Volume 238, number 1, 180–184 FEB 06356 September 1988

Molecular cloning and sequence analysis of cDNA encoding human kidney D-amino acid oxidase

Kyoko Momoi, Kiyoshi Fukui, Fusao Watanabe and Yoshihiro Miyake

Department of Biochemistry, National Cardiovascular Center Research Institute, Fujishirodai 5-chome, Suita, Osaka 565, Japan

Received 20 July 1988; revised version received 8 August 1988

cDNA clones encoding D-amino acid oxidase were isolated from a human kidney cDNA library by hybridization with cDNA for the pig enzyme. The cDNA insert of 2.0 kilobase pairs long provided coding information for a protein consisting of 347 amino acids. The molecular mass of the enzyme was calculated to be 39 410 Da. The amino acid sequence similarity between the pig and human enzymes is 84.4%, and among the active site residues proposed from chemical modification studies, methionine-110 of the pig enzyme was replaced by threonine. Northern blot analysis confirmed the expression of an mRNA of 2.0 kilobases encoding the D-amino acid oxidase in human kidney.

D-Amino acid oxidase; cDNA cloning; Nucleotide sequence; (Human)

1. INTRODUCTION

D-Amino acid oxidase (EC 1.4.3.3) is one of the principal enzymes in peroxisomes and is mainly localized in kidney proximal tubules. The enzyme, containing FAD as a prosthetic group, catalyzes the oxidative deamination of a wide range of D-amino acids. Since the first description by Krebs [1], extensive characterization of the enzyme has been performed. The enzyme exists in peroxisomes of various mammalian tissues and organs [2]. The amino acid sequence [3] and cDNA nucleotide sequence [4] for pig kidney D-amino acid oxidase have been reported and its reaction mechanism has been well documented. However, the physiological role of the enzyme is not known. In humans, D-amino acid oxidase activity was observed in kidney, liver, brain and other tissues [5]. Although the properties of the human enzyme have not been studied in detail, the enzyme seems to be similar to the pig enzyme with respect to substrate specificity and molecular mass. The enzyme is known to decrease significantly in patients with a subtype of Zellweger syndrome, a disorder of the peroxisome formation [6]. In order to determine the physiological function and pathological significance of this enzyme, we attempted to analyze human D-amino acid oxidase on a molecular basis. This paper describes the isolation, nucleotide sequencing and characterization of cDNA encoding human kidney D-amino acid oxidase. The deduced amino acid sequence is compared with that of the pig enzyme to discuss the structure-function relationship of these enzymes.

Correspondence address: Y. Miyake, Dept of Biochemistry, National Cardiovascular Center Research Institute, Fujishirodai 5-chome, Suita, Osaka 565, Japan

2. MATERIALS AND METHODS

2.1. *Isolation of cDNA for human kidney D-amino acid oxidase*

A piece of human kidney was obtained by autopsy at the National Cardiovascular Center. Total RNA was prepared by the guanidium isothiocyanate method [7]. Poly(A)-rich RNA was purified by oligo(dT)-cellulose column chromatography [8], details of the procedure being given previously [4]. Double-stranded cDNA was synthesized and inserted into the *Eco*RI site of λgt10 DNA to construct a human kidney cDNA library. The recombinant phage DNA was packaged in vitro and then screened by plaque hybridization [9] using a ^{32}P-labelled cDNA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

fragment of pDAO-10, a cloned pig D-amino acid oxidase cDNA, as a probe [4].

2.2. *DNA sequence determination*

The nucleotide sequence of the cloned cDNA was determined by the dideoxy method with the M13mp18 and M13mp19 phages as cloning vectors, and a modified bacteriophage T_7 DNA polymerase (US Biochemical Corp.) was used for the sequencing reaction [10]. In addition to the M13 universal sequencing primer, we synthesized three oligonucleotide primers by the phosphoramidite method with an automated DNA synthesizer (Applied Biosystems, model 380A).

2.3. *Northern blot hybridization*

The size of the human D-amino acid oxidase mRNA was estimated by Northern blot analysis using pig ribosomal RNAs as markers. Glyoxal- and dimethylsulfoxide-treated kidney poly(A)-rich RNAs were electrophoresed on 1.0% agarose gel and then transferred to a nitrocellulose membrane (Schleicher and Shuell, West Germany). The filter was hybridized with a ^{32}P-labelled cDNA fragment (*Apa*LI-*Pvu*II) of λHD3 (see below).

3. RESULTS AND DISCUSSION

3.1. *Isolation and characterization of cDNAs for human D-amino acid oxidase*

A cDNA library of 0.8×10^6 independent clones of the λgt10 phage was constructed from 5 μg of human kidney poly(A)-rich RNA. The library was screened with the ^{32}P-labelled *Sal*I-*Pvu*II fragment of pDAO-10, a cDNA clone for pig D-amino acid oxidase [4]. Four positive hybridization clones were isolated from the library. Two clones (1.4 and 1.0 kb cDNA inserts), called λHD1 and λHD2, were subjected to further characterization. The restriction endonuclease mapping of these cDNA inserts is shown in fig.1. Comparison of the restriction endonuclease sites and partial sequencing revealed that these two clones were completely identical with each other except for a deletion of the 5′-region in λHD2. To obtain a full-length cDNA clone, another cDNA library of 1.0×10^6 independent clones was constructed and screened with the 1.4 kbp insert of λHD1. Six positive hybridization clones were isolated in the second screening. One of these clones, called λHD3, was found to comprise a 2.0 kbp insert and was subjected to sequence determination. The insert was isolated by digestion with *Hin*dIII (5′) and *Bgl*II (3′), and then subcloned into the *Hin*dIII-*Bam*HI site of the M13mp18 and M13mp19 vectors. The cDNA insert of λHD1 could not be excised with *Eco*RI, although the library was constructed using *Eco*RI linkers. Nucleotide sequence analysis involved a combination of subcloning of a restriction enzyme-digested fragment into the M13 phage vector and the use of synthetic sequencing primers. The restriction map and sequencing strategy are shown in fig.1. The restriction mapping and nucleotide sequencing indicated that λHD3 covered the whole λHD1 sequence and extended to 5′-upstream. Comparison of λHD3 and pig kidney D-amino acid oxidase cDNA revealed that λHD3 was a full-length cDNA copy of human kidney D-amino acid oxidase.

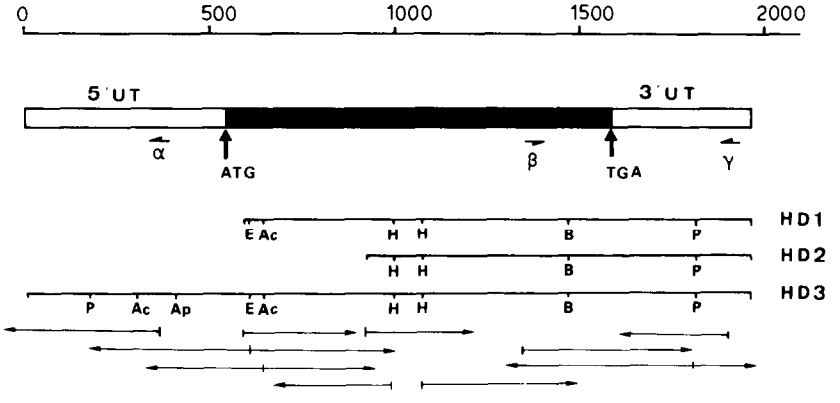

Fig.1. Restriction map and sequencing strategy for human D-amino acid oxidase cDNA. The protein coding region is indicated by a closed box. The 5′- and 3′-noncoding regions are indicated by open boxes. Arrows under the box indicate the direction of use of the synthetic oligonucleotide sequencing primer. α, 5′-TGCAATGGACCCCAACTGCT-3′; β, 5′-CTGGCTTCCGGCCAGTA-3′; γ, 5′-ATTTTCTGTGGCTCTGG-3′. The restriction maps only show the relevant restriction sites. Ac, *Acc*II; B, *Bal*I; E, *Eco*RI; H, *Hin*cII; P, *Pvu*II; 5′UT, 5′-untranslated region; 3′UT, 3′-untranslated region.

```
                                                                    •••
TTGGGGTCCATTGCAACCCGAGGCGAGACTAGAGTTCCCAAGCGAGAAGGGAAGAGGCAGTGGGTGCACGTGGAAGGCGGACAGAGGGCTGGAAACAAGA -101

CGCTCCAGAATCAGGAGCTTCCCCTCAGGAAATAGCATCCTGTGTCCCCGCACTGCAGTTGTCTGGTCTCTCCAGCAGTTTGGTACTTCCGGCTGCTGCA   -1

ATG CGT GTG GTG GTG ATT GGA GCA GGA GTC ATC GGG CTG TCC ACC GCC CTC TGC ATC CAT GAG CGC TAC CAC TCA    75
Met Arg Val Val Val Ile Gly Ala Gly Val Ile Gly Leu Ser Thr Ala Leu Cys Ile His Glu Arg Tyr His Ser
  1               5                  10                  15                  20                  25

GTC CTG CAG CCA CTG GAC ATA AAG GTC TAC GCG GAC CGC TTC ACC CCA CTC ACC ACC ACC GAC GTG GCT GCC GGC   150
Val Leu Gln Pro Leu Asp Ile Lys Val Tyr Ala Asp Arg Phe Thr Pro Leu Thr Thr Thr Asp Val Ala Ala Gly
 26              30                  35                  40                  45                  50

CTC TGG CAG CCC TAC CTT TCT GAC CCC AAC AAC CCA CAG GAG GCG GAC TGG AGC CAA CAG ACC TTT GAC TAT CTC   225
Leu Trp Gln Pro Tyr Leu Ser Asp Pro Asn Asn Pro Gln Glu Ala Asp Trp Ser Gln Gln Thr Phe Asp Tyr Leu
 51              55                  60                  65                  70                  75

CTG AGC CAT GTC CAT TCT CCC AAC GCT GAA AAC CTG GGC CTG TTC CTA ATC TCG GGC TAC AAC CTC TTC CAT GAA   300
Leu Ser His Val His Ser Pro Asn Ala Glu Asn Leu Gly Leu Phe Leu Ile Ser Gly Tyr Asn Leu Phe His Glu
 76              80                  85                  90                  95                  100

GCC ATT CCG GAC CCT TCC TGG AAG GAC ACA GTT CTG GGA TTT CGG AAG CTG ACC CCC AGA GAG CTG GAT ATG TTC   375
Ala Ile Pro Asp Pro Ser Trp Lys Asp Thr Val Leu Gly Phe Arg Lys Leu Thr Pro Arg Glu Leu Asp Met Phe
101             105                 110                 115                 120                 125

CCA GAT TAC GGC TAT GGC TGG TTC CAC ACA AGC CTA ATT CTG GAG GGA AAG AAC TAT CTA CAG TGG CTG ACT GAA   450
Pro Asp Tyr Gly Tyr Gly Trp Phe His Thr Ser Leu Ile Leu Glu Gly Lys Asn Tyr Leu Gln Trp Leu Thr Glu
126             130                 135                 140                 145                 150

AGG TTA ACT GAG AGG GGA GTG AAG TTC TTC CAG CGG AAA GTG GAG TCT TTT GAG GAG GTG GCA AGA GAA GGC GCA   525
Arg Leu Thr Glu Arg Gly Val Lys Phe Phe Gln Arg Lys Val Glu Ser Phe Glu Glu Val Ala Arg Glu Gly Ala
151             155                 160                 165                 170                 175

GAC GTG ATT GTC AAC TGC ACT GGG GTA TGG GCT GGG GCG CTA CAA CGA GAC CCC CTG CTG CAG CCA GGC CGG GGG   600
Asp Val Ile Val Asn Cys Thr Gly Val Trp Ala Gly Ala Leu Gln Arg Asp Pro Leu Leu Gln Pro Gly Arg Gly
176             180                 185                 190                 195                 200

CAG ATC ATG AAG GTG GAC GCC CCT TGG ATG AAG CAC TTC ATT CTC ACC CAT GAC CCA GAG AGA GGC ATC TAC AAT   675
Gln Ile Met Lys Val Asp Ala Pro Trp Met Lys His Phe Ile Leu Thr His Asp Pro Glu Arg Gly Ile Tyr Asn
201             205                 210                 215                 220                 225

TCC CCG TAC ATC ATC CCA GGG ACC CAG ACA GTT ACT CTT GGA GGC ATC TTC CAG TTG GGA AAC TGG AGT GAA CTA   750
Ser Pro Tyr Ile Ile Pro Gly Thr Gln Thr Val Thr Leu Gly Gly Ile Phe Gln Leu Gly Asn Trp Ser Glu Leu
226             230                 235                 240                 245                 250

AAC AAT ATC CAG GAC CAC AAC ACC ATT TGG GAA GGC TGC TGC AGA CTG GAG CCC ACA CTG AAG AAT GCA AGA ATT   825
Asn Asn Ile Gln Asp His Asn Thr Ile Trp Glu Gly Cys Cys Arg Leu Glu Pro Thr Leu Lys Asn Ala Arg Ile
251             255                 260                 265                 270                 275

ATT GGT GAA GCA ACT GGC TTC CGG CCA GTA CGC CCC CAG ATT CGG CTA GAA AGA GAA CAG CTT CGC ACT GGA CCT   900
Ile Gly Glu Ala Thr Gly Phe Arg Pro Val Arg Pro Gln Ile Arg Leu Glu Arg Glu Gln Leu Arg Thr Gly Pro
276             280                 285                 290                 295                 300

TCA AAC ACA GAG GTC ATC CAC AAC TAT GGC CAT GGA GGC TAC GGG CTC ACC ATC CAC TGG GGA TGT GCC CTG GAG   975
Ser Asn Thr Glu Val Ile His Asn Tyr Gly His Gly Gly Tyr Gly Leu Thr Ile His Trp Gly Cys Ala Leu Glu
301             305                 310                 315                 320                 325

GCA GCC AAG CTC TTT GGG AGA ATC CTG GAA GAA AAG AAA TTG TCC AGA ATG CCA CCA TCC CAC CTC TGA AGA CTC  1050
Ala Ala Lys Leu Phe Gly Arg Ile Leu Glu Glu Lys Lys Leu Ser Arg Met Pro Pro Ser His Leu END
326             330                 335                 340                 345

CAGTGACTGCTGCCTCCCCCCACAAGAACTCCCTTCTCCCCTCAGCCAATGAATCAATGTGCTCCTTCATAAGCCATTGCTTCTCCCTCACTTCTTTCCT 1150

CAAAGAAGCATGAGGTGAGAGAAAGCCACAAAGTCAGTGCCTGGAGAAGGGTTCAGCCCAACATGGGGCCCCTCTCATCACTGAAATCCCTCTACCTTCT 1250

CTGGGTCTGGCATTATAAAGAACAGCTGAGGCTGTCATTCCATGAGTCTTCAGAAGAAAGGACAGCTCAGAAAATCAAAGAGGCCAACTGCCCAGAGCCA 1350

CAGAAAATGGAGGATAATTGAGGCTAAGTAACCTGATTACAAGTTGTACTAACATATTAAAGGTTCTGAAAAGTCCTGCAAAA -----
```

Fig.2. Nucleotide and deduced amino acid sequences of cDNA encoding human D-amino acid oxidase. The DNA sequence is numbered from the first nucleotide of the ATG triplet encoding the predicted initiation methionine. The amino acid sequences are also numbered from the initiator methionine. The ATTAAA sequence for the polyadenylation signal is underlined. The upstream in-frame stop codon at -171 to -169 is indicated by closed circles.

3.2. *Nucleotide sequence and deduced amino acid sequence*

The resulting nucleotide sequence of the λHD3 insert and deduced amino acid sequence are shown in fig.2. There is an open reading frame starting from the initiation codon, ATG, at nucleotides 1–3 and ending at the termination codon, TGA, at nucleotides 1042–1044. On comparison with the nucleotide sequence of cDNA for pig D-amino acid oxidase, this ATG was deduced to be the initiation codon for human D-amino acid oxidase. The open reading frame encodes a polypeptide of 347 amino acid residues, as in the case of pig D-amino acid oxidase. The molecular mass of this polypeptide was calculated to be 39 410 Da. The predicted molecular mass of human D-amino acid oxidase compared well with the results of Western blot analysis of the human kidney homogenate (not shown). The assumed initiation codon is preceded by sequences that fulfill Kozak's criteria [11]. These sequences are located downstream from the in-frame terminator, TAG (−171 to −169). The 3′-noncoding region contained a polyadenylation signal, ATTAAA (1406–1411), at 18 nucleotides upstream from the poly(A) tail.

3.3. *Amino acid sequence similarity between human and pig D-amino acid oxidases*

The amino acid sequence deduced for human D-amino acid oxidase was compared with that of pig kidney D-amino acid oxidase (fig.3). The similarity in amino acid sequence between the human and pig kidney D-amino acid oxidases is 84.4%. The amino-terminal 54 residues of these proteins are extensively similar, the amino-terminal 31 residues being identical. This region contains a stretch of highly hydrophobic residues and was predicted to interact with the adenosyl moiety of the FAD

```
                10        20        30        40        50
Human     MRVVVIGAGVIGLSTALCIHERYHSVLQPLDIKVYADRFTPLTTTDVAAG
          ********************************* ********* ********
Pig       MRVVVIGAGVIGLSTALCIHERYHSVLQPLDVKVYADRFTPFTTTDVAAG
                10        20        30        40        50
                60        70        80        90        100
Human     LWQPYLSDPNNPQEADWSQQTFDYLLSHVHSPNAENLGLFLISGYNLFHE
          ***** * * ***** * **** *****  **** * **   ****** *
Pig       LWQPYTSEPSNPQEANWNQQTFNYLLSHIGSPNAANMGLTPVSGYNLFRE
                60        70        80        90        100
                110       120       130       140       150
Human     AIPDPSWKDTVLGFRKLTPRELDMFPDYGYGWFHTSLILEGKNYLQWLTE
          * *** *** ******************* **** *******  *******
Pig       AVPDPYWKDMVLGFRKLTPRELDMFPDYRYGWFNTSLILEGRKYLQWLTE
                110       120       130       140       150
                160       170       180       190       200
Human     RLTERGVKFFQRKVESFEEVAREGADVIVNCTGVWAGALQRDPLLQPGRG
          ********** *********** ***** ******** ** *********
Pig       RLTERGVKFFLRKVESFEEVARGGADVIINCTGVWAGVLQPDPLLQPGRG
                160       170       180       190       200
                210       220       230       240       250
Human     QIMKVDAPWMKHFILTHDPERGIYNSPYIIPGTQTVTLGGIFQLGNWSEL
          ** ****** * ** *** ************* * ***** ** *** *
Pig       QIIKVDAPWLKNFIITHDLERGIYNSPYIIPGLQAVTLGGTFQVGNWNEI
                210       220       230       230       250
                260       270       280       290       300
Human     NNIQDHNTIWEGCCRLEPTLKNARIIGEATGFRPVRPQIRLEREQLRTGP
          ********************* * * ** ********* **********
Pig       NNIQDHNTIWEGCCRLEPTLKDAKIVGEYTGFRPVRPQVRLEREQLRFGS
                260       270       280       290       300
                310       320       330       340
Human     SNTEVIHNYGHGGYGLTIHWGCALEAAKLFGRILEEKKLSRMPPSHL*
          ************************ *****  ***  *  *******
Pig       SNTEVIHNYGHGGYGLTIHWGCALEVAKLFGKVLEERNLLTMPPSHL*
                310       320       330       340
```

Fig.3. Comparison of the amino acid sequences of human and pig D-amino acid oxidases. Amino acids are denoted by a one-letter code. Identical amino acid residues are indicated by asterisks.

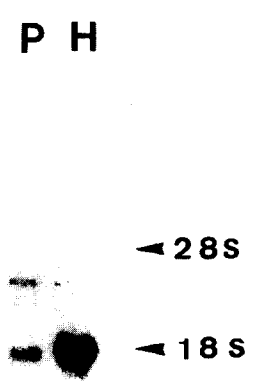

Fig.4. Northern blot hybridization of poly(A)-rich RNAs from pig and human kidney. 5 μg of each RNA was subjected to blot analysis and then hybridized with the *Apa*LI-*Pvu*II digested fragment of the λHD3 insert. As a molecular mass marker, pig kidney rRNA was used. P, pig kidney poly(A)-rich RNA; H, human kidney poly(A)-rich RNA.

molecule [3,12,13]. Several amino acid residues of pig D-amino acid oxidase have been suggested to participate in the enzyme reaction from the results of chemical modification of pig D-amino acid oxidase, and the positions of the modified residues were determined [3,14–17]. These residues are almost the same in human D-amino acid oxidase. However, methionine-110 of the pig enzyme is replaced by threonine in human D-amino acid oxidase. D'Silva et al. [17] recently reported that this methionine-110 may participate in the enzyme reaction, on the basis of the results of chemical modification and peptide mapping.

3.4. *Northern blot analysis*

The size of mRNA for D-amino acid oxidase was determined by Northern blot analysis. The *Apa*LI-*Pvu*II fragment of λHD3 (see fig.1) was used as a hybridization probe. As shown in fig.4, only a single intense hybridization band was detected around the position of 18 S rRNA (about 2 kb). This coincided quite well with the size of the λHD3 insert. This result confirmed that λHD3 comprises a full-length cDNA for human D-amino acid oxidase. Barker and Hopkinson [5] reported electrophoretic variant phenotypes of human kidney D-amino acid oxidase, but we did not detect any other type of enzyme on Northern blot or Western blot analysis.

Acknowledgements: This study was supported in part by a Research Grant for Cardiovascular Diseases (62S-1) from the Ministry of Health and Welfare of Japan, and Grants-in-Aid for Special Project Research (62122005) and for Scientific Research (63570137, 63770170, 63770188) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

[1] Krebs, H.A. (1935) Biochem. J. 29, 1620–1644.
[2] Krebs, H.A. (1952) in: The Enzymes (Sumner, J.B. and Myrback, K. eds) vol.II, pp.500–514, Academic Press, New York.
[3] Ronchi, S., Minchiotti, L., Galliano, M., Curti, B., Swenson, R.P., Williams, C.J. and Massey, V. (1982) J. Biol. Chem. 257, 8824–8834.
[4] Fukui, K., Watanabe, F., Shibata, T. and Miyake, Y. (1987) Biochemistry 26, 3612–3618.
[5] Barker, R.F. and Hopkinson, D.A. (1977) Ann. Hum. Genet. 41, 27–42.
[6] Goldfischer, S., Collins, J., Rapin, I., Neumann, P., Neglia, W., Spiro, A.J., Ishii, T., Roels, F., Vamecq, J. and Van Hoof, F. (1986) J. Pediat. 108, 25–32.
[7] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[8] Aviv, H. and Leder, P. (1972) Proc. Natl. Acad. Sci. USA 69, 1408–1412.
[9] Benton, W.D. and Davis, R.W. (1977) Science 196, 180–182.
[10] Tabor, K. and Richardson, C. (1987) Proc. Natl. Acad. Sci. USA 84, 4767–4771.
[11] Kozak, M. (1981) Nucleic Acids Res. 9, 5233–5252.
[12] Hofsteenge, J., Vereijeken, J.M., Weijer, W.J., Beintema, J.J., Wierenga, R.K. and Drench, J. (1980) Eur. J. Biochem. 113, 141–150.
[13] Fukui, K., Watanabe, F., Shibata, T., Momoi, K. and Miyake, Y. (1987) in: Flavins and Flavoproteins (Edmondson, D.E. and MacCormick, eds) pp.509–512, Walter de Gruyter, Berlin.
[14] Swenson, R.P., Williams, C.H., jr and Massey, V. (1982) J. Biol. Chem. 257, 1937–1944.
[15] Ronchi, S., Galliano, M., Minchiotti, L., Curti, B., Rudie, N.G., Porter, D.J.T. and Bright, H.J. (1980) J. Biol. Chem. 255, 6044–6046.
[16] D'Silva, C., Williams, C.H., jr and Massey, V. (1986) Biochemistry 25, 5602–5688.
[17] D'Silva, C., Williams, C.H., jr and Massey, V. (1987) Biochemistry 26, 1717–1722.