Purification and molecular cloning of rat 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase

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2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD; EC 4.1.1.45) is one of the important enzymes regulating tryptophan–niacin metabolism. In the present study, we purified the enzyme from rat liver and kidney, and cloned the cDNA encoding rat ACMSD. The molecular masses of rat ACMSDs purified from the liver and kidney were both estimated to be 39 kDa by SDS/PAGE. Analysis of N-terminal amino acid sequences showed that these two ACMSDs share the same sequence. An expressed sequence tag (EST) of the mouse cited from the DNA database was found to be identical with this N-terminal sequence. Reverse transcription-PCR (RT-PCR) was performed using synthetic oligonucleotide primers having the partial sequences of the EST, and then cDNAs encoding rat ACMSDs were isolated by using subsequent 3′-rapid amplification of cDNA ends and RT-PCR methods. ACMSD cDNAs isolated from liver and kidney were shown to be identical, consisting of a 1008 bp open reading frame (ORF) encoding 336 amino acid residues with a molecular mass of 38091 Da. The rat ACMSD ORF was inserted into a mammalian expression vector, before transfection into human hepatoma HepG2 cells. The transfected cells expressed ACMSD activity, whereas the enzyme activity was not detected in uninfected parental HepG2 cells. The distribution of ACMSD mRNA expression in various tissues was investigated in the rat by RT-PCR. ACMSD was expressed in the liver and kidney, but not in the other principal organs examined.

Key words: tryptophan–niacin conversion, picolinate, quinolinate.

INTRODUCTION

In the biosynthetic pathway of nicotinamide–adenine dinucleotide (NAD) from tryptophan, as shown in Scheme 1, 2-amino-3-carboxymuconate-6-semialdehyde (ACMS) decarboxylase (ACMSD; EC 4.1.1.45) catalyses the decarboxylation of ACMS to 2-aminomuconate-6-semialdehyde (AMS), the former compound being an intermediate metabolite of tryptophan generated by 3-hydroxyanthranilate 3,4-dioxygenase (EC 1.13.11.6). In the absence of ACMSD, ACMS changes non-enzymically to quinolinic acid, which is then modified by quinolinate phosphoribosyltransferase (or nicotinate-nucleotide pyrophosphorylase; EC 2.4.2.19) and metabolized further to NAD by a separate series of reactions. Thus an increase in ACMSD activity leads to a lower level of conversion of tryptophan into quinolinate and NAD. In the rat, ACMSD activity has been detectable only in the liver and kidney, and that of the latter was found to be generally higher than the former [1].

It is known that several nutritional factors and hormones affect the activity of ACMSD in rat. Its activity has been observed to increase in rats fed with high-protein diet [2,3] or administered with prednisolone, a synthetic adrenal cortical hormone [3,4]. Ingestion of polyunsaturated fatty acids by rats leads to a decrease in their hepatic ACMSD activity [5–7]. Furthermore, in streptozotocin-induced diabetic rats, the enzyme activity is elevated markedly, and the injection of insulin reduces the increased activity [8]. These reports simultaneously showed an inverse relationship between ACMSD activity and the amount of NAD converted from tryptophan, indicating that ACMSD has an important role in the metabolism of tryptophan.

Quinolinic acid has been reported to be associated with the pathogenesis of certain neurodegenerative diseases, such as Huntington's disease [9,10], since it acts as an excitotoxic agonist of the *N*-methyl-D-aspartate receptor [11,12]. ACMSD was postulated to affect the production of quinolinate [13]; however, it was not investigated whether ACMSD is involved in such neurological disorders, and furthermore there is still no evidence for the existence of ACMSD expression in the brain.

The various important enzymes in the kynurenine pathway, except for ACMSD, have already been cloned [14–19]. ACMSD was purified from pig kidney [20]; however, the procedure used in this purification was not applicable for the enzyme from rat tissues, indicating that rat ACMSD is structurally different from the pig enzyme. Therefore we modified the method to render it suitable for the rat enzyme, and succeeded in purifying ACMSD from rat liver and kidney, which led to the identification of cDNA encoding rat ACMSD on the basis of its amino acid sequence.

EXPERIMENTAL

Materials

Rats purchased from CLEA Japan, Inc. (Tokyo, Japan) were fed on a commercial diet, CE-2 (CLEA Japan, Inc.), and housed in individual cages at 22±1 °C with a 12 h light/12 h dark cycle

Abbreviations used: ACMS, 2-amino-3-carboxymuconate-6-semialdehyde; ACMSD, ACMS decarboxylase; AMS, 2-aminomuconate-6-semialdehyde; EST, expressed sequence tag; 3-HA, 3-hydroxyanthranilic acid; ORF, open reading frame; 3′-RACE, 3′-rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB069781.

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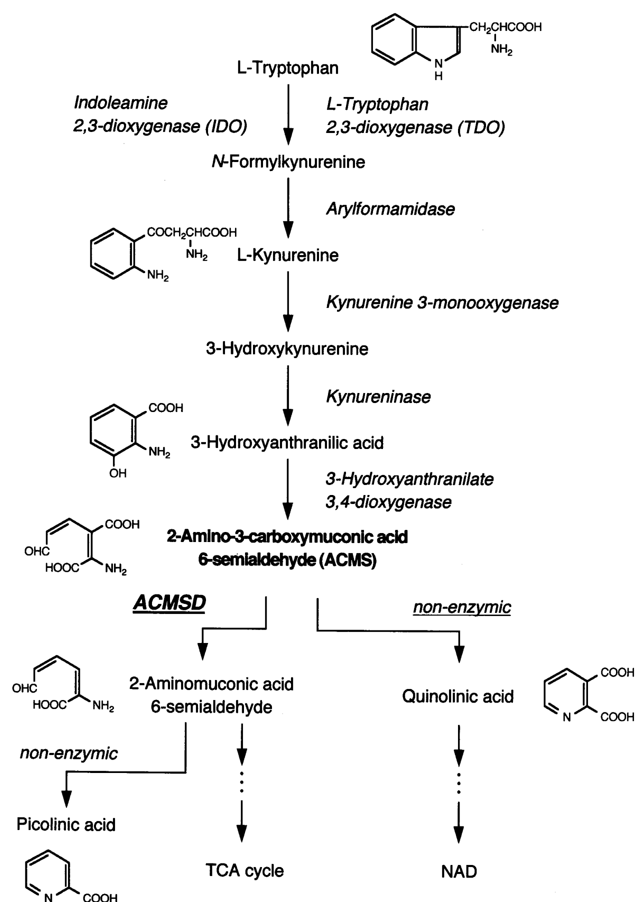

Scheme 1 Metabolic conversion of tryptophan into NAD

TDO and IDO act in the liver and extrahepatic tissues respectively.

until being killed by decapitation. HepG2 (RCB No. 0459), a human hepatoma cell line, was purchased from the Riken Cell Bank (Tsukuba, Japan), and cells were maintained in α-minimal essential medium (Gibco BRL, Life Technologies, Inc., Rockville, MD, U.S.A.) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of air/CO_2 (19 : 1). Synthetic oligonucleotides for PCR primers were purchased from Sawady Technology Co. Ltd. (Tokyo, Japan). 3-Hydroxyanthranilic acid was obtained from Sigma (St Louis, MO, U.S.A.), and all other chemicals were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), unless otherwise indicated.

Assay for ACMSD activity

Activity of ACMSD was determined by measuring the conversion of ACMS into product, as described previously [8,21]. In brief, the pre-assay mixture consisted of 25 μM 3-hydroxyanthranilic acid (3-HA), and 10–20 m-units/ml 3-HA dioxygenase solution prepared from rat liver in 50 mM Mes/NaOH buffer solution, pH 6.0, was incubated at 25 °C, with monitoring of the increase in absorbance at 360 nm due to the formation of ACMS from 3-HA. After the reaction was completed within approx. 2 min, a small aliquot of ACMSD solution was added, and the decrease in absorbance at 360 nm was followed at 15 s intervals. The rate of the decrease in absorbance caused by ACMSD was calculated by subtracting that of the control reaction mixture without ACMSD from that described above. One unit of ACMSD activity was defined as the amount of enzyme that converts 1 μmol of ACMS · min^{-1} at 25 °C. For the calculation of activity, a molar absorption coefficient of 47 500 $M^{-1} \cdot cm^{-1}$ for ACMS was used [21,22].

Purification of ACMSD from rat liver and kidney

Hepatic ACMSD was purified from livers of diabetic rats expressing a high activity of ACMSD [8]. In order to induce diabetes, 9-week-old male Wistar rats were given a single intraperitoneal injection of streptozotocin (50 mg/kg of body weight) and, 2 weeks later, their livers were removed and collected. Kidneys for the enzyme purification were collected from normal male Wistar rats.

All steps for the enzyme purification were performed at 4 °C, unless otherwise indicated. Of each tissue, 50 g was homogenized in 4 vol. of ice-cold 50 mM potassium phosphate buffer solution, pH 7.0, containing 0.14 M potassium chloride, 5 mM 2-mercaptoethanol, 1 mM dithiothreitol, 1 mM EDTA and 1 mM PMSF with a Polytron homogenizer. In this and the buffer solutions described below, both 2-mercaptoethanol and dithiothreitol were present in order to protect free thiol groups, even when these existed in isolation, since we were aware that the combination of these reagents stabilized the activity of ACMSD. The homogenate was centrifuged at 9000 ***g*** for 1 h. Solution of protamine sulphate (0.4 %, w/v) was added to the supernatant (0.8 ml/g of tissue) and centrifuged at 9000 ***g*** for 1 h. The supernatant was collected and subjected to ammonium sulphate fractionation. The fraction precipitated between 50 % and 60 % of its saturation was resuspended in a small amount of the buffer solution described above. Of the sample solution, 10 ml was applied to a Butyl-Toyopearl (Tosoh Corp., Tokyo, Japan) column (2.5 cm × 50 cm) equilibrated with 50 mM potassium phosphate buffer solution, pH 7.0, containing 0.38 M ammonium sulphate, 0.14 M potassium chloride, 5 mM 2-mercaptoethanol, 1 mM dithiothreitol and 1 mM EDTA. The column was eluted with this buffer solution. The fractions containing ACMSD activity were pooled and dialysed against 10 mM potassium phosphate solution, pH 7.0, containing 0.2 M potassium chloride, 5 mM 2-mercaptoethanol and 1 mM dithiothreitol. The enzyme solution was applied to a hydroxyapatite (Seikagaku Corp., Tokyo, Japan) column (2.5 cm × 15 cm) equilibrated with the same buffer, and the column was washed with 200 ml of the buffer and eluted with a linear gradient of 10–200 mM potassium phosphate (total vol. of 400 ml). The active fractions were dialysed against 20 mM Tris/HCl solution, pH 8.0, containing 50 mM sodium chloride, 5 mM 2-mercaptoethanol and 1 mM dithiothreitol, and then concentrated to 2 ml by ultrafiltration using a PM-10 membrane (Amicon, Inc., Beverly, MA, U.S.A.). This enzyme solution was applied to a Mono Q HR 5/5 column (Amersham Biosciences, Little Chalfont, Bucks., U.K.) for ion-exchange chromatography. The enzyme was eluted with a linear gradient of 50–80 mM NaCl in 20 min, at a flow rate of 0.5 ml/min, at room temperature. The active fractions were pooled, concentrated to 1 ml, and applied to a Superdex 200 column (1.6 cm × 60 cm; Amersham Biosciences) equilibrated with 50 mM potassium phosphate solution, pH 7.0, containing 0.15 mM sodium chloride, 5 mM 2-mercaptoethanol, 1 mM dithiothreitol and 1 mM EDTA. ACMSD was eluted at a flow rate of 0.8 ml/min with the same buffer solution at room temperature (20–25 °C).

Protein concentration was determined by the method of Bradford [23] with BSA as a standard; concentrations of semi-

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purified enzyme (after the Mono Q-treatment step) and purified enzyme (after the Superdex-treatment step) were determined by the method of Kalckar [24]. SDS/PAGE was performed on 12.5 % polyacrylamide slab gels, as described by Laemmli [25].

Kinetic characterization of rat ACMSD

In order to determine the optimal pH for enzyme activity, the following buffers were used to assay the ACMSD activity (at a final concentration of 50 mM): Mes/NaOH buffer for the pH range of 5.5–7.0; potassium phosphate buffer for the pH range of 6.5–7.5; and Tris/HCl for the pH range of 7.5–8.5 [20]. Before assaying, purified ACMSD was treated at 40, 50 and 60 °C in a water-bath incubator for 0, 5, 10, 20 and 30 min to examine heat stability. During the kinetic analyses, the concentration of the substrate ACMS was used in the range of 1.2–17.6 μM. K_m values were calculated from Lineweaver–Burk plots [26].

Determination of partial amino acid sequences of ACMSD

For N-terminal amino acid sequencing, each purified ACMSD (1 μg of protein per lane) was subjected to SDS/PAGE and subsequently electroblotted on to a PVDF membrane (Immobilon-PSQ, Millipore, Bedford, MA, U.S.A.) for 90 min at 180 mA. To obtain internal peptides of ACMSD, 1 μg of the purified protein was digested with endoproteinase Glu-C (Sigma), separated with SDS/PAGE and then electroblotted on to PVDF membranes [27]. The membranes adsorbing ACMSD or ACMSD peptides were subjected to sequence analysis [28] using the PPSQ-10 protein sequencing system (Shimadzu Corp., Kyoto, Japan).

Preparation of nucleic acids

Total RNA was extracted from rat tissues or the cultured cells using the SV total RNA isolation system (Promega, Madison, WI, U.S.A.). For the extraction of plasmid DNA, the Wizard® Plus SV Minipreps DNA purification system (Promega) was used. In order to purify the PCR products from the agarose gel and PCR solution, the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany) and QIAquick PCR purification kit (Qiagen GmbH) were used respectively. RNA and DNA concentrations of the preparation were evaluated by measuring A_{260} (the ratio of A_{260}:A_{280} was in the range of 1.7–2.0).

Reverse transcription (RT)-PCR and 3′-rapid amplification of cDNA ends (3′-RACE)

For RT-PCR, 1 μg of total RNA was reverse-transcribed in a final vol. of 20 μl by using the First-Strand cDNA synthesis kit for RT-PCR (AMV; Boehringer Mannheim, Indianapolis, IN, U.S.A.), and PCR was subsequently performed in a final vol. of 100 (or 50) μl containing an aliquot of the RT reaction, 2.5 (or 1.25) units of TaKaRa Ex *Taq*™ polymerase (Takara Shuzo Co., Kyoto, Japan), 0.2 mM dNTPs, and 0.5 μM each sense and antisense primer, together with the buffer supplied with the commercial DNA polymerase preparation. Each cycling program was performed immediately following incubation at 94 °C for 2 min.

The 3′-Full RACE core set (Takara Shuzo Co.) was used for the synthesis of cDNA from 1 μg of total RNA in 3′-RACE, followed by the first round of PCR performed in a final vol. of 100 μl containing 20 μl of the RT reaction, 2.5 units of TaKaRa Ex *Taq*™ (polymerase), 0.2 mM dNTPs and 0.2 μM each sense and antisense primer, together with the buffer supplied with the polymerase preparation. Immediately after the incubation at 94 °C for 2 min, the following cycle was used for PCR: denaturation for 1 min at 94 °C, annealing for 1 min at 55 °C and extension for 2 min at 72 °C. These steps were repeated for 35 cycles. The second round of PCR ('nested' PCR) was performed in a final vol. of 100 μl containing 2 μl of the first PCR solution, 2.5 units of TaKaRa Ex *Taq*™ (polymerase), 0.2 mM dNTPs and 0.2 μM each sense and antisense primer. The conditions for the PCR steps were the same as those described above (for the first round of PCR).

DNA sequencing of PCR products

PCR products were subjected to agarose-gel electrophoresis and extracted from the gel. The purified products were subcloned into pGEM®-T Easy vector (Promega), and competent *Escherichia coli* JM109 cells of high efficiency (Promega) were transformed with the vectors, according to the manufacturer's instructions. Plasmids were prepared, and the inserts were sequenced by the dideoxynucleotide chain-termination method [29] using the Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences) and a DSQ-2000L DNA sequencer (Shimadzu Corp.), according to the manufacturer's instructions.

Isolation of cDNA encoding rat ACMSD

A BLAST (TblastN) homology search of the expressed sequence tag (EST) database revealed a mouse EST (GenBank® accession no. AA062380) to be 100 % identical with the N-terminal amino acid sequence of rat ACMSD. On the basis of this partial sequence of the EST, which we could browse on the database (see Figure 4), the following synthetic oligonucleotides were designed: ASp0, 5′-CGT GTC TCC TCT GGT CCT GTG GAG-3′; and AAp0, 5′-CCC AGT AGC TAA ACA TGA CAG G-3′.

Liver and kidney total RNAs were extracted from 8-week-old male Wistar rats, and RT-PCR was performed to isolate the cDNA encoding the 5′-region of the open reading frame (ORF) of rat ACMSD, encompassing the start codon, using ASp0 and AAp0 as primers. The PCR mixtures were set up in a final vol. of 100 μl containing 10 μl of the RT reaction, and the following cycle was used for PCR: denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 45 s at 72 °C; these steps were repeated for 35 cycles. The PCR products were then sequenced, before 3′-RACE was performed under the same conditions as those described above. As sense primers, ASp1 (5′-ATG AAA ATT GAC ATC CAC ACT C-3′) and ASp2 (5′-CTA CCA AAG GAA TGG CCT GAT-3′) were used in the first and second (nested) rounds of 3′-RACE respectively. The oligonucleotide included in the 3′-RACE kit (3sites Adaptor Primer) was used as the antisense primer in both amplification reactions. The amplified products were then sequenced. In order to confirm the nucleotide sequences of the ORF, RT-PCR was performed using ASp1 and AAp1 (5′-TCA TTC AAA TAG TTT TCT CTC AAG ACC C-3′), encompassing the start (ATG) and stop (TGA) codons respectively. The PCR mixtures in a final vol. of 100 μl containing 10 μl of the RT reaction were heated at 94 °C for 2 min, and immediately subjected to 35 cycles of PCR, with a denaturation step for 1 min at 94 °C, an annealing step for 1 min at 55 °C and an extension step for 90 s at 72 °C.

Expression of rat ACMSD in HepG2 cells

cDNA encoding rat ACMSD ORF was amplified by RT-PCR using ASp1 and AAp1 primers using rat kidney total RNA as the template. This cDNA was subcloned into pTARGET™ mammalian expression vector (Promega), and HepG2 cells were

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transfected with the vector by the cationic-lipid method using Tfx™-20 reagent (Promega), according to the manufacturer's instructions. The stable transfectants were selected in the medium containing G418 (Geneticin) sulphate for 4 weeks. As a control, parental HepG2 cells and the cells transfected with the self-ligated vector were examined similarly. The collected cells were washed with PBS, and then homogenized in ice-cold 50 mM potassium phosphate buffer, pH 7.0, containing 0.14 M potassium chloride, 5 mM 2-mercaptoethanol, 1 mM dithiothreitol, 1 mM EDTA and 1 mM PMSF. The cytosolic fractions were obtained from the homogenate by ultracentrifugation at 105000 ***g*** for 60 min, and then assayed for ACMSD activity.

To examine the expression of rat ACMSD mRNA in the cells, RT-PCR was performed using ASp2 (5′-CTA CCA AAG GAA TGG CCT GAT-3′) and AAp2 (5′-TGG TCT CCG ATG GCA TTC CTA-3′) as the sense and antisense primers respectively. The following cycle was used for PCR: denaturation for 30 s at 94 °C, annealing for 30 s at 55 °C and extension for 45 s at 72 °C. These steps were repeated for 40 cycles, and 5 μl of the resulting PCR solution was subjected to agarose-gel electrophoresis.

Distribution of ACMSD mRNA in various tissues of rat

cDNA derived from ACMSD mRNA was amplified by RT-PCR to analyse the expression of ACMSD mRNA in various rat tissues: whole brain, thymus, lung, heart, pancreas, liver, kidney, adrenal gland, spleen, testis, retro-peritoneal white adipose tissue and femoral skeletal muscle (quadriceps femoris). The tissues were excised from a male Wistar rat at the age of 10 weeks.

The PCR primers, ASp2 and AAp2, were used for detection of ACMSD mRNA. The reaction mixtures for PCR in a final vol. of 50 μl contained 5 μl of the RT reaction, and PCR was performed for 40 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s). β-Actin, as an internal control, was also detected by RT-PCR from the same RNA samples, using the primers 5′-GTG GGC CGC CCT AGG CAC CAG-3′ (sense) and 5′-CTC TTT AAT GTC ACG CAC GAT TTC-3′ (antisense) to amplify 540 bp of cDNA derived from β-actin mRNA. The reaction mixtures in a final vol. of 50 μl contained 1 μl of the RT reaction, and PCR was performed for 30 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s). Each PCR product (5 μl/lane) was electrophoresed on a 1.5% (w/v) agarose gel.

RESULTS

Purification of rat ACMSD

Table 1 summarizes the purification of ACMSD from rat liver and kidney. In terms of the various purification steps, liver and kidney ACMSDs were fractionated similarly. The purified ACMSD preparations from both tissues each generated a single

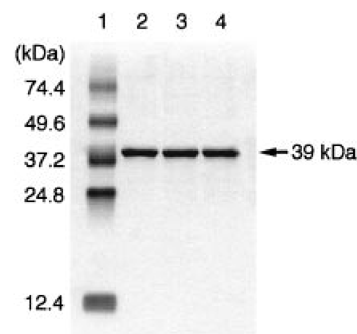

Figure 1 SDS/PAGE analysis of purified rat ACMSD

In lanes 2–4, approx. 1 μg of each protein was subjected to electrophoresis on a 12.5% (w/v) polyacrylamide gel. Protein bands were detected with silver staining. Lane 1, molecular-size marker; lane 2, ACMSD from liver; lane 3, ACMSD from kidney; lane 4, mixture of ACMSDs from liver and kidney. The molecular mass of each ACMSD was estimated to be 39 kDa, as indicated with an arrow on the right of the gel.

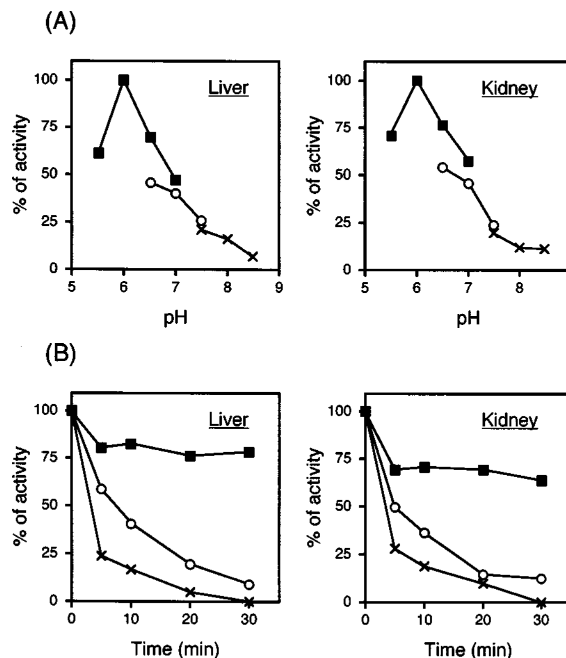

Figure 2 Characteristics of purified ACMSD from rat liver and kidney

(**A**) ACMSD activity was measured at different pH values in Mes/NaOH (■), potassium phosphate (○) or Tris/HCl (×) buffers, all at a final concentration of 50 mM. (**B**) The purified ACMSDs were incubated at 40 (■), 50 (○) or 60 (×) °C for 0–30 min before assay.

Table 1 Purification of ACMSD from rat liver and kidney

Step	Total protein (mg)		Total activity (m-units)		Specific activity (m-units · mg of protein^{-1})		Yield (%)	
	Liver	Kidney	Liver	Kidney	Liver	Kidney	Liver	Kidney
Homogenate supernatant	3080	3530	5620	16200	1.82	4.58	100	100
Ammonium sulphate	579	910	1970	6080	3.40	6.68	35	38
Butyl-Toyopearl	9.59	33.5	1580	6820	165	203	28	42
Hydroxyapatite	0.53	2.48	1490	7520	2780	3030	27	47
Mono Q	0.057	0.262	451	1937	6920	7400	8	12
Superdex	0.045	0.165	396	1330	8820	8070	7	8

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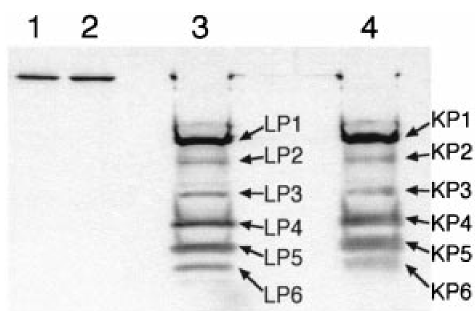

Figure 3 Digestion of purified rat ACMSD from liver and kidney with endoproteinase Glu-C

SDS/PAGE of ACMSD and its peptides (LP1–6 and KP1–6) cleaved with endoproteinase Glu-C. Lane 1, liver ACMSD; lane 2, kidney ACMSD; lane 3, liver ACMSD digested with endoproteinase Glu-C; lane 4, kidney ACMSD digested with endoproteinase Glu-C. Each peptide fragment was subjected to amino acid sequence analysis.

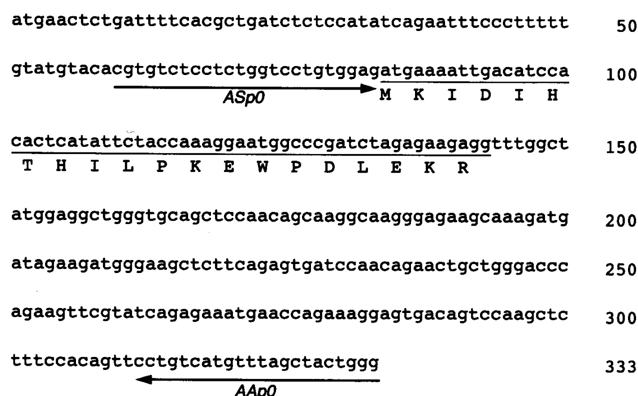

Figure 4 Nucleotide sequence of mouse EST AA062380

The predicted amino acid sequence that is in agreement with that of the N-terminus of rat ACMSD examined in the present study (20-amino-acid stretch) is underlined. Sequences of synthetic oligonucleotides (*ASp0* and *AAp0*) used as primers for RT-PCR are shown by the arrows.

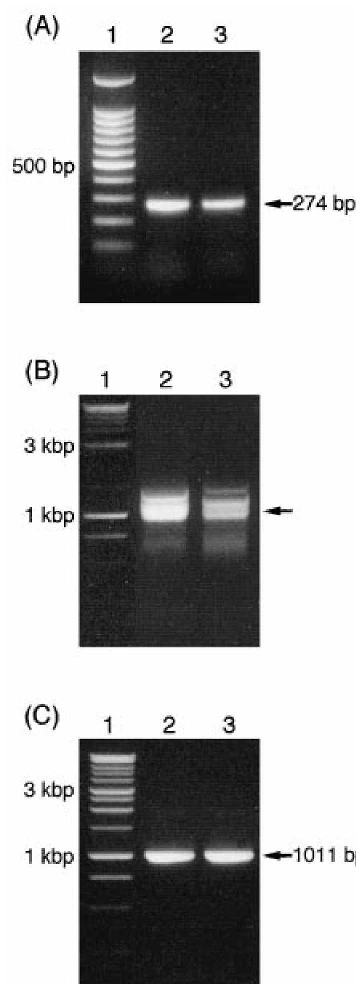

Figure 5 Amplification of rat ACMSD cDNA fragments by RT-PCR and 3′-RACE

Each product (10 μl of PCR solution) was subjected to agarose gel electrophoresis. (**A**) RT-PCR using ASp0 and AAp0 as the sense and antisense primers respectively. The 274 bp fragments, indicated with the arrow, were sequenced. Lane 1, size marker (100 bp DNA ladder); lane 2, total RNA from the kidney; lane 3, total RNA from the liver. (**B**) Second (nested) 3′-RACE products, using ASp2 as the sense primer. As an antisense primer, the oligonucleotide included in the 3′-RACE kit was used in both PCRs. Products proximal to the 1–1.5 kbp band, indicated with an arrow, were sequenced. Lane 1, 1 kb DNA ladder; lane 2, total RNA from the kidney; lane 3, total RNA from the liver. (**C**) RT-PCR for amplification of cDNA encoding ACMSD ORF. ASp1 and AAp1 were used as sense and antisense primers respectively. Each 1011 bp fragment (indicated with the arrow) was amplified and subsequently sequenced. Lane 1, 1 kb DNA ladder; lane 2, total RNA from the kidney; lane 3, total RNA from the liver.

band on SDS/PAGE, and the molecular mass of both was estimated to be 39 kDa (Figure 1).

The pH optimum for activity of the purified ACMSD was between pH 5.5 and 6.5 in 50 mM Mes/NaOH buffer, and the activities were discernibly higher in Mes/NaOH buffer than in potassium phosphate buffer for the same measurements of pH (Figure 2A). ACMSD activity was decreased by 25 % during the heat treatment at 40 °C for 5 min, but thereafter was stable for up to 30 min at the same temperature (Figure 2B). The activities were completely lost by incubation at 60 °C for 30 min. The K_m values of the purified enzymes from liver and kidney were calculated to be 1.4×10^{-5} M and 1.3×10^{-5} M respectively.

Isolation of ACMSD cDNA

The N-terminal amino acid sequences of the ACMSDs were determined. Liver and kidney ACMSD shared the same sequence in a 20-amino-acid residue stretch (MKIDIHTHILPKEWPD-LEKR). Moreover, the peptides, LP1–6 and KP1–6, which were obtained by the digestion of the purified ACMSDs with endoproteinase Glu-C with subsequent separation on SDS/PAGE (Figure 3), were subjected to amino acid sequencing analysis. The sequences of LP1 and KP1 were determined, and they also shared the same sequence in an N-terminal 17-amino-acid residue stretch (LGFPGIQIQGSHINMWDL). Other bands, LP2–6 and KP2–6, appeared to be mixtures of the digested peptides, and their sequences could not be identified.

A mouse EST AA062380 was found to be 100 % identical with the N-terminal sequence of ACMSD from a homology search (TblastN). A partial nucleotide sequence of the EST that we were able to retrieve from the database is shown in Figure 4. Two oligonucleotides, ASp0 and AAp0, were synthesized as sense and antisense primers respectively, and their sequences are also shown in Figure 4.

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atgaaaattgacatccacactcacattctaccaaaggaatggcctgatctagaaaagagg   60
M  K  I  D  I  H  T  H  I  L  P  K  E  W  P  D  L  E  K  R     20
 N-terminus
tttggctatggaggctgggtgcagctccaacagcaaggcaagggagaagcaaagatgatg  120
F  G  Y  G  G  W  V  Q  L  Q  Q  Q  G  K  G  E  A  K  M  M     40

aaagacgggaaggtcttcagagtgatccaacagaactgctgggacccagaagtccgcatc  180
K  D  G  K  V  F  R  V  I  Q  Q  N  C  W  D  P  E  V  R  I     60

agagaaatgaaccagaaaggagtgacagtgcaagctctttccacagttcccgtcatgttt  240
R  E  M  N  Q  K  G  V  T  V  Q  A  L  S  T  V  P  V  M  F     80

agttactgggccaaacctaaggacactttggaactgtgccagtttttaaacaatgacctc  300
S  Y  W  A  K  P  K  D  T  L  E  L  C  Q  F  L  N  N  D  L    100

gctgccacggtggccagataccctcgaaggtttgtgggtttggggacattgcctatgcaa  360
A  A  T  V  A  R  Y  P  R  R  F  V  G  L  G  T  L  P  M  Q    120

gccccggggctggctgtcgaggagatggagcgttgtgtaaaggagctaggatttccagga  420
A  P  G  L  A  V  E  E  M  E  R  C  V  K  E  L  G  F  P  G    140
                                             LP1 and KP1
atccagattggatcccacatcaacatgtgggacctgaatgacccggaactcttccccatc  480
I  Q  I  G  S  H  I  N  M  W  D  L  N  D  P  E  L  F  P  I    160

tacacggcggctgagaggctgaattgttctctgttcgtgcatccctgggatatgcagatg  540
Y  T  A  A  E  R  L  N  C  S  L  F  V  H  P  W  D  M  Q  M    180

gatggacggatggccaaatactggctgccttggctcgtaggaatgccatcggagaccacc  600
D  G  R  M  A  K  Y  W  L  P  W  L  V  G  M  P  S  E  T  T    200

acggccatttgctccatgatcatgggtggggtgttcgagaagtttcccaaactcaaagtg  660
T  A  I  C  S  M  I  M  G  G  V  F  E  K  F  P  K  L  K  V    220

tgttttgcacatggaggtggtgctttccccttcaccataggaagaattgctcatggattt  720
C  F  A  H  G  G  G  A  F  P  F  T  I  G  R  I  A  H  G  F    240

aacatgcgcccagatctctgtgcccgggacaattcatctgaccccagaaaataccttggc  780
N  M  R  P  D  L  C  A  R  D  N  S  S  D  P  R  K  Y  L  G    260

tccttctacacggactccctggttcatgatcctctgtctctcaagctattgacagatgtt  840
S  F  Y  T  D  S  L  V  H  D  P  L  S  L  K  L  L  T  D  V    280

atagggaaggatagagttattctgggaactgattacccctttccactaggtgagcaggag  900
I  G  K  D  R  V  I  L  G  T  D  Y  P  F  P  L  G  E  Q  E    300

cctgggaagttgatagagtctatggcagactttgatgaagaaacaaaggataaacttaca  960
P  G  K  L  I  E  S  M  A  D  F  D  E  E  T  K  D  K  L  T    320

gctggcaatgccttgactttttgggtcttgagagaaaactatttgaatga          1011
A  G  N  A  L  T  F  L  G  L  E  R  K  L  F  E  *            336
```

Figure 6 Nucleotide and deduced amino acid sequences of rat ACMSD

The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence. The sequenced N-terminus and the internal peptides (LP1 and KP1) are underlined. The asterisk denotes the stop codon.

RT-PCR, using the ASp0 and AAp0 primers and total RNA species from the liver and kidney, was used to amplify the 274 bp fragments accordingly (Figure 5A). Subsequently, the oligonucleotides, ASp1 and ASp2, which were based on the sequences of the obtained fragments, were used for nested 3′-RACE as sense primers, and the products (Figure 5B) were sequenced. Finally, cDNAs encoding the ACMSD ORF were isolated by RT-PCR using ASp1 and AAp1 as primers, encompassing the start and stop codons respectively (Figure 5C).

As shown in Figure 6, the cDNA of 1011 bp contained a single

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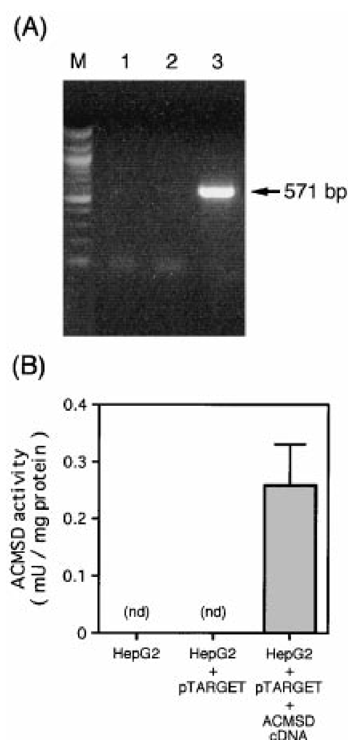

Figure 7 Expression of recombinant rat ACMSD in HepG2 cells

(**A**) RT-PCR analysis of rat ACMSD stably expressed in HepG2 cells. Lane M, 100 bp DNA ladder; lane 1, total RNA extracted from normal HepG2 cells (non-transfected); lane 2, total RNA from the cells transfected with pTARGET™ vector only; lane 3, total RNA from the cells transfected with pTARGET™ including rat ACMSD cDNA. The arrow shows the rat ACMSD cDNA fragment. (**B**) Enzyme activity on the basis of the recombinant rat ACMSD stably expressed in HepG2 cells. ACMSD activity was measured in the cytosolic fraction of the transfected or non-transfected cells. The error bars indicate the S.D. for three samples. nd, not detected. mU, m-units.

ORF encoding a protein of 336 amino acids, with a predicted molecular mass of 38091 Da, and the amino acid sequences of KP1 and LP1 were found internally at residue positions 136–152.

Stable expression of rat ACMSD in HepG2 cells

To confirm that the isolated cDNA indeed encodes rat ACMSD, the rat ACMSD cDNA amplified by RT-PCR from kidney total RNA was subcloned into the mammalian expression vector pTARGET™, and HepG2 cells were transfected with the vector containing rat ACMSD cDNA. By RT-PCR using total RNA isolated from these stably transfected cells, the 571 bp fragment corresponding to nt 28–598 in ACMSD cDNA was amplified, which indicated that ACMSD mRNA was expressed in the cells (Figure 7A). Furthermore, appreciable ACMSD activity was detected in these transfected cells, whereas the host HepG2 cells and those transfected with the control vector lacking ACMSD cDNA did not demonstrate any enzyme activity (Figure 7B).

Tissue distribution of ACMSD mRNA expression

In order to detect ACMSD mRNA in various rat tissues, RT-PCR was used. The PCR products derived from ACMSD mRNA were found in liver and kidney, but were not detected in other tissues examined (Figure 8). The results showed that ACMSD mRNA was expressed only in the liver and kidney in the normal rat, a result consistent with the fact that ACMSD activity was generally detected in liver and kidney, but not in other tissues (results not shown).

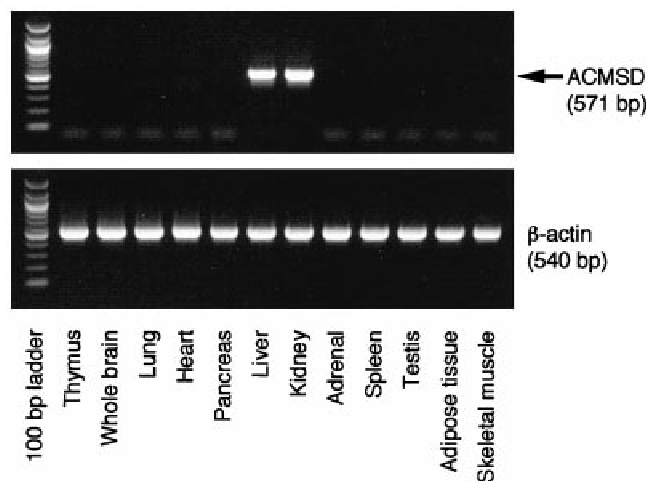

Figure 8 RT-PCR analysis of ACMSD expression in rat tissues

ACMSD cDNA fragments (571 bp), shown by the arrow, were amplified in the liver and kidney, but not in other tissues.

DISCUSSION

ACMSD activity, which is affected *in vivo* by nutritional status [2,5–7] and the administration of various hormones [3,4,8,30–32], has been studied mostly in rats. In the present study, we have purified the enzyme from rat liver and kidney, and cloned the cDNA encoding rat ACMSD.

The purification of ACMSD from pig kidney has been reported previously [20]; however, we were unable to purify the enzyme from rat organs by the same method. Therefore we explored the use of other methods, and succeeded in purifying the enzyme by a simplified alternative method, which resulted in a higher yield of the purified enzyme. ACMSDs from rat liver and kidney were purified similarly, and both enzymes, upon purification, had essentially the same properties. However, compared with a previous study [20] on the pig ACMSD, rat ACMSD differed with respect to the optimal pH for activity (pH of 6.0 for rat enzyme, compared with pH 7.5 for the pig enzyme). Another notable difference between the enzymes was that the molecular mass of the rat ACMSD was estimated in the present study to be 39 kDa by SDS/PAGE, whereas that of the pig enzyme was reported to be 41 kDa and 58 kDa by SDS/PAGE and gel filtration respectively [20]. However, when rat and pig ACMSD were subjected to SDS/PAGE simultaneously, the result indicated that their relative molecular masses were almost identical (results not shown). As far as the sequence of the N-termini of the ACMSDs that we were able to determine is concerned (a stretch of 17 amino acids), the residue at position 7 was identified as threonine in the rat ACSMD, but was serine in the pig enzyme (results not shown). Structural differences such as these might be responsible for the differences in certain properties that were observed between these enzymes, e.g. behaviours of the enzymes when subjected to chromatography and the optimal pH values required for activity.

Sequencing of the rat ACMSD cDNA isolated from liver and kidney showed that this cDNA has an ATG-initiated ORF of

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1008 bp, which encodes a 336-amino-acid polypeptide containing the N-terminal and internal (LP1 and KP1 peptide) amino acid sequences of ACMSD that were examined. Furthermore, ACMSD activity was detected in HepG2 cells transfected with a vector containing ACMSD cDNA, which was amplified by RT-PCR using total RNA from rat kidney as a template, but activity was not apparent in host cells or cells transfected with the control vector alone. These results corroborate that this cDNA encodes ACMSD. However, the increased amount of protein corresponding to rat ACMSD was not observed in the homogenates of these cells upon analysis by SDS/PAGE with Coomassie Brilliant Blue R250 staining, indicating that the expression of the enzyme might be insufficient in these cells to allow detection as the enzyme protein (results not shown).

The mouse EST AA062380 has a sequence that is 100% identical with the N-terminal 20-amino-acid sequence of rat ACMSD, and nt 84–333 in the EST (333 bp fragment in the database) were found to correspond to rat ACMSD cDNA (nt 1–250). Since a comparison of the EST with rat ACMSD cDNA revealed that only 12 nucleotides and 3 deduced amino acids were different, mouse EST AA062380 might encode ACMSD.

During the metabolism of tryptophan, ACMSD is able to influence directly the production of quinolinate and picolinate. Quinolinate is non-enzymically generated from ACMS in the absence of ACMSD activity. This metabolite is a potent endogenous excitotoxin, which functions as an agonist of the *N*-methyl-D-aspartate receptor [11,12]. Intra-striatal injection of quinolinic acid, which provokes neuronal death, is used in an animal model of Huntington's disease [9,10,33,34]. Moreover, quinolinate is considered to have roles in the pathogenesis of epilepsy [35,36], in Alzheimer's disease [37] and in dementia resulting from AIDS [38]. It has also been reported that levels of quinolinate are elevated in cerebrospinal fluid and brain tissue of patients with the AIDS dementia complex [39]. Picolinic acid, which is generated non-enzymically from AMS following the ACMSD-catalysed decarboxylation of ACMS, induces nitric oxide synthase synergistically in murine macrophages treated with interferon-γ [40,41], and may influence the immune system in macrophages [42,43] and apoptosis in human leukaemia cell lines [44,45].

In spite of these studies, it has not been investigated whether or not ACMSD participates in these processes. Until now, ACMSD activity has been detected only in the liver and kidney [1]. This finding is consistent in part with the results of the present study, indicating that ACMSD mRNA is expressed in the liver and kidney, but not in other tissues in the normal rat. However, differences in ACMSD expression relative to specific regions of the brain, or alterations in ACMSD expression in animal tissues resulting from conditions of morbidity or in cells stimulated with reagents, such as cytokines and mitogens [46], are still unknown. The results from the present study will be important for obtaining new information not only on the regulation of NAD synthesis *de novo*, but also on the new physiological functions of ACMSD and related compounds.

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