review

Liver mitochondrial P450 involved in cholesterol catabolism and vitamin D activation

Kyu-Ichiro Okuda[1]

Department of Surgery I, Miyazaki Medical College, Kiyotake, Miyazaki 889-16, Japan

Abstract The isolation, purification, and cloning of the mitochondrial P450 enzyme catalyzing not only the 27-hydroxylation of 5β-cholestane-3α,7α-diol and cholestane-3α,7α,12α-triol, but also the 25-hydroxylation of vitamin D_3 are reviewed. The sterol hydroxylase was shown to be present on the mitochondrial inner membrane-matrix, to be inactivated by carbon monoxide, and to be activated by 450 nm radiation, establishing its membership in the P450 class. Characterization of the reaction product indicated that hydroxylation occurred on the 27 methyl group; the enzyme also mediated 25-hydroxylation of vitamin D_3. Cloning of the enzyme confirmed that it was of mitochondrial origin and that it catalyzed both sterol and vitamin D hydroxylation. Enzymatic activity was deficient in fibroblasts from patients with cerebrotendinous xanthomatosis. Genetic analysis of patients with cerebrotendinous xanthomatosis has shown several genetic defects: *1)* two different point mutations in which arginine codons were replaced by cysteine codons; *2)* deletion of thymidine in exon 4; and *3)* a guanosine to adenosine substitution at the 3' splice acceptor site of intron 4 of the gene. The mitochondrial 27-hydroxylase is a key enzyme in bile acid biosynthesis and can be distinguished from microsomal hydroxylases that also catalyze hydroxylation of the side chain of cholesterol and intermediates in bile acid biosynthesis. In the rat, a microsomal enzyme catalyzes 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol, but the importance of this pathway in bile acid biosynthesis is unclear.—**Okuda, K-I.** Liver mitochondrial P450 involved in cholesterol catabolism and vitamin D activation. *J. Lipid Res.* 1994. **35:** 361–372.

Supplementary key words 27-hydroxylase • cerebrotendinous xanthomatosis • 26-hydroxylase

The P450 "system" is a super family of membrane bound *b*-type cytochrome enzymes that contain more than 200 different molecular species. This system was first identified in liver endoplasmic reticulum (microsomes) (1) and subsequently identified in cell fractions from many other tissues, e.g., lung microsomes, skin microsomes, adrenocortical microsomes, adrenocortical mitochondria, kidney microsomes, kidney mitochondria, intestinal microsomes, bacterial cell fluid, etc. Later, it was found that these cytochromes function as hydroxylases or oxidases, but not as components of the electron transport system. As a result, the name was changed from cytochrome P-450, to simply "P450" (2).

Abbreviations and trivial names: cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid; chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid; C-triol, 5β-cholestane-3α,7α,12α-triol; 27-OH-C-triol, 5β-cholestane-3α,7α,12α-27-tetrol; THCA, 3α,7α,12α-trihydroxy-5β-cholestan-27-oic acid; 25-OH-D_3, 25-hydroxyvitamin D_3; 1α,25-$(OH)_2$-D_3, 1α,25-dihydroxyvitamin D_3.

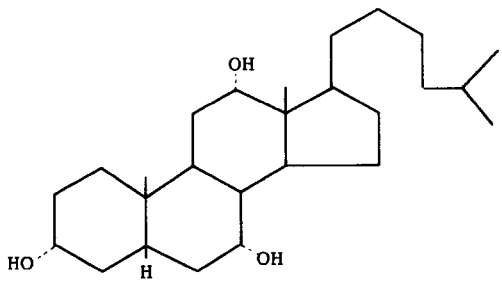
[1]Formerly from the Department of Biochemistry, Hiroshima University, School of Dentistry. Name was previously spelled as Kyuichiro Okuda.

Early attempts to show that P450 was present in liver mitochondria were unsuccessful (3, 4). Subsequently, evidence was obtained by this and other laboratories that P450 was present in the liver mitochondrial inner membrane-matrix. This P450 was shown to catalyze the 27-hydroxylation of 5β-cholestane-3α,7α,12α-triol as well as the 25-hydroxylation of vitamin D_3. Here, the history, properties and physiological significance of this liver mitochondrial P450 are reviewed. A deficiency of this enzyme has recently been shown to cause cerebrotendinous xanthomatosis, a rare inherited disorder of metabolism that causes severe clinical disease.

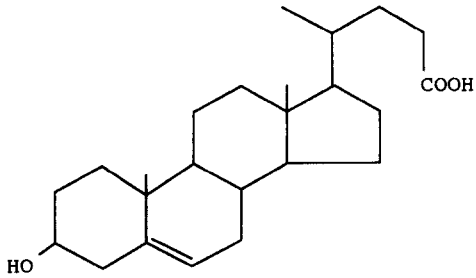
I. STEROL 27-HYDROXYLASE

A. Early studies

In 1955 Bergstrom, Danielsson, and Samuelsson (5) proposed that in the conversion of cholesterol to cholic acid, nuclear biotransformations are completed before side chain biotransformations begin. In this scheme, 5β-cholestane-3α,7α,12α-triol (C-triol) is formed, a molecule that may be considered as "chimeric," having the nucleus of cholic acid yet retaining the side chain of cholesterol. Were side chain biotransformations to precede nuclear biotransformations, a molecule with the converse structure would be formed, having a cholesterol nucleus and a cholic acid-type side chain (3β-hydroxy-5-cholen-24-oic acid) (**Fig. 1**).



5β-Cholestane-3α,7α,12α-triol (C-triol)



3β-Hydroxy-5-cholen-24-oic acid

Fig. 1. Hypothetical intermediates in the conversion of cholesterol to bile acids.

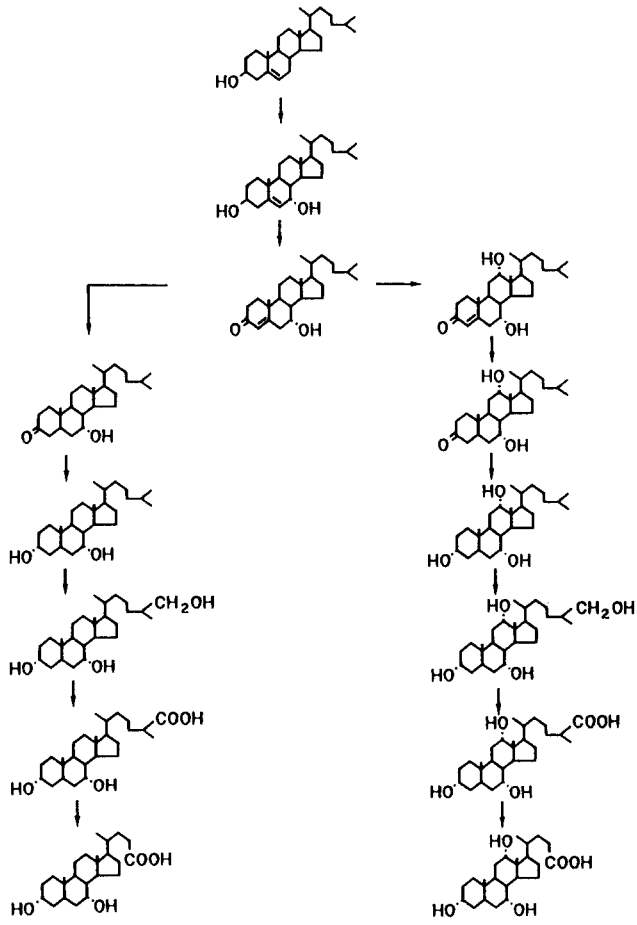


Fig. 2. Metabolic pathway of cholesterol catabolism to bile acids.

In 1956 Fredrickson and Ono (6) incubated cholesterol with rat liver mitochondria and obtained both 25-hydroxycholesterol and 26-hydroxycholesterol. Subsequently, Danielsson (7) incubated C-triol with liver mitochondria and obtained the 27-hydroxy derivative (5β-cholestane-3α,7α,12α,27-tetrol, 27-OH-C-triol) as well as a small amount of the C_{27} bile acid (3α,7α,12α-trihydroxy-5β-cholestan-27-oic acid, THCA) (**Fig. 2**).

At that time, two column chromatographies were used to separate the substrate and products. Because of the long time and elaborate procedures required to assay the reaction products by this method, enzyme kinetics, cofactor requirements, and subcellular localization of the enzymes involved were not defined. The Swedish research group did note that the addition of a 100,000 *g* supernatant to the reaction mixture accelerated the reaction. Later, Okuda and Hoshita (8) devised an improved analytical method which used only a single thin-layer chromatographic step to resolve the substrate (C-triol) and the products (27-OH-C-triol and THCA). This methodological advance facilitated definition of the enzyme kinetics of

the reaction. Okuda and Hoshita (8) found that either "boiled extract of liver homogenate" or NADPH, but not a 100,000 *g* supernatant, was required for 27-hydroxylation of C-triol by a mitochondrial fraction. Further experiments indicated that the co-factor had a low molecular weight (based on gel permeation chromatography), and had a strongly negative charge (as it was adsorbed by an anion exchange column and required high ionic strength for elution) (K-I. Okuda, unpublished observations). Because of these properties, constituents of the citric acid cycle were considered likely candidates for the unidentified cofactor. A few years later, isocitrate was found to be as effective as the "boiled extract" and was thought likely to be the key cofactor (9).

In 1970, Cronholm and Johansson (10) examined the hydroxylation of C-triol using an assay system fortified with a 100,000 *g* supernatant or NADPH. They found that the endoplasmic reticulum showed much higher 27-hydroxylation activity than mitochondria. Furthermore, when they measured the activities of marker enzymes of each subcellular fraction, they found that on a protein basis their mitochondrial preparation was contaminated to about 25% with the endoplasmic reticulum, whereas their microsomal preparation was only contaminated to about 1% with mitochondria. They proposed that if uncontaminated mitochondria were used, C-triol would undergo still less 27-hydroxylation, and suggested that the mitochondrial hydroxylation reported by the Okuda group was caused by contaminated subcellular fractions. Björkhem and Gustafsson (11) performed a similar experiment using 5β-cholestane-3α,7α-diol (C-diol) as a substrate. (This molecule is the hypothetical precursor of chenodeoxycholic acid.) These workers confirmed the experiments of Cronholm and Johansson, finding that 27-hydroxylation activity was greater in microsomes. They concurred with the view that 27-hydroxylation mediated by mitochondrial fractions was explained by contamination with microsomes. However, when these workers examined the 27-hydroxylation of cholesterol, they observed that mitochondrial fractions were more active than microsomal enzymes, suggesting that cholesterol 27-hydroxylase differed from C-triol and C-diol 27-hydroxylase. They also reported that the enzymes catalyzing the 27-hydroxylation of cholesterol were inhibited by carbon monoxide (CO). Nonetheless, because Sottocassa et al. (3) had reported that P450 could not be detected in liver mitochondria, these workers did not think that the mitochondrial enzyme mediating the 27-hydroxylation of cholesterol was a P450 enzyme.

B. Presence in mitochondria

In 1973, Taniguchi, Hoshita, and Okuda (9) obtained convincing evidence for the original assertion of Okuda and Hoshita (8). To test whether the Japanese results were caused by impure subcellular fractions, Taniguchi et al. (9) repeatedly washed the mitochondrial fraction, measuring 27-hydroxylation activity together with those of marker enzymes for microsomes (glucose 6-phosphatase) and mitochondria (cytochrome oxidase for inner membrane) after each wash. They found that the activity of glucose 6-phosphatase dropped after the second washing to about 1/10th of the original activity, whereas that of cytochrome oxidase and the index enzyme, 27-hydroxylase, did not. To confirm the identity of the cofactor, they reexamined the influence of isocitrate on 27-hydroxylation activity. They confirmed that isocitrate sustained 27-hydroxylation activity almost as effectively as the "boiled extract" (see above).

Taniguchi et al. (9) also examined the localization of the 27-hydroxylation activity in the mitochondrion. They reasoned that in view of the fact that $NADP^+$-specific isocitrate dehydrogenase and functional nicotinamide nucleotides are localized in the matrix (12), an endogenous electron-donating system generating NADPH for 27-hydroxylation in vivo must interact with the M-side (13) of the inner membrane. Further, it was known that 27-hydroxylation activity was decreased in isotonic or hypertonic reaction media, but well preserved if mitochondria were pre-lysed by digitonin. An inner membrane-matrix fraction (mitoplast) was found to retain as much of the hydroxylation activity as was present in lysed mitochondria. Taniguchi et al. (9) therefore concluded that mitochondria were ruptured by hypotonic reaction media in the standard hypotonic assay medium, and that such rupture was essential for 27-hydroxylation activity. Additional experiments were also performed to define the location of the 27-hydroxylation activity within the mitochondrion. The 27-hydroxylase was assigned to the inner membrane-matrix region because of its behavior in relation to other mitochondrial marker enzymes during stepwise solubilization with digitonin.

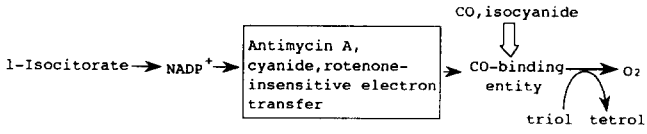
C. Properties of the mitochondrial enzyme

Experiments were performed to determine the exact site of hydroxylation of C-triol, and to determine whether the hydroxylation catalyzed by mitochondrial enzymes gave the same product as that catalyzed by microsomal enzymes (9). To define the site of hydroxylation, the tetrol fractions obtained by incubation of C-triol with mitochondria or microsomes were subjected to 27-dehydrogenation using a rat liver soluble fraction which was known to contain a 27-OH-C-triol dehydrogenase as well as a 3α,7α,12α-trihydroxy-5β-cholestan-27-al dehydrogenase. The activity of these enzymes is restricted to the primary alcohol group and aldehyde group at C-27, respectively (14). Taniguchi et al. (9) found that 90% of the mitochondrial product was converted to THCA and its derivatives, indicating that hydroxylation had occurred on a terminal methyl group of the C-triol side chain. In contrast, only a minor portion of the microsomal products was con-

verted to THCA, indicating that microsomal hydroxylation occurred at positions other than C-26 or C-27. The 27-hydroxylase was also shown to be sensitive to CO and phenylisocyanate whereas it was totally insensitive to KCN. Taniguchi et al. (9) also extended their earlier studies on the kinetics of 27-hydroxylation. They determined an apparent Michaelis constant (K_m) for oxygen of 10–20 mM, a value similar to that observed with other P450 enzymes.

D. Evidence that the 27-hydroxylase is a P450 enzyme

Together, these results established unequivocally that C-triol 27-hydroxylase activity was present in the inner membrane of liver mitochondria. Nonetheless, Taniguchi et al. (9) were unable to restore the enzyme activity by white light after CO-inactivation, probably because no powerful light source was available to them. They proposed the following sequence for the electron transfer involved in hydroxylation of C-triol in liver mitochondria.



The electron transfer involved in the mitochondrial 27-hydroxylase system was similar in nature to the P450 residing in the inner membrane in the adrenal mitochondria that was known to catalyze hydroxylation. In addition, the finding that isocitrate could serve as an electron donor and that the reaction was totally inhibited by phenylisocyanate and CO [both of which are characteristic inhibitors of P450 (1, 15)], led Taniguchi et al. (9) to propose that 27-hydroxylase was a "P450-like entity" analogous to microsomal P450.

Additional experiments confirmed the existence of this enzyme in rat liver mitochondria (16, 17). Björkhem et al. (18) had also reported the presence of this enzyme in human liver mitochondria. Later, as will be discussed, it was observed that rat and human liver mitochondria catalyze not only 27-hydroxylation of C-triol but also 25-hydroxylation of vitamin D_3, an indispensable reaction for activation of vitamin D_3 (19).

The conclusive evidence that the liver mitochondrial 27-hydroxylase is indeed a member of P450 was obtained by Okuda, Weber, and Ullrich (16). These workers examined the effect of monochromatic light of varying wavelength on the reaction products of isocitrate-dependent mitochondrial 27-hydroxylation of C-triol after prior inhibition by a CO–O_2 gas mixture. They found that the enzyme activity was maximally reversed by light at a wavelength of 450 nm. As isocitrate produces NADPH by a specific isocitrate dehydrogenase in mitochondria but cannot be an electron donor to microsomal P450, the possibility that hydroxylation was mediated by contamination of the mitochondrial fraction by a microsomal P450 was excluded.

At almost the same time Sato et al. (17) solubilized liver mitochondria and partially purified the enzyme by their newly developed P450-purification method (20, 21). The preparation contained a heme group and showed spectral properties characteristic of the heme proteins of the P450 family. The isolated P450 revealed a typical CO-difference spectrum characteristic of P450. The preparation could be reconstituted to catalyze CO-sensitive and NADPH-dependent 27-hydroxylation of C-triol when supplemented with NADPH-adrenodoxin reductase and adrenodoxin, both purified from bovine adrenocortical mitochondria. Sato et al. (17) presented additional evidence in favor of P450 being of mitochondrial origin. To do this, they prepared an inner membrane-matrix fraction free of microsomes (based on activity of the marker enzyme NADPH-cytochrome P450 reductase) and chose this fraction as a starting material for enzyme purification. C-triol 27-hydroxylation activity was reconstituted from the purified P450 with addition of adrenodoxin and NADPH-adrenodoxin reductase which are the characteristic electron donor components for mitochondrial P450 (22). The purified P450 was unable to N-demethylate benzphetamine, a typical microsomal P450 biotransformation. They concluded that rat liver inner mitochondrial membrane contains a species of P450 that catalyzes C-triol 27-hydroxylation. Okuda, Ruf, and Ullrich (23) performed additional experiments on the spectroscopic and electron spin resonance properties of the liver mitochondrial P450. They observed a type I substrate binding spectrum for C-triol, one of the typical binding spectra of P450 (24).

E. Stereospecificity of the sterol 27-hydroxylase

In 1965 Berseus (25) incubated [1,7,15,22,26-^{14}C]cholesterol with mouse liver mitochondria and isolated [1,7,15,22,26-^{14}C]cholest-5-ene-3β,27-diol. When the diol was converted to 3-oxo-5α-cholestanoic acid and subjected to decarboxylation, radioactivity was not present in the liberated carbon dioxide. He therefore concluded that hydroxylation occurred stereospecifically in the methyl group derived from C-3' of mevalonate. Stereospecificity of the product of mitochondrial sterol hydroxylase was studied by Atsuta and Okuda (26). They identified the product of the enzyme incubated with C-triol as (25R)-5β-cholestane-3α,7α,12α,26-tetrol. Carbon 25 of C-triol is prochiral. Kienle et al. (27) deduced that in the reduction of the double bond (Δ^{24}) of lanosterol (or of desmosterol), both hydrogens are added to the *re* face of the double bond, so that one of the C-26 atoms (derived from C-2 of mevalonate) becomes the pro-(R) methyl group,

whereas the other (derived from C-3' of mevalonate) becomes the pro-(S) methyl group in the side chain of cholesterol. When the pro-(S) methyl group is hydroxylated, the C-25 carbon atom becomes 25-R; conversely, when the pro-(R) methyl group is hydroxylated, the C-25 carbon atom becomes 25-S. Popjak et al. (28) proposed a nomenclature in which the pro-(R)-methyl group at C-25 (derived from C-2 of mevalonate) be numbered 26, and the pro-(S) methyl group (derived from C-3' of mevalonate) be numbered 27. The terminal hydroxylases may also be named according to this proposal, pro-(S) methyl hydroxylase as 27-hydroxylase and pro-(R) methyl hydroxylase as 26-hydroxylase.

F. Other properties of the sterol 27-hydroxylase

Atsuta and Okuda (29) compared the 27-hydroxylation of C-diol with that of C-triol by rat liver mitochondria. They found that the two enzyme activities had similar properties. In addition, they observed that the two enzyme activities in the same P450 preparation copurified when Sato's method (17) was used. Based on criteria proposed by Dixon and Webb (30) they showed that C-diol is 27-hydroxylated by the same enzyme as C-triol. They proposed that both enzyme activities are catalyzed by one active site of a single protein (31). Their final preparation showed a turnover number of 23 min^{-1} for C-triol 27-hydroxylation and 11 min^{-1} for C-diol 27-hydroxylation; the specific content of P450 was 2.0 nmol/mg of protein.

Recently a new function of P450 of sterol 27-hydroxylase (P45027) was proposed. In this proposal P450 catalyzes the further hydroxylation of 27-OH-C-triol to give *gem*-diol which undergoes dehydration to form the aldehyde (32); and the aldehyde is then further oxidized by the same enzyme to THCA. Whether all THCA is formed by this mechanism or by a pathway involving dehydrogenation (14) is unclear.

Atsuta and Okuda (33) also purified rat liver mitochondrial iron-sulfur protein (liver ferredoxin) and NADPH-liver ferredoxin reductase. The purified liver ferredoxin showed a typical absorption spectrum characteristic of adrenodoxin and molecular weight of 12,400, and the partially purified NADPH-liver ferredoxin reductase showed a spectrum characteristic of flavoprotein. C-triol 27-hydroxylation activity could be reconstituted using these proteins and the purified liver mitochondrial P450. Liver ferredoxin showed much higher affinity toward P450 than adrenodoxin. At about the same time, Pedersen, Oftebro, and Vanngaard (34) and Pedersen and Godager (35) independently purified liver mitochondrial adrenodoxin and NADPH-liver ferredoxin reductase, respectively, and used these proteins for reconstitution of cholesterol 27-hydroxylation. Ichikawa and Hiwatashi (36) purified NADPH-ferredoxin reductase from rabbit liver mitochondria and found that the purified enzyme contained FAD but not FMN as a prosthetic group. Mitochondrial C-triol hydroxylase was thus similar to other mitochondrial NADPH-adrenodoxin reductases, but differed from NADPH-cytochrome P450 reductase which contains both FMN and FAD. Waki, Hiwatashi, and Ichikawa (37) also purified NADPH-ferredoxin reductase from bovine mitochondria.

G. Enzyme purification

In 1984, Wikvall (38) reported the further purification of rabbit liver mitochondrial 27-hydroxylase by essentially the same method as Sato et al. (17); the enzyme ran as a single band on polyacrylamide gel electrophoresis and a specific content of P450 of 10 nmol/mg of protein. Dahlbäck (39) further purified C-triol 27-hydroxylase activity and isolated a preparation with a higher turnover number and a higher specific content. She determined a partial NH_2-terminal amino acid sequence and found that it differed from that of all P450s previously isolated.

The homogeneous preparation of C-triol 27-hydroxylase with the highest turnover number was isolated from rat liver mitochondria by Okuda, Masumoto, and Ohyama in 1988 (40). These workers used an HPLC method that enabled the chromatographic purification to be completed within 1 h, and thus markedly reduced the time required for purification of the labile enzyme. The specific content of P450 of the purified enzyme was 12 nmol/mg

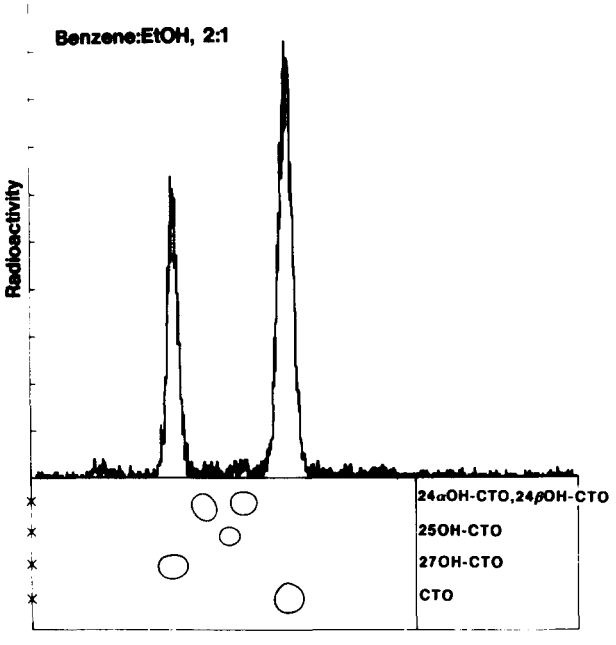


Fig. 3. Thin-layer chromatogram of the products obtained by incubation of C-triol with P45027 purified from rat liver mitochondria to compare activities of the purified enzyme toward two different possible substrates. Incubation mixture contained 0.05 nmol of P45027, 4 nmol of adrenodoxin, 0.1 unit of NADPH-cytochrome P450 reductase, 10 mmol of NADPH, and 14 nmol of [^{3}H]C-triol in a final volume of 1 ml. Incubation was conducted for 15 min at 37°C.

of protein and the molecular weight was 52,500. The turnover number toward C-triol was 35.5 min^{-1} (**Fig. 3**). The enzyme also showed slight activity in the hydroxylation of cholesterol. [The product was 27-hydroxycholesterol, based on its having the same R_f value as the 27-hydroxycholesterol prepared from kryptogenin (41) (**Fig. 4**)]. Although the activity of the purified enzyme toward cholesterol was extremely low compared to that for C-triol (turnover number for cholesterol was 0.7 min^{-1} compared to 35.5 min^{-1} for C-triol), it was sufficient to explain the activity observed by Björkhem and Gustafsson (11, 42) in rat liver mitochondria. Owing to its very low activity toward cholesterol, this hydroxylation activity is unlikely to be of physiological significance in cholesterol metabolism per se. Nonetheless, this hydroxylation could be important if the product were to play a regulatory role in cholesterol biosynthesis (43). The lower value for P450 specific content could also have been caused by loss of the prosthetic group of heme from the protein as the purified protein had only one major NH_2-terminal amino acid (44).

H. Hydroxylation of vitamin D_3

Okuda et al. (40) examined the substrate specificity of their highly homogeneous preparation of 27-hydroxylase. They were astonished to find that the preparation showed the same hydroxylation activity toward vitamin D_3 as the homogeneous preparation of vitamin D_3 25-hydroxylase prepared by Masumoto, Ohyama, and Okuda (45) who had purified the enzyme based on its vitamin D_3 25-hydroxylation activity (turnover number: 0.31 min^{-1}) (**Table 1**). Since Okuda's preparation showed the same turnover number toward vitamin D_3 as the homogeneous preparation, the activity of the 27-hydroxylase toward vitamin D_3 could not be attributed to contamination by microsomes. (The astonishingly close agreement in the turnover number for the two enzyme preparations suggested that a single enzyme was involved.) Independently, Dahlbäck and Wikvall (46) also noticed that both C-triol 27-hydroxylase and vitamin D_3 25-hydroxylase activities were present in their mitochondrial P450 preparation. However, they considered that the two activities were due to separate enzymes (see below).

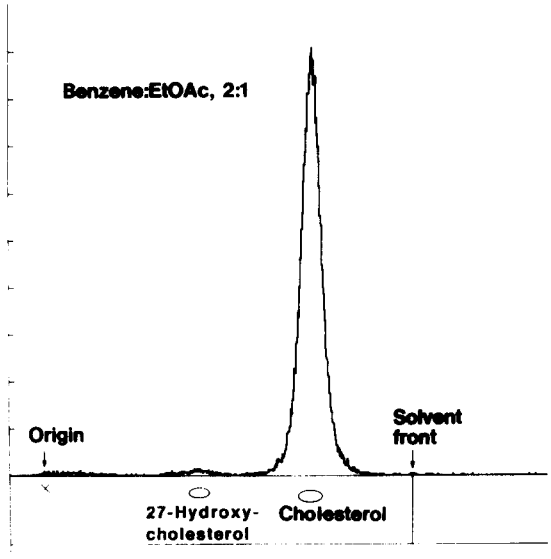


Fig. 4. Thin-layer chromatogram of the product obtained by incubation of cholesterol with P45027 purified from rat liver mitochondria to compare the activity with that toward C-triol. Incubation conditions are the same as described in the legends to Fig. 3.

TABLE 1. Enzymatic activities of the purified rat liver mitochondrial P450[a]

Substrate	Vitamin D_3	5β-Cholestane-3α,7α,12α-triol (C-triol)
Enzymatic biotransformation	25-Hydroxylation	27-Hydroxylation
Turnover Number	0.36 min^{-1}	35.5 min^{-1}

[a]The reaction mixture contained 0.025 nmol P45027, 2 nmol of adrenodoxin, 0.05 unit of NADPH-adrenodoxin reductase, and 10 mmol of NADPH in a final volume of 0.5 ml. Incubations were conducted for 15 min at 37°C.

I. Cloning of the sterol 27-hydroxylase

A complementary DNA for rabbit C-triol 27-hydroxylase was isolated by Andersson et al. (47) from a rabbit liver cDNA library using a synthetic DNA encoding the NH_2-terminal amino acid sequence. The cDNA contained 1725 bp open reading frame encoding 535 amino acid residues, of which a fraction of sequence consisting of 36 amino acids was processed when the nascent protein was transported to mitochondria. The total number of amino acids of the mature enzyme was therefore 499, corresponding to a molecular weight of 56,657. Andersson et al. (47) thus confirmed that the P450 is indeed of mitochondrial origin using molecular biology techniques. Blotting experiments revealed that the mRNA for this enzyme was expressed in many tissues. In their report, they proposed that the mRNA may be involved in generating intracellular sterols that could be endogenous regulators of enzymes involved in cholesterol homeostasis, as they are powerful suppressors of the transcription of genes involved in expression of the low density lipoprotein receptor (48). However, they were not aware at the time that the same enzyme is also involved in production of the active form of vitamin D_3, 1α,25-$(OH)_2$-D_3 (see below).

The C-triol 27-hydroxylase present in rat liver was cloned by Usui, Noshiro, and Okuda (44) in 1990, in an independent study.

II. HUMAN LIVER MITOCHONDRIAL 27-HYDROXYLASE AND ITS GENETIC DEFICIENCY

A. Human sterol 27-hydroxylase

The first report on human mitochondrial 27-hydroxylase was by Björkhem et al. (18) who reported that human hepatic C-triol 27-hydroxylase was located predominantly in mitochondria and that its activity in microsomes was extremely weak. These workers observed vigorous C-triol 25-hydroxylation activity in microsomes. However, the relative importance of the two hydroxylases in the metabolism of cholesterol was not determined.

A major advance was made by Cali and Russell (49) who reported isolation of complementary DNA for human liver C-triol 27-hydroxylase. The protein encoded consisted of a 33-amino acid mitochondrial signal sequence followed by a mature protein of 498 amino acids.

B. Cerebrotendinous xanthomatosis

1. *Clinical and biochemical features.* Cerebrotendinous xanthomatosis (CTX) is a rare inherited disease characterized by abnormal deposition of cholesterol and cholestanol in body tissues. Deposition in the central nervous system leads to neurological dysfunction marked by dementia, spinal cord dysfunction, and cerebellar ataxia. Deposition in other tissues causes tendon xanthomatosis, premature atherosclerosis, and cataracts (50). In 1974, Setoguchi et al. (51) reported that a major metabolic defect associated with this disease was present in the pathway of cholesterol side-chain cleavage. They observed an unusual accumulation of 5β-cholestane-3α,7α,12α,25-tetrol as well as its metabolites, such as 5β-cholestane-3α,7α,12α,24ξ,25-pentol and others, in the bile of the CTX patients. In addition, 27-hydroxy sterols were completely absent from the patient's bile. They therefore proposed two possible interpretations for defective bile acid formation in the CTX patient: the first is a lack of an enzyme that catalyzes the further transformation of 5β-cholestane-3α,7α,12α,24ξ,25-pentol to cholic acid; the second is the lack of an enzyme that catalyzes the transformation of C-triol into 27-OH-C-triol.

2. *The biochemical defect in CTX.* Salen et al. (52) proposed that the defect in CTX was due to the impaired oxidation of the side chain of cholesterol, a step that is necessary for bile acid biosynthesis. They attributed the reduced bile acid formation in the CTX patient to a defect in the ability to hydroxylate 25-hydroxylated C_{27}-steroids in the 24β-position. (Because the hydroxylases involved in this pathway are entirely in microsomes, a further description of their properties is beyond the scope of this review.) However, their results could be interpreted differently. For example, under normal conditions, C-triol is hydroxylated by the 27-hydroxylase in liver mitochondria to give 27-OH-C-triol. In CTX, if 27-hydroxylation were blocked, C-triol could undergo additional side-chain hydroxylation at a variety of sites by P450s existing in endoplasmic reticulum that are known to act on xenobiotics. Such hydroxylation would constitute a salvage pathway, even if incomplete, and would be similar to the biochemical events known to occur in many inborn errors of metabolism where intermediates accumulate and are biotransformed to uncommon metabolites. Taniguchi et al. (9) had previously suggested that there was a functional difference between the P450 hydroxylases present in liver mitochondria and those present in the endoplasmic reticulum when C-triol was used as a substrate. Oftebro et al. (53) examined these possibilities. When C-triol was incubated with liver subcellular fractions obtained from a CTX patient, they observed no C-triol 27-hydroxylation activity in either mitochondria or endoplasmic reticulum. Then, in 1986 Skrede et al. (54) made the seminal observation that 27-hydroxylation activity was barely detectable in fibroblasts from CTX patients, but was present in cultured skin fibroblasts from healthy individuals.

Together, these results established that the metabolic defect of the CTX patient was the absence of C-triol 27-hydroxylation activity in liver mitochondria. Oftebro et al. (53, 55) proposed that even though the alternative pathway to bile acid formation was present in CTX patients (involving a microsomal sterol 25-hydroxylase), such hydroxylation activity was not sufficient to compensate for the lack of the mitochondrial enzyme. They therefore concluded that the liver mitochondrial sterol 27-hydroxylation is essential for normal synthesis of bile acids in humans.

Deficiency in mitochondrial C_{27}-steroid 27-hydroxylation could be the result of either an enzyme defect per se, or a defect in one or more mechanisms responsible for transporting the C_{27}-steroid substrates to the active site of the enzyme, e.g., a lack of a transport protein (9). To investigate this problem, Oftebro (55) solubilized liver mitochondria of a CTX patient and a normal human subject and compared the 27-hydroxylation activity of these preparations. In all preparations from the control subject, P450 was detectable by spectroscopy and all were catalytically active in both 27-hydroxylation of C-triol and vitamin D_3 25-hydroxylation. In contrast, in the solubilized preparation from the liver biopsy of the CTX patient, no P450 spectrum was observed and no 27-hydroxylation of either C-triol or C-diol could be detected. On the other hand, the liver mitochondrial fraction of the CTX patient did catalyze 25-hydroxylation of vitamin D_3 in a reconstituted enzyme system containing adrenodoxin and NADPH-adrenodoxin reductase. (The absolute dependency upon ferredoxin for this hydroxylation activity proved once again that 25-hydroxylation of vitamin D_3 is catalyzed by a cytochrome P450 of mitochondrial origin.) The findings of Oftebro (55) indicated clearly that CTX is caused by a failure to synthesize a functional species of

liver mitochondrial P450, normally catalyzing 27-hydroxylation of C_{27}-steroids in human liver.

3. The genetic defect in CTX. Recently, Cali et al. (56) have identified different point mutations in the gene encoding C-triol 27-hydroxylase in two unrelated patients. Both mutations involved single base pair substitutions that resulted in an arginine codon (CGPy) being replaced by a cysteine codon (TGPy). In the first patient, the aberrant cysteine was at position 446, and two residues away from a cysteine at position 444, which is known to be a ubiquitously conserved P450 residue in protein, where it serves as a ligand for the prosthetic group of heme. In the second patient, the aberrant cysteine was located at position 362 in the sequence. This region is highly conserved among mitochondrial P450s and is considered to bind the adrenodoxin cofactor. In both cases, the observed mutations were shown to be present in functional domains of the enzyme; transfection of mutant cDNAs into culture cells by Cali et al. (56) resulted in the synthesis of immunoreactive sterol 27-hydroxylase protein with greatly diminished activity. Two other mutations underlying this disease were recently found in Jews of Moroccan origin (57). In the first case, there was a frame-shift mutation due to deletion of thymidine in exon 4; in the second, a splice-junction mutation was present. This was caused by a guanosine to adenosine substitution at the 3' splice acceptor site of intron 4 of the gene.

III. VITAMIN D_3 HYDROXYLATION ACTIVITY OF THE STEROL 27-HYDROXYLASE

A. Vitamin D_3 hydroxylation

Vitamin D_3 is converted into its active form by hydroxylation at C25 in the liver and subsequent 1α-hydroxylation in the kidney (58). The product, 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2$-D_3), is the active form of the vitamin. DeLuca (59) reported that the 25-hydroxylation step was catalyzed by liver mitochondria. Later, however, Bhattacharyya and DeLuca (60, 61) reported that vitamin D_3 25-hydroxylase is present in microsomes and that its activity is dependent on the concentration of ionized calcium in serum.

Björkhem and Holmberg (19, 62) reinvestigated the subcellular location of the enzyme using mass fragmentography. They found that NADPH-dependent 25-hydroxylating activity was present in both mitochondria (specific activity: 3 pmol/mg of protein/min) and microsomes. NADPH could be replaced by isocitrate in mitochondria, probably because the former was produced by the activity of mitochondrial NADP-dependent isocitrate dehydrogenase. When the mitochondrial enzyme activity was incubated in an atmosphere of $^{18}O_2$, ^{18}O was incorporated into the product. In 1983 Andersson, Holmberg, and Wikvall (63) purified microsomal P450 catalyzing vitamin D_3 to homogeneity. However, these workers did not sequence the enzyme, so it could not be ascertained whether this enzyme was new or identical to those already described. In 1984, Hayashi, Noshiro, and Okuda (64, 65) independently purified the microsomal P450 that catalyzes vitamin D_3 25-hydroxylation. They found that the enzyme had the same NH_2-terminal amino acid sequence as P4502C11 which was known to exist in male liver microsomes but not in female microsomes. In fact, the female liver microsomes showed a very low activity toward vitamin D_3 25-hydroxylation (about 1/5th that of male liver microsomes) (66).

B. Attempts to distinguish vitamin D_3 hydroxylase from sterol 27-hydroxylase

Saarem et al. (67) studied the 27-hydroxylation activities of human liver mitochondria toward C-triol and C-diol, as well as 25-hydroxylation activity toward vitamin D_3. They found that all three hydroxylation activities were present in mitoplast. They therefore concluded that all three hydroxylation activities are probably exclusively located in the inner mitochondrial membrane of human liver.

Björkhem et al. (68) studied the hydroxylation activity of P450 solubilized from rat liver mitochondria. They reported that phenobarbital treatment caused 25-hydroxylation of vitamin D_3 to increase, whereas it caused 27-hydroxylation activity to decrease. A rachitogenic diet caused a marked stimulation of 25-hydroxylation of vitamin D_3 activity but had little effect on 27-hydroxylation of C-triol. They considered that these differences in "inducibility" suggested the existence of at least two species of P450 in liver mitochondria, one or more involved in vitamin D_3 25-hydroxylation and another involved in sterol 27-hydroxylation. Furthermore, Oftebro et al. (53) detected 25-hydroxylation activity in mitochondria from CTX liver, while no 27-hydroxylation activity was detectable, providing additional evidence for at least two enzymes being involved in 25-hydroxylation of vitamin D_3. Saarem and Pedersen (69) observed that both vitamin D_3 25-hydroxylase and C-triol 27-hydroxylase activities are higher in female rat liver mitochondria than those in the male; they also reported that both enzyme activities increased in a parallel fashion when estradiol (valerate) was injected into male rats. In contrast, injection of testosterone into female rats decreased the activity of both of these mitochondrial enzymes. Dahlbäck (39) has prepared a monoclonal antibody against rabbit liver mitochondrial C-triol 27-hydroxylase. The antibody inhibited C-triol 27-hydroxylation but did not inhibit vitamin D_3 25-hydroxylation. She suggested that the two hydroxylase activities were due to separate enzymes. Differing results were obtained by Ohyama et al. (70) who studied these reactions using criteria proposed by Dixon and Webb (30) for testing the specificity of an enzyme. Ohyama et al. (70) observed that during successive purification steps, there

was a constant ratio between the two activities at all stages of purification. In addition, it was impossible to separate two activities by a number of procedures such as hydrophobic chromatography on an ω-aminohexyl Sepharose column or high performance liquid chromatography using TSK-gel DEAE-5PW column. When the enzyme was inactivated by controlled heating or by controlled modification by N-bromosuccinimide, the two enzyme activities declined in a similar fashion. When C-triol 27-hydroxylation activity was measured in the presence of vitamin D_3, it was inhibited competitively with vitamin D_3, and vice versa, suggesting that there is only one active site for both substrates.

As it was hardly conceivable that two different protein entities behave exactly in a parallel fashion to all these treatments, Ohyama et al. (70) proposed that vitamin D_3 25-hydroxylation and C-triol 27-hydroxylation are catalyzed at a common active site of a single protein. This finding seems to explain why abnormal vitamin D metabolism is observed in CTX patients (71). The discrepancy with the results of previous authors has not been explained.

C. Cloning of vitamin D_3 hydroxylase

Masumoto et al. (45) purified rat liver mitochondrial vitamin D_3 25-hydroxylase to homogeneity based on its enzyme activity. They used an HPLC technique and obtained a homogeneous preparation of P450 that had the highest turnover number so far reported for this enzyme. It showed a single band on polyacrylamide gel electrophoresis and had a turnover number of 0.31 min^{-1}. Usui et al. (44) prepared antibodies against this protein prepared according to Masumoto's method. Using these antibodies as a probe, they isolated a cDNA clone encoding this enzyme. The isolated cDNA showed no similarity to any P450s known, except for that of the C-triol 27-hydroxylase isolated by Andersson et al. (47) from rabbit liver mitochondria with which it had 73% similarity. It contained a mitochondria-specific presequence consisting of 32 amino acid residues. The mature enzyme therefore consisted of 501 amino acid residues corresponding to a molecular weight of 57,182.

Usui et al. (72) prepared an expression plasmid encoding rat liver mitochondrial vitamin D_3 25-hydroxylase (the C-triol 27-hydroxylase) and transfected it into COS cells. After transfection, they observed both C-triol 27-hydroxylation as well as vitamin D_3 25-hydroxylation activities in the solubilized mitochondria of the transfected cells; the reaction mixture was supplemented with adrenodoxin and NADPH-adrenodoxin reductase. Akiyoshi-Shibata et al. (73) transfected the cDNA encoding for the precursor protein of rat liver mitochondrial vitamin D_3 25-hydroxylase (P450LMT25) and expressed it under the control of the yeast alcohol dehydrogenase I promoter and terminator in *Saccharomyces cerevisiae* cells. The mitochondrial fraction prepared from the transformed yeast cells exhibited both 25-hydroxylation activity toward vitamin D_3 and 27-hydroxylation activity toward C-triol in a reconstituted system containing bovine adrenodoxin and NADPH-adrenodoxin reductase. They thus ruled out a possibility that the mammalian cell line contained endogenous C-triol 27-hydroxylase activity. Su et al. (74) independently isolated a cDNA encoding a rat ovary mitochondrial P450 catalyzing both cholesterol 27-hydroxylation and vitamin D_3 25-hydroxylation.

IV. MICROSOMAL 26-HYDROXYLASE

Although microsomal P450 is out of the scope of this review, it may be useful to compare its properties with mitochondrial P450. Cronholm and Johansson (10) observed strong C-triol ω-hydroxylation activity[2] in microsomes and Björkhem and Gustafsson (11) observed a similar reaction product when C-diol was used as substrate. They also noted the existence of C-triol and C-diol 27-hydroxylase in liver mitochondria; however, the microsomal hydroxylation activity was not characterized in any detail. It was reported that the major product(s) were likely to be secondary or tertiary alcohols (9–11). Gustafsson and Sjöstedt (75) showed that rat liver microsomes hydroxylate C-triol exclusively at the 25-pro-(R) methyl carbon. In contrast, Atsuta and Okuda (26) found that C-triol was hydroxylated at the 25-pro-(S) methyl carbon by mitochondria. Considering the exquisite stereospecificity of enzymes in general, these results strongly suggested that the microsomal enzyme differed from the mitochondrial P450 enzyme (P45027) characterized by Okuda's group. Further work on the microsomal enzyme, such as purification and isolation of its cDNA, is needed. Because the rate of microsomal hydroxylation might exceed that of mitochondrial hydroxylation (10, 11), the physiological function of microsomal P450 as well as its chemical distinction from the mitochondrial P450 should be clarified.

V. CONCLUSION

The existence of a "P450-like entity" in liver mitochondria was unequivocally established by Taniguchi et al. (9). Its assignment to the P450 class was confirmed based on its photochemical action spectrum (16). The functional P450 in liver mitochondria was purified by Sato et al. (17) for the first time. They established that its absorbance spectrum was characteristic of P450 and succeeded in reconstituting the activity using NADPH-adrenodoxin

[2]As the chirality of the product was not known at the time, it was described as ω-hydroxylase.

reductase and adrenodoxin, both prepared from bovine adrenal mitochondria, as electron donors. Over the past two decades, these studies, using classical biochemical techniques, have culminated in the cloning of the enzyme. It is now clearly established that a species of P450 (P45027; gene symbol *CYP27*) is present in rat, rabbit, and human liver mitochondria which not only catalyzes the 27-hydroxylation of C-triol, an intermediate in the conversion of cholesterol to bile acids, but also catalyzes 25-hydroxylation of vitamin D_3 to give 25-hydroxyvitamin D_3, the precursor of the active form of vitamin D_3.

It will be fascinating to learn how two physiologically important reactions, C-triol 27-hydroxylation and vitamin D_3 25-hydroxylation, are carried out by a single P450; what is the physiological meaning of these differing hydroxylation pathways; and how are these two reactions regulated under physiological and pathological conditions. It will also be interesting to learn whether the anomaly of vitamin D metabolism observed in CTX patients is due to the lack of vitamin D_3 25-hydroxylase, what is its pathophysiological significance, and whether alternative pathways for 25-hydroxylation of vitamin D_3 are present in these patients.

CTX is now firmly established as a genetic disease caused by deficient C-triol 27-hydroxylation. Four genetic defects have been elucidated and more are likely to be discovered.

Studies are also needed to define the physiological significance of P45027 that is present in extrahepatic tissues such as skin, duodenum, adrenals, and lungs.

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