Cloning and functional expression of human kynurenine 3-monooxygenase

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Abstract Kynurenine 3-monooxygenase, an NADPH-dependent flavin monooxygenase, catalyses the hydroxylation of L-kynurenine to L-3-hydroxykynurenine. By hybridization screening using a cDNA probe encoding the entire exon 2 of *Drosophila melanogaster* kynurenine 3-monooxygenase, we isolated a 2.0 kb cDNA clone coding for the corresponding human liver enzyme. The deduced amino acid sequence of the human protein consists of 486 amino acids with a predicted molecular mass of 55 762 Da. Transfection of the human cDNA in HEK-293 cells resulted in the functional expression of the enzyme with kinetic properties similar to those found for the native human protein. RNA blot analysis of human tissues revealed the presence of a major mRNA species of ~2.0 kb in liver, placenta and kidney.

Key words: Kynurenine 3-monooxygenase; Flavin monooxygenase; Sequence comparison; Kynurenine pathway; *Drosophila melanogaster*; Human liver

1. Introduction

The interest in the kynurenine pathway has grown steadily over the past few years and stems from several findings suggesting a role of this metabolic route in some pathological conditions. Accumulation of kynurenine metabolites may, in fact, constitute a potentially hazardous event. Quinolinic acid is a potent excitotoxin [1] and its overproduction by activated brain-invading macrophages and/or microglial cells has been linked to neuronal damage occurring in neuroinflammatory disorders [2–4]. Relatively high concentrations of L-3-hydroxykynurenine may also be neurotoxic and a recent report suggests that this metabolite mediates neuronal cell death through H_2O_2 production [5]. In addition, kynurenic acid may represent an endogenous neuroprotectant species [1]. On the other hand, because of the antioxidant properties of some L-tryptophan metabolites, activation of the kynurenine pathway may represent a defence against the excessive production of reactive oxygen and nitrogen species in inflammation [6].

The primary structure of most enzymes constituting this metabolic route has been elucidated. These include tryptophan 2,3-dioxygenase [7], indoleamine 2,3-dioxygenase [8], kynureninase [9], 3-hydroxyanthranilic-acid dioxygenase [10] and kynurenine aminotransferase isoforms [11–13].

Kynurenine 3-monooxygenase (KMO, EC 1.14.13.9) is an NADPH-dependent flavin monooxygenase, catalysing the hydroxylation of the L-tryptophan metabolite L-kynurenine to form L-3-hydroxykynurenine. KMO is a membrane protein located on the outer membrane of mitochondria [14]. Tissue distribution studies have revealed that, in rats, highest enzyme activity is found in kidney and liver, with brain having the least activity in comparison to peripheral organs [14]. Notably, KMO appears to be upregulated in inflammatory conditions [3,15]. It has been proposed that inhibitors of KMO may provide new neuroprotective agents and KMO inhibitors have been found to protect against chemically and electrically induced seizures in rodents and to reduce infarct size in a global ischemia model in gerbils [16,17].

Recently, genomic DNA analysis of chromosome 2 of *Drosophila melanogaster* has resulted in the identification of the *cinnabar* gene region (accession number U56245) encoding KMO from this insect species [18]. By using a cDNA probe derived from the *D. melanogaster* KMO (dmKMO) gene, we report here the molecular cloning and the functional expression of a cDNA clone encoding human KMO (hKMO).

2. Materials and methods

2.1. Isolation of human KMO cDNA

To generate a hybridization probe for screening, we performed PCR amplification of the *D. melanogaster* genomic DNA χK8 clone (1 ng) [18] using 10 pmol each of sense and antisense primers (sense: 5′-GGGTTCTCTGGCAGCCTTGAACT-3′, antisense: 5′-CTCCACATAATTGTACATGGCCAG-3′) as previously described [10]. The final PCR product of 1042 bp (dmKMO cDNA) was isolated and subcloned into the pBluescript vector. A human liver cDNA library constructed in the λ uni-zap vector (Stratagene, La Jolla, CA) was screened following previously described procedures [19]. Briefly, the filters were hybridized at low stringency with a ^{32}P-labeled nick-translated 1042 bp dmKMO cDNA probe for 24 h at 37°C in 6× NET (1× NET = 150 mM NaCl, 15 mM Tris-HCl, pH 8.3, 1 mM EDTA), 1× Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), 0.1% SDS, 1 mM $Na_4P_2O_7$, 100 mM ATP, 20% deionized formamide, and 500 μg/ml salmon sperm DNA. The filters were then washed twice at room temperature for 1 h in 6× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.4), containing 0.1% SDS.

A human KMO partial cDNA (hKMOF4), obtained from low stringency screening, served then as hybridization probe for screening the same human liver cDNA library, at higher stringency [19]. From a total of 2×10^6 plaques, 15 positive clones were obtained and purified through an additional round of screening at lower density plaque. Bluescript plasmids (pBSK, Stratagene), carrying the cDNA inserts,

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Abbreviations: KMO, kynurenine 3-monooxygenase; hKMO, human kynurenine 3-monooxygenase; dmKMO, *Drosophila melanogaster* kynurenine 3-monooxygenase; bp, base pair(s); kb, kilobases; ORF, open reading frame

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were isolated from positive phages via in vivo excision. The nucleotide sequence of the cDNA clones was determined by automated cycle sequencing (Applied Biosystems, California).

2.2. Northern blot analysis

Multiple tissue Northern blots from different human tissues were purchased from Clontech. After prehybridization for 4 h at 42°C, the RNA blots were hybridized for 18 h with a ^{32}P-labeled nick-translated PCR derived 739 bp cDNA probe (nucleotides 625–1364) as previously described [10]. A ^{32}P-labeled nick-translated PCR-amplified fragment of human S12 ribosomal protein was used [9] as an internal standard probe. The blots were exposed to X-ray film for 22 h.

2.3. Expression of recombinant hKMO in HEK-293 cells

The 2.0 kb cDNA encoding hKMO was subcloned in the *Eco*RI site of the expression vector pBC/CMV [20], placing transcription of the cDNA under the control of the strong immediate early promoter of human cytomegalovirus. Human embryonic kidney fibroblast cells (HEK-293, ATCC CRL 1573) were transfected as previously described [10]. Two days after transfection, the cells were harvested and washed twice with phosphate-buffered saline and stored at −80°C until analysis. For enzyme activity determination, the transfected cells were homogenized in 1 ml of 10 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM phenylmethanesulfonyl fluoride.

2.4. Determination of KMO activity

KMO activity was measured using a microplate version of the radioenzymatic method described by Erickson et al. [14], based on the release of tritiated water after hydroxylation of L-[3-^{3}H]kynurenine. The assay was performed in a final volume of 100 µl of 0.1 M potassium phosphate buffer, pH 7.4. The reaction mixture consisted of 100 µM L-kynurenine sulfate, 0.2 µCi of L-[3-^{3}H]kynurenine (spec. act. 14.4–16.1 Ci/mmol; 1 Ci = 37 GBq, F. Hoffmann-La Roche, Basel), 200 µM NADPH, 0.4 U/ml glucose-6-phosphate dehydrogenase and 3 mM glucose-6-phosphate. After addition of 5–10 µl of the cell homogenates, the plate was incubated for 10 min at 37°C. Incubation

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   1  GAATTCGGCACGAGCAGAAGCAACAATAATTGTGAAAAATACTTCAGCAGTTATGGACTCATCTGTCATTCAAAGGAAAA    80
   1                                                          M  D  S  S  V  I  Q  R  K  K    10

  81  AAGTAGCTGTCATTGGTGGTGGCTTGGTTGGCTCATTACAAGCATGCTTTCTTGCAAAGAGGAATTTCCAGATTGATGTA   160
  11    V  A  V  I  G  G  G  L  V  G  S  L  Q  A  C  F  L  A  K  R  N  F  Q  I  D  V    36

 161  TATGAAGCTAGGGAAGATACTCGAGTGGCTACCTTCACACGTGGAAGAAGCATTAACTTAGCCCTTTCTCATAGAGGACG   240
  37  Y  E  A  R  E  D  T  R  V  A  T  F  T  R  G  R  S  I  N  L  A  L  S  H  R  G  R    63

 241  ACAAGCCTTGAAAGCTGTTGGCCTGGAAGATCAGATTGTATCCCAAGGTATTCCCATGAGAGCAAGAATGATCCACTCTC   320
  64    Q  A  L  K  A  V  G  L  E  D  Q  I  V  S  Q  G  I  P  M  R  A  R  M  I  H  S  L    90

 321  TTTCAGGAAAAAAGTCTGCAATTCCCTATGGGACAAAGTCTCAGTATATTCTTTCTGTAAGCAGAGAAAATCTAAACAAG   400
  91    S  G  K  K  S  A  I  P  Y  G  T  K  S  Q  Y  I  L  S  V  S  R  E  N  L  N  K    116

 401  GATCTATTGACTGCTGCTGAGAAATACCCCAATGTGAAAATGCACTTTAACCACAGGCTGTTGAAATGTAATCCAGAGGA   480
 117   D  L  L  T  A  A  E  K  Y  P  N  V  K  M  H  F  N  H  R  L  L  K  C  N  P  E  E   143

 481  AGGAATGATCACAGTGCTTGGATCTGACAAAGTTCCCAAAGATGTCACTTGTGACCTCATTGTAGGATGTGATGGAGCCT   560
 144   G  M  I  T  V  L  G  S  D  K  V  P  K  D  V  T  C  D  L  I  V  G  C  D  G  A  Y   170

 561  ATTCAACTGTCAGATCTCACCTGATGAAGAAACCTCGCTTTGATTACAGTCAGCAGTACATTCCTCATGGGTACATGGAG   640
 171    S  T  V  R  S  H  L  M  K  K  P  R  F  D  Y  S  Q  Q  Y  I  P  H  G  Y  M  E   196

 641  TTGACTATTCCACCTAAGAACGGAGATTATGCCATGGAACCTAATTATCTGCATATTTGGCCTAGAAATACCTTTATGAT   720
 197  L  T  I  P  P  K  N  G  D  Y  A  M  E  P  N  Y  L  H  I  W  P  R  N  T  F  M  M   223

 721  GATTGCACTTCCTAACATGAACAAATCATTCACATGTACTTTGTTCATGCCCTTTGAAGAGTTTGAAAAACTTCTAACCA   800
 224    I  A  L  P  N  M  N  K  S  F  T  C  T  L  F  M  P  F  E  E  F  E  K  L  L  T  S   250

 801  GTAATGATGTGGTAGATTTCTTCCAGAAATACTTTCCGGATGCCATCCCTCTAATTGGAGAGAAACTCCTAGTGCAAGAT   880
 251    N  D  V  V  D  F  F  Q  K  Y  F  P  D  A  I  P  L  I  G  E  K  L  L  V  Q  D   276

 881  TTCTTCCTGTTGCCTGCCCAGCCCATGATATCTGTAAAGTGCTCTTCATTTCACTTTAAATCTCACTGTGTACTGCTGGG   960
 277  F  F  L  L  P  A  Q  P  M  I  S  V  K  C  S  S  F  H  F  K  S  H  C  V  L  L  G   303

 961  AGATGCAGCTCATGCTATAGTGCCGTTTTTTGGGCAAGGAATGAATGCGGGCTTTGAAGACTGCTTGGTATTTGATGAGT  1040
 304   D  A  A  H  A  I  V  P  F  F  G  Q  G  M  N  A  G  F  E  D  C  L  V  F  D  E  L   330

1041  TAATGGATAAATTCAGTAACGACCTTAGTTTGTGTCTTCCTGTGTTCTCAAGATTGAGAATCCCAGATGATCACGCGATT  1120
 331    M  D  K  F  S  N  D  L  S  L  C  L  P  V  F  S  R  L  R  I  P  D  D  H  A  I   356

1121  TCAGACCTATCCATGTACAATTACATAGAGATGCGAGCACATGTCAACTCAAGCTGGTTCATTTTTCAGAAGAACATGGA  1200
 357  S  D  L  S  M  Y  N  Y  I  E  M  R  A  H  V  N  S  S  W  F  I  F  Q  K  N  M  E   383

1201  GAGATTTCTTCATGCGATTATGCCATCGACCTTTATCCCTCTCTATACAATGGTCACTTTTTCCAGAATAAGATACCATG  1280
 384   R  F  L  H  A  I  M  P  S  T  F  I  P  L  Y  T  M  V  T  F  S  R  I  R  Y  H  E   410

1281  AGGCTGTGCAGCGTTGGCATTGGCAAAAAAAGGTGATAAACAAAGGACTCTTTTTCTTGGGATCACTGATAGCCATCAGC  1360
 411    A  V  Q  R  W  H  W  Q  K  K  V  I  N  K  G  L  F  F  L  G  S  L  I  A  I  S   436

1361  AGTACCTACCTACTTATACACTACATGTCACCACGATCTTTCCTCTGCTTGAGAAGACCATGGAACTGGATAGCTCACTT  1440
 437  S  T  Y  L  L  I  H  Y  M  S  P  R  S  F  L  C  L  R  R  P  W  N  W  I  A  H  F   463

1441  CCGGAATACAACATGTTTCCCCGCAAAGGCCGTGGACTCCCTAGAACAAATTTCCAATCTCATTAGCAGGTGATAGAAAG  1520
 464   R  N  T  T  C  F  P  A  K  A  V  D  S  L  E  Q  I  S  N  L  I  S  R  *           486

1521  GTTTTGTGGTAGCAAATGCATGATTTCTCTGTGACCAAAATTAAGCATGAAAAAAATGTTTCCATTGCCATATTTGATTC  1600
1601  ACTAGTGGAAGATAGTGTTCTGCTTATAATTAAACTGAATGTAGAGTATCTCTGTATGTTAATTGCAATTACTGGTTGGG  1680
1681  GGGTGCATTTTAAAAGATGAAACATGCAGCTTCCCTACATTACACACACTCAGGTTGAGTCATTCTAACTATAAAAGTGC  1760
1761  AATGACTAAGATCCTTCACTTCTCTGAAAGTAAGGCCCTAGATGCCTCAGGGAAGACAGTAATCATGCCTTTTCTTTAAA  1840
1841  AGACACAATAGGACTCGCAACAGCATTGACTCAACACCTAGGACTAAAAATCACAACTTAACTAGCATGTTAACTGCACT  1920
1921  TTTCATTACGTGAATGGAACTTACCTAACCACAGGGCTCAGACTTACTAGATAAAACCAGAAATGGAAATAAGGAATTC  1999
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Fig. 1. Nucleotide and predicted amino acid sequence of cloned human liver KMO (hKMOC15 cDNA clone). The deduced amino acid sequence of the encoded polypeptide is shown in single-letter code below the nucleotide sequence and is numbered beginning with the initiating methionine. Nucleotides are numbered in the 5′ to 3′ direction. The asterisk denotes the 3′-terminal stop codon.

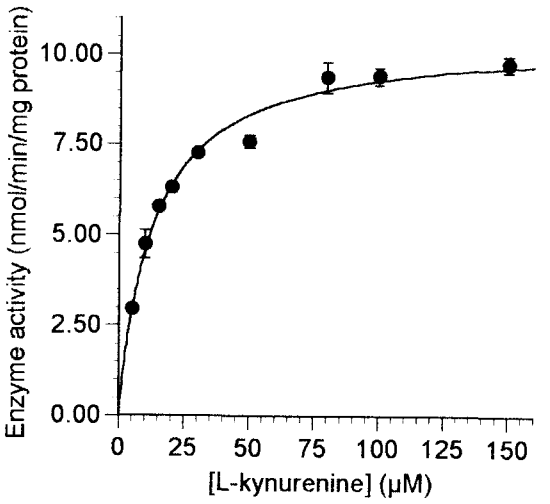

Fig. 2. Kinetic properties of recombinant hKMO expressed in HEK-293 cells. Saturation curves were calculated by fitting the data to Michaelis-Menten equation using the Ultrafit (Biosoft) computer program. Data are the means ± SEM of three experiments.

was terminated by addition of 150 μl of a 10% (w/v) suspension of activated charcoal (Norit A) and, after shaking for 2 min, the plates were centrifuged for 10 min at 2000×g. An aliquot (50 μl) of the sample was then transferred to the well of a second microtiter plate containing 200 μl/well of scintillation fluid (Microscint 40, Packard). After vigorous shaking, the plates were counted for radioactivity using a Top-Count (Packard) counter.

3. Results and discussion

3.1. Molecular cloning of human KMO cDNA

To isolate a cDNA encoding hKMO, we generated by PCR amplification a 1042 bp cDNA probe encoding the entire exon 2 (nucleotides 4447–5488) [18] of KMO from *D. melanogaster* (see Section 2). The dmKMO cDNA probe was then used to screen a λ uni-zap cDNA library of human liver. In an initial screening of 1.2×10^6 recombinants at very low stringency, five weak positive clones were detected. Translation of the DNA sequence of the largest hybridizing clone (hKMOF4) was found to code for a peptide sequence of 315 amino acids with 65% similarity with the predicted amino acid sequence of dmKMO (not shown). This indicated that the isolated cDNA

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      hKMO MDSSVIQRKKVAVIGGGLVGSLQACFLAKRNFQIDVYEAREDTR..VATFTRGRSINLALSHRGRQALKAVGLE..DQIVSQGIPMR  83
    Q21795 .......MPSVAIAGAGLVGALNACFFAQKGWDVSVYEFRKDIR..TMKHVQGRSINLALSQRGKSALEAVGLK..EYIVNQGVPLY  76
     dmKMO RDERHGRRRRVAVIGAGLVGSLAALNFARMGNHVDLYEYREDIR..QALVVQGRSINLALSQRGRKALAAVGLE..QEVLATAIPMR 162
  YBJ8_YEAST ......MSESVAIIGAGLVGCLAALAFSKEGYNVTLYDFRQDPRLDTTKNKNLKSINLAISARGIDALKSIDPDACEHILQDMIPMK  81
    Q21794 .......MPSVVIAGGGLVGSANACFFGQKGWIVDVYESRFDPRGNHME..NGKSINLALGVRAMSTMKRIGLK..EKVIHIGVPIR  76
 Consensus           V***G*GLVG * A         * *Y- R D R        *+SINLA*  R*   *  * *      **  **P*

      hKMO ARMIHSLSGK.K.SAIPYGTKSQYILSVSRENLNKDLLTAAEKYPNVKMHFNHR.....LLKCNPEEGMITVLGSDKVPKD 157
    Q21795 ARLIHNKDGK.TYSRQPYGKPGEHIVSINRRHLNEVMITQAEKSPNVKFFFEHKVKNVDYDKKQLVVQCTSQPSKIPTFGNKSPPQE 162
     dmKMO GRMLHDVRGN.SSVVLYDPINNQCLYSVGRRQLNEVLLNACDKLPNIRCHFEHKL.....TSANLREGSMEFRNPAKE 234
  YBJ8_YEAST GRMIHDLKGR.QESQL.YGLHGEAINSINRSVLNNSLLDELEK.STTELKFGHKLVKIEWTDDKQICHFAIGEDLKTPHTEK..... 160
    Q21794 DQIAHFGDTKGKLKRLPVLNDDDFILTINRQELSQILINEAEKYNNVKFHFNCKATKFDLKSESLIVQNSDNLST..... 151
 Consensus   **H            *    * ** R  L*  **   -K  *    F  +

      hKMO .....VTCDLIVGCDGAYSTVRSHLMKKPRFDYSQQYIPHGYMELTIPPK.....NGDYAMEPNYLHIWPRNTFMMIALPNMNKS 232
    Q21795 HAEFHVEADLILACDGAYSAVRRSLMTIPRFDFSQEYIEHGYVELNIMAN.....NNEFAFEENVFHLWPRGHFTLIALANRDKT 242
     dmKMO AVAAH..ADLIVGCDGAFSSVRQXXVRLPGFNYSQEYIETGYLELCIPSK.....SGDFQMPANYLHIWPRNTFMMIALPNQDKS 312
  YBJ8_YEAST .......YDFVIGCDGAYSATRSQMQRKVEMDFSQEYMNLRYIELYIPPTEEFKPNYGGNFAIAPDHLHIWPRHKFMLIALANSDGS 240
    Q21794 .....VDGDLFLACDGAHSSIRRSLLKAPGFNFSQKYSEFGYIDLSVNSTQQCDLKLGTHYS.....WRRRGIILVAIVNKDQS 225
 Consensus         D  **CDGA S  R      *   :SQ Y    Y*-L *            :         W R    **A**N  °

      hKMO FTCTLFMPFEEFEKLL.TSNDVVDFFQKYFPDAIPLIGEKLLVQDFFLLPAQPMISVKCSSFHF.KSHCVLLGDAAHAIVPFFGQGM 317
    Q21795 FTVTIFAPFSEFEKHMSTSEDVLSFFEENFPDAFLLLGKEHIADTFNRVKPQPLVSIKCSPHSF.FDNLVLMGDAAHAMVPFYGQGM 328
     dmKMO FTVTLSMPFEIFAG.IQNQNDLLEFFKLNFRDALPLIGEQQLIKDFFKTRPQFLVSIKCRPYHY.ADKALILGDAAHAMVPYYGQGM 397
  YBJ8_YEAST FTSTFFGSKDQISDLITSKSRVREFLIENFPDIINIMDLDDAVKRFITYPKESLVCVNCKPYDVPGGKAILLGDAAHAMVPFYGQGM 327
    Q21794 LTVSMFATFSEFESNLVGPVESVLFFKNNFYEIFKILGEDHIRNTIARNKPQAIISVQCSQHVF.FDKLVLMGDAAHAMVPFNGQGV 311
 Consensus  T °  *        *        F    F -*  **    *           ** * C          ***GDAAHA*VP: GQG*

      hKMO NAGFEDCLVFDELMDKFSND.LSLCLPVFSRLRIPDDHAISDLSMYNYIEMRAHVNSSWFIFQK...NMERFLHAIMPSTFIPLYTM 400
    Q21795 NCGFEDCLVFSETLEEYGND.IAKAVKVYSDGRVNDAHSINDLAMYNYEELKDLVNKSSYKLRK...KFDTIMNSIFPKSWIPLYSM 411
     dmKMO NAGMEDVTLLTDILAK.QLP.LDETLALFTESRWQDAFAICDLAMYNYVEMRDLTKRWTFRLRK...WLDTLLFRLFP.GWIPLYNS 478
  YBJ8_YEAST NCGFEDVRILMALLKKHSGD.RSRAFTEYTQTRHKDLVSITELAKRNYKEMSHDVTSKRFLLRK...KLDALFSIIMKDKWIPLYTM 410
    Q21794 NCGFEDCLVLQEIMDQYEEDELEDVIKEYSKVRTNETNIINQMEWDVNLTLTSSVHGSLGWIREIRAHIKLLLCFIFPSS....FTL 394
 Consensus N G ED  *    *              :°  R  -   I  *       *                              *       :°

      hKMO VTF.SRIRYHEAVQRWHWQKKVINKGLFFLGSLIAISSTYLLIHYMSPRSFLCLRRPWNWIAHFRNTTCFPAKAVDSLEQISNLISR 486
    Q21795 VTF.SRIPYSEVIERRKRQDKILSRIMTTTSTLALIGAAAGIYVNRGKLGL..... 461
     dmKMO VSF.SSMPYRQCIANRKWQDQLLKRIFGAT.FLAAIVTGGAIYAQRFL..... 524
  YBJ8_YEAST ISFRSDISYSRALERAGKQTRILKFLESLTLGMLSIGGYKLFKFLTRERS..... 460
    Q21794 AAF.SREKYSEI....ARKAQIVEKCTSLFTNVFIVFIISVISLFWYLLGYCRGNLQAYCLQCAVVSGVCVQEVS..... 464
 Consensus * F S   Y             **          * *    *
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Fig. 3. Multiple alignment of the amino acid sequence of human KMO with proteins from the SwissProt and translated EMBL databases. hKMO, human kynurenine 3-monooxygenase; dmKMO, kynurenine 3-monooxygenase from *D. melanogaster*; YBJ8_Yeast, hypothetical protein in ATP1-ROX3 intergenic region of *S. cerevisiae*; Q21794 and Q21795, two sequences contained in chromosome III of *C. elegans*. In the consensus sequence, upper-case letters and symbols indicate identical amino acids and amino acids conserved in their physico-chemical properties, respectively; *, hydrophobic amino acids; :, aromatic amino acids; °, polar amino acids; +, positively charged amino acids; −, negatively charged amino acids. Note that the KMO amino acid sequence of *D. melanogaster*, shown in the figure, starts with the Arg at position 80.

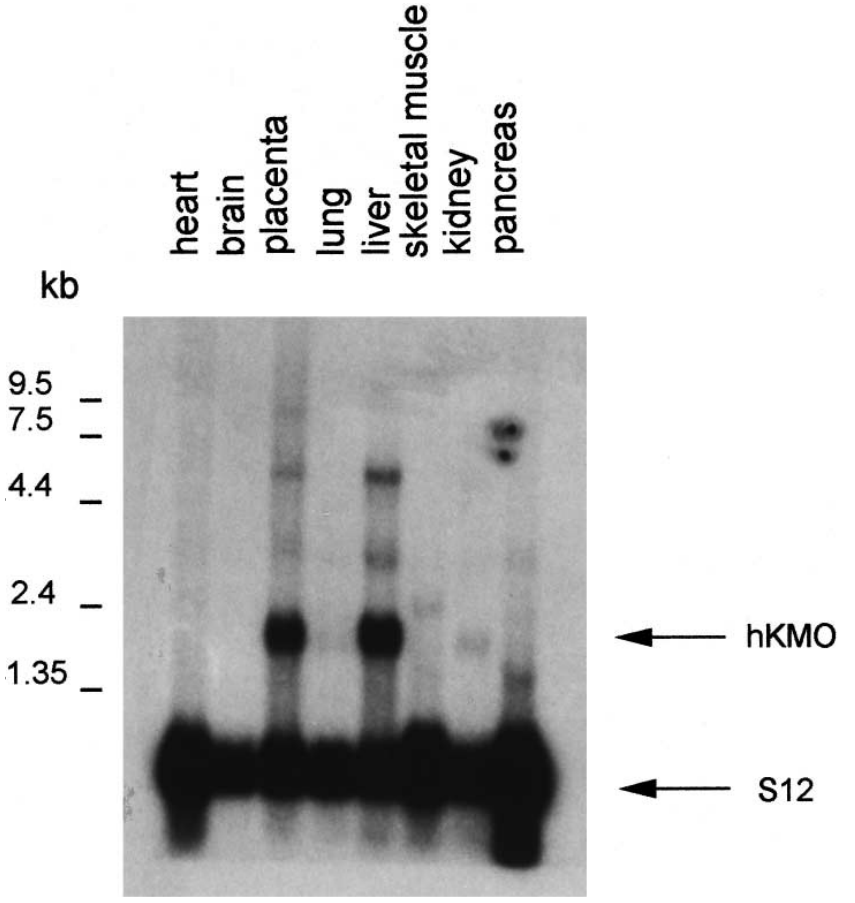

Fig. 4. Northern-blot hybridization of poly (A)-rich RNAs from various human tissues. The blot was hybridized with a ^{32}P-labeled nick-translated 739 bp hKMO cDNA and ribosomal S12 cDNA as internal standard. The scale on the ordinate indicates kilobases as determined by RNA size markers. The arrows indicate KMO and S12 mRNAs. The blot was exposed to X-ray film for 22 h.

was likely a partial sequence of hKMO. To isolate a full length clone of hKMO, the human liver λ uni-zap cDNA library was submitted to a second screening with the hKMOF4 human partial cDNA probe. Fifteen positive clones were identified after screening of 2×10^6 plaques at high stringency. The inserts from the positive clones were excised from pBluescript and, after analysis by Southern blot, were found to range from 700 to 5000 bp in size. DNA sequence analysis revealed that the majority of the clones were identical except for the length of 3′-untranslated region. The nucleotide and deduced amino acid sequences of the EcoRI insert isolated from one of the clones, named hKMOC15, are shown in Fig. 1. Analysis of the DNA sequence (nucleotide 1–1999) indicated the presence of a single open reading frame (ORF) with a predicted initiation codon (ATG) at nucleotide 53 and termination at nucleotide 1513 with the stop codon TAG. This ORF encodes a polypeptide of 486 amino acid residues with a predicted molecular mass of 55 762 Da. The ORF is preceded by a 52-nucleotide 5′-untranslated region containing no in-frame stop codon and followed by a 489-nucleotide untranslated region at the 3′-end.

3.2. Transient expression of human liver KMO in HEK-293 cells

To confirm that the isolated human cDNA indeed encoded an enzyme with KMO activity, the human liver hKMOC15 2.0 kb cDNA was subcloned in the expression vector pBC/CMV [20]. HEK-293 cells, which do not exhibit detectable KMO activity, were used for transient transfection. A relatively high level of functional expression of the enzyme was observed in the homogenates of the transfected cells. Kinetic analysis of recombinant hKMO showed a K_m value for L-kynurenine (13.0 ± 3.3 μM) (Fig. 2) similar to that observed by us for the native protein in human liver mitochondrial preparations (24.1 ± 4.6 μM) (see also Ref. [14]). In addition, the KMO inhibitor *m*-nitrobenzoylalanine [16] inhibited the activity of the recombinant enzyme (measured in the presence of 50 μM L-kynurenine as substrate) with an IC_{50} value very close to that measured for the human enzyme (303 ± 2 and 332 ± 86 nM (mean ± SEM, $n = 3$), respectively).

In accordance with the mitochondrial location of this enzyme, an enrichment (approximately 4-fold) in KMO specific activity was measured in the crude mitochondrial fraction of the transfected cells in comparison to cell homogenate, whereas in the other subcellular fractions (i.e. nuclei, microsomes and cytosol) the enzyme activity detected was much lower (not shown).

3.3. hKMO amino acid sequence analysis

Comparison of the amino acid sequence of hKMO with that deduced for KMO from *D. melanogaster*, using the GAP computer program contained in the Genetics Computer

Group sequence analysis software (GCG, version 8, September 1994, University of Wisconsin, USA), indicated 47% and 66% amino acid identity and similarity, respectively (not shown). The N-terminus of the *D. melanogaster* KMO appears to be 79 residues longer that the human protein (not shown). Analysis of the human enzyme sequence showed the presence of three potential *N*-glycosylation sites (Asn, Xaa, Ser/Thr) at Asn residues 230, 372 and 465. No information, however, is presently available as to whether the native protein is glycosylated. The N-terminal region of hKMO presents an AMP/ADP-binding sequence fingerprint (from residue 10–38 in hKMO) found in NAD- and FAD-dependent enzymes [21]. The Gly-x-Gly-x-x-Gly motif (residues 15–20 in hKMO), the hydrophobic amino acid residues (positions 11, 13, 24, 27, 34 and 36) required for the correct fold of the β_1-α-β_2- structure of the dinucleotide binding domain and the acid sidechain (Glu38) forming an hydrogen bond with the 2′-OH of the ribose moiety of FAD, are all present in the hKMO sequence. In addition, the fact that this domain is at the N-terminal of the protein is in accordance with what was found for several other known monooxygenases belonging to the subclass E 1.14.13 [18]. Analysis of the hydropathy pattern of hKMO did not reveal any evident hydrophobic amino acid stretch which could be responsible for association to the outer mitochondrial membrane. It has been proposed that the first 70 residues of the *D. melanogaster* protein may contain the sequence for mitochondrial targeting and/or anchoring [18]. However, as mentioned above, this sequence is absent in hKMO where the N-terminal consists of the AMP/ADP binding site. Therefore, the N-terminus of hKMO is unlikely to be involved in membrane anchoring. Whether, in analogy to e.g. monoamine oxidase, another outer membrane mitochondrial protein [22], the C-terminus of hKMO, which contains relatively hydrophobic regions, may be responsible for mitochondrial anchoring is matter of speculation.

3.4. Human KMO sequence comparison

When the SwissProt (Release 34) and translated EMBL database (Release 2) were searched using the program Blast, the most significant hits, besides KMO from *D. melanogaster*, were two gene products from *Caenorhabditis elegans* (protein identification, PID, numbers Q21795 and Q21794) contained in the central gene cluster of chromosome III of this nematode, and YBJ8_Yeast, a hypothetical protein in the ATP1-ROX3 intergenic region of *Saccharomyces cerevisiae* chromosome 2 (accession number Z35859). A multiple alignment of the predicted amino acid sequences of hKMO, dmKMO and the above mentioned gene products is shown in Fig. 3. Several residues were found to be identical or conserved in their physico-chemical properties in all the sequences, therefore suggesting that these *C. elegans* and yeast genes encode the corresponding KMO homologues in these organisms. In addition to the N-terminal AMP/ADP binding motif, the alignment also showed the presence of a highly conserved stretch, including the totally conserved sequence GDAAHA (starting at position 303 in hKMO), which, according to the crystal structure of the NADPH-dependent flavin monooxygenase *p*-hydroxybenzoate hydroxylase (from *Pseudomonas fluorescens*; 1PBE in Brookhaven protein data bank), corresponds to the binding site for the riboflavin moiety of FAD [23]. It appears, therefore, that the active site of KMO shares similar structural features to that of *p*-hydroxybenzoate hydroxylase.

3.5. Blot hybridization

A Northern-blot analysis of poly(A)-rich RNAs from various human tissues is shown in Fig. 4. A major band of ~2.0 kb was detected in liver and placenta using the ^{32}P-labeled nick-translated PCR-derived 743 bp cDNA probe from hKMO. A weaker but detectable band could also be observed in kidney whereas in the other tissues, including brain, no hybridization signal could be detected under our experimental conditions. Overall, these results would indicate a rather specific expression pattern of the enzyme in the various organs. The higher abundance of hKMO mRNA in liver than in kidney is in accordance with the enzyme activity levels measured in these two tissues from humans (unpublished results).

3.6. Conclusions

The successful cloning of human KMO will allow the investigation of several aspects of enzyme function, including the regulation of its expression in pathophysiological conditions. In addition, it will enable the production of the enzyme in sufficient quantities for further structural and functional analysis. Due to the growing interest in KMO inhibitors as potential neuroprotective agents, this will be instrumental for the rational design of potent and selective KMO inhibitors.

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