A NEW PROTEASE INACTIVATING δ-AMINOLEVULINIC ACID SYNTHETASE IN MITOCHONDRIA OF HUMAN BONE MARROW CELLS

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Summary;

A new protease which inactivates specifically apo-forms of certain pyridoxal phosphate enzymes was found in the human bone marrow cells. The enzyme, which is located in the mitochondrial fraction, was purified so as to be nearly homogeneous as judged by polyacrylamide disc gel electrophoresis. It is considered to be a seryl protease, because diisopropylfluorophosphate (10^{-5} M) inhibited the protease activity completely, while p-chloromercuribenzoate (10^{-3} M) had no effect. It had some resemblance to elastase, but had no elastinolytic activity.

INTRODUCTION

δ-Aminolevulinic acid synthetase* which requires pyridoxal-5'-phosphate as prosthetic group is known to be rate limiting enzyme in the heme biosynthetic pathway. The enzyme activity in the erythroblasts of patients with primary sideroblastic anemia was found to be decreased (1). Almost all the hematological features of this disease could be explained by a decrease of this enzyme activity (1). In the course of investigations on the regulatory mechanism of ALA-S activity in the erythropoietic organ, a protease which inactivates specifically the apo-form of pyridoxal phosphate enzymes, including ALA-S, was found in mitochondria of bone marrow cells. Although some properties of the protease resemble those of the proteases reported by Katunuma et al (2-6) from small intestine, liver and skeletal muscle of rat, this protease is to be considered as different from these latter proteases with respect to many properties described in Results and Discussion.

The protease also has some properties similar to elastase, it is however, not identical with elastase, because this protease showed no elastinolytic activity.

From the results described above, the present protease is considered to be

* Abbreviation; ALA-S, δ-aminolevulinic acid synthetase; OTA, ornithine aminotransferase

a new protease. The protease was purified so as to be nearly homogeneous as judged by polyacrylamide disc gel electrophoresis and the present paper describes the purification method and some properties of this new protease. Furthermore, since the activity of this protease increases several-fold by high protein intake, it is important to discuss its medical significance in relation to hematological diseases caused by disturbance of heme synthesis.

MATERIALS AND METHODS

Preparation and assay of substrate enzymes The enzyme (ALA-S) was assayed and purified as described by Aoki et al (1,7). Apo-ALA-S was obtained by dialysing the enzyme against 0.2 M Tris buffer (pH 7.0), containing 10^{-4} M of NH_2OH, for 6 hours. Crystalline OTA, homoserine deaminase and serine dehydratase were obtained and converted into their apo-form by the method of Matsuzawa et al (8), Matsuo et al (9), and Suda et al (10), respectively. These enzyme activities were assayed by the method of Katunuma et al (11), Matsuo et al (9), and Suda et al (10), respectively. Crystalline glutamic dehydrogenase (bovine liver), lactic dehydrogenase (pig muscle), and Malate dehydrogenase (pig muscle) were purchased commercially, and assayed spectrophotometrically at 25°C (12-14). Crystalline α-amylase, asparaginase, glucose-6-phosphate dehydrogenase, and arginase were also obtained commercially and were assayed by the method of Somogi (15), Meister (16), Kornberg et al (17), and Greenberg (18), respectively.

Assay of the protease activity Synthetic substrates for proteases were purchased from Protein Research Foundation, Osaka, Japan. Protease activity using synthetic esters as substrates was measued by the method of Roberts (19). When amide substrates were used, the enzyme activity was measured by monitering continuously, at 410 nm, the p-nitroaniline liberated. The protease activity using apo-OTA as a substrate was measured according to Katunuma (2,4). One unit was defined as the amount of protease inactivating 50 % of the respective substrate enzyme in 30 min. In the case of enzymes other than apo-OTA as substrate, values illustrate inactivations of these substrate enzymes as percentages of that of OTA. Measurement of elastase activity using congored-elastin as substrate was conducted according to the method of Shotton (20).

Purification of the protease Bone marrow cells were collected into a 1.15 % KCl solution, containing 0.01 M potassium phosphate buffer, pH 7.5 (buffer A) after cracking the human resected ribs. Approximately 10 ml of the pellet, obtained after centrifugation at 10,000 xg for 10 min, was resuspended in 300-400 ml of buffer A. After sonication for 1 min at 80 W (Branson, Model W 185), the solution was centrifuged at 20,000 xg for 20 min. The enzyme was extracted from the precipitate at 37°C for 30 min with 80 ml of 0.5 M potassium phosphate buffer, pH 7.0. The supernatant, obtained after centrifugation at 20,000 xg for 10 min, was dialysed against 50 volumes of water for 3 hours. The enzyme solution was applied to a DEAE-cellulose column (2.6 x 10 cm) equilibrated with 0.01 M potassum phosphate buffer, pH 7.5. The enzyme was eluted with 0.5 M potassium phosphate buffer, pH 7.5, after washing the column with 200 ml of 0.01 M potassium phosphate buffer, pH 7.5. Approximately 70 ml of the eluate which contained high enzyme activity was precipitated with ammonium sulfate (75 %). The precipitate was redissolved in 2 ml of o.o5 M phosphate buffer, pH 7.5. Then 0.2 ml of 1 % protamine sulfate solution was added slowly and sirred for 20 min at 4°C. After centrifugation at 15,000 xg for 10 min, the supernatant was applied to a Sephadex G-75 column (2.6 x 45 cm). The fractions which contained high enzyme activity were put on a CM-cellulose column (2.6 x 10 cm) equilibrated with 0.01 M potassium phosphate buffer, pH 7.5, and then with 100 ml of 0.1 M potassium phosphate buffer, pH 7.5. Elution was performed with 0.3 M potassium phosphate buffer, pH 7.5. The enzyme fraction was precipitated with ammonium sulfate (75 %). The precipitate was dissolved in 1 ml of 0.05 M potassium phosphate

buffer, pH 7.5. The enzyme was then applied to a Sephadex G-75 column (1.9 x 45 cm) as a final step of purification. Protein concentrations were determined by the method of Lowry et al. (21).

Procedure for subcellular fractionation Bone marrow cells (ca. 6 x 10^8) from rats fed on high protein diet (70 % casein) were used as the starting material for subcellular fractionation. The cells were suspended in 10 ml of 0.25 M sucrose containing 0.05 % bovine serum albumin and 0.01 M Tris, pH 7.5 buffer. The suspension was homogenized in a Potter-Elvehjem homogenizer (Teflon type, 1,000 rpm, 5 min). The following preparation methods were used; the method of Muramatsu et al. (22) for nuclear fraction, Johnson et al. (23) for mitochondria, Ragab et al. (24) for lysosomal fraction, Hogeboom's method (25) for microsomes. Succinate-cytochrome C reductase activity, acid phosphatase activity, and NADPH-cytochrome C reductase activity were measured by the method of Tisdale (26), Kind et al. (27), and Williams et al. (28), respectively.

Separation of hematological cells Separation of bone marrow cells was conducted by the modified method of Kinoshita et al. (29). Bone marrow cells were layered on the separating medium consisting of three parts which were made by mixing appropriate amount of Lymphoprep (Nyegaard and CO.AS.Oslo) and saline. Specific gravity of each part was 1.045 (upper part), 1.060 (middle part), and 1.065 (lower part). After centrifugation at 400 xg for 30 min, the cell layers formed between upper and middle part, and between middle and lower part were collected to obtain young granulocytes and erythroblasts, respectively. Lymphocytes and mature granulocytes were obtained by the method of Thorby et al. (30), and Bøyum (31), respectively.

RESULTS AND DISCUSSION

The purification of the protease is shown in Table 1. The purified enzyme was analyzed by polyacrylamide disc gel electrophoresis. A main band accompanying several minor bands is found. The enzyme activity was demonstrated only

Table 1. Purification of the protease

Steps	Vol. (ml)	Protein (mg)	Activity (x 10^4 units)	Specific activity (units/mg protein)	Yield (%)	Purification
Sonication mixture	352	3625	35.2	97	100	1
Phosphate buffer extract	80	200	15.7	784	45	8.1
DEAE-cellulose	74	74	6.1	820	17	8.5
Sephadex G-75	70	30	5.8	1930	16	20
CM-cellulose	87	7.8	2.3	2900	6	30
Sephadex G-75	39	2.5	0.9	3730	3	38.5

in the main band. The molecular weight of this enzyme was estimated to be approximately 18,000, judging from Sephadex G-75 column chromatography. The optimum pH was around 8.5. Diisopropylfluorophosphate inhibited the activity completely at 10^{-5} M. Neither ethylene diamine tetraacetic acid (10^{-3} M) nor p-chloromercuribezoate (10^{-3} M) inhibited the enzyme activity. While OTA activity in the incubation mixture decreased rapidly, the amount of trichloroacetic acid insoluble enzyme protein did not change appreciably with small amount of ninhydrin positive substance released, as shown in Fig. 1. From this result it is concluded that the protease inactivates apo-OTA by splitting close to the C and/or NH_2 terminus of the protein. The substrate specificity of the protease is shown in Table 2. Inactivation of nonpyridoxal enzymes was negligible or small, however, pyridoxal enzymes in their apo-form were inactivated rapidly. The specificity of the protease for synthetic substrates is also illustrated in Table 2, and the protease hydrolyzed only synthetic esters for elastase (N-acetyl-(ℓ-alanyl)$_3$ methyl ester and N-benzoyl-ℓ-alanine methyl ester).

The effect of several protease inhibitors from bacteria was examined. Chymostatin, Pepstatin, Leupeptin, and Antipain did not inhibit the enzyme activity at a concentration of 100 μg/ml. On the other hand, Elastatinal (32) which is known to be a specific inhibitor for elastase inhibited approximately 80 % of the activity at a concentration of 20 μg/ml. These facts

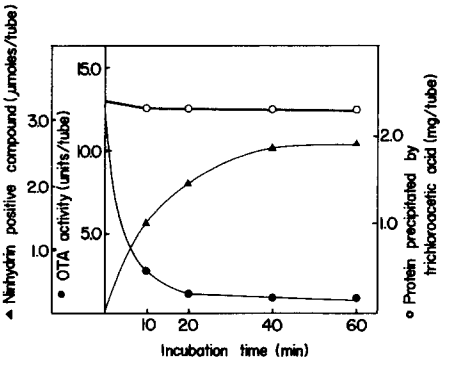

Fig. 1. Relationship between inactivation of OTA and protein degradation by the protease. Each incubation mixture contained apo-OTA, 2.4 mg; potassium phosphate buffer (pH 8.0), 80 μmoles; and enzyme, 180 units in a final volume of 1.0 ml. At the end of incubation an aliquot of 0.02 ml of the reaction mixture was removed to measure OTA activity, and then 0.2 ml of 50 % trichloroacetic acid (TCA) was added. The reaction mixture was then centrifuged at 3,000 xg for 10 min. The amount of both protein precipitated by TCA and ninhydrin positive material in the supernatant were measured by the method of Lowry et al. (21) and Rosen (33), respectively. ●——●, OTA activity; ▲——▲, ninhydrin positive material; o——o, protein precipitated by TCA.

Table 2. Substrate specificity of the protease

Substrates	Sources	Percent activity
Apo-ornithine transaminase	rat liver	100
Apo-homoserine deaminase	rat liver	185
Apo-serine dehydratase	rat liver	21
Apo-ALA-S	rabbit reticulocytes	9.7
Glutamic dehydrogenase	bovine liver	<1
Lactic dehydrogenase	pig muscle	<1
Malic dehydrogenase	pig heart	<1
α-Amylase	swine pancreas	3.2
ℓ-Asparaginase	E. Coli	6.5
Arginase	bovine liver	<1
N-acetyl-ℓ-tyrosine ethyl ester		<0.1
N-acetyl-ℓ-phenylalanine ethyl ester		<0.1
N-acetyl-ℓ-tryptohpan ethyl ester		<0.1
L-tyrosine ethyl ester		<0.1
N-benzoyl-ℓ-arginine ethyl ester		<0.1
N-tosyl-ℓ-arginine methyl ester		<0.1
N-acetyl-(ℓ-alanyl)$_3$ methyl ester		3.2
N-benzoyl-ℓ-alanine methyl ester		0.8
N-acetyl-(ℓ-alanyl)$_3$ p-nitroanilide		<0.1
N-succinyl-(ℓ-alanyl)$_3$ p-nitroanilide		<0.1

* The protease activity using apo-OTA as substrate was measured according to the method of Katunuma (2,4). When enzymes other than apo-OTA or synthetic substrates were used as substrates for this protease, values illustrate inactivations or hydrolysis of these enzymes or synthetic substrates as percentages of that of OTA.

indicate that the protease closely resemble elastase. However, both the fact that the protease had no elastinolytic action when congored-elastin was used as substrate, and the fact that amide substrates for elasrase (N-acetyl-(ℓ-alanyl)$_3$ p-nitroanilide and N-succinyl-(ℓ-alanyl)$_3$ p-nitroanilide) were not hydrolyzed by this protease (Table 2) suggest that the protease is not identical with elastase. These results indicate that the protease is different from proteases reported by Katunuma et al. (2) previously. As demonstrated by the distribution pattern of the marker enzymes tested in the different fractions a good separation of the subcellular elements was achieved. The protease is most active in the mitochondrial fraction as shown in Table 3.

The activity and/or amount of the protease are markedly influenced by dietary conditions. The increase of protease activity in bone marrow cells of rats fed a high protein diet, 70 % casein, for 1, 4 and 8 weeks were observed as

Table 3. Subcellular fractionation of bone marrow cells

Fractions	Succinate cytochrome C reductase (nmoles cytochrome C reduced/min/mg protein)	Acid phosphatase (King-Armstrong units/mg protein)	NADPH-cytochrome C reductase (units/mg protein) ($\times 10^{-3}$)	New protease (units/mg protein)
Nuclear fraction	18	497	25	325
Mitochondrial fraction	900	864	67	2314
Lysosomal fraction	11	2023	230	54
Microsomal fraction	5	982	600	6
Supernatant fraction	3	242	18	2

* Each fraction was sonicated at 70 W for 10 sec (Barnson, Model W185), and then enzyme activities were measured. Measurement of new protease activity using apo-OTA as substrate was conducted according to the method of Katunuma (2,4).

Table 4. Distribution of protease activity in hematological cells

Cell types	Enzyme activity* (units/10^8 cells)
Young granulocytes	230
Mature granulocytes	59
Erythroblasts	310
Red blood cells	<1
Lymphocytes	<20

* Separation of hematological cells was conducted as described in Materials and Methods. Enzyme was prepared as follows; 10-20 x 10^6 of hematological cells were suspended in 0.9 % NaCl containing 0.01 M potassium phosphate buffer (pH 7.5), and sonicated at 70 W for 15 sec (Branson, Model W 185). From the precipitate, which was obtained by centrifuging the sonicated solution at 20,000 xg for 10 min, the protease was extracted with 0.5 M potassium phosphate buffer (pH 7.0) for 30 min at 37°C. Enzyme activity using apo-OTA as substrate was measured according to the method of Katunuma (2,4).

640, 1270 and 4390 units/10^8 cells, respectively, while the activity of rats fed 20 % casein diet showed only 108 units/10^8 cells under these conditions. While this protease is regulated by various dietary conditions, the activity

of the protease reported by Katunuma et al. (2) did not change significantly under different dietary or hormonal conditions. The cell distribution pattern of the protease activity in bone marrow is analyzed in Table 4. The protease activity is high both in erythroblasts and in young granulocytes, and apparently decreases during the course of maturation. It may be related to the content of mitochondria in the different cell types. Preliminary data suggest that the protease activity in the bone marrow of patients suffering from rheumatoid arthritis and chronic myelogeneous leukemia is increased markedly. These fingings indicate a possible role of this enzyme in various hematological diseases.

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