Porcine Microsomal Vitamin D₃ 25-Hydroxylase (CYP2D25)

CATALYTIC PROPERTIES, TISSUE DISTRIBUTION, AND COMPARISON WITH HUMAN CYP2D6*

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Received for publication, May 16, 2000, and in revised form, July 19, 2000
Published, JBC Papers in Press, August 2, 2000, DOI 10.1074/jbc.M004185200

The metabolic activation of the prohormone vitamin D₃ requires a 25-hydroxylation that has been reported to be catalyzed by both mitochondrial CYP27A and a microsomal vitamin D₃ 25-hydroxylase in the liver. CYP27A has been extensively studied, but its role as a physiologically important vitamin D₃ 25-hydroxylase has been questioned. The present paper reports that the microsomal vitamin D₃ 25-hydroxylase, purified from pig liver, converted vitamin D₃ into 25-hydroxyvitamin D₃ in substrate concentrations which are within the physiological range (apparent Kₘ = 0.1 μM). The enzyme 25-hydroxylated vitamin D₃, 1α-hydroxyvitamin D₃ and vitamin D₂ and also converted tolcudine, a substrate for human CYP2D6, into its 5-hydroxymethyl metabolite. Tolerodine inhibited the microsomal 25-hydroxylation, whereas quinidine, an inhibitor of CYP2D6, did not markedly inhibit the reaction. The primary structure of the microsomal vitamin D₃ 25-hydroxylase, designated CYP2D25, shows 77% identity with that of human CYP2D6. Northern blot and reverse transcription-polymerase chain reaction experiments revealed that CYP2D25 mRNA is expressed in higher levels in liver than in kidney and in small amounts in adrenals, brain, heart, intestine, lung, muscle, spleen, and thymus. Experiments with human liver microsomes and recombinantly expressed CYP2D6 strongly indicate that the microsomal 25-hydroxylation of vitamin D₃ in human liver is catalyzed by an enzyme different from CYP2D6.

Vitamin D₃ (cholecalciferol) is essential for the absorption of calcium and phosphate in the intestine and has effects on the regulation of growth and differentiation of certain specialized cell types. The prohormone vitamin D₃ is formed from 7-dehydrocholesterol in the skin under the influence of ultraviolet irradiation. Vitamin D₃ can also be derived from dietary sources. Vitamin D₂ (ergocalciferol), which differs structurally from vitamin D₃ in the side chain, has been frequently used to treat and prevent vitamin D deficiency and is used in parenteral vitamin formulations. A cytochrome P450-dependent 25-hydroxylation in the liver is the first step in the metabolic activation of both vitamin D₃ and vitamin D₂ into their active hormonal forms. It has been reported that extracts and enzyme preparations from other tissues, e.g. kidney, also contain this activity. The 25-hydroxylation is followed by the tightly regulated 1α-hydroxylation in the kidney, to form the biologically active compounds 1α,25-dihydroxyvitamin D₃ and 1α,25-dihydroxyvitamin D₂, respectively (for review, see Ref. 1).

A mitochondrial vitamin D₃ 25-hydroxylase, CYP27A,¹ has been purified from several species and characterized (2–7). It is well established that this enzyme catalyzes the obligatory 27-hydroxylation of cholesterol and C₂₇-sterols in bile acid biosynthesis. The tissue distribution of this mitochondrial enzyme is widespread, its mRNA is found not only in liver but in most other tissues (3). Leading authorities in the vitamin D field have expressed skepticism that a single enzyme, i.e. CYP27A, is responsible for the hepatic 25-hydroxylation as a whole (1). There are several unexplained observations suggesting that a cytochrome P450 other than CYP27A performs 25-hydroxylation of vitamin D. Thus, studies on patients having a mutated CYP27A gene demonstrated a lack of 27-hydroxylation of C₂₇-sterols in bile acid biosynthesis but not decreased serum levels of 25-hydroxyvitamin D₃ (8). Mice with the disrupted sterol 27-hydroxylase gene showed markedly reduced bile acid biosynthesis, but the circulating 25-hydroxyvitamin D₃ levels were increased more than 2-fold (9). Furthermore, the mitochondrial CYP27A does not 25-hydroxylate vitamin D₃ (2), evoking the question: which cytochrome P450 enzyme synthesizes 25-hydroxyvitamin D₂ (1)?

A microsomal vitamin D₃ 25-hydroxylase was purified from pig liver by A xen et al. (10). The microsomal 25-hydroxylase catalyzed 25-hydroxylation of both vitamin D₃ and vitamin D₂ (11). An antibody raised against the pig enzyme recognized a protein of similar apparent molecular weight in human liver microsomes (10). A cDNA encoding the pig liver microsomal enzyme was recently isolated (12). The structure of microsomal vitamin D₃ 25-hydroxylase, as deduced by both DNA sequence analysis and protein sequence analysis, showed 70–80% identity with members of the CYP2D subfamily. The enzyme has been assigned the name CYP2D25.² The only CYP2D enzyme known to be expressed in man is CYP2D6. This enzyme is polymorphically expressed and is lacking in 5–10% of the caucasian population. It is not known whether vitamin D₃ 25-hydroxylation in man could be catalyzed by CYP2D6.

The objective of the current study was to characterize the microsomal vitamin D₃ 25-hydroxylase (CYP2D25) by studying its catalytic properties, effect of inhibitors, and tissue distribution.

* This work was supported by the Swedish Medical Research Council Project 03X-218. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank TM/EBI Data Bank with accession number(s) Y16417.

1 The abbreviations used are: CYP, cytochrome P450; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; HLM, human liver microsome.

2 The sequence reported for the porcine microsomal vitamin D₃ 25-hydroxylase was designated CYP2D25 by the P450 nomenclature committee and has been deposited in the GenBank TM with accession number Y16417.

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tion and to make comparisons with the structurally related human CYP2D6.

EXPERIMENTAL PROCEDURES

Materials—Vitamin D$_3$, vitamin D$_2$, sulfaphenazole, troleandomycin, quinidine, cyclosporin A, and 2-phenylcyclopropylamine were from Sigma. 25-Hydroxyvitamin D$_3$ and 1a,25-dihydroxyvitamin D$_3$ were obtained from Solvay (Duphar, The Netherlands), and 1a-hydroxyvitamin D$_3$ was a gift from Dr. Lise Binderup Leo Pharma (Copenhagen, Denmark). Unlabeled and $^{14}$C-labeled adrenodoxin were donated by Dr. Hans Postlind, Pharmacia & Upjohn (Stockholm, Sweden) and [guanidine-14]$^{14}$]debrisoquin was from Amersham Pharmacia Biotech.

RNA Isolation—Tissues were obtained from 6-month-old, castrated male pigs. Total RNA was extracted from liver, kidney, lung, heart, muscle, thymus, spleen, intestine, adrenal, and brain using RNaseasy Midi Kit (Qiagen) following the manufacturer’s instructions.

Northern Blot Analysis—Total RNA, 30 µg, from each tissue was denatured for analysis of CYP2D25 and cyclophillin transcripts, separated on 1% denaturing agarose gels, and subjected to Northern blot and hybridization with a $^{32}$P-labeled 1624-bp Nkpr1-Xbal fragment of pig liver microsomal CYP2D25 cDNA and a 400-bp fragment of rat cyclophilin cDNA as described by Axe & et al. (12).

RT-PCR—Total RNA was extracted from different tissues of castrated male pigs as described above. First-strand cDNA synthesis was performed using 1 µg of RNA from liver and kidney and 10 µg of RNA from brain, lung, thymus, intestine, muscle, heart, adrenal, and spleen and 30 pmol of a CYP2D25-specific antisense primer. The negative controls containing RNA without reverse transcriptase enzyme were included in each experiment. The resulting cDNA was subjected to PCR amplification using 30 pmol of each of sense and antisense primers for CYP2D25. The following primers (purchased from Life Technologies, Inc.) were used to obtain the 245-bp cDNA sequence of CYP2D25: sense, 5-GGTTGGTGATAAGTGGTCC-3 (nucleotides 1269–1248). The PCR profile was: 94 °C denaturation for 1 min, 59 °C annealing for 1 min, 72 °C extension for 1 min, followed by 26 cycles with 94 °C denaturation for 1 min, 72 °C extension for 1 min, and 72 °C for 10 min. The 245-bp product was analyzed by electrophoresis on 2% agarose gels containing 0.5 µg/ml ethidium bromide. The identity of the DNA product isolated from the electrophoretic gel was verified by restriction enzyme analysis (BsiHKA I from BioLabs Inc).

Source of Enzymes—Microsomal vitamin D$_3$ 25-hydroxylase was purified from pig liver as described by Axe & et al. (10). The apparently homogeneous enzyme showed a single protein band upon SDS-PAGE.

Human CYP27A, recombinantly expressed in Escherichia coli, was purified as described previously (13). Human liver microsome samples from different donors were obtained from Gentest Corp. (Woburn, MA) and used in immunoblotting and incubation experiments. Recombinantly expressed human CYP2D6 (SUPERSONESTM) and control microsomes from nontransfected insect cells were obtained from Gentest Corp. (Woburn, MA).

SDS-PAGE and Immunoblotting—SDS-PAGE was carried out as described previously (10, 16). Electrophoresis and immunoblotting were performed with CYP2D25 and different human liver microsome samples from various donors, some of them with polymorphism for CYP2D6. The monoclonal and polyclonal antibodies raised against purified pig liver microsomal vitamin D$_3$ 25-hydroxylase (CYP2D25) in the Western blot experiments, were prepared in this laboratory (10). The monoclonal antibody raised against human CYP2D6 was obtained from Gentest Corp.

Incubations with Vitamin D$_3$—Incubations with vitamin D$_3$, 1a-hydroxyvitamin D$_3$, and Vitamin D$_2$—Incubations with vitamin D$_3$, 1a-hydroxyvitamin D$_3$, and vitamin D$_2$, and analysis of incubation mixtures were carried out essentially as described previously (2, 10, 11). The substrate concentration was 25 µM. In some experiments with vitamin D$_3$ and the microsomal vitamin D$_3$ 25-hydroxylase (CYP2D25), the substrate concentrations were in the range 62–500 nM. Incubations were carried out for 10–30 min at 37 °C with 0.025–0.1 nM of CYP2D25, 1 unit of NADPH-cytochrome P450 reductase (14), and 1.2 µmol of NADPH in a final volume of 1 ml of 50 mM Tris acetate, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The incubations were terminated by addition of 125 µl of acetone, and the mixtures were analyzed for product formation by HPLC as described previously (10).

RESULTS AND DISCUSSION

Catalytic Properties of Pig Liver Microsomal Vitamin D$_3$ 25-Hydroxylase—The catalytic properties of the microsomal vitamin D$_3$ 25-hydroxylase was studied with the native enzyme purified to apparent homogeneity from pig liver. The enzyme catalyzed 25-hydroxylation of various vitamin D compounds, at saturating concentrations (25 µM), in the order of efficiency: 1α-hydroxyvitamin D$_3$ (760 pmol/nmol cytochrome P450/min) > vitamin D$_2$ (200 pmol/nmol cytochrome P450/ min) > vitamin D$_3$ (110 pmol/nmol cytochrome P450/min).

In previous studies, the rate of hydroxylation by microsomal and mitochondrial vitamin D$_3$ 25-hydroxylases, purified from rat and pig liver, has been analyzed with micromolar concentrations of vitamin D$_3$, and apparent Km values of 5–10 µM have
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been reported (2, 10, 18, 19). Since the concentrations of vitamin D₃ in liver have been estimated to be much lower, about 0.1 μM (20), experiments were carried out to analyze the ability of the purified microsomal 25-hydroxylase to convert nanomolar concentrations of vitamin D₃ into 25-hydroxyvitamin D₃.

The enzyme efficiently 25-hydroxylated vitamin D₃ in the concentration range of 62–500 nM. With 100 nM vitamin D₃ and an incubation time of 10 min, the rate of 25-hydroxylation was linear up to 0.1 nmol of purified 25-hydroxylase. Using the incubation time of 10 min, the rate of 25-hydroxylation was measured for various concentrations of tolterodine (0-75 μM) or quinidine (0–125 μM) in a final volume of 1 ml of 50 mM Tris acetate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. In incubations with 0.25 nmol of mitochondrial vitamin D₃ 25-hydroxylase (CYP27A), 4 nmol of adrenodoxin, and 0.4 nmol of adrenodoxin reductase was added instead of microsomal NADPH-cytochrome P450 reductase. The activities in control incubations (without tolterodine) with the microsomal and mitochondrial 25-hydroxylases were 185 and 170 pmol/nmol P450/min, respectively.

Since the primary structure of the microsomal vitamin D₃ 25-hydroxylase (CYP2D25) is 70–80% identical with other members of the CYP2D subfamily (12), the activity of CYP2D25 toward some known CYP2D substrates was also studied. Debrisoquine is a commonly used marker substrate for CYP2D6, which catalyzes the 4-hydroxylation of this compound (21, 22). Tolterodine, a muscarinic receptor antagonist, is converted into the 5-hydroxymethyl metabolite by CYP2D6 (17). Debrisoquine 4-hydroxylation was very low with the microsomal vitamin D₃ 25-hydroxylase, less than 12 pmol/nmol P450/min. Tolterodine, on the other hand, was efficiently converted into its 5-hydroxymethyl metabolite at a rate of 360 pmol/nmol/min, which is comparable with that of 25-hydroxyvitamin D₃.

FIG. 2. HPLC analysis of incubations with tolterodine and microsomal vitamin D₃ 25-hydroxylase. [¹⁴C]Tolterodine, 10 μM, was incubated for 30 min at 37 °C with 0.1 nmol of CYP2D25, 2 units of NADPH-cytochrome P450 reductase, and 0.5 μmol of NADPH in a total volume of 250 μl of 100 mM phosphate buffer, pH 7.4. The incubations were terminated by addition of 125 μl of acetonitrile and the mixtures were analyzed by HPLC as described previously (17). The product formed had the same retention time as the authentic 5-hydroxymethyl metabolite of tolterodine (17).

FIG. 4. Northern blot analysis of CYP2D25 mRNA from various porcine tissues. Total RNA, 30 μg, from each tissue was denatured and separated on 1% denaturing agarose gels containing formaldehyde and then transferred to nitrocellulose filter. Lane 1, liver; lane 2, kidney; lane 3, adrenal; lane 4, lung; lane 5, brain; lane 6, heart; lane 7, muscle; lane 8, spleen; lane 9, thymus; lane 10, intestine. The hybridization was carried out with a 32P-labeled 1624-bp pig liver microsomal CYP2D25 cDNA (12) by standard procedures. After autoradiography for 48 h at −70 °C, the filter was stripped and probed with a 400-bp fragment of rat cyclophilin cDNA as control.

FIG. 5. RT-PCR analysis of CYP2D25 mRNA from various porcine tissues. First-strand cDNA synthesis using total RNA from various tissues and PCR reactions with primers specific to CYP2D25 were performed as described under “Experimental Procedures.” The amplified PCR products (240 bp) were analyzed by electrophoresis on 2% agarose gels containing formaldehyde and then transferred to nitrocellulose filter. Lane 1, intestine (10 μg); lane 2, thymus (10 μg); lane 3, spleen (10 μg); lane 4, muscle (10 μg); lane 5, heart (10 μg); lane 6, brain (10 μg); lane 7, lung (10 μg); lane 8, adrenal (10 μg); lane 9, liver (1 μg); lane 10, kidney (1 μg). Right lane, DNA size ladder (516, 394, 344, 298, 220, 200 bp). The identity of the DNA products from liver, kidney, and spleen were verified by restriction enzyme analysis. Negative controls containing RNA without reverse transcriptase enzyme and without cDNA template, respectively, were included in each experiment and did not show any product formation.
formation (Fig. 2). The results show that CYP2D25 is not specific for vitamin D₃ compounds. The very low activity toward debrisoquine is not unique to CYP2D25 among members of the CYP2D subfamily. Other CYP2D enzymes, such as those of the mouse and some of the rat CYP2D enzymes, have been reported to be inactive toward debrisoquine but catalyze hydroxylations of endogenous substrates, i.e. testosterone (21–23).

Effect of Various Cytochrome P450 Inhibitors on the 25-Hydroxylation of Vitamin D₃—To obtain further information on the catalytic properties of the microsomal vitamin D₃ 25-hydroxylase, the effects of various P450 inhibitors were studied. The CYP2D6 substrate tolterodine inhibited the microsomal 25-hydroxylation of vitamin D₃ (25 μM) by more than 80% at a 50 μM concentration (Fig. 3). This marked inhibition is to be expected since tolterodine was shown to be a substrate also for the microsomal vitamin D₃ 25-hydroxylase (cf. above). Tolterodine had no effect on the 25-hydroxylation catalyzed by the mitochondrial vitamin D₃ 25-hydroxylase (CYP27A). Quinidine, an inhibitor of CYP2D6, inhibited the microsomal 25-hydroxylation at most by 50% in concentrations up to 125 μM (Fig. 3). Quinidine is a potent inhibitor of CYP2D6-catalyzed reactions in much lower concentrations, about 1 μM (17, 21). In this concentration, the inhibition of the 25-hydroxylation was less than 20% (Fig. 3). A lack of inhibition by quinidine has been reported for the N-demethylation of imipramine by CYP2D18 (24). Cyclosporin A (30 μM), which has been reported to be an inhibitor of mitochondrial CYP27A-mediated 25-hydroxylation of vitamin D₃ (25), inhibited the microsomal 25-hydroxylation by about 45%. For comparison, the 25-hydroxylation catalyzed by mitochondrial CYP27A was inhibited by 60% (data not shown).

Inhibitors of other cytochrome P450 subfamilies had no or very little effect on microsomal vitamin D₃ 25-hydroxylation. Thus, sulfaphenazole (50 μM), a competitive inhibitor of CYP2C9 (21), tranylcypromine (50 μM), an inhibitor of CYP2C19 (26), and tolterodine (50 μM), a specific inhibitor of CYP3A isozymes (27), inhibited the reaction only slightly, 20–30%. α-Naphthoflavone (50 μM), an inhibitor of CYP1A (28), did not affect the reaction.

Northern Blot and RT-PCR Experiments on the Tissue Distribution of mRNA for Pig Microsomal CYP2D25—RNA from various pig tissues was isolated and examined in Northern blot experiments using the 32P-labeled pig liver CYP2D25 cDNA as a probe (12). With this method, mRNA for CYP2D25 was detected in liver and kidney but not in adrenals, brain, heart, intestine, lung, muscle, spleen, or thymus (Fig. 4). The presence of mRNA expressing CYP2D25 in kidney is supported by previous studies reporting the purification from pig kidney of a microsomal enzyme active in 25-hydroxylation of vitamin D₃ (29, 30).

The results of the Northern blot experiments showed that CYP2D25 is expressed predominantly in liver and in lower amounts in kidney. However, a low expression in other tissues could not be excluded. Indeed, experiments with RT-PCR revealed that mRNA for CYP2D25 is present not only in liver and kidney, but also in small amounts in adrenals, brain, heart, intestine, lung, muscle, spleen, and thymus (Fig. 5). The presence of mRNA expressing CYP2D25 in kidney is supported by previous studies reporting the purification from pig kidney of a microsomal enzyme active in 25-hydroxylation of vitamin D₃ (29, 30).

The comparison of the primary structures of CYP2D25 and CYP2D6—Fig. 6 shows the deduced amino acid sequence of porcine CYP2D25 and alignment with the sequence of human CYP2D6. The overall amino acid identity between the two proteins was 77%. The putative substrate recognition sites for CYP2 proteins (SRS-1 through SRS-6) described by Gottoh (34) are underlined.
zyme but also protein of the same apparent M, as described previously (10). The amount of human liver microsomal protein applied to the gel was 50 μg. Lane 1, pig liver CYP2D25 (1 μg); lane 2, HLM 8; lane 3, HLM 9; lane 4, HLM 1; lane 5, HLM 4; lane 6, HLM 3; lane 7, HLM 5; lane 8, CYP2D25 (2 μg). The positions to which the M, standards, CYP1A2 (apparent M, = 55,000) and CYP2B4 (apparent M, = 48,000), migrated are indicated on the right.

Fig. 7. Western blot analysis of different human liver microsome samples with antibodies directed against CYP2D25 (A) and CYP2D6 (B). SDS-PAGE and immunoblotting experiments with various human liver microsome (HLM) samples and polyclonal antibodies raised against pig liver microsomal CYP2D25 (A) and a monoclonal antibody directed against human CYP2D6 (B) were carried out as described previously (10). The antibody directed against human CYP2D6 did not react with human liver microsomes. Liver microsome samples from different donors were separately incubated with 1α-hydroxyvitamin D3 and reconstitutively expressed CYP2D6 (A) and purified pig liver microsomal CYP2D25 (A). 1α-Hydroxyvitamin D3 (25 μl) was incubated for various periods of time with reconstituted pig liver microsomal CYP2D25 (0.1 nmol) or human CYP2D6 (0.05 nmol), which reconstitutively overexpressed in insect microsomes.

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Table I

| Liver microsome sample | 25-Hydroxylase activity pmol/mg protein/min | CYP2D6 content pmol/mg protein |
|------------------------|--------------------------------------------|-------------------------------|
| HLM 1                  | 5.0 (4.5–5.2)                               | 8                             |
| HLM 2                  | 3.4 (3.1–3.6)                               | 10                            |
| HLM 3                  | 2.8 (2.5–3.0)                               | 10                            |
| HLM 4                  | 2.4 (1.9–2.6)                               | 1                             |
| HLM 5                  | 0.9 (0.8–1.0)                               | 0                             |
| HLM 6                  | 0.9 (0.7–1.1)                               | 6                             |
| HLM 7                  | 0.9 (0.8–0.9)                               | 4                             |
| HLM 8                  | 0.8 (0.6–1.0)                               | 11                            |
| HLM 9                  | 0.7 (0.6–1.0)                               | 12                            |

*There is a sequencing error in the cDNA sequence for the porcine microsomal vitamin D3 25-hydroxylase as reported in a previous communication (12). The reported CAC codon should be a GAC codon, and thus there is an aspartate in position 304 in the vitamin D3 25-hydroxylase and not histidine as reported previously (12).
activity was detected in liver microsomes lacking or having very low concentrations of CYP2D6, and the microsomal CYP2D6 samples with the highest concentration of CYP2D6 showed the lowest 25-hydroxylase activity (Table I). Experiments with Recombinantly Expressed Human CYP2D6—CYP2D6, recombinantly overexpressed in insect microsomes, was incubated with 1α-hydroxyvitamin D₃ for various periods of time. No 25-hydroxylated product could be detected in these incubation mixtures. In contrast, incubations with microsomal 25-hydroxylase and 1α-hydroxyvitamin D₃ resulted in a time-dependent increase in the formation of 25-hydroxylated product (Fig. 8). It may be concluded from these results that CYP2D6 is not a vitamin D₃ 25-hydroxylase and that microsomal 25-hydroxylation in human liver is catalyzed by a cytochrome P450 enzyme different from CYP2D6.

The results of the present study describing structural and catalytic properties and tissue distribution of the porcine microsomal vitamin D₃ 25-hydroxylase should be of value in studies on the identification and characterization of the human liver microsomal vitamin D₃ 25-hydroxylase.

Acknowledgments—We are grateful to Drs. Hans Postlind and Eva Axén, Pharmacia & Upjohn, Stockholm, for providing tolterodine and for analysis of products formed from incubations with tolterodine.

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