Identification and Expression of a cDNA Encoding Human α-Amino-β-carboxymuconate-ε-semialdehyde Decarboxylase (ACMSD)

A KEY ENZYME FOR THE TRYPTOPHAN-NIACINE PATHWAY AND "QUINOLINATE HYPOTHESIS"∗

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Quinolinate (quinolic acid) is a potent endogenous excitotoxin of neuronal cells. Elevation of quinolinate levels in the brain has been implicated in the pathogenesis of various neurodegenerative disorders, the so-called “quinolinate hypothesis.” Quinolinate is non-enzymatically derived from α-amino-β-carboxymuconate-ε-semialdehyde (ACMS). α-Amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD) is the only known enzyme that can process ACMS to a benign catabolite and thus prevent the accumulation of quinolinate from ACMS. ACMSD seems to be regulated by nutritional and hormonal signals, but its molecular mechanism has, to date, been largely unknown. Utilizing partial amino acid sequences obtained from highly purified porcine kidney ACMSD, a cDNA encoding human ACMSD was cloned and characterized. The cDNA encodes a unique open reading frame of 336 amino acids and displays little homology to any known enzymes or motifs in mammalian databases, suggesting that ACMSD may contain a new kind of protein fold. Real-time PCR-based quantification of ACMSD revealed very low but significant levels of the expression in the brain. Brain ACMSD messages were down- and up-regulated in response to low protein diet and streptozocin-induced diabetes, respectively. The enzyme activities measured from partially purified brains were closely correlated with the changes in the message levels. Expression of quinolinate phosphoribosyltransferase (QPRT), another enzyme that catabolizes quinolinate, was also found in the brain. This suggests that a pathway does exist by which the levels of quinolinate in the brain are regulated. In this report, we address the molecular basis underlying quinolinate metabolism and the regulation of ACMSD expression.

Quinolinate (quinolic acid) is an intermediate in the de novo synthesis of NAD from tryptophan and acts as a potent endogenous neurotoxin through hyperstimulation of the N-methyl D-aspartate (NMDA) receptor (1–3). When quinolinate is loaded into rat brains by autodialysis, the striatal region is specifically severely damaged (4–6). An autoradioreceptor assay showed that the number of NMDA glutamate receptors in patients of Huntington’s disease was reduced by 93% (7), supporting a hypothesis that an endogenous agonist of the receptor is primarily responsible for the neural degradation associated with the disease. Unlike kainate or ibotenate, quinolinate is thought to be the only physiological agonist for the NMDA receptors involved in the disorder (1–3). Thus, a dysfunction of quinolinate metabolism in the human brain has been postulated to be involved in the pathogenesis of such neurodegenerative disorders as epilepsy, Alzheimer’s disease and Huntington’s disease (“quinolinate hypothesis”) (8, 9).

As shown in Fig. 1, quinolinate is non-enzymatically derived from α-amino-β-carboxymuconate-ε-semialdehyde (ACMS). ACMS is generated from 3-hydroxyanthranilic acid (3HA) by the catalysis of 3HA oxygenase. 3HA oxygenase activity in the brains of Huntington’s disease patients was found to be 3.5-fold higher than in normal controls (10), suggesting an association between elevated levels of quinolinate and the disease. ACMS can be diverted to a benign catabolite, α-aminomuconate-ε-semialdehyde (AMS), by α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD) (E.C. 4.1.1.45). Therefore, in terms of the quinolinate hypothesis, ACMSD may play an important role in prevention of progression to disease. Because the conversion rate of tryptophan to quinolinate (and then to NAD) and ACMSD activity are quite inversely correlated, conditions that reduce ACMSD expression may increase the risk of quinolinic toxicity. ACMSD has been shown to be down-regulated by low protein and low polyunsaturated fatty-acid diets (11), clofibrate (a peroxisome proliferate activator) (12), tyrosine (13), pyrazinamide, and pyrazinoic acid (14). On the other hand, ACMSD is up-regulated by adrenaline, glucocorticoid, female hormones, (13) and in diabetes (15). In this study, as a first step toward elucidating the molecular basis underlying quinolinate metabolism and regulation of ACMSD expression, a cDNA encoding human ACMSD was cloned utilizing partial amino acid sequences obtained from highly purified ACMSD. Functional expression of the cDNA in mammalian cells proved that the cDNA encodes ACMSD.

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The nucleotide sequences of cloned ACMSD cDNAs for human, mouse, C. elegans, and rat reported in this paper have been submitted to GenBank with the accession numbers AB07418, AB07419, AB07420, and AB069781, respectively.

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† The abbreviations used are: NMDA, N-methyl D-aspartate; ACMS, α-amino-β-carboxymuconate-ε-semialdehyde; 3HA, hydroxyanthranilic acid; AMS, α-aminomuconate-ε-semialdehyde; ACMSD, α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; QPRT, quinolinate phosphoribosyltransferase; SH, Src homology.

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**EXPERIMENTAL PROCEDURES**

**General Recombinant DNA Methods—DNA modification enzymes and restriction enzymes were purchased from standard commercial sources and used according to the manufacturers’ instructions.** PCR was performed as described (16) except that Taq polymerase was used with Pfu polymerase (1:1 unit ratio) to increase the fidelity of template amplification. The recombinant plasmids constructed as described below were confirmed by a combination of restriction enzyme mapping and DNA sequencing. The oligonucleotides used are listed below.

**Purification of Porcine Kidney ACMSDase—**The enzyme was purified as described previously (17). Homogeneity of the sample was confirmed by SDS-PAGE and N-terminal amino acid sequencing.

**Determination of Partial Amino Acid Sequences—**About 50 pmol of purified porcine kidney ACMSDase spotted on polyvinylidene difluoride membrane was subjected to direct N-terminal sequencing with a HP G1005A protein sequencing system (Hitachi). A separate portion of the purified enzyme was carboxymethylated and then cleaved by achro- 

**Quantitation of ACMSD Expression with Real-time PCR—**Quantitation of ACMSD expression was measured by a previously described method (21). Host COS7 cells and transfectants with the self-ligated vector were subjected to the same examinations as controls.

**Quantification of ACMSD Expression with Real-time PCR—**Quantitation of ACMSD expression in mouse kidney, liver, and brain was performed by an iCycler PCR (BioRad), a real-time PCR machine, using SYBR Green dye for target DNA quantitation. Rat total kidney, liver, and whole brain tissues were subjected to ACMSD and QPRT expression quantitation. The ACMSD-specific primers used were 5'-GAGGGAGAAATCAAGGGCTGG-3' (forward) and 5'-CACGTGGCAGGCACGAGGAGG-3' (reverse) and 5'-TGAAAATTGACATCCATAGT-3' (reverse). Expression of quinolinate phosphoribosyl-transferase (QPRT) was quantitated by the same method. The QPRT-specific primers used were 5'-AGCTGGTGTCAGCTGACAAG-3' (forward) and 5'-GGAGGAGAAATCAGGCTGG-3' (reverse) per a previous report (19).

**Animal Studies—**Male 8-week-old ICR mice were fed a low protein diet (5% casein, 84% sucrose, 5% corn oil, 5% mineral mixture, 1% vitamin mixture) or a normal protein diet (20% casein, 69% sucrose, 5% corn oil, 5% mineral mixture, 1% vitamin mixture) as described by Shibata and Matsuo (22) for 10 days and sacrificed, whereupon various tissues were subjected to ACMSD and QPRT expression quantitation. In a separate study, mice fed with the normal diets were induced to be diabetic by intraperitoneal injection of 70 mg/kg streptozocin. After 100,000 × g for 15 min to sediment insoluble materials. Supernatants were used for enzyme assays. The ACMSD activity was measured by an iCycler PCR (BioRad), a real-time PCR machine, using SYBR Green dye for target DNA quantitation. Rat total kidney, liver, and whole brain tissues were subjected to ACMSD and QPRT expression quantitation. The ACMSD-specific primers used were 5'-GAGGGAGAAATCAAGGGCTGG-3' (forward) and 5'-CACGTGGCAGGCACGAGGAGG-3' (reverse) and 5'-TGAAAATTGACATCCATAGT-3' (reverse). Expression of quinolinate phosphoribosyl-transferase (QPRT) was quantitated by the same method. The QPRT-specific primers used were 5'-AGCTGGTGTCAGCTGACAAG-3' (forward) and 5'-GGAGGAGAAATCAGGCTGG-3' (reverse) per a previous report (19).
The solution was dialyzed against 10 mM potassium phosphate solution, pH 7.0, containing 0.2 M potassium chloride, 5 mM 2-mercaptoethanol, and 1 mM dithiothreitol and then further concentrated by ultrafiltration using a PM-10 membrane at 4 °C. Using the sample, brain QPRT activity was measured as described (19). For brain QPRT activity detection, a portion of the homogenate was heated at 65 °C for 2 min. The sample was centrifuged at 10,000 × g for 1 h. The supernatant was subjected to ammonium sulfate fractionation.

**RESULTS**

Cloning and Characterization of Human ACMSD cDNA—Direct N-terminal sequencing of 50 pmol of highly purified porcine kidney ACMSD yielded a sequence of up to 51 amino acids starting with intact methionine (uncertain readings in parentheses): MKIDISHLKPDELKKRFQG(YG)-XGVELQHHSFGAKMLE(D)/G/K/YFR/V/R/VQF51. The yields of the first five amino acid cleavages were 26.3 pmol: Lys (23.4), Ile (18.9), Asp (28.0), and Ile (17.1), and the backgrounds of other amino acids were less than 2 pmol in each cycle. The presence of a single N terminus indicated high purity of the protein. Internal amino acid sequences were also obtained from peptides liberated from the purified enzyme by limited proteolysis: RFVGLGTLPMQAP, XSLFVHPWDMQ, YWLPWLVGMPAETTIAIC-SMIMGGVFKE, VYFAHGGGSPFPPPGRI, and VILGTDDYPPFLGGLEPQG. Based on the assumption that the apparent 40-kDa molecular mass of ACMSD would translate to roughly 300–330 amino acids, ~40% of the primary structure was most likely obtained by amino acid sequencing, indicating that the preparation was highly purified. Utilizing this information and frequent human codon usage (23) to design oligonucleotide probes, a full-length ACMSD cDNA was cloned from a human brain cDNA library. The cDNA clone encodes 1237 nucleotides (nt) with a single noncoding sequence and a 172-nt 3'-noncoding region (Fig. 2). The presence of the presumed initiator ATG codon at base 58 was confirmed in the eukaryotic consensus sequence and is nearly the entire 3'-noncoding region starting at position 1208, indicating that the molecular mass data (38033.36) deduced from the open reading frame is consistent with the molecular mass data observed from purified mammalian ACMSD (40 kDa). A typical polyadenylation signal (AATAAA) can be seen in the 3'-untranslated region. Over these 137 amino acids, the human and porcine enzymes share 98% identity.

Searches of known functional domains and motifs for enzyme binding using PROSITE and MOTIFIC software did not return any significant matches. Using PSORT, a piece of software that analyzes sorting signals in protein sequences, it was predicted that human ACMSD is a cytosolic protein. Using FASTA and BLAST, both homology search algorithms, no homologous protein-coding sequences were found in mammalian genomic databases, indicating that this is the first reporting of...
TABLE II
Expression of QPRT and ACMSD in mouse tissues quantitated by real-time PCR

| Tissue*       | QPRT       | Enzyme Activity | ACMSD       | Enzyme activity |
|---------------|------------|----------------|-------------|----------------|
|               | mRNA       | unit*           | pmol/hr/mg*  | mRNA           | unit*           | pmol/hr/mg*  |
| Kidney        |            |                 |             |                |
| Liver         | 3.26 ± 0.15| 2250 ± 190      | 51 ± 5      | 0.023 ± 0.002  |
| Brain         |            |                 |             |                |

* Mouse tissues were prepared from male 8-week-old ICR mice fed a normal protein diet (see “Experimental Procedures”) for 10 days.

** Units of mRNA expression relative to GAPDH (internal standard) when QPRT/GAPDH in kidney is expressed as 1.000. Values are shown as means ± S.E. (four animals were used).

** 2. QPRT activity is shown as the amount of enzyme that generates a given pmol of nicotinamido mononucleotide, a reaction product hr mg of protein of the tissue homogenates. Values are shown as means ± S.E. (four animals were used). Brain QPRT activities measured and calculated from concentrated QPRT fractions that were partially purified from four pools of 10 mice brains (see “Experimental Procedures” for detail); values for the brain are shown as means ± S.E. (four pools of 10 animals were used). The brain enzyme activities (pmol/hr/mg) refer to the mg of protein in the original brain homogenates. Therefore, comparisons to kidney and liver can be made on an equal basis.

** ACMSD activity is shown as the amount of enzyme that generates a given nmol of a-nimmonunonate-c-semialdehyde (AMS), a reaction product mg of protein of the tissue homogenates. Values are shown as means ± S.E. (four animals were used). Brain ACMSD activities were measured and calculated from concentrated ACMSD fractions that were partially purified from four pools of 10 mice brains (see “Experimental Procedures” for detail); values for the brain are shown as means ± S.E. (four pools of 10 animals were used). The brain enzyme activities (nmol/hr/mg) refers to mg of protein in the original brain homogenates.

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** Statistical significant (p < 0.001) when compared between the control and the test groups calculated using Student’s t-test.
up-regulated by 80%. On the other hand, brain QPRT expression remained unchanged.

To confirm whether the changes in brain ACMSD at the message levels reflect those in the protein levels, we have developed a micro enzyme assay for partially purified samples from a pool of 10 mice brains. ACMSD activity was found to be specifically concentrated into a fraction precipitated between 50 and 60% of ammonium sulfate. After the dialysis and ultrafiltration, brain ACMSD was concentrated about 50-fold at the activity/volume and purified about 10-fold at the specific activity. The yields of the activities were confirmed to be reproducible at about 55 ± 5% (means ± standard deviations) in four experiments using the known amount of purified kidney ACMSD samples and the liver or kidney tissues. Brain ACMSD activities shown in Tables II and III were calculated and normalized with this value of the yield. Statistical analysis was done for values obtained from four enzyme assays using four pools of 10 mice brains for each experimental condition. Relationship between ACMSD message levels measured by the real-time PCR technique and the enzyme activities in the liver, kidney, and brain samples were highly correlated as shown in Fig. 4. The correlation coefficient value was 0.84.

Brain QPRT activity was also measured using similarly purified mice brains. QPRT activity was found to be specifically concentrated into a fraction after heat treatment and precipitation by 40% of ammonium sulfate. Brain QPRT was concentrated about 60-fold at the activity/volume and purified about 20-fold at the specific activity. The yields of the activities were confirmed to be reproducible at about 260 ± 18% (means ± standard deviations) in four experiments using the known amount of purified kidney QPRT samples and the liver or kidney tissues. The apparent increase in the yield was reported previously and suggested that the purification removed inhibitory substances in the homogenates (19). Brain QPRT activities shown in Tables II and III were calculated and normalized with this value of the yield. Statistical analysis was done for values obtained from four enzyme assays using four pools of 10 mice brains for each experimental condition.

The changes of brain ACMSD in different nutritional conditions and diabetes measured at the enzyme activity levels were clearly correlated to the changes measured at the message levels by the real-time PCR technique. The changes of brain QPRT measured at the enzyme activity levels remained unchanged in the different conditions, indicating that the correlation between protein and message levels existed.

**DISCUSSION**

This report describes the cloning of human ACMSD and helps to characterize its structure and functional expression. The identification of this enzyme was achieved by a) purification and determination of partial amino acid sequences, b) cloning and structural characterization of cDNAs encoding human, rat, mouse, and C. elegans ACMSD, and c) functional expression of human ACMSD in COS 7 cells and measurement of enzymatic activity.

ACMSD is a unique enzyme that react with ACMS, the primary oxidation product of 3-hydroxyanthranilate, to produce AMS with the simultaneous formation of CO2. Without the enzyme, ACMS is unstable and rapidly cyclizes spontaneously to produce quinolinate, a potent excitotoxin, which is thought to be involved in the pathogenesis of neurodegenerative disorders such as epilepsy, Alzheimer’s disease, and Huntington’s disease (the quinolinate hypothesis) (8, 9). Although genes responsible for Alzheimer’s and Huntington’s diseases have been identified, the mechanism by which malfunction of these genes causes neural death is still largely unknown. Because quinolinate is the only known physiological excitotoxin, which acts via NMDA receptors, the quinolinate hypothesis should be tested in the molecular context. This paper provides information toward this end.

The primary structure of ACMSD does not share any particular homology to known enzymes or proteins in databases, suggesting that ACMSD represents a novel type of protein structure-fold. No typical consensus sequences for nucleotide, metal, or any other co-factor binding sites were detected with motif search programs. This is consistent with the finding that ACMSD is not affected by metal-chelating agents.

ACMSD activity has been shown to be inhibited by various SH inhibitors. p-Chloromercuribenzoate inhibited its activity nearly 100% at a concentration of 0.1 mM. This inhibition is prevented by either reduced glutathione or cysteine. Alignment of the mammalian and C. elegans ACMSD sequences indicated that Cys (2), all in close proximity, are conserved in four species beyond the phylogenetic boundary (Fig. 3, boxed), suggesting that these Cys residues may be involved in the reaction center of ACMSD.

Utilizing homologous regions shared by human, rat, and mouse ACMSD sequences, we were able to design a common set of primers by which the expression of ACMSD mRNA could be quantitated by real-time PCR. Using glyceraldehyde-3-phosphate dehydrogenase as an internal standard, the ratio of ACMSD expression in mouse kidney, liver, and brain was found to be 370:15:1. The expression of brain ACMSD was found to be nutritional condition-sensitive: a low protein diet decreased and a streptozocin-induced diabetes increased the expression levels. The mode of action was similar to those in kidney and liver (11, 15). Quinolinate synthesized in peripheral tissues, however, could not pass through the blood brain barrier (25), proving that the metabolism of quinolinate and its regulation is brain-specific. Because the changes in ACMSD expression at the message levels are shown to be highly correlated to those at the enzyme activity levels, it is suggested that the quantification of the message levels with the real-time PCR technique is useful to address the regulation of ACMSD expression and quinolinate levels.

In conclusion, this study provides a molecular definition of mammalian ACMSD and essential information for future studies designed to reveal the physiological function of the enzyme both in the central nervous system and in peripheral tissues.

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