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Molecular cloning of rat kynurenine aminotransferase: identity with glutamine transaminase K

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Abstract The enzyme kynurenine aminotransferase (KAT) catalyses the conversion of L-kynurenine to kynurenic acid. A combination of polymerase chain reaction techniques and hybridization screening was used to isolate a cDNA clone encompassing the entire coding region of KAT from rat kidney. Identification of the cDNA as coding for KAT was based both on the comparison of amino acid sequences obtained from purified rat KAT and on the expression of KAT activity in COS-1 cells transfected with the cDNA. RNA blot analysis indicated that KAT mRNA is widely expressed in rat tissues. Cultured cells transfected with the cDNA for KAT also showed glutamine transaminase K activity. Based mainly on sequence data, these results demonstrate that rat kidney KAT is identical with glutamine transaminase K.

Key words: Kynurenine aminotransferase; Glutamine transaminase K; Cysteine conjugate β-lyase; Excitatory amino acid; Neuroprotection; Kynurenic acid

1. Introduction

Indoleamine 2,3-dioxygenase (IDO) (EC 1.13.11.11) opens the tryptophan indole ring, initiating the formation of a series of compounds collectively called kynurenines [1]. Among these, kynurenic acid (KYNA) is known as an endogenous antagonist of all three ionotropic excitatory amino acid receptors in the mammalian brain [1]. KYNA has attracted particular attention for its ability to block quinolinic acid-induced neurotoxicity and seizures [2], to decrease ischemic brain damage and associated deficits [3], and to protect against hypoxia-induced cerebral edema [4]. In mammalian peripheral organs, KYNA is biosynthesized from L-kynurenine by several rather unspecific aminotransferases [5,6]. Studies in the rat brain have shown that at physiological kynurenine concentrations a single kynurenine aminotransferase (KAT) is responsible for KYNA production [7]. In the course of our investigations of KYNA metabolism and function [8], we now report the isolation of a full length cDNA clone which contains the entire protein coding region of rat KAT.

2. Materials and methods

2.1. Partial amino acid sequence of rat KAT

Rat kidney KAT was prepared essentially as described previously [9]. The enzyme eluted from a Sephacryl S-200 column was further purified by HPLC using a reverse-phase column (SC18, 250 × 4.6 mm, Japan Spectro. Co. Ltd). Elution was performed with a gradient of 70% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid (TFA) and 0.1% TFA, applied for 40 min at a flow rate of 1 ml/min. 500 pmol of HPLC-purified rat KAT were then digested by trypsin and CNBr as described [10]. Subsequently, the samples were subjected to reverse-phase HPLC, and the resulting polypeptide fragments were identified and collected. Peptides 9–25 and 194–225 were obtained by trypsin digestion while peptide 354–367 was obtained after CNBr cleavage. Sequence analysis was performed by Edman degradation in a gas-phase sequenator (Applied Biosystems, Foster City, CA, USA) [11]. Cys-203 and Cys-210 in peptide 194–225 were determined as carboxymethylcysteine. The first two amino acids in peptide 354–367 were not determined due to high background of the first two cycles of the analysis.

2.2. PCR cloning and library screening

Total RNA from rat kidney was extracted from small quantities of tissue according to the RNAzol method (RNAzol-Cinna/Biotex Lab, TX, USA). Reverse transcription and PCR were performed as described [12]. Since the relative position of the two tryptic fragments obtained from purified rat kidney KAT (peptides 9–25 and 194–225) along KAT's primary structure was unknown, four degenerated 26-bp-long oligonucleotides were designed and synthesized using a DNA/RNA synthesizer (380B Applied Biosystems), and the reaction products were purified on a Sephadex G-50 column (Nap 25 Column, Pharmacia). The sense orientation oligonucleotide, OligoA: (AAYYTNTGYCARCARCAYGAYGTNGT), and the anti-sense orientation oligonucleotide, OligoC: (ACNACRTCRTGYTGYTGRCANARRTT) based on the peptide sequence Asn-Leu-Cys-Gln-Gln-His-Asp-Val-Val (residues 8–16 of peptide 194–225), and the sense orientation oligonucleotide, OligoB: (ACNGANARRTTYTGRTCXATNCCRTC) and the corresponding anti-sense oligonucleotide, oligoD: (GAYGGNATZGAYCARAAYYTNTCNGT), based on the peptide sequence Asp-Gly-Ile-Asp-Gln-Asn-Leu-Ser-Val (residues 3–11 of peptide 9–25) (N = TCAG; Z = TCA; R = AG; Y = TC; X = TGA), were synthesized. The first strand cDNA was divided in two aliquote and amplified by PCR. The two oligonucleotide mixtures PCR1: oligoA and oligoD and PCR2 oligoB and oligoC were used as primers in the PCR reaction. A specific amplification product was observed only with PCR1. The product of the amplification was a DNA molecule of about 550 bp. The PCR1-amplification product was gel-purified and sequenced by the dideoxy chain termination method [13], using Sequenase (United States Biochemicals Corp., Cleveland, OH). The PCR product was used as a probe to screen under stringent conditions a λgt11 rat kidney cDNA library (Clontech, Palo Alto, CA). The phage DNA from the positive plaques was digested with *Eco*RI, and the insert was ligated to the *Eco*RI site of pUC18. Sequencing was carried out with universal and forward primer and subsequently with a series of synthetic oligonucleotide primers according to the dideoxy termination method.

2.3. Expression of cloned KAT in COS-1 cells

The expression plasmid encoding rat KAT was constructed as follows. PCR amplification was performed using two specific oligonucleotides with *Xho*I linkers. The sense orientation oligonucleotide

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(5'-TGTCCTCGAGACCATGACCAAACGGCTGCAGGCTCGG-A-3') began at +4 of the coding strand, whereas the antisense-orientation oligonucleotide (5'-GTACCTCGAGTCAGGGTTGGAGCTC-TTTCCACTTG-3') complemented the sequence starting from the end of the coding sequence. The *Xho*I-digested fragment, after being controlled by sequencing, was cloned into the *Xho*I site of a pSVL expression vector (Pharmacia biotechnology). COS-1 cells were transfected with 10 μg of pSVL-KAT plasmid by the calcium phosphate method [14]. 72 hours after transfection, cells were disrupted by freezing and thawing. After centrifugation (15,000 *g* for 30 min), the supernatant was used for the determination of KAT and glutamine transminase K (GTK) activities.

2.4. Transaminase assays

The reaction mixture (100 μl) contained 70 μM pyridoxal phosphate, 5 mM pyruvate, 3 mM kynurenine, and enzyme preparation in 0.17 M potassium phosphate buffer, pH 8.1, and was incubated at 37°C for 30 min. The reaction was stopped by adding 20 μl 50% trichloroacetic acid, and the precipitate was removed by centrifugation. The KYNA content of the supernatant was analyzed by HPLC (flow rate: 1 ml/min) using a C18 column (Vydac 201TP54, 25 × 4.6 cm × mm) equilibrated with 5 mM acetic acid, 5% methanol, 0.1% heptane sulfonic acid, pH 3.0 KYNA was eluted with 50 mM acetic acid, 5% methanol, 0.5% heptane sulfonic acid, pH 4.5, and its absorbance was measured at 243 nm. GTK activity was measured in the same cell extract as described previously [15], using 10 mM phenylalanine and 5 mM α-keto-γ-methiolbutyrate.

3. Results and discussion

Positive plaques were isolated by the screening of approx. 5×10^5 plaques from a rat kidney cDNA library, and the inserts were analyzed by restriction analysis and DNA sequencing. Fig. 1 shows the full-length cDNA sequence of rat KAT. The partial amino acid sequences obtained from the NH_2 terminus and internal peptides of the purified rat kidney enzyme, includ-

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CTC ACC ATG ACC AAA CGG CTG CAG GCT CGG AGG CTG GAC GGG ATT GAT CAA AAC CTC TGG GTG GAG TTT GGC AAA CTG ACC AAG GAG TAT   90
      Met Thr Lys Arg Leu Gln Ala Arg Arg Leu Asp Gly Ile Asp Gln Asn Leu Trp Val Glu Phe Gly Lys Leu Thr Lys Glu Tyr

GAC GTC GTG AAC TTG GGT CAG GGC TTC CCT GAC TTC TCG CCT CCG GAC TTT GCA ACG CAA GCT TTT CAG CAG GCT ACC AGT GGG AAC TTC  180
Asp Val Val Asn Leu Gly Gln Gly Phe Pro Asp Phe Ser Pro Pro Asp Phe Ala Thr Gln Ala Phe Gln Gln Ala Thr Ser Gly Asn Phe

ATG CTC AAC CAG TAC ACC AGG GCA TTT GGT TAC CCA CCA CTG ACA AAC GTC CTG GCA AGT TTC TTT GGC AAG CTG CTG GGA CAG GAG ATG  270
Met Leu Asn Gln Tyr Thr Arg Ala Phe Gly Tyr Pro Pro Leu Thr Asn Val Leu Ala Ser Phe Phe Gly Lys Leu Leu Gly Gln Glu Met

GAC CCA CTC ACG AAT GTG CTG GTG ACA GTG GGT GCC TAT GGG GCC TTG TTC ACA GCC TTT CAG GCC CTG GTG GAT GAA GGA GAT GAG GTC  360
Asp Pro Leu Thr Asn Val Leu Val Thr Val Gly Ala Tyr Gly Ala Leu Phe Thr Ala Phe Gln Ala Leu Val Asp Glu Gly Asp Glu Val

ATC ATC ATG GAA CCT GCT TTT GAC TGT TAT GAA CCC ATG ACA ATG ATG GCT GGA GGT TGC CCT GTG TTC GTG ACT CTG AAG CCG AGC CCT  450
Ile Ile Met Glu Pro Ala Phe Asp Cys Tyr Glu Pro Met Thr Met Met Ala Gly Gly Cys Pro Val Phe Val Thr Leu Lys Pro Ser Pro

GCT CCT AAG GGG AAA CTG GGA GCC AGC AAT GAT TGG CAA CTG GAT CCT GCA GAA CTG GCC AGC AAG TTC ACA CCT CGC ACC AAG GTC CTG  540
Ala Pro Lys Gly Lys Leu Gly Ala Ser Asn Asp Trp Gln Leu Asp Pro Ala Glu Leu Ala Ser Lys Phe Thr Pro Arg Thr Lys Val Leu

GTC CTC AAC ACA CCC AAC AAC CCT TTA GGA AAG GTA TTC TCT AGG ATG GAG CTG GAG CTG GTG GCT AAT CTG TGC CAG CAG CAC GAT GTC  630
Val Leu Asn Thr Pro Asn Asn Pro Leu Gly Lys Val Phe Ser Arg Met Glu Leu Glu Leu Val Ala Asn Leu Cys Gln Gln His Asp Val

GTG TGC ATC TCT GAT GAG GTC TAC CAG TGG CTG GTC TAT GAC GGG CAC CAG CAC GTC AGC ATC GCC AGC CTC CCT GGC ATG TGG GAT CGG  720
Val Cys Ile Ser Asp Glu Val Tyr Gln Trp Leu Val Tyr Asp Gly His Gln His Val Ser Ile Ala Ser Leu Pro Gly Met Trp Asp Arg

ACC CTG ACC ATC GGC AGT GCA GGC AAA AGC TTC AGT GCC ACT GGC TGG AAG GTG GGC TGG GTC ATG GGT CCA GAT AAC ATC ATG AAG CAC  810
Thr Leu Thr Ile Gly Ser Ala Gly Lys Ser Phe Ser Ala Thr Gly Trp Lys Val Gly Trp Val Met Gly Pro Asp Asn Ile Met Lys His

CTG AGG ACA GTG CAC CAG AAT TCT ATC TTC CAC TGC CCC ACC CAG GCC CAG GCT GCA GTA GCC CAG TGC TTT GAG CGG GAG CAG CAA CAC  900
Leu Arg Thr Val His Gln Asn Ser Ile Phe His Cys Pro Thr Gln Ala Gln Ala Ala Val Ala Gln Cys Phe Glu Arg Glu Gln Gln His

TTT GGA CAA CCC AGC AGC TAC TTT TTG CAG CTG CCA CAG GCC ATG GAG CTG AAC CGA GAC CAC ATG ATC CGT AGC CTG CAG TCA GTG GGC  990
Phe Gly Gln Pro Ser Ser Tyr Phe Leu Gln Leu Pro Gln Ala Met Glu Leu Asn Arg Asp His Met Ile Arg Ser Leu Gln Ser Val Gly

CTC AAG CTC TGG ATC TCC CAG GGG AGC TAC TTC CTC ATT GCA GAC ATC TCA GAC TTC AAG AGC AAC ATG CCT GAC CTG CCC GGA GCT GAG 1080
Leu Lys Leu Trp Ile Ser Gln Gly Ser Tyr Phe Leu Ile Ala Asp Ile Ser Asp Phe Lys Ser Lys Met Pro Asp Leu Pro Gly Ala Glu

GAT GAG CCT TAT GAC AGA CGC TTT GCC AAG TGG ATG ATC AAA AAC ATG GGC TTG GTG GGC ATC CCT GTC TCC ACA TTC TTC AGT CGG CCC 1170
Asp Glu Pro Tyr Asp Arg Arg Phe Ala Lys Trp Met Ile Lys Asn Met Gly Leu Val Gly Ile Pro Val Ser Thr Phe Phe Ser Arg Pro

CAT CAG AAG GAC TTT GAC CAC TAC ATC CGA TTC TGT TTT GTC AAG GAC AAG GCC ACA CTC CAG GCC ATG GAT GAG AGA CTG CGC AAG TGG 1260
His Gln Lys Asp Phe Asp His Tyr Ile Arg Phe Cys Phe Val Lys Asp Lys Ala Thr Leu Gln Ala Met Asp Glu Arg Leu Arg Lys Trp

AAA GAG CTC CAA CCC TGA GGA GGC TGC CCT CAG CCC CAC CTC GAA CAC AGG CCT CAG CTA TGC CTT AGC ACA GGG ATG GCA CTG GAG GGC 1350
Lys Glu Leu Gln Pro

CCA GCT GTG TGA CTG CGC ATG TTT CCA GAA AAG AGG CCA TGT CTT GGG GGT TGA AGC CAT CCT TTC CCA GTG TCC ATC TGG ACT ATT GGG 1440

TTG GGG GCC AGT TCT GGG TCT CAG CCT ACT CCT CTG TAG GTT GCC TGT AGG GTT TTG ATT GTT TCT GGC CTC TCT GCC TGG GGC AGG AAA 1530

GGG TGG AAT ATC AGG CCC GGT ACC ACC TTA GCC CTG CCG AGG CTC TGT GGC TTC TCT ACA TCT TCT CCT GTG ACC TCA GGA TGT TGC TAC 1620

TGT TCC TAA TAA AGT TTT AAG TTA TTA GG(A)                                                                              1667
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Fig. 1. Nucleotide sequence of KAT cDNA and its derived amino acid sequence. Regions of alignment with trypsin and CNBr cleavage fragments as well as N-terminal sequence are underlined. Note that peptide 9–25 is included in the N-terminal sequence. The N-terminal was determined after PCR cloning. The putative PLP binding site is underlined twice. Triplets differing from the rat β-lyase sequence [16] are boxed.

ing a total of 70 amino acids, were consistent with the deduced amino acid sequence of the cDNA. The predicted molecular weight (47,743) was in good agreement with that determined for the native rat kidney enzyme [9]. The nucleotide sequence surrounding the first methionine corresponded well to the Kozak's consensus sequence for an eukaryotic initiation site, so that it is likely that translation begins at the ATG site shown in Fig. 1. The first amino acid of mature KAT determined by protein sequencing is leucine. It is therefore likely that removal of four amino acids at the N-terminus of rat KAT occurs in vivo due to the proteolytic cleavage of endopeptidases acting at dibasic residues. A typical polyadenylation signal, AATAAA, was observed in the 3'-untranslated region at nucleotides 1628–1633. A consensus sequence for pyridoxal phosphate (PLP) binding [15] was present at amino acids 244–249 of the deduced KAT sequence. Northern gel analysis of multiple tissues identified heterogeneous rat KAT transcripts. A 2.3 kb major mRNA was expressed, albeit at different levels, in all tissues except testis where a slightly larger mRNA could be detected. Two bands of about 2.3 and 3.0 kb, at a relative abundance of about 3:1, were detected in mRNA extracted from liver. To assess functional activity of the cDNA, rat KAT was subcloned into a mammalian expression vector and transfected to COS-1 cells. A significant increase in KAT activity was observed in sense-transfected cells as compared to antisense-transfected control cells (Fig. 3B). Comparison of the nucleotide sequence with the GenBank DNA sequence database indicated that the sequence was virtually identical with rat kidney cytosolic cysteine conjugate β-lyase [16]. Two differences, likely explained by rat strain heterogeneity, were observed: alanine instead of arginine (residue 107) and valine instead of isoleucine (residue 177). Since cysteine conjugate β-lyase possesses GTK activity [17], KAT-positive COS-1 cells transfected with the sense cDNA were examined for the presence of GTK activity (Fig. 3A). The results demonstrated the identity of KAT and GTK. Molecular

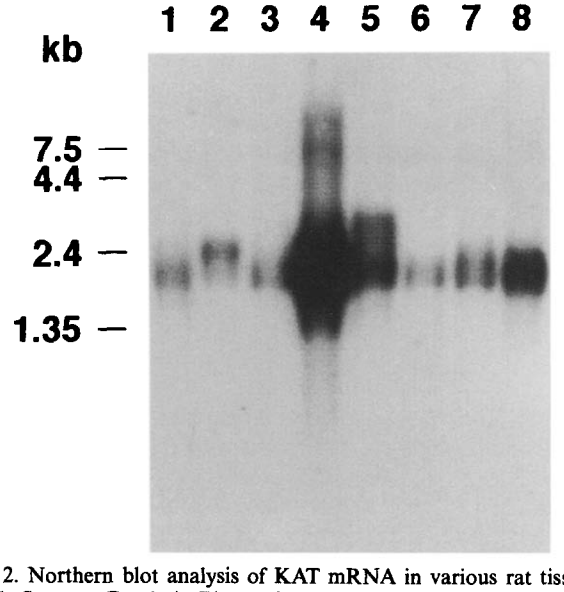

Fig. 2. Northern blot analysis of KAT mRNA in various rat tissues (adult Sprague–Dawley). The probe was the 550 bp PCR fragment corresponding to the N-terminus part of the KAT coding region. Lane 1, ovary; 2, testis; 3, retina; 4, kidney; 5, liver; 6, lung; 7, brain; 8, heart. The positions of RNA size markers are shown on the left, and the numbers indicate size in kilobases.

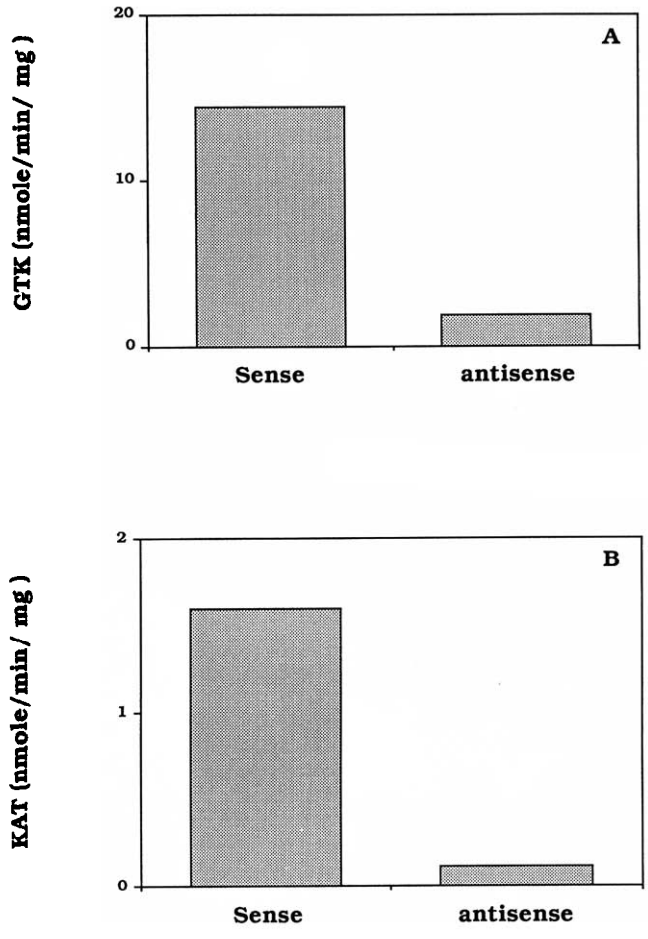

Fig. 3. Transaminase activities in transfected cells. Enzyme activities are expressed per mg of protein. A, glutamine transaminase K activity; B, kynurenine transaminase activity. Each value is the mean of three experiments.

probes derived from the cloned cDNA can therefore be expected to provide useful tools for the further study of the role of this multifunctional enzyme en physiology and pathology.

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