The sequential oxidation and cleavage of the side chain of 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) initiated by the hydroxylase at C-24 is considered to be the major pathway of this hormone in the target cell metabolism. In this study, we examined renal metabolism of a synthetic analog of 1α,25(OH)₂D₃, 24,24-difluoro-1α,25-dihydroxyvitamin D₃ (F₂-1α,25(OH)₂D₃) C-24 of which was designed to resist metabolic hydroxylation. When kidney homogenates prepared from 1α,25(OH)₂D₃-supplemented rats were incubated with F₂-1α,25(OH)₂D₃, it was mainly converted to a more polar metabolite. We isolated and unequivocally identified the metabolite as 24,24-difluoro-1α,25,26-trihydroxyvitamin D₃ (F₂-1α,25,26(OH)₃D₃) by ultraviolet absorption spectrometry, vitrifact atom bombardment liquid chromatography/mass spectroscopy analysis, and direct comparison with chemically synthesized F₂-1α,25,26(OH)₃D₃. Metabolism of F₂-1α,25(OH)₂D₃ into F₂-1α,25,26(OH)₃D₃ by kidney homogenates was induced by the prior administration of 1α,25(OH)₂D₃ into rats. The C-24 oxidation of 1α,25(OH)₂D₃ in renal homogenates was inhibited by F₂-1α,25(OH)₂D₃ in a concentration-dependent manner. Moreover, F₂-1α,25,26(OH)₃D₃ was formed in ROS17/2.8 cells transfected with a plasmid expressing 1α,25(OH)₂D₃-24-hydroxylase (CYP24) but not in the cells transfected with that expressing vitamin D₃-25-hydroxylase (CYP27) or containing inverted CYP27 cDNA. These results show that CYP24 catalyzes not only hydroxylation at C-24 and C-23 of 1α,25(OH)₂D₃ but also at C-26 of F₂-1α,25(OH)₂D₃, indicating that this enzyme has a broader substrate specificity of the hydroxylation sites than previously considered.

Metabolic inactivation of 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), the biologically active metabolite of vitamin D₃, in its target cells is initiated by side chain hydroxylation at C-23, C-24, and C-26 (1–4). Of these hydroxylation sites, it is now accepted that the sequential oxidation and cleavage of the side chain initiated by the hydroxylase at C-24 catalyzed by mitochondrial 1α,25(OH)₂D₃-24-hydroxylase (CYP24) is the major pathway by which the hormone is inactivated (5). Because transcription of the CYP24 gene is highly up-regulated by 1α,25(OH)₂D₃ in its target cells (6–8), CYP24 is regarded as the key enzyme for the breakdown of the hormone (9). Metabolism of 1α,25(OH)₂D₃ initiated by C-23 hydroxylation is induced by 1α,25(OH)₂D₃ itself (10), and recombinant human CYP24 also catalyzes C-23 hydroxylation of 25-hydroxyvitamin D₃ (25(OH)D₃) to yield 23S,25-dihydroxyvitamin D₃ (23S,25(OH)₂D₃) (11). Therefore, it is likely that CYP24 initiates both C-24 and C-23 hydroxylation pathways of 1α,25(OH)₂D₃. In contrast, mitochondrial vitamin D₃-25-hydroxylase (CYP27) catalyzes the hydroxylation at C-25 and C-26 of vitamin D₃ and 1α-hydroxyvitamin D₃ (1α(OH)D₃) (12), but it is not clear whether or not this enzyme hydroxylates C-26 of 1α,25(OH)₂D₃. Recently, a model for the mechanism of the hydroxylation site selection by CYP24 and CYP27 was proposed. This model postulates that CYP24 directs its hydroxylation site(s) by the distance of C-24 and C-23 from the vitamin D ring structure and that CYP27 does so by the distance between the hydroxylation sites and the end of the side chain (13).

Of numerous synthetic analogs of 1α,25(OH)₂D₃, 24,24-difluoro-1α,25-dihydroxyvitamin D₃ (F₂-1α,25(OH)₂D₃) (14) was the first that had a higher biological activity than the parental 1α,25(OH)₂D₃ (15–17). Although the biological activity of F₂-1α,25(OH)₂D₃ was higher, the binding affinity of this analog to VDR was almost identical to that of 1α,25(OH)₂D₃ (16, 17). It is accepted that the resistance of the C-F bond at C-24 of this analog to metabolic inactivation contributes to its higher biological activity. The metabolic fate of F₂-1α,25(OH)₂D₃, however, has not yet been clarified. We recently reported that F₂-1α,25(OH)₂D₃ is metabolized into a more polar compound(s) D₃, in its target cells is initiated by side chain hydroxylation at C-23, C-24, and C-26 (1–4). Of these hydroxylation sites, it is now accepted that the sequential oxidation and cleavage of the side chain initiated by the hydroxylase at C-24 catalyzed by mitochondrial 1α,25(OH)₂D₃-24-hydroxylase (CYP24) is the major pathway by which the hormone is inactivated (5). Because transcription of the CYP24 gene is highly up-regulated by 1α,25(OH)₂D₃ in its target cells (6–8), CYP24 is regarded as the key enzyme for the breakdown of the hormone (9). Metabolism of 1α,25(OH)₂D₃ initiated by C-23 hydroxylation is induced by 1α,25(OH)₂D₃ itself (10), and recombinant human CYP24 also catalyzes C-23 hydroxylation of 25-hydroxyvitamin D₃ (25(OH)D₃) to yield 23S,25-dihydroxyvitamin D₃ (23S,25(OH)₂D₃) (11). Therefore, it is likely that CYP24 initiates both C-24 and C-23 hydroxylation pathways of 1α,25(OH)₂D₃. In contrast, mitochondrial vitamin D₃-25-hydroxylase (CYP27) catalyzes the hydroxylation at C-25 and C-26 of vitamin D₃ and 1α-hydroxyvitamin D₃ (1α(OH)D₃) (12), but it is not clear whether or not this enzyme hydroxylates C-26 of 1α,25(OH)₂D₃. Recently, a model for the mechanism of the hydroxylation site selection by CYP24 and CYP27 was proposed. This model postulates that CYP24 directs its hydroxylation site(s) by the distance of C-24 and C-23 from the vitamin D ring structure and that CYP27 does so by the distance between the hydroxylation sites and the end of the side chain (13).

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in rat osteoblastic ROB-C26 cells (17). The metabolism was initiated after transcription of the CYP24 gene, which was induced by the substrate, F$_2$-1a,25(OH)$_2$D$_3$ itself (17).

In this study, we examined whether CYP24 can metabolize vitamin D analogs, the C-24 of which is resistant to hydroxylation. We identified 24,24-difluoro-1a,25,26-trihydroxyvitamin D$_3$ (F$_2$-1a,25,26(OH)$_3$D$_3$) as a major metabolite of F$_2$-1a,25(OH)$_2$D$_3$. Moreover, the enzyme catalyzing the conversion of F$_2$-1a,25(OH)$_2$D$_3$ into F$_2$-1a,25,26(OH)$_3$D$_3$ was CYP24.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals were of highly purified analytical grade. F$_2$-1a,25(OH)$_2$D$_3$ (18), (25R)- and (25S)-F$_2$-1a,25(OH)$_2$D$_3$ were synthesized in our laboratory. The cDNA for CYP27 (21), human 1,25-dihydroxyvitamin D$_3$ (1a,25(OH)$_2$D$_3$), and 25-hydroxyvitamin D$_3$ (25(OH)D$_3$) (specific activity, 202 GBq/mmol) and 24,24-difluoro-1a,25-dihydroxy(1a-H)vitamin D$_3$ (F$_2$-1a,25(OH)$_2$D$_3$) (specific activity, 396 GBq/mmol) were prepared by Daiichi Pure Chemicals, Co., Ltd. (Tokyo, Japan) as described (19). 1a,25(OH)$_2$D$_3$ was purchased from Wako Pure Chemicals (Osaka, Japan). 1,24R,24-trihydroxyvitamin D$_3$ (1a,24R,25(OH)$_2$D$_3$) was a gift from Kureha Chemical Industries, Co., Ltd. (Tokyo, Japan). [3H]-25(OH)D$_3$ (specific activity, 111 TBq/mmol) was from Amersham International plc (Buckinghamshire, UK).

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Guanidine isothiocyanate was from Fluka Biochemika (Buchs, Switzerland). A rat osteosarcoma cell line, ROS17/2.8, was donated by Dr. M. Noda (Tokyo Medical and Dental University, Tokyo, Japan). A rat renal cell line, 407, was obtained by Roy N. Pompa (New York, NY). A rat osteosarcoma cell line, ROS 17/2.8, was donated 16-23 h prior to transfection and seeded in 100-mm plates at a density of 1 × 10$^6$ cells/dish. Cells were transfected with pSVL-CYP24, pSVL-CYP27, and pSVL-CYP27(-) as described in the manufacturer's manual.

**RESULTS**

**Metabolism of F$_2$-1a,25(OH)$_2$D$_3$ in CYP24- and CYP27-transfected ROS 17/2.8 Cells—**ROS 17/2.8 cells were subcultured 16-20 h prior to transfection and seeded in 100-mm plates at a density of 1 × 10$^6$ cells/dish. Cells were transfected with pSVL-CYP24, pSVL-CYP27, and pSVL-CYP27(-) as described in the manufacturer's manual.

**Purification and Identification of the Major Metabolite (Peak X)**—(Peak X) was purified by reverse phase HPLC (column, YMC-Pack ODS-AM 4.6 × 150 mm; mobile phase, 45% acetonitrile/water; flow rate, 1 ml/min) to give 1.36 µg of the major metabolite (peak X) based on the absorption at 265 nm.

**Northern Blots—**RNA was prepared and Northern blotted as described (18). Total RNA was extracted with acid guanidinium thiocyanate/phenol/chloroform. Poly(A)$^+$ RNA was isolated by a batch method using Oligotex-dT30 super (Takara Biochemicals, Shiga, Japan). Samples (0.8 µg) of poly(A)$^+$ RNA were resolved by electrophoresis, transferred onto a Hybond-N membrane (Amerham International plc), and probed using [a-32P]dCTP-labeled cDNAs for CYP24, CYP27, and β-tubulin obtained by a random primer extension system.

**Conclusions—**To isolate the major metabolite (peak X), 30 µg of F$_2$-1a,25(OH)$_2$D$_3$ was incubated with 150 µl of 10% (v/v) kidney homogenates obtained from rats injected with the vehicle (Fig. 1, top). On the contrary, a lipid-soluble metabolite (peak X) was detected in kidney homogenates from rats given 1a,25(OH)$_2$D$_3$ (Fig. 1, bottom). The HPLC profile of peak X indicated that it was a more polar metabolite than F$_2$-1a,25(OH)$_2$D$_3$. Experiments to assess the kinetics of lowering the substrate in rat kidney homogenates indicated that the Km value for F$_2$-1a,25(OH)$_2$D$_3$ was 2 µM, which is similar to that for 1a,25(OH)$_2$D$_3$ (1.7 µM). On the other hand, the Vmax for the former was 75 pmol/min/g tissue, whereas that for the latter was 510 pmol/min/g tissue.

**Identification of the Major Metabolite (Peak X)**—To isolate the major metabolite (peak X), 30 µg of F$_2$-1a,25(OH)$_2$D$_3$ was incubated with 150 µl of 10% (w/v) kidney homogenates obtained from rats given 1a,25(OH)$_2$D$_3$. The lipid extract of the metabolite was purified by a Sep-pak cartridge followed by two HPLC separations (straight and reverse phase) to yield 1.7 µg of the homogeneous metabolite (peak X). The UV spectrum of peak X was typical of the vitamin D triene system (λ$_{max}$ at 265 nm, λ$_{min}$ at 228 nm in ethanol). The HPLC profile of peak X indicated that it was a more polar metabolite than F$_2$-1a,25(OH)$_2$D$_3$.

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mental Procedures" for details.

The substrate F2-1 was incubated with kidney homogenates from vehicle-treated rats (upper panel) and NaIO4-oxidized rats (lower panel). The total lipid fraction of the reaction mixture was analyzed by straight phase HPLC. The peaks for the substrate F2-1 and peak X are indicated. See “Experimental Procedures” for details.

FIG. 2. Mass spectra of the major metabolite of F2-1 (A), the 25-epimers of F2-1 (B) and (C).

H2O). This spectrum indicates that an oxygen atom was introduced to the side chain of the substrate F2-1. To examine whether a vicinal diol function is present in the metabolite, peak X was treated with NaIO4, and the product was analyzed by frit-FAB LC/MS. HPLC showed a single major peak, and the MS spectrum of the peak showed a parent ion at m/z 437 (M + H) and fragment ions at m/z 419 (M + H - H2O) (relative intensity, 11.5%) and m/z 401 (M + H - 2H2O) (relative intensity, 10.2%). These results indicate that a formyl group was removed by NaIO4 oxidation and that there is a terminal vicinal diol function in the side chain of peak X. Thus, we identified peak X as one of the 25-epimers of F2-1.

FIG. 3. Co-chromatography of the chemically synthesized F2-1, 25-epimers and the lipid-soluble fraction of the reaction mixture of kidney homogenates and F2-1, 25-epimers. A, UV absorption at 265 nm. B, radioactivity. Elution profiles of a mixture of the lipid fraction and synthetic standards on straight phase HPLC (column, Finepak SIL (4.6 × 250 mm); mobile phase, n-hexane:chloroform:ethanol, 10:2.5:1.5; flow rate, 1 ml/min). C, UV absorption at 265 nm. D, radioactivity. Elution profiles of the mixture of the lipid fraction and synthetic standards on reverse phase HPLC (column, J' sphere ODS-AM (4.6 × 150 mm); mobile phase, a linear gradient of acetonitrile concentration (40 to 100% in 30 min); flow rate, 1 ml/min). Radioactivity corresponding to peak X comigrated with F2-1, 25-epimers on both HPLC systems.

peak X was confirmed by direct comparison with two chemically synthesized 25-epimers of F2-1, 25-epimers of F2-1, 25-epimers of F2-1 by HPLC and frit-FAB LC/MS. The metabolite comigrated with the two synthesized compounds (epimers were not separated under the HPLC conditions) (Fig. 3), and the mass spectra of the isolated and synthetic epimers were identical (Fig. 2).

Effects of F2-1, 25-epimers on the Metabolism of 1,25(OH)2D3 by Renal Homogenates—The results shown in Fig. 1 suggested that 1,25(OH)2D3 induced the enzyme that catalyzed the conversion of F2-1, 25-epimers into peak X (F2-1, 26-epimers). Until now, no enzymes other than CYP24 involved in vitamin D metabolism are known to be induced by 1,25(OH)2D3. CYP24 catalyzes the following series of reactions: 1,25(OH)2D3 → 1,24R,25(OH)2D3 → 24-oxo-1,25-dihydroxyvitamin D3 (24-oxo-1,25(OH)2D3) → 24-oxo-1,23S,25-trihydroxyvitamin D3 (24-oxo-1,23S,25(OH)2D3) (25). The side chain of 24-oxo-1,23S,25(OH)2D3 is then cleaved and oxidized to yield water-soluble calcitroic acid (5). Therefore we examined the ability of F2-1, 25-epimers to compete for the metabolism of 1,25(OH)2D3 in rat kidney homogenates. F2-1, 25-epimers inhibited the conversion of 1,25(OH)2D3 into water-soluble metabolite(s) in a concentration-dependent manner (Fig. 4, left). Furthermore, HPLC analysis of the lipid-soluble fraction revealed that F2-1, 25-epimers inhibited the 24-hydroxylation of 1,25(OH)2D3 (Fig. 4, right). These results indicated that F2-1, 25-epimers competes with the interaction of CYP24 and 1,25(OH)2D3.

Metabolism of F2-1, 25-epimers in the CYP24-transfected ROS 17/2.8 Cells—To confirm that CYP24 can catalyze the hydroxylation of F2-1, 25-epimers at C-26, we examined the metabolism of F2-1, 25-epimers in ROS17/2.8 cells transfected with the plasmid expressing CYP24. Expression of CYP24 and CYP27 was confirmed by Northern blotting (Fig. 5D). Control ROS17/2.8 cells expressed neither CYP24 mRNA (Fig. 5D) nor CYP24 activity (data not shown). Incubation of F2-1, 25-epimers
25-Hydroxylation of Vitamin D Compounds by CYP24

DISCUSSION

CYP24 was discovered as the enzyme responsible for the hydroxylation at C-24 in the metabolism of 1α,25(OH)₂D₃ and 25(OH)D₃ (25). Recently, it was found that human recombinant CYP24 also catalyzes the C-23 hydroxylation of 25(OH)D₃ (11), indicating that this enzyme has multicatalytic functions. However, there is no evidence that CYP24 hydroxylates any other carbons than C-24 or C-23 of vitamin D compounds. In this study, we showed that CYP24 catalyzes the 26-hydroxylation of 1α,25(OH)₂D₃ (or 25(OH)D₃) (lanes a, b, and c) in the cells transfected with pSVL-CYP24 (A). The expression was also examined in the cells transfected with pSVL-CYP27 (B) and pSVL-CYP27 (−) (C). The retention times of F₂-1α,25(OH)₂D₃ (Fig. 5A) also comigrated with the synthetic F₂-1α,25,26(OH)₃D₃ on the straight phase HPLC (column, Finepak SIL 4.6 × 250 mm; mobile phase, n-hexane/chloroform/methanol, 10:2.5:1; flow rate, 1 mL/min) (data not included). In contrast, F₂-1α,25,26(OH)₃D₃ was not generated in the cells transfected with pSVL-CYP27 or pSVL-CYP27 (−) under the same conditions (Fig. 5, B and C). These results showed that CYP24 catalyzes the 26-hydroxylation of F₂-1α,25(OH)₂D₃.

From metabolic hydroxylation. Therefore, it is highly likely that CYP24 is also responsible for the C-26 hydroxylation of the vitamin D₃ metabolites in vivo. At present, the possibility cannot be excluded that enzymes other than CYP24, such as CYP27, hydroxylate C-26 of 1α,25(OH)₂D₃ in vivo. In fact, CYP27 reportedly hydroxylates C-25 and C-26 of vitamin D₃ and 1α(OH)D₃ (12). Under our conditions, however, kidney homogenates obtained from rats given either 1α,25(OH)₂D₃ or vehicle did not metabolize vitamin D₃ into 25(OH)D₃ or other metabolites (data not shown), indicating that no CYP27 is present in the kidney. In addition, CYP27-transfected ROS17/2.8 cells did not metabolize F₂-1α,25(OH)₂D₃ into F₂-1α,25,26(OH)₃D₃ (Fig. 5B). Therefore, the C-26 hydroxylation of F₂-1α,25(OH)₂D₃ in kidney homogenates does not appear to be mediated by CYP27.

Two methyl groups at C-25 of 1α,25(OH)₂D₃ (or 25(OH)D₃) are heteroergic. Hydroxylation of one of the methyls yields a new chiral center at C-25. Hydroxylation of the pro-S-methyl group produces 25R configuration and pro-R-methyl group 25S configuration. Two types of 26-oxygenated vitamin D₃ metabolites have been found; one is the metabolites with 25-methyl and the other catalyzes the pro-25-hydroxyvitamin D₃-26,23-dihydroxyvitamin D₃ (29), and their precursors. It has also been reported that vitamin D₃ is a mixture of 25R- and 25S-isomers (30). These results suggest that there are two C-26 hydroxylation enzymes; one catalyzes the hydroxylation of the pro-S-methyl and the other catalyzes the pro-R-methyl. It may be likely that these two types of hydroxylation at C-26 are catalyzed by CYP24 and CYP27, respectively. The stereochemical configuration at C-25 of the metabolite X, F₂-1α,25,26(OH)₃D₃, is now under investigation.

CYP24 has been found in all the target tissues of vitamin D that possess VDR (31). Cloning the cDNA and characterizing
the CYP24 gene (20, 32) has allowed the mechanism of regulation of its gene expression to be studied. Northern blotting has revealed that the expression of this enzyme is induced exclusively by 1α,25(OH)₂D₃ at the transcriptional level (6–8). Three groups independently identified functional but different vitamin D-responsive elements (VDRE-I and VDRE-II) in the antisense strand in rat CYP24 gene promoter at −151 to −137 (VDRE-I) (33, 34) and at −259 to −245 (VDRE-II) (35) in rats. The presence of the two VDREs in the CYP24 gene promoter may be important for regulating intracellular concentration as well as the half-life of 1α,25(OH)₂D₃. Makin et al. reported that the target cells of vitamin D metabolize 1α,25(OH)₂D₃ sequentially into calcitriol by the 24-oxidation pathway (5). Using a bacterially expressed enzyme, Akiyoshi-Shibata et al. showed that CYP24 alone can catalyze all of the following reactions; 1α,25(OH)₂D₃ → 1α,24R,25(OH)₂D₃ → 24-oxo-1α,25(OH)₂D₃ → 24-oxo-1α,23S,25(OH)₂D₃ (25). The ability of CYP24 to catalyze not only 24-hydroxylation but also its successive reactions implies that the role of this enzyme is to decrease the binding affinity of vitamin D compounds to VDR in the target cells, because 1α,24R,25(OH)₂D₃ still has about 40% of the affinity of 1α,25(OH)₂D₃ for VDR (36). Although the bacterially expressed CYP24 also catalyzed the sequential metabolism of 25(OH)D₃, namely 25(OH)D₃ → 24R,25-dihydroxyvitamin D₃ → 24-oxo-25-hydroxyvitamin D₃ → 24-oxo-23S,25-dihydroxyvitamin D₃, the Kₘ value of the enzyme for 1α,25(OH)₂D₃ was one-tenth of that for 25(OH)D₃ (25), suggesting that the former is the real substrate of CYP24.

In conclusion, CYP24 appears to solely regulate the intracellular concentration of the VDR ligand and hence the VDR-mediated transactivation in the target cells of vitamin D. It is highly likely that CYP24 catalyzes all three known catabolic hydroxylation pathways, further emphasizing the importance of this enzyme in regulating vitamin D metabolism and function.

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