Primary Structure Deduced from Complementary DNA Sequence and Expression in Cultured Cells of Mammalian 4-Hydroxyphenylpyruvic Acid Dioxygenase

EVIDENCE THAT THE ENZYME IS A HOMODIMER OF IDENTICAL SUBUNITS HOMOLOGOUS TO RAT LIVER-SPECIFIC ALLOANTIGEN F*

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4-Hydroxyphenylpyruvic acid dioxygenase is an important enzyme in tyrosine catabolism in most organisms. From porcine and human liver cDNA libraries we isolated complementary DNA inserts for the enzyme. Protein sequence analysis of the porcine enzyme revealed a block of the amino terminus of the mature enzyme. Comparison of the amino acid sequence determined by Edman degradation of peptides derived from porcine liver 4-hydroxyphenylpyruvic acid dioxygenase with the nucleotide sequences revealed the primary structure of the porcine and human enzymes. The mature mature human and porcine enzymes have an 89% amino acid sequence identity in amino acid residues and are composed of 392 amino acid residues. A computer-assisted homology search revealed that the enzyme is 88% identical in amino acid sequence to rat liver-specific alloantigen F.

A monoclonal antibody (mob 51), which can immunoprecipitate both the human and porcine enzymes, was developed. Cultured BMT-10 cells transfected with the cDNA insert of the human enzyme, using the expression vector pCAGGSneoE, produced a polypeptide with an M, of 43,000, which was immunoprecipitated with mob 51. Enzymic activity of the enzyme was detected in the transfected cells but not in the mock transfected cells. These findings suggest that the human 4-hydroxyphenylpyruvic acid dioxygenase is a homodimer of two identical subunits with an M, of 43,000. Liver-specific alloantigen F seems to be closely related to the enzyme or possibly to the subunit of the enzyme itself. Elucidation of the complete amino acid sequence of the enzyme is expected to reveal structure-function relationships of this metabolically important enzyme and to shed light on inherited disorders related to tyrosine metabolism, especially tyrosinemia types 1 and 3.

4-Hydroxyphenylpyruvic acid dioxygenase (HPD)1 (EC 1.13.11.27), an enzyme that participates in the catabolism of tyrosine in most organisms, is present in liver and kidney. Homogentisic acid is produced from 4-hydroxyphenylpyruvic acid by HPD, and the reaction involves decarboxylation, oxidation, and rearrangement. The enzyme has been isolated from porcine (1), human (2), and avian livers (3), and the properties of the enzyme have been extensively characterized (2, 4–12). Purification studies suggested that the human enzyme is a homodimer of identical subunits with an M, of 43,000 (2), whereas the porcine liver enzyme was found to be composed of two nonidentical subunits with a similar M, of 44,000 (1). The enzyme from these sources contains iron; part of the enzymic activity is restored by the addition of Fe2+ and the amino terminus of the enzyme is apparently blocked. The primary structure of the enzyme, from any source, has not been reported. Molecular cloning of the mammalian HPD and elucidation of the primary structure of the enzyme is expected to provide a basis for elucidating molecular mechanisms involved in the oxidation of 4-hydroxyphenylpyruvic acid.

EXPERIMENTAL PROCEDURES

Isolation of Porcine Liver HPD and Development of Antibodies—For antibody production and sequence analysis, porcine liver HPD was purified as previously described (5) but with some modifications (13). The enzyme activity was eluted from a Mono Q column as three peaks of isozymes, the same as for the human liver enzyme (2). The enzyme in the first peak was additionally purified on a Superose column (13).

Antiserum and specific IgG directed to the enzyme protein were prepared as described (13). For preparation of mouse monoclonal antibody directed against the enzyme protein, mice were immunized with Freund’s adjuvant, and hybrid cells were isolated, as described (14). These hybrid cells were monitored for production of IgG that cross-reacted with the porcine liver enzyme. Mouse IgG was purified from

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) D13390.

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1 The abbreviations used are: HPD, hydroxyphenylpyruvic acid dioxygenase; HPLC, high performance liquid chromatography; PE, pyridylethyl.

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ascites fluid, as described (15) and was used for additional experiments.

Protein Sequence Analysis—Porcine HPD was reduced with tri-n-butylphosphine (Wako Pure Chemical Co., Osaka, Japan) (16) and pyridylethylated with 4-vinylpyridine (Tokyo Kasei, Kogyo, Japan) (17) in the presence of 7 M guanidine hydrochloride containing 0.1 M Tris•HCl (pH 6.8) and 1 M EDTA (pH 8.0). The reduced peptides were desalted by reversed-phase HPLC using a Bakerbond WP-C4 column (4.6 X 50 mm, J. T. Baker Inc.,Phillipsburg, NJ). Methionyl bonds of the PE-protein were cleaved with cyanogen bromide in 70% formic acid, as described by Gross (18). Peptides

ATGGC(G/A/T/C)AA(T/C)TA(T/C)GA(G/A)GA

were primarily separated by gel permeation HPLC on a TSK G2000 butylphosphine (Wako Pure Chemical Co., Osaka, Japan) (16) and using lipofection (Bethesda Research Laboratories, Gaithersburg, MD) (15) as described (15), and then the proteins bound to the agarose were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (34). Immunoblot analyses with anti-HPD rabbit IgG were carried out by the method of Towbin et al. (35) as described (15).

RESULTS

The conventionally purified HPD from porcine liver contained a single species of polypeptide with an Mr of 43,000 (Fig. 1). The results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration on a calibrated Superose column suggested that the purified porcine HPD was a homodimer of subunits with an Mr of 43,000. The purified porcine liver HPD was used for antibody production.

An attempt at immunoaffinity isolation of porcine HPD was made using themob 51, one of the monoclonal IgGs that recognizes porcine HPD. In this experiment, a partially purified HPD preparation (DEAE-Sephadex step, Ref. 5) was applied on a Sepharose gel column immobilized with mob 51 (2 mg/ml gel). The column was washed with 50 mM Tris-HCl (pH 7.4) containing 500 mM NaCl, and proteins were eluted with 100 mM Na2CO3 (pH 11.0). A single species of protein with an Mr of 43,000 was found in the eluate obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Immunoprecipitation of human HPD from crude human liver homogenates was carried out using the immobilized mob 51. When the immunoprecipitated HPD was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblots were analyzed using conventional antiserum, an immunostained protein migrated slightly faster than did the porcine HPD (Fig. 1). Among the monoclonal mouse IgGs, only mob 51 cross-reacted with human HPD in the liver homogenate. Thus, the immunochemical procedure provided evidence that porcine and human HPD are composed of a single subunit with an Mr of 42,500-43,000.

![Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified protein and immunoprecipitation of porcine and human enzyme.](image-url)

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified protein and immunoprecipitation of porcine and human enzyme. The purified protein (3 μg) (lane 1), immunoimmobilized HPD (lanes 2–4) were separated on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, as described under “Experimental Procedures.” The gel was stained with Coomassie Brilliant Blue G 250 (lane 1) or analyzed by immunoblotting (lanes 2–4). For immunoprecipitation, crude extracts of human and porcine liver were incubated with mob 51 (0.5 μg) for 60 min at room temperature, and 10 μl of TDI (0.5 μg) agarse (Pharmacia; suspended in Tris-HCl buffer (pH 7.4, 1:1, v/v)) was added to the mixture. The incubation was continued for another 60 min, and then the preparation was centrifuged at low speed. The gel pellet was washed three times with ice-cold phosphate-buffered saline solution, and the protein associated with the gel was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting, using anti-HPD rabbit IgG (22). Lane 2, purified porcine liver HPD; lane 3, immunoprecipitated human HPD; lane 4, mob 51 alone. The arrow indicates the subunit of HPD.
Sequenator analysis of the porcine S-PE-protein (70 pmol) yielded no phenylthiohydantoin in three cycles of Edman degradation. Thus, the amino terminus of porcine HPD was blocked.

Amino acid sequences of seven peptides (denoted by the prefix M: M2, M3, M4, M5, M6, M7, and M8) were determined partially or completely through the carboxyl terminus (Table I). On the basis of the amino acid sequences, several oligonucleotide probes were synthesized and used to screen the porcine liver cDNA library. Among the oligonucleotide probes, the oligonucleotides named probe 4, 5, and 6 produced positive signals with the three oligonucleotide probes. The nucleotide sequences of the cDNA inserts of these phage clones were determined. The three positive clones. Each of these recombinant phage clones contained additional phage clones. The nucleotide sequences of the inserts from these clones were synthesized and used to screen the human liver cDNA library. The human HPD (Fig. 2) was obtained from the porcine liver library to obtain phage clones carrying inserts that covered the entire coding region of human HPD (Fig. 2).

Two phage clones named λhdpD1 from the porcine library and λhdpH31 from the human library were analyzed in detail.

| Table I | Summary of amino acid sequence analysis of cyanogen bromide (M) peptides |
|---------|---------------------------------------------------------------|
| Cycle   | M2     | M3   | M4    | M5     | M6    | M7    | M8    | M8-K2 |
| 1       | Gly    | Asn  | Glu   | Arg    | Ala    | Pro   | Gln   | Ala    |
| 2       | Asp    | Tyr   | Ser   | Asn    | Asn    | Ile   | Asp   | Phe    |
| 3       | His    | Thr   | Ala   | Leu    | Tyr    | Asn   | Arg   | Glu    |
| 4       | Leu    | Gln   | Gln   | Gln    | Gln    | Glu   | Pro   | Glu    |
| 5       | Val    | Cys*  | Trp   | Phe    | Glu    | Glu   | Pro   | Thr    |
| 6       | Lys    | Phe   | Tyr   | His    | Ser    | Ala   | Val   | Gln    |
| 7       | His    | Leu   | Metb  | Arg    | Ile    | Pro   | Phe   | Glu    |
| 8       | Gly    | Pro   | Phe   | Lys    | Gly    | Leu   | Leu   | Leu    |
| 9       | Asp    | Gly   | Trp   | Metb   | Lys    | Gly   | Arg   | Arg    |
| 10      | Gly    | Phe   | Ser   | Lys    | Val    | Val   | Gly   | Lys    |
| 11      | Val    | Gln   | Val   | Lys    | Ile    | Asn   | Asn   | Asn    |
| 12      | Lay    | Ala   | Asp   | Ser    | Glu    | Leu   | Ile   | Glu    |
| 13      | Asp    | Pro   | Asp   | Gln    | Arg    | Thr   | Ile   | Thr    |
| 14      | Ile    | Thr   | Thr   | Thr    | Asn    | Asp   | Asp   | Asp    |
| 15      | Ala    | Phe   | Gln   | Gln    | Asn    | Thr   | Phe   | Asn    |
| 16      | Phe    | Thr   | Ile   | Glu    | His    | Asp   | Ile   | Asp    |
| 17      | Gln    | Asp   | His   | Val    | Gly    | Asn   | Arg   | Arg    |
| 18      | Val    | Pro   | Thr   | Asp    | Phe    | Ile   | Thr   | Asp    |
| 19      | Gln    | Leu   | Glu   | Tyr    | Asn    | Ala   | Gln   | Glu    |
| 20      | Asp    | Leu   | Tyr   | Asn    | Ala    | Ile   | Thr   | Glu    |
| 21      | Cys*   | Ser   | Ser   | Gly    | ND     | Asp   | Asp   | Asp    |
| 22      | Asp    | Lys   | Ala   | Gly    | Asn    | Thr   | Gln   | Gln    |
| 23      | Tyr    | Leu   | Leu   | Ala    | Phe    | Glu   | Asn   | Asn    |
| 24      | Ile    | Pro   | Gln   | Asn    | Asn    | Asn   | Asn   | Asn    |
| 25      | Val    | Val   | Val   | Val    | Val    | Val   | Val   | Val    |
| 26      | Gln    | Gln   | Gln   | Gln    | Gln    | Gln   | Gln   | Gln    |
| 27      | Lys    | ND    | ND    | ND     | ND     | ND    | ND    | ND     |
| 28      | Ala    | Ile   | Ile   | Ile    | Ile    | Ile   | Ile   | Ile    |
| 29      | Arg    | Ala   | Ala   | Ala    | Ala    | Ala   | Ala   | Ala    |
| 30      | Gln    | Leu   | Leu   | Leu    | Leu    | Leu   | Leu   | Leu    |
| 31      | Arg    | Lys   | Lys   | Lys    | Lys    | Lys   | Lys   | Lys    |
| 32      | Gly    | Thr   | Thr   | Thr    | Thr    | Thr   | Thr   | Thr    |
| 33      | Ala    | Glu   | Glu   | Glu    | Glu    | Glu   | Glu   | Glu    |
| 34      | Ile    | Asp   | Asp   | Asp    | Asp    | Asp   | Asp   | Asp    |
| 35      | Ile    | Ile   | Ile   | Ile    | Ile    | Ile   | Ile   | Ile    |
| 36      | Val    | Val   | Val   | Val    | Val    | Val   | Val   | Val    |

* Cys was identified as phenylthiohydantoin of the S-pyridylethyl derivative.
* Met was identified as phenylthiohydantoin of homoserine.
ND, not determined.

The predicted primary structures of porcine and human HPD precursors were compared with the partial amino acid sequences of peptides of the porcine enzyme. As shown in Fig. 3, these sequences were the same in six regions, residues 83-118, 151-174, 194-224, 230-273, 341-365, and 341-365 of the predicted sequence, thereby confirming that the isolated cDNA insert codes for porcine HPD. Among eight peptides (denoted by the prefix M) isolated from a digest of the S-PE-protein (1.5 nmol) with cyanogen bromide, the amino acid composition of peptide M1 resembled that of residues 2-82 of the amino acid sequence predicted from the mRNA. The first clone λhdpD1 carried a cDNA insert of 1.3 kilobases and covered the entire coding sequence of porcine HPD mRNA. The λhdpH31 carried a 1.5-kilobase insert with 21-base 5'-untranslated, full-length coding, and 202-base 3'-untranslated regions.

Both the porcine and human cDNA inserts contained an open reading frame that could be translated to 393 amino acid residues (Figs. 3 and 4). The nucleotide sequence surrounding the putative initiation codon in the human cDNA insert was similar to the consensus sequence described by Kozak (36). A polyadenylation signal was present at 180 base pairs downstream of the stop codon of TAG in human cDNA. Northern blot analysis of mRNA indicated only a single species of mRNA was present in the human liver, and size of the mRNA for human HPD was estimated to be 1.7-1.8 kilobases long (data not shown).

The predicted structures of porcine and human HPD inserts showed that those of human HPD were 1.5 kilobases long and covered the entire coding region of human HPD. After digestion of peptide M1 by Achromobacter protease I, another amino-terminal blocked peptide (M1-K1) was isolated by reversed phase HPLC. Molecular mass of this blocked peptide was estimated to be 743.08, using a PE-SCIEX API biomolecular mass analyzer, in agreement with experimental error within the value (742.76) calculated for the acetyl group; hence the amino terminus of the enzyme was acetylated. Sequenator analysis (Table I) and mass spectrometric measurement (data not shown) of the carboxyl-terminal peptide (M8-K2) generated by Achromobacter protease I indicated that 6 amino acid residues at the carboxyl terminus were missing in the purified enzyme. It is not clear whether this cleavage occurred during steps of purification.
rat F antigen

human HPD and rat antigen F. Identical amino acids are boxed.

These results taken together show that the translation product of porcine HPD is processed at both the amino and carboxyl termini; the putative initiation methionyl residue is removed, and the newly formed amino-terminal threonyl residue seems to be blocked by an acetyl group (37).

Comparison of the predicted amino acid sequences of the porcine and human enzymes revealed that both the enzymes are highly homologous, with an 87.5% overall identity. The homology of nucleotide sequences between the porcine and human HPD cDNAs was 88.6% in the coding region. Transfection and expression experiments of HPD were done to determine whether the cDNA of HPD was indeed coded for the mammalian HPD protein. When the cDNA of HPD was transfected into the transfected cells synthesized a polypeptide with an immunoreactive band (Fig. 5). On the other hand the mock transfected cells produced no detectable amount of the immunoreactive polypeptide. Thus, the transfected cells synthesized a polypeptide with an M, of 43,000, which was immunoprecipitated with mob Y1 (Fig. 5). On the other hand the mock transfected cells produced no detectable amount of the immunoreactive polypeptide. Thus, the transfected cells synthesized a polypeptide with an M, of 43,000, which was immunoprecipitated with mob Y1.
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activity, whereas the mock transfected cells showed little activity (Fig. 6).

These results show that the cDNA insert carried all of the sequence information necessary for full expression of the enzyme protein and activity. It is also evident that the mature human of protein was composed of two identical subunits with an M, of 43,000.

**DISCUSSION**

We obtained evidence that mammalian HPD is a homodimer of the identical subunit composed of 386 amino acid residues. Purification of the human HPD by other workers (8, 12) indicated that the relative molecular mass of the active human enzyme is 87,000, and the subunit mass is 43,000, determined using polyacrylamide gels containing SDS. These data are in good accord with our results. It was reported that the amino terminus of human and avian HPD was blocked (2, 6); and our present data suggest that the amino-terminal threonyl residue seems to be acetylated.

Microheterogeneity of purified enzymes from various sources revealed three major forms of avian enzyme that are enzymatically active with isoelectric points of I = 6.0, II = 6.2, and III = 6.4 (6). The porcine enzyme was eluted from the Mono Q column as three peaks (5). A similar heterogeneity was noted for the human enzyme, and these forms were immunologically identical (2). Processing of the carboxyl-terminal amino acids seen in the present study might relate to heterogeneity of the enzyme protein.

A computer-assisted search for homology of the amino acid sequences suggested that the sequences are not homologous to any known oxidases, including pyruvate dehydrogenase (38) or branched-chain keto acid dehydrogenase (39). The human and porcine HPD are highly homologous to rat liver-specific alloantigen F (40, Fig. 4).

Liver-specific antigen F, first reported in 1968, was immunoprecipitated by sera from mice immunized with water-soluble liver extracts of allogenic mice (41). Although biological function of the antigen is not well understood, this antigen is widely distributed among species of mammals (42-46). Part of the primary structure of rat antigen F was deduced by molecular cloning of the cDNA obtained by immunoscreening of the rat liver cDNA library with allo-antisera to the antigen (40). The present study revealed that the amino acid sequence of rat antigen F is highly homologous to human and porcine HPD, with an approximately 90% identity. The relative molecular masses of antigen F from mouse and human liver were reported to be 43,000 (45) and 44,000 (42), respectively, which is similar to the M, of 43,000 noted for mouse and human liver HPD (8, 13). In addition, both antigen F and HPD (47) are expressed in the liver and kidney. These data suggested that HPD is a protein closely related to antigen F or is antigen F itself.

There are three known types of hereditary tyrosinemia. In a mouse model for the type III tyrosinemia, HPD activity is genetically defective, and the subunit protein of HPD is undetectable by immunoblot analysis of liver extracts (13). Our preliminary study on tyrosinemic mice indicated that (i) tyrosinemic mice lack the F antigen, and (ii) the hypertyrosinemic gene in the mice is located on chromosome 5 as is the F antigen (48). Molecular analysis of tyrosinemic mice indicated that mRNA related to the HPD cDNA was absent in the liver. All of these data support our working hypothesis that HPD enzyme protein is F antigen itself.

Molecular cloning of the mammalian HPD and elucidation of the primary structure of the enzyme are expected to provide a basis for elucidating molecular mechanisms involved in the oxidation of 4-hydroxyphenylpyruvic acid and the molecular events related to disorders of tyrosine metabolism.

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