Published in final edited form as:
Metabolism. 2007 September ; 56(9): 1248–1255. doi:10.1016/j.metabol.2007.04.023.

Mutational Analysis of CYP27A1: Assessment of 27-Hydroxylation of Cholesterol and 25-Hydroxylation of Vitamin D

Ram P. Gupta[1], **Kennerly Patrick**[2], and **Norman H. Bell**[1]

[1] Department of Medicine, Medical University of South Carolina, Strom Thurmond Research Building, 114 Doughty Street Charleston, SC 29403

[2] Department of Pharmaceutical Sciences, Medical University of South Carolina, Strom Thurmond Research Building, 114 Doughty Street Charleston, SC 29403

Abstract

The *CYP27A*1 gene encodes a mitochondrial enzyme which modulates the acidic biosynthetic pathway for bile acids beginning with the 27-hydroxylation of cholesterol. CYP27A1 also 25-hydroxylates vitamin D_3. Gene mutations cause cerebrotendinous xanthomatosis (CTX), an autosomal recessive disorder, and may cause 25-hydroxyvitamin D deficiency and early onset osteoporosis and fractures in affected patients. To examine the effects of mutations of CYP27A1 on vitamin D and cholesterol hydroxylating activity, recombinant CYP27A1 and mutant cDNAs produced by site-directed mutagenesis were stably expressed in either *Escherichia coli* or COS-1 cells. Activities of wild type and mutant enzymes were determined with cholesterol, vitamin D_3, and 1a-hydroxyvitamin D_3 ($1aOHD_3$) as substrates. Of the 15 mutants tested, 11 expressed protein and 4 expressed little or no protein. Functional heme activity, estimated by reduced CO difference spectra at 450 nm, was absent in 12 mutants. When expressed in *E. coli*, three mutants, K226R, D321G, and P408S, each known to cause clinically CTX, showed modest decreases in reduced CO spectra peak and either no change or decreases of less than 50% in hydroxylation of cholesterol, vitamin D_3 and $1aOHD_3$ compared to wild type. When expressed transiently in COS-1 cells, each of these mutants showed 25-hydroxylation activity for $1aOHD_3$ as well as wild type. Thus, 3 mutants, K226R, D321G, and P408S, known to occur clinically with non-functioning mutants, hydroxylated cholesterol, vitamin D_3 and $1aOHD_3$. How they contribute to the pathogenesis of CTX despite being biologically active in vitro remains to be determined.

Keywords

CYP, cytochrome P450; CTX, cerebrotendinous xanthomatosis; $25\text{-}OHD_3$, 25-hydroxyvitamin D_3; 1, $25\text{-}(OH)_2D_3$; 1, 25-dihydroxyvitamin D_3; $1\alpha OHD_3$, 1α-hydroxyvitamin D_3

In humans, 25-hydroxylation of vitamin D takes place in both liver microsomes and mitochondria. 25-Hydroxylation of vitamin D_3 in human liver was first demonstrated in partially purified mitochondrial membranes (1,2). CYP27A1 was later identified as the mitochondrial enzyme responsible for 25-hydroxylation of vitamin D_3 and not vitamin D_2

Address all correspondence and requests for reprints to: Norman H. Bell, M.D., Department of Medicine, Medical University of South Carolina, Strom Thurmond Research Building, Room 550, 114 Doughty Street, Charleston, SC 29403, TEL: (843) 876-5162, FAX (843) 876-5163, Email: E-mail: belln@musc.edu.

(3,4). Whether CYP27A1 was the sole or the major enzyme responsible for 25-hydroxylation of vitamin D was unclear. Recent studies identified CYP2R1 as human microsomal vitamin D 25-hydroxylase (5), an enzyme which hydroxylates both vitamin D_2 and vitamin D_3 (5,6). The finding of a homozygous inactivating mutation of *CYP2R1* gene in two Nigerian brothers with rickets caused by isolated 25-hydroxyvitamin D (25-OHD) deficiency established its physiologic role in vitamin D biology (7,8). A role for CYP27A1 in this regard is not established.

In mammalian liver, conversion to bile acids through two pathways, the neutral pathway and the acidic pathway (9) is the principal means for catabolism and removal of cholesterol from the body. The acidic pathway begins with 27-hydroxyation by the mitochondrial enzyme CYP27A1 (10). CYP27A1 further oxidizes the side chain of 27-hydroxycholesterol to an aldehyde and to 3β-hydroxy-5-cholestenoic acid (11). As noted, the enzyme also 25-hydroxylates vitamin D_3 but not vitamin D_2 (3,4). In humans, mutations of the *CYP27A1* gene cause cerebrotendinous xanthomatosis (CTX), an autosomal recessive disorder characterized by abnormal synthesis of bile acids with development of cataracts, tendon xanthomas, and progressive neurological deterioration (12–14). Some patients with CTX have a low or low normal serum 25-OHD and a low bone mass which lead to severe early onset osteoporosis and fractures (15,16). A phenotype-genotype analysis of 79 patients with CTX having 23 different homozygous mutations from 45 families did not find any correlation (13). The purpose of the present studies was to determine whether missense mutations that cause CTX alter 25-hydroxylation of vitamin D_3 and 1α-hydroxyvitamin D_3 ($1\alpha OHD_3$) and whether active sites for 27-hydroxylation of cholesterol and 25-hydroxylation of vitamin D_3 are different. To perform molecular mapping of active sites, enzyme activities of recombinant wild type and mutant CYP27A1 proteins produced by site-directed mutagenesis and expressed in *Escherichia coli* and COS-1 cells were compared.

MATERIALS AND METHODS

Materials

Vitamin D_3, 1,25-dihydroxyvitamin D_3 [$1,25\text{-}(OH)_2D_3$] and cholesterol were purchased from Sigma Aldrich (St. Louis, MO) and acetonitrile, dichloroethane, hexanes, methanol and 2-propanol from Fisher Scientific (Norcross, GA). [^{3}H-26,27-methyl]-$1,25\text{-}(OH)_2D_3$, [^{3}H-26,27-methyl]-$25\text{-}OHD_3$ and [^{14}C-4]-cholesterol from Amersham Biosciences (Piscataway, NJ) and 27-hydroxycholesterol from Research Plus, Inc. (Manasquan NJ). $1\alpha OHD_3$ was generously provided by Leo Pharmaceutical Products, Ltd. (Ballerup, Denmark). Micro-to-Midi Total RNA Purification System was purchased from Invitrogen Life Technologies (Carlsbad, CA). Recombinant bovine adrenodoxin and adrenodoxin reductase were expressed and purified by the methods of Sagara et al. (17,18).

Site-directed mutagenesis

Site-directed mutagenesis was carried out with the QuikChange site-directed mutagenesis kit as described by the manufacturer (Stratagene, LaJolla, CA). Oligonucleotide primers used for each site-mutation in wild-type plasmid CYP27A1 DNA are shown in Table 1. Mutations were confirmed by sequencing the mutated portion of DNA. The mutated DNA and wild-type CYP27A1 were then digested with restriction enzymes (Table 1), and the fragment containing the mutation was cloned into the digested wild-type CYP27A1 plasmid cDNA. Both ends of mutated cDNA fragments were sequenced to confirm correct ligation at restriction sites as well as the sequence of mutated fragment.

Preparation of membranes from wild-type CYP27A1 and its mutants

pTrcCYP27A1 expression vector (CYP27A1 in pTrc99A vector and CYP27A1 K226R mutant in pTre99A vector) (generous gifts of Irene Pikuleva, University of Texas Medical Branch, Galveston, TX) were prepared as described (19). Competent *E. coli* DH5αF′IQ cells (Invitrogen, Grand Island, NY) were transformed with either wild-type CYP27A1 or mutant cDNAs. Recombinant proteins were expressed, membrane extracts were purified essentially as described (19,20), and membranes were isolated and suspended in 10 mM phosphate buffer pH 7.4 containing 20% glycerol, and protease inhibitors. The heme content was measured by reduced CO difference spectra (21). Protein concentration was determined by the bicinchoninic acid method with a kit (Pierce Chemicals, Rockford, IL) (22).

RT-PCR of CYP27A1 and its mutants

Total RNA was extracted from the overnight cultures of *E. coli* containing recombinant CYP27A1 and its mutants with the RNeasy Mini Kit (Qiagen, Valencio, CA). cDNAs were synthesized by the Reverse Transcription System (Promega, Madison, WI) at 48° C for 1 hr followed by incubation at 95° C for 5 min as recommended by the manufacturer. Amplification of CYP27A1 was performed with True Allele PCR Premix (PE Applied Biosystems, Foster City, CA) as follows: 10 min at 95° C, one cycle; 45 sec at 95° C, 45 sec at 60° C, and 1.5 min at 72° C, 30 cycles. Sense and anti-sense primers for CYP27A1 were: 5′-AACACCCAGTTTGTGTTCTGCCAC-3′ and 5′-AGGAACTGCAGGCCCACTTTCTTA-3′, respectively. The PCR bands were quantified by analysis performed on a computer by using the public domain NIH Image program developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

Western blot analysis

Purified membrane proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred onto PVDF (polyvinylidene difluoride) membrane, blocked, and incubated with polyclonal anti-CYP27A1 antibody (a generous gift of David Russell, University of Texas Southwestern Medical Center, Dallas, TX, 1:2000 dilution) overnight at 4°C. The blots were washed, blocked, and again incubated with HRP-conjugated anti-rabbit IgG (1:2000) at room temperature for 1.5 h. Proteins were visualized with SuperSignal West Pico chemiluminescent substrate as described by the manufacturer (Pierce, Rockford, IL).

Assay for vitamin D 25-hydroxylase activity

Membrane fractions of CYP27A1 and its mutants expressed in *E. coli*, which showed protein expression and functional heme activity as determined by reduced CO difference spectra, were used to assay 25-hydroxylation of $1\alpha OHD_3$ and vitamin D_3. Wild type and mutant CYP27A1 proteins were reconstituted by incubating CYP450 (0.002–0.004 μM), adrenodoxin (2.0 μM), and adrenodoxin reductase (0.25–0.5 μM) in 70 μl at room temperature for 10 min. The reconstituted enzyme complex was used to hydroxylate 1α $(OH)D_3$ (10 μM) or vitamin D_3 (25 μM) in a total volume of 1 ml as described (23). The reaction was carried out for 10 min. The reaction mixtures were extracted with acetonitrile and subjected to solid phase extraction. The extracts containing the hydroxylated products were evaporated and estimated by HPLC analysis (23,24).

Extraction of samples

The acetonitrile mixture containing $1\alpha OHD_3$ or vitamin D_3 metabolites was mixed with a vortex, and 1,000 cpm $[^3H]$-1,25-$(OH)_2D_3$ or $[^3H]$-25-OHD_3 was added, respectively, for recovery and then centrifuged at 4° C and 2,000 × g for 15 min (23). The supernatant was decanted to another 13 × 100 mm glass tube containing 1 ml of 0.4 M K_2HPO_4 (pH 10.4, pH

adjusted with KOH). The solution containing 1,25-$(OH)_2D_3$ was mixed and transferred to a silica C18OH Cartridge (DiaSorin, Stillwater, MN) that had been conditioned twice with 1.5 ml of methanol. The cartridge was washed with 5 ml solvent A (methanol: water, 70:30), 5 ml solvent B (hexanes: dichloromethane, 88:12) and eluted with 5 ml solvent C (hexanes:2-propanol, 95:5) (23). The cartridges were washed with 1 ml 2-propanol and conditioned with methanol as described above for further use. The solution containing 25-OHD_3 was mixed and transferred to a silica C18 Cartridge (DiaSorin, Stillwater, MN) that had been conditioned as described above. The column was washed with 5 ml of solvent A and then eluted with 3.5 ml of acetonitrile. The eluted extracts were evaporated, the residue was dissolved in 200 μl dichloromethane:hexanes:2-propanol, 50:50:2.5 and subjected to HPLC.

Isolation and measurement of 1,25$(OH)_2D_3$

Extracts containing 1αOHD_3 metabolites in 200 μl solution were loaded onto a Zorbax Sil 4.6 X 250 mm column and then separated by HPLC with hexanes:2-propanol, 85:15. 1,25 $(OH)_2D_3$ was quantified by measuring the area of the separated peak which eluted at 8.5 min. Recovery was assessed with [^{3}H]-1,25-$(OH)_2D_3$ as previously described (23). Extracts containing vitamin D_3 metabolites in 200 μl solution were also loaded onto a Zorbax Sil 4.6 X 250 mm column, but were separated by running hexanes:2-propanol (96:4) for 9 min, followed by hexanes:2-propanol (85:15) for 16 min. The column was again equilibrated for 3 min with hexanes:2-propanol (96:4) before loading the next sample for analysis. 25-OHD_3 was quantified by measuring the area of the separated peak which eluted at 10.1 min. Recovery was assessed with [^{3}H]-25-OHD_3 as described (23). Results are expressed as pmol/mg protein/min for recombinant cytochrome P-450 enzymes expressed in *E. coli.*

Assay for cholesterol 27-hydroxylase activity

Active membrane fractions containing wild type or mutant CYP27A1 were reconstituted by incubating them with adrenodoxin and adrenodoxin reductase in 70 μl at 4° C for 10 min. The reaction mixture contained 0.004 μM P450, 2.0 μM adrenodoxin, 0.5 μM adrenodoxin reductase, 100 μl of 0.5 M Na_2HPO_4, 100 μl of 3% poly(vinyl alcohol) (Mw. 13000–23000), 50 μl of NADPH regenerating solution A (BD Gentest), 10 μl of regenerating solution B (BD Gentest), 0.025 μCi of [4-^{14}C]cholesterol (specific activity 57.0 mCi/mmol), and 25 μM cholesterol in a total volume of 1 ml. Cholesterol was added as a 20 μl solution in 45% aqueous solution of 2-hydroxypropyl-β-cyclodextrin. The reaction was carried out at 37° C for 60 min, and the reaction mixture was extracted with 3 volumes of ethyl acetate. The extract was evaporated under N_2 and subjected to HPLC on a Develosil ODS-UG-5 column (4.6 X 150 mm). A linear gradient from solvent A (acetonitrile: methanol: water: 40:40:20) to 100% methanol (1.5 ml/min) was run for 15 min. This was followed by the flow of 100% methanol for 15 min. The column was again equilibrated with solvent A for 4 min before loading the next sample for HPLC. One ml fractions were collected from 0 to 30 min, evaporated, and counted for ^{14}C radioactivity. The fraction of cholesterol converted into 27-hydroxycholesterol was calculated from the fraction of total radioactivity appearing as 27-hydroxycholesterol. The total radioactivity represented 25 nmol of cholesterol. The retention times for 27-hydroxycholesterol and cholesterol were 12 and 22 min, respectively. Results are expressed as nmole/mg protein/min.

Gas chromatography-mass spectrometry (GC-MS)

An Agilent Model 6890 GC-5973N MS with automatic sampler and MSD Productivity Chem Station were used for GC-MS analyses. The MS was calibrated with perfluorotributylamine (FC-43), ionization was carried out by electron impact, and detection by total ion monitoring. The HPLC eluate fraction corresponding to the elution time of 27-hydroxycholesterol established in the radiolabeled experiment was collected, evaporated to dryness under N_2, then

derivatized in a silanized microvial insert within a Teflon-capped automated sampler vial. 20 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA): trimethyl-chlorosilane (TMC) 99:1 (Supelco, Bellefonte, PA) was used for derivatization to form bis-trimethylsilyl (TMS) products. GC injection (2 μl) was by the pulsed-splitess mode. A 30 m × 0.32 mm, 0.25 μm film thickness (DB-5; J & W Scientific, Folsom, CA) phenylmethy-polysiloxanes fused silica column held at 70° C for 1.5 min, then ramped at 20° C/min and held at 300° C was used for chromatographic separation. The injector port was operated at 250° C. The helium carrier gas flow rate was 1.2 mL/min.

Measurement of reduced CO difference spectra

Reduced CO difference spectra was determined by the method of Omura and Sato (21).

Expression of CYP27A1 and its mutants in COS-1 cells

pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA) was modified to express CYP27A1 and its mutants in COS-1 cells. pcDNA3.1(+) vector was digested with HindIII and BamHI, blunted with Klenow fragment (New England BioLabs, Beverly, MA), and ligated to form pcDNAm1 vector. pcDNAm1-CYP27A1 was prepared by digesting pUC27A1 with EcoRI and cloning CYP27A1 fragment in pcDNAm1 vector digested with the same restriction enzyme. The orientation of CYP27A1 was confirmed by sequencing with primer 7 T7. The expression mutants of CYP27A1 (pcDNAm1D321G, pcDNAm1P408S, pcDNAAm1K226R) were prepared by digesting the corresponding mutants in pTrc99A vector with KpnI and HindIII and cloning the mutated fractions in pcDNAm1-CYP27A1 digested with the same restriction enzymes. The ligations at restriction sites were confirmed by sequencing.

Transfection of COS-1 cells

COS-1 cells (American Type Tissue Cells, Manassas, VA) were seeded in 60-mm dishes at a density of 0.5×10^6 cells/dish in DMEM medium with glutamate (2 mM), sodium pyruvate (1 mM), gentamycin (10 μg/ml), and 10% fetal bovine serum. The cells were transfected next day with the vector (pcDNAm1, 2.5 μg) alone, or CYP27A1 (2.5 μg), D321G (2.5 μg), P408S (2.5 μg), and K226R (2.5 μg) in the same vector. The transfection was performed for 3 h in serum-free medium without antibiotic as recommended by the manufacturers (Invitrogen, Carlsbad, CA) with Lipofectamine and PLUS reagent. Half the volume of medium with 3-fold FBS concentration was added and cells were incubated at 37° C in 95% air and 5% CO_2.

Assay of vitamin D 25-hydroxylase in COS-1 cells

After 24 h, the transfected COS-1 cells were washed once with 3 ml of serum-free medium and treated with $1\alpha OHD_3$ (8.5 μM) in 4 ml of serum-free medium for 48 h at 37° C in 95% air and 5% CO_2. The $1\alpha OHD_3$ solution was evaporated to dryness under N_2, dissolved in DMSO, and diluted in medium before adding to the dishes. The final concentration of DMSO in the medium was 0.3 percent. The reaction was stopped by adding 4 ml of acetonitrile. [^{3}H]-1,25-$(OH)_2D_3$ (2,000 cpm) was added to the cells after termination of reaction to calculate the recovery of 1,25-$(OH)_2D_3$. The metabolites were extracted and subjected to HPLC as described (23). The peak containing 1,25-$(OH)_2D_3$ was evaporated under nitrogen and dissolved in 1 ml of ethanol. Aliquots were used for estimation of 1,25-$(OH)_2D_3$ by radioimmunoassay and liquid scintillation counting as previously described (23).

RESULTS

Wild type CYP27A1 and 15 recombinant missense mutant cDNAs, identified as causing CTX, were expressed in *E. coli.* Their locations in the protein and putative functions are summarized in Table 2 (25), and locations are shown graphically in a model modified from Prosser et al.

(26) in Figure 1. All of them expressed mRNA as indicated by RT-PCR (Fig. 2A). Western blot analyses showed all but four (A183P, R362H, R362H, and R372G) expressed protein levels comparable to the wild-type enzyme (Fig. 2B), suggesting that these four mutations may result in unstable proteins that were probably rapidly degraded. Twelve mutants did not produce a reduced CO difference (data not shown), indicating possible impaired interaction between the cysteinyl thiolate ligand and heme iron. Three remaining mutants, K226R, D321G, and P408S, showed a reduced CO difference spectra and had vitamin D 25-hydroxylase and cholesterol 27-hydroxylase activities that were the same or lower by as much as 50 percent compared to wild-type CYP27A1 when expressed in *E. coli* (Table 3). The mutants also showed vitamin D 25-hydroxylase activity which was the same or higher by as much as 54 percent compared to wild type when expressed in COS-1 cells (Table 4). Thus, the mutant enzymes either were active as regards hydroxylation of vitamin D_3, $1aOHD_3$ and cholesterol or presumably were inactive because of diminished or absent expression of protein, or more commonly because of impaired heme function as indicated by an inability to produce a reduced CO difference spectra.

Under the prescribed conditions for GC-MS, both the reference standard and metabolically formed 27-hydroxycholesterol, as their TMS derivatives, eluted at 19.9 min after injection (data not shown). In both cases, the mass spectra included the molecular ion *m/z* 546 (45% relative abundance), 456 (80%; M-TMS), 417 (75%) and 129 (100%) as described by others (27), confirming the identity of the sterol. BSTFA derivatizing reagent TMC was required to effectively silylate the 27-hydroxycholesterol.

Thus, three mutants of CYP27A1 which are causally related to CTX show hydroxylating activity for vitamin D and cholesterol which is tends to be lower than wild type when expressed in *E. coli* and higher than wild type when expressed in COS-1 cells.

DISCUSSION

Some 38 different mutations of the *CYP27A1* gene causing CTX have been reported (12-14). The majority of these mutations do not result in predicted protein expression. Most disrupt splicing and either cause instability of the message, abnormal splicing resulting in rapid degradation of the mRNA or frame-shift and premature chain termination. A few produce a nonsense stop codon and premature translational stop. Missense mutations are ones that may result in protein expression, but since they are causative for CTX are assumed to be functionally null. In patients with CTX, phenotype and genotype do not correlate, and there is considerable intrafamilial variation in phenotype (13).

In the present study, we report that of 15 recombinant missense mutant cDNAs in *E. coli,* all expressed mRNA, 11 expressed protein, and only four expressed little or no protein. Twelve mutants did not show a reduced CO difference spectra, suggesting that these mutations disrupt the heme-binding domains (14). These proteins are essentially inactive, as heme binding is necessary for this P450 enzyme to function. These mutations may impair the interaction between the cysteinyl thiolate ligand and heme iron. The remaining three mutants (K226R, D321G and P408S) showed a reduction in CO difference spectra and had vitamin D 25-hydroxylase and cholesterol 27-hydroxylase activities which were the same or lower compared to wild type enzyme. In patients with CTX, the K226R, D312G and P408S mutations occur as heterozygous mutations in association with R362S or R372G (13,14). These mutant proteins have been reported to show little or no biologic activity compared to CYP27A1 when expressed in COS cells (29,30). We found that the K226R, D312G and P408S mutations expressed in COS-1 cells showed vitamin D 25-hydroxylase activity that was the same or higher compared to wild type. It appears therefore that despite demonstrating enzyme activity in vitro, these mutant proteins are unable to prevent CTX. This suggests that the enzymatic site responsible

for cholesterol oxidation in mitochondria is somehow compromised in affected patients. In addition, patients with these mutations may be of interest with respect to their circulating 25-OHD values and whether they are less predisposed to osteoporosis.

Prosser et al. (26) constructed a homology-based model of CYP27A1 which was derived from a systemic analysis of hydrogen bonding patterns of 11 prokaryotic and mammalian cytochrome P450 crystal structures (Fig. 1). Potential contact residues in the F-helix, β-3 sheet and β-5 sheet were identified in computational docking studies with vitamin D_3 and $1\alpha OHD_3$. Verification of these sites as important in regulating enzyme activity was accomplished in studies with site-directed mutagenesis of non-naturally occurring mutations expressed in COS-1 cells which altered the metabolism of $1\alpha OHD_3$ to favor either 25 or 27 hydroxylation. The findings indicated that conserved hydrophobic residues in the β-5 hairpin interact with residue F215 in the F-helix and influence the shape of the substrate binding cavity. A possible heme-binding interaction at residue N370 and a structural role for T369 and S371 were implicated by mutations in the β-3a strand (26).

Mast et al. (31) utilized computer modeling and studies of site-directed mutagenesis of CYP27A1 with non-naturally occurring mutants expressed in *E. coli* and cholesterol, 5β-cholestane-3a,7a,12a-triol, and their derivatives as substrates to identify active site residues and substrate orientation. Results of modeling studies indicated that some residues bound both substrates and others only one of them. Mutations of overlapping substrate-contacting residues W100, H103, T110, M301C, V367, I481 and V482 were found to influence binding and enzyme activity in a substrate-dependent manner and to permit identification of interaction with key side chains. For example, T110 was thought to interact with the 12a-hydroxyl of 5β-cholestane-3a,7a,12a-triol and V367 to be critical for optimal positioning of the cholesterol C26 methyl group and for regioselective hydroxylation of this sterol (31).

With regard to the naturally occurring mutations, R94, R372 and R441 are thought to be involved in heme binding, R446 in ferredoxin binding, A138 and T306 in either the O_2 binding site and its stability, respectively, R362 and P408 in the ERR triad, a structural motif between the K-helix and meander regions, or its stability, and P368 in stabilization of the β-3 sheet (Table 2) (25,26). The ERR-triad apparently serves as a folding motif which stabilizes heme-binding and may play a role in redox-partner binding (32). R104, K226, and D321 are thought to provide structural integrity for the B′-helix, F-G loop, and I-J loop, respectively.

In a previous study, the K226R mutation was found to show a reduced CO difference spectra and an almost 80 percent reduction in cholesterol 27-hydroxylase activity compared to wild type when expressed in *E. coli* (20). In the present study, no alteration in cholesterol 27-hydroxylase activity was found. Differences in methods may account for the differences in results. Two other mutants, D321G and P408S, showed a reduced CO difference spectra and vitamin D 25-hydroxylase and cholesterol 27-hydroxylase activities which were modestly reduced compared to wild type CYP27A1.

As noted, CYP2R1 was identified as human microsomal vitamin D 25-hydroxylase and is likely the vitamin D 25-hydroxylase (5). Its physiological importance was documented by the finding that an inactivating homozygous L99P gene mutation was the cause of isolated 25-OHD deficiency and rickets in two young Nigerian brothers (7,8). An argument can be made that CYP27A1 may also play a role in vitamin D metabolism. Again, some patients with CTX have a low bone mass and early onset of severe osteoporosis and fractures associated with a low or low normal serum 25-OHD (15,16). Bile acid metabolism is altered in CTX. Since vitamin D requires solubilization by bile acids for absorption, impaired absorption of the vitamin could occur. However, the possibility that alteration of bile acid synthesis could interfere with the enterohepatic circulation of vitamin D or its metabolites is unlikely. Studies

with radioactive vitamin D in patients with bile fistulas showed that less than 4 percent of metabolites excreted in bile were present as 25-OHD or its glucuronide conjugate (33). Since vitamin D_3 is synthesized in skin and it and its metabolites are the major circulating form, it is unlikely that interference with enterohepatic circulation of vitamin D caused by abnormal bile acid metabolism would contribute to abnormal vitamin D metabolism in patients with CTX. Expression of CYP27A1 in human liver and kidney was found to vary significantly with season and to correlate with serum 25-OHD (34). The fact that an inactivating mutation of CYP2R1 produces a greater reduction in serum 25-OHD and hypocalcemia and more overt bone disease than mutations of CYP27A1 may be reflected in part by the relative biologic activities of the two enzymes. Thus, 25-hydroxylation of vitamin D_3 is 3.6-fold higher and affinity for vitamin D_3 is 7-fold higher for CYP2R1 than for CYP27A1 (6).

In summary, three of 15 mutations of CYP27A1 associated with CTX, K226R, D321G, and P408S, showed the same or lower values in 25 hydroxylation of vitamin D_3 and 27 hydroxylation of cholesterol compared to wild type when expressed in *E. coli*, and the same or higher values in hydroxylation of vitamin D_3 than wild type when expressed in COS-1 cells. In patients with CTX, the three mutants are known to occur in association with mutations R362A or R372G which have little or no biologic activity (28,29). The remaining twelve mutant proteins were either poorly expressed or did not produce a peak for reduced CO difference spectra. In most patients with CTX, CYP27A1 is either not expressed or is biologically inactive as regards 25 hydroxylation of vitamin D. Life-long deficiency of circulating 25-OHD may account for the early development of osteoporosis and fractures in some patients with this disorder. Evaluation of vitamin D metabolism and possible osteoporosis in patients with CTX should be considered.

Acknowledgments

These studies were supported by National Institute of Diabetes and Digestive and Kidney Disease Grant DK-56603-01A1 (to N. H. Bell).

References

1. Oftebro H, Saarem K, Bjorkhem I, et al. Side chain hydroxylation of C27-steroids and vitamin D_3 by a cytochrome P-450 enzyme system isolated from human liver mitochondria. J Lipid Res 1981;22:1254–1264. [PubMed: 6274987]
2. Saarem K, Bergseth S, Oftebro H, et al. Subcellular localization of vitamin D_3 25-hydroxylase in human liver. J Biol Chem 1984;259:10936–10940. [PubMed: 6469991]
3. Guo Y-D, Strugnell S, Back DW, et al. Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. Proc Natl Acad Sci USA 1993;90:8668–8672. [PubMed: 7690968]
4. Sawada N, Sakaki T, Ohta M, et al. Metabolism of vitamin D(3) by human CYP27A1. Biochem Biophys Res Commun 2000;273:977–984. [PubMed: 10891358]
5. Cheng JB, Motola DL, Mangelsdorf DJ, et al. Deorphanization of cytochrome P450 CYP2R1, a microsomal vitamin D 25-hydroxylase. J Biol Chem 2003;278:38,084–38,093.
6. Shinkyo R, Sakaki T, Kamakura M, et al. Metabolism of vitamin D by human microsomal CYP2R1. Biochem Biophys Res Commun 2004;324:451–457. [PubMed: 15465040]
7. Cheng JB, Levine MA, Bell NH, et al. Genetic evidence that human CYP2R1 is a key vitamin D 25-hydroxylase. Proc Natl Acad Sci USA 2005;101:711–715.
8. Dong Q, Miller W. Vitamin D 25-hydroxylase deficiency. Mol Genet Metab 2004;83:197–198. [PubMed: 15464435]
9. Fuchs M. Bile acid regulation of hepatic physiology: III. Regulation of bile acid synthesis: past progress and future challenges. Am J Physiol Gastroenterol Liver 2003;284:551–557.

10. Cali JJ, Russell DW. Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P-450 that catalyzes multiple oxidation reaction in bile acid biosynthesis. J Biol Chem 1991;266:7774–7778. [PubMed: 1708392]
11. Pikuleva IA, Babiker A, Waterman MR, et al. Activities of recombinant human cytochrome P450c27 (CYP27) which produce intermediates of alternative bile acid biosynthetic pathways. J Biol Chem 1981;273:18153–18160. [PubMed: 9660774]
12. Cali JJ, Hsieh CL, Francke U, et al. Mutations in the bile acid biosynthetic enzyme sterol 27-hydroxylase underlie cerebrotendinous xanthomatosis. J Biol Chem 1991;266:7779–7783. [PubMed: 2019602]
13. Verrips A, Hoefsloot LH, Steenbergen GCH, et al. Clinical and molecular genetic characteristics of cerebrotendinous xanthomatosis. Brain 2000;123:908–919. [PubMed: 10775536]
14. Lee MH, Hazard S, Carpten JD, et al. Fine-mapping, mutation analyses, and structural mapping of cerebrotendinous xanthomatosis in U.S. pedigrees. J Lipid Res 2001;42:159–169. [PubMed: 11181744]
15. Berginer VM, Shany S, Aklalay D, et al. Osteoporosis and increased bone fractures in cerebrotendinous xanthomatosis. Metabolism 1993;42:69–74. [PubMed: 8446051]
16. Federico A, Dotti MT, Lore F, et al. Cerebrotendinous xanthomatosis: pathophysio-logical study on bone metabolism. J Neurol Sci 1993;115:67–70. [PubMed: 8468594]
17. Sagara Y, Hara T, Ariyasu Y, et al. Direct expression in Escherichia coli and characterization of bovine adrenodoxins with modified amino-terminal regions. FEBS Lett 1992;300:208–212. [PubMed: 1555645]
18. Sagara Y, Wada A, Takata Y, et al. Direct expression of adrenodoxin reductase in Escherichia coli and the functional characterization. Bio Pharm Bull 1993;16:627–637. [PubMed: 8401393]
19. Murtazina D, Puchkaev AV, Schein CH, et al. Membrane-protein interactions contribute to efficient 27-hydroxylation of cholesterol by mitochondrial cytochrome P450 27A1. J Biol Chem 2002;277:37582–7589. [PubMed: 12124390]
20. Pikuleva IA, Cao C, Waterman MR. An additional electrostatic interaction between adrenodoxin and P450c27 (CYP27A1) results in tighter binding than between adrenodoxin and p450scc (CYP11A1). J Biol Chem 2002;274:2045–2052. [PubMed: 9890963]
21. Omura T, Sato R. The carbon monoside-binding pigment of liver microsomes II. Solubilization, purification and properties. J Biol Chem 1964;239:2379–2385. [PubMed: 14209972]
22. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85. [PubMed: 3843705]
23. Gupta RP, Hollis BW, Patel SP, et al. CYP3A4 is a human microsomal vitamin D-25-hydroxylase. J Bone Miner Res 2004;19:680–688. [PubMed: 15005856]
24. Gupta RP, He YA, Patrick KS, et al. CYP3A4 is a vitamin D-24- and 25-hydroxylase: analysis of structure function by site-directed mutagenesis. J Clin Endocrinol Metab 2005;90:1210–1219. [PubMed: 15546903]
25. Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci 2004;29:664–673. [PubMed: 15544953]
26. Prosser DE, Guo YD, Jia Z, et al. Structural motif-based homology modeling of CYP27A1 and site-directed mutational analyses affecting vitamin D hydroxylation. Biophys J 2006;90:3389–3409. [PubMed: 16500955]
27. Axelson M, Larsson O. Low density lipoprotein (LDL) cholesterol is converted to 27-hydroxycholesterol in human fibroblasts. Evidence that 27-hydroxycholesterol can be an important intracellular mediator between LDL and the suppression of cholesterol production. J Biol Chem 1995;270:15102–15110. [PubMed: 7797494]
28. Chen W, Kubota S, Ujike H, et al. A novel Arg362Ser mutation in the sterol 27-hydroxylase gene (CYP27): its effects on pre-mRNA splicing and enzyme activity. Biochemistry 1998;37:15050–15056. [PubMed: 9790667]
29. Chen W, Kubota S, Kim K-S, et al. Alternative pre-mRNA splicing of the sterol 27-hydroxylase gene (CYP 27) caused by a G to A mutation at the last nucleotide of exon 6 in a patient with cerebrotendinous xanthomatosis (CTX). J Lipid Res 1997;38:870–879. [PubMed: 9186905]

30. Sawada N, Sakaki T, Kitanaka S, et al. 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 2001;268:6607–6615. [PubMed: 11737215]

31. Mast N, Murtazina D, Liu H, et al. Distinct binding of cholesterol and 5β-cholestane-3a,7a,12a-triol to cytochrome P450-27A1: Evidence from modeling and site-directed mutagenesis studies. Biochemistry 2006;45:4396–4404. [PubMed: 16584175]

32. Hasemann CA, Kurumbail RG, Bodddupalli SS, et al. Structure and function of cytochromes P450: A comparative analysis of three crystal structures. Structure 1995;3:41–62. [PubMed: 7743131]

33. Clements MR, Chalmers TM, Fraser DR. Enterohepatic circulation of vitamin D: a reappraisal of the hypothesis. Lancet 1984;1:1376–1379. [PubMed: 6145834]

34. Gascon-Barre M, Demers C, Ghrab O, et al. 2001 Expression of CYP27A, a gene encoding a vitamin D-25 hydroxylase in human liver and kidney. Clin Endocrinol 2001;54:107–115.

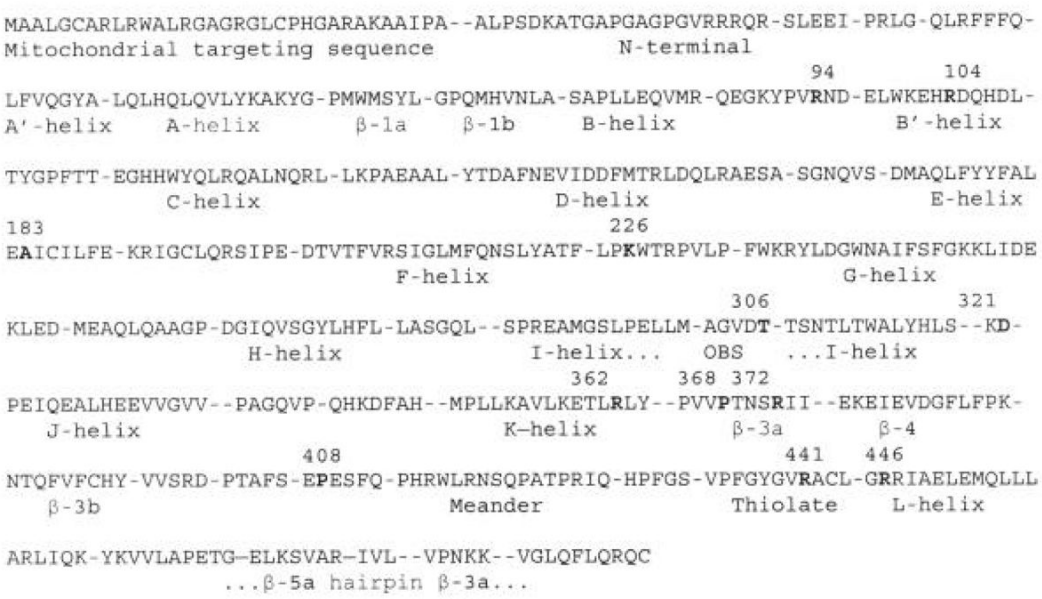

Fig. 1.

Model of CYP27A1 with sites of the naturally occurring mutations. Modified from Prosser et al. (26). The sites of the mutations are shown in bold letters.

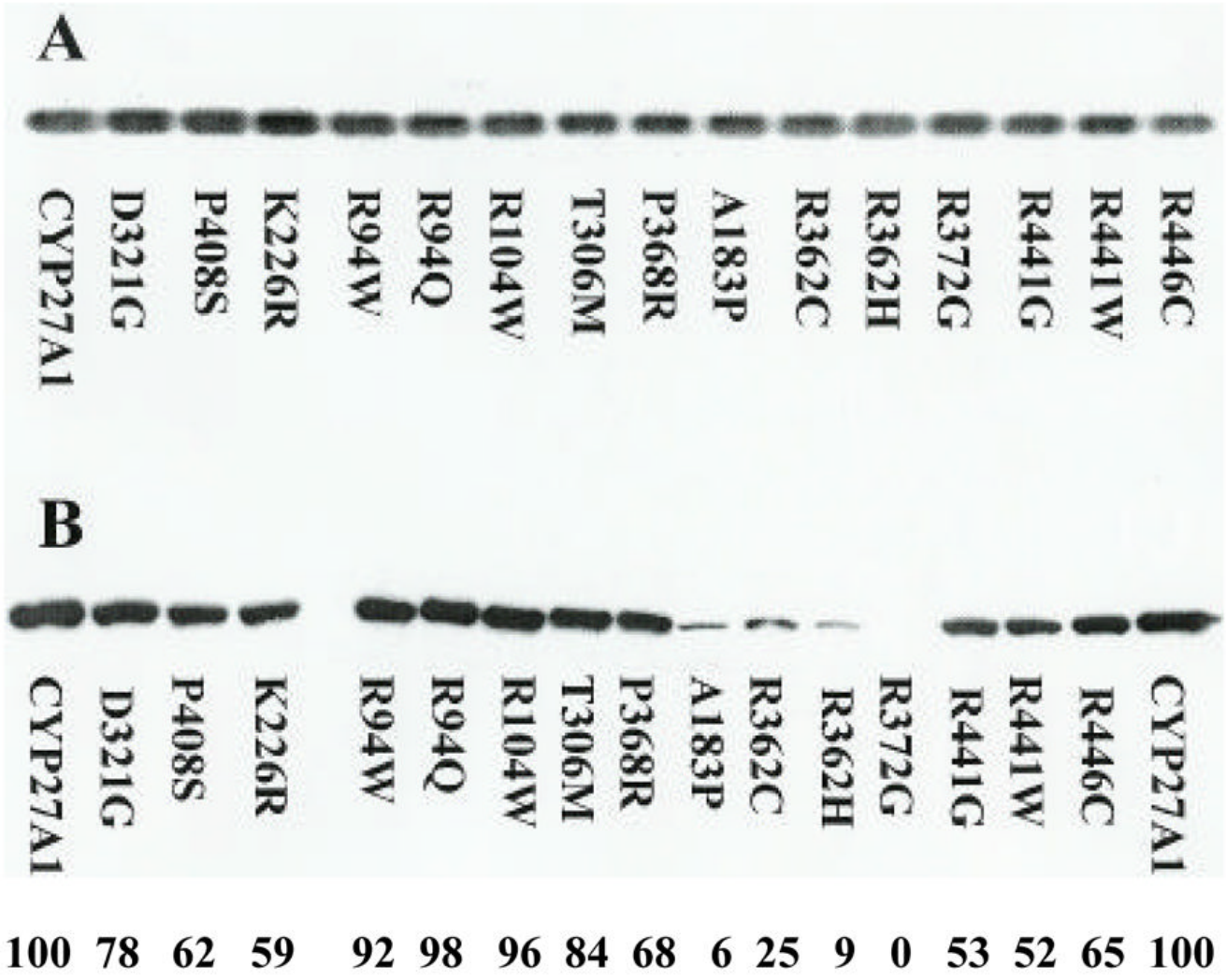

Fig. 2.

mRNA (A) and protein (B) of expressed wild type and mutant CYP27A1. mRNA was determined by RT-PCR. Note that all mutants expressed mRNA, 10 expressed protein, and 4 expressed little or no protein. Figures represent percent of protein expressed compared to that of CYP27A1.

Table 1

Oligonucleotides and restriction enzymes used to generate mutants of CYP27A1

Mutation	Oligonucleotide sequences	Restriction enzymes
R94W	5′-GGGAAAGTACCCAGTATGGAACGACATGGAGCTA-3′	KpnIv, SacI
	5′-TAGCTCCATGTCGTTCCATACTGGGTACTTTCCC-3′	
R94Q	5′-GGAAAGTACCCAGTACAGAACGACATGGAGCTAT-3′	KpnI, SacI
	5′-ATAGCTCCATGTCGTTCTGTACTGGGTACTTTCC-3′	
R104W	5′-ATGGAAGGAGCACTGGGACCAGCACGAC-3′	KpnI, SacI
	5′-GTCGTGCTGGTCCCAGTGCTCCTTCCAT-3′	
A183P	5′-CTACTACTTTGCCTTGGAACCTATTTGCTACATCCTGT-3′	KpnI, StuI
	5′-ACAGGATGTAGCAAATAGGTTCCAAGGCAAAGTAGTAG-3′	
K226R	5′-TATGCCACCCTTCCTCCCCCGGTGGACTCGCCCCGTG-3′	KpnI, StuI
	5′-CACGGGGCGAGTCCACCGGGGGAGGAAGCTGGCATA-3′	
T306M	5′-GGCTGGAGTGGACATGACATCCAACACGCT-3′	BsmI, AflII
	5′-AGCGTGTTGGATGTCATGTCCACTCCAGCC-3′	
D321G	5′-CACCTCTCAAAGGGCCCTGAGATCCAGG-3′	KpnI, StuI
	5′-CCTGGATCTCAGGGCCCTTTGAGAGGTG-3′	
R362C	5′-GCTTAAGGAGACTCTGTGTCTCTACCCTGTGG-3′	StuI, XbaI
	5′-CCACAGGGTAGAGACACAGAGTCTCCTTAAGC-3′	
R362H	5′-GCTTAAGGAGACTCTGCATCTCTACCCTGTGGTC5′-3′	StuI, XbaI
	5′-GACCACAGGGTAGAGATGCAGAGTCTCCTTAAGC-3′	
P368R	5′-ACCCTGTGGTCCGCACAAACTCCCGG-3′	StuI, XbaI
	5′-CCGGGAGTTTGTGCGGACCACAGGGT-3′	
R372G	5′-GTCCCCACAAACTCCGGGATCATAGAAAAGGAAA-3′	StuI, XbaI
	5′-TTTCCTTTTCTATGATCCCGGAGTTTGTGGGGAC-3′	
R441G	5′-GCTATGGGGTCGGGGCCTGCCTGG-3′	StuI, XbaI
	5′-CCAGGCAGGCCCCGACCCCATAGC-3′	
R441W	5′-GCTATGGGGTCTGGGCCTGCCTGG-3′	StuI, XbaI
	5′-CCAGGCAGGCCCAGACCCCATAGC-3′	
R446C	5′-GCCTGCCTGGGCTGCAGGATTGCAGA-3′	StuI, XbaI
	5′-TCTGCAATCCTGCAGCCCAGGCAGGC-3′	

Mutants were prepared from a wild type CYP27A1 template. The bases changed in the oligonu- cleotides used for site mutation are underlined. The corresponding restriction enzymes were used to clone mutated DNA fragments into digested wild type CYP27A1.

Table 2

Site of the naturally occurring mutations of human CYP27A1 and their proposed function

Mutation	Location	Putative function
R94W, R94Q	B′-B loop	heme binding
R104W	B′-helix	structural
A183P	E-helix	OBS stability
K226R	F-G loop	structural
T306M	I-helix	OBS
D321G	I-J loop	structural
R362C, R362H	K-helix	ERR triad
P368R	β3a	stabilizes β3
R372G	β3a	heme binding
P408S	meander	stabilizes ERR triad
R441G, R441W	L-helix	heme binding
R446C	L-helix	ferredoxin binding

OBS is an oxygen binding site within the I-helix. The ERR triad is a structural motif between the K-helix and meander regions. Adapted from refs. 25 and 26.

Table 3

Effect of mutations expressed in *E. coli* on reduced CO difference spectra, vitamin D 25-hydroxylase activity and cholesterol 27-hydroxylase activity

CYP27A1 preparation	Reduced CO difference spectra (nmol/L)	vitamin D_3	substrate 1a(OH)D_3	cholesterol (nmol/mg protein/min)
		(pmol/mg protein/min)		
WT	235	171 ± 7 (100)	1,225 ± 21 (100)	4.1 ± 0.2 (100)
K226R	182	126 ± 5 (−26)	916 ± 23[a] (−25)	3.5 ± 0.2 (−15)
D321G	108	124 ± 3[b] (−27)	863 ± 31[a] (−30)	3.6 ± 0.1 (−12)
P408S	104	85 ± 4[a] (−50)	709 ± 12[a] (−42)	2.4 ± 0.3[b] (−41)

Results with substrates are mean ± SE of 3 observations. Figures in parentheses are percent change in activity compared to WT. Substrate concentration was 25 μM for vitamin D3 and cholesterol and 10 μM for 1a(OH)D3. Incubations were carried out for 10 min with vitamin D compounds and 60 min with cholesterol. To convert metric to SI units, multiply by 2.5 for 25-hydroxyvitamin D3, 2.5 for 27-hydroxycholesterol, and 2.4 for 1,25-dihydroxyvitamin D3.

[a] P <0.01 vs WT CYP27A1.

[b] P <0.05 vs WT CYP27A1.

Table 4

Effect of mutations expressed in COS-1 cells on vitamin D 25-hydroxylase activity

CYP27A1 preparation	substrate 1a(OH)D_3(pmol/10^6 cells/24 h)	
Vector	5.7 ± 0.5	
WT	5.7 ± 0.5	(100)
K226R	8.5 ± 2.8	(+49)
D321G	7.8 ± 0.5 [a]	(+37)
P408S	8.8 ± 1.6	(+54)

Results are mean ± SE of 4 observations. The value for the vector was subtracted from the total value of each transfection. Figures in parentheses are percent change in activity compared to WT. Substrate concentration was 8.5 μM. Incubations were carried out for 24 h. To convert metric to SI units for 1,25-dihydroxyvitamin D3 multiply by 2.4.

[a] P <0.02 vs WT CYP27A1.