Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 320 (2004) 156–164
BBRC
www.elsevier.com/locate/ybbrc

Conversion of vitamin D_3 to 1α,25-dihydroxyvitamin D_3 by *Streptomyces griseolus* cytochrome P450SU-1☆

Natsumi Sawada,[a] Toshiyuki Sakaki,[a,b,*] Sachiyo Yoneda,[a] Tatsuya Kusudo,[a] Raku Shinkyo,[a] Miho Ohta,[c] and Kuniyo Inouye[a]

[a] *Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*
[b] *Biotechnology Research Center, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Kosugi, Toyama 939-0398, Japan*
[c] *Laboratory of Nutrition, Koshien College, 4-25 Kawarabayashi-cho, Nishinomiya 663-8107, Japan*

Received 13 April 2004
Available online 9 June 2004

Abstract

Streptomyces griseolus cytochrome P450SU-1 (CYP105A1) was expressed in *Escherichia coli* at a level of 1.0 μmol/L culture and purified with a specific content of 18.0 nmol/mg protein. Enzymatic studies revealed that CYP105A1 had 25-hydroxylation activity towards vitamin D_2 and vitamin D_3. Surprisingly, CYP105A1 also showed 1α-hydroxylation activity towards 25(OH)D_3. As mammalian mitochondrial CYP27A1 catalyzes a similar two-step hydroxylation towards vitamin D_3, the enzymatic properties of CYP105A1 were compared with those of human CYP27A1. The major metabolite of vitamin D_2 by CYP105A1 was 25(OH)D_2, while the major metabolites by CYP27A1 were both 24(OH)D_2 and 27(OH)D_2. These results suggest that CYP105A1 recognizes both vitamin D_2 and vitamin D_3 in a similar manner, while CYP27A1 does not. The K_m values of CYP105A1 for vitamin D_2 25-hydroxylation, vitamin D_3 25-hydroxylation, and 25-hydroxyvitamin D_3 1α-hydroxylation were 0.59, 0.54, and 0.91 μM, respectively, suggesting a high affinity of CYP105A1 for these substrates.

Keywords: Hydroxylation of vitamin D_3; CYP105A1; *Streptomyces* P450; 1α-Hydroxylation of 25-hydroxyvitamin D_3; CYP27A1

A gene cluster for biosynthesis of antibiotics in *Streptomyces* often contains a cytochrome P450 gene [1–5]. The cytochrome P450 genes are also involved in the biosynthesis of polyketide epothilone [6] and daunorubicin [7] which are potential anticancer agents. These facts suggest that *Streptomyces* P450s are practically useful for the production of medicines. To date, one of the most successful applications of cytochrome P450 reaction on the industrial scale appears to be the bioconversion process for Pravastatin (Sankyo, Japan), an inhibitor of HMG-CoA reductase, formation using a *Streptomyces* P450. Matsuoka et al. [8,9] reported that the 6β-hydroxylase of *Streptomyces carbophilus* for the lead compound ML-236BNa was a water-soluble P450 named P450sca (CYP105A3). Sasaki et al. [10,11] reported the transformation of vitamin D_3 to 1α,25-dihydroxyvitamin D_3 via 25-hydroxyvitamin D_3 using *Amycolata* sp. strains, and cloned a gene encoding 25-hydroxylase (CYP105A2) for vitamin D_3 [12]. Omer et al. [13] cloned the gene coding *Streptomyces griseolus* P450SU-1 (CYP105A1), which can metabolize sulfonylurea herbicides. They succeeded in the functional expression of CYP105A1 in the chloroplast of higher plants by the addition of a targeting signal sequence to the N-terminus of CYP105A1 [14]. Recently, Taylor et al. [15] indicated the possible application of CYP105D1 to bioremediation, because CYP105D1 is involved in degradation of agrochemicals and environmental pollutants. These studies strongly suggest that *Streptomyces* P450s have great potential for a wide range of practical applications.

☆ *Abbreviations:* P450, cytochrome P450; CYP, cytochrome P450; ADR, NADPH-adrenodoxin reductase; ADX, adrenodoxin; 25(OH)D_3, 25-hydroxyvitamin D_3; 1α(OH)D_3, 1α-hydroxyvitamin D_3; 1α,25(OH)$_2D_3$, 1α,25-dihydroxyvitamin D_3; 25(OH)D_2, 25-hydroxyvitamin D_2; 1α(OH)D_2, 1α-hydroxyvitamin D_2; 1α,25(OH)$_2D_2$, 1α,25-dihydroxyvitamin D_2.

* Corresponding author. Fax: +81-766-56-2498.
E-mail address: tsakaki@pu-toyama.ac.jp (T. Sakaki).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.05.140

In this study, we express CYP105A1 in *Escherichia coli*, and revealed that CYP105A1 had 25-hydroxylation activity towards both vitamin D_2 and vitamin D_3 as well as CYP105A2 which had a 55% identity of amino acid sequence with CYP105A1. In addition, we show the production of $1\alpha,25(OH)_2D_3$ by CYP105A1 from vitamin D_3 via 25-hydroxyvitamin D_3, which was not observed in the CYP105A2-dependent metabolism of vitamin D_3 [12]. As described previously [16], mitochondrial CYP27A1 has the ability to convert vitamin D_3 to $1\alpha,25(OH)_2D_3$. In this report, the enzymatic properties of CYP105A1 toward vitamin D_2 and vitamin D_3 are compared with those of human mitochondrial CYP27A1.

Materials and methods

Materials. DNA modifying enzymes and restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan). Primer DNAs were purchased from GENSET KK (Kyoto, Japan). *E. coli* JM109 (Takara Shuzo) and DH5α were used as host strains. Ferredoxin and NADPH-ferredoxin reductase from spinach were purchased from Sigma (St. Louis, USA). Vitamin D_3, vitamin D_2, $1\alpha(OH)D_3$, glucose dehydrogenase, and catalase were purchased from Wako Pure Chemical Industries (Osaka, Japan). $25(OH)D_3$ was purchased from Funakoshi (Tokyo, Japan). $1\alpha(OH)D_2$ was purchased from Calbiochem (La Jolla, CA, USA). $25(OH)D_2$ and $1\alpha,25(OH)_2D_2$ were kindly given by Dr. K. Yamamoto of Tokyo Medical and Dental University. Vitamin D receptor binding kit containing calf-thymus cytosol was purchased from Yamasa Shoyu (Chiba, Japan). *S. griseolus* CYP105A1 and ferredoxin genes were kindly provided by Sumitomo Chemical (Takarazuka, Japan). Bovine adrenodoxin and NADPH-adrenodoxin reductase were kindly given by Dr. Y. Nonaka of Koshien University. NADPH was purchased from Oriental Yeast (Tokyo, Japan). Other chemicals used were of the highest quality commercially available.

Construction of expression plasmids. The expression plasmid for CYP105A1 was constructed as follows. The PCR fragment containing CYP105A1 gene and the ferredoxin gene encoding Fd1 [13] with *Nde*I (CATATG) and *Hin*dIII (AAGCTT) sites was obtained using the primers 5′-ATATAAGCTTAAACATATGACCGATACCGCCACG AC-3′ and 5′-ATATAAGCTTCACCAGGTGACCGGGAGTT-3′. The PCR fragment was inserted into *Nde*I and *Hin*dIII sites of pKSNdl [17] that was obtained from pKK223-3. The resultant expression plasmid was introduced into *E. coli* JM109 cells. The expression plasmid for Fd-2 was constructed as follows. The PCR fragment containing ferredoxin gene encoding Fd-2 [13] with *Nde*I (CATATG) and *Hin*dIII (AAGCTT) sites was obtained using the primers 5′-TTATATAAGCTTAGGAGGCATATGCGCATCCAC GTCGACCAGGAC-3′ and 5′-TAATTAAAGCTTTCAGTCGGTC AC CGTGATCGCGGC-3′. The PCR fragment was inserted into *Nde*I and *Hin*dIII sites of pKSNdl. The resultant expression plasmid was introduced into *E. coli* DH5α cells.

Cultivation of the recombinant E. coli cells. Recombinant *E. coli* cells were grown in TB medium [17] containing 50 μg/ml ampicillin at 26 °C under good aeration. The induction of transcription of CYP105A1 under the tac promoter was initiated by addition of isopropyl-thio-β-D-galactopyranoside (IPTG) at a final concentration of 1 mM when the cell density (OD_{660}) reached 0.5. δ-Aminolevulinic acid was also added at final concentration of 0.5 mM. The recombinant *E. coli* cells were gently shaken at 26 °C under good aeration by bubbling for 24 h. The cultivation of the recombinant *E. coli* cells expressing Fd-2, and induction of Fd-2 were carried out according to the methods mentioned above except for no addition of δ-aminolevulinic acid.

Preparation of cytosolic fractions. Subcellular fractionation of *E. coli* cells was carried out basically according to our previous study [17]. Twenty millimolars of Tris–HCl (pH 7.4) buffer was used for all the procedures.

Measurement of reduced CO difference spectra and substrate-induced difference spectra. The reduced CO difference spectra of CYP105A1 and CYP27A1 were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously [18]. The P450 content was estimated from the reduced CO difference spectrum using a difference of the extinction coefficients at 446 and 490 nm of $91\ mM^{-1}\ cm^{-1}$ [19]. The P450 content of the purified CYP105A1 was estimated using a molar extinction coefficient of $110\ mM^{-1}\ cm^{-1}$ at 417 nm [20]. The substrate-induced difference spectra of the purified CYP105A1 were measured in the presence of 2.0 μM CYP105A1 and 2.0 μM of vitamin D_3 or $1\alpha(OH)D_3$.

Purification of CYP105A1. Cytosolic fraction was applied to a DEAE–Sepharose CL-6B column (1.6×30 cm) equilibrated with 20 mM Tris–HCl (pH 7.4) and eluted by the linear gradient of NaCl (0–400 mM) in 20 mM Tris–HCl (pH 7.4). The fractions with spectrophotometric index $A_{417}/A_{280} > 1.2$ were collected and concentrated by Centriprep YM-30 (Millipore, Bedford, USA) and applied to a hydroxylapatite column (Bio-Rad, Hercules, USA) equilibrated with 10 mM KPi (pH 7.4). CYP105A1, which passed through the column, was collected and used as the purified sample.

Purification of Fd-2. Cytosolic fraction was applied to a DEAE–Sepharose CL-6B equilibrated with 20 mM Tris–HCl (pH 7.4) and eluted by the linear gradient of NaCl (0–500 mM) in 20 mM Tris–HCl (pH 7.4) at a flow rate of 2 ml/min. The fractions with spectrophotometric index $A_{410}/A_{280} > 0.6$ were pooled and dialyzed against 20 mM Tris–HCl (pH 7.4). Solid $(NH_4)_2SO_4$ was added to the dialyzed fraction at 25% saturation. After centrifugation at 10,000*g* for 30 min, the supernatant was applied on a TOYO PEARL phenyl-650M (3×14 cm, Tosoh, Japan) equilibrated with 25% $(NH_4)_2SO_4$ in 20 mM Tris–HCl (pH 7.4). Fd-2, which passed through the column, was collected and brought to 90% saturation with solid $(NH_4)_2SO_4$. After centrifugation at 10,000*g* for 30 min, the precipitate was resuspended in 4 ml of 20 mM Tris–HCl (pH 7.4) and applied to Sephadex G-50 (1.6×40 cm) equilibrated with 20 mM Tris–HCl (pH 7.4) at a flow rate of 0.5 ml/min. The fractions with spectrophotometric index $A_{410}/A_{280} > 0.66$ were collected and used as the purified sample.

Comparison of Fd-2 and spinach Fdx in the CYP105A1-dependent hydroxylation of vitamin D_2. The activity of spinach Fdx and the purified Fd-2 was compared by measuring the hydroxylation activity towards vitamin D_2 in a reconstituted system containing 0.5 μM of the purified CYP105A1, 4.0 or 20.0 μM of spinach ferredoxin or the purified Fd-2, 0.2 or 2.0 μM spinach ferredoxin reductase, 1 U/ml glucose dehydrogenase (Wako), 1% glucose, 0.1 mg/ml catalase, 1 mM NADPH, 5.0 or 10.0 μM of the substrate, 100 mM Tris–HCl (pH 7.4), and 1 mM EDTA at 30 °C.

Measurement of the activity of CYP105A1. Each of the hydroxylation activity towards vitamin D_2, $1\alpha(OH)D_2$, vitamin D_3, $1\alpha(OH)D_3$, and $25(OH)D_3$ was measured in the reconstituted system containing 0.5 μM of the purified CYP105A1, 0.1 mg/ml spinach Fdx, 0.1 U/ml spinach Fdr, 1 U/ml glucose dehydrogenase (Wako), 1% glucose, 0.1 mg/ml catalase, 1 mM NADPH, 5.0 or 10.0 μM of the substrate, 100 mM Tris–HCl (pH 7.4), and 1 mM EDTA at 30 °C. The purified Fd-2 was also used as an electron donor of CYP105A1 instead of spinach Fdx.

For the determination of K_m and V_{max} values for 25-hydroxylation towards vitamin D_2 and vitamin D_3, and 1α-hydroxylation towards $25(OH)D_3$, the concentration of CYP105A1 was reduced to 0.1 μM, and the substrate concentrations varied from 0.5 to 10.0 μM. The kinetic parameters were calculated by the nonlinear regression analysis using the Kaleida-Graph (Synergy software).

The reaction was initiated by addition of NADPH. Aliquots of the reaction mixture were collected after varying time intervals and extracted with four volumes of chloroform–methanol (3:1). The organic phase was recovered and dried up. The resulting residue was

solubilized with acetonitrile and applied to HPLC under the following conditions: column, YMC-Pack ODS-AM (4.6 × 300 mm) (YMC, Tokyo, Japan); UV detection, 265 nm; flow rate, 1.0 ml/min; column temperature, 40 °C; and mobile phase, acetonitrile/water (50: 50, v/v) for 5 min followed by a linear gradient of 70–100% acetonitrile aqueous solution per 15 min followed by 100% acetonitrile for 25 min for the analysis of the metabolites.

Preparation of membrane fraction containing human CYP27A1. The expression plasmid for mature form of human CYP27A1 was constructed as described previously [16], and the resultant plasmid pKH27A1 was introduced into *E. coli* JM109. The recombinant *E. coli* JM109/pKH27A1 cells were cultivated at 29 °C with the conditions similar to those for CYP105A1. Membrane fraction containing CYP27A1 was prepared as described previously. The P450 content in the membrane fraction was estimated to be 1.1 nmol/mg protein.

Measurement of the activity of CYP27A1. The activity of CYP27A1 was measured in the recombinant system containing the membrane fraction containing 0.5 μM CYP27A1, 5.0 or 10.0 μM of a substrate, 0.5 μM NADPH-adrenodoxin reductase, 5 μM adrenodoxin, 1.0 mM NADPH, 100 mM Tris–HCl, pH 7.4, and 1 mM EDTA at 37 °C as described previously [16]. For the determination of K_m and V_{max} values for 25-hydroxylation towards vitamin D_3 and 1α-hydroxylation towards 25(OH)D_3, the concentrations of CYP27A1, ADX, and ADR were reduced to 0.1, 2.0, and 0.2 μM, respectively [16]. The reaction mixture was extracted with four volumes of chloroform–methanol (3:1, v/v) and analyzed as described above.

Mass spectrometric analysis of the metabolites. Isolated metabolites from HPLC effluents were subjected to mass spectrometric analysis using a Finnegan mat TSQ-70 with atmospheric pressure chemical ionization (APCI), positive mode. The conditions of LC were described below: column, reverse-phase ODS column (6 × 150 mm) (μBondapak C18, Waters); mobile phase, 90% methanol aqueous solution; flow rate; 1.0 ml/min; and UV detection, 265 nm.

Binding assay for calf-thymus vitamin D receptor. Displacement of [^{3}H]-1α,25(OH)$_2$$D_3$ from calf-thymus cytosol receptor (Yamasa Shoyu, Japan) by metabolites of 25(OH)D_3 and 25(OH)D_2, or authentic standard of 1α,25(OH)$_2$$D_3$ was determined as described previously [22]. Various amounts of each of 1α,25(OH)$_2$$D_3$, 1α,25(OH)$_2$$D_2$, 25(OH)$D_3$, 25(OH)$D_2$, and the metabolites in 20 μl ethanol were added to 500 μl of the calf-thymus cytosol diluted with 50 mM potassium phosphate buffer (pH 7.4) containing 0.3 M KCl, and incubated for 1 h at 20 °C. Next, 34 fmol of [^{3}H]-1α,25(OH)$_2$$D_3$ in 25 μl ethanol was added and incubated for 1 h at 20 °C. Then, 200 μl of dextran–charcoal (0.05% dextran T-150, 0.5% charcoal decolorizing neutral) in 50 mM sodium phosphate buffer (pH 7.5), which was freshly prepared and stirred well before addition, was added to separate bound and free [^{3}H]-1α,25(OH)$_2$$D_3$. The assay tube was shaken with a vortex mixer and centrifuged at 1000*g* for 10 min at 4 °C. The radioactivity in the supernatant was measured with a liquid scintillation counter.

Other methods. The concentrations of vitamin D_3 derivatives were estimated by their molar extinction coefficient of 1.80×10^4 M^{-1} cm^{-1} at 264 nm [23]. Protein concentration was determined by the method of Lowry et al. [24], using bovine serum albumin as a standard. The N-terminal amino acid sequence of CYP105A1 was determined by automated Edman degradation using an Applied Biosystems (Foster City, CA, USA) Model 494 gas-phase protein/peptide sequencer.

Results

Expression of CYP105A1

Although cultivation of the recombinant *E. coli* cells at 37 °C resulted in the formation of inclusion body of CYP105A1, cultivation at 26 °C for 48 h produced active CYP105A1 at an expression level of 1.0 μmol/L culture. Recently, Hussain and Ward [25] reported the expression of CYP105A1 by using the modified form of *E. coli* BL21(DE3) as a host strain. In this study, *E. coli* JM109 was used as a host strain. Most of the CYP105A1 was localized in cytosolic fraction. By both DEAE cellulose and hydroxylapatite chromatography, CYP105A1 was purified as shown in Fig. 1. The specific content was increased from 2.8 to 18.0 nmol/mg protein (Table 1). The final preparation of CYP105A1 had a specific content of 18.0 nmol/mg protein. The purified sample showed almost a single band with an apparent molecular weight of 44,000 as shown in Fig. 1. The determined N-terminal 10-amino acid sequence was Thr-Asp-Thr-Ala-Thr-Thr-Pro-Gln-Thr-Thr, which coincided with that of a 2- to 11-amino acid sequence of CYP105A1 deduced from the DNA sequence [13]. The CO-reduced difference spectrum of CYP105A1 was measured in a 1.0 M potassium phosphate buffer (pH 7.4) instead of a 100 mM Tris–HCl buffer (pH 7.4) in which P450 was readily converted to P420. As shown in Fig. 2, the CO-reduced difference spectrum of CYP105A1 showed a peak at 447 nm. The oxidized spectrum showed a Soret

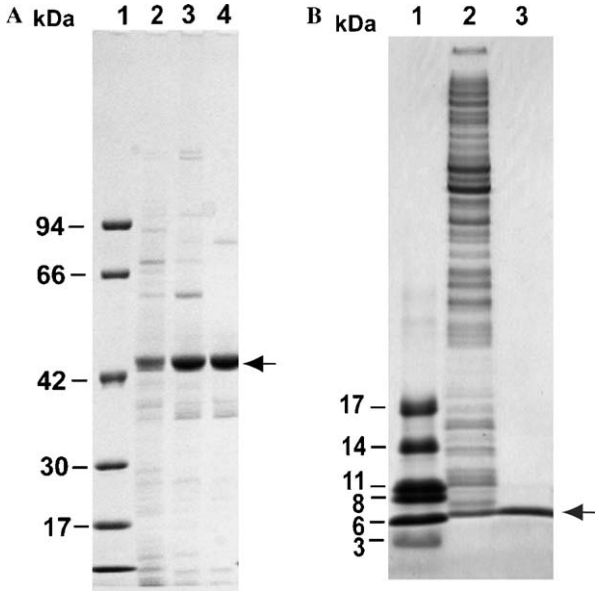

Fig. 1. SDS–PAGE analysis of cytosolic fraction of the recombinant *E. coli* cells and the purified samples of CYP105A1 (A) and Fd-2 (B). The cytosolic fraction (lane A-2) and the elute from DEAE–Sepharose CL-6B (lane A-3) and hydroxyapatite column (lane A-4) were analyzed. Lane A-1 shows relative mass molecular markers (rabbit phosphorylase b, M_r 94,000; bovine serum albumin, M_r 66,000; rabbit aldolase, M_r 42,000; bovine carbonic anhydrase, M_r 30,000; and horse myoglobin, M_r 17,000). The cytosolic fraction (lane B-2) and the purified Fd-2 (lane B-3). Lane B-1 shows peptide molecular weight markers (myoglobin, M_r 16,949; myoglobin peptides I and II, M_r 14,404; myoglobin peptides I and III, M_r 10,700; myoglobin peptide I, M_r 8159; myoglobin peptide II, M_r 6214; and myoglobin peptide III, M_r 2512).

Table 1
Purification of CYP105A1 from the recombinant *E. coli* cells

Step and fraction	P450 (nmol)	Total protein (mg)	Specific content (nmol P450/mg protein)	Yield (%)
Cytosolic fraction	491	176	2.8	100
DEAE–Sepharose	354	24	14.6	72
Hydroxylapatite	245	14	18.0	50

peak at 417 nm, suggesting that the heme iron of CYP105A1 was in a low spin state. The substrate-induced difference spectra of CYP105A1 with each of vitamin D_3 and 1α(OH)D_3 showed Type I spectra, suggesting that the heme iron of CYP105A1 was shifted to a high spin by the substrate binding (Fig. 2). It should be noted that the magnitude of the absorbance difference between 490 and 420 nm by 1α(OH)D_3 was twice more than that by vitamin D_3 (Fig. 2).

Expression and purification of Fd-2

The expression level of Fd-2 was 3.8 μmol/L culture. The specific content of Fd-2 was increased from 7.0 to 75.0 nmol/mg protein by DEAE–Sepharose CL-6B, TOYO PEARL phenyl-650M column chromatography, ammonium sulfate precipitation, and Sephadex G-50 column chromatography. The purified sample showed almost a single band with an apparent molecular weight of 7000, as shown in Fig. 1. The optical spectrum of Fd-2 showed that of typical [3Fe-4S] which has an absorption maximum near 300 and 410 nm (data not shown).

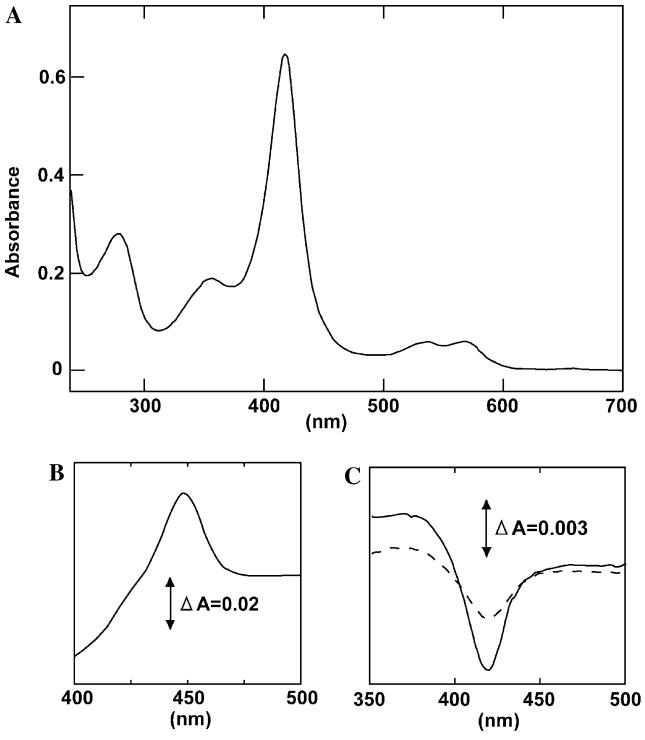

Fig. 2. Oxidized (A), reduced CO difference (B), and substrate-induced (C) spectra of the purified CYP105A1. The substrate-induced difference spectra were measured in the presence of 2.0 μM CYP105A1 and each of 2.0 μM vitamin D_3 (----) and 2.0 μM 1α(OH)D_3 (——).

Comparison of Fd-2 and spinach Fdx in the CYP105A1-dependent hydroxylation of vitamin D_2

Under all the conditions examined, no significant difference was observed between Fd-2 and spinach Fdx in the CYP105A1-dependent 25-hydroxylation of vitamin D_2 (data not shown). These results strongly suggest that the efficiency of electron transport from spinach Fdx to CYP105A1 is nearly the same as that from Fd-2 to CYP105A1. Thus, we used spinach Fdx as an electron donor of CYP105A1 instead of Fd-2 for the further analysis of vitamin D metabolism by CYP105A1.

HPLC and LC-MS analyses of metabolites of vitamin D

The CYP105A1-dependent reaction was dramatically prolonged up to 180 min by using this NADPH regeneration system. Hydroxylation activity of CYP105A1 towards vitamin D_3, 1α(OH)D_3, 25(OH)D_3, and 1α(OH)D_2 was measured in the reconstituted system.

On the metabolism of vitamin D_3 and 1α(OH)D_3, CYP105A1 showed a metabolite at the same retention time as 25(OH)$_3D_3$ (Fig. 3) and 1α,25(OH)$_2D_3$ (data not shown). The metabolite 1 (M1) showed a molecular ion at *m/z* 401 (M + H), and fragment ions at 383 (401-H_2O) and 365 (401-2H_2O) (data not shown). Ion at *m/z* 430 (M + H + 29) was also observed [16]. The spectrum coincided with that of authentic standard of 25(OH)D_3. These results strongly suggest that the metabolite 1 (M1) is 25(OH)D_3. Similarly, the metabolite from 1α(OH)D_3 showed nearly the same mass spectrum as the authentic standard of 1α,25(OH)$_2D_3$. CYP27A1 showed similar metabolism to CYP105A1 towards vitamin D_3 and 1α(OH)D_3 as described previously [16].

On the metabolism of vitamin D_2 and 1α(OH)D_2, CYP105A1 showed metabolites at the same retentions time as 25(OH)D_2 (Fig. 3) and 1α,25(OH)$_2D_2$ (data not shown). The metabolite 2 (M2) showed a molecular ion at *m/z* 413 (M + H), and fragment ions at 395 (413-H_2O) and 377 (413-2H_2O) (data not shown). The spectrum coincided with that of the authentic standard of 25(OH)D_2. These results strongly suggest that the metabolite 2 (M2) is 25(OH)D_2. The mass spectrum of the

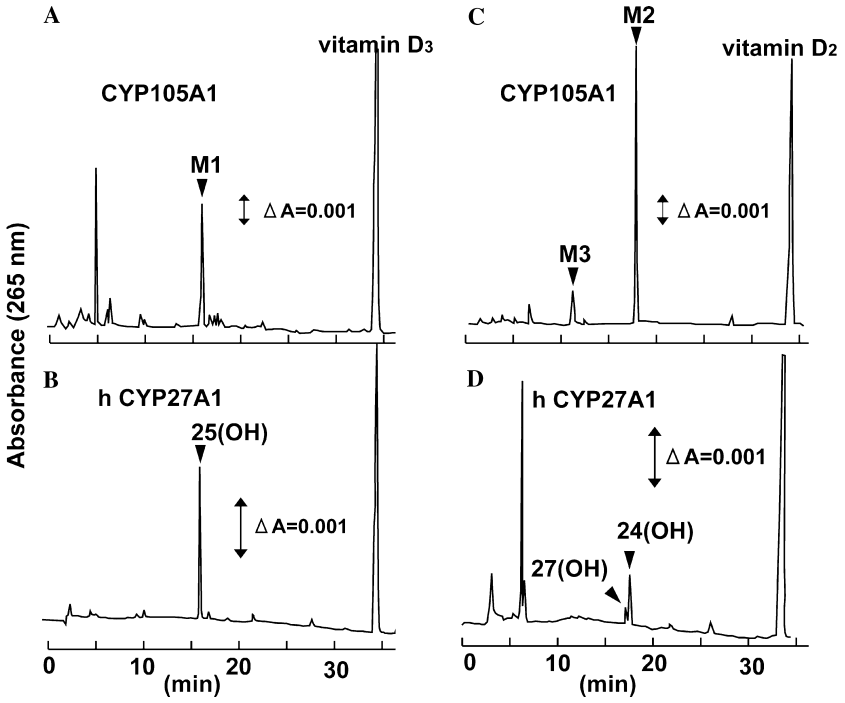

Fig. 3. HPLC profiles of vitamin D_3 and its metabolites by CYP105A1 (A) and CYP27A1 (B), and those of vitamin D_2 and its metabolites by CYP105A1 (C) and CYP27A1 (D). After incubation with 5.0 μM vitamin D_2 (A,B) or 5.0 μM vitamin D_3 (C,D) for 180 min (A,C) or 30 min (B,D), the reaction mixture was extracted and analyzed by HPLC under Materials and methods. 25(OH) indicates the elution position of authentic standard of 25(OH)D_3. 24(OH) and 27(OH) indicate the putative elution positions of 24(OH)D_2 and 27(OH)D_2 based on Guo et al. [26].

metabolite of 1α(OH)D_2 was quite similar to that of the authentic standard of 1α,25(OH)$_2D_2$. On the other hand, CYP27A1 showed two major metabolites of vitamin D_2 distinct from 25(OH)D_2 (Fig. 3). Judging from the results described by Guo et al. [26] that the major metabolites were 27- and 24-hydroxylated vitamin D_2 in turn, the metabolites appeared to be 27(OH)D_2 and 24(OH)D_2, respectively. Mass spectra of these metabolites were clearly different from that of 25(OH)D_2. An extremely small peak at *m*/*z* 377 (413-2H_2O) of 24(OH)D_2 indicates that the 24-hydroxyl group is hardly lost. It was found that CYP105A1 and CYP27A1 were clearly different on the metabolism of vitamin D_2 and 1α(OH)D_2. The minor metabolite 3 (M3) was considered to be a two-hydroxylated compound of vitamin D_2 on the basis of HPLC and mass analyses, and appeared to be produced via 25(OH)D_2. The metabolite 3 (M3) showed a molecular ion at *m*/*z* 429 (M + H), and fragment ions at 411 (429-H_2O), 393 (429-2H_2O), and 375 (429-3H_2O) (data not shown). The mass spectrum was clearly different from that of 1α,25(OH)$_2D_2$, suggesting that a hydroxyl group was added to the side chain of 25(OH)D_2.

On the metabolism of 25(OH)D_3, CYP105A1 showed a metabolite at nearly the same retention time as 1α,25(OH)$_2D_3$ (Fig. 4). The metabolite 4 (M4) showed fragment ions at 399 (417-H_2O), 381 (417-2H_2O), and 363 (417-3H_2O) (data not shown). The mass spectrum

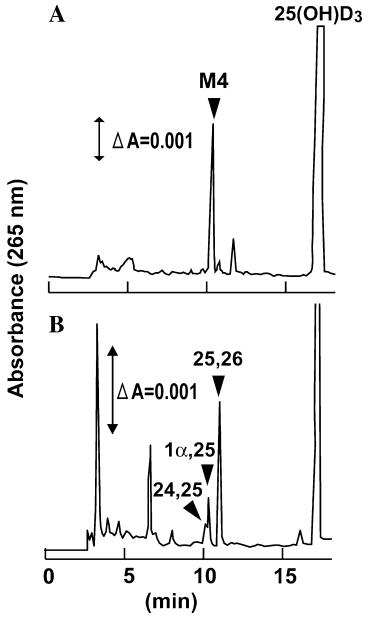

Fig. 4. HPLC profiles of 25(OH)D_3 and its metabolites by CYP105A1 (A) and those by CYP27A1 (B). After incubation with 5.0 μM of 25(OH)D_3 for 180 min (A) or 30 min (B), the reaction mixture was extracted and analyzed by HPLC under Materials and methods. 1α,25 and 24,25 indicate the elution positions of authentic standards of 1α,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$, respectively. 25,26 indicates the elution position of 25,26(OH)$_2D_3$ based on our previous report [16].

was nearly the same as that of the authentic standard of 1α,25(OH)$_2$D$_3$. These results strongly suggest that the metabolite 4 is 1α,25(OH)$_2$D$_3$. Vitamin D receptor (VDR) binding assay confirmed this assumption.

Comparison of vitamin D hydroxylation activity between CYP105A1 and CYP27A1

Table 2 summarizes the hydroxylation activity of CYP105A1 and CYP27A1 towards vitamin D$_3$, 1α(OH)D$_3$, vitamin D$_2$, and 1α(OH)D$_2$ at a substrate concentration of 10 μM. The 25-hydroxylation activity of CYP105A1 towards vitamin D$_3$ was much lower than that of CYP27A1. The 1α-hydroxyl group of the substrate considerably increased the 25-hydroxylation activity of CYP105A1 as well as the magnitude of $\Delta A_{490-420}$ in Fig. 2. Because the displacement of H_2O as the distal ligand by the substrate is essential for P450 reaction, a good correlation between the magnitude of $\Delta A_{490-420}$ and the activity in Table 2 is quite reasonable. Interestingly, similar results showing higher activity towards 1α(OH)D$_3$ were also obtained on the CYP27A1-dependent activity. It is noted that CYP27A1 catalyzes not 25-hydroxylation but 24- and 27-hydroxylation towards vitamin D$_2$.

On the metabolism of 25(OH)D$_3$, CYP105A1 catalyzes 1α-hydroxylation, while CYP27A1 catalyzes not only 1α-hydroxylation but also 26(27)-hydroxylation and 24-hydroxylation, as described previously [16]. In the experimental conditions used in this study, 26- and 27-hydroxylations are not distinguishable.

Table 3 shows the comparison of the kinetic parameters of CYP105A1 and CYP27A1. Judging from the V_{max}/K_m values for vitamin D$_3$ and vitamin D$_2$, vitamin D$_2$ is a better substrate for CYP105A1. It is noted that the K_m values of CYP105A1 are significantly lower than those of CYP27A1 for both VD$_3$ and 25(OH)D$_3$. As the reconstituted system of mitochondrial CYP27A1 contains membrane fraction while the reconstituted system of soluble CYP105A1 has no lipids, the kinetic parameters of both CYPs cannot be compared directly. However, it is certain that CYP105A1 has a high affinity for these substrates.

Binding assay for calf-thymus vitamin D receptor

To examine whether the metabolites are the active forms of vitamin D or not, a binding assay using calf-thymus VDR was performed. The concentrations of 1α,25(OH)$_2$D$_3$, 1α,25(OH)$_2$D$_2$, the metabolites M3 and M4, 25(OH)D$_3$, and 25(OH)D$_2$ for 50% B/B0 were 40, 28, 800, 53, and 44,000, and 70,000 pM, respectively

Table 2
Comparison of vitamin D hydroxylation activity between CYP105A1 and human CYP27A1

Substrate	Position	CYP105A1 activity (mmol/min/mol P450)	CYP27A1 activity (mmol/min/mol P450)
VD$_2$	24	—	33 ± 4
	25	78 ± 14	—
	27 (26)	—	14 ± 1
1α(OH)D$_2$	24	—	114 ± 18
	25	131 ± 20	—
	27 (26)	—	106 ± 16
VD$_3$	25	20 ± 7	196 ± 31
1α(OH)D$_3$	25	54 ± 12	473 ± 41
25(OH)D$_3$	1α	4 ± 1	12 ± 5

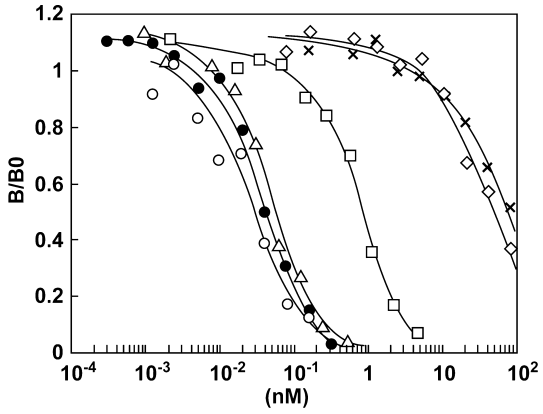

Fig. 5. VDR-binding assay of the metabolite 3 (□), metabolite 4 (△), and authentic standards of 1α,25(OH)$_2$D$_3$ (●), 1α,25(OH)$_2$D$_2$ (○), and 25(OH)D$_3$ (◇), and 25(OH)D$_2$ (×). The designated concentrations are the final concentrations of the metabolites and authentic standards in the reaction mixture. B and B0 mean the concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ bound to VDR, and the concentration of [^{3}H]-1α,25(OH)$_2$D$_3$ added in the reaction mixture, respectively.

Table 3
Kinetic parameters of CYP105A1 and human CYP27A1 for vitamin D hydroxylation activity

CYP	Substrate	Position	K_m (μM)	V_{max} (mmol/min/mol P450)	V_{max}/K_m
105A1	VD$_2$	25	0.59 ± 0.08	84 ± 3	142
105A1	VD$_3$	25	0.54 ± 0.09	16 ± 1	30
27A1[a]	VD$_3$	25	3.2 ± 0.5	270 ± 30	84
105A1	25(OH)D$_3$	1α	0.91 ± 0.26	3.6 ± 0.3	3.9
27A1[a]	25(OH)D$_3$	1α	3.5 ± 0.4	21 ± 2	6.0

[a] The values were reported in our previous study [16].

(Fig. 5). It is noted that M4 showed nearly the same affinity for VDR as 1α,25(OH)$_2$D$_3$. Together with HPLC and LC-MS analyses, M4 was identified to be 1α,25(OH)$_2$D$_3$. On the other hand, the metabolite M3 showed significantly lower affinity for VDR than the authentic standard of 1α,25(OH)$_2$D$_2$, indicating that M3 is not 1α,25(OH)$_2$D$_2$. These results and the LC-MS analysis suggest that M3 is a hydroxylated product of 25(OH)D$_2$ at its side chain portion. If so, however, the affinity of M3 for VDR would not be so different from that of 25(OH)D$_2$. The fact that M3 showed an 80-fold higher affinity for VDR than 25(OH)D$_2$ might suggest that the M3 contains 1α,25(OH)$_2$D$_2$ at a level of a few percent.

Discussion

Omer et al. [13] cloned the gene coding *S. griseolus* CYP105A1 which metabolized sulfonylurea herbicides. They succeeded in the functional expression of CYP105A1 in the chloroplast of higher plants by the addition of a targeting signal sequence to the N-terminus of CYP105A1 [14]. On the other hand, Kawauchi et al. [12] cloned a gene encoding 25-hydroxylase (CYP105A2) for vitamin D$_3$ from *Amycolata autotrophica*. The identity of the amino acid sequence of CYP105A1 and CYP105A2 is approximately 55%. We examined the vitamin D metabolism of CYP105A1 in the reconstituted system and revealed that CYP105A1 had a catalytic activity toward vitamin D$_2$ and vitamin D$_3$. Our recent studies revealed that human mitochondrial CYP27A1 catalyzes the multi-step hydroxylation toward vitamin D$_3$ [16]. Thus, we compared the metabolism of vitamin D by CYP105A1 with that by human mitochondrial CYP27A1. As described previously [16], CYP27A1 can produce 1α,25(OH)$_2$D$_3$ from vitamin D$_3$ via 25(OH)D$_3$, although another P450 species CYP27B1 is physiologically essential as 1α-hydroxylase of 25(OH)D$_3$ [26–28]. One of our interests is whether CYP105A1 can produce 1α,25(OH)$_2$D$_3$ from vitamin D$_3$ via 25(OH)D$_3$ as well as CYP27A1. The major metabolite of vitamin D$_3$ by CYP105A1 was found to be 25(OH)D$_3$, indicating that its enzymatic properties are similar to those of CYP27A1 and CYP105A2. When 25(OH)D$_3$ was added as a substrate, the metabolite (M4) was observed (Fig. 4). Mass analysis and VDR-binding assay strongly suggested that M4 is 1α,25(OH)$_2$D$_3$. Thus, the enzymatic properties of CYP105A1 for vitamin D$_3$ appear to be quite similar to those of CYP27A1. In addition, a putative trihydroxylated metabolite was observed in the metabolism of 1α(OH)D$_3$, suggesting that CYP105A1 can add a hydroxyl group at another position besides the C-25 and C-1α positions (data not shown). Similar results were observed in the metabolism of vitamin D$_3$ by CYP27A1, showing the addition of a hydroxyl group at the C-24 and C-26 (27) positions besides the C-25 and C-1α positions as described previously [16]. In contrast, a clear difference was observed between CYP105A1 and CYP27A1 on their substrate specificity. CYP105A1 showed a significantly higher activity toward vitamin D$_2$ than vitamin D$_3$, opposite to that of CYP27A1. The mass spectrum indicated that the major metabolite was 25(OH)D$_2$, while the metabolites by CYP27A1 were 24(OH)D$_2$ and 27(OH)D$_2$. Thus, the conformation and/or mobility of the side chain of vitamin D$_2$ in the substrate-heme pocket of CYP27A1 appears to be different from that of vitamin D$_3$. On the other hand, CYP105A1 may recognize vitamin D$_2$ and vitamin D$_3$ in a similar manner.

Dihydroxylated metabolites were also observed in the metabolism of vitamin D$_2$ by CYP105A1. First, we assumed that the dihydroxylated metabolite is 1α,25(OH)$_2$D$_2$ based on the formation of 1α,25(OH)$_2$D$_3$ from vitamin D$_3$. However, mass analysis and VDR-binding assay of the metabolite rejected this assumption. For the hydroxylation at the C-1α position, the side chains of vitamin D$_2$ and D$_3$ should play an important role in fixing the substrate at a proper position. A significant difference in the conformation and/or mobility of the side chain between vitamin D$_2$ and vitamin D$_3$ may explain the difference of the metabolism between 25(OH)D$_2$ and 25(OH)D$_3$. Judging from our results, CYP105A1 might be practically useful for the production of 25(OH)D$_2$ and 25(OH)D$_3$. In addition, practical application of CYP105A1 for production of 1α,25(OH)$_2$D$_3$ might be also possible, although the 1α-hydroxylation activity toward 25(OH)D$_3$ is considerably lower than the 25-hydroxylation activity toward vitamin D$_3$.

As reported by O'Keefe et al. [21], the activity of CYP105A1 with Fd-2 had been about 15 times more than that with spinach Fdx [20] in the metabolism of sulfonylurea R7402. However, in the metabolism of vitamin D$_2$, no significant difference was observed between Fd-2 and spinach Fdx. These results suggest that the efficiency of electron transfer to CYP105A1 from spinach Fdx is nearly the same as that from Fd-2 in the metabolism of vitamin D$_2$. Although we cannot explain the discrepancy between sulfonylurea R7402 and vitamin D$_2$, it is possible to assume that the electron transport efficiency and/or the rate-determining step differ depending upon the substrate. It should be noted that the activity with 4 μM spinach Fdx (or Fd-2) and 2.0 μM Fdr was lower than that with 4 μM spinach Fdx (or Fd-2) and 0.2 μM Fdr, indicating that excessive addition of Fdr inhibits the activity. These results strongly suggest that the interaction site of spinach Fdx (or Fd-2) with CYP105A1 is the same as that with Fdr.

X-ray crystallographic analysis revealed the tertiary structures of water-soluble microbial P450s, P450cam

(CYP101) [29], P450BM-3 (CYP102) [30], P450terp (CYP108) [31], P450eryF [32], P450nor (CYP55A1) [33], CYP119 [34], CYP51 [35], and CYP154C1 [36]. Although these P450s showed low homology with each other in their amino acid sequences, they showed similar tertiary structures. Recently, Williams et al. [37] showed a tertiary structure of chimeric P450 between rabbit microsomal CYP2C3 and CYP2C5 as a first report showing the structure of membrane-bound P450. However, the tertiary structure of mammalian mitochondrial P450 has not been determined yet. As we have studied structure–function relationships of mitochondrial CYP27A1 and CYP27B1 [16,27,38–40], information on the tertiary structure of those P450s is invaluable for our studies. We expect that the tertiary structure of CYP105A1 with vitamin D_2 or D_3 will help in the understanding of the substrate binding of CYP27A1. In addition, information on the tertiary structure of CYP105A1 will help one to design a modified CYP105A1 with a higher catalytic activity or different substrate specificity.

Acknowledgments

We express our gratitude to Dr. I. Ogawa, Dr. F. Mukumoto, and Dr. T. Sato of Sumitomo Chemical Co., Ltd. for amino acid sequencing and useful discussions. We also thank Dr. K. Yamamoto of Tokyo Medical and Dental University for providing 25(OH)D_2 and 1α,25$(OH)_2D_2$. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

[1] D.C. Lamb, T. Skaug, H.L. Song, C.J. Jackson, L.M. Podust, M.R. Waterman, D.E. Kelly, S.L. Kelly, The cytochrome P450 complement (CYPome) of *Streptomyces coelicolor* A3(2), J. Biol. Chem. 277 (2002) 24000–24005.

[2] F.G. Healy, S.B. Krasnoff, M. Wach, D.M. Gibson, R. Loria, Involvement of a cytochrome P450 monooxygenase in thaxtomin A biosynthesis by *Streptomyces acidiscabies*, J. Bacteriol. 184 (2002) 2019–2029.

[3] C. Bruntner, B. Lauer, W. Schwarz, V. Mohrle, C. Bormann, Molecular characterization of co-transcribed genes from *Streptomyces tendae* Tu901 involved in the biosynthesis of the peptidyl moiety of the peptidyl nucleoside antibiotic nikkomycin, Mol. Gen. Genet. 262 (1999) 102–114.

[4] R. Fouces, E. Mellado, B. Diez, J.L. Barredo, The tylosin biosynthetic cluster from *Streptomyces fradiae*: genetic organization of the left region, Microbiology 145 (Pt. 4) (1999) 855–868.

[5] N. Lomovskaya, S.L. Otten, Y. Doi-Katayama, L. Fonstein, X.C. Liu, T. Takatsu, A. Inventi-Solari, S. Filippini, F. Torti, A.L. Colombo, C.R. Hutchinson, Doxorubicin overproduction in *Streptomyces peucetius*: cloning and characterization of the dnrU ketoreductase and dnrV genes and the doxA cytochrome P-450 hydroxylase gene, J. Bacteriol. 181 (1999) 305–318.

[6] L. Tang, S. Shah, L. Chung, J. Carney, L. Katz, C. Khosla, B. Julien, Cloning and heterologous expression of the epothilone gene cluster, Science 287 (2000) 640–642.

[7] R.J. Walczak, J.V. Hines, W.R. Strohl, N.D. Priestley, Bioconversion of the anthracycline analogue desacetyladriamycin by recombinant DoxA, a P450-monooxygenase from *Streptomyces* sp. strain C5, Org. Lett. 3 (2001) 2277–2279.

[8] T. Matsuoka, S. Miyakoshi, K. Tanzawa, K. Nakahara, M. Hosobuchi, N. Serizawa, Purification and characterization of cytochrome P-450sca from *Streptomyces carbophilus*. ML-236B (compactin) induces a cytochrome P-450sca in *Streptomyces carbophilus* that hydroxylates ML-236B to pravastatin sodium (CS-514), a tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme-A reductase, Eur. J. Biochem. 184 (1989) 707–713.

[9] N. Serizawa, T. Matsuoka, A two component-type cytochrome P-450 monooxygenase system in a prokaryote that catalyzes hydroxylation of ML-236B to pravastatin, a tissue-selective inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, Biochim. Biophys. Acta 1084 (1991) 35–40.

[10] J. Sasaki, A. Mikami, K. Mizoue, S. Omura, Transformation of 25- and 1 alpha-hydroxyvitamin D_3 to 1 alpha, 25-dihydroxyvitamin D_3 by using *Streptomyces* sp. strains, Appl. Environ. Microbiol. 57 (1991) 2841–2846.

[11] J. Sasaki, A. Miyazaki, M. Saito, T. Adachi, K. Mizoue, K. Hanada, S. Omura, Transformation of vitamin D_3 to 1alpha,25-dihydroxyvitamin D_3 via 25-hydroxyvitamin D_3 using *Amycolata* sp. strains, Appl. Microbiol. Biotechnol. 38 (1992) 152–157.

[12] H. Kawauchi, J. Sasaki, T. Adachi, K. Hanada, T. Beppu, S. Horinouchi, Cloning and nucleotide sequence of a bacterial cytochrome P-450VD25 gene encoding vitamin D_3 25-hydroxylase, Biochim. Biophys. Acta 1219 (1994) 179–183.

[13] C.A. Omer, R. Lenstra, P.J. Litle, C. Dean, J.M. Tepperman, K.J. Leto, J.A. Romesser, D.P. O'Keefe, Genes for two herbicide-inducible cytochromes P-450 from *Streptomyces griseolus*, J. Bacteriol. 172 (1990) 3335–3345.

[14] D.P. O'Keefe, K.R. Bozak, R.E. Christoffersen, J.A. Tepperman, C. Dean, P.A. Harder, Endogenous and engineered cytochrome P-450 mono-oxygenases in plants, Biochem. Soc. Trans. 20 (1992) 357–361.

[15] M. Taylor, D.C. Lamb, R. Cannell, M. Dawson, S.L. Kelly, Cytochrome P450105D1 (CYP105D1) from *Streptomyces griseus*: heterologous expression, activity, and activation effects of multiple xenobiotics, Biochem. Biophys. Res. Commun. 263 (1999) 838–842.

[16] N. Sawada, T. Sakaki, M. Ohta, K. Inouye, Metabolism of vitamin D_3 by CYP27A1, Biochem. Biophys. Res. Commun. 273 (2000) 977–984.

[17] M. Akiyoshi-Shibata, T. Sakaki, Y. Ohyama, M. Noshiro, K. Okuda, Y. Yabusaki, Further oxidation of hydroxycalcidiol by calcidiol 24-hydroxylase, Eur. J. Biochem. 224 (1994) 335–343.

[18] S. Kondo, T. Sakaki, H. Ohkawa, K. Inouye, Electrostatic interaction between cytochrome P450 and NADPH-P450 reductase: comparison of mixed and fused systems consisting of rat cytochrome P4501A1 and yeast NADPH-P450 reductase, Biochem. Biophys. Res. Commun. 257 (1999) 273–278.

[19] T. Omura, R. Sato, The carbon monoxide-binding pigment of liver microsomes. II. Solubilisation, purification, and properties, J. Biol. Chem. 239 (1964) 2379–2385.

[20] D.J. Waxman, C. Walsh, Phenobarbital-induced rat liver cytochrome P-450. Purification and characterization of two closely related isozymic forms, J. Biol. Chem. 257 (1982) 10446–10457.

[21] D.P. O'Keefe, K.J. Gibson, M.H. Emptage, R. Lenstra, J.A. Romesser, P.J. Litle, C.A. Omer, Ferredoxins from two sulfonylurea herbicide monooxygenase systems in *Streptomyces griseolus*, Biochemistry 30 (1991) 447–455.

[22] K. Nakagawa, Y. Sowa, M. Kurobe, K. Ozono, M.L. Siu-Caldera, G.S. Reddy, M.R. Uskokovic, T. Okano, Differential activities of 1α,25-dihydroxy-16-ene-vitamin D_3 analogs and their

3-epimers on human promyelocytic leukemia (HL-60) cell differentiation and apoptosis, Steroids 66 (2001) 327–337.
[23] A. Hiwatashi, Y. Nishii, Y. Ichikawa, Purification of cytochrome P-450D1α (25-hydroxyvitamin D_3-1α-hydroxylase) of bovine kidney mitochondria, Biochem. Biophys. Res. Commun. 105 (1982) 320–327.
[24] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[25] H.A. Hussain, J.M. Ward, Enhanced heterologous expression of two *Streptomyces griseolus* cytochrome P450s and *Streptomyces coelicolor* ferredoxin reductase as potentially efficient hydroxylation catalysts, Appl. Environ. Microbiol. 69 (2003) 373–382.
[26] Y.D. Guo, S. Strugnell, D.W. Back, G. Jones, Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions, Proc. Natl. Acad. Sci. USA 90 (1993) 8668–8672.
[27] S. Kitanaka, K. Takeyama, A. Murayama, T. Sato, K. Okumura, M. Nogami, Y. Hasegawa, H. Niimi, J. Yanagisawa, T. Tanaka, S. Kato, Inactivating mutations in the 25-hydroxyvitamin D_3 1alpha-hydroxylase gene in patients with pseudovitamin D-deficiency rickets, N. Engl. J. Med. 338 (1998) 653–661.
[28] G.K. Fu, D.L. Martin, Y.H. Zhang, D.D. Bikle, C.H.L. Shackleton, W.L. Miller, A.A. Portale, Cloning of human 25-hydroxyvitamin D_3-1α-hydroxylase and mutations causing vitamin D dependent rickets type I, Mol. Endocrinol. 11 (1997) 1961–1970.
[29] T.L. Poulos, B.C. Finzel, I.C. Gunsalus, G.C. Wagner, J. Kraut, The 2.6-Å crystal structure of *Pseudomonas putida* cytochrome P-450, J. Biol. Chem. 260 (1985) 16122–16130.
[30] K.G. Ravichandran, S.S. Boddupalli, C.A. Hasermann, J.A. Peterson, J. Deisenhofer, Crystal structure of hemoprotein domain of P450BM-3, a prototype for microsomal P450's, Science 261 (1993) 731–736.
[31] C.A. Hasemann, K.G. Ravichandran, J.A. Peterson, J. Deisenhofer, Crystal structure and refinement of cytochrome P450terp at 2.3 Å resolution, J. Mol. Biol. 236 (1994) 1169–1185.
[32] J.R. Cupp-Vickery, T.L. Poulos, Structure of cytochrome P450eryF involved in erythromycin biosynthesis, Nat. Struct. Biol. 2 (1995) 144–153.
[33] S.Y. Park, H. Shimizu, S. Adachi, A. Nakagava, I. Tanaka, K. Nakahara, H. Shoun, E. Obayashi, H. Nakamura, T. Iizuka, Y. Shiro, Crystal structure of nitric oxide reductase from denitrifying fungus *Fusarium oxysporum*, Nat. Struct. Biol. 4 (1997) 827–832.
[34] J.K. Yano, L.S. Koo, L.D.J. Schuller, H. Li, P.R.O. Montellano, T.L. Poulos, Crystal structure of a thermophilic cytochrome P450 from the Archaeon *Sulfolobus solfataricus*, J. Biol. Chem. 275 (2000) 31086–31092.
[35] L.M. Podust, T.L. Poulos, M.R. Waterman, Crystal structure of cytochrome P450 14alpha-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors, Proc. Natl. Acad. Sci. USA 98 (2001) 3068–3073.
[36] L.M. Podust, Y. Kim, M. Arase, B.A. Neely, B.J. Beck, H. Bach, D.H. Sherman, D.C. Lamb, S.L. Kelly, M.R. Waterman, The 1.92-Å structure of *Streptomyces coelicolor* A3(2) CYP154C1. A new monooxygenase that functionalizes macrolide ring systems, J. Biol. Chem. 278 (2003) 12214–12221.
[37] P.A. Williams, J. Comse, V. Sridhar, E.F. Johnson, D.E. McRee, Mammalian microsomal cytochrome P450 monooxygenase: structural adaptations for membrane binding and functional diversity, Mol. Cell 5 (2000) 121–131.
[38] T. Sakaki, N. Sawada, K. Takeyama, S. Kato, K. Inouye, Enzymatic properties of mouse 25-hydroxyvitamin D_3 1α-hydroxylase expressed in *Escherichia coli*, Eur. J. Biochem. 259 (1999) 731–738.
[39] N. Sawada, T. Sakaki, S. Kitanaka, K. Takeyama, S. Kato, K. Inouye, Enzymatic properties of human 25-hydroxyvitamin D_3 1α-hydroxylase, Eur. J. Biochem. 265 (1999) 950–956.
[40] N. Sawada, T. Sakaki, S. Kitanaka, S. Kato, K. Inouye, Structure–function analysis of CYP27B1 and CYP27A1. Studies on mutants from patients with vitamin D-dependent rickets type I (VDDR-I) and cerebrotendinous xanthomatosis (CTX), Eur. J. Biochem. 268 (2001) 6607–6615.