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Mutational analysis of CYP27A1: assessment of 27-hydroxylation of cholesterol and 25-hydroxylation of vitamin D

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Abstract

The *CYP27A1* gene encodes a mitochondrial enzyme that 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 complementary DNAs 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 1α-hydroxyvitamin D_3 ($1\alpha OHD_3$) 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,* 3 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 $1\alpha OHD_3$ compared with wild type. When expressed transiently in COS-1 cells, each of these mutants showed 25-hydroxylation activity for $1\alpha OHD_3$ as well as wild type. Thus, 3 mutants, K226R, D321G, and P408S, known to occur clinically with nonfunctioning mutants, hydroxylated cholesterol, vitamin D_3, and $1\alpha OHD_3$. How they contribute to the pathogenesis of CTX despite being biologically active in vitro remains to be determined.

1. Introduction

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 [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 that hydroxylates both vitamin D_2 and vitamin D_3 [5,6]. The finding of a homozygous inactivating mutation of *CYP2R1* gene in 2 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 2 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-hydroxylation 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 neurologic 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

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Table 1
Oligonucleotides and restriction enzymes used to generate mutants of CYP27A1

Mutation	Oligonucleotide sequences	Restriction enzymes
R94W	5′-GGGAAAGTACCCAGTATGGAACGACATGGAGCTA-3′ 5′-TAGCTCCATGTCGTTCCATACTGGGTACTTTCCC-3′	*Kpn*I, *Sac*I
R94Q	5′-GGAAAGTACCCAGTACAGAACGACATGGAGCTAT-3′ 5′-ATAGCTCCATGTCGTTCTGTACTGGGTACTTTCC-3′	*Kpn*I, *Sac*I
R104W	5′-ATGGAAGGAGCACTGGGACCAGCACGAC-3′ 5′-GTCGTGCTGGTCCCAGTGCTCCTTCCAT-3′	*Kpn*I, *Sac*I
A183P	5′-CTACTACTTTGCCTTGGAACCTATTTGCTACATCCTGT-3′ 5′-ACAGGATGTAGCAAATAGGTTCCAAGGCAAAGTAGTAG-3′	*Kpn*I, *Stu*I
K226R	5′-TATGCCACCCTTCCTCCCCCGGTGGACTCGCCCCGTG-3′ 5′-CACGGGGCGAGTCCACCGGGGGAGGAAGCTGGCATA-3′	*Kpn*I, *Stu*I
T306M	5′-GGCTGGAGTGGACATGACATCCAACACGCT-3′ 5′-AGCGTGTTGGATGTCATGTCCACTCCAGCC-3′	*Bsm*I, *Afl*II
D321G	5′-CACCTCTCAAAGGGCCCTGAGATCCAGG-3′ 5′-CCTGGATCTCAGGGCCCTTTGAGAGGTG-3′	*Kpn*I, *Stu*I
R362C	5′-GCTTAAGGAGACTCTGTGTCTCTACCCTGTGG-3′ 5′-CCACAGGGTAGAGACACAGAGTCTCCTTAAGC-3′	*Stu*I, *Xba*I
R362H	5′-GCTTAAGGAGACTCTGCATCTCTACCCTGTGGTC5′-3′ 5′-GACCACAGGGTAGAGATGCAGAGTCTCCTTAAGC-3′	*Stu*I, *Xba*I
P368R	5′-ACCCTGTGGTCCGCACAAACTCCCGG-3′ 5′-CCGGGAGTTTGTGCGGACCACAGGGT-3′	*Stu*I, *Xba*I
R372G	5′-GTCCCCACAAACTCCGGGATCATAGAAAAGGAAA-3′ 5′-TTTCCTTTTCTATGATCCCGGAGTTTGTGGGGAC-3′	*Stu*I, *Xba*I
R441G	5′-GCTATGGGGTCGGGGCCTGCCTGG-3′ 5′-CCAGGCAGGCCCCGACCCCATAGC-3′	*Stu*I, *Xba*I
R441W	5′-GCTATGGGGTCTGGGCCTGCCTGG-3′ 5′-CCAGGCAGGCCCAGACCCCATAGC-3′	*Stu*I, *Xba*I
R446C	5′-GCCTGCCTGGGCTGCAGGATTGCAGA-3′ 5′-TCTGCAATCCTGCAGCCCAGGCAGGC-3′	*Stu*I, *Xba*I

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

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.

2. Materials and methods

2.1. Materials

Vitamin D_3, 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] 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]-1,25-$(OH)_2D_3$, [^{3}H-26,27-*methyl*]-25-OHD_3, and [^{14}C-4]-cholesterol from Amersham Biosciences (Piscataway, NJ) and 27-hydroxycholesterol from Research Plus (Manasquan, NJ). $1\alpha OHD_3$ was generously provided by Leo Pharmaceutical Products (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].

2.2. Site-directed mutagenesis

Site-directed mutagenesis was carried out with the QuikChange site-directed mutagenesis kit as described by the manufacturer (Stratagene, La Jolla, 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 complementary DNA (cDNA). Both ends of mutated cDNA fragments were sequenced to confirm correct ligation at restriction sites as well as the sequence of mutated fragment.

2.3. 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 mmol/L 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].

2.4. Reverse transcription-polymerase chain reaction 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, Valencia, CA). cDNAs were synthesized by the Reverse Transcription System (Promega, Madison, WI) at 48°C for 1 hour followed by incubation at 95°C for 5 minutes as recommended by the manufacturer. Amplification of CYP27A1 was performed with True Allele PCR Premix (PE Applied Biosystems, Foster City, CA) as follows: 10 minutes at 95°C, 1 cycle; 45 seconds at 95°C, 45 seconds at 60°C, and 1.5 minutes at 72°C, 30 cycles. Sense and antisense 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 US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

2.5. Western blot analysis

Purified membrane proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto 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 horseradish peroxidase–conjugated antirabbit IgG (1:2000) at room temperature for 1.5 hours. Proteins were visualized with SuperSignal West Pico chemiluminescent substrate as described by the manufacturer (Pierce).

2.6. 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 μmol/L), adrenodoxin (2.0 μmol/L), and adrenodoxin reductase (0.25-0.5 μmol/L) in 70 μL at room temperature for 10 minutes. The reconstituted enzyme complex was used to hydroxylate $1\alpha(OH)D_3$ (10 μmol/L) or vitamin D_3 (25 μmol/L) in a total volume of 1 mL as described [23]. The reaction was carried out for 10 minutes. The reaction mixtures were extracted with acetonitrile and subjected to solid-phase extraction. The extracts containing the hydroxylated products were evaporated and estimated by high-performance liquid chromatography (HPLC) analysis [23,24].

2.7. Extraction of samples

The acetonitrile mixture containing $1\alpha OHD_3$ or vitamin D_3 metabolites was mixed with a vortex, 1000 cpm [^{3}H]-1,25-$(OH)_2D_3$ or [^{3}H]-25-OHD_3 was added for recovery, and the mixture was centrifuged at 4°C and 2000*g* for 15 minutes [23]. The supernatant was decanted to another 13 × 100-mm glass tube containing 1 mL of 0.4 mol/L 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) and 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.

2.8. Isolation and measurement of 1,25(OH)$_2$D$_3$

Extracts containing $1\alpha OHD_3$ metabolites in 200 μL solution were loaded onto a Zorbax Sil 4.6 × 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 that eluted at 8.5 minutes. 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 × 250-mm column, but were separated by running hexanes-2-propanol (96:4) for 9 minutes, followed by hexanes-2-propanol (85:15) for 16 minutes. The column was again equilibrated for 3 minutes 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 that eluted at 10.1 minutes. Recovery was assessed with [^{3}H]-25-OHD_3 as described [23]. Results are expressed as picomoles per milligram protein per minute for recombinant cytochrome P-450 enzymes expressed in *E. coli.*

2.9. 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 minutes. The reaction mixture contained 0.004 μmol/L P450, 2.0 μmol/L adrenodoxin, 0.5 μmol/L adrenodoxin reductase, 100 μL of 0.5 mol/L Na_2HPO_4, 100 μL of 3% poly(vinyl alcohol) (molecular weight, 13 000-23 000), 50 μL of NADPH regenerating solution A (BD Gentest BD Biosciences, San Jose, CA), 10 μL of regenerating solution B (BD Gentest), 0.025 μCi of [4-^{14}C] cholesterol (specific activity, 57.0 mCi/mmol), and 25 μmol/L 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 minutes, and the reaction mixture was extracted with 3 volumes of ethyl acetate. The extract was evaporated under nitrogen and subjected to HPLC on a Develosil ODS-UG-5 column (4.6 × 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 minutes. This was followed by the flow of 100% methanol for 15 minutes. The column was again equilibrated with solvent A for 4 minutes before loading the next sample for HPLC. One-milliliter fractions were collected from 0 to 30 minutes, evaporated, and counted for carbon 14 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 minutes, respectively. Results are expressed as nanomoles per milligram protein per minute.

2.10. Gas chromatography-mass spectrometry

An Agilent Model 6890 GC-5973N MS with automatic sampler and MSD Productivity Chem Station were used for as chromatography-mass spectrometry (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 nitrogen, then derivatized in a silanized microvial insert within a Teflon-capped automated sampler vial. Twenty microliters of bis(trimethylsilyl)trifluoroacetamide (BSTFA)-trimethylchlorosilane (TMC) (99:1, Supelco, Bellefonte, PA) was used for derivatization to form bis-trimethylsilyl (TMS) products. GC injection (2 μL) was by the pulsed splitless mode. A 30-m × 0.32-mm, 0.25-μm film thickness (DB-5; J & W Scientific, Folsom, CA) phenylmethylpolysiloxanes fused silica column held at 70°C for 1.5 minutes, 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.

2.11. Measurement of reduced CO difference spectra

Reduced CO difference spectra was determined by the method of Omura and Sato [21].

2.12. 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 *Hin*dIII and *Bam*HI, blunted with Klenow fragment (New England BioLabs, Beverly, MA), and ligated to form pcDNAm1 vector. pcDNAm1-CYP27A1 was prepared by digesting pUC27A1 with *Eco*RI and cloning the 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 *Kpn*I and *Hin*dIII and cloning the mutated fractions in pcDNAm1-CYP27A1 digested with the same restriction enzymes. The ligations at restriction sites were confirmed by sequencing.

2.13. Transfection of COS-1 cells

COS-1 cells (American Type Culture Collection, Manassas, VA) were seeded in 60-mm dishes at a density of 0.5 × 10^6 cells per dish in Dulbecco's minimum Eagle's medium with glutamate (2 mmol/L), sodium pyruvate (1 mmol/L), 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 hours 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% carbon dioxide.

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

After 24 hours, the transfected COS-1 cells were washed once with 3 mL of serum-free medium and treated with 1αOHD_3 (8.5 μmol/L) in 4 mL of serum-free medium for 48 hours at 37°C in 95% air and 5% carbon dioxide. The 1αOHD_3 solution was evaporated to dryness under nitrogen, dissolved in dimethyl sulfoxide, and diluted in medium before adding to the dishes. The final concentration of dimethyl sulfoxide in the medium was 0.3%. The reaction was stopped by adding 4 mL of acetonitrile. [^{3}H]-1,25-$(OH)_2D_3$ (2000 cpm) was added to the cells after termination of the reaction to calculate the recovery of 1,25-$(OH)_2D_3$. The metabolites were extracted and subjected to HPLC as

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 references [25] and [26].

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].

3. 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 Fig. 1. All of them expressed mRNA as indicated by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 2A). Western blot analyses showed all but 4 (A183P, R362H, R362H, and R372G) expressed protein levels

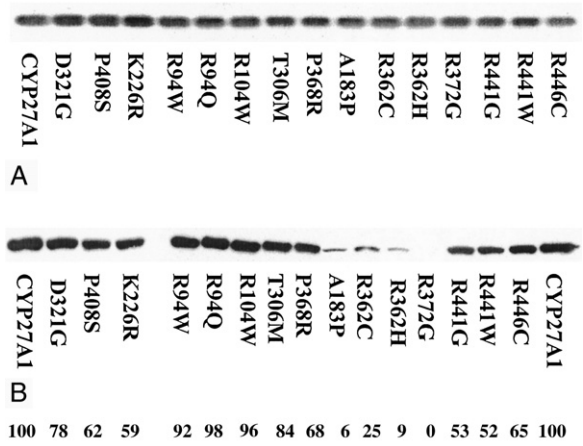

Fig. 2. mRNA (A) and protein (B) of expressed wild-type and mutant CYP27A1. mRNA was determined by RT-PCR. Note that all mutants expressed mRNA, 10 expressed protein, and 4 expressed little or no protein. Figures represent percent of protein expressed compared with that of CYP27A1.

comparable to the wild-type enzyme (Fig. 2B), suggesting that these 4 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% compared with wild-type CYP27A1 when expressed in *E. coli* (Table 3). The mutants also showed vitamin D 25-hydroxylase activity that was the same or higher by as much as 54% compared with wild type when expressed in COS-1 cells (Table 4). Thus, the mutant enzymes either were active as regards hydroxylation of vitamin D_3, 1αOHD_3, and cholesterol, or presumably were

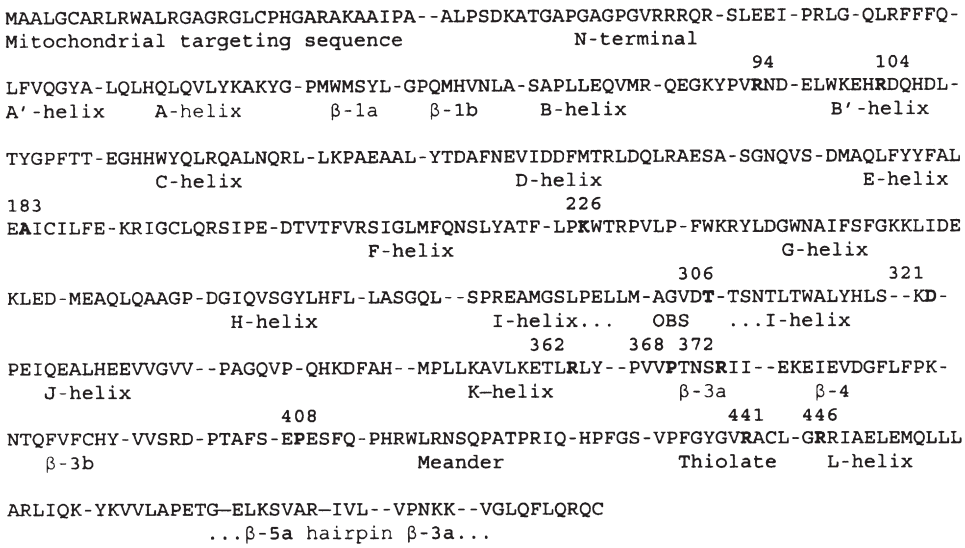

Fig. 1. Model of CYP27A1 with sites of the naturally occurring mutations. Adapted from Prosser et al [26]. The sites of the mutations are shown in bold letters.

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)	Substrate		
		Vitamin D_3 (pmol/mg protein per minute)	1α(OH)D_3 (pmol/mg protein per minute)	Cholesterol (nmol/mg protein per minute)
WT	235	171 ± 7 (100)	1225 ± 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 with wild type (WT). Substrate concentration was 25 μmol/L for vitamin D_3 and cholesterol and 10 μmol/L for 1α(OH)D_3. Incubations were carried out for 10 minutes with vitamin D compounds and 60 minutes with cholesterol. To convert metric to SI units, multiply by 2.5 for 25-hydroxyvitamin D_3, 2.5 for 27-hydroxycholesterol, and 2.4 for 1,25-dihydroxyvitamin D_3.

[a] $P < .01$ vs WT CYP27A1.

[b] $P < .05$ vs WT CYP27A1.

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 minutes 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, 3 mutants of CYP27A1 that are causally related to CTX show hydroxylating activity for vitamin D and cholesterol that tends to be lower than wild type when expressed in *E. coli* and higher than wild type when expressed in COS-1 cells.

4. Discussion

Some 38 different mutations of the *CYP27A1* gene causing CTX have been reported [12-14]. Most of these mutations do not result in predicted protein expression. Most disrupt splicing and cause instability of the message, abnormal splicing resulting in rapid degradation of the mRNA, or frameshift 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 because 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 4 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 3 mutants (K226R, D321G, and P408S) showed a reduction in CO difference spectra and had vitamin D 25-hydroxylase and cholesterol 27-hydroxylase activities that were the same or lower compared with 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 with CYP27A1 when expressed in COS cells [28,29]. 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 with 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.

Table 4
Effect of mutations expressed in COS-1 cells on vitamin D 25-hydroxylase activity

CYP27A1 preparation	Substrate 1α(OH)D_3 (pmol/10^6 cells per 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 with WT. Substrate concentration was 8.5 μmol/L. Incubations were carried out for 24 hours. To convert metric to SI units for 1,25-dihydroxyvitamin D_3 multiply by 2.4.

[a] $P < .02$ vs WT CYP27A1.

Prosser et al [26] constructed a homology-based model of CYP27A1 that 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 that 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 [30] used computer modeling and studies of site-directed mutagenesis of CYP27A1 with non–naturally occurring mutants expressed in *E. coli* and cholesterol, 5β-cholestane-3α,7α,12α-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 12α-hydroxyl of 5β-cholestane-3α,7α,12α-triol and V367 to be critical for optimal positioning of the cholesterol C26 methyl group and for regioselective hydroxylation of this sterol [30].

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 that stabilizes heme binding and may play a role in redox-partner binding [31]. 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% reduction in cholesterol 27-hydroxylase activity compared with 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 that were modestly reduced compared with 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 physiologic importance was documented by the finding that an inactivating homozygous L99P gene mutation was the cause of isolated 25-OHD deficiency and rickets in 2 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. Because 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% of metabolites excreted in bile were present as 25-OHD or its glucuronide conjugate [32]. Because 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 [33]. 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 2 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, 3 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 with 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 3 mutants are known to occur in association with mutations R362A or R372G, which have little or no biologic activity [28,34]. The remaining 12 mutant proteins were either poorly expressed or did not produce a peak for reduced CO difference spectra. In most patients with CTX, CYP27A1 either is not expressed or is biologically inactive as regards 25-hydroxylation of vitamin D. Lifelong 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.

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References

[1] Oftebro H, Saarem K, Bjorkhem I, et al. Side chain hydroxylation of C27-steroids and vitamin D3 by a cytochrome P-450 enzyme system isolated from human liver mitochondria. J Lipid Res 1981;22: 1254-64.

[2] Saarem K, Bergseth S, Oftebro H, et al. Subcellular localization of vitamin D3 25-hydroxylase in human liver. J Biol Chem 1984;259: 10936-40.

[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 U S A 1993;90:8668-72.

[4] Sawada N, Sakaki T, Ohta M, et al. Metabolism of vitamin D(3) by human CYP27A1. Biochem Biophys Res Commun 2000;273: 977-84.

[5] Cheng JB, Motola DL, Mangelsdorf DJ, et al. Deorphanization of cytochrome P450 CYP2R1, a microsomal vitamin D 25-hydroxylase. J Biol Chem 2003;278:38084-93.

[6] Shinkyo R, Sakaki T, Kamakura M, et al. Metabolism of vitamin D by human microsomal CYP2R1. Biochem Biophys Res Commun 2004; 324:451-7.

[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 U S A 2005;101:711-5.

[8] Dong Q, Miller W. Vitamin D 25-hydroxylase deficiency. Mol Genet Metab 2004;83:197-8.

[9] Fuchs M. Bile acid regulation of hepatic physiology: III. Regulation of bile acid synthesis: past progress and future challenges. Am J Physiol Gastrointest Liver Physiol 2003;284:551-7.

[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;266:7774-8.

[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;273:18153-60.

[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;266:7779-83.

[13] Verrips A, Hoefsloot LH, Steenbergen GCH, et al. Clinical and molecular genetic characteristics of cerebrotendinous xanthomatosis. Brain 2000;123:908-19.

[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;42:159-69.

[15] Berginer VM, Shany S, Aklalay D, et al. Osteoporosis and increased bone fractures in cerebrotendinous xanthomatosis. Metabolism 1993;42:69-74.

[16] Federico A, Dotti MT, Lore F, et al. Cerebrotendinous xanthomatosis: pathophysiological study on bone metabolism. J Neurol Sci 1993;115: 67-70.

[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;300:208-12.

[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;16:627-37.

[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: 37582-9.

[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;274:2045-52.

[21] Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes II. Solubilization, purification and properties. J Biol Chem 1964;239:2379-85.

[22] Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76-85.

[23] Gupta RP, Hollis BW, Patel SP, et al. CYP3A4 is a human microsomal vitamin D-25-hydroxylase. J Bone Miner Res 2004;19:680-8.

[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;90:1210-9.

[25] Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. Trends Biochem Sci 2004;29:664-73.

[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;90:3389-409.

[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;270:15102-10.

[28] 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;38:870-9.

[29] 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;268:6607-15.

[30] Mast N, Murtazina D, Liu H, et al. Distinct binding of cholesterol and 5β-cholestane-3α,7α,12α-triol to cytochrome P450-27A1: Evidence from modeling and site-directed mutagenesis studies. Biochemistry 2006;45:4396-404.

[31] Hasemann CA, Kurumbail RG, Boddupalli SS, et al. Structure and function of cytochromes P450: a comparative analysis of three crystal structures. Structure 1995;3:41-62.

[32] Clements MR, Chalmers TM, Fraser DR. Enterohepatic circulation of vitamin D: a reappraisal of the hypothesis. Lancet 1984;1:1376-9.

[33] Gascon-Barre M, Demers C, Ghrab O, et al. Expression of CYP27A, a gene encoding a vitamin D-25 hydroxylase in human liver and kidney. Clin Endocrinol 2001;54:107-15.

[34] 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;37:15050-6.