Isolation and Characterization of a Cytochrome P-450 from Rat Kidney Mitochondria That Catalyzes the 24-Hydroxylation of 25-Hydroxyvitamin D$_3$*

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A cytochrome P-450 that catalyzes the 24-hydroxylation of 25-hydroxyvitamin D$_3$ (P-450oc24: P-450cholecalciferol24) was purified to electrophoretic homogeneity from the kidney mitochondria of female rats treated with vitamin D$_3$ (Ohyama, Y., Hayashi, S., and Okuda, K. (1989) FEBS Lett. 255, 405-408). The molecular weight was 53,000, and its absorption spectrum showed peaks characteristic of cytochrome P-450. The turnover number was 22 min$^{-1}$ and the specific content was 2.8 nmol/mg protein. The N-terminal amino acid sequence, Arg-Ala-Pro-Lys-Glu-Val-Pro-Leu-, is different from the N-terminal sequence of any other cytochrome P-450s so far reported. Upon reconstitution with the electron-transferring system of the adrenal mitochondria, the enzyme showed a high activity in hydroxylating 25-hydroxyvitamin D$_3$ as well as 1a,25-dihydroxyvitamin D$_3$ at position 24. However, the purified enzyme hydroxylated neither vitamin D$_3$ nor 1a-hydroxyvitamin D$_3$. The enzyme was also inactive toward xenobiotics. The enzyme hydroxylated 25-hydroxyvitamin D$_3$ at position 24 but not at 1a, indicating that the enzyme is distinct from that catalyzing 1a-hydroxylation. The reaction followed Michaelis-Menten kinetics, and the $K_m$ value for 25-hydroxyvitamin D$_3$ was 2.8 $\mu$m. Both vitamin D$_3$ and 1a-hydroxyvitamin D$_3$ inhibited the 24-hydroxylation of 25-hydroxyvitamin D$_3$ in a competitive, concentration-dependent manner. 25-Hydroxyvitamin D$_3$ 24-hydroxylase activity was significantly inhibited by 7,8-benzoflavone, ketocazole, and CO$_2$, whereas it was only slightly inhibited by aminoglutethimide, metyrapone, and SKF-525A. Mouse antibodies raised against the cytochrome P-450 inhibited the reaction about 70% and reacted with the P-450oc24 in immunoblotting but did not react with other kinds of cytochrome P-450 in rat liver microsomes and mitochondria.

In 1972 Omdahl et al. (1972) found that a metabolite of 25-hydroxyvitamin D$_3$ other than 1a,25-dihydroxyvitamin D$_3$, named peak Va, could be synthesized in the kidney, depending on the concentration of calcium in the diet, i.e. mitochondria isolated from high calcium-fed animals produced peak Va, whereas those isolated from low calcium-fed animals metabolized 25-hydroxyvitamin D$_3$ to 1a,25-dihydroxyvitamin D$_3$. Subsequently, the structure of peak Va was identified as 24,25-dihydroxyvitamin D$_3$ by Holick et al. (1972), Lam et al. (1973), and Tanaka et al. (1975). Holick et al. (1972) speculated that when the animal is hypocalcemic, the need for calcium is interpreted in some way by the kidney, resulting in the "turning on" of the 25-hydroxyvitamin D$_3$ 1a-hydroxylase in order to increase the flux of calcium concentration, while when the animal is normocalcemic or hypercalcemic, there is little need for a supply of calcium and therefore the kidney "shuts off" the 25-hydroxyvitamin D$_3$ 1a-hydroxylase system and produces instead another metabolite, 24,25-dihydroxyvitamin D$_3$. Accordingly, clarification of how this switchover of the two enzyme activities occurs may lead to elucidation of the mechanism of calcium homeostasis in the animal body, and for that matter purification and characterization of the individual enzymes are of pivotal importance. However, owing to their extremely low contents and lability, it was difficult to purify these enzymes.

As to the properties of 25-hydroxyvitamin D$_3$ 24-hydroxylase, several reports have been published to date. Knutson and DeLuca (1974) observed that almost all of the recovered 24-hydroxylase activity was located in the crude nuclear debris and heavy mitochondrial fraction of chickens raised on a high calcium, vitamin D$_3$-supplemented diet. They also showed that the reaction required NADPH and oxygen, suggesting that the enzyme is a mixed function oxidase. When NADPH was supplied through the oxidation of succinate and malate, the electron transport inhibitors, cyanide, antimycin, and carbon monoxide, inhibited the reaction. However, when NADPH was supplied directly, these inhibitors had no effect on the 24-hydroxylase activity. More direct evidence that 25-hydroxyvitamin D$_3$ 24-hydroxylase is a mixed function oxidase was obtained by the work of Madhok et al. (1977), who observed that the oxygen enzymatically inserted as a hydroxyl group by chick kidney homogenates into the 24-position of 25-hydroxyvitamin D$_3$ to give 24,25-dihydroxyvitamin D$_3$ is derived exclusively from $^{18}$O$_{2}$. Kulkowski et al. (1979) observed that the enzyme is inhibited by carbon monoxide and metyrapone. Pedersen et al. (1983) also observed that 24-hydroxylase in rat kidney is inhibited by metyrapone. Subsequently, solubilization and reconstitution of the chick renal enzyme were achieved by Burgos-Trinidad et al. (1986). Although these results are highly suggestive that the enzyme is a member of the cytochrome P-450 family, no conclusive evidence has been published so far either by taking photochemical action spectra or by purifying the enzyme to a homogenous state.

Recently, the cytochrome P-450 involved in this reaction was highly purified in this laboratory based on the catalytic activity, and the enzyme activity was reconstituted from the

*This study was supported in part by Grants-in-Aid for Scientific Research 61771449 (to Y. O.) and 63835004 (to K. O.). 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.
cytochrome P-450, adrenodoxin, and NADPH-adrenodoxin reductase (Ohyama et al., 1989). In this paper the details of purification and some properties of this cytochrome P-450 (tentatively named cytochrome P-450cc24) are described.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Induction of 25-Hydroxyvitamin D₃ 24-Hydroxylase—The activity of 25-hydroxyvitamin D₃ 24-hydroxylation could be increased by intraperitoneal injection of vitamin D₃ into rats. In this experiment, daily injection of 50,000 IU of vitamin D₃ elicited about a 10-fold increase of enzyme activity.

Purification of 25-Hydroxyvitamin D₃ 24-Hydroxylase—25-Hydroxyvitamin D₃ 24-hydroxylase was solubilized from kidney mitochondria of female rats with cholate and Lubrol (Ohyama et al., 1989). The solubilized fraction was then subjected to a pentyl-Sepharose column. Of the several derivatives of Sepharose tested, including o-hexyl-, propyl-, and pentyl-Sepharose, the last one was found to be the most suitable for the present purpose. In our experience, preliminary separation of the enzyme at this stage seems to be important for further purification. Furthermore, owing to the very low content of cytochrome P-450 and relative abundance of other kinds of hemoprotein in kidney mitochondria, it was important to pursue the enzyme protein by measuring the catalytic activity throughout the preparation.

A sodium dodecyl sulfate-polyacrylamide gel electrophoretogram of the eluate from high performance liquid chromatography with the activity is shown in Fig. 6 (lane 7). The active fraction revealed a single major band. Molecular weight of the enzyme calculated from the electrophoretogram was 55,000. The specific activity was 54.6 nmol/min/mg protein corresponding to 316-fold purification from the cholate-solubilized fraction, and specific content was 2.8 nmol of cytochrome P-450/mg of protein. The major band on the electrophoretogram is the cytochrome P-450 responsible for 25-hydroxyvitamin D₃ 24-hydroxylase was confirmed by Western blotting analysis with antibody raised against the purified enzyme, which inhibited the enzyme activity.

Absorption Spectra of 25-Hydroxyvitamin D₃ 24-Hydroxylase—As shown in Fig. 1, the oxidized form exhibited an intense Soret absorption peak at 417 nm, typical of a low spin state of P-450, and α and β bands at 570 and 535 nm, respectively. On addition of CO to the reduced form, a Soret peak characteristic of heme proteins of the cytochrome P-450 family was observed at 453 nm (inset).

N-terminal Amino Acid Sequence—The N terminus of 25-hydroxyvitamin D₃ 24-hydroxylase was sequenced by Dr. Yoshiyasu Yabusaki (Biotechnology Laboratory, Takarazuka Research Center, Sumitomo Chemical Co., Ltd.) as follows: Arg-Ala-Pro-Lys-Glu-Val-Pro-Leu-.

The sequence is different from that of any other cytochrome P-450 so far reported, suggesting that 25-hydroxyvitamin D₃ constitutes a unique family of cytochrome P-450.

Identification of the Reaction Product—Identification of the product obtained by incubating 25-hydroxyvitamin D₃ with the purified P-450cc24 together with adrenodoxin and NADPH-adrenodoxin reductase was performed by HPLC using two regular and one reversed phase system. A peak was observed at a retention time apparently corresponding to 24,25-dihydroxyvitamin D₃, whereas no peak was observed at the retention time corresponding to 1α,25-dihydroxyvitamin D₃. When the product eluted from HPLC was treated with periodate, the product could no longer be detected. From these results the product was identified as 24,25-dihydroxyvitamin D₃ (Ohyama et al., 1989). Although the absolute configuration at position 24 is not known at this time, it is presumed to be 24R since it was established that the natural form of 24,25-dihydroxyvitamin D₃ has the 24R-configuration (Tanaka et al., 1975).

Kinetic Properties of 25-Hydroxyvitamin D₃ 24-Hydroxylase—The enzyme reaction proceeded in a time-linear fashion up to 10 min under the assay conditions described. The pH activity curve showed a broad peak at pH 7.7. When the substrate concentration was varied, the reaction followed Michaelis type kinetics, as shown in Fig. 2. The Michaelis constant calculated from the Lineweaver-Burk plot was 2.8 μM. The turnover number calculated from the Vₘₐₓ value was 22 min⁻¹. Interestingly, dilauroylglyceryl-3-phosphorylcholine, which is required for reconstitution of liver microsomal cytochromes P-450, enhanced the turnover number about 50%.
(Table I). This effect was only observed in the purified preparation.

Substrate Specificity—Substrate specificity is shown in Table I. As shown in the table the enzyme showed the highest activity toward 25-hydroxyvitamin D₃. However, when the enzyme was incubated with vitamin D₃ and 1α-hydroxyvitamin D₃, no peak was observed in the region corresponding to monohydroxylamin D₃ and dihydroxylamin D₃, respectively, being consistent with the postulation of Tanaka et al. (1977) that a 25-hydroxyl group is required for kidney mitochondrial 24-hydroxylation of vitamin D₃ derivatives. In fact, the enzyme hydroxylates 1α,25-dihydroxylamin D₃ to give 1α,24,25-trihydroxylamin D₃. Further kinetic studies with this substrate were, however, not attempted at this point. The enzyme did not show any activity toward xenobiotics such as benzphetamine, 7-ethoxycoumarin, and benzo[a]pyrene, suggesting the unique nature of P₄₅₀occ₂₄.

Inhibition of Enzyme Activity—Table II shows the effect of various P₄₅₀ inhibitors on 25-hydroxyvitamin D₃ 24-hydroxylase. As shown in the table neither metyrapone, which is known to inhibit beef adrenal mitochondrial cytochrome P-45₀₁₁₂ (Harding et al., 1989), nor aminoglutethimide, which is known to inhibit beef adrenal mitochondrial cytochrome P-45₀₁₁₂ (Dexter et al., 1967), inhibited the reaction appreciably. SKF-525A also showed very little inhibitory action. By contrast, 7,8-benzofluavone, which is known to inhibit arylhydrocarbon hydroxylase (Wiebel et al., 1971), and ketoconazole, a synthetic fungicide known to inhibit aromatase (Mason et al., 1985), inhibited the enzyme activity significantly. Some vitamin D₃ analogs also inhibited the enzyme activity, of which 1α-hydroxyvitamin D₃ showed the highest inhibition. The type of inhibition was competitive (Fig. 3), Kᵢ value being 0.27 μM (inset). Vitamin D₃ inhibited less strongly than 1α-hydroxyvitamin D₃, and vitamin D₂ much less strongly. When the 3β-hydroxy group of vitamin D₃ was tosylated, the derivative revealed no inhibition, suggesting that the 3β-hydroxy group is important for binding of substrates or inhibitors to the cytochrome.

The activity of 25-hydroxyvitamin D₃ 24-hydroxylase was also inhibited by carbon monoxide (Fig. 4). The Warburg partition constant, Kᵢ was determined since the sensitivity of the 25-hydroxyvitamin D₃ to CO is one of a critical point to differentiate this enzyme from 25-hydroxyvitamin D₃ 1α-hydroxylase as described under "Discussion" and was found to be 0.46, a value similar to that of other mixed function oxidases (Trulzisch et al., 1973).

Effect of Antibodies on the Reconstituted 25-Hydroxyvitamin D₃ 24-Hydroxylase—Mice were immunized by injecting the purified P₄₅₀cc₂₄, and their serum was collected at the time when their spleens were removed for preparation of monoclonal antibodies. Fig. 5 shows that the antiserum inhibited the 24-hydroxylation by about 70% at a concentration of 400 μg of serum protein/0.5 ml of reaction mixture. However, three different monoclonal antibodies inhibited the reaction.

- *Incubation conditions were as described in the legend to Table I.
- *Reaction was conducted under an atmosphere of N₂/O₂/C0₂, 40:20:40.
The reciprocal of the Warburg partition coefficient.

In the figure, *n* is the ratio of the rate when inhibited by CO to the rate without CO. The gradient of the plot is the reciprocal of the Warburg partition coefficient.

The content of 25-hydroxyvitamin D₃ 24-hydroxylase in normal rat kidney mitochondria is extremely low. To purify the enzyme, therefore, it was important to enrich the enzyme concentration of the starting material. For this purpose the enzyme was induced by injecting vitamin D₃ into animals before they were killed. By this procedure the activity was more, owing to its extremely low concentration in the tissue, cytochrome P-450cc₂₄ (2.4 pmol), as described under “Experimental Procedures” using partially purified sample was used (activity of 1.6 nmol/min/mg protein).

only slightly, although they reacted with P-450cc₂₄ on immunoblotting analysis (data not shown).

Immunoblotting Analysis—Fig. 6 shows SDS-polyacrylamide gel electrophoretogram and immunoblotting of various P-450 preparations. Anti-P-450cc₂₄ was used as a probe. Panel A, silver staining; panel B, immunoblotting with anti-P-450cc₂₄. 1, P-450cc₂₄ (crude preparation, 0.3 µg); 2, P-450cc₂₄ (Hayashi et al., 1986); 3, P-450b; 4, P-450c; 5, P-450ch₉₆ (Ogishima et al., 1987); 6, P-450₉₅₉₉ (Masumoto et al., 1988); 7, the purified P-450cc₂₄ (0.05 µg); 8, molecular weight standards (phosphorylase *B*, *M* 94,000; bovine serum albumin, *M* 67,000; ovalbumin, *M* 43,000; carbamic anhydrase, *M* 30,000); migration was from top to bottom.

**Fig. 6.** SDS-polyacrylamide gel electrophoretogram and immunoblotting of various P-450 preparations. Anti-P-450cc₂₄ was used as a probe. Panel A, silver staining; panel B, immunoblotting with anti-P-450cc₂₄. 1, P-450cc₂₄ (crude preparation, 0.3 µg); 2, P-450cc₂₄ (Hayashi et al., 1986); 3, P-450b; 4, P-450c; 5, P-450ch₉₆ (Ogishima et al., 1987); 6, P-450₉₅₉₉ (Masumoto et al., 1988); 7, the purified P-450cc₂₄ (0.05 µg); 8, molecular weight standards (phosphorylase *B*, *M* 94,000; bovine serum albumin, *M* 67,000; ovalbumin, *M* 43,000; carbamic anhydrase, *M* 30,000); migration was from top to bottom.

**Fig. 5.** Effect of anti-P-450cc₂₄ on the 25-hydroxyvitamin D₃ 24-hydroxylase activity. The reaction was conducted as described under “Experimental Procedures” using 17.5 pmol. In the figure, *n* is the ratio of the rate when inhibited by CO to the rate without CO. The gradient of the plot is the reciprocal of the Warburg partition coefficient.

The specific activity of the purified 25-hydroxyvitamin D₃ 24-hydroxylase was several thousand-fold higher than those of solubilized preparations so far reported (Burgos-Trinidad et al., 1986; Gray and Ghazarian, 1989). However, one of the problems of the present method is its relatively low yield. The loss of activity occurs at the final stage of high performance liquid chromatography on DEA-5PW. However, to obtain a homogeneous preparation it was the most important step and could not be omitted. The loss of enzyme activity seemed to be due to removal of heme during this chromatography. The yield of the enzyme may be raised by further improvement of this stage. Cytochrome P-450cc₂₄ may constitute a novel family of cytochrome P-450, since the N-terminal amino acid sequence, as so far determined, is different from that of any such cytochrome P-450s so far reported. 24-Hydroxylase activity was inhibited by vitamin D₃ and 1α-hydroxyvitamin D₃ and at least the latter was a competitive inhibitor from kinetic studies. These results indicate that these compounds could occupy the active site of the enzyme, while they are not hydroxylated at all. The requirement for the 25-hydroxyl group as postulated by Tanaka et al. (1975) was thus confirmed from kinetic studies as well.

**Fig. 4.** The effect of carbon monoxide on 25-hydroxyvitamin D₃ 24-hydroxylation activity. Incubations were conducted as described under “Experimental Procedures” using the purified P-450cc₂₄ (2.4 pmol). In the figure, *n* is the ratio of the rate when inhibited by CO to the rate without CO. The gradient of the plot is the reciprocal of the Warburg partition coefficient.

**DISCUSSION**

The content of 25-hydroxyvitamin D₃ 24-hydroxylase in normal rat kidney mitochondria is extremely low. To purify the enzyme, therefore, it was important to enrich the enzyme concentration of the starting material. For this purpose the enzyme was induced by injecting vitamin D₃ into animals before they were killed. By this procedure the activity was increased about 10-fold. Even after this treatment, however, the concentration of the enzyme was not as high as that of most cytochrome P-450s in liver microsomes. It was therefore necessary to select a highly efficient column for hydrophobic chromatography. None of the columns commonly used for hydrophobic chromatography, *e.g.* ω-aminohexyl-Sepharose or ω-aminooctyl-Sepharose, was effective for this purpose. We therefore prepared and tested various kinds of hydrophobic columns. As a result it was found that pentyl-Sepharose was the most effective one for the purpose of this study. Furthermore, owing to its extremely low concentration in the tissue, the P-450 was too precious to use by measuring its concentration by the CO difference spectrum, as is commonly employed in purification of most cytochrome P-450s acting on xenobiotics or carcinogens. Other kinds of hemoproteins existing in kidney mitochondria also interfered with this measurement. The present purification was therefore performed based on enzymatic activity. It was recently shown in this laboratory that such a method is important in purifying endogenous cytochrome P-450s that are minor components (Ogishima et al., 1987).

The specific activity of the purified 25-hydroxyvitamin D₃ 24-hydroxylase was several thousand-fold higher than those of solubilized preparations so far reported (Burgos-Trinidad et al., 1986; Gray and Ghazarian, 1989). However, one of the problems of the present method is its relatively low yield. The loss of activity occurs at the final stage of high performance liquid chromatography on DEA-5PW. However, to obtain a homogeneous preparation it was the most important step and could not be omitted. The loss of enzyme activity seemed to be due to removal of heme during this chromatography. The yield of the enzyme may be raised by further improvement of this stage. Cytochrome P-450cc₂₄ may constitute a novel family of cytochrome P-450, since the N-terminal amino acid sequence, as so far determined, is different from that of any such cytochrome P-450s so far reported. 24-Hydroxylase activity was inhibited by vitamin D₃ and 1α-hydroxyvitamin D₃ and at least the latter was a competitive inhibitor from kinetic studies. These results indicate that these compounds could occupy the active site of the enzyme, while they are not hydroxylated at all. The requirement for the 25-hydroxyl group as postulated by Tanaka et al. (1975) was thus confirmed from kinetic studies as well.

It is well known that in kidney mitochondria an enzyme exists which catalyzes the 1α-hydroxylation of 25-hydroxyvitamin D₃, an active metabolite of vitamin D₃. A question has been raised whether 25-hydroxyvitamin D₃ 24-hydroxylation and 1α-hydroxylation are catalyzed by a single enzyme. Knutson and DeLuca (1974) have shown several reasons that 24-hydroxylase is distinct from 1α-hydroxylase, one of which is the failure of inhibition of 24-hydroxylation by a carbon monoxide to oxygen ration of 3:1. However, the value of the
vitamin D3 has not so far been isolated. The biological role of
creases the activity of 25-hydroxyvitamin D3 24-hydroxylase.
interrelated as pointed out by Chandler encoding this enzyme.
we have purified and characterized 25-hydroxyvitamin D3 24-
min D3 in curing rickets. It is less active on a weight for
have observed the fact that 1α,25-dihydroxyvitamin D3 in-
24,25-dihydroxyvitamin D3 is, therefore, not yet entirely clear.
catalyze the 1α-hydroxylation highly suggests that 25-hy-
animals at a molecular biological level.
hydroxylation and 1α-hydroxylation more or less (Paulson
in 25-hydroxyvitamin D3. Determination of N-terminal amino acid
from 1,25-dihydroxyvitamin D3 so that the action of surplus
1,24,25-trihydroxyvitamin D3. Determination of N-terminal amino acid sequence end preparation of antibodies will make it possible to isolate a cDNA encoding this enzyme and will pave the way for study of the mechanism of calcium homeostasis in animals at a molecular biological level.

Acknowledgments—We wish to thank Dr. Minor J. Coon of The University of Michigan Medical School for reading our manuscript and correcting the English style.
25-Hydroxyvitamin D$_3$ 24-Hydroxylase

**Experimental Procedures**

Material-Antibodies were purified from bovine antihuman antibodies according to the method described by Balch et al. [1971] and anti-hexahydrophospho-cholesterol antibodies were purified by immunopurification (Ikeba et al. 1977). 25-Hydroxyvitamin D$_3$ was synthesized from 1,25-Dihydroxycholecalciferol (Kanto Chemical Co., Osaka, Japan). 7,24-Dihydroxyvitamin D$_3$ was from Sigma (St. Louis, Mo., U.S.A.), and hexahydrophosphocholesterol (Cambridge, CA.) was from Merck K. G. (Darmstadt, Germany). All other reagents used were of the highest quality commercially available. 

**Preparation of Antibodies**

To prepare antibodies, 150,000 of 1 ml of calf thymus and 150,000 of 1 ml of calf thymus were emulsified in Freund's complete adjuvant (Institute of Immunology, Tokyo, Japan). High performance liquid chromatography (HPLC) of 25-Hydroxyvitamin D$_3$ (Sigma, St. Louis, Mo., U.S.A.) was performed using a Waters Model 500-HPLC equipped with a Waters 486-UV detector. The analysis was conducted for 24 h at the same temperature and terminated by adding 0.1 ml of 1 N HCl. The products were extracted by ether. The residue was dissolved in a small volume of chloroform and dried under reduced pressure. The residue was identified by thin-layer chromatography (TLC) with a mixture of 1:1:1 (v/v) chloroform/methanol/2N HCl. The products were further purified by preparative HPLC. The yields were determined by the production of human parathyroid hormone (HPT) and by the increase of an ultraviolet absorption at 210 nm.

**Preparation of Mammalian Antibodies**

Female mice of the BALB/c strain were immunized by intraperitoneal injection of the mixture of the particulate and the purified antibodies in Freund's complete adjuvant. After three months, the sera were collected. The sera were used for the preparation of anti-serum and the antisera were used in the subsequent steps. The antisera were then applied to a protein A-Sepharose 4B column (2 x 15 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% sodium cholate, 0.1% SDS, and 1% diethylthioctlate. The column was washed with equilibration buffer containing 0.1% sodium cholate, 0.1% SDS, and 1% diethylthioctlate. After immunopurification, the biologically active preparation was dialyzed against distilled water and stored at -20°C until use.

**Immunoblotting**

After the immunopurification, the proteins were transferred to an immunoblot paper with 10% acrylamide gel and electrophoresis was conducted (4°C) for 2 h, followed by treatment with 5% nonfat dry milk in phosphate-buff ered saline (PBS). After the staining with the primary antibody, the blots were labeled with a secondary antibody conjugated with horseradish peroxidase and visualized with chemiluminescence reagents.