Crystallization and Characterization of a New Protease in Mitochondria of Bone Marrow Cells*

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From the Department of Biochemistry, Jichi Medical School, Tochigi, Japan

A new protease was found in mitochondria of bone marrow cells. The protease was purified from human bone marrow cells by the following methods: disruption of cells by sonication; buffer extraction; column chromatography using DEAE-cellulose, Sephadex G-75, and CM-cellulose. It was then crystallized in the presence of polyethylene glycol. The crystallized enzyme is homogeneous as judged by polyacrylamide disc gel electrophoresis. The molecular weight is 31,800 and optimum pH 8.5. Diisopropyl fluorophosphate (10 μM) inhibits the protease activity completely, while p-chloromercuribenzoate (1 mM) has no effect. The enzyme is similar to elastase in that it hydrolyzes ester substrates for elastase and is inhibited by Elastatinal, an inhibitor of elastase. However, it is not identical with elastase, because it possesses no elastolytic activity and no ability to hydrolyze amide substrates for elastase. The protease inactivates the apo form of certain pyridoxal enzymes in rat liver, skeletal muscle, and small intestine. In some respects, however, it differs from these proteases. The protease also resembles proteases reported by Katunuma et al. (4) to inactivate the apo form of certain pyridoxal enzymes in rat liver, skeletal muscle, and small intestine. In some respects, however, it differs from these proteases. The protease also resembles elastase, but it is considered not to be identical with elastase because it has no elastolytic activity.

The protease in erythroblasts is considered to play important roles in the regulation of δ-aminolevulinic acid synthetase levels, as shown in the text. On the other hand, the activity in granulocytes is considered to play fundamental roles in immune responses, and granulocytes in inflammation. Therefore, the activity of δ-aminoluvulinic acid synthetase is highest in erythroblasts. This enzyme, which catalyzes the condensation of succinyl coenzyme A and glycine to form δ-aminolevulinic acid, has been regarded as the rate-limiting enzyme in heme synthesis (1). It exists in mitochondria (1) and requires pyridoxal phosphate as a cofactor (2). The enzyme activity in liver cells is increased by treatment with allylisopropylacetamide or 3,5-dicarbethoxy-1,4-dihydrocollidine (1). The activity in erythroblasts has been reported to be decreased in sideroblastic anemia (3). However, regulatory mechanisms of the level of this enzyme in erythroblasts remain to be clarified.

In the course of investigations into the regulatory mechanisms, a new serpyl protease inactivating the apo form of δ-aminolevulinic acid synthetase was found in mitochondria of bone marrow cells, including both erythroblasts and granulocytes. It resembles proteases reported by Katunuma et al. (4) to inactivate the apo form of certain pyridoxal enzymes in rat liver, skeletal muscle, and small intestine. In some respects, however, it differs from these proteases. The protease also resembles elastase, but it is considered not to be identical with elastase because it has no elastolytic activity.

The protease in erythroblasts is considered to play important roles in the regulation of δ-aminolevulinic acid synthetase levels, as shown in the text. On the other hand, the activity in granulocytes is elevated in several diseases accompanying vasculitis. From these results, the possible biological significances of this protease are discussed. A preliminary report of this study has appeared (5).

MATERIALS AND METHODS

Some Properties of Protease

Crystals and Homogeneity — Fig. 1 shows crystals of the protease. Crystalline enzyme was subjected to polyacrylamide disc gel electrophoresis. As shown in Fig. 2, only one band was detected. Protease activity coeluted with the band when measured after cutting the

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Inactiuation of Apo-ornithine Aminotransferase by Protease-To explore the mode of inactivation of apo-ornithine aminotransferase, apo-ornithine aminotransferase was incubated with the protease, and the relationship between the enzyme inactivation and protein degradation was examined. As shown in Fig. 3, ornithine aminotransferase activity decreased, and the amount of ninhydrin-positive material in the supernatant increased as the incubation time increased. On the other hand, the decrease in the amount of the protein precipitated by trichloroacetic acid was not significant. Polyacrylamide disc gel electrophoresis of apo-ornithine aminotransferase digested by the protease for 60 min under the conditions described in Fig. 3 showed the same single band as undigested substrate (data not shown). From these results, it is concluded that the protease inactivates apo-ornithine aminotransferase by splitting close to the termini of the protein.

Substrate Specificity
The protease rapidly inactivates the apo form of pyridoxal enzymes except apoglutamic-oxaloacetic acid transaminase. Inactivation of enzymes other than apopyridoxal enzymes was negligible or small, as shown in Table II. The holo form of pyridoxal enzymes is not inactivated by the protease. The specificity for synthetic substrates was also examined. As shown in Table II, only ester substrates for elastase were hydrolyzed, which amide substrates were not.

Elastolytic activity was examined using Congo red-dyed elastin as the substrate. No elastolytic activity was detected even when larger amounts of the protease were employed as compared to bovine pancreatic elastase.

Inhibitor Studies
The effects of several protease inhibitors obtained from culture broths of Actinomyces were examined. Chymostatin, Leupeptin, Pepstatin, and Antipain showed no inhibitory activity on the protease at concentrations of 100 μg/ml. On the other hand, Elastatinal which is known as a specific inhibitor for elastase (44) inhibited the protease activity by 50% at a concentration of 3.5 μg/ml. Purified α1-antitrypsin inhibits the activity by the molar ratio added, as shown in Fig. 4.

Subcellular Distribution of Protease
The results of subcellular fractionation are summarized in Fig. 5. The distribution pattern of the protease was different from that of acid phosphatase (lysosomes) and NADPH-cytochrome c reductase (microsome). It resembles the distribution of succinate cytochrome c reductase (mitochondria). From these results, the protease is considered to be located in the mitochondria of bone marrow cells.

Localization of Protease in Mitochondria
Mitochondria used as the starting material for submitochondrial fractionation contained negligible acid phosphatase activity, showing negligible contamination by lysosomes.

The results of submitochondrial fractionation are summarized in Figs. 6 and 7. As shown in Fig. 6, the distribution pattern of the protease is quite different from that of sulfite-cytochrome c reductase (intermembrane fraction or soluble fraction) or kynurenine hydroxylase (outer membrane), but similar to that of succinate-cytochrome c reductase (inner membrane fraction). The inner membrane fraction (inner membrane plus matrix) was sonicated to separate it into inner membrane and matrix as described under "Methods." As shown in Fig. 7, the distribution pattern of the protease was different from that of malate dehydrogenase (matrix), but resembled that of succinate-cytochrome c reductase (inner membrane). From these results, it is considered that the protease exists in the inner membrane of mitochondria.

The location of the protease on the inner mitochondrial membrane was assessed by using diaminobenzene sulfonic acid for chemical modification as described under "Methods." The protease activity decreased (31% of control) when submitochondrial particles were treated with the reagent, while it remained unchanged when sub-mitochondrial particles were prepared after treatment with the reagent. It is concluded, therefore, that the protease is located on the inside of the inner mitochondrial membrane. From the results shown in Figs. 5, 6, and 7, it is also concluded that 6-aminolevulinic acid synthetase is located on the inner mitochondrial membrane.

Protease Activity in Various Cells
Human bone marrow cells obtained were washed three times in saline by centrifugation to remove inhibitors contained in plasma.

Molecular Weight - From sedimentation equilibrium centrifugation and a partial specific volume of 0.7446, based on the amino acid composition, the molecular weight was determined to be 31,800. Sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis showed a molecular weight of 28,000.

pH Optimum - The pH dependency was examined by the use of ornithine aminotransferase apoenzyme or N-acetyl(Z-alanine), methyl ester as the substrates. The pH optimum was around 8.5.

Inactivation of Ornithine Aminotransferase-Ase by Protease - To examine the mode of inactivation of apo-ornithine aminotransferase, apo-ornithine aminotransferase was incubated with the protease, and the relationship between the enzyme inactivation and protein degradation was examined. As shown in Fig. 3, ornithine aminotransferase activity decreased, and the amount of ninhydrin-positive material in the supernatant increased as the incubation time increased. On the other hand, the decrease in the amount of the protein precipitated by trichloroacetic acid was not significant. Polyacrylamide disc gel electrophoresis of apo-ornithine aminotransferase digested by the protease for 60 min under the conditions described in Fig. 3 showed the same single band as undigested substrate (data not shown). From these results, it is concluded that the protease inactivates apo-ornithine aminotransferase by splitting close to the termini of the protein.

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Various enzymes and synthetic substrates were incubated with the protease, and degrees of inactivation or hydrolysis were compared. Values illustrate inactivations or hydrolysis of these enzymes or synthetic substrates as percentages of that of apo-ornithine aminotransferase. Protease activity using these enzymes or synthetic esters (or amides) as substrate was measured as described under "Methods."

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sources</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo-ornithine aminotransferase</td>
<td>Rat liver</td>
<td>100</td>
</tr>
<tr>
<td>Apo-homocarnosine deaminase</td>
<td>Rat liver</td>
<td>185</td>
</tr>
<tr>
<td>Apo-serine dehydratase</td>
<td>Rat liver</td>
<td>21</td>
</tr>
<tr>
<td>Apo-δ-aminolevulinic acid synthetase</td>
<td>Rabbit reticulocytes</td>
<td>9.7</td>
</tr>
<tr>
<td>Apoglutamic oxaloacetic transaminase</td>
<td>Pig heart</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Glutamic dehydrogenase</td>
<td>Beef liver</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>Pig muscle</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Malic dehydrogenase</td>
<td>Pig heart</td>
<td>&lt;1</td>
</tr>
<tr>
<td>α-Amylase</td>
<td>Swine pancreas</td>
<td>3.2</td>
</tr>
<tr>
<td>γ-Aminopeptidase</td>
<td>E. coli</td>
<td>6.5</td>
</tr>
<tr>
<td>Arginase</td>
<td>Rovno liver</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>Leuconostoc mesenteroides</td>
<td>5.9</td>
</tr>
</tbody>
</table>

### DISCUSSION

**Amino Acid Analysis**

The amino acid composition of the protease is shown in Table IV. Relatively high content of arginine and a low content of lysine are considered the outstanding features.

**Chemical Modifications**

Diisopropyl fluorophosphate inhibited the protease activity completely at a concentration of 10 μM. The effect of modification of histidine residues by diethylpyrocarbonate is summarized in Fig. 8. The relationship between the number of modified histidine residues and loss of activity reveals that complete inhibition would be achieved by modifying 1 histidine residue/mol. Absorption spectra also showed that no modification of tyrosine or tryptophan residues occurred.

Trinitrobenzenesulfonic acid modified amino groups, but the activity remained unchanged. No tyrosines were affected when the protease was reacted with a 600 M excess of tetranitromethane at 25° for 60 min. Moreover, no tyrosines were modified with N-acetyl-

**Effect of Elastatinal on Rate of Decay of δ-Aminolevulinic Acid Synthetase Activity in Erythroblasts**

Bone marrow aspirates from normal persons were incubated with cycloheximide at 37° for various times. δ-Aminolevulinic acid synthetase activity was then measured. As shown in Fig. 11, the rate of decay of the enzyme activity was significantly decreased by the addition of Elastatinal, a specific inhibitor for elastase and for the protease.

**Protease Activity in Bone Marrow Cells from Various Animals**

Bone marrow cells were collected from several animals. Table V shows the protease activity in such cells. The activity in human cells proved to be highest.

**Relationship between Protease Activity and δ-Aminolevulinic Acid Synthetase Activity in Human Erythroblasts**

The protease activity was elevated in bone marrow cells from patients with rheumatoid arthritis, central vein thrombosis, and Behçet's disease in the acute phase (details of these results will be published elsewhere). In order to examine whether the protease regulates δ-aminolevulinic acid synthetase levels in mitochondria of erythroblasts, the relationship between δ-aminolevulinic acid synthetase activity and protease activity was examined by the use of erythroblasts from patients with rheumatoid arthritis and normal controls. An inverse relationship was obtained between δ-aminolevulinic acid synthetase activity and protease activity, as shown in Fig. 10.

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**DISCUSSION**

The protease described here is considered to be a serine protease because diisopropyl fluorophosphate (10 μM) inhibits the activity, while p-chloromercuribenzoate (1 mM) has no effect. It closely resembles elastase because it hydrolyzes ester substrates for elastase and is inhibited by Elastatinal. However, it does not hydrolyze synthetic amide substrates for elastase and shows no elastolytic activity. Although the present protease inactivates apopyridoxal enzymes, it is different from proteases that inactivate the apo form of certain pyridoxal enzymes (4) from the small intestines, skeletal muscles, and livers of rats in several respects. These proteases hydrolyze esters other than those affected by elastase, and are inhibited by Antiapin or Chymostatin but not inhibited by Elastatinal (4). Because of this, the present protease is considered to be a new enzyme.

In contrast to other intracellular proteases, the present enzyme was shown to be located in mitochondria of bone marrow cells. It occurs in erythroblasts and granulocytes, but not in lymphocytes, thrombocytes, or mature erythrocytes. Although proteases inactivating the apo form of certain pyridoxal enzymes have been found in the small intestines, skeletal muscles, and livers of rats (4), they are different from the protease reported here. Therefore, at present, the protease is considered to be specific to bone marrow cells. The activity was highest in human bone marrow cells as compared with that of other animals.

Amino acid analysis revealed a large content of arginine but small content of lysine. The basic properties of the
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protease are considered to be due to the large content of arginine. The composition also indicates that the protease is not identical with pancreatic elastase (46) nor with granulocyte elastase (47). The present protease showed an entirely different amino acid composition from that of the protease reported by Katunuma et al. (4) from the small intestines of rats.

The protease is stable over a wide range of pH values. This property resembles somewhat that of elastase, as reported by Lewis et al. (48).

Modification using diethylpyrocarbonate demonstrated that the protease loses activity when 1 histidine residue is modified. One carboxyl group was shown to be necessary for activity by modification with carbodiimide and [14C]glycine ethyl ester. Chemical modification also showed that tyrosine residues are buried in the protein. Two amino groups were modified with 2,4,6-trinitrobenzenesulfonic acid with no loss of activity. The NH₂-terminal amino group in the active center of elastase is known to be buried above pH 4.2 and difficult to modify (49). The same situation may apply to the present protease. From these results, we conclude that 1 histidine, 1 carboxyl, and 1 or more serine residues are essential to the activity of the protease. Serine, histidine, and a carboxyl group are considered as the essential units in the active center of serine proteases such as chymotrypsin (50, 51), elastase (52), and subtilisin (53). The active center of the present protease is considered similar to those of these serine proteases. However, 16 of 16 carboxyl groups of chymotrypsin were easily modified, by the same method as employed here, by Carraway et al. (54). They also showed that the carboxyl group in the active center is not modified because it is buried. The same behavior was shown for elastase by Shotton and Watson (52). In contrast to chymotrypsin and elastase, one carboxyl group essential to the activity of the present protease is easily modified with a water-soluble carbodiimide and [14C]glycine ethyl ester. Therefore, the environment around the carboxyl group essential to the protease activity must be rather different from those in chymotrypsin and elastase.

The protease activity was elevated in the erythroblasts of patients with rheumatoid arthritis. On the other hand, δ-a-methylselenic acid synthetase activity was demonstrated to be decreased in this disease. An inverse relationship was obtained between the protease activity and δ-a-methylselenic acid synthetase activity in erythroblasts. Taking into consideration that the protease is located on the inside of the inner mitochondrial membrane and that δ-a-methylselenic acid synthetase is also located there, it is probable that the protease attacks the apo form of δ-a-methylselenic acid synthetase. Together with these results, the fact that the addition of Elastatinal to bone marrow suspensions diminishes the rate of decrease of δ-a-methylselenic acid synthetase activity indicates that the protease regulates δ-a-methylselenic acid synthetase levels in the mitochondria of erythroblasts by degrading the apo form of the enzyme.

As shown under "Results," the protease activity is also high in granulocytes. The activity in granulocytes is elevated in the acute phase of Behçet's disease, in rheumatoid arthritis, and in central vein thrombosis. These facts, together with the observation that injection of human immunoglobulin G digested by this protease into rabbit skin induces inflammation (details will be published elsewhere), suggest that the protease in the granulocytes plays other important roles besides the regulation of pyridoxal enzyme levels in mitochondria.

Acknowledgments—I thank Professor N. Katunuma, Tokushima University, Tokushima, and Dr. K. Nakao, President of Jichi Medical School, for their advice and encouragement. Sedimentation equilibrium experiments were kindly conducted by Y. Igarashi, Dokkyo University, Tochigi. The technical assistance of Mitsu K. Ikeda is also gratefully acknowledged.

REFERENCES
A New Protease in Mitochondria of Bone Marrow Cells

Chemical modification of some enzymological material with diisopropylfluorophosphate and 3-mercaptoethanol.

Methods

There are several different methods for isolating mitochondria, and they are summarized in the following section. The method used here is the one described by Ovadi et al. (1967). The method involves the use of a differential centrifugation technique, with the mitochondria being isolated from the cytosol by a series of incubations and centrifugations. The mitochondria are then purified by a series of additional centrifugations, and the final yield is determined by measuring the activity of the enzyme being studied.

Results

The purification of the protease resulted in a 10-fold increase in specific activity, with a corresponding increase in the yield of the enzyme. The final yield was determined to be 3.5 mg per g of tissue, which is comparable to the yield of other proteases isolated from mammalian cells.

Discussion

The results presented here indicate that a new protease is present in the mitochondria of bone marrow cells. This protease is distinct from other proteases previously described, and its presence in the mitochondria suggests a role in the regulation of mitochondrial function.

References


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Table I

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
<th>Purification</th>
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<tr>
<td>Crude extract</td>
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<td>1,493</td>
<td>1.3</td>
<td>160</td>
<td>1</td>
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<tr>
<td>Dialyzed extract</td>
<td>115</td>
<td>708</td>
<td>3.5</td>
<td>54</td>
<td>2.9</td>
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<tr>
<td>DEAE-cellulose</td>
<td>450</td>
<td>530</td>
<td>4.6</td>
<td>42</td>
<td>1.3</td>
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<tr>
<td>Sodium phosphate A500</td>
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<td>306</td>
<td>15.5</td>
<td>22</td>
<td>12.9</td>
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<tr>
<td>Osmium chloride</td>
<td>32</td>
<td>307</td>
<td>20.0</td>
<td>35</td>
<td>17.3</td>
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<tr>
<td>Carbohydrate extract</td>
<td>2</td>
<td>23</td>
<td>25.0</td>
<td>7</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* The unit was defined as the protease activity which reduces or inhibits trypsine activity by 50% in 5 min at 37°C in the presence of 0.05 M potassium phosphate buffer, pH 8.0, at 37°C. The unit of specific activity was defined as 100 mg of protein/30 min read at 260 nm.

Table II

The protease activity in various sodium phosphate buffers

<table>
<thead>
<tr>
<th>Sodium phosphate buffer</th>
<th>protease activity (unit/mg protein)</th>
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<tr>
<td>50 mM sodium phosphate</td>
<td>10</td>
</tr>
<tr>
<td>100 mM sodium phosphate</td>
<td>15</td>
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<td>200 mM sodium phosphate</td>
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Table III

Table IV

<table>
<thead>
<tr>
<th>Molar ratio</th>
<th>NPN/Phosphate</th>
<th>NPN/Tris cations</th>
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<tr>
<td>1:1</td>
<td>0.45</td>
<td>1.6</td>
</tr>
<tr>
<td>2:1</td>
<td>2.35</td>
<td>5.6</td>
</tr>
<tr>
<td>3:1</td>
<td>3.91</td>
<td>10.8</td>
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<tr>
<td>4:1</td>
<td>4.98</td>
<td>18.3</td>
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Table V

The protease activity in bone marrow cells

<table>
<thead>
<tr>
<th>Animal</th>
<th>protease activity (unit/mg protein)</th>
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<tbody>
<tr>
<td>Mouse</td>
<td>0.33</td>
</tr>
<tr>
<td>Rat</td>
<td>0.49</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.29</td>
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<tr>
<td>Guinea pig</td>
<td>0.26</td>
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Table VI

<table>
<thead>
<tr>
<th>Enzyme added (mg/ml)</th>
<th>Activity (unit/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>N</td>
<td>1.2</td>
</tr>
<tr>
<td>C</td>
<td>0.8</td>
</tr>
<tr>
<td>A</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Fig. 1. Relationship between the concentration of protease added and the inhibition of the protease activity. 1. The protease activity expressed as the protease activity that reduced or inhibited trypsine activity by 50% in 5 min at 37°C in the presence of 0.05 M potassium phosphate buffer, pH 8.0, at 37°C. The unit of specific activity was defined as 100 mg of protein/30 min read at 260 nm.

Fig. 2. Effect of a protease on the protease activity. The protease activity expressed as the protease activity that reduced or inhibited trypsine activity by 50% in 5 min at 37°C in the presence of 0.05 M potassium phosphate buffer, pH 8.0, at 37°C. The unit of specific activity was defined as 100 mg of protein/30 min read at 260 nm.

Fig. 3. Inhibitory effect of a protease on the protease activity. The protease activity expressed as the protease activity that reduced or inhibited trypsine activity by 50% in 5 min at 37°C in the presence of 0.05 M potassium phosphate buffer, pH 8.0, at 37°C. The unit of specific activity was defined as 100 mg of protein/30 min read at 260 nm.
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