OXIDATION MECHANISM OF 7-HYDROXY-Δ8-TETRAHYDROCANNABINOL AND 8-HYDROXY-Δ9-TETRAHYDROCANNABINOL TO THE CORRESPONDING KETONES BY CYP3A11

TAMIHIDE MATSUNAGA, HIROYUKI TANAKA, SHINSUKE HIGUCHI, KINYA SHIBAYAMA, NOBUYUKI KISHI, KAZUHITO WATANABE, AND IKUO YAMAMOTO

Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Hokuriku University

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ABSTRACT:

A cDNA isolated from a C57BL/6 mouse liver cDNA library had the identical nucleotide sequence in coding region with the mouse CYP3A11, and the NH2-terminal sequence was also identical to that of cytochrome P450 (P450) MDX-B, a microsomal alcohol oxygenase. The COS-7 cells transfected with the CYP3A11 expression vector formed 7-oxo-Δ8-tetrahydrocannabinol (7-oxo-Δ8-THC) from 7α- and 7β-hydroxy-Δ8-THC. An immunologically related protein with P450 MDX-B was expressed in the COS-7 cell microsomes. The cell microsomes expressed CYP3A11; COS-3A11 catalyzed the oxidation of 7-hydroxy-Δ8-THC and 8-hydroxy-Δ9-THC to 7-oxo-Δ8-THC and 8-oxo-Δ9-THC, respectively, in a reconstituted system. 18O derived from atmospheric oxygen was incorporated into about 30% of the corresponding ketones formed from 7α-hydroxy-Δ8-THC and 8β-hydroxy-Δ8-THC by mouse hepatic microsomes, P450 MDX-B, and COS-3A11, although incorporation of the stable isotope into the oxidized metabolites from 7β-hydroxy-Δ8-THC and 8α-hydroxy-Δ8-THC was negligible. 18O, however, was not incorporated into 7-oxo-Δ8-THC formed from 7α-hydroxy-Δ8-THC by using cumene hydroperoxide instead of NADPH under 18O2. When 18O-labeled 7α-hydroxy-Δ8-THC and 8β-hydroxy-Δ8-THC were incubated with above enzymes under air, about 30% of the ketones formed released 18O from a hydroxy group at the 7 and 8 positions in the course of the oxidation. These results suggest that 7α-hydroxy-Δ8-THC and 8β-hydroxy-Δ8-THC may be oxidized to the corresponding ketones by CYP3A11 via a gem-diol pathway. 7β-Hydroxy-Δ8-THC and 8α-hydroxy-Δ8-THC may be also converted to the ketones through a stereoselective dehydration of an enzyme-bound gem-diol rather than through a direct hydrogen extraction as a peroxyl form of the enzyme.

Tetrahydrocannabinol (THC), a psychoactive constituent of marijuana, is known to be extensively metabolized in various animal species (Harvey, 1984; Harvey and Paton, 1984). Two isomers of THC, Δ8- and Δ9-THC, have been isolated from marijuana. Many studies concerning metabolism of Δ8-THC and Δ9-THC have been reported, whereas the content of Δ9-THC in Mexican origin is 10% at most of total THC (Hivelé et al., 1966), and it may be actually formed by isomerization (Turner et al., 1980). Because it shows comparable pharmacological activity to Δ9-THC and is chemically more stable, it is a useful substance for studies of metabolism (Yamamoto et al., 1988; Watanabe et al., 1991, 1992).

The major metabolic sites in the isomers are allylic position at the C-7 (for Δ8-THC) and C-8 (for Δ9-THC), and C-11 (both of Δ8- and Δ9-THC) (Harvey and Paton, 1984). It has been generally known that secondary alcohols, such as hydroxysteroids, 3-hydroxyhexobarbital, and morphine, are oxidized to the corresponding ketones by dehydrogenases in cytosol and microsomes (Kageura and Toki, 1975; Masere and Bannenberg, 1994; Yamamoto et al., 1997). However, we have found for the first time that a microsomal enzyme, called microsomal alcohol oxygenase (MALCO), is able to oxidize the secondary alcohols, 7α- and 7β-hydroxy-Δ8-THC to 7-oxo-Δ8-THC (Narimatsu et al., 1988) (Fig. 1). It has been reported that the aliphatic, benzyl, 1-phenylethyl, and allylic alcohols are oxidized to the corresponding carbonyl compounds by numerous cytochrome P450 (P450) enzymes with overlapping substrate specificity (Morgan et al., 1982; Vaz and Coon, 1994; Bellucci et al., 1996). We have purified two P450 enzymes, P450PFB-B and P450 MDX-B, the major enzymes of MALCO in hepatic microsomes of guinea pig (Matsunaga et al., 1997) and mouse (Matsunaga et al., 1998), respectively. These enzymes are estimated to be CYP3A1 isoforms from catalytic properties and NH2-terminal amino acid sequences, especially since the sequence of P450 MDX-B is the same as that of CYP3A11 (Yanagimoto et al., 1992). We have recently clarified that 7α- and 7β-hydroxy-Δ8-THC MALCO activities in human liver are catalyzed by CYP3A4. When the substrates were incubated with P450PFB-B and CYP3A4 under an 18O-gas phase, atmospheric oxygen was incorporated into 7-oxo-Δ8-THC formed from 7α-hydroxy-Δ8-THC, whereas incorporation of the stable isotope into the oxidized metabolites from 7β-hydroxy-Δ8-THC was negligible (Matsunaga et al., 1997, 2000).
A general mechanistic scheme of reactions catalyzed by P450 accounts for the insertion of an oxygen atom derived from atmospheric oxygen into the oxidized product. In the oxidation of alcohols to carbonyl products, however, some exceptions to the predicted incorporation of an atom of O₂ into the carbonyl product have been observed by various laboratories. Partial or complete lack of incorporation of oxygen derived from O₂ into the carbonyl product has been observed, which is apparently not explainable by exchange with water (Akhtar et al., 1982; Cheng and Schenkman, 1983; Suhara et al., 1984; Wood et al., 1988). This has resulted in various mechanistic hypotheses for the oxidation of alcohols by P450, such as oxidative dehydrogenation (Cheng and Schenkman, 1983; Wood et al., 1988) or stereospecific dehydration of a transient gem-diol, such that the inserted oxygen is specifically lost (Suhara et al., 1984). Vaz and Coon (1994) reported the mechanism of oxidation of alcohol to the corresponding carbonyl compounds by a reconstituted system of CYP2B4 and CYP2E1 in more detail using benzyl and 1-phenyl alcohols as substrates.

The intention of the present study was to answer the question of whether oxidation of the α- and β-epimers of 7-hydroxy-Δ⁸-THC and 8-hydroxy-Δ⁷-THC in fact reside within the same protein by using recombinant enzymes and to characterize the oxidation mechanism of MALCO.

Experimental Procedures

Materials. 7α- and 7β-Hydroxy-Δ⁸-THC (Mechoulam et al., 1972), 7-oxo-Δ⁸-THC (Narimatsu et al., 1984), 8α- and 8β-hydroxy-Δ⁹-THC (Pitt et al., 1975) and 8-oxo-Δ⁹-THC (Pitt et al., 1975) were prepared by the methods previously reported. Purities of the cannabinoids were checked to be more than 98% by gas chromatography. The C57BL/6 mouse liver cDNA library and pCMV4 were generous gifts from Dr. Masahiko Negishi (Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC) and Dr. Frank J. Gonzalez (Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD), respectively. Restriction enzymes, the DNA ligation kit, PCR kit, M13 mp19, BcaBEST dyeoxy sequencing kit, and PCR primers were purchased from Takara Shuzo (Tokyo, Japan). The DIG DNA labeling kit was obtained from Roche Molecular Biochemicals (Summerville, NJ). ISOGEN was obtained from Nippon Gene (Tokyo, Japan). ¹⁸O₂ (97 atom %) was purchased from Amersham Pharmacia Biotech UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Other chemicals and solvents used were of the highest quality commercially available.

Fig. 1. Oxidation metabolism of Δ⁹-THC at the C-7 position by hepatic microsomes.

Labeled 7α-hydroxy-Δ⁸-THC and 8β-hydroxy-Δ⁹-THC were synthesized and isolated as follows. Δ⁹-THC (1 mg) and Δ⁸-THC (1 mg) were incubated with hepatic microsomes (2 g of liver equivalent) of guinea pigs and mice, respectively, and an NADPH-generating system as described below (final, 10.0 ml) at 37°C for 30 min under ¹⁸O₂. The metabolites were extracted into ethyl acetate (25 ml × 2 times), and the solvent was combined and evaporated in vacuo. The residue was dissolved in a small portion of ethyl acetate subjected to preparative thin-layer chromatography on a silica gel plate (0.25-mm thickness; 20 × 20 cm) with a solvent system of chloroform/acetone (40:1, v/v), and the metabolites were extracted with ethyl acetate (25 ml × 2 times). After evaporation of the solvent, small portions of the isolated metabolites were examined by gas chromatography/mass spectrometry (GC/MS) after conversion to the trimethylsilyl derivative, as described previously (Matsunaga et al., 1997). The ratios of relative intensities of molecular ions at m/z 476 [(M + 2)⁺] to m/z 474 (M⁺) were 4.35 and 7.69 for 7α-hydroxy-Δ⁸-THC and 8β-hydroxy-Δ⁹-THC, respectively.

Animals. Male mice of the dY strain (8-weeks old; Hokuriku Experimental Animal Lab, Kanazawa, Japan) were used in all experiments.

RNA Preparation. Total RNAs were prepared from mouse liver by using ISOGEN according to the manufacturer’s instructions. The resulting RNA was dissolved in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, quantified spectrophotometrically, and stored in aliquots at −70°C before use.

cDNA Cloning and Sequencing. Approximately 6.7 × 10⁶ plaques were screened by plaque hybridization using the PCR product of CYP3A11 as probes. The 16 positive clones were obtained after the second screening. The cDNAs were subcloned into pBluescript SK(–) multifunctional phagemids according to the manufacturer’s protocols (Toyobo, Tokyo, Japan). Among them, the longest clone isolated (approximately 2.1 kilobase pair) was cut out as BamHI and/or EcoRI fragments. These fragments were subcloned into M13 mp19, and DNA sequences of both strands were determined by the dyeodeoxy method (Sanger et al., 1977). Comparison of the nucleotide and the deduced amino acid sequences was carried out using GENETYX software (Software Development, Tokyo, Japan).

Construction of CYP3A11 Expression Vector. A plasmid vector capable of expressing CYP3A11 in mammalian cells (pCMV4-3A11) was constructed via standard methods of genetic engineering. The oligonucleotides for PCR primers used to amplify a CYP3A11 cDNA fragment, including the complete coding region, were 5'-GGCTGACCATTGGACCTTGGTTC-3' (sense primer) and 5'-GGCTGACCTGGAGGCTTC-3' (antisense primer) that were added to the restriction site of KpnI and XhoI, respectively, as underlined. Amplification of the DNA fragment from the CYP3A11 inserted into pBlue-
script SKI(−) was carried out by use of TaKaRa Ex Taq polymerase (Takara Shuzo). The DNA fragment was inserted into the KpnI and XhoI sites of pCMV4 (Andersson et al., 1989), which is a transient mammalian expression vector transformed into Escherichia coli HB101, and amplified the vector plasmid. The desired recombinant was characterized by hybridization and restriction enzyme mapping.

**Heterologous Expression of Mouse CYP3A11 in COS-7 Cells.** COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum and transfected by electroporation method using CellPorator Electroporation System I (Invitrogen, Rockville, MD). The cells were suspended in 1 ml of ice-cold Hepes-buffered saline (40 mM Hepes, pH 7.2, 0.8% NaCl) and mixed with 80 μg of pCMV4 or pCMV4–3A11. The cells were exposed to a single pulse of 1800 μF and 200 V and suspended in 25 ml of culture medium. The transfected cells were seeded to a 75-cm² flask and then incubated.

**Assay of Enzyme Activity.** Two days after transfection of pCMV4 or pCMV4–3A11 into COS-7 cells, the cells were washed with Dulbecco’s phosphate-buffered saline (Wako Pure Chemicals, Osaka, Japan), and the medium was replaced with 10 ml of Dulbecco’s modified Eagle’s medium containing 10%(v/v) fetal bovine serum. 7-Hydroxy-Δ⁴⁻THC (120 μg) was added directly to the culture medium (final substrate concentration, 36.4 μM). At 12 h after addition of the substrate, the medium was removed for assay of product formation, and then the cells were harvested to take a count of cell number. To assay the amount of products formed by the transfected cells, the medium was extracted twice with 25 ml of ethyl acetate after the addition of 5α-cholestan as an internal standard. The resulting organic phases were evaporated in vacuo, and analyzed by GC/MS after conversion to the trimethylsilyl derivatives. GC/MS was carried out at 70 eV with a JEOL CGC-06 gas chromatograph coupled with a JEOL JMS-DX 300 mass spectrometer (JEOL, Tokyo, Japan). The conditions of GC/MS were as follows: column, 5% SE-30 on Chromosorb W (60–80 mesh, 3 mm × 2 m); column temperature, 250°C; carrier gas, He (40 mI/min); ionizing current, 300 μAmp.

To prepare the microsomes, the transfected COS-7 cells were harvested at 48 h after the transfection. The cells were lysed by sonication and then homogenized by a Teflon-homogenizer. The microsomal pellets were prepared by centrifugation of the homogenate and suspended in 100 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 5 mM EDTA. The reaction mixture for recombiant forms (0.5 ml) contained 200 μg of microsomal protein, 100 mM potassium phosphate buffer, pH 7.4, 50 pmol of cytochrome b₅, 0.5 units of NADPH-P450 reductase, 0.46 mM sodium cholate, and 1 mM NADPH. The reaction was performed at 37°C for 15 min with 72.7 μM 7-hydroxy-Δ⁴⁻THC or 8-hydroxy-Δ⁴⁻THC as substrate. In the reconstitution of P450 MDX-B, the formation of 7-oxo-Δ⁴⁻THC and 8-oxo-Δ⁴⁻THC were measured essentially as described above, except for using P450 MDX-B (50 pmol) and microsomal lipids (50 μg) instead of microsomal protein. Hepatic microsomes of mice were prepared by the method reported previously (Matsunaga et al., 1996). The NADPH-dependent 7-oxo-Δ⁴⁻THC forming activity in the hepatic microsomes was determined essentially as previously described (Matsunaga et al., 1997). Cumene hydroperoxide (CuOOH)-mediated 7-oxo-Δ⁴⁻THC formation was performed as described by Rahimtulla and O’Brien (1974). 7α-Hydroxy-Δ⁴⁻THC (60.6 μM) was incubated with the hepatic microsomes (0.5 mg of protein), 75 μM CuOOH, and 100 mM potassium phosphate buffer, pH 7.4, to make a final volume of 0.5 ml. The mixture was incubated at 37°C for 15 min. Metabolites were extracted with 2.5 ml of ethyl acetate after addition of 5α-cholestan and analyzed by the method described above.

To examine oxygen incorporation from atmospheric oxygen into the resulting ketone, 7-hydroxy-Δ⁴⁻THC or 8-hydroxy-Δ⁴⁻THC was incubated with mouse hepatic microsomes, purified P450, or COS-7 cell microsomes expressed CYP3A11 in the incubation system, as described above at 37°C for 20 min under 18O₂. After incubation, the metabolites extracted with ethyl acetate were converted to trimethylsilyl derivative and analyzed by the same methods described above using GC/MS. The isotopic incorporation values were calculated using an equation [% incorporation = (A – B)/(1 + A – B) × 1/C × 10⁴], where A was the ratio in relative intensities of ions at [M + 2]⁺ to M⁺ of the corresponding ketone formed from 7-hydroxy-Δ⁴⁻THC or 8-hydroxy-Δ⁴⁻THC under 18O₂, B was the ratio in relative intensities of ions at [M + 2]⁺ to M⁺ of the corresponding ketone formed from 7-hydroxy-Δ⁴⁻THC or 8-hydroxy-Δ⁴⁻THC under 18O₂.

To examine oxygen release from a hydroxyl group at the 7- and 8-positions of 18O-labeled 7α-hydroxy-Δ⁴⁻THC (7α-18OH-Δ⁴⁻THC) and 8β-hydroxy-Δ⁴⁻THC (8β-18OH-Δ⁴⁻THC) in the course of the oxidation, 18O-labeled 7α-hydroxy-Δ⁴⁻THC (7α-18O-Δ⁴⁻THC) or 8β-hydroxy-Δ⁴⁻THC (8β-18O-Δ⁴⁻THC) was incubated with mouse hepatic microsomes, purified P450 or COS-7 cell microsomes expressed CYP3A11 in the incubation system, as described above at 37°C for 20 min under 18O₂. After incubation, the metabolites extracted with ethyl acetate were converted to trimethylsilyl derivative and analyzed by GC/MS. The isotopic incorporation values were calculated using an equation, % release = (A – B) × 100, where A and B were the apparent atom percentage of 18O in 7α-18OH-Δ⁴⁻THC or 8β-18OH-Δ⁴⁻THC and the corresponding ketone, respectively.

**Western Blot Analysis and Quantification of Recombinant Protein Expression.** Western blot analysis was carried out according to the method reported previously (Towbin et al., 1979). Microsomal protein of mouse livers and COS-7 cells were separated by SDS-polyacrylamide gel electrophoresis, which was carried out as described by Laemmli (1970). The blotted membrane was probed with polyclonal anti-P450 MDX-B antibody. The content of CYP3A11 protein in microsomes of COS-7 cells was determined from densitometric analysis (NIH Image Software, by Dr. W. Rasband) of Western blotting membrane.

Other Methods. P450 MDX-B was purified from hepatic microsomes of dexamethasone-treated mice by the method reported previously (Matsunaga et al., 1998). NADPH-P450 reductase and cytochrome b₅ were purified from hepatic microsomes of mice by the methods of Yasukochi and Masters (1976), and Funae and Imaoka (1985), respectively. One unit of the reductase was defined as the amount of reductase catalyzing the reduction of 1 μmol of cytochrome c/min. Polyclonal antibody against the purified P450 was raised in rabbits, as described previously (Narimatsu et al., 1990). Protein concentration was estimated by the method of Lowry et al. (1951), using bovine serum albumin as a standard. P450 content in mouse hepatic microsomes was determined by the methods of Omura and Sato (1964).

**Results**

**CYP3A11 cDNA Cloning and Heterologous Expression.** A cDNA clone encoding a P450 enzyme was isolated from a C57BL/6 mouse liver cDNA library. The nucleotide sequence of the coding region of this cDNA was identical with that of the mouse CYP3A11 (Yanagimoto et al., 1992), and the deduced amino acid sequence of NH₂-terminal was also identical to that of P450 MDX-B (Matsunaga et al., 1998). To determine the enzyme activity for 7-hydroxy-Δ⁴⁻THC of CYP3A11, we performed transient expression experiments in COS-7 cells. As described under Experimental Procedures, a cDNA fragment corresponding to the coding region of CYP3A11 was inserted into the pCMV4 expression vector to create plasmid pCMV4–3A11. This plasmid or the parental vector pCMV4 was transfected into COS-7 cells using an electroporation procedure. At 48 h after transfection, 7α- and 7β-hydroxy-Δ⁴⁻THC were added to the cell medium, and the ability of the cells to oxidize these substrates over the next 12 h was measured. As shown in Fig. 2, the formation of 7-oxo-Δ⁴⁻THC by the cells transfected with the pCMV4 vector alone was negligible. In contrast, introduction of the pCMV4–3A11 into the cells significantly enhanced the formation of 7-oxo-Δ⁴⁻THC from 7α- and 7β-hydroxy-Δ⁴⁻THC, and the catalytic activities were 4.1 and 4.8 nmol/12 h/2.5 × 10⁵ cells, respectively. The cells were harvested at 48 h after transfection, and microsomes were prepared. Antibody against P450 MDX-B detected a protein in microsomes of the pCMV4–3A11-transformed cells that comigrated with a single band.
in mouse hepatic microsomes (Fig. 3). In each case, the size was approximately 51 kDa, as expected.

**Enzymatic Characterization of Recombinant CYP3A11.** Table 1 shows the 7-hydroxy-Δ⁸-THC and 8-hydroxy-Δ⁹-THC activities in microsomes of mouse liver and CYP3A11-expressed COS-7 cells (COS-3A11), and P450 MDX-B reconstituted with reductase and cytochrome b₅. 7α- and 7β-Hydroxy-Δ⁸-THC and 8α- and 8β-hydroxy-Δ⁹-THC MALCO activities of COS-3A11 were found to be 1.85 and 2.74 nmol/min/nmol of P450, respectively. These catalytic activities are comparable to those of P450 MDX-B. COS-3A11 also showed oxidative activity for 8α- and 8β-hydroxy-Δ⁹-THC, and the activities were 1.44 and 2.18 nmol/min/nmol of P450, respectively. The forming activity of 8-oxo-Δ⁸-THC from 8β-hydroxy-Δ⁹-THC by COS-3A11 was about 1.5-fold higher than that from 8α-hydroxy-Δ⁹-THC and the same as 7-hydroxy-Δ⁸-THC.

**Incorporation of Atmospheric Oxygen into 7-Oxo-Δ⁸-THC and 8-Oxo-Δ⁹-THC.** Both the α- and β-epimers of 7-hydroxy-Δ⁸-THC and 8-hydroxy-Δ⁹-THC were incubated with COS-3A11 under ¹⁸O₂, and the trimethylsilyl derivative of metabolites was analyzed by GC/MS. The relative intensities of molecular ions at m/z 400 and 402 were shown in Table 2. The ratio in relative intensities of ions at m/z 402/400 of 7-oxo-Δ⁸-THC formed from 7α-hydroxy-Δ⁸-THC was 0.47 showing that ¹⁸O derived from an atmospheric oxygen molecule was incorporated into 26.9% of the oxidized metabolite. In the case of 7β-hydroxy-Δ⁸-THC, the ratio was 0.23, and ¹⁸O was incorporated into 9.7% of the metabolites. On the other hand, the ratios of 8-oxo-Δ⁹-THC formed from 8α- and 8β-hydroxy-Δ⁹-THC were 0.28 and 0.53, and ¹⁸O was incorporated into 14.7 and 31.5%, respectively. These results were consistent with the results using mouse hepatic microsomes and P450 MDX-B as the enzyme source.

Moreover, ¹⁸O-labeled 7α-hydroxy-Δ⁸-THC (7α-¹⁸OH-Δ⁸-THC) and 8β-hydroxy-Δ⁹-THC (8β-¹⁸OH-Δ⁹-THC) were incubated with microsomes under air. Table 3 lists the ratio in the relative intensities of the molecular ions [(M + 2)/M⁺] of substrates and metabolites. When 7α-¹⁸OH-Δ⁸-THC (ratio 4.35) and 8β-¹⁸OH-Δ⁹-THC (7.69) were incubated with COS-3A11, the ratios were changed to 1.45 and 1.61, indicating that 27.2 and 30.3% of the corresponding ketones formed released ¹⁸O from a hydroxyl group at the 7- and 8-positions, respectively, in the course of the oxidation. On the other hand, when...

![Figure 2](image2.png)  
**Fig. 2.** 7-Oxo-Δ⁸-THC forming activity in COS-7 cells expressed CYP3A11.

Expression plasmids, pCMV4 or pCMV4-3A11, were transfected into COS-7 cells, as described under Experimental Procedures. Two days after transfection, 7-hydroxy-Δ⁸-THC (final concentration, 36.4 μM) was added directly to the culture medium. At 12 h after addition of the substrate, the amount of products formed by the transfected cells was determined by GC/MS. The data are expressed as the mean ± S.E. of three experiments.

![Figure 3](image3.png)  
**Fig. 3.** Western blot analysis of COS-7 cell microsomes expressed CYP3A11, recombinant CYP3A11.

Microsomal proteins were separated by electrophoresis, transferred to membrane, and reacted with a polyclonal antibody against P450 MDX-B. Lane 1 contains hepatic microsomes of mice (3 μg). Lanes 2 and 3 contain microsomes of COS-7 cells (20 μg each) transfected with pCMV4 and pCMV4-3A11, respectively. The arrow indicates the molecular size of 51 kDa.

**TABLE 1**  
7-Oxo-Δ⁸-THC and 8-oxo-Δ⁹-THC forming activities of mouse hepatic microsomes, P450MDX-B and COS-3A11

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Forming Activity</th>
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<tr>
<td></td>
<td>Hepatic Microsomes</td>
</tr>
<tr>
<td>7α-OH-Δ⁸-THC</td>
<td>0.13</td>
</tr>
<tr>
<td>7β-OH-Δ⁸-THC</td>
<td>0.39</td>
</tr>
<tr>
<td>8α-OH-Δ⁹-THC</td>
<td>0.13</td>
</tr>
<tr>
<td>8β-OH-Δ⁹-THC</td>
<td>0.20</td>
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Each value represents the mean of duplicate determinations.  
N.D., not detected; —, not determined.
7β-hydroxy-Δ⁸-THC was incubated with mouse hepatic microsomes using CuOOH instead of NADPH under ¹⁸O₂, none of ¹⁸O was incorporated into 7-oxo-Δ⁸-THC (data not shown). In mouse hepatic microsomes, the corresponding ketones formed from 7α-¹⁸O-Δ⁸-THC and 8β-¹⁸O-Δ⁸-THC released about 30% of the ¹⁸O as COS-3A11 and CYP3A11.

Discussion

7-Hydroxy-Δ⁸-THC is NADPH- and O₂-dependently oxidized to 7-oxo-Δ⁸-THC by hepatic microsomes (Narimatsu et al., 1988). P450 MDX-B purified from mouse hepatic microsomes as a major enzyme of 7-hydroxy-Δ⁸-THC MALCO has been estimated to be CYP3A11 from the NH₂-terminus amino acid sequences and catalytic properties (Matsunaga et al., 1998). In the present study, we isolated CYP3A11 cDNA from a C57BL/6 mouse liver cDNA library. 7-Oxo-Δ⁸-THC was significantly formed when 7α- or 7β-hydroxy-Δ⁸-THC was added into the culture medium of COS-7 cells transfected with pcMV4-3A11. Furthermore, the COS-3A11 catalyzed the oxidation of 7α- and 7β-hydroxy-Δ⁸-THC and P450 MDX-B. 8α- and 8β-Hydroxy-Δ⁸-THC were also oxidized to the corresponding ketone by the COS-3A11. The recombinant CYP3A11 was immunologically reacted with the antibody against P450 MDX-B. 7α- and 7β-Hydroxy-Δ⁸-THC MALCO activities in hepatic microsomes of mice were almost completely inhibited by antibody against P450 MDX-B (Matsunaga et al., 1998). These results indicate that P450 MDX-B is CYP3A11 and that 7-hydroxy-Δ⁸-THC and 8-hydroxy-Δ⁸-THC are oxidized to the corresponding ketones by recombinant CYP3A11.

Several mechanistic pathways, which can be in part distinguished by use of ¹⁸O₂-gas phase or ¹⁸O-labeled substrates, can be proposed for the oxidation of 7-hydroxy-Δ⁸-THC (Fig. 4). The reaction is considered to occur by a mechanism involving an oxygen atom abstraction from the carbinol carbon (path A) or by electron abstraction from the oxygen (path B) by the oxo ferryl porphyrin π-cation radical [(Por⁺⁺)FeIV=O], which is believed to be the active oxidant of P450 (Groves and Han, 1995). The formed radical should be very short lived because no rearrangement to products other than 7-oxo-Δ⁸-THC appears to occur before rebound to the metal-bound hydroxy radical (effectively a hydroxy radical) to form a gem-diol, which finally gives the ketone by water loss (path D). If a basic residue in the active site is participating for holding of the intermediate during gem-diol breakdown, the molecular oxygen-derived oxygen atom will be completely lost. The degree of selectivity in the loss of either hydroxy from the bound gem-diol intermediate would depend on the rate constant for diol dehydration and the equilibrium constant for ligand exchange at the heme iron. The constant for ligand exchange would depend on the freedom of movement of the substrate within the active site and/or the intensity of interaction between the intermediate and active site, resulting in varying degrees of incorporation of oxygen from ¹⁸O₂ into the carbonyl group. A complete isotopic scrambling would be expected for this mechanism if complete conformational equilibration occurs in the gem-diol because H₂¹⁸O and H₂¹⁶O should be eliminated with the same probability. When the substrates were incubated with COS-3A11 under ¹⁸O₂, the ratios in relative intensities of ions at [(M + 2)⁺] to (M⁺) of the corresponding ketone formed from 7α-hydroxy-Δ⁸-THC were 0.47. These ratios may not be affected by oxygen exchange between the ketone and water because, in our previous experiment, we have demonstrated that ¹⁸O derived from water has not been taken up into 7-oxo-Δ⁸-THC formed from 7α-hydroxy-Δ⁸-THC (Narimatsu et al., 1988). This shows that the gem-diol pathway is certainly operative in the oxidation of 7α-hydroxy-Δ⁸-THC, although the preference for the ¹⁸O release from the metabolic intermediate was observed. There may not be a difference between the isotopes ¹⁸O and ¹⁶O for abstraction of water from gem-diol because the mass spectrometric analysis of the resulting ketone from ¹⁸O-labeled 7α-hydroxy-Δ⁸-THC also shows that about 30% of the resulting ketone released ¹⁸O from a hydroxy group. This result means that ¹⁶O derived from atmospheric oxygen was incorporated into about 30% of the resulting ketone. The observed preference could be due to incomplete conformational equilibration of the gem-diol before loss of water.

In the formation of 7-oxo-Δ⁸-THC from 7β-hydroxy-Δ⁸-THC, alternatively double hydrogen abstraction pathways may be involved, consisting in the reaction of the metal-bound hydroxy radical with the hydroxyl hydrogen (path E) or with a hydrogen attached to the C-6α position to give the enolic form of the ketone (path F). The last pathway has been proposed as the mechanism in the oxidative conversion of ethyl carbonate to vinyl carbamate catalyzed by CYP2E1 (Guegenerich and Kim, 1991). In path C, hydrogen atom abstraction from the hydroxy group followed by hydrogen abstraction from carbonyl carbon results in the ketone. Unlike above paths, electron abstraction from the carbonyl carbon radical (path G) or oxygen (path H) followed by proton loss yields the products. None of molecular oxygen must be incorporated into the resulting ketone by paths C, E, F, G, and H. However, GC/MS analyses of the resulting ketones from 7β-hydroxy-Δ⁸-THC incubated with hepatic microsomes, P450 MDX-B, and COS-3A11 under ¹⁸O₂ shows that molecular oxygen was incorporated into the resulting ketones, although the degree of incorporation of ¹⁸O from molecular oxygen is only about 10%. Furthermore, allylic positions are the major site of hydroxyl catalyzed by CYP3A (Smith and Jones, 1992). These results suggest that 7β-hydroxy-Δ⁸-THC might be also converted to the ketone through a stereoelectrotopic dehydration of an enzyme-bound gem-diol rather than through a direct hydrogen abstraction as a peroxy form of the enzyme, although the oxidation mechanisms for 7α-hydroxy-Δ⁸-THC might be different.

Wood et al. (1988) have reported that in the oxidation of testosterone and epistosterone to androstenedione by CYP2B1 a gem-diol intermediate is formed, which undergoes a stereoselective loss of water from the α-face. It might be speculated that in the CYP3A11-catalyzed oxidation of 7β-hydroxy-Δ⁸-THC to 7-oxo-Δ⁸-THC in an ¹⁸O₂ atmosphere a gem-diol intermediate formed also undergoes
stereoselective dehydration from the α-face. However, the degree of retention of labeled oxygen following the metabolism of 7α-hydroxy-Δ⁸-THC is significantly lower than that of epitestosterone (84% enriched) (Wood et al., 1988). Interestingly, the degree in incorporation of molecular oxygen into 8-oxo-Δ⁸-THC is different from that into 7-oxo-Δ⁸-THC. When the 8-hydroxy-Δ⁸-THC was incubated with microsomes or CYP3A11 under ¹⁸O₂, atmospheric oxygen was incorporated into about 30% of 8-oxo-Δ⁸-THC formed from β-epimer (8β-hydroxy-Δ⁸-THC) but only about 10% of 8-oxo-Δ⁸-THC formed from α-epimer (8α-hydroxy-Δ⁸-THC). This result is consistent with the result using 8β-¹⁸OH-Δ⁸-THC as substrate. If 7β-hydroxy-Δ⁸-THC undergoes C-7 hydroxylation at the pseudoaxial position of the more stable conformation of the substrate with pseudoequatorial hydroxy group and if water is lost stereoselectively from the pseudoaxial position before a complete conformational equilibration of the formed gem-diol has occurred, an excess of ¹⁶O over ¹⁸O ketone will be formed. 8α-Hydroxy-Δ⁸-THC with hydroxy group at the pseudoequatorial position in the more stable conformation (Archer et al., 1970) may also undergo C-8 hydroxylation at the pseudoxial position, and the retention of oxygen atom derived from molecular oxygen would be negligible.

These results suggest that 7α-hydroxy-Δ⁸-THC and 8β-hydroxy-Δ⁸-THC may be oxidized to the corresponding ketone by CYP3A11 via a gem-diol pathway. On the other hand, 7β-hydroxy-Δ⁸-THC and 8α-hydroxy-Δ⁸-THC may also be converted to the ketone through a stereoselective dehydration of an enzyme-bound gem-diol. Further extensive studies of steady-state deuteration isotope effects are required to clarify the rate-limiting step in the oxidation mechanism of 7-hydroxy-Δ⁸-THC and 8-hydroxy-Δ⁸-THC.

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References


Kageura E and Toki S (1975) Guinea pig liver 3-hydroxy-2-oxobutaryl dehydrogenase: purifica-

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