Identification of a Novel Rat Microsomal Vitamin D3 25-Hydroxylase*†

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Tomoaki Yamasaki, Shunsuke Izumi, Hiroshi Ide, and Yoshihiko Ohyama‡
From the Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739-8526, Japan. Tel./Fax: 81-82-424-7458; E-mail: ohyama@sci.hiroshima-u.ac.jp.

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Vitamin D3 requires the 25-hydroxylation in the liver and the subsequent 1α-hydroxylation in the kidney to exert its biological activity. Vitamin D3 25-hydroxylation is hence an essential modification step for vitamin D3 activation. Until now, three cytochrome P450 molecular species (CYP27A1, CYP2C11, and CYP2D25) have been characterized well as vitamin D3 25-hydroxylases. However, their physiological role remains unclear because of their broad substrate specificities and low activities toward vitamin D3 relative to other substrates. In this study, we purified vitamin D3 25-hydroxylase from female rat liver microsomes. The activities of the purified fraction toward vitamin D3 and 1α-hydroxyvitamin D3 were 1.1 and 13 nmol/min/nmol of P450, respectively. The purified fraction showed a few protein bands in a 50–60-kDa range on SDS-PAGE, typical for a cytochrome P450. The tryptic peptide mass fingerprinting of a protein band (56 kDa) with matrix-assisted laser desorption ionization/time of flight mass spectrometry identified this band as CYP2J3. CYP2J3 was heterologously expressed in Escherichia coli. Purified recombinant CYP2J3 showed strong 25-hydroxylation activities toward vitamin D3 and 1α-hydroxyvitamin D3 with turnover numbers of 3.3 and 22, respectively, which were markedly higher than those of P450s previously characterized as 25-hydroxylases. Quantitative PCR analysis showed that CYP2J3 mRNA is expressed at a level similar to that of CYP27A1 without marked sexual dimorphism. These results strongly suggest that CYP2J3 is the principal P450 responsible for vitamin D3 25-hydroxylation in rat liver.

Vitamin D3 25-hydroxylation, which mainly occurs in the liver, is the initial step of vitamin D3 activation (1). The 25-hydroxylated vitamin D3 product circulates in blood as a complex with the vitamin D-binding protein. The complex is filtered once through the glomerulus and reabsorbed by the endocytic receptor megalin into the proximal tubular cell in the kidney, where 25-hydroxyvitamin D3 (25-OH-D3)1 is 1α-hydroxylated to yield 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) (2–5). The most important physiological function described for 1,25-(OH)2D3 is maintenance of calcium homeostasis (a process that involves calcium absorption from the intestine, reabsorption from the kidney, and mobilization from the bone) (2, 3, 5). In addition, involvement in a variety of processes such as cell differentiation and proliferation has been reported (1, 3).

In the 1990s, two important kidney mitochondrial cytochrome P450s involved in vitamin D3 metabolism were identified. One is CYP27B1, which catalyzes the 1α-hydroxylation reaction, essential for vitamin D3 activation. Its expression is regulated by parathyroid hormone, 1,25-(OH)2D3, and calcitomin (6–11). The genetic defect of the gene results in pseudovitamin D deficiency rickets (12). The other is CYP24, which directs the catabolism of 1,25-(OH)2D3 and 25-OH-D3 by the hydroxylation at position C-24. The expression of this gene is primarily controlled by 1,25-(OH)2D3 via a vitamin D receptor (13, 14). These two enzymes are apparently regulated in a reciprocal manner depending on physiological factors concerning calcium homeostasis, by which the biological activity of vitamin D3 is maintained at appropriate levels in the body (1).

In contrast to the 1α-hydroxylase and 24-hydroxylase in kidney, the 25-hydroxylase of vitamin D3 in liver remains not fully understood, although 25-OH-D3 is the major circulation form of vitamin D3 in mammals. It has been established that 25-hydroxylation is catalyzed by cytochrome P450 enzymes present in microsomes and mitochondria of liver (15, 16). Until now, one mitochondrial and three microsomal cytochrome P450 enzymes have been reported as vitamin D3 25-hydroxylases: CYP2C11 (17, 18), CYP27A1 (19–22), CYP2D25 (23, 24), and CYP2R1 (25). They show broad substrate specificities and low catalytic activities toward vitamin D3 relative to other substrates. CYP2C11 is a rat male-specific P450 (26) and catalyzes the 2α- and 16α-hydroxylation of testosterone 40-fold more efficiently than vitamin D3 25-hydroxylation (18). CYP27A1 is a mitochondrial P450 catalyzing not only vitamin D3 25-hydroxylation but also sterol 27-hydroxylation. The latter reaction is 100-fold more efficient than vitamin D3 25-hydroxylation and is essential for the catabolism of cholesterol to bile acid (27). Mutation of this gene causes cerebrotendinous xanthomatosis, leading to accumulation of cholesterol and cholesterol in most tissues (28). The disruption of the Cyp27a1 gene in mice severely compromises cholesterol metabolism but not vitamin D3 metabolism (29). CYP2D25 was purified from pig liver and kidney microsomes, and its cDNA was isolated (23, 24). A series of reports by Wikvall and co-workers have shown that (i) this enzyme hydroxylates not only at the C-25 of vitamin D3 but...
also at the C-1α and C-26 of 25-OH-D₃ (30); (ii) it is expressed in kidney as well as in liver (31); and (iii) a possible human ortholog, CYP2D6, exhibits no 25-hydroxylation activity (32). Very recently, Cheng et al. (25) have reported a microsomal P450 (CYP2R1) in mice and humans as a 25-hydroxylase. They successfully isolated this P450 by expression cloning from a CYP22A1-deficient mouse cDNA library, and the 25-hydroxylation activity of the recombinant enzyme was demonstrated. However, a detailed analysis of the enzyme activity including substrate specificity and kinetic parameters has not yet been reported.

The aim of the present study is to investigate whether other P450 enzymes with a 25-hydroxylation activity higher than those identified exist in mammals. Previous studies showed that CYP2C11 is a male-specific P450 expressed in rat liver and scarcely expressed in female rat liver (26). However, female rat liver microsomes exhibit substantial vitamin D₃ 25-hydroxylation activity (17, 33), suggesting the existence of a yet unidentified vitamin D₃ 25-hydroxylase. In the present study, we have identified the P450 enzyme responsible for this 25-hydroxylation activity from female rat liver microsomes. Here we report CYP2J3 as a principal microsomal vitamin D₃ 25-hydroxylase in rat.

**EXPERIMENTAL PROCEDURES**

**Materials**—Vitamin D₃ and testosterone were obtained from Katayama Chemical (Osaka, Japan), and 25-OH-D₃, 1,25-(OH)₂D₃, 1α-OH-D₃, cholic acid, and isopropyl-1-thio-β-galactoside from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CHAPS and MEGA-10 (Amersham Biosciences) column (1.6 x 2.5 cm) (the DEAE-Sepharose I step). The column was washed with buffer D and then eluted stepwise with the same buffer containing 100, 200, and 300 mM NaCl. The fractions (100 mM NaCl) containing relatively high activity were used for purification of Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% cholate, and 0.1% CHAPS (the DEAE-Sepharose II step). The column was washed with buffer D and then eluted stepwise with the same buffer containing 40, 80, and 300 mM NaCl. The fractions (40 mM NaCl) containing high activity were pooled and dialyzed against buffer F (20 mM potassium phosphate buffer (pH 7.4), 0.1% CHAPS) and dialyzed against buffer F (20 mM potassium phosphate buffer (pH 7.4), 0.5 mM EDTA, and 0.1% CHAPS). The dialyzed fraction (4.2 ml) was applied to a Bio-Gel HTP hydroxypatite Fast Flow (Amersham Biosciences) column (1.6 x 2.5 cm) (the DEAE-Sepharose I step). The column was washed with buffer E and eluted stepwise with the same buffer containing 40, 80, and 300 mM NaCl. The fractions (40 mM NaCl) containing high activity were pooled and dialyzed against buffer F (20 mM potassium phosphate buffer (pH 7.4), 0.5 mM EDTA, and 0.1% CHAPS). The dialysate (2.5 ml) was applied to a Bio-Gel HTP hydroxypatite column (1.0 x 2.0 cm) (the hydroxypatite II step). The column was washed with buffer E and then eluted stepwise with 40, 80, and 300 mM NaCl. The fractions (40 mM NaCl) containing high activity were pooled and dialyzed against buffer F (20 mM potassium phosphate buffer (pH 7.4), 0.5 mM EDTA, and 0.1% CHAPS). The dialysate (4.2 ml) was applied to a Bio-Gel HTP hydroxypatite column (1.6 x 2.5 cm) equilibrated with buffer F, consisting of 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1% cholate, and 0.1% MEGA-10 (the DEAE-Sepharose II step). The column was washed with buffer F and then eluted with 400 mM potassium phosphate buffer (pH 7.5), containing 0.5% cholate. The fractions with relatively high activity were stored at -80°C and used for enzyme characterization.

**Enzyme Assay—25-Hydroxylase activity was assayed with a reconstituted method (35). Typically, the assay mixture (0.49 ml) consisted of 10–300 µg of protein, NADPH-P450 reductase (0.5 units based on cytochrome c reducing activity (36)), 100 mM Tris-HCl (pH 7.7), 1 µM EDTA, and 10 nmol of 1α-OH-D₃ dissolved in 5 µl of ethanol. The assay mixture was preincubated for 2 min at 37°C, and then the reaction was started by adding 10 µl of 50 mM β-NADPH. The incubation was carried out for 10 min at 37°C, and the reaction was terminated by the addition of 100 µl of 1 N NaOH. The reaction products were extracted with 4 ml of ethyl acetate. The organic phase (3 ml) was evaporated to dryness under reduced pressure. The residue was dissolved in chloroform and ethyl acetate (4:1, v/v). An aliquot was subjected to HPLC analysis (PU-980 pump, UV-975 detector, JASCO Co., Ltd., Tokyo, Japan) using a Finepak SIL-5 column (4.6 x 250 mm; JASCO). The sample was eluted with isopropyl alcohol/methanol/hexane (7.9.96, v/v/v) at a flow rate of 1.4 ml/min, and effluents were monitored by absorbance at 265 nm (A₂₆₅ of vitamin D derivatives). The amount of the product was calculated according to the standard curve based on the peak height. In the assay of microsomal activity, the NADPH-P450 reductase was omitted from the assay mixture. The hydroxylation activity toward vitamin D₃ was assayed as follows. 0.98 ml of a typical assay mixture consisting of P450 fraction, 1α-OH-D₃, NADPH-P450 reductase, 100 µM Tris-HCl (pH 7.7), 1.0 µM EDTA, and 100 nmol of vitamin D₃ dissolved in 10 µl of ethanol was preincubated for 2 min at 37°C, and then the reaction was started by adding 20 µl of 50 mM β-NADPH. The incubation was carried out for 10 min at 37°C, and the reaction was terminated by adding 1 ml of ethanol. Subsequent procedures were similar to those for the 1α-OH-D₃ assays. HPLC analysis were performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a substrate by the method similar to that used for 25-hydroxylase activity. HPLC analysis was performed with isopropyl alcohol/methanol/hexane (5:2.93, v/v/v) at a flow rate of 1.2 ml/min. The testosterone hydroxylation activity was measured using 50 nmol of testosterone as a
The gel pieces were destained by washes with either 100 μl of 50% acetonitrile and 25 μM ammonium bicarbonate repeatedly (in the case of Coomassie Brilliant Blue staining) or with 100 μl of 15 μM potassium ferricyanide and 50 mM sodium thiosulfate once and water repeated (for silver staining) (39). After shaking with 100 μl of acetonitrile for 5 min, acetonitrile was removed, and the gel pieces were dried in a vacuum centrifuge for 20 min at 30 °C. The gel pieces were shaken with 100 μl of reducing solution (10 mM dithiothreitol and 25 mM ammonium bicarbonate) for 60 min at 56 °C and then washed with 100 μl of 25 mM ammonium bicarbonate for 10 min. Subsequently, the gel pieces were shaken under dark conditions with 100 μl of alkylating solution (55 mM iodoacetamide and 25 mM ammonium bicarbonate) for 45 min and washed with 100 μl of 25 mM ammonium bicarbonate. The gel pieces were dried again. The gel pieces were immersed in 90 mM 50 mM ammonium bicarbonate and incubated on ice for 30 min. After the gel pieces were dried, the gel pieces were destained by washes with either 100 μl of 15 mM potassium ferricyanide and 50 mM ammonium bicarbonate (Sigma) in 0.1% trifluoroacetic acid, 50% acetonitrile and 5% trifluoroacetic acid. The extracts were combined, and the solvent was evaporated to a volume of ~20 μl. The concentrated samples were subjected to MALDI-TOF mass analysis as described by Barnes et al. (42). The gel pieces were incubated for 18 h at 37 °C. Digested peptides were extracted twice from gel pieces with 50 μl of 50% acetonitrile and 5% trifluoroacetic acid. The extracts were combined, and the solvent was evaporated to a volume of ~20 μl. The concentrated samples were subjected to MALDI-TOF mass analysis as described below.

**Peptide Mass Fingerprinting**—One μl of matrix solution (10 mg/ml α-cyano-4-hydroxy-trans-cinnamic acid (Sigma) in 0.1% trifluoroacetic acid, 50% acetonitrile and 5% trifluoroacetic acid) was added to 1 μl of sample in the Eppendorf tube, and then 1 μl of the sample-matrix solution was spotted onto a stainless steel probe tip and allowed to air-dry for 10 min at room temperature. Samples were measured on a Bruker Biflex II MALDI-TOF mass spectrometer (Bruker) equipped with an ion source with visualization optics and an N2 laser (337 nm). Mass spectra were recorded in the reflector positive mode at a 28.5-kV acceleration voltage (sample was remeasured as described. All MALDI spectra were calibrated externally using a standard peptide mixture (angiotensin II (Mw = 1047.2), adrenocorticotropic hormone fragment 18–39 (Mw = 2466.7), and insulin (Mw = 5734.6)).

**Construction of Expression Plasmids**—Standard methods for construction of plasmids were as described by Sambrook et al. (40). Rat liver cDNA libraries (for male and female, separately) were prepared by using a reverse transcription reaction with total RNA, oligo(DT)12–18 primer and SuperScript II (Invitrogen). CYP2J3 cDNA (41) (GenBank™ accession number U39983) was isolated by PCR from the cDNA library according to the following conditions (annealing temperature 74 °C) containing 0.5 mM each of dNTP, 0.1 mg/ml ampicillin, chloramphenicol (final 20 μg/ml), and 1 mM dithiothreitol. The amplified products were pooled (total 3.0 μl) and dialyzed with 200 mM potassium phosphate buffer (pH 7.4) to remove cell debris. The supernatant was centrifuged at 230,000 x g for 1 h. The resulting supernatant was used as a lysate. The plasmid was purified with equal volume of phenol and then eluted stepwise with the same buffer containing 0.05, 0.15, and 0.3% Emulgen 911. The fractions showing absorption at 417 nm (0.15 and 0.3% Emulgen 911 eluates) were pooled (30 μl) and dialyzed with 20 μl of a dilution solution (20% glycerol, 1 mM dithiothreitol, and 1 mM EDTA). The dialyzed sample was subjected to a Bio-Gel HTP hydroxypatite (Bio-Rad) column (1.0 × 2.0 cm). The column was extensively washed with equilibration buffer (50 mM potassium phosphate (pH 7.4)) to remove Emulgen 911 completely until no absorption at 276 nm was detected. Then the protein was eluted stepwise with 200 and 700 mM potassium phosphate buffer (pH 7.4) and finally with 10 mM potassium phosphate buffer (pH 7.4). The fractions containing 0.5 mg of protein were pooled (total 3.0 μl) and dialyzed against 10 mM potassium phosphate buffer (pH 7.4), 20% glycerol, 0.5 mM EDTA, and 0.5 mM dithiothreitol. The purified sample was stored at 80 °C until use.

**Isolation of Total RNA**—Total RNA was isolated by a lithium chloride precipitation method (44). Rat tissues (~1.2–1.5 g) were minced and homogenized with 30 ml of lithium urea solution (3 M lithium chloride, 6 M urea, 0.2% SDS, 0.2% sodium phosphate buffer) and then disrupted by ultrasonic treatment. The tissue samples were then treated with RNase H. The cDNAs of glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GenBank™ accession number A017801), CYP2J3, CYP2C11 (GenBank™ accession number J02657), rat CYP2TA1 (GenBank™ accession number Y07534), and rat CYP2R1 were quantified by LightCycler (Roche Applied Science) ac-
According to the supplier's instruction. A real time PCR mixture (20 μl) consisted of 2 μl of reverse transcription products (after 10–300 times dilution with TE buffer), 1.6 μl of 25 mM MgCl₂ (in the case of CYP27A1 and GAPDH) or 2.4 μl of 25 mM MgCl₂ (for CYP2J3, CYP2C11, and CYP2R1), 10 pmol of each primer, and 2 μl of LightCycler-FastStart DNA Master SYBR Green I (Roche Applied Science) and was set up in a microcapillary tube. The PCR conditions for GAPDH, CYP2J3, and CYP27A1 were as follows: denaturation at 95 °C for 10 min and 35 cycles at 95 °C for 15 s, 55 °C for 10 s, and 72 °C for 15 s. Conditions for CYP2C11 and CYP2R1 consisted of denaturation at 95 °C for 10 min and 35 cycles at 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 15 s. The expression levels of CYP2J3, CYP2C11, CYP27A1, and CYP2R1 were normalized with respect to that of GAPDH. The sequences of primers for real time PCR were as follows: CYP2J3, 5'-CCT GGA TTT TGC TAA CAT TC-3'; 5'-CTA AGC TCT TTC TTA GT-3'; CYP2C11, 5'-GCC ACA TCA TGG GAA ACA GCA ACC-3' and 5'-AAG ACC ACC AAC AAG GCC ATC TTC ACC-3'; CYP27A1, 5'-ATG TGG GAC ATC TTC ACC-3' and 5'-GGG AAG GAA AGC GAT ACA GAC-3'; CYP2R1, 5'-GAA GGC ATA TCA ACT GTG-3' and 5'-ATC CAT CCT CGT CCA TAT CTG-3'. GAPDH, 5'-TGT TGG TGC GTA GTA TGT GCT G-3' and 5'-AGT GAT GAT ATG ATC GAT G-3'. The primers amplify fragments of 227 bp (CYP2J3), 124 bp (CYP2C11), 164 bp (CYP27A1), 162 bp (CYP2R1), and 277 bp (GAPDH) that encompass two exons of the gene.

Under these conditions, a single amplified product expected from the primer set was detected on an agarose gel electrophoresis, and the calculated product size was confirmed by DNA sequencing. Calculated ratios of mRNA of CYP2J3, CYP2C11, CYP27A1, and CYP2R1 relative to that of GAPDH were presented as relative mRNA level in Fig. 6 after correction with difference of the cycle numbers to reach a certain fluorescence intensity level and the fragment lengths of GAPDH and test mRNA, since the fluorescence intensity depends on the size of the amplified product.

**SDS-Polyacrylamide Gel Electrophoresis—SDS-PAGE** was performed according to the method of Laemmli (45). The polyacrylamide gel was stained with 2D-SILVER STAIN II (Daiichi Pure Chemicals, Tokyo, Japan) except for the case of peptide mass fingerprinting. Coomassie Brilliant Blue and silver staining for fingerprinting were carried out as described (37).

**Other Methods**—ω-Aminohexyl-Sepharose was prepared by cross-linking between Sepharose CL-4B (200 ml; Amersham Biosciences) and hexamethylenediamine (25 g) with cyanogen bromide (25 g) as described (46). NADPH-P450 reductase was purified from rat liver microsomes as reported (36). Cytochrome P450 concentration was determined by a reduced CO difference spectrum using an extinction coefficient of 91 nmol⁻¹ cm⁻¹ (47). Protein concentrations were determined by the method of Lowry et al. (48) using bovine serum albumin as a standard.

### RESULTS

**Purification of Vitamin D₃ 25-Hydroxylase from Female Rat Liver Microsomes**—Cytochrome P450s previously identified as the 25-hydroxylase exhibit 4.5–8.2 times higher 25-hydroxylation activity toward 1α-OH-D₃ than that toward vitamin D₃ (18, 19, 23). In this study, we also found that female rat liver microsomes exhibited a similar preference (data not shown). Therefore, 1α-OH-D₃ 25-hydroxylation activity was measured to monitor the elution of the enzyme throughout the purification step.

Since the hydrophobic column step is generally the first step for the purification of P450 enzymes, we carefully tested several chromatographic conditions to minimize the loss of enzyme activity. These included hydrophobic gels (ω-aminooctyl-ω-aminohexyl-, and pentyl-Sepharose with different ligand densities) and detergents (CHAPS, Emulgen 911, Emulgen 913, Lubrol PX, MEGA-10, n-octyl-β-D-thiogalactoside, sucrose monolaurate, Triton X-100, Tween 20). On the basis of these results, the resolubilized PEG fraction (PEG 4–16%) was applied to an ω-aminohexyl-Sepharose column. The activity was eluted with a buffer containing 0.05% Emulgen 911, resulting in an increase of total activity and a 15-fold increase in the turnover number (Table I and Fig. 1A). The active fractions were subjected to the hydroxyapatite I step. The activity was eluted with 60 mM potassium phosphate buffer, whereas most of the heme proteins (probably P450s) were eluted with 100 and 300 mM potassium phosphate buffer (Fig. 1B). The turnover number after the hydroxyapatite I column was 3.8 times higher than that of ω-aminohexyl-Sepharose (Table I). In the following purification steps, DEAE-Sepharose columns (I and II) were successively used with different detergents (Fig. 1, C and D). In the first DEAE-Sepharose column chromatography I with CHAPS, the most activity was eluted with 100 mM NaCl. In the second DEAE-Sepharose column chromatography II, the detergent was changed to MEGA-10. 25-Hydroxylase activity was mainly eluted with 40 mM NaCl with a remarkable increase of the turnover number (Table I). Finally, the active fractions were applied to a hydroxyapatite II column to concentrate the activity and remove the nonionic detergent. The hydroxyapatite II fraction showed a specific activity of 6.4 nmol of product/min/mg of protein and a turnover number of 13 nmol/min/mg of P450, which were 910- and 1100-fold higher than those of microsomes, respectively. It should be noted that the total activity of ω-aminohexyl-Sepharose was 1.4 times higher than that of microsomes, implying masking of the 25-hydroxylase activity at the early stage of purification. A similar phenomenon was also reported in the purification of male rat vitamin D₃ 25-hydroxylase (CYP2C11) (18, 49). Fig. 2 shows SDS-PAGE analysis of the pooled fractions after each purification step. The final preparation, the hydroxyapatite II fraction, showed many protein bands (lane 6). Therefore, the 25-hydroxylase activity could not be attributed to a particular protein band. However, the turnover number of 25-hydroxylase in the final preparation was comparable with those of most P450s (usually 2–20 min⁻¹) involved in the metabolism of endogenous compounds such as CYP19 for androstenedione (50), CYP24 for 25-OH-D₃ (51), and CYP27A1 for 5α-cholestan-3α,7α,12α-triol (27), suggesting that limited numbers of P450s, if any, were present in the final preparation.

**Enzymatic Properties of 25-Hydroxylase from Rat Liver Microsomes**—It has been demonstrated that cytochrome P450 enzymes require the specific electron-transferring proteins (NADPH-P450 reductase for microsomal activity and adrenodoxin and adrenodoxin reductase for mitochondrial activity) to reconstitute the enzyme activities. Consistent with these
facts, the 25-hydroxylation activity purified from female rat liver microsomes was reconstituted only in the presence of NADPH-P450 reductase (data not shown). The hydroxyapatite II fraction (final preparation in this experiment) was highly active and showed the turnover numbers of 1.1 and 13 nmol/min/nmol of P450 toward vitamin D3 and 1α-H9251-OH-D3, respectively. CYP2C11, a 25-hydroxylase purified from male rat liver microsomes, showed strong 2α- and 16α-hydroxylation activities to testosterone that were about 40 times higher than 25-hydroxylation activity to vitamin D3 (18). In contrast, the purified fraction in this study exhibited no 2α- and 16α-hydroxylation activities toward testosterone.

Identification of Female Rat Microsomal 25-Hydroxylase by Peptide Mass Fingerprinting—The final fraction of the 25-hydroxylase activity in column chromatographies exhibited many protein bands in SDS-PAGE (Fig. 2). Of the observed bands, those between 50 and 60 kDa were identified as CYP2J3 and epoxide hydrolase 1 by tryptic peptide mass fingerprinting.
mass analysis for peptide mass fingerprinting. The mass spectrum from one of the major bands (apparent \( M_r = 56,000 \) in Fig. 2) was identified as CYP2J3 by comparison of the observed peptides with the NCBI nr data base through the search engine Mascot (available on the World Wide Web at www.matrixscience.com/index.html) (see Fig. B and Table A in Supplemental Materials). The number of matched peptides was 17 for predicted trypptic peptides of CYP2J3, and they covered 41% of the protein. Three partially cleaved peptides were included in the 17 peptides. The predicted molecular weight of the rat CYP2J3 protein is 57,966, which is comparable with the apparent molecular size of the band in SDS-PAGE.

Construction and Expression of Recombinant CYP2J3 Proteins—We designed two cDNAs to express recombinant CYP2J3 proteins according to previous reports (42, 52, 53) (see Fig. A in Supplemental Materials). In mN2J3, the second codon was changed from CTT (Leu) to GCT (Ala) at the second codon, and the codons for the N-terminal region were enriched from codon for Ala to Leu. dN2J3, the N-terminal hydrophobic region of mN2J3 was truncated, and the codons for the N-terminal region were enriched with AT sequences. dN2J3 was purified using the membrane and cytosol fractions after subfractionation (Fig. 3). The expression of dN2J3 was detected only when \( \delta \)-ALA, a precursor for heme synthesis, was added to the culture medium (see Table B in Supplemental Materials). In contrast, the expression of mN2J3 was not observed even in the presence of \( \delta \)-ALA. To increase the expression level of dN2J3, chaperone proteins were coexpressed, since GroEL has been reported to increase P450 expression (54). In the experiment, we used an expression plasmid pGro7 that codes GroES-GroEL to increase P450 expression (55, 56). Coexpression of GroES-GroEL (pK-dN2J3/pGro7) markedly increased the dN2J3 level (12-fold) (see Table B in Supplemental Materials). The accumulation of the recombinant dN2J3 protein in cells was maximized at 44 h.

**Purification of Recombinant Enzymes**—E. coli cells expressing dN2J3 were converted to spheroplasts, disrupted by sonication, and then fractionated by centrifugation. The dN2J3 was detected in both the membrane and cytosolic fractions with almost equal amounts as judged from the CO differential spectra (Fig. 3). These results indicate that the N-terminal region of microsomal P450 is a critical region for membrane anchoring, as reported previously (57). dN2J3 was purified using the membrane fraction, since the specific content (P450/protein) was higher than that of the supernatant (Fig. 3). The specific content of the purified sample was 2.5 nmol of P450/mg of protein.
The SDS-PAGE analysis of the sample showed a major band with a expected size, together with a few minor bands (Fig. 4). The final recovery of P450 was about 20%. The relatively low specific content and recovery would be attributable to the extensive washing with a high salt buffer in the hydroxapatite column step to remove Emulgen 911, which might have resulted in an inactive heme-free P450. MALDI-TOF mass spectrometry confirmed that the major band was dN2J3 protein (data not shown). The purified dN2J3 was used for the further analysis of enzyme activity.

**Analysis of Enzyme Activity**—Initially, enzyme activity was measured in the reconstituted system using the solubilized membrane and cytosolic fractions prepared from *E. coli* expressing dN2J3. Both fractions clearly showed vitamin D$_3$ and 1α-OH-D$_3$ 25-hydroxylation activities. However, these activities were significantly lower than those of the purified fraction from female rat liver microsomes (cytosolic fraction, 0.02 nmol/min/nmol of P450 toward vitamin D$_3$ and 3.3 nmol/min/nmol of from female rat liver microsomes (cytosolic fraction, 0.02 nmol/min/nmol of P450 toward vitamin D$_3$ (at a substrate concentration of 20 μM) and 18 nmol/min/nmol of P450 toward 1α-OH-D$_3$; membrane fraction, 0.19 nmol/min/nmol of P450 toward vitamin D$_3$ and 4.7 nmol/min/nmol of P450 toward 1α-OH-D$_3$). Therefore, we purified dN2J3 for better characterization. The hydroxylation activities of the purified dN2J3 toward vitamin D$_3$ and 1α-OH-D$_3$ were quantified based on the HPLC analyses (Fig. 5). A major peak and a few minor peaks were observed in a range of the elution time (6–10 min) for monohydroxylated metabolites in each case. The major products were identified by comparison of the retention time with authentic 25-OH-D$_3$ and 1,25-(OH)$_2$D$_3$ on normal and reversed phase column chromatographies. The minor products were not subjected to further analysis. The activity of purified dN2J3 was 2.7 nmol/min/nmol of P450 toward vitamin D$_3$ (at a substrate concentration of 20 μM) and 3.3 nmol/min/nmol of P450 toward 1α-OH-D$_3$ in both sexes (Fig. 6). The estimated Km values determined by the Michaelis-Menten equation were as follows. For vitamin D$_3$, $V_{\text{max}}$ was 3.3 nmol/min/nmol of P450, $K_m$ was 0.79 μM; for 1α-OH-D$_3$, $V_{\text{max}}$ was 22 nmol/min/nmol of P450, $K_m$ was 0.60 μM. Testosterone 2α- and 16α-hydroxylation activities, characteristics of CYP2C11, were not detected. CYP27A1 and CYP2D25 hydroxylated not only vitamin D$_3$ at C-25 but also 25-OH-D$_3$ at positions of C-26, C-24, and C-1α (30, 58). However, CYP2J3 did not show these hydroxylation activities toward 25-OH-D$_3$ (data not shown), demonstrating that CYP2J3 is a vitamin D$_3$ 25-hydroxylase. In comparison with other reported 25-hydroxylases, dN2J3 exhibited remarkably high hydroxylation activities and specificity (Table II).

**Tissue Distribution and Sexual Dimorphism of CYP2J3 and CYP2C11**—To evaluate the significance of CYP2J3 as the 25-hydroxylase in rat liver microsomes, the expression level of CYP2J3 was measured and compared with that of CYP2C11. CYP2J3 has been reported to be involved in the oxidation of arachidonic acid in rat heart (41). In the report, the measurement of tissue distribution of CYP2J3 by Western and Northern blotting analyses provided somewhat controversial results; strong expression in heart, kidney, and liver was detected by the Western blotting, whereas predominant expression in liver was detected by the Northern blotting. A possible reason for this discrepancy may be due to the cross-reaction of the antibodies in Western analysis or the cross-hybridization of the probe in Northern analysis with other proteins or mRNA such as those of homologous P450 enzymes. Therefore, we used a real time PCR method, which is more specific and then eliminated false signals from those other than CYP2J3.

In the experiment, liver, heart, and kidney total RNA were prepared separately from two adult rats of each sex. The mRNA levels of CYP2J3 in male rat livers were 0.32 and 0.41 relative to GAPDH, and those of female rat livers were 0.16 and 0.21 (Fig. 6B). In contrast, the expression levels in kidney and heart were quite low in both sexes (less than 0.005). The very low expression of CYP2J3 in the heart and kidney relative to the liver agrees with the reported data of Northern analysis (41). On the other hand, the expression of CYP2C11 was detected only in male liver but not in kidney, heart, and female liver (Fig. 6A). These results have further confirmed that CYP2C11 is a male-specific P450 (26). Unlike CYP2C11, analysis of CYP2J3 expression levels in liver showed no marked sexual dimorphism, although the levels appeared slightly higher in male. The mRNA levels of CYP2J3 in heart and kidney were independent of sex, although they were extremely low.

**Comparison of mRNA Levels of Rat Liver 25-Hydroxylases**—To estimate physiological relevance of the P450s (CYP2J3, CYP27A1, and CYP2R1) exhibiting vitamin D$_3$ 25-hydroxylation activity, the mRNA levels of those P450s in two male and two female rat livers were analyzed by a real time PCR method. mRNA levels of CYP27A1 and CYP2R1 were similar between both sexes (Fig. 6, C and D). The estimated relative expression levels of mRNA were roughly 1:1:0.3 for CYP2J3 (female), CYP27A1, and CYP2R1 (Fig. 6, B–D). The result suggests that the level of the CYP2J3 protein is comparable with that of CYP27A1 but is higher than that of CYP2R1 in rat liver.

**DISCUSSION**

25-Hydroxylation is the initial step in the activation of vitamin D$_3$. Although three P450s, CYP27A1 (mitochondrial), CYP2C11 (microsomal), and CYP2D25 (microsomal), have been reported as 25-hydroxylases, their catalytic activities for vitamin D$_3$ were relatively low as compared with those of P450s...
involved in metabolism of endogenous substrates such as cholesterol and steroid hormones. To explore the existence of other P450s exhibiting 25-hydroxylation activity, we have carried out the purification from female liver microsomes that exhibit significant 25-hydroxylation activity.

The purified fraction from female rat liver (hydroxyapatite II) showed a turnover number of 1.1 and 13 for vitamin D$_3$ and 1α-OH-D$_3$, respectively. These values were at least 3.5-fold higher than those of other known 25-hydroxylases (CYP27A1, CYP2C11, and CYP2D25) (Table II). The fraction showed no 2α- and 1α-hydroxylation activities toward testosterone, which are characteristics of CYP2C11 (18). Therefore, our purification data indicate that the rat has at least three P450s possessing 25-hydroxylation activity (i.e., CYP2C11 (male-specific, microsomes), CYP27A1 (both sexes, mitochondria), and a novel P450 (at least female, microsomes). Furthermore, this novel P450 has a higher 25-hydroxylation activity toward vitamin D$_3$ and 1α-OH-D$_3$ than other previously reported P450s.

To identify the novel P450, we used a peptide mass fingerprinting method. The final preparation showed many protein bands on SDS-PAGE. However, only a few bands were observed in the molecular mass region of 50–60 kDa, where most P450s are usually found (Fig. 2). Therefore, two major bands (56 and 50 kDa) in the region were excised from the gel and subjected to MALDI-TOF mass spectrometry after trypsin digestion. The peptide fragments of the band (apparent molecular mass 56 kDa) (Fig. 2) matched with the predicted peptide profile of CYP2J3 (GenBank accession number U39943). The analysis of the other band (molecular mass 50 kDa) identified epoxide hydrolase 1 (GenBank accession number M26125). This result strongly suggests that the novel P450 with the vitamin D$_3$ 25-hydroxylase is CYP2J3.

To confirm the CYP2J3 activity, we expressed recombinant CYP2J3 in E. coli. The purified recombinant dN2J3 has shown strong 25-hydroxylation activity toward vitamin D$_3$ and 1α-OH-D$_3$ with the turnover number of 2.7 and 18, respectively (Table II). The values were somewhat higher than those of the purified sample from rat liver. These data have confirmed that vitamin D$_3$ 25-hydroxylation activity in the female rat liver microsomes is indeed CYP2J3. In the analysis of CYP2J3 activity, an N-terminal deleted protein of CYP2J3 was expressed and used for the assessment of activity. It should be noted that the truncations of the N-terminal region of P450s do not severely affect the catalytic activities and substrate specificities of the P450 (59, 60). Therefore, the reason why dN2J3 showed higher catalytic activity than the purified fraction of CYP2J3 will be attributed to inhibition by the contaminating proteins in the purified fraction of CYP2J3 (Fig. 2, lane 6).

It has been previously reported that CYP2J3 is involved in the oxidation of arachidonic acid in the rat heart (41). However, many P450s other than CYP2J3, such as other CYP2 family and CYP4A subfamily members, are involved in arachidonic acid metabolism in liver (61, 62), suggesting that oxidation of arachidonic acid is not solely dependent on CYP2J3 activity. Furthermore, the reported activity of CYP2J3 for arachidonic acid is about 10 times lower than that for vitamin D$_3$ determined in this study (41). Therefore, the major physiological function of CYP2J3 in the liver is very likely vitamin D$_3$ hydroxylation rather than arachidonic acid oxidation.

The present study has revealed that in addition to CYP2C11, CYP2J3 catalyzes the 25-hydroxylation of vitamin D$_3$ in rat microsomes. These two P450s are probably major contributors for 25-hydroxylation, since the purification of the activity from male rats based on the activity led to CYP2C11, a male-specific major microsomal P450, and the purification from female rats reached CYP2J3. To estimate the contribution of these two P450s to vitamin D$_3$ activation, we measured their mRNA level by a real-time PCR method. CYP2C11 mRNA was detected predominantly in male liver, whereas that of CYP2J3 was detected in both sexes at comparable levels (Fig. 6, A and B). Since the real-time PCR method used in this study detects the fluorescense of SYBR Green binding to DNA, the strength of the fluorescence depends on the size of the amplified product. In this case, the amplification products for CYP2J3 and CYP2C11 are 227 and 124 bp, respectively. Therefore, after consideration for the PCR fragment sizes, CYP2C11 is expressed roughly 20-fold higher than CYP2J3 in male (Fig. 6, A and B). 25-Hydroxylation activity for male liver microsomes is reported to be 2–3-fold higher than that of female microsomes (63). If the mRNA level determines the protein level (i.e., activity), the ratio of the activities predicted for male and female liver microsomes agrees well with that observed. Namely, the CYP2J3 expression level is slightly higher in the male than in the female (Fig. 6B). CYP2C11 expressed only in male rat shows a 20-fold higher mRNA level but a 15-fold lower activity than CYP2J3. Therefore, the ratio of the predicted activities of male (CYP2J3 and CYP2C11) and female (CYP2J3) becomes a similar value (2–3 versus 1) to that reported (63). In light of these considerations, it appears reasonable that 25-hydroxylation activity in rat liver microsomes is attributable to CYP2C11 and CYP2J3.

Very recently, Cheng et al. (25) have reported CYP2R1 as a microsomal vitamin D$_3$ 25-hydroxylase of mouse (GenBank accession number AY332318) and human (GenBank accession number AY323817). They expertly demonstrated that CYP2R1 is a 25-hydroxylase by using an expression cloning method. The CYP2R1 gene is conserved throughout vertebrates (64). Therefore, CYP2R1 may function as a conserved 25-hydroxylase in vertebrates. However, the enzyme activity of CYP2R1 has not been fully analyzed, although the reporter gene assay used in the experiment suggests that 25-hydroxylation activity of CYP2R1 is comparable with that of CYP27A1 (25). A search for rat CYP2R1 in the DNA data base has shown a cDNA (GenBank accession number CB777152) highly homologous to that of mouse. Therefore, rats express three microsomal P450s (CYP2C11, CYP2J3, and CYP2R1) in a mitochondrial P450 (CYP27A1) with vitamin D$_3$ 25-hydroxylase activity. To estimate the physiological significance of CYP2J3, CYP2C11, and CYP2R1, we compared their mRNA levels by a real-time PCR method. The relative expression levels of CYP2J3 (female), CYP27A1, and CYP2R1 were roughly 1:1:0.3 (Fig. 6, B–D). Considering their putative expression levels and 25-hydroxylation activities, it is very likely that CYP2J3 is the principal P450 contributing vitamin D$_3$ 25-hydroxylation in rat liver. Wikvall’s group has characterized CYP2D25 as a 25-hydroxylase of pig microsomes (23, 24, 30, 65). Taken together, it seems that different species use different P450 molecular species in the activation of vitamin D$_3$. For a more comprehensive picture, it is essential to characterize CYP2R1 enzyme activity and to evaluate other CYP2J3 subfamily members in different species with respect to their substrate specificity.

In conclusion, the present study has revealed a novel vitamin D$_3$ 25-hydroxylase activity in rat liver and the importance of CYP2J3 as a vitamin D$_3$ 25-hydroxylase.

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Tomoaki Yamasaki, Shunsuke Izumi, Hiroshi Ide and Yoshihiko Ohyama

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