Four intracellular proteases partially purified from liver preferentially degraded the oxidatively modified (catalytically inactive) form of glutamine synthetase. One of the proteases was cathepsin D which is of lysosomal origin; the other three proteases were present in the cytosol. Two of these were calcium-dependent proteases with different calcium requirements. The low-calcium-requiring type (calpain I) accounted for most of the calcium-dependent activity of both mouse and rat liver. The calcium-independent cytosolic protease, referred to as the alkaline protease, has a molecular weight of 300,000 determined by gel filtration. Native glutamine synthetase was not significantly degraded by the cytosolic proteases at physiological pH, but oxidative modification of the enzyme caused a dramatic increase in its susceptibility to attack by these proteases. In contrast, trypsin and papain did degrade the native enzyme and the degradation of modified glutamine synthetase was only 2- to 4-fold more rapid. Adenylylation of glutamine synthetase had little effect on its susceptibility to proteolysis. Although major structural modifications such as dissociation, relaxation, and denaturation also increased the rate of degradation, the oxidative modification is a specific type of covalent modification which could occur in vivo. Oxidative modification can be catalyzed by a variety of mixed function oxidase systems present within cells and causes inactivation of a number of enzymes. Moreover, the presence of cytosolic proteases which recognize the oxidized form of glutamine synthetase suggests that oxidative modification may be involved in intracellular protein turnover.

Intracellular proteins continuously turn over with widely differing half-lives. Although correlations have been made between protein half-life and various structural features (1), the mechanism by which a protein molecules is selected for proteolytic degradation is not understood. It has been suggested with respect to enzymes that inactivation precedes proteolysis (2-10). The degradation of other proteins may also occur by a two-step mechanism in which the protein is first modified such that it becomes more susceptible to proteolytic attack (11-13).

Studies in this laboratory (14) have suggested that oxidative modification of enzymes may mark them for degradation. Oxidative modification of Escherichia coli glutamine synthetase is a specific modification (15) which can be catalyzed by a variety of mixed-function oxidase systems (14-17). Substrates protect against this type of covalent modification, providing the potential for regulation (14, 17). The modification causes inactivation of the glutamine synthetase and increases its susceptibility to proteolysis by subtilisin (18) and E. coli proteases (16, 19).

Since several mammalian enzymes have been shown to be oxidatively modified (17, 20-22), it was of interest to determine whether there is an intracellular mammalian protease which could specifically recognize the oxidative modification of protein. E. coli glutamine synthetase was chosen as the substrate protein because its oxidative modification has been well-characterized (14, 15) and it can be naturally and efficiently labeled by addition of 14C-aminoacids to the growth medium of an overproducing strain of E. coli. The results of this study show that several mammalian proteases selectively degrade the oxidized form of glutamine synthetase.

**Preparation of 14C-labeled Glutamine Synthetase**

Glutamine synthetase with a low state of adenylylation was purified from a strain of E. coli (YMClO/pglnG) which has a plasmid containing the glutamine synthetase structural gene (23). Cells were grown in a medium containing 0.5% (w/v) glucose with 4 mM glutamine as the nitrogen source. MnCl2 (1 mM) was included to prevent oxidative modification of the glutamine synthetase (14). One mCi of 14C-aminoacids were added to each flask containing 200 ml of culture medium.

Cells were harvested in late logarithmic phase and ruptured by a French pressure cell. The glutamine synthetase was purified by the zinc precipitation and acetone ammonium sulfate procedures (24, 25) and assayed by the γ-glutamyltransferase method at pH 7.57 (26). The specific activity of the enzyme was 135–140 units/mg.

**EXPERIMENTAL PROCEDURES**

**Materials**

L-[U-14C]Amino acid mixture (specific activity, approximately 1.8 mCi/mg) was obtained from ICN Pharmaceuticals, Inc. L-Ascorbic acid was from J. T. Baker Chemical Co. and acetic anhydride was from Aldrich. Mouse liver acetone powder, bovine pancreatic trypsin (type III, 11230 BAEE* units/mg), papaya latex papain (type III, 17 units/mg), bovine spleen cathepsin D (13 units/mg), pepstatin A, leupeptin, N'-benzoyl-arginine ethyl ester and concanavalin A-agarose were purchased from Sigma. Sephacyrl S-300 and Sephadex G-100S were from Pharmacia and Cm-cellulose (CM52) and DEAE-cellulose (DE52) were from Whatman.

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1. J. E. Roseman and R. L. Levine, personal communication.
2. The abbreviations used are: BAEE, N'-benzoyl-L-arginine ethyl ester; EGTA, ethylene glycol bis[β-aminoethyl ether]-N,N',N'-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MRS, 5-(N-morpholino)ethanesulfonic acid.
Purified glutamine synthetase was stored at 4 °C in 10 mM imidazole buffer, pH 7.0, containing 100 mM KCl and 1 mM MnCl₂. It was dialyzed into 50 mM Hepes buffer, pH 7.2, containing 100 mM KCl and 10 mM MgCl₂ prior to use.

Oxidative Modification of Glutamine Synthetase

¹⁴C-labeled glutamine synthetase (approximately 5 mg/ml) was placed in a 75,000 molecular weight cut-off collagen bag (Schleicher & Schuell) and incubated at 37 °C (with shaking) in the presence of 25 mM ascobate and 100 μM FeCl₃ in 50 mM Hepes buffer, pH 7.2, containing 100 mM KCl and 10 mM MgCl₂. The resulting oxidative modification (14) was monitored by the loss of enzyme activity, which was complete within 2 h. The oxidized glutamine synthetase was dialyzed into 25 mM ascorbate and 100 μM FeCl₃ in 50 mM Hepes buffer, pH 7.2, removed from the ascorbate system after 90-150 min and dialyzed against Hepes/KCl/MgCl₂ buffer containing 1 mM EDTA. The enzyme was then dialyzed against the same buffer with no EDTA.

Adenylation of Glutamine Synthetase

¹⁴C-labeled glutamine synthetase (9 mg/ml) was adenylylated by incubation at 37 °C for 15 min with purified adenyltransferase in 50 mM Hepes buffer, pH 7.2, containing 150 mM KCl, 50 mM MgCl₂, 2 mM ATP, and 12.7 mM glutamine. The extent of adenylation as measured by the constant concentration of 1.6 M urea. Heat denaturation of native and oxidized enzyme was carried out as described above.

Other Modifications of Native and Oxidized Glutamine Synthetase

¹⁴C-labeled glutamine synthetase was acetylated with acetic anhydride (28). Native or oxidized forms of glutamine synthetase (9 mg/ml) were placed in collagen bags in 50 mM Hepes buffer, pH 7.2, containing 100 mM KCl and 10 mM MgCl₂. Acetic anhydride (10 mM) was added to the buffer which was being stirred on ice. A constant pH was maintained by addition of 1 N NaOH. The reaction was allowed to go to completion (within 30 min) and then the samples were dialyzed against 50 mM Hepes buffer, pH 7.2, containing 100 mM KCl and 10 mM MgCl₂. The extent of acetylation of amino groups was determined to be approximately 50% by fluorescamine reaction with free amino groups (29). The activity of native glutamine synthetase was reduced to about 20% of its original value during the acetylation procedure.

Glutamine synthetase subunits were dissociated by urea treatment (30). Native or oxidized ¹⁴C-labeled glutamine synthetase, 0.25 mg/ml in 50 mM Hepes buffer, pH 8.0, containing 5 mM KCl, 0.5 mM MgCl₂, and 8 M urea, were left at 4 °C for 2 h prior to the assay. To prevent reassociation of the subunits during the assay, the samples were diluted directly into the protease assay mixtures to give a final concentration of 1.6 M urea. Heat denaturation of native and oxidized ¹⁴C-labeled glutamine synthetase (0.25 mg/ml) was carried out at 90 °C for 3 min.

Protease Assays

Protease activity was routinely assayed by measurement of trichloroacetic acid-soluble products released from the native or oxidized form of unadenylated ¹⁴C-labeled glutamine synthetase.

Assays were carried out in closed Eppendorf tubes in a total volume of 0.1 ml using 2-10 μg of glutamine synthetase/assay. Incubations were at 37 °C for 1 h (unless stated otherwise) and were stopped by addition of 0.5 ml 10% trichloroacetic acid and 0.1 ml of 5 ml of bovine serum albumin in 0.1 N HCl. The tubes were cooled for 10-15 min at then spun in a microfuge. A 0.5 ml aliquot of the supernatant was added to 10 ml of Aquasol (New England Nuclear) for determination of radioactivity in a Beckman liquid scintillation counter. Protease activity is expressed as the per cent conversion of ¹⁴C-labeled glutamine synthetase to trichloroacetic acid-soluble products.

Protease activity in crude mouse liver preparations was measured by incubation with oxidized ¹⁴C-labeled glutamine synthetase in 50 mM Hepes buffer, pH 7.5, and in 50 mM sodium acetate buffer, pH 5.0 or 5.5, for 1-4 h. Assay buffers for the purified proteases were usually as follows: casein in 0.1 M sodium citrate buffer, pH 3.5; calcium-dependent proteases, 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM 2-mercaptoethanol and 1.4 mM CaCl₂; alkaline protease, 50 mM Hepes buffer, pH 8.0; trypsin, 50 mM Hepes buffer, pH 7.5; and papain, 50 mM MES buffer, pH 6.0, containing 3 mM 2-mercaptoethanol. Hydrolysis of benzoyl-arginine ethyl ester (0.25 mM) by trypsin was assayed by monitoring the change in absorbance at 253 nm.

In studies with highly purified protease preparations, assays were generally started by addition of the protease to minimize loss of activity in the absence of substrate. In the case of the calcium-dependent proteases, calcium was added last. Assay conditions were chosen such that the production of trichloroacetic acid-soluble products was approximately linear with time and protease concentration. However, it was not always possible to achieve the latter condition using crude protease preparations.

Blank values were determined in the absence of enzyme preparation except for the calcium-dependent proteases where CaCl₂ in the assay was replaced by 2 mM EDTA.

Tissue Preparation

Mice (NIH strain, 22-25 g, body weight) were starved overnight and then killed by cervical dislocation. Livers were washed in 0.3 M sucrose, 1 mM EDTA, pH 7.4, and were then homogenized in 9 volumes (v/v) of the sucrose solution in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 1,500 × g for 10 min. The pellet was discarded and the supernatant fraction was centrifuged at 17,000 × g for 20 min. The pellet from this step was resuspended in sucrose solution and again centrifuged at 17,000 × g for 20 min. The washed pellet was resuspended in a small volume of 5 mM Hepes buffer, pH 7.0, for use as a crude lysosomal preparation. The supernatant from the first 17,000 × g spin was spun at 39,000 × g for 2 h in order to obtain s soluble fraction.

Purification and Identification of Lysosomal Protease Activity

Protease activity was purified from crude lysosomal preparations by the following procedures.

Step 1: Acid Treatment—For acid treatment (pH 5.0 in sodium acetate buffer) or acetone powder preparation and extraction in ion-exchange buffer see Step 2.

Step 2: Ion-exchange Chromatography—Either DEAE-cellulose equilibrated in 10 mM Tris-HCl buffer, pH 7.2, or CM-cellulose equilibrated in 20 mM Na acetate buffer, pH 5.0. In each case the gradient for elution of protease activity was 0-0.2 M KCl in the appropriate buffer.

Step 3: Concanavalin A-Agarose Chromatography—The column was equilibrated in 20 mM sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl, 1 mM CaCl₂, and 1 mM MnCl₂. Elution was achieved with 0.1 M α-methylmannoside in buffer. The enzyme was concentrated by ultrafiltration using an Amicon PM-10.

Step 4: Sephadex G-100SF Gel Filtration—The buffer was 20 mM sodium phosphate, pH 6.0, containing 0.1 M NaCl. The standard proteins used to calibrate the column were hexokinase, hemoglobin, ovalbumin, chymotrypsinogen, and cytochrome C. For studies with protease inhibitors, pepstatin A was dissolved in dimethyl sulfoxide. Inhibition was compared to a control with an equal final concentration of the solvent (0.1%).

Purification of the Alkaline Protease

The alkaline protease activity of mouse liver acetone powder was purified by the following procedure: Step 1, acetone powder extraction in 20 mM sodium pyrophosphate buffer, pH 8.0; Step 2, ammonium sulfate fractionation, 30-60%, followed by dialysis; Step 3, DEAE-cellulose chromatography using 10 mM Tris-HCl buffer, pH 7.2, with a gradient to 0.35 M KCl in buffer; and Step 4, Sepharose S-300 gel filtration in 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. This protease has subsequently been purified to apparent homogeneity from both mouse and rat liver acetone powders and further details of the purification procedure will be presented elsewhere.

Purification of Calcium-dependent Protease Activity

Calcium-dependent protease activity was partially purified from a soluble protein preparation from fresh mouse liver by a DEAE-cellulose batch procedure and Sephacryl S-300 gel filtration (31). The two forms of the protease were then separated by ion-exchange chromatography (31). Calcium-dependent protease activity was isolated from rat (Fischer 344, male, 9 months) liver by the same method except that the batch procedure was omitted.
RESULTS

Identification of Some Intracellular Proteases Which Degrade Oxidized Glutamine Synthetase—Most of the oxidized \(^{14}\)C-labeled glutamine synthetase degrading activity in crude tissue preparations was found in the mitochondrial/lysosomal fraction using the acid pH assay. The major lysosomal protease activity was identified as the carboxyl protease, cathepsin D. Gel filtration gave a single peak of protease activity with a molecular weight of 43,000. This activity was highest between pH 3 and 4 and the pH profile was similar to that for bovine spleen cathepsin D shown below (Fig. 1). The specific activity of the partially purified mouse liver preparation was 11.6% conversion of oxidized \(^{14}\)C-labeled glutamine synthetase to trichloroacetic acid-soluble products/µg of protein/h (32). In this preparation protease activity at pH 5.5 was almost completely inhibited by 10 µM pepstatin A; however, 10 µM leupeptin (an inhibitor of the lysosomal thiol proteases) had no significant effect. Cathepsin D has a pH optimum between 3 and 4, and a molecular weight around 43,000 (34). It is inhibited by pepstatin A, a specific inhibitor of carboxyl proteases (35).

Little activity was detected with the pH 7.5 assay in any of the subcellular fractions. However, some neutral cytosolic protease activity is calcium-dependent, and addition of calcium was found to stimulate the degradation of oxidized glutamine synthetase by soluble fractions prepared from mouse liver. The calcium-dependent activity was partially purified and was then separated into two peaks (the first, calpain I, and the second, calpain II, which differ in their calcium requirement (31, 36)) by ion-exchange chromatography. Calpain I accounted for most of the calcium-dependent degradation of oxidized glutamine synthetase. No activity was detected in the absence of calcium in the partially purified preparations.

Since extracts of acetone powder prepared from crude lysosomal fractions contained protease activity (cathepsin D), a whole liver acetone powder (Sigma) was also tested for activity. In this case, unlike that of the lysosomal acetone powder, substantial degradation of oxidized glutamine synthetase was observed at alkaline pH values. The protease responsible for this activity did not require calcium for activity. It was subsequently purified from mouse liver acetone powder and will be referred to simply as the alkaline protease. The apparent molecular weight of the protease determined by gel filtration was 300,000.

Comparison of the Rate of Degradation of Oxidized and Native Glutamine Synthetase—Oxidatively modified glutamine synthetase was degraded more rapidly than the native enzyme by all of the proteases tested. The ratio of the degradation of oxidized glutamine synthetase to that of native enzyme for cathepsin D was found to be pH-dependent. Results for the bovine spleen enzyme are shown in Fig. 1. At pH values around the optimum, there was only a 2- to 3-fold difference, whereas around pH 4–5 the oxidized enzyme was degraded about 10 times more rapidly. Similar results were obtained with the purified mouse liver cathepsin D preparation.

Typical results for the proteolysis of native and oxidized glutamine synthetase by calcium-dependent protease activity is shown in Fig. 2. Both of the calcium-dependent protease activities degraded oxidized glutamine synthetase, although less than 10% of the substrate was converted to trichloroacetic acid-soluble products. Neither protease significantly degraded native glutamine synthetase. The ratio of the rate of degradation of oxidized glutamine synthetase to the rate of degradation of the native enzyme was around 30 (Table I).

The alkaline protease purified from mouse liver acetone powder could degrade the oxidized form of glutamine synthetase about 40- to 80-fold more rapidly than the native enzyme under the standard assay conditions. The results of a typical experiment are shown in Fig. 3. At higher pH values, native glutamine synthetase was also degraded (Fig. 4). In an experiment with the protease isolated from fresh liver, 3.93% of the oxidized form of glutamine synthetase was converted to trichloroacetic acid-soluble products in 1 h while only 0.06% of the native enzyme became acid-soluble. For that experiment the assays were carried out at pH 8.0 with Hepes, MgCl\(_2\), EDTA, and KCl concentrations of 50, 0.2, 0.1, and 2 mM, respectively.

Trypsin and papain were able to degrade native glutamine synthetase.

![Fig. 1. Preferential degradation of the oxidized form of glutamine synthetase by cathepsin D. Assays were carried out in 50 mM sodium citrate buffer containing 4 µg of oxidized (O) or native (●) \(^{14}\)C-labeled glutamine synthetase (GS), 0.2 mM MgCl\(_2\), 2 mM KCl, and 0.08 µg of bovine spleen cathepsin D.](image)

![Fig. 2. Preferential degradation of oxidatively modified glutamine synthetase by calcium-dependent protease activity. Rat liver calcium-dependent protease activity was partially purified by Sephacryl S-300 gel filtration in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA. Assays were carried out in 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM MgCl\(_2\), 1 mM KCl, 2 mM CaCl\(_2\), 10 mM 2-mercaptoethanol, and 4 µg of oxidized (O) or native (●) \(^{14}\)C-labeled glutamine synthetase (GS) with varying amounts of protease. Incubations were at 30 °C. Protein determination was by the method of Bradford (33) using bovine serum albumin as standard.](image)
Intracellular Proteases Recognize Oxidized Enzyme

TABLE I

Proteolysis by the isolated calcium-dependent proteases

The two calcium-dependent protease activities, calpain I and II, were separated by ion-exchange chromatography. Mouse liver protease assays were carried out at 30°C in 50 mM Hepes, pH 8.0, containing 1.4 mM CaCl₂ and 10 mM 2-mercaptoethanol, 0.2 mM MgCl₂, and 2 mM KCl. Assays for the rat proteases were carried out as described in the legend of Fig. 2. The amount of protease preparation used gave less than the maximum per cent conversion of oxidized glutamine synthetase (GS) to acid-soluble products and the activities do not directly reflect the relative amounts of protease present.

| Protease   | Conversion to acid soluble products | Oxidized/native* |
|------------|-------------------------------------|------------------|
| Mouse Liver|                                     |                  |
| Calpain I  | 0.18                                | 4.71             | 26               |
| Calpain II | 0.07                                | 2.22             | 32               |
| Rat Liver  |                                     |                  |
| Calpain I  | 0.14                                | 3.89             | 28               |
| Calpain II | 0.06                                | 1.92             | 32               |

* Relative rates of degradation of oxidized and native glutamine synthetase.

Fig. 3. Preferential degradation of oxidatively modified glutamine synthetase by the mouse liver alkaline protease. Assays were carried out in 50 mM Hepes buffer, pH 8.0, containing 4 μg of oxidized (0) or native (●) ¹⁴C-labeled glutamine synthetase (GS), 1 mM MgCl₂, 2 mM KCl, 0.02 mM EDTA, and varying amounts of a partially purified protease preparation from mouse liver acetone powder. Protein concentrations were determined by the method of Lowry et al. (32) using bovine serum albumin as standard.

Fig. 4. pH profile for the degradation of oxidized and native glutamine synthetase by the mouse liver alkaline protease. Assays were carried out in 50 mM buffer containing 4 μg of oxidized ¹⁴C-labeled glutamine synthetase (GS), 0.2 mM MgCl₂, 2 mM KCl, and 0.02 mM EDTA. Buffers were MES (○), Hepes (●), Tris (△), and glycine (100 mM, ■). Assays with native glutamine synthetase (×) were carried out under the same conditions.

Fig. 5. Degradation of native and oxidized forms of glutamine synthetase by trypsin. Assays were carried out at 30°C in 50 mM Hepes buffer, pH 7.5, containing 0.25 mM MgCl₂, 2.5 mM KCl, 5 μg of oxidized (0) or native (●) ¹⁴C-labeled glutamine synthetase (GS) and 0.05 μg of trypsin.

Fig. 6. Degradation of native and oxidized forms of glutamine synthetase by papain. Assays were carried out in 50 mM MES buffer, pH 6.0, containing 3 mM 2-mercaptoethanol, 4 μg of oxidized (0) or native (●) ¹⁴C-labeled glutamine synthetase (GS), and 0.11 μg of papain.

synthetase and the observed increase in the rate of degradation following oxidative modification of the substrate protein (Figs. 5 and 6) was much less dramatic than for the intracellular proteases. For trypsin the difference was 2- to 4-fold and for papain only about 2-fold.

Effect of Other Modifications on the Rate of Degradation of Glutamine Synthetase—During the course of this study it became apparent that divalent metal ions decreased the degradation of both native and oxidized glutamine synthetase by the mouse liver alkaline protease and also by trypsin (Table II). Degradation was generally stimulated by EDTA. ATP added alone also enhanced the rate of proteolysis but Mg²⁺ATP had little effect (Table II). The magnitude of the stimulation by EDTA and inhibition by MgCl₂ depended on the MgCl₂ concentration in the control assay. The effects on the hydrolysis of benzoyl-arginine ethyl ester by trypsin were compared with those on the degradation of native and oxidized glutamine synthetase. The results suggest that the opposing effects of divalent metal ions and their chelators on glutamine synthetase degradation are due to structural changes in the glutamine synthetase molecule rather than direct effects on the proteases. KCl was found to stimulate degradation of both native and oxidized forms of glutamine synthetase. Substrates
TABLE II
Effect of divalent metals, EDTA, and substrates on the proteolysis of native and oxidized glutamine synthetase

| Addition | Alkaline protease | Trypsin* | Trypsin* |
|----------|-------------------|----------|----------|
|          | Native GS | Oxidized GS | Native GS | Oxidized GS | BAEE |
| None     | 0.19 | 9.7 | 2.47 | 6.1 | 100 |
| MgCl₂    | 0.12 | 4.7 | 1.06 | 5.8 | 111 |
| EDTA     | 9.81 | 19.2 | 4.79 | 13.7 | 64 |
| MgCl₂ + EDTA | 0.11 | 8.6 | 3.0 | 6.6 | 109 |
| ATP      | 0.7 | 18.6 | 2.82 | 12.9 | 109 |
| MgCl₂ + ATP | 0.12 | 8.8 | 1.46 | 6.1 | 109 |
| MgCl₂ + ATP + Glu | 1.39 | 6.2 | 1.66 | 11.5 | 120 |
| Glu      | 2.47 | 6.8 | 1.28 | 6.1 | 109 |
| ADP      | 0.26 | 13.4 | 2.43 | 8.0 | 109 |
| KCl      | 0.22 | 12.8 | 3.70 | 11.5 | 120 |

*Protease activity is expressed as per cent conversion of glutamine synthetase to trichloroacetic acid-soluble products/h.

**Trypsin activity with BAEE is given as per cent of control activity.

TABLE III
Proteolysis of modified glutamine synthetase

| Conversion to acid-soluble products | Alkaline protease | Cathepsin D | Trypsin |
|------------------------------------|-------------------|-------------|--------|
| Native GS₃₅ | 0.13 | 0.53 | 5.3 |
| Oxidized | 5.6 | 8.1 | 21 |
| Native deneylated GS₃₅ | 0.16 | 0.66 | 7.2 |
| Oxidized deneylated | 4.0 | 6.3 | 21 |
| Acetylated GS₃₅ | 2.9 | 2.8 | 11 |
| Oxidized acetylated | 3.8 | 15 | 34 |
| Urea-treated GS₃₅ | 4.5 | 4.8* | 52 |
| Oxidized urea-treated | 5.2 | 6.0* | 49 |
| Heat-denatured GS₃₅ | 1.3 | 23 | 53 |
| Oxidized heat-denatured | 3.0 | 23 | 50 |

provided some protection against proteolysis by trypsin for native but not for oxidatively modified glutamine synthetase (Table II).

Table III summarizes the results obtained with some other modified forms of glutamine synthetase. Acenylhalation had little effect on the rate of degradation. Structural modifications caused a marked increase in the rate of degradation of the native enzyme comparable to that observed following oxidative modification. Further modifications to the oxidized form of the enzyme had less effect on its susceptibility to proteolysis.

Incorporation of radioactive amino acids during protein synthesis is the best method of radiochemically labeling a protein for this type of study. Other methods such as iodination or methylation may affect the susceptibility of the protein to proteolysis (37). The use of the oxidized form of generally labeled glutamine synthetase provided a very sensitive assay for the identification and purification of proteases.

Protease activity, detected in crude liver preparations, appeared to be of lysosomal origin; most of this activity was due to the carboxyl protease, cathepsin D. The pH profile of cathepsin D was very similar for the enzyme from bovine spleen and mouse liver. Optimum activity is usually observed between pH 3 and 4 (34). The pH value inside lysosomes is around 4.7 (38) and the ratio of degradation of oxidized to native glutamine synthetase was higher at this pH than at the pH optimum of the protease. Denaturation of native glutamine synthetase at pH values below pH 4.5 may account for the decreased difference in the degradation rate for the two forms at low pH values. The decreased difference in the rate of degradation of oxidized and native glutamine synthetase by cathepsin D following heat denaturation of the substrate is consistent with the preference of this protease for acid-denatured substrates.

It was difficult to measure cytosolic protease activity in crude preparations, presumably due to the presence of intracellular protease inhibitors and/or of competing protein substrates. However, three cytosolic proteases were partially purified using oxidized glutamine synthetase as the substrate. Since the alkaline protease does not appear to have been described previously, it will be the subject of a subsequent report. The other two proteases, the calcium-dependent thiol proteases, have been described: one (calpain I) has a low calcium requirement and the other (calpain II) has a higher calcium requirement (36). The two forms may be related (39-42). The specific intracellular protease inhibitor of the calcium-dependent proteases (36) was removed by the gel filtration procedure. Both proteases were found to degrade oxidized glutamine synthetase, but less than 10% of the substrate was rendered trichloroacetic acid-soluble. This may simply be due to interference by contaminating proteins in the protease preparations, but limited proteolysis has been suggested to be a characteristic of the calcium-dependent proteases (36). In contrast to other reports (31, 36) which suggest that the low-calcium-requiring protease is present in relatively small amounts in liver, the results of this study indicated that calpain 1 accounts for most of the calcium-dependent oxidized glutamine synthetase degrading activity in both mouse and rat liver. This apparent discrepancy could possibly be explained by differences in substrate specificity of calpain I and II. They were assayed at the same high calcium concentration.

Trypsin and papain, a serine and a thiol protease, respectively, were chosen for comparison with the intracellular mammalian proteases. In contrast to the intracellular proteases, both trypsin and papain were able to degrade native glutamine synthetase. The increase in the rate of degradation following oxidative modification of the glutamine synthetase was only about 2- to 4-fold. Similar results have previously been obtained with subtilisin (18). Although very little degradation of native glutamine synthetase was observed with the alkaline protease at physiological pH values, degradation of the native enzyme was found to be almost as rapid as that of oxidized glutamine synthetase at high pH values. The glutamine synthetase dodecamer dissociates under these conditions (30). Native glutamine synthetase was also degraded more rapidly following urea treat-
Intracellular Proteases Recognize Oxidized Enzyme

11. Ramponi, G., Nasi, P., Liguri, G., Cappugi, G., and Grisoli, S. (1978) FEBS Lett. 90, 228–232
12. Harshko, A., and Ciechanover, A. (1982) Annu. Rev. Biochem. 51, 335–364
13. Toyo-Oka, T. (1982) Biochim. Biophys. Res. Commun. 107, 44–50
14. Levine, R. L., Oliver, C. N., Fulks, R. M., and Stadtman, E. R. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2120–2124
15. Levine, R. L. (1983) J. Biol. Chem. 258, 11823–11827
16. Oliver, C. N., Levine, R. L., and Stadtman, E. R. (1981) in Experiences in Biochemical Perception (Ornstein, L. N., ed) pp. 233–249, Academic Press, New York
17. Fucci, L., Oliver, C. N., Coon, M. J., and Stadtman, E. R. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 1521–1525
18. Farber, J. M., and Levine, R. L. (1982) Fed. Proc. 41, 865
19. Roseman, J. E., and Levine, R. L. (1983) Fed. Proc. 42, 1812
20. Bray, R. C., Cockle, S. A., Fielden, E. M., Roberts, P. B., Rotillo, G., and Calabrese, L. (1974) Biochem. J. 139, 43–48
21. Gurnieri, C., Lugavessi, A., Flanigin, F., Muscaria, C., and Caldeva, C. M. (1982) Biochim. Biophys. Acta 718, 157–164
22. Shinav, E., Navok, T., and Chevion, M. (1983) J. Biol. Chem. 258, 14778–14783
23. Blackman, K., Chen, Y. M., and Magasanik, B. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 3743–3747
24. Miller, R. E., Shelton, E., and Stadtman, E. R. (1974) Arch. Biochem. Biophys. 163, 155–171
26. Wolfolk, C. A., Shapiro, B., and Stadtman, E. R. (1966) Arch. Biochem. Biophys. 116, 177–192
27. Stadtman, E. R., Smyrniotis, P., Davis, J. N., and Wittenberger, M. E. (1979) Anal. Biochem. 95, 275–285
28. Shapiro, B. M., and Stadtman, E. R. (1970) Methods Enzymol. 17, 910–922
29. Riordan, J. F., and Valle, B. L. (1972) Methods Enzymol. 25, 494–499
30. Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 153, 213–220
31. Ginsburg, A. (1972) Adv. Protein Chem. 28, 1–79
32. De Martino, G. N. (1981) Arch. Biochem. Biophys. 211, 253–257
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
34. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
35. Barrett, A. J. (1977) in Proteinases in Mammalian Cells and Tissues (Barrett, A. J., ed) pp. 209–245, North-Holland Publishing Co., Amsterdam
36. Takahashi, K., Chang, W.-J., and Ko, J. S. (1974) J. Biochem. 76, 897–899
37. Murachi, T. (1983) Trends Biochem. Sci. 8, 167–169
38. Oprea, L., Wiley, H. S., and Wallace, R. A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1566–1560
39. Ohkuma, S., and Poole, B. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 3327–3331
40. Pomporelli, S., Melloni, E., Salamino, F., Spasatore, B., Michetti, M., and Horecker, B. L. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 53–56
41. Imahori, K., Suzuki, K., and Kawashina, S. (1983) in Proteinase Inhibitors: Medical and Biological Aspects (Katunuma, N., Uma- zawa, H., and Holzer, H., eds) pp. 173–179, Japan Scientific Societies Press, Tokyo
42. Meilgern, R. L., Repetti, A., Muck, T. C., and Easyly, J. (1982) J. Biol. Chem. 257, 7203–7209
43. Hathaway, D. R., Werth, D. K., and Haeberle, J. R. (1982) J. Biol. Chem. 257, 9072–9077
44. Nakamura, K., and Stadtman, E. R. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 2011–2015
45. Stadtman, E. R., and Ginsburg, A. (1974) in The Enzymes (Boyer, P. D., ed) 3rd Ed., Vol. 10, pp. 755–807, Academic Press, New York
46. Oliver, C. N., Fulks, R., Levine, R. L., Fucci, L., Rivett, A. J., Roseman, J. E., and Stadtman, E. R. (1984) in Molecular Basis of Aging (Roy, A. K., and Chatterjee, B., eds) Academic Press, New York, in press
47. Robinson, A. B. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 885–888

Acknowledgments—I thank Earl Stadtman for his support, advice, and encouragement. I also thank Rodney Levine and Jo Ellen Roseman for helpful discussions.

REFERENCES

1. Ballard, F. J. (1977) Essays Biochem. 13, 1–37
2. Holzer, H., and Heinrich, P. C. (1980) Annu. Rev. Biochem. 49, 63–91
3. Goldberg, A. L., and St. John, A. C. (1976) Annu. Rev. Biochem. 45, 747–803
4. Reynolds, R. D., and Thompson, S. D. (1974) Arch. Biochem. Biophys. 164, 43–51
5. Odenbrader, M. F., and Prouty, W. F. (1977) J. Biol. Chem. 252, 2869–2872
6. Hemmings, B. A. (1980) FEBS Lett. 122, 297–302
7. Holzer, H. (1983) in Proteinase Inhibitors: Medical and Biological Aspects (Katunuma, N., Umezawa, H., and Holzer, H., eds) pp. 181–190, Japanese Scientific Societies Press, Tokyo
8. Ballard, F. J., and Hopgood, M. F. (1976) Biochem. J. 154, 717–724
9. Francis, G. L., and Ballard, F. J. (1980) Biochem. J. 186, 581–590
10. Bond, J. S., and Offerman, M. K. (1981) Acta Biol. Med. Ger. 40, 1365–1374

3 A. J. Rivett, unpublished results.