Purification and Properties of α -Amino- β -Carboxymuconate- ϵ -Semialdehyde Decarboxylase (ACMSD), Key Enzyme of Niacin Synthesis from Tryptophan, from Hog Kidney

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Summary α -Amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase (ACMSD) (EC 4.1.1.45) was purified to a homogeneous state from hog kidney cytosol by ammonium sulfate fractionation, Butyl-Toyopearl 650, hydroxyapatite, DEAE-Sephadex, Toyopearl HW55, Superdex 200 and TSK-gel G3000SW chromatographies. The molecular weight of the enzyme was estimated to be 58,000 by TSK-gel G3000SW gel filtration. The optimum pH (constant concentration) was 7.5. The K_m for α -amino- β -carboxymuconate- ϵ -semialdehyde was 1.61×10^{-5} M. The activity of purified enzyme was inhibited by some chemical modifying reagents such as monoiodoacetic acid and *p*-(chloromercuri)benzoic acid. A sulfhydryl group was deduced to exist in the active site of the enzyme.

Key Words tryptophan, niacin, aminocarboxymuconate semialdehyde decarboxylase, hog kidney, picolinic carboxylase, decarboxylase

α -Amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase (ACMSD) (EC 4.1.1.45) decarboxylates α -amino- β -carboxymuconate- ϵ -semialdehyde (ACMS) to form α -aminomuconate- ϵ -semialdehyde (AMS). In 1956, Mehler termed it picolinic carboxylase (1).

It is well known that tryptophan is converted to niacin derivatives such as NAD and NADP in many kinds of mammals (2,3). The tryptophan-NAD pathway is very important from a nutritional standpoint because of the conversion of an amino acid into a vitamin. This pathway is known to be affected by many nutritional factors such as carbohydrate (4), lipid (5) and protein (6). ACMSD plays a key role in regulating NAD biosynthesis from tryptophan. If ACMSD activity is high enough, most tryptophan is metabolized to enter the glutarate pathway as shown in Fig. 1. On the other hand, if the activity is low, tryptophan is metabolized to enter the NAD pathway (3,7), that is, the apparent tryptophan-niacin conversion [urinary excretion of nicotinamide + N^1 -methyl-

Fig. 1. Conversion of tryptophan to NAD. 3HA: 3-hydroxyanthranilate, ACMS: α -amino- β -carboxymuconate- ϵ -semialdehyde, ACMSD: α -amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase.

nicotinamide + N^1 -methyl-2-pyridone-5-carboxamide + N^1 -methyl-4-pyridone-3-carboxamide ($\mu\text{mol/day}$)/daily tryptophan intake ($\mu\text{mol/day}$)] is increased.

ACMSD is present in the liver and kidney of many species. It has been reported that liver ACMSD activity in the cat, cow, mouse and human is 32, 5, 3, and 2 times higher, respectively, than that of the rat liver (3).

On the other hand, ACMSD activity is altered by many factors; dietary protein level (8), dietary fatty acid (9–11) and hormones (12,13). Moreover, in streptozotocin diabetic rats, the enzyme activity is markedly increased (14,15). However, the mechanism for these phenomena has still not been completely clarified. From the point of view of nutritional chemistry, it is important to elucidate such phenomena at the molecular level. It is also necessary to purify ACMSD and clone cDNA of ACMSD in order to elucidate the regulatory mechanism of ACMSD in various nutritional conditions.

In 1970, Nishizuka et al. partially purified ACMSD from the cat liver and examined its properties (16). In the present experiment, we purified and examined some properties of ACMSD from hog kidney, because it seemed to be easy to obtain a large amount of the materials for purification and to purify it as a single enzyme protein.

MATERIALS AND METHODS

Materials. Fresh hog kidney was obtained from a slaughterhouse in Tokyo and was stored at -20°C until use. 3-Hydroxyanthranilic acid (3-HA) and gel filtration molecular weight markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Molecular weight markers on sodium dodecyl sulfate polyacrylamide gel disk electrophoresis (SDS-PAGE) and polyacrylamide gel (Pagel AE-6000, NPU-12.5L) were purchased from Oriental Yeast Kogyo Co. (Tokyo) and Atto Co. (Tokyo), respectively. Silver stain kit, iodoacetamide, idoacetic acid, *N*-acetylimidazole (AI), *p*-(chloromercuri)benzoic acid (PCMB), *N*-ethylmaleimide (NEM) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) were obtained from Wako Pure Chemicals (Osaka, Japan). Diethyl pyrocarbonate (DEP) and 1,2-naphthoquinone 4-sulfonic acid (NQS) were obtained from Sigma Chemicals Co. All reagents used were of the highest grade.

Columns. Butyl-Toyopearl 650M and TSKgel G3000SW were obtained from Tosoh Co. (Tokyo). DEAE-Sephadex A-50 and Superdex 200 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). DIAFLO Ultrafiltration Membranes 10PM10 and Econo-Pac 10DG were purchased from Amicon Co. (Ireland) and Bio Rad Laboratories (Richmond, CA, USA), respectively.

Enzyme assay. The activity of ACMSD was estimated by measuring the decrease in absorbance of ACMS at 360 nm (14, 16). In a quartz cuvette of 1-cm light path with a 3-ml capacity, 10 μl of 3-HA (1 mg/ml, ethanol), 1.0 ml of Tris-acetate buffer (0.2 M, pH 8.0), 0.1 ml of 3-HA oxygenase solution (5.34×10^{-7} mol ACMS/min/ml) was added to water to a total volume of 2.8 ml (reaction mixture solution). This reaction mixture was prepared immediately before use. The reaction performed at 25°C was followed spectrophotometrically at 360 nm. After the formation of ACMS, judged as completed by its absorbance at 360 nm, 40 μl of ACMSD solution was added. The decrease in absorbance at 360 nm was followed at 15-s intervals against a blank incubation that contained all ingredients except the ACMSD solution. Each velocity was corrected for the spontaneous decrease in absorbance due to the formation of quinolinic acid.

Definition of unit and specific activity. One unit of ACMSD is defined as the amount of enzyme which causes the reduction of 1 μmol of ACMS per min at 360 nm. Since the molar extinction coefficient of ACMS at 360 nm is 4.5×10^4 under these conditions, 1 unit of the enzyme activity corresponds to the disappearance of 1 μmol of the substrate per min.

Protein concentration. Protein concentration was determined by the method

of Lowry et al., using bovine serum albumin as a standard (17) and purified enzyme (TSK G3000SW gel filtration treatment) was determined by the method of Kalckar (18).

Optimum pH determination. The standard assay for the enzyme using 25 μ l of Tris-acetate buffer (0.2 M, pH 8.0) and 975 μ l water instead of 1 ml of Tris-acetate buffer (0.2 M, pH 8.0), except for various buffers (final concentration, 0.05 M) at indicated pH values, was performed.

Kinetics. A quartz spacer (9 mm $\times$ 10 mm $\times$ 50 mm) and a quartz cuvette of 1-cm light path with a 3-ml capacity were used to make a light path of reaction mixture 1 mm, that enable the concentration of ACMS to be in the range of 0–1.4 $\times$ 10⁻⁴ M.

Chemical modifying reagents. AI was dissolved in 0.1 M Tris-HCl buffer solution (pH 7.5). PCMB was first dissolved in 0.1 M NaOH and the solution was then diluted with 0.05 M potassium phosphate buffer solution (pH 7.0). DEP was dissolved ethanol and then diluted with 0.1 M cold potassium phosphate buffer solution (pH 6.0).

Composition of buffer. Buffer A: 50 mM potassium phosphate buffer (pH 7.0) containing 0.14 M KCl, 5 mM 2-mercaptoethanol (2-ME), 1 mM dithiothreitol (DTT) and 1 mM EDTA.2Na. Buffer B: buffer A 10 ml and (NH₄)₂SO₄ 3.9 g. Buffer C: (NH₄)₂SO₄ 176 g and buffer A 1 liter and glycerin 10 ml. Buffer D: 10 mM potassium phosphate buffer (pH 7.0) containing 0.2 M KCl, 5 mM 2-ME 1 mM DTT. Buffer E: 120 mM potassium phosphate buffer (pH 7.0) containing 0.2 M KCl, 5 mM 2-ME, 1 mM DTT. Buffer F: 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M KCl, 5 mM 2-ME and 1 mM DTT. Buffer G: 10 mM Tris-HCl buffer, pH 8.0, containing 0.35 M KCl, 5 mM 2-ME and 1 mM DTT. Buffer H: 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M KCl, 5 mM 2-ME, 1 mM DTT and 1 mM EDTA.2Na. Buffer I: 50 mM potassium phosphate buffer (pH 7.0) containing 0.14 M KCl, 1 mM DTT and 1 mM EDTA.2Na.

Purification. All steps were performed at 0–4°C.

Step 1. Frozen hog kidney (300 g) was cut finely and homogenized in a Waring blender in 4 volumes of buffer A. Connective tissues were removed by passing through two-layer gauze. The homogenate was centrifuged for 60 min at 15,000 $\times$ g.

Step 2. Treatment with protamine sulfate. To the supernatant solution, 240 ml (0.8 ml/g tissue) of 0.4% solution of protamine sulfate (pH 7.0) was added slowly. The mixture was stirred for 30 min, and the precipitate was removed by centrifugation (8,000 $\times$ g for 50 min).

Step 3. Ammonium sulfate fractionation. (NH₄)₂SO₄ was added to the supernatant with stirring to give 50% saturation (added 313 g (NH₄)₂SO₄ against 1 liter eluate). After 3 h, the precipitate was removed by centrifugation (8,000 $\times$ g for 50 min). To the supernatant solution, (NH₄)₂SO₄ was added to 65% saturation (added 101 g (NH₄)₂SO₄ to 1 liter supernatant solution). After 3 h, the precipitate was collected by centrifugation (8,000 $\times$ g for 50 min) and was dissolved in a

minimal amount of buffer B. The same amount of buffer A was added to obtain a 30% saturated $(\text{NH}_4)_2\text{SO}_4$ solution.

Step 4. Butyl-Toyopearl column chromatography. The enzyme solution was put on a Butyl-Toyopearl column (diameter, 5.0 cm; length, 20 cm) equilibrated with buffer C. After washing the column with 2 liter of buffer C, the enzyme was eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ from 30% (buffer C) to 0% saturation [buffer A containing 0.4% (v/v) glycerin] (total volume of 2 liter). The active fractions were pooled and the solution was desalted by ultrafiltration using an Amicon PM10 membrane filter. The Econo-Pac 10DG column was used to exchange the solvent of enzyme for buffer D.

Step 5. Hydroxyapatite column chromatography. The active fraction of step 4 was put on a hydroxyapatite column (diameter, 5 cm; length, 20 cm) equilibrated with buffer D. After the column was washed with 2 liter of buffer D, the enzyme was eluted with a linear gradient of 10 mM (buffer D) to 120 mM potassium phosphate buffer (buffer E) (total volume of 2 liter). The enzyme solution was collected by the fraction tube containing 8 μl 2-ME and 20 μl of 1 mM EDTA.2Na (pH 7.0) (one fraction tube contained 18 ml enzyme solution). The active fractions were pooled and the solution was desalted by ultrafiltration. The Econo-Pac 10DG column was used to exchange the solvent of enzyme for buffer F.

Step 6. DEAE-Sephadex column chromatography. The active fraction of step 5 was put on a DEAE-Sephadex A-50 column (diameter, 2.8 cm; length, 20 cm) equilibrated with buffer F. After the column was washed with 400 ml of buffer F, the enzyme was eluted with a linear gradient of 100 mM (buffer F) to 350 mM KCl buffer (buffer G) (total volume of 800 ml). Active fractions were pooled and desalted by ultrafiltration. The Econo-Pac 10DG column was used to exchange the solvent of enzyme for buffer H.

Step 7. Toyopearl HW55 column chromatography. The active fraction of step 6 was put on a Toyopearl HW55 column (diameter, 1.6 cm; length, 70 cm) equilibrated with buffer A. The enzyme solution was eluted with 300 ml of buffer A. Active fractions were pooled and concentrated by ultrafiltration.

Step 8. Superdex 200 column chromatography (FPLC). The active fraction of step 7 was applied to a Superdex 200 column (diameter, 1.6 cm; length, 60 cm) equilibrated with buffer H. Active fractions were pooled and concentrated by ultrafiltration.

Step 9. TSKgel G3000SW column chromatography (HPLC). The active fraction of step 8 was put on a TSK gel G3000 SW column (diameter, 21.5 mm; length, 60 cm) equilibrated with buffer I. Active fractions were pooled and concentrated by ultrafiltration.

Storage of the enzyme. When the partially purified or purified enzyme was stored for at least 1 month, the enzyme could be stored without loss of activity at -20°C in buffer A containing 20% glycerol.

RESULTS

Purification

A summary of the purification is presented in Table 1. ACMSD was purified 2200-fold from the first step. The recovery was 1.8%. In regard to purification of ACMSD from hog kidney, DEAE-Sephadex column chromatography and Superdex 200 column chromatography were very effective. The purified enzyme gave a single band on PAGE, as shown in Fig. 2.

Molecular weight

The molecular weight of the enzyme was estimated to be 58,000 and 41,000 by gel filtration on a TSK-gel G3000SW column and by SDS-PAGE, respectively (Fig. 2). The molecular weight obtained by gel filtration and SDS-PAGE were different.

pH optimum

Effect of pH on the ACMSD activity is shown in Fig. 3. Maximum enzyme activity was attained at pH 7.5 in 0.05 M Tris-HCl buffer. The ACMSD activity in the potassium phosphate buffer was observed to be lower than that in the Tris-HCl buffer at the same pH value.

Kinetics

The Lineweaver-Burk plots of ACMSD are shown in Fig. 4. The K_m value for ACMS was calculated as 1.61×10^{-5} M.

Table 1. Summary of the purification of α -amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase (ACMSD) from hog kidney.

Purification step	Protein (mg)	Specific activity (munits/mg protein)	Total activity (units)	Recovery (%)
Crude extract	22,037	1.68	37.0	100
Protamine treatment	16,416	1.66	27.3	74
(NH ₄) ₂ SO ₄ fraction	4,680	1.76	8.24	22
Butyl-Toyopearl	291	21.2	6.17	17
Hydroxyapatite	29	248	7.19	19
DEAE-Sephadex	2.39	1,522	3.64	9.8
Toyopearl HW-55	2.31	1,545	3.57	9.6
Superdex 200 (FPLC)	0.50	3,511	1.76	4.8
TSK-G3000SW (HPLC)	0.18	3,789	0.68	1.8

One unit equals the amount of enzyme needed to consume $1 \mu\text{mol}$ of the α -amino- β -carboxymuconate- ϵ -semialdehyde (ACMS) per min at 25°C under standard assay conditions.

Fig. 2. Native and SDS-polyacrylamide gel electrophoresis of the purified enzyme. Native and SDS-polyacrylamide disk gel electrophoresis was performed according to the method of Ornstein-Davis (22,23) and Laemmli (24). 12.5% gel was used. The enzyme protein was electrophoresed at 20 mA per gel for 70 min at room temperature. Protein was detected with silver stain kit.

Fig. 3. Effect of pH on activity of ACMSD. The enzyme assay was performed at 25°C in 50 mM buffer. ■ Potassium phosphate buffer; ● Tris-HCl buffer; ◆ Glycine-NaOH buffer.

Fig. 4. Lineweaver-Burk plots of initial velocity against substrate concentration.

Table 2. Effect of SH, amino and imidazole groups modifying reagents on ACMSD activity.

Reagents	Inhibition (%)
Iodoacetamide	100
Monoiodoacetic acid	100
NEM	99
PCMB	100
AI	49
DEP	99

ACMSD ($2.9 \times 10^{-6} \text{ M}$, $6 \mu\text{l}$) was incubated with iodoacetamide (100 mM, $28 \mu\text{l}$), monoiodoacetic acid (100 mM), NEM (100 mM), PCMB (10 mM) or AI (100 mM) for 40 min at 25°C . When the effect of DEP (100 mM) was investigated, the incubation was carried out for 30 min at 0°C (ACMSD solution containing chemical modifying reagent). ACMSD activity was determined using the standard assay conditions (reaction mixture solution $2.8 \text{ ml} + \text{ACMSD solution containing chemical modifying reagent } 34 \mu\text{l}$). Values are expressed as a percentage for the activity preincubated without chemical modifying reagents at 25°C .

Effect of chemical modifying reagents

Shown in Table 2, the enzyme was strongly inhibited by iodoacetamide, monoiodoacetic acid, NEM, PCMB and DEP. It is possible that the SH and amino or imidazole groups may exist in the active site of ACMSD of hog kidney.

DISCUSSION

The conversion ratio of tryptophan to niacin is altered by nutritional conditions (5, 6, 8, 9). These phenomena are mainly attributable to the alteration in the activity of ACMSD. ACMSD plays a key role in the tryptophan-NAD pathway. In this experiment, we purified ACMSD from hog kidney to a single protein band on PAGE. In purification steps, recovery of ACMSD was increased by hydroxy-

apatite chromatography (Table 1). Because the eluate from Butyl-Toyopearl column contained ammonium sulfate. A preliminary experiment revealed that ACMSD activity was suppressed by ammonium sulfate and that it was recovered by the removal of ammonium sulfate (data not shown). On the other hand, MgCl_2 slightly stimulates hog kidney ACMSD activity (130% at $5 \times 10^{-4} \text{ M}$) as well as cat liver ACMSD activity (130% at $2 \times 10^{-4} \text{ M}$) (16).

Many decarboxylases such as glutamate decarboxylase (EC 4.1.1.15), lysine decarboxylase (EC 4.1.1.18), arginine decarboxylase (EC 4.1.1.19) and ornithine decarboxylase (EC 4.1.1.17) require pyridoxal phosphate (19). On the other hand, orotidine-5'-phosphate decarboxylase (EC 4.1.1.23) does not require pyridoxal phosphate (19). The tryptophan catabolic pathway to nicotinic acid requires pyridoxal phosphate at the metabolic step of kynureninase (20). So we examined whether ACMSD requires pyridoxal phosphate protein or not. The purified ACMSD did not exhibit absorption at 340–430 nm at neutral pH. The purified enzyme was not inhibited by hydroxylammonium chloride, a pyridoxal phosphate inhibitor, at a final concentration of 1 mM (data not shown). So ACMSD was found not to require pyridoxal phosphate. Nishizuka et al. partially purified ACMSD from the cat liver (16). They reported there was no cofactor of cat liver ACMSD.

The molecular weight of ACMSD from hog kidney was estimated by gel filtration and SDS-PAGE to be about 56,000 and 41,000, respectively. There are two possible reasons for the difference of the values of molecular weight between these methods. One is that ionic bonds may affect the binding between ACMSD and the gel bed in the column. However, even when the gel filtration buffer contained 0.15, 0.2 and 0.3 M KCl, the relative retention time of ACMSD against dextran blue of each buffer were the same, indicating that ACMSD did not form ionic bonds with the gel bed. The other possibility is that ACMSD from hog kidney may be elliptical in shape, not spherical. Because in gel filtration method of analysis, the elution pattern of highly asymmetric molecules and those containing carbohydrate deviate significantly from the curve generated from elution volumes of normal (globular) proteins (21). For example, fibrinogen and ferritin deviate significantly from the gel filtration standard curve (21).

The active site of hog kidney ACMSD has the amino, sulfhydryl and imidazole groups. Partially purified cat liver ACMSD activity was inhibited by various SH-inhibitors, too (16). The K_m value of hog kidney ACMSD for ACMS was $1.6 \times 10^{-5} \text{ M}$. The K_m value of cat liver ACMSD for ACMS was on the order of 10^{-6} M . Thus, hog kidney ACMSD has lower affinity for ACMS than cat liver enzyme.

In this experiment, we purified ACMSD from hog kidney to a homogeneous state on PAGE. Next time, we want to make monoclonal antibody and clone the cDNA for the enzyme to elucidate ACMSD regulation by many nutrients.

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