Communication

Oxygenation Mechanism in Conversion of Aldehyde to Carboxylic Acid Catalyzed by a Cytochrome P-450 Isozyme*

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The oxygenation of an aldehyde, 11-oxo-Δ²-tetrahydrocannabinol to a carboxylic acid, Δ⁹-tetrahydrocannabinol-11-oic acid was catalyzed by cytochrome P-450 P-450 MUT-2 purified from hepatic microsomes of male ddN mice. The oxygenation mechanism was confirmed by the incorporation of oxygen-18 from molecular oxygen into the carboxylic acid formed. An aldehyde form but not a hydrated form of 11-oxo-Δ²-tetrahydrocannabinol may be a substrate for the cytochrome P-450. The oxygenation of aldehyde catalyzed by cytochrome P-450 might be a common metabolic reaction in biological systems, and should be considered as an additional role of cytochrome P-450 in biotransformation of endogenous compounds and xenobiotics.

The biological oxidation of aldehydes to carboxylic acids, in general, is catalyzed by aldehyde dehydrogenase (aldehyde:NAD⁺ oxidoreductase, EC 1.2.1.3 and aldehyde:NAD⁺ oxidoreductase, EC 1.2.1.5). The enzyme has been studied extensively and reviewed (1). The mechanism of this reaction has been postulated as involving the initial formation of the binary complex of the enzyme with an oxidized pyridine nucleotide, followed by formation of the ternary complex of the enzyme, cofactor, and an aldehyde substrate, and finally by hydrolysis of this complex to form a carboxylic acid (2, 3). In the course of metabolic study with tetrahydrocannabinol (THC), a constituent of marijuana, we found for the first time that the microsomal aldehyde oxygenase could catalyze the oxygenation of aldehydes to carboxylic acids (4, 5). Cytochrome P-450 (P-450s) are involved in a variety of metabolic reactions in biological systems, and should be considered as an additional role of cytochrome P-450 in biotransformation of endogenous compounds and xenobiotics.

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‡ The abbreviations used are: THC, tetrahydrocannabinol; P-450, cytochrome P-450; GC-MS, gas chromatography-mass spectrometry; TMS, trimethylsilyl; HPLC, high performance liquid chromatography.

The oxygenation of an aldehyde, 11-oxo-Δ²-tetrahydrocannabinol to a carboxylic acid, Δ⁹-tetrahydrocannabinol-11-oic acid was catalyzed by cytochrome P-450. The oxygenation of aldehyde catalyzed by cytochrome P-450 might be a common metabolic reaction in biological systems, and should be considered as an additional role of cytochrome P-450 in biotransformation of endogenous compounds and xenobiotics.

This communication describes a rather novel role and a part of mechanism of P-450 isozyme in the oxidation of a xenobiotic aldehyde to a carboxylic acid.

Materials and Methods

Enzymes—An enzyme designated P-450 MUT-2 which has a catalytic activity for the oxidation of 11-oxo-Δ²-THC to Δ⁹-THC-11-oic acid was purified from hepatic microsomes of male ddN mice using the methods described by Funae and Imaoka (7) with a slight modification. Briefly, the microsomes (1050 mg of protein) were solubilized with sodium cholate and applied to an a-aminoocetyl Sepharose 4B column. A fraction containing P-450 (338 nmol, 125 mg of protein) was eluted with 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol, 0.4% (w/v) sodium cholate, and 0.08% (w/v) Emulgin 913. The P-450 fraction was subjected to HPLC using a DEAEP-5PW column (7). The microsomal aldehyde oxygenase activity monitored 11-oxo-Δ²-THC as a substrate was eluted in a pass-through fraction. When reconstituted with NADPH, l-α-diauroylglycero-3-phosphocholine and NADP⁺, P-450 reductase purified from hepatic microsomes of male ddN mice by the method of Shephard et al. (8), the pass-through fraction after hydroxyapatite column chromatography showed the specific activity of 12.2 nmol of Δ⁹-THC-11-oic acid formed per min/nmol P-450. The pass-through fraction was further purified by HPLC using a DEAEP-5PW column (7). P-450 MUT-2 was finally purified using CM-Sephadex C-50 and hydroxylapatite columns by stepwise elution with potassium phosphate buffer. The purified enzyme (2.76 nmol, 0.19 mg of protein) showed a single protein-staining band with a minimum molecular weight of 50,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9), and strong peaks at 417 and 451 nm respectively, on the oxidized and reduced CO-complex forms. The NH₂-terminal amino acid sequence of P-450 MUT-2 was determined to be following order: M-D-L-V-V-F-L-A-L-T-L-S-X-L-I-L-L-S-L-W. The NH₂-terminal amino acid sequence of P-450 MUT-2 is highly homologous with those of P-450f, P-450g, P-450h, and P-450i (10) suggesting that P-450 MUT-2 is a member of IIC gene subfamily of P-450s, which are constitutively expressed (11).

Chemicals—Oxygen-18 gas was purchased from Amersham International plc. 11-Oxo-Δ³-THC was prepared by the method of Inayama et al. (12). 11-Oxo-Δ³-THC dimethyl acetal was prepared by the method of Luche and Genel (13). The reaction of 11-o xo-Δ³-THC (60 mg, 0.18 nmol) with 0.5 ml of methyl orthoformate and 350 mg of CeCl₃ in 1 ml of methanol gave 11-o xo-Δ³-THC dimethyl acetal (12 mg, 0.3 nmol) which was purified by column chromatography on alumina with a solvent system of n-hexane:ethyl acetate (8:1). NMR, δ (CDCI₃), 0.88, 1.10, 1.36 (three methyl groups), 3.32 (two methoxy methyl groups), 4.60 (s, CH₃-on proton), 5.90 (m, CH₂-on proton), 6.10, 6.24 (two aromatic protons). 11-Oxo-Δ³-THC containing oxygen-18 (79 atom %) was prepared by oxygen-exchange from 11-o xo-Δ³-THC with a solution of NaOH in ethanol/H₂O (9). Major fragment ions in mass spectrum of the aldehyde prepared were at m/z 330 (M⁺, 100%), 328 (M⁺-2), 276 (M⁺-28), 274 (M⁺-30), 272 (M⁺-32), 261 (M⁺-33) and 193 (94%).

Assays—A typical incubation system consisted of 15 µmol of P-450 MUT-2, 0.33 units of NADPH P-450 reductase, 1 mM NADPH, 15 µg of L-α-diauroyl glycerophosphocholine, 120 µmol of 11-o xo-Δ³-THC, and 100 mM sodium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. Incubation was at 37 °C for 30 min, and Δ⁹-THC-11-oic acid was extracted with 5 ml of ethyl acetate after the addition of 0.5 µg of S'-not-Δ³-THC-4'-oic acid methyl ester as an internal standard (14). The ethyl acetate extract was subjected to gas chromatography-mass spectrometry (GC-MS) after derivatization to methyl ester and trimethylsilyl ether (TMS) as described previously (4). The incubation under oxygen-18 gas (97 atom %) was performed similarly as described in the previous papers (4, 5). NMR spectra were recorded on a JEOL JNM-MH-100 spectrometer. GC-MS was carried out at 70 eV with a JEOL GCG-06 gas chromatograph coupled with a JEOL JMS-DX-300 mass spectrometer and a JEOL JMA-DA 5000 mass data system. The conditions were as follows: column, 5% SE-30 on Chromosorb W (60-80 mesh, 3 mm x 2 m); column temperature, 250 °C; carrier gas, He 40 ml/m
GC-MS analyses of methyl ester and TMS derivatives of Δ⁶-THC-11-oic acid formed under different conditions

The incubation system was same as described in the text except for experiment 4 where the incubation volume was reduced to 0.2 ml.

| Experiment No. | Substrates                  | Conditions     | Relative abundance of M⁺ ions |
|---------------|-----------------------------|----------------|-------------------------------|
|               |                             | m/z 430        | m/z 432                       |
| 1.            | 11-Oxo-Δ⁶-THC               | Air            | 100                           |
| 2.            | 11-Oxo-Δ⁶-THC               | ⁴⁰O₂ (97 atom %) | 6                             |
| 3.            | 11-Oxo-Δ⁶-THC dimethyl acetal | ⁴⁰O₂ (97 atom %) | 7                             |
| 4.            | 11-Oxo-Δ⁶-THC               | H²¹⁸O (44 atom %) | 100                           |
| 5.            | 11-¹³CO₂-Δ⁶-THC (79 atom %) | Air            | 24                            |

* Methylation process was not required.

RESULTS AND DISCUSSION

GC-MS analysis indicated that 11-oxo-Δ⁶-THC was transformed to Δ⁶-THC-11-oic acid by the reconstituted system involving P-450 MUT-2. When either P-450 MUT-2 or NADPH P-450 reductase was omitted from the incubation mixture, the carboxylic acid metabolite could not be detected. This indicates an obligatory requirement of the P-450 and NADPH P-450 reductase for the reaction. The specific activity for the oxidation of 11-oxo-Δ⁶-THC was 29.8 nmol/min/nmol P-450 under the optimal conditions. The addition of cytochrome b₅ did not significantly affect the specific activity. P-450 MUT-2 could also catalyze the oxidation of other aldehyde substrates such as 9-anthraldehyde, veratraldehyde, and cinnamic aldehyde. The catalytic activity of P-450 MUT-2 for 9-anthraldehyde was 6.3 nmol of 9-anthracene carboxylic acid formed per min/nmol P-450. The formation of the carboxylic acid metabolites of cinnamic aldehyde and veratraldehyde was qualitatively confirmed by GC-MS analysis.

To clarify the reaction mechanism, several lines of experiments were conducted. The results are summarized in Table I. In Experiment 2, 11-oxo-Δ⁶-THC was incubated with the reconstituted system under oxygen-18 (97 atom %). The methyl ester and TMS derivative of the carboxylic acid metabolite formed was analyzed by GC-MS, which showed molecular ions at m/z 430 and 432 with relative abundance of 6:100, indicating that the reaction is exclusively monoxygenation.

The formation of hydrates (gem-diol) is well known for many aldehydes in aqueous medium (17). It is therefore important for understanding the mechanism to know a preferable substrate for P-450 MUT-2 whether an aldehyde form or its hydrated form. Thus, 11-oxo-Δ⁶-THC dimethyl acetal was prepared as a model substrate for the hydrated form and incubated with the reconstituted system. The dimethyl acetal was transformed to a methyl ester of Δ⁶-THC-11-oic acid, in which the specific activity was 8.1 nmol/min/nmol P-450. Under oxygen-18 gas (97 atom %), one atom of oxygen-18 was exclusively incorporated into the methyl ester of the carboxylic acid metabolite formed from the a (Table I, Experiment 3). These results suggest that the drated form could be a substrate for the enzyme and an acid might be an intermediate (Scheme 1).

However, the hydrated form cannot be the predominant substrate for this P-450, because the specific activity for acetate was much lower than that for 11-oxo-Δ⁶-THC.

As shown in experiment 4, oxygen-18 was not significantly enriched into Δ⁶-THC-11-oic acid formed from 11-oxo-THC with the reconstituted system in phosphate buffer containing H₂¹⁸O (44 atom %). In addition, the aldehyde containing oxygen-18 (79 atom %) in the aldehyde group was prep and incubated with the reconstituted system (Table I, Experiment 5). GC-MS analysis revealed that molecular ion methyl ester and TMS derivative of the carboxylic acid formed were observed at m/z 430 and 432 with relative abundance of 24:100. The content of oxygen-18 in the carboxylic acid was almost same as that in the substrate. The results obtained from the above experiments indicate that 11-oxo-Δ⁶-THC is hardly hydrated in the incubation medium under the time scale of these experiments does not exchange solvent water and that the aldehyde form is preferentially oxygenated to the carboxylic acid by the P-450 as shown in Scheme 1.

In conclusion, it seems very likely that this P-450 isoform could catalyze the oxygenation of lipophilic aldehyde carboxylic acids in common, and might play some role in the biotransformation of both xenobiotic and endogenous aldehydes. The oxygenation of aldehydes catalyzed by P-450 should be considered as an additional function of the enzyme because of its physiological and biological importance.
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