Cloning and recombinant expression of rat and human kynureninase

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Abstract Kynureninase [E.C.3.7.1.3.] is one of the enzymes involved in the biosynthesis of NAD cofactors from tryptophan through the kynurenine pathway. By tryptic and CNBr digestion of purified rat liver kynureninase, we obtained about 28% of the amino acid sequence of the enzyme. The rat kynureninase cDNA, isolated by means of reverse-transcribed polymerase chain reaction and hybridization screening, codes for a polypeptide of 464 amino acids. Northern blot analysis revealed the synthesis of a 2.0 kb rat kynureninase mRNA. A cDNA encoding human liver kynureninase was also isolated. The deduced amino acid sequence is 85% identical to that of the rat protein. COS-1 cells were transfected with both cDNAs. The K_m values of the rat enzyme, for L-kynurenine and DL-3-hydroxykynurenine, were 440 ± 20 μM and 32 ± 5 μM and of the human enzyme 440 ± 20 μM and 49 ± 6 μM, respectively. Interestingly, COS-1 cells transfected with the cDNA coding for rat kynureninase also display cysteine-conjugate β-lyase activity.

Key words: Kynurenine; Kynurenic acid; Excitatory amino acid; Neuroprotection; Cysteine-S-conjugate β-lyase; Quinolinic acid

1. Introduction

Kynureninase [L-kynurenine hydrolase E.C.3.7.1.3] catalyzes the conversion of L-3-hydroxykynurenine (3-OHKYN) and L-kynurenine (L-KYN) to 3-hydroxyanthranilic acid (3-OHAA) and anthranilic acid (AA), respectively [1]. The reaction is pyridoxal-5′-phosphate (PLP) dependent and is sensitive to nutritional vitamin B6 deprivation in mammals [2]. The biochemical properties of kynureninase were studied in mouse, rat and pig liver extracts [3–6] showing that this enzyme is an homodimer of around 95 kDa. It is present in all major classes of vertebrates [7] and can be regarded as a 3-hydroxykynureninase-type enzyme, since it hydrolyzes preferentially 3-OHKYN [8]. The product of this reaction, 3-OHAA is further utilised to synthesize quinolinic acid (QUINA), whose neurotoxic effect in the CNS is well documented [9]. On the other hand, L-KYN can be converted by kynurenine aminotransferase to produce kynurenic acid (KYNA), a neuroprotective agent [1]. Therefore, increases in KYNA concentrations within the central nervous system could mitigate the neurotoxic effects of QUINA while reductions in KYNA levels could promote excitotoxicity. Intraperitoneal injection of a kynureninase inhibitor *o*-methoxybenzoylalanine (oMBA), increases the concentration of KYNA in the hippocampal extracellular space and is able to prevent audiogenic convulsions in DBA/2 mice [10]. This, among other evidence [11], suggests that the pharmacological modulation of the kynurenine pathway could have neuroprotective effects. A substantial increase in the kynureninase activity has been observed in several cerebral and systemic inflammatory conditions [12]. In addition, it has been recently found that interferon-γ induces kynureninase activity in murine macrophages [13] and kynureninase mRNA in human monocyte/macrophages [14]. Moreover, recent in vivo data [15] suggested either the existence of two kynureninase enzymes or the presence of different regulatory co-factors of the enzymatic activity in liver and brain tissues. The cloning and characterization of kynureninase cDNA expression is therefore instrumental for clarifying the function of this enzyme, the existence of possible tissue-specific isoforms, the regulation of its expression by immune stimuli and its possible involvement in disorders associated with unbalanced levels of QUINA and KYNA. To gain insights into the physiology and pathology of the kynurenine pathway we have recently isolated the cDNAs encoding two other enzymes: kynurenine aminotransferase [16] and kynurenine 3-hydroxylase [17]. Here we report the molecular cloning of rat and human kynureninase and their biochemical characterization in transiently transfected COS-1 cells. The constitutive expression of rat kynureninase mRNA is also demonstrated in liver, kidneys, brain and other tissues by reverse-transcribed polymerase chain reaction (RT-PCR) and Northern blot analysis.

2. Materials and methods

2.1. Partial amino acid sequence of rat liver kynureninase

Rat liver kynureninase was purified to homogeneity as previously described [18]. Both the intact enzyme and the protein electroblotted onto PDVF membrane, were subjected to Edman's degradation. Next, 500 pmol of purified rat kynureninase were digested by trypsin and CNBr, and the samples were subjected to reverse-phase HPLC (column 4.6×250 mm, Biofine RPC-SC18 or YMC-c4). The resulting polypeptide fragments were identified and collected. Sequence analysis was performed by Edman's degradation in a gas-phase sequencer (Applied Biosystems, Foster City, CA). Several peptide sequences were obtained: *M1*: CSYKYLNSGAGGLAGAFIHEKHAHTIKPALVGX; *M2*: FGHELSTRFNM; *M3*: VKTYLEELDK; *M4*: AKIGAYGHEVGKRPX; *M5*: IIGDESI; *M6*: IKPREGEETLR; *M7*: AKIGAY; *M8*: KDIVGAHEKEIA; *M9*: EPSPLELPVDAVRRIATELNCDP; *T1*: HAHTIKPALVGXFGHELSTR; *T2*: FNMDNK; *T3*: TYLEELDKXAKIGAYGHEVGK; *T4*: PXIIGDESI; *T5*: LDEEDKLK; *T6*: VAPVPLYNSFHDVYK; *T7*: LQLIPGVNGFR; *T8*: AFPSDHYAIEXQI; *T9*: DLPSIDLSLVNEDDNAIYFLGN; *T10*: SILLTGYLEYL. CNBr peptides are de-

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noted with a prefix *M*, and tryptic peptides with a prefix *T*. Amino acids are named using the one-letter code.

2.2. Rat and human kynureninase cDNA clones selection from λgt11 phage libraries of liver cDNA

A 96-bp-long fragment of the rat kynureninase cDNA was obtained by RT-PCR as follows. Reverse transcription was performed using rat liver poly(A)$^+$ mRNA, obtained using oligo (dT)-cellulose spin columns (Pharmacia Biotech, Uppsala, Sweden) on total RNA, extracted according to the RNAzol method (Cinna/Biotex Lab, TX). Reverse transcription and PCR were performed as previously described [19,20]. Two amino acid sequences from the least degenerated regions of the following overlapping peptides: M1, M2, T1 and T2, were selected to design degenerated oligonucleotides: 5′-GC(G,A,T,C)-TT(T,C)AT(A,T,C)CA(T,C)GA(A,G)AA(A,G)CA-3′ (sense orientation) based on the peptide sequence AFIHEKH and 5′-(T,C)-TT(A,G)TT(A,G)TCCAT(A,G)TT(A,G)AA(G,A,C,G)C-3′ (antisense orientation) based on the peptide sequence RFNMDNK. The product of the amplification was gel-purified and sequenced using the Sequenase Kit (United States Biochemicals Corp., Cleveland, OH).

Rat and human cDNA libraries were constructed starting from 1 μg of liver poly(A)$^+$ mRNA in λgt11 essentially as previously described [21]. The 96-bp PCR product was used as a probe to screen under stringent conditions the rat liver λgt11 cDNA library. More than 20 positive clones were obtained and the one with the longest insert was selected. The λ phage DNA was digested with *Eco*RI and the insert was purified by gel electrophoresis and subcloned into a Bluescript SK phagemid (Stratagene, La Jolla, CA). Sequencing was carried out with universal and gene specific primers. Oligonucleotides were synthesised using the 394 DNA/RNA Synthesizer (Applied Biosystem, Foster City, CA) and purified with Sephadex G25 columns (Pharmacia Biotech, Uppsala, Sweden). The screening of the human liver cDNA library was performed using the rat full-length cDNA as ^{32}P-labelled probe. The insert was cleaved from the positive human clones with *Eco*RI restriction enzyme and subcloned into a Bluescript SK vector (Stratagene, La Jolla, CA).

2.3. RT-PCR study and Northern blot analysis of rat kynureninase mRNA expression in different tissues

Rat poly(A)$^+$ mRNA from different tissues (Clontech, Palo Alto, CA) was treated for 30 min with RNase-free DNase (Stratagene, La Jolla, CA) at 37°C to remove any residual genomic DNA. Messenger RNA samples were reverse transcribed at 37°C for 60 min. The reaction mixture contained 250 ng of poly(A)$^+$ mRNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 4 mM DTT, 0.5 mM each dNTP, 30 U of RNase Inhibitor (Promega, Madison, WI), 100 pmol of random examers (Promega, Madison, WI) and 200 U Superscript Reverse Transcriptase (GIBCO Life Tech., Gaithersburg, MA), in a final volume of 30 μl. The reverse transcription step was omitted in PCR controls from each RNA sample to check for the presence of contaminating genomic DNA. Three microliters of each cDNA were used for the PCR reaction. The PCR mix contained 0.2 μM of upstream and downstream primers 5′-CAACTATGACTGCGCT-GAGG-3′; 5′-GTTCTCGCTTGTCACAGACG-3′, 200 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl, 2.5 U of Amplitaq (Perkin Elmer Cetus, Norwalk, CT). Thermal cycling was performed as follows: 1 min at 94°C, 1 min at 60°C, 2 min at 72°C for 30 cycles. For the Northern Blot analysis: a commercial filter (Clontech, Palo Alto, CA) containing 2 μg per lane of poly(A)$^+$ mRNA from different rat tissues was hybridised under stringent conditions with the ^{32}P-labelled 96-bp PCR product used for the screening of the rat library.

2.4. Recombinant expression of rat and human kynureninases in COS-1 cells and enzymatic assays

The rat and human kynureninase cDNAs were inserted into the *Xho*I site of the eukaryotic expression vector pSVL (Pharmacia Biotech, Uppsala, Sweden). Inserts were obtained by means of PCR and specific 5′ and 3′ primers. The following oligonucleotides were used: 5′-TGTCTCGAGCTGATGGAGCCCTCGCCT-3′ (sense) and 5′-TGTCTCGAGGGCTAGTTTCTTTCTGTAG-3′ (antisense) for rat kynureninase; 5′-TGTCTCGAGGTAATGGAGCCTTCATCT-3′ (sense); 5′-TGTCTCGAGTGCTAATTTTTTGTTTCTG-3′ (antisense) for human kynureninase. The identity of the PCR fragments was verified by DNA sequencing of both strands. COS-1 cells (1 500 000/75 cm^2), grown in DMEM medium, were transfected with 10 μg of pSVL-rat or pSVL-human kynureninase DNA using the calcium phosphate method [22]. Seventy-two hours after transfection, cells were disrupted by 4 cycles of freezing and thawing. After centrifugation of the lysate (15 000 × *g* for 30 min) the supernatant was stored at −20°C and used for the enzymatic assay.

Kynureninase activity was assayed according to Kawai et al. [23]. The assay mixture contained 2 mM of L-KYN or DL-3OHKYN, 20 μM of PLP in 50 mM Tris buffer (pH 8.5), and the reaction was stopped with TCA 8.3% (w/v), at different times after incubation at 37°C and centrifuged. The supernatant was applied to a C18 reversed-phase column (201TP54, 4.6×25 cm, Vydac Separation Group, CA) and eluted isocratically at a flow rate of 1 ml/min with a mobile phase consisting of 80 mM potassium phosphate (pH 2.5), 17% (v/v) acetonitrile. AA or 3-OHAA were measured with a fluorescence detector on line (Waters 414, Waters Corporation, MA) set at λ_{ex} = 313 nm and λ_{em} = 420 nm. The detection was about 60-fold less sensitive for 3-HOAA than for AA in these experimental conditions. Kinetic constants were calculated with a non-linear regression program (SigmaPlot) using the Michaelis-Menten equation. Notably, kynureninase has been shown to be specific for the L-forms of 3OHKYN whereas the D-forms are neither substrates nor inhibitors of the enzyme [1], Since in this work the racemic (DL) form of 3OHKYN was used, the reported K_m values should accordingly be divided by a factor of two to obtain the correct K_m for L-3OHKYN.

Cysteine-conjugate β-lyase activity was measured using a coupled assay as described by Abraham and Cooper [24]. The reaction mixture, consisting of 50 mM Tris buffer (pH 8.5), 2 mM S-(1,2-dichlorovinyl)-L-cysteine (DCVC), 0.5 mM α-keto-γ-methiolbutyrate, 0.1 mM PLP, was incubated for 0, 30, 60 min prior to the incubation of the reaction product with 1.8 U alanine dehydrogenase with addition of 0.3 mM NADH and 0.1 M ammonium acetate. Absorbance at 340 nm was measured using a microplate reader. All reagents were from Sigma if not otherwise indicated.

3. Results and discussion

Rat liver kynureninase was purified to homogeneity from the liver homogenate supernatant with a 1286-fold enrichment of the kynureninase activity in the final fraction [18]. No sequence information could be obtained after Edman's degra-

Table 1
Kinetic properties of recombinant human and rat kynureninase expressed in COS-1 cells and measured in presence of 20 μM PLP

	Human kynureninase		Rat kynureninase		
	V_{max} (μM/min)	K_m (μM)	V_{max} (μM/min)	K_m (μM)	K_i (μM)
L-KYN	0.7 ± 0.07	438 ± 20	1.46 ± 0.01	440 ± 20	2.4 ± 0.2
DL-3OHKYN	4.3 ± 0.2	49 ± 6	4.2 ± 0.1	32 ± 5	13 ± 4

K_m were determined measuring kynureninase activity at 10 different concentrations of l-KYN (range 10 μM to 10 mM) and six different concentrations of DL-3HOKYN (range 10 μM to 0.5 mM). Protein concentration for rat and human kynureninase assay were 0.78 mg/ml and 0.18 mg/ml, respectively.

For the rat enzyme, reaction rate was also measured in presence of *o*-(methoxybenzoyl)alanine (5 μM) with L-KYN and DL-3OHKYN concentrations in the range of the K_m values.

Results are the mean ± SEM of three experiments.

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TTG AAA AGG TAC TGG AAA CTG AGG ACC CTA TCT GGA TCA AAG CAG TTT CTG ATG GAG CCC TCG CCT CTT GAG CTA CCA GTT GAT GCA GTG    90
                                                                Met Glu Pro Ser Pro Leu Glu Leu Pro Val Asp Ala Val

CGG CGC ATC GCG GCT GAA CTC AAT TGT GAC CCA ACC GAT GAG AGG GTG GCT CTC CGC TTG GAT GAG GAA GAT AAA CTG AAG CGT TTT AAG   180
Arg Arg Ile Ala Ala Glu Leu Asn Cys Asp Pro Thr Asp Glu Arg Val Ala Leu Arg Leu Asp Glu Glu Asp Lys Leu Lys Arg Phe Lys

GAC TGT TTT TAT ATC CCC AAA ATG CGG GAC CTG CCT TCA ATT GAT CTA TCT TTA GTG AAT GAG GAT GAT AAT GCC ATC TAT TTC CTG GGA   270
Asp Cys Phe Tyr Ile Pro Lys Met Arg Asp Leu Pro Ser Ile Asp Leu Ser Leu Val Asn Glu Asp Asp Asn Ala Ile Tyr Phe Leu Gly

AAT TCC CTT GGT CTT CAA CCG AAG ATG GTT AAA ACA TAC CTG GAG GAA GAG CTA GAT AAG TGG GCC AAA ATA GGA GCC TAT GGC CAT GAG   360
Asn Ser Leu Gly Leu Gln Pro Lys Met Val Lys Thr Tyr Leu Glu Glu Glu Leu Asp Lys Trp Ala Lys Ile Gly Ala Tyr Gly His Glu

GTA GGG AAA CGT CCT TGG ATT ATA GGA GAT GAG AGC ATT GTA ACC CTT ATG AAG GAC ATT GTA GGA GCC CAT GAG AAA GAA ATA GCT CTA   450
Val Gly Lys Arg Pro Trp Ile Ile Gly Asp Glu Ser Ile Val Thr Leu Met Lys Asp Ile Val Gly Ala His Glu Lys Glu Ile Ala Leu

ATG AAT GCT TTG ACT GTT AAT TTA CAT CTC CTG CTG TTA TCA TTC TTT AAG CCT ACA CCA AAG CGG CAC AAA ATT CTT CTA GAA GCC AAA   540
Met Asn Ala Leu Thr Val Asn Leu His Leu Leu Leu Leu Ser Phe Phe Lys Pro Thr Pro Lys Arg His Lys Ile Leu Leu Glu Ala Lys

GCC TTC CCT TCT GAT CAT TAT GCG ATC GAG TCA CAG ATT CAA CTT CAT GGA CTT GAT GTT GAG AAA AGT ATG CGG ATG ATA AAG CCA CGA   630
Ala Phe Pro Ser Asp His Tyr Ala Ile Glu Ser Gln Ile Gln Leu His Gly Leu Asp Val Glu Lys Ser Met Arg Met Ile Lys Pro Arg

GAG GGG GAA GAG ACC TTA AGA ATG GAG GAC ATA CTG GAA GTA ATT GAG AAG GAA GGA GAC TCA ATT GCT GTG GTC CTG TTC AGT GGC CTG   720
Glu Gly Glu Glu Thr Leu Arg Met Glu Asp Ile Leu Glu Val Ile Glu Lys Glu Gly Asp Ser Ile Ala Val Val Leu Phe Ser Gly Leu

CAC TTT TAT ACT GGA CAG CTG TTC AAC ATT CCT GCC ATT ACA CAA GCC GGA CAT GCA AAG GGC TGT TTT GTT GGC TTT GAC CTA GCG CAT   810
His Phe Tyr Thr Gly Gln Leu Phe Asn Ile Pro Ala Ile Thr Gln Ala Gly His Ala Lys Gly Cys Phe Val Gly Phe Asp Leu Ala His

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GCG GTT GGA AAT GTT GAA CTC CAC TTA CAT GAC TGG GAT GTT GAC TTT GCC TGC TGG TGC TCC TAC AAG TAT TTA AAT TCA GGA GCT GGA   900
Ala Val Gly Asn Val Glu Leu His Leu His Asp Trp Asp Val Asp Phe Ala Cys Trp Cys Ser Tyr Lys Tyr Leu Asn Ser Gly Ala Gly

GGT CTG GCT GGT GCC TTC ATC CAT GAG AAA CAC GCT CAC ACG ATC AAG CCA GCG TTA GTG GGA TGG TTC GGC CAT GAA CTC AGT ACA AGA   990
Gly Leu Ala Gly Ala Phe Ile His Glu Lys His Ala His Thr Ile Lys Pro Ala Leu Val Gly Trp Phe Gly His Glu Leu Ser Thr Arg

TTT AAC ATG GAT AAC AAA CTA CAA TTA ATC CCC GGG GTC AAT GGA TTC CGA ATT TCC AAC CCT CCC ATT CTG TTG GTC TGC TCC TTG CAT  1080
Phe Asn Met Asp Asn Lys Leu Gln Leu Ile Pro Gly Val Asn Gly Phe Arg Ile Ser Asn Pro Pro Ile Leu Leu Val Cys Ser Leu His

GCC AGT TTA GAG ATC TTT CAG CAA GCA ACT ATG ACT GCG CTG AGG AGA AAA TCC ATT CTG CTG ACA GGT TAT CTG GAA TAC TTG CTC AAA  1170
Ala Ser Leu Glu Ile Phe Gln Gln Ala Thr Met Thr Ala Leu Arg Arg Lys Ser Ile Leu Leu Thr Gly Tyr Leu Glu Tyr Leu Leu Lys

CAT TAC CAT GGC GGA AAT GAC ACA GAA AAC AAG AGG CCA GTT GTG AAC ATA ATC ACC CCA TCC AGA GCA GAG GAA CGA GGC TGC CAG CTG  1260
His Tyr His Gly Gly Asn Asp Thr Glu Asn Lys Arg Pro Val Val Asn Ile Ile Thr Pro Ser Arg Ala Glu Glu Arg Gly Cys Gln Leu

ACA CTG ACC TTT TCC ATT TCC AAG AAA GGC GTT TTT AAG GAA CTA GAA AAA AGA GGA GTC GTC TGT GAC AAG CGA GAA CCA GAA GGC ATC  1350
Thr Leu Thr Phe Ser Ile Ser Lys Lys Gly Val Phe Lys Glu Leu Glu Lys Arg Gly Val Val Cys Asp Lys Arg Glu Pro Glu Gly Ile

CGG GTG GCC CCG GTT CCT CTC TAT AAT TCT TTC CAT GAT GTT TAT AAG TTC ATC AGA CTG CTT ACT GCC ATA CTC GAC TCT ACA GAA AGA  1440
Arg Val Ala Pro Val Pro Leu Tyr Asn Ser Phe His Asp Val Tyr Lys Phe Ile Arg Leu Leu Thr Ala Ile Leu Asp Ser Thr Glu Arg

AAC TAG CCA TGC TTT CTA AAT AAC TCA AGT AAA TCT CAC ACA CTG GGG GTT CCA CTT CTA CTG CAT TTT AGT CAT TCA AAA GTC TCC AGA  1530
Asn

AAT TGA TGG CAT AGA AAT GAT GAT GAT TTT ATA AAC TTA CAT AAA ACC TGG TAC ATG TTT TAA TAT CTG TGT CGC TGA TGT GCT GTG GAC  1620

TAA GAA GTC ACA TTT TAC ATG ACT CCA ACC TAC AGA TGA CTG TCT TGA TCA GCT GTC ACC TTC CAT GGT CAC TGA AAG GTT GTG TGT TTA  1710

ATT TGT GAC TGA AAT GAC AAC ATT AAA ATG TAT CTG GAC TTC TTG TAT AAA AAA A                                               1765
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Fig. 1. Nucleotide sequence and predicted primary amino acid sequence of rat kynureninase. Regions of alignment with trypsin and CNBr cleavage fragments are underlined. The PLP binding region is underlined twice. The asterisk shows the lysine involved in the PLP binding. Triplets coding for amino acids which differ from those observed by Takeuchi et al. [25] are boxed (GeneBank accession number U68168).

dation of the purified protein, indicating that the NH_2-terminus is probably modified as a result of a post-transcriptional event. This data is in agreement with the study of Takeuchi et al. [25] which demonstrates the presence of a *N*-acetylmethionine at the N-terminal residue of the protein purified from rat liver. Several peptide sequences were obtained from the purified rat kynureninase (Fig. 1). Degenerated oligonucleotides were designed from the amino acid sequences of peptides M1, M2, T1 and T2 and they were used as primers for RT-PCR of rat liver cDNA (see Section 2 for details). The resulting 96-bp cDNA product was used to screen a rat liver cDNA library and to isolate a full-length rat kynureninase cDNA clone (Fig. 1). The rat kynureninase cDNA is 1765 nucleotides long. It contains a unique translation initiation site in position 52 and an open reading frame coding for a protein of 464 amino acid residues. The comparison of the rat kynureninase predicted sequence and the primary sequence of the native protein [25], shows two major differences: alanine instead of threonine (residue 18) and threonine instead of serine (residue 118). Notably, we also found an alanine residue in position 18 in the native protein (see peptide M9 sequence). Moreover, the-

onine in position 18 was found to be modified with an unknown group [25]. Whether these differences reflect the presence of two functionally distinct isoforms of the enzyme deserve further investigation. The tissue distribution of rat kynureninase mRNA was analyzed by Northern blot and RT-PCR. Fig. 2 shows the constitutive presence in liver and kidney, of a single kynureninase transcript, approximately 2.0 kb long. The RT-PCR analysis (Fig. 3), which is more sensitive than Northern blot, shows that rat kynureninase mRNA is also detectable in heart, retina, ovary, lung, testis and brain, consistent with the organ distribution of the kynureninase activity [23].

The rat kynureninase cDNA was used as probe to screen a human liver cDNA library and to isolate a cDNA clone corresponding to the human kynureninase (see Section 2 for details). The rat and human kynureninase cDNAs show a 70% identity at the nucleotide level (not shown) and 85% of identity at the protein level (Fig. 4). While this manuscript was in preparation, the cDNA sequence of the human kynureninase was reported [26]. No differences can be observed between the published human kynureninase cDNA sequence [26] and the

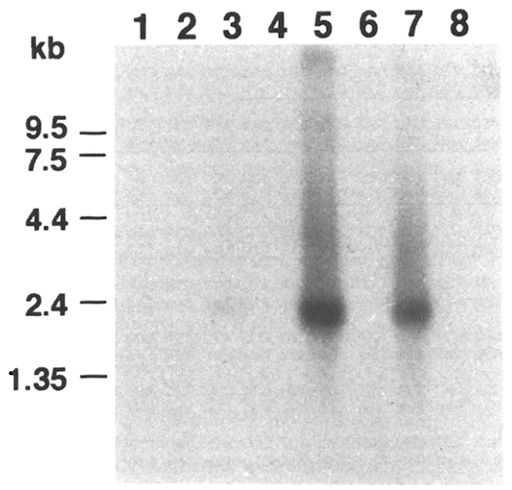

Fig. 2. Northern blot analysis of rat kynureninase mRNA in various rat tissues (adult Sprague-Dawley). Lanes: 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. RNA size (kb) is shown on the left.

one we refer to. A comparison of the human kynureninase cDNA sequence with the EST databank at NIH resulted in the detection of several sequences (accession No. R27138, R11005, among others) containing either the 5′ or the 3′ untranslated region of human kynureninase. These cDNA fragments were obtained from placenta and liver/spleen of a male fetus indicating the constitutive expression of the kynureninase mRNA in these human tissues. Interestingly, two putative proteins (Fig. 4) predicted on the base of the *Caenorhabditis elegans* chromosome III (accession No. U56965) and of the *Saccharomyces cerevisiae* chromosome XII (accession No. U19027) DNA sequence, exhibit high degree of homology with rat and human kynureninase. In particular, the region of the proteins containing the PLP-binding site (Lys^{276} in the rat sequence): FACWCSYKY is conserved among the four sequences (Fig. 4). Based upon sequence comparison we propose that these unidentified sequences encode *S. cerevisiae* and *C. elegans* kynureninase.

To further confirm that the isolated cDNAs encoded rat and human kynureninase the two cDNAs were subcloned into the pSVL expression vector and transfected to COS-1 cells. Kynureninase and hydroxykynureninase activities were detected in transfected COS-1 cells (Table 1). No activity was observed in cells transfected with the antisense cDNAs. Table 1 shows the kinetic properties of the two enzymes. Kynureninase activity was measured at different concentrations of L-KYN (from 0.01 to 10 mM). K_m values obtained for both recombinant proteins are similar to the values published for the rat native [5] and human recombinant enzymes [26]. Notably, when DL-3OHKYN was used as substrate, the kinetic of the reaction could be considered linear only during the first 6 min of reaction. At longer reaction time we could observe a decrease of the reaction velocity compatible, as reported for the native enzyme [3], with an inhibitory effect of the reaction product: 3-OHAA. With DL-3OHKYN as substrate, the K_m for both rat and human enzymes was about 10-fold lower and the V_{max} about 5-fold higher than using L-KYN. The rat kynureninase and hydroxykynureninase activities were inhibited by oMBA [10], a competitive inhibitor of kynureninase, with a half-maximal inhibitory value of 2.4 μM (L-KYN) and 13 μM (DL-3OHKYN) respectively. Optimum pH was found to be around 9.0 for both rat and human kynureninase using L-KYN and around 8.5 with DL-3OHKYN confirming the property of the native protein [7].

A major route of detoxication for many halogenated xenobiotics is by conjugation to the tripeptide glutathione. Subsequent metabolic processing of such conjugates yields the cysteine conjugate of the original chemicals, which then may serve as substrate for enzymes that express β-lyase activity. Recently, attention has been directed toward the toxicological importance of these enzymes because β-lyase action can yield toxic metabolites [27]. The PLP-binding site of rat kynureninase (Fig. 1) is similar to the one present in the γ family of PLP-dependent enzymes, which contains proteins with β-lyase activity [25]. Notably, COS-1 cells transfected with the rat kynureninase cDNA showed a cysteine-conjugate β-lyase specific activity of 1.97 nmol min^{-1} mg^{-1} using DCVC as substrate. No activity was present in cells transfected with the antisense cDNA. Fig. 4 shows that the four polypeptide sequences contain an almost identical PLP-binding site region. Being this region common also to aminotransferases with cysteine conjugate β-lyase activity, it is likely that also human, and putative *S. cerevisiae* and *C. elegans* kynureninases posses such an enzymatic activity. Apart from kidney, β-lyase activity is also found predominantly in the liver. As shown in Fig. 2, these are the tissues with higher levels of kynureninase mRNA. Cysteine S-conjugates readily cross the blood–brain barrier [28]. Inactivation of kynureninase in the brain by cysteine S-conjugates could therefore profoundly affect the cerebral level of kynurenine and its neurotoxic/neuroprotective metabolites.

Chiarugi and coworkers investigated the effect of oMBA on kynurenine metabolism in vivo [15]. As expected, oMBA significantly decreased the brain content of AA, the product of kynureninase activity. Unexpectedly, brain content of 3-OHAA, the product of hydroxykynureninase, was not modified by the treatment. Moreover, in vitro inhibition studies with oMBA indicated different IC_{50} values when enzyme

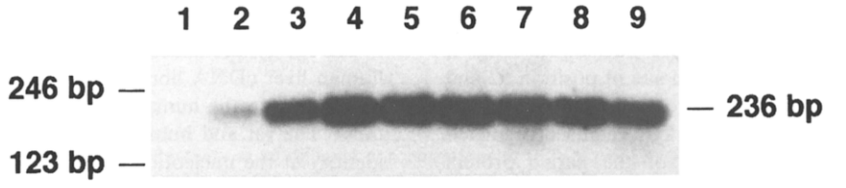

Fig. 3. RT-PCR analysis of rat kynureninase mRNA in different rat tissues (adult Sprague-Dawley). Lanes: 1, negative control; 2, brain; 3, heart; 4, retina; 5, testis; 6, ovary; 7, liver; 8, lung; 9, kidney. PCR products were electrophoresed on 2% agarose gel, blotted into nitrocellulose membrane and hybridized with 5′-end-P^{32} labelled oligonucleotide: 5′-TCTCCGCTTGGATGAGGAAGAT-3′. Southern Blot results are shown. This a qualitative assay and the relative amount of the PCR products obtained cannot be compared among different tissues.

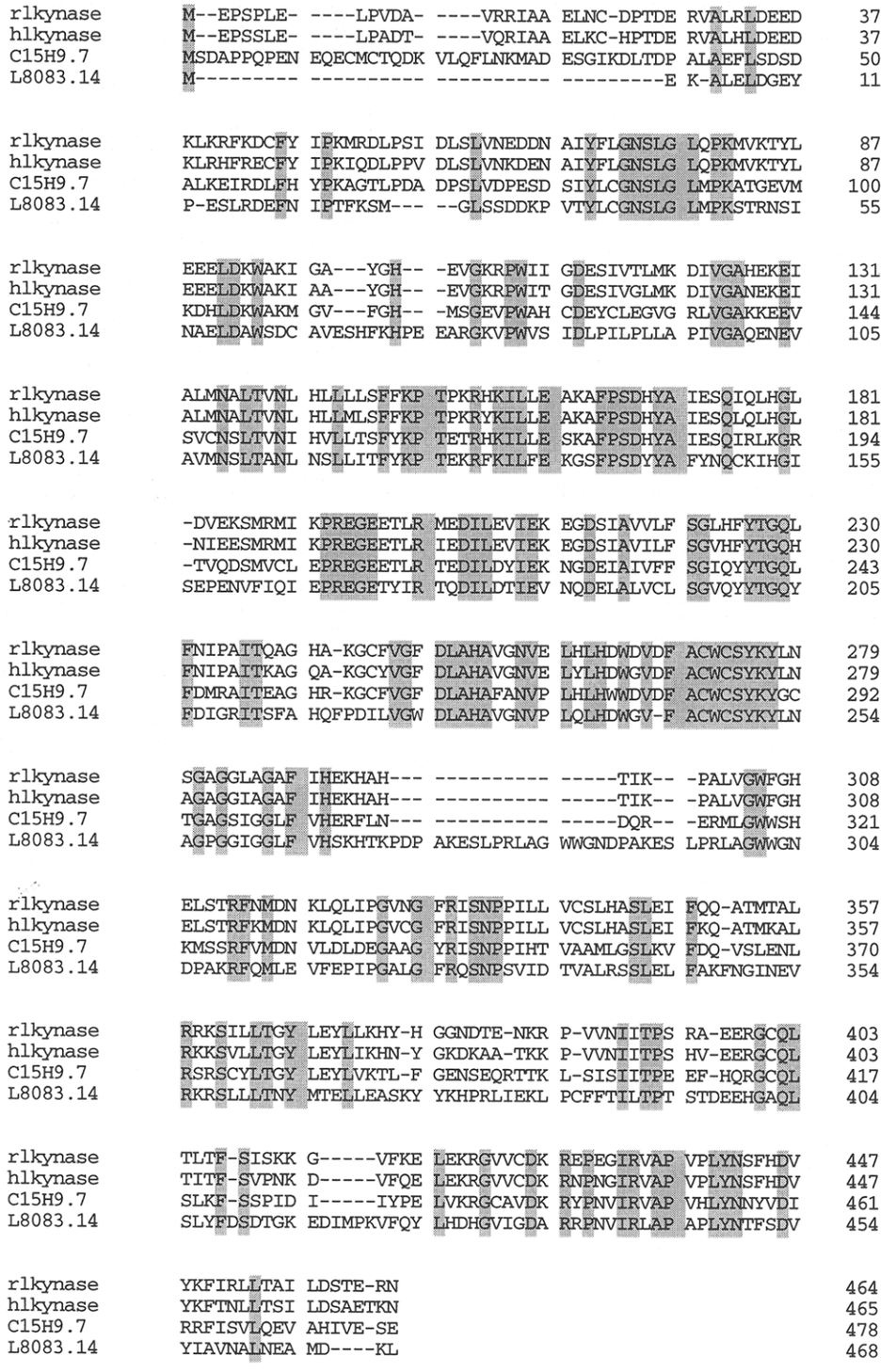

Fig. 4. Alignment of the rat and human kynureninase primary amino acid sequences with the predicted sequence of two putative proteins: L8083.14 and C15H9.7 obtained from the *Saccharomyces cerevisiae* chromosome XII and the *Coenorabditis elegans* chromosome III, respectively. *rlkynase*, rat kynureninase; *hlkynase*, human kynureninase. Conserved amino acids among all proteins are shaded.

preparation from liver and brain tissues where used. This would suggests either the existence of two kynureninase enzymes or the presence of different enzyme modulators in peripheral and brain tissues. Our data clearly demonstrate that the isolated liver cDNA, when transfected to COS-1 cells, possess both kynureninase and hydroxykynureninase activities. However, we cannot exclude the presence of brain-specific kynureninase isoform(s) displaying different substrate specificity. This study analyses the molecular and biochemical properties of rat and human liver kynureninases providing an

initial step to understand the pathophysiological role of this enzyme and to design more specific inhibitors with neuroprotective potential.

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