The \( E.\ coli \) protease initially cleaves oxidized glutamine synthetase near the carboxyl terminus to yield products of molecular weights 4,500 and 47,000. The small peptide was trichloroacetic acid soluble, permitting assay of the protease by following the generation of trichloroacetic acid-soluble radioactivity. The 47,000 \( M\), product was insoluble in trichloroacetic acid and not further degraded by the purified protease. Crude extracts from \( E.\ coli \) degrade oxidized glutamine synthetase quite extensively (Oliver et al., 1982). Hence, other proteases must participate in the degradation of glutamine synthetase.

The properties of the protease described here distinguish it from previously purified proteases of \( E.\ coli \). Most of these proteases have been classified as serine proteases based on their inhibition by disopropyl fluorophosphate or phenylmethylsulfonyl fluoride; however, this protease is not inhibited by these nor by most other serine protease inhibitors. Its molecular weight, estimated by gel filtration and SDS-polyacrylamide gel electrophoresis, is 75,000 in contrast to smaller \( E.\ coli \) proteases 1 (43,000) (Pacaud and Uriel, 1971), II (58,000) (Pacaud and Richaud, 1975), and leader peptidase (39,000) (Zwizinski and Wickner, 1980), and larger enzymes such as protease IV (650,000) of Pacaud (1982) and Do (520,000) and La (350,000) previously studied in Goldberg's laboratory (Goldberg et al., 1981). Protease La, the product of the lon gene, is stimulated by ATP whereas this protease is not. In addition to glutamine synthetase, this protease degrades both insulin and casein, and it, therefore, can be contrasted with the proteases purified by Goldberg and his co-workers (Goldberg et al., 1981) which degrade either iodinated insulin or methylated casein but not both. The major sites of cleavage of insulin B chain by this \( E.\ coli \) protease differ from those of \( E.\ coli \) protease III (Cheng and Zipser, 1979). The cleavage site of the other two \( E.\ coli \) proteases, P, and C, which degrade insulin has not been reported (Goldberg et al., 1981).

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targeted, with cells able to degrade specific proteins very rapidly without attacking others. Steady-state turnover rates vary widely among proteins, and these rates can change rapidly in response to changes in the nutritional or hormonal state of the organism. At present, there is little known of the biochemical mechanisms which regulate intracellular protein turnover. It seems unlikely that each protein in the cell would be associated with a protease acting only on that protein. Such a mechanism would provide specificity but at an enormous metabolic expense and a burdensome genetic load. Such specific proteases will probably prove uncommon.

Alternatively, one might conceive of a smaller number of proteases with less rigid specificity. Regulation could then occur through several mechanisms, including 1) alteration of the substrate protein to render it susceptible; 2) activation of the protease by metabolic effectors; and 3) compartmentation of the proteins, for example, in the lysosome. The first mechanism operates in *E. coli* through an oxidative modification which renders glutamine synthetase susceptible to proteolysis (Levine et al., 1981; Rivett, 1985b). Glutamine synthetase is susceptible to multiple covalent modifications by mixed function oxidation (Levine, 1983a; Nakamura and Stadtman, 1984; Oliver et al., 1985; Rivett and Levine, 1985). Initial modification leads to loss of catalytic activity. With additional exposure to the oxidizing system, the protein is rendered susceptible to the protease described here as well as to pro-

Susceptibility of glutamine synthetase (and other proteins) to mixed function oxidation is modulated by metabolic effectors. Susceptibility is also modulated by adenylylation of the enzyme, a well-studied reversible covalent modification which is intricately tied to the nutritional status of the cell (Chock et al., 1980). In particular, glutamine synthetase is adenylylated when glutamine is available in the growth medium. Unadenylated glutamine synthetase is susceptible to oxidative modification in the absence of substrates but is resistant to oxidation in the presence of both glutamate and ATP (Levine, 1983b). However, adenylylated glutamine synthetase is susceptible to oxidative modification even in the presence of these substrates. Thus, oxidative modification of glutamine synthetase occurs under conditions when the enzyme is not required by the cell: 1) substrates are lacking; or 2) glutamine is present.

Intracellular proteolysis of glutamine synthetase can thus be viewed as a two-step process (Levine, 1985).

**REFERENCES**


E. coli Protease


