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Microsomal Aldehyde Oxygenase (MALDO): Purification and Characterization of a Cytochrome P450 Isozyme Responsible for Oxidation of 9-Anthraldehyde to 9-Anthracenecarboxylic Acid in Monkey Liver[1]

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Oxidative activity of 9-anthraldehyde (9-AA) to 9-anthracenecarboxylic acid in monkey liver was mainly located in microsomes. The reaction required NADPH as an essential cofactor and was significantly inhibited by SKF 525-A, metyrapone, disulfiram, and CO, potent inhibitors of microsomal aldehyde oxygenase (MALDO). Two cytochrome P450 isozymes, named P450JM-A and P450JM-C, which mediate the oxidative biotransformation of 9-AA were purified from hepatic microsomes of untreated male and female Japanese monkeys, respectively. These isozymes each showed a single band of molecular mass 51,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The NH_2-terminal amino acid sequences of P450JM-A and P450JM-C are highly homologous with those of several P450s belonging to the CYP2A and CYP2B subfamilies, respectively. The anti-P450JM-C antibody significantly suppressed 9-AA MALDO activity in monkey liver, but anti-P450JM-A antibody did not. The antibody against CYP2C11, which is a major isozyme responsible for 9-AA MALDO in male rat liver, also inhibited the activity. These results indicate that P450JM-C and isozyme(s) immunologically related to CYP2C11 predominantly possess MALDO activity toward 9-AA.

Key words: 9-anthraldehyde, cytochrome P450, microsomal aldehyde oxygenase, monkey, purification.

It is generally known that aldehydes are oxidized to the corresponding carboxylic acids by aldehyde dehydrogenase [EC 1.2.1.3] and/or aldehyde oxidase [EC 1.2.3.1] in mitochondria or cytosol. We have previously found for the first time that mouse hepatic microsomal enzyme is able to oxidize xenobiotic aldehydes to carboxylic acids *via* an oxygenation mechanism (*1-4*). 9-Anthraldehyde (9-AA) and 11-oxo-Δ^8-tetrahydrocannabinol (11-oxo-Δ^8-THC) are good substrates for this microsomal aldehyde oxygenase, named MALDO, catalyzed by cytochrome P450 (P450) of mice (*5*) and rats (*6*). Recently, Terelius *et al.* reported that CYP2E1 purified from rat hepatic microsomes catalyzed the oxidation of acetaldehyde (*7*). Roberts *et al.* also showed that CYP1A2 from rabbits catalyzed the oxidation of retinal to retinoic acid (*8*). We have reported that P450MUT-2 (CYP2C29) from mice played a major role in MALDO activity for 11-oxo-Δ^8-THC (*3, 9-11*) and P450-UT-2 (CYP2C11) from adult male rats also played an important part in the oxidation of 9-AA (*12*) and 11-oxo-Δ^8-THC (*13*). Several P450 isozymes have been purified and characterized from hepatic microsomes of untreated or inducer-treated cynomolgus monkeys, marmosets, and baboons (*14-23*). However, it is difficult to identify the isozyme responsible for a particular reaction because of species differences in the properties of the P450s (*24, 25*).

In the present study, we purified and characterized a P450 isozyme, which plays a major role in the formation of 9-anthracenecarboxylic acid (9-ACA) from 9-AA, from hepatic microsomes of untreated Japanese female monkeys.

MATERIALS AND METHODS

Materials—NADP and glucose 6-phosphate were purchased from Boehringer-Mannheim GmbH (Darmstadt, Germany); cytochrome *c* (type V from horse heart), molecular weight markers (Dalton Mark VII-L), NADPH, glucose 6-phosphate dehydrogenase (type V, EC 1.1.1.49), phosphatidylserine (PS), and dilauroylphosphatidylcholine (DLPC) were from Sigma Chemical (St. Louis, MO); 9-AA,

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Abbreviations: P450, cytochrome P450; 9-AA, 9-anthraldehyde; 9-ACA, 9-anthracenecarboxylic acid; Δ^8-THC, Δ^8-tetrahydrocannabinol; PS, phosphatidylserine; DLPC, dilauroylphosphatidylcholine; EDTA, ethylenediamine tetraacetate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

9-ACA, and octamethylenediamine were from Aldrich Chemical (Milwaukee, WI); cholic acid, dithiothreitol, 7-ethoxycoumarin, 7-hydroxycoumarin, and coumarin were from Wako Pure Chemicals (Osaka); Sepharose 4B, CM-Sephadex C-50, and 2′,5′-ADP-Sepharose 4B were from Pharmacia Fine Chemicals (Uppsala, Sweden); hydroxylapatite for an open column was from Bio-Rad (Richmond, CA): preparative DEAE-5PW and hydroxylapatite columns for HPLC were from Tosoh (Tokyo). Emulgen 911 and SKF 525-A were kindly provided by Kao-Atlas (Tokyo), and Smith, Kline and French Laboratories (Philadelphia, PA), respectively. 11-Oxo-Δ^8-THC and Δ^8-THC-11-oic acid were prepared by the methods of Inayama *et al.* (*26*) and Mechoulam *et al.* (*27*), respectively. Other chemicals and solvents used were of the highest quality commercially available.

Animals and Preparation of Subcellular Fractions—Liver samples from untreated male rhesus monkey (6 years old), and male [2, 4 (69Mf and 70Mf) and 6 years old] and female (7 and 10 years old) Japanese monkeys were provided by Osaka Bioscience Institute (Suita) and by the Primate Research Institute, Kyoto University (Inuyama), respectively. The livers were homogenized as described previously (*12*). Subcellular fractions were prepared by centrifugation as follows: $600\times g$ (for 10 min) pellet as nuclear fraction, $9{,}000\times g$ (for 20 min) pellet as mitochondrial fraction, $105{,}000\times g$ (for 60 min) pellet as microsomes, and supernatant as cytosolic fraction. Each pellet was suspended in the same volume of 50 mM Tris HCl buffer (pH 7.5) containing 0.15 M KCl and recentrifuged at the same speed described above. The pellets obtained were resuspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 5mM EDTA as each subcellular fraction.

Purification of P450JM-A from Hepatic Microsomes of Untreated Male Japanese Monkeys (2 and 6 Years Old)—Microsomes (P450: 1.13 nmol/mg protein, total 3,760 nmol) were suspended in buffer A [0.1 M potassium phosphate buffer (pH 7.2) containing 20% glycerol, 1 mM EDTA, and 0.5 mM dithiothreitol]. Then 20% sodium cholate solution (pH 7.4) was added to a final concentration of 0.7%. This mixture was stirred for 30 min at 0°C to solubilize microsomes and the resulting suspension was centrifuged at $105{,}000\times g$ for 60 min.

The supernatant fraction of the cholate-solubilized hepatic microsomes was put on an ω-aminooctyl-Sepharose 4B column (4×30 cm) equilibrated with buffer A containing 0.5% sodium cholate. The column was washed with equilibration buffer, and P450 was eluted with buffer A containing 0.4% sodium cholate and 0.1% Emulgen 911. The P450 fractions were pooled, concentrated with an ultrafiltration membrane (UK-50, Toyo Roshi, Tokyo), and dialyzed against 20 mM Tris-acetate buffer (pH 7.5) containing 20% glycerol. The dialyzed solution was subjected to HPLC with a preparative DEAE-5PW anion-exchange column (2.15×15 cm, Tosoh, Tokyo), previously equilibrated with buffer B [20 mM Tris-acetate buffer (pH 7.5) containing 20% glycerol and 0.4% Emulgen 911]. The column chromatography was performed with a linear gradient of sodium acetate from 0 to 0.2 M in buffer B. The elution profiles of heme and protein were monitored at 417 and 244 nm, respectively, as described by Funae and Imaoka (*28*). The fractions that had the highest aldehyde oxygenase activity for 9-AA were combined and concentrated by ultrafiltration. After dialysis against buffer C [5 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol, and 0.4% Emulgen 911], the solution was applied to a CM-Sephadex C-50 column (1.2×30 cm), previously equilibrated with buffer C. P450 was eluted with 100 ml each of 5, 10, 20, 40, 80, or 160 mM buffer C. The fractions eluted with the 40 and 80 mM phosphate buffers were combined, concentrated and dialyzed against buffer D [10 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.2% sodium cholate]. The dialyzed sample was subjected to HPLC with a hydroxylapatite column (0.75×7 cm, Tosoh), previously equilibrated with buffer D. P450 was eluted with a linear gradient of potassium phosphate buffer (pH 7.4) from 10 to 350 mM containing 20% glycerol, 0.2% sodium cholate, and 0.4% Emulgen 911. The fractions eluted with about 150 mM potassium phosphate buffer were electrophoretically homogeneous, and they were combined, and concentrated. The detergent was removed by using a small hydroxylapatite column as previously described (*10*).

Purification of P450JM-C from Hepatic Microsomes of Untreated Female Japanese Monkeys (7 and 10 Years Old)—Microsomes (P450: 0.84 nmol/mg protein, total 4,682 nmol) were suspended in buffer A and solubilized by the method described above. ω-Aminooctyl-Sepharose 4B and DEAE-5PW column chromatographies of the solubilized microsomes were carried out using the same procedures as described above. The fraction that had the highest aldehyde oxygenase activity for 9-AA was dialyzed against buffer D and subjected to HPLC with a hydroxylapatite column, previously equilibrated with buffer D. P450 was eluted with a linear gradient of potassium phosphate buffer (pH 7.4) from 10 to 350 mM containing 20% glycerol, 0.2% sodium cholate, and 0.4% Emulgen 911. The fractions eluted with about 100 mM potassium phosphate buffer were electrophoretically homogeneous.

Purification of Other Enzymes, and Preparation of Antibodies—NADPH-cytochrome *c* (P450) reductase was purified from hepatic microsomes of ddN mice by the method of Yasukochi and Masters (*29*). One unit of the reductase was defined as the amount of reductase catalyzing the reduction of 1 μmol of cytochrome *c* per min. Cytochrome b_5 was purified from hepatic microsomes of rhesus monkey according to the methods of Funae and Imaoka (*28*). Polyclonal antibodies against the purified P450JM-A and P450JM-C were raised in female New Zealand White rabbits as described previously (*30*). The IgG fraction from the rabbit serum was obtained by our reported method (*31*).

Marker Enzyme Assays—Marker enzymes were used to follow the separation of subcellular fractions. The mitochondrial membrane marker enzyme, succinate-cytochrome *c* reductase, was assayed according to the method of Mackler *et al.* (*32*). NADPH-cytochrome *c* (P450) reductase, an endoplasmic reticulum marker, was assayed according to the methods of Omura and Takesue (*33*). Alcohol dehydrogenase, a marker for the cytosol, was assayed at 340 nm using ethanol as the substrate according to published procedures (*34*).

Enzyme Assays—In the studies of subcellular fractions, the substrate was incubated with each fraction, an NADPH-generating system (0.5 mM NADP, 10 mM glucose 6-phos-

phate, 1 unit of glucose 6-phosphate dehydrogenase, 10 mM magnesium chloride), and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 0.5 ml. In the reconstitution studies, the substrate was incubated with purified P450 (30 pmol), 0.33 units of NADPH-cytochrome *c* (P450) reductase, 30 pmol of cytochrome b_5, 10 μg of DLPC or DLPC : PS (1 : 1), 100 μg of sodium cholate, 1 mM NADPH, and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 0.5 ml. 9-AA (44.5 μM), 11-oxo-Δ^8-THC (0.15 mM), 7-ethoxycoumarin (50 μM), coumarin (50 μM), and benzphetamine (1 mM) were used as substrates. The reaction was allowed to proceed for 10 min at 37°C. The metabolites of 9-AA (*5*), 11-oxo-Δ^8-THC (*6*), 7-ethoxycoumarin (*35*), coumarin (*35*), and benzphetamine (*36*) were determined by the reported methods.

Inhibition of 9-AA MALDO Activity by Antibodies—The antibody was added to medium containing microsomes and 100 mM potassium phosphate buffer (pH 7.4), and preincubated at 37°C for 30 min. The NADPH-generating system described above was added to the medium, and the reaction was then started by adding substrate. After incubation at 37°C for 10 min, the metabolites were assayed by the same methods described above.

Other Methods—Protein concentration was estimated by the method of Lowry *et al.* (*37*), using bovine serum albumin as a standard. P450 and cytochrome b_5 contents were determined by the methods of Omura and Sato (*38*), and Omura and Takesue (*33*), respectively. ω-Aminooctyl-Sepharose 4B was synthesized as described previously (*39*). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (*40*). The statistical significance of differences was determined by means of the Bonferroni test.

RESULTS

Subcellular Localization of Enzyme Activity and Cofactor Requirements in Oxidation of 9-AA to 9-ACA—Table I summarizes the distribution of marker enzymes and NADPH-dependent oxidative activity of 9-AA in the subcellular fractions of monkey liver. Succinate-cytochrome *c* reductase activity was recovered predominantly in the mitochondrial and nuclear fractions (Table I). This activity in the nuclei was probably due to contamination by mitochondria or incomplete disruption of cellular material. Low succinate-cytochrome *c* reductase levels in the microsomal and cytosolic fractions indicated minimal contamination by mitochondria. Alcohol dehydrogenase, a marker enzyme for the cytosol, was predominantly located in the 105,000 × *g* supernatant fraction (Table I). Low levels of the activity were recovered in the nuclear and mitochondrial fractions with lesser activity in the microsomal fractions. NADPH-cytochrome *c* (P450) reductase, a marker enzyme for the endoplasmic reticulum, was present in the highest amounts in the microsomal fraction. Significant levels of the enzyme, however, were also observed in the mitochondrial and nuclear fractions. In the oxidation of 9-AA to 9-ACA, the highest level of activity was found in the microsomal fraction. Significant levels of the activity were also present in the mitochondrial and nuclear fractions, 14-17 and 14-27% of the microsomal specific activity, respectively. The cofactor requirement for microsomal oxidation of 9-AA was examined (Table II). NADPH was most effective, followed by NADH and NAD. 9-ACA was scarcely detected when these cofactors were not added and NADP was added to the incubation mixture.

Effects of Various Inhibitors on 9-ACA Formation—Table III summarizes the effects of various inhibitors on the microsomal formation of 9-ACA in the presence of an NADPH-generating system. SKF 525-A and metyrapone, inhibitors of P450, inhibited the formation of 9-ACA from 9-AA by 91-95 and 84-88%, respectively. The reaction was also significantly inhibited by addition of disulfiram. However, α-naphthoflavone, a P450 inhibitor different from SKF 525-A and metyrapone, had no significant effect on the formation of 9-ACA. Barbital and pyrazole, inhibitors of aldehyde reductase and alcohol dehydrogenase, respectively, did not show significant inhibition of the

TABLE II. **Cofactor requirements for the formations of 9-ACA with hepatic microsomes of monkeys.** The data are expressed as the mean of two experiments. Each cofactor was added to the incubation mixture at 1 mM. Numbers in parentheses represent the relative activities.

Cofactor	9-ACA-forming activity		
	Japanese monkey 69Mf (nmol/min/mg protein)	Japanese monkey 70Mf (nmol/min/mg protein)	Rhesus monkey (nmol/min/mg protein)
None	0.08 (1)	0.08 (2)	0.01 (0.4)
NAD	0.35 (6)	0.28 (5)	0.14 (6)
NADP	0.09 (2)	0.06 (1)	0.01 (0.4)
NADH	0.80 (15)	0.62 (12)	0.32 (13)
NADPH	5.49 (100)	5.16 (100)	2.40 (100)

TABLE I. **Distribution of NADPH-dependent oxidative activity of 9-AA and marker enzymes in subcellular fractions of monkey livers.** The data are expressed as the mean of two experiments.

Subcellular fractions	Japanese monkey 69Mf					Japanese monkey 70Mf					Rhesus monkey	
	Protein	9-ACA-forming activity	NADPH-cytochrome *c* (P450) reductase	Succinate-cytochrome *c* reductase	Alcohol dehydrogenase	Protein	9-ACA-forming activity	NADPH-cytochrome *c* (P450) reductase	Succinate-cytochrome *c* reductase	Alcohol dehydrogenase	Protein	9-ACA-forming activity
	(mg/g liver)	(nmol/min/mg protein)				(mg/g liver)	(nmol/min/mg protein)				(mg/g liver)	(nmol/min/mg protein)
Homogenate	193	0.89	18.7	24.1	45.9	203	0.82	14.8	22.0	50.1	193	0.63
Nuclei	25	0.71	4.8	56.9	10.6	26	0.72	6.30	60.7	12.2	26	0.99
Mitochondria	24	0.76	15.9	118	21.6	17	0.71	16.5	140	37.9	26	0.64
9,000 × *g* supernatant	103	0.91	17.2	2.86	67.1	108	0.89	15.2	4.91	66.9	112	0.81
Microsomes	18	4.52	79.8	6.79	4.02	18	5.09	69.3	7.47	2.98	16	3.73
Cytosol	82	0.03	1.0	2.44	83.8	90	0.03	0.56	2.03	90.3	86	0.21

oxidation of the aldehyde. When 9-AA was incubated with microsomes in an atmosphere of CO/O_2 (4 : 1), the reaction was inhibited by 74–81% compared with that under air.

Purification of P450 from Hepatic Microsomes of Untreated Japanese Monkeys—On the basis of the above results, we carried out the purification of MALDO from hepatic microsomes of monkeys, using the formation activity of 9-ACA from 9-AA as an indicator. Tables IV and V show a summary of the purification of P450JM-A and P450JM-C from male and female Japanese monkeys, respectively. The P450-containing fraction was eluted as one peak by washing the column of ω-aminooctyl-Sepharose 4B with phosphate buffer (pH 7.4) containing 0.1% Emulgen 911. Further purification was conducted by HPLC with a DEAE-5PW column.

The eluate in the case of male monkeys could be divided into five peak fractions based on the absorbance at 417 nm (Fig. 1A). Among these fractions, the highest activity for 9-ACA formation was found in fraction III, followed by fractions IV, V, II, and I (Table IV). We carried out further purification of P450JM-A from fraction III by CM-Sephadex C-50 and hydroxylapatite column chromatographies. The oxidative activity of 9-AA by P450JM-A was found to be 14.3 nmol/min/nmol P450. The purified P450 showed a single protein band on SDS-PAGE and the apparent molecular weight of the isozyme was estimated to be 51,000 (Fig. 2A). Specific content and recovery of P450JM-A were 9.6 nmol/mg protein and 0.07%, respectively.

The eluate in the case of female monkeys could be divided into four peak fractions (Fig. 1B). Among these fractions, the highest activity for 9-ACA formation in the absence of cytochrome b_5 was found in fraction IV, followed by fractions II, III, and I in the reconstituted system using DLPC as lipids (Table V). However, addition of cytochrome b_5 in the reconstituted system resulted in about 2-fold increase of the oxidative activity towards 9-AA in fraction II (Table V). We carried out further purification of P450 from fraction II by hydroxylapatite column chromatography. The eluate from the hydroxylapatite column could be divided into four fractions. Among these fractions, the highest activity was found in fraction II, designated P450JM-C, which showed a single protein band on SDS-PAGE (Fig. 2B). The apparent molecular weight of this isozyme was estimated to be 51,000. The oxidative activity of 9-AA by P450JM-C was found to be 19.6 nmol/min/nmol P450. The activity was increased about 2-fold by using a mixture of DLPC and PS instead of DLPC as lipids, and was 13-fold higher than that in microsomes. The purified P450JM-C showed a single protein band on SDS-PAGE and the apparent molecular weight of this isozyme was estimated to be 51,000 (Fig. 2B). Specific content and recovery of P450JM-C were 3.2 nmol/mg protein and 0.2%, respectively. The oxidized and reduced CO-complex forms of P450JM-C showed Soret peaks at 417 and 450 nm, respectively (data not shown).

NH_2-Terminal Amino Acid Sequence—The NH_2-terminal amino acid sequences of P450JM-A and P450JM-C

TABLE III. **Effects of various inhibitors on the formation of 9-ACA with hepatic microsomes of monkeys.** 9-AA was incubated with the microsomes for 10 min at 37°C in the presence of various inhibitors. Each inhibitor was added to the incubation mixture at 1 mM. The data are expressed as the mean of two experiments. Numbers in parentheses represent the relative activities.

Inhibitor	9-ACA-forming activity		
	Japanese monkey 69Mf (nmol/min/mg protein)	Japanese monkey 70Mf (nmol/min/mg protein)	Rhesus monkey (nmol/min/mg protein)
Experiment 1			
Control	4.28 (100)	3.82 (100)	1.97 (100)
SKF 525-A	0.20 (5)	0.19 (5)	0.17 (9)
α-Naphthoflavone	4.34 (101)	3.61 (95)	1.98 (101)
Metyrapone	0.59 (14)	0.45 (12)	0.31 (16)
Disulfiram	0.51 (12)	0.27 (7)	0.09 (5)
Barbital	3.69 (86)	3.31 (87)	1.97 (100)
Pyrazole	3.31 (77)	2.80 (73)	1.53 (78)
Experiment 2			
Air	5.60 (100)	4.84 (100)	1.69 (100)
$CO/O_2=4$	1.18 (21)	0.94 (19)	0.44 (26)

TABLE IV. **Purification of P450JM-A from hepatic microsomes of untreated male Japanese monkeys.** The 9-ACA-forming activity was expressed as the mean of two experiments.

Purification step	P450 contents			9-ACA-forming activity (nmol/min/nmol P450)
	Total (nmol)	Specific (nmol/mg protein)	Recovery (%)	
Microsomes	3,760	1.13	100	1.96
ω-Aminooctyl-Sepharose 4B	2,698	4.29	71.8	4.77[a]
DEAE-5PW				
Fraction I	979	5.13	26.0	2.84[a]
Fraction II	326	5.04	8.7	3.61[a]
Fraction III	160	5.67	4.3	8.88[a]
Fraction IV	299	5.69	8.0	6.79[a]
Fraction V	118	4.85	3.1	3.84[a]
(from DEAE-5PW fraction III)				
CM-Sephadex C-50	—[b]	—[b]	—[b]	—[b]
Hydroxylapatite P450JM-A	2.7	9.60	0.07	14.3[a]

[a]Cytochrome b_5 added. [b]Not determined.

TABLE V. **Purification of P450JM-C from hepatic microsomes of untreated female Japanese monkeys.** The 9-ACA-forming activity was expressed as the mean of two experiments.

Purification step	P450 contents			9-ACA-forming activity (nmol/min/nmol P450)	
	Total (nmol)	Specific (nmol/mg protein)	Recovery (%)	$-b_5$	$+b_5$
Microsomes	4,682	0.84	100	3.01	—[a]
ω-Aminooctyl-Sepharose 4B	3,164	2.63	67.6	1.33	—[a]
DEAE-5PW					
Fraction I	1,937	4.32	41.4	1.30	2.00
Fraction II	185	3.60	4.0	3.34	7.48
Fraction III	643	2.56	13.7	2.91	4.11
Fraction IV	132	3.02	2.8	4.28	4.38
(from DEAE-5PW fraction II)					
Hydroxylapatite					
Fraction I	27	5.34	0.6	—[a]	2.7
Fraction II (P450JM-C)	9	3.20	0.2	—[a]	19.6
					38.5[b]
Fraction III	33	4.81	0.7	—[a]	3.6
Fraction IV	36	5.62	0.8	—[a]	2.0

[a]Not determined. [b]Lipid mixture system of DLPC and PS was used as lipids instead of DLPC.

were compared with those of previously reported P450 (Tables VI and VII). The amino acid sequence of P450JM-A was highly homologous with those of P450 isozymes belonging to the 2A subfamily and the same as that of P450FI purified from baboon (Table VI). The sequence of P450JM-C was similar to those of P450 isozymes belonging to the 2B subfamily, especially to that of human CYP2B6 (identical in 49 of the 51 amino acid residues) (Table VII). The characterized P450JM-C sequence up to the first 34 residues was identical to the recently reported sequence of a P450 isozyme termed P450CMLa which was purified from hepatic microsomes of untreated cynomolgus monkeys (*20*).

Catalytic Properties of Purified P450—The component requirements for the oxidation of 9-AA to 9-ACA in the reconstituted system containing P450JM-C were estimated, because its specific content was low (Table VIII). In the complete system, we used a lipid mixture (consisting of DLPC and PS) system in which monkey cytochrome b_5 and sodium cholate are also included. The oxidative activity in the complete system was 34.9 nmol/min/nmol P450. However, the activity could not detected in the absence of P450JM-C, NADPH-cytochrome *c* (P450) reductase or NADPH and was dependent on the presence of lipids in the reconstituted system. The activity was about 2-fold higher in the complete system than in the reactions where cytochrome b_5 was omitted. When NADH was used as a cofactor instead of NADPH, the activity was 11% of that of the complete system. In an atmosphere of CO/O_2 (4 : 1), the reaction was almost completely inhibited. The formation of Δ^8-THC-11-oic acid from 11-oxo-Δ^8-THC was not detected in the reconstituted system, even when the substrate was

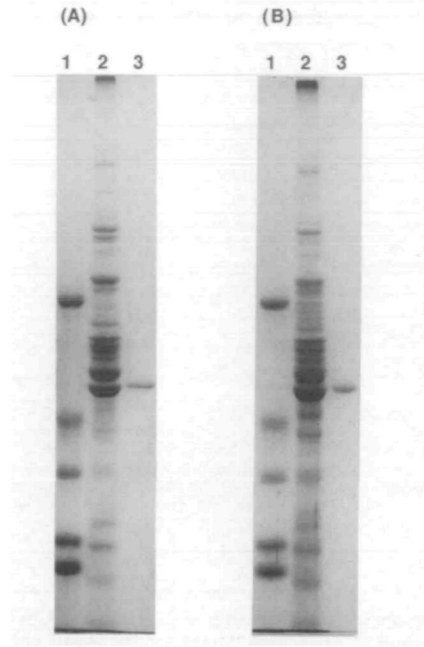

Fig 2 **SDS-PAGE of purified P450JM-A and P450JM-C.** Proteins were stained with Coomassie Brilliant Blue R250 The cathode is at the top. To lane 1 were applied standard proteins consisting of bovine serum albumin (molecular mass, 66,000), egg albumin (45,000), glyceraldehyde 3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), and trypsinogen (24,000). A· Lanes 2 and 3 contained 20 μg of male monkey hepatic microsomes and 1.0 μg of P450JM-A, respectively. B. Lanes 2 and 3 contained 20 μg of female monkey hepatic microsomes and 1 0 μg of P450JM-C, respectively.

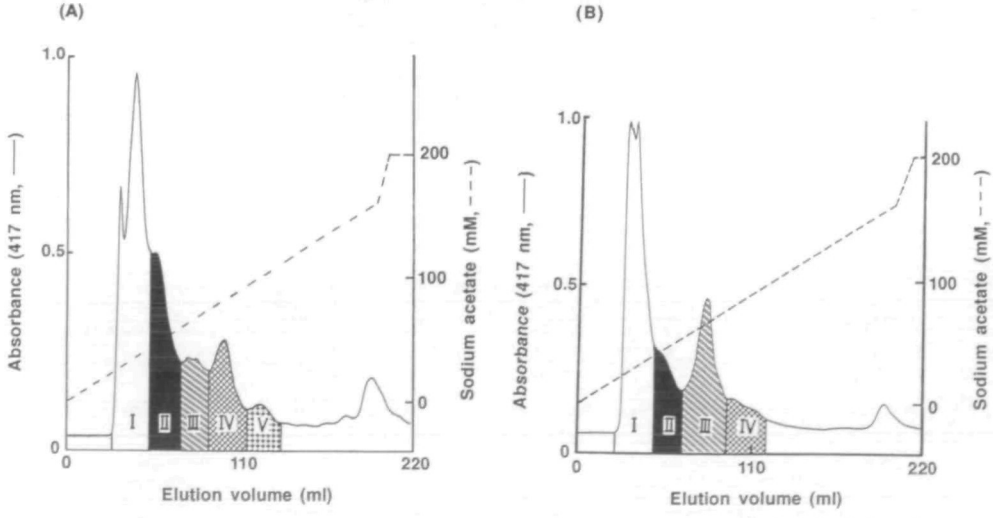

Fig. 1. **Elution profiles of P450s from a DEAE-5PW column.** Solubilized hepatic microsomes of male (A) or female (B) Japanese monkeys were applied to an ω-aminooctyl-Sepharose 4B column A fraction eluted with 0 1% Emulgen 911 from the ω-aminooctyl-Sepharose 4B column was subjected to HPLC with a DEAE-5PW column Proteins were eluted at a flow rate of 2 ml/min with a linear gradient of sodium acetate from 0 to 0.2 M over 90 min in 20 mM Tris-acetate buffer (pH 7.5) containing 20% glycerol and 0.4% Emulgen 911 Heme proteins were continuously monitored at 417 nm.

TABLE VI. **Comparison of NH_2-terminal amino acid sequence of P450JM-A with those of P450 2A isozymes.** Numbers in parentheses represent percent similarity with the sequence of P450JM-A

Isozyme	Animal	1 5 10 15 20	Homology (%)	Reference
P450JM-A	Monkey	M L A S G L L L V A L L A X L T V M V L		
P450FI	Baboon	— — — — — — — — — — — — — X — — — — —	(100)	*19*
CYP2A7	Human	— — — — — — — — — — — — — C — — — — —	(100)	*42*
CYP2A6	Human	— — — — — M — — — — — — V C — — — — —	(89)	*43*
CYP2A1, 2A2	Rat	— — D T — — — — — V I — — S — S — — F —	(68)	*44*

X, unidentified amino acid. —, the same amino acid as in P450JM-A.

TABLE VII. **Comparison of NH_2-terminal amino acid sequence of P450JM-C with those of P450 2B isozymes.** Numbers in parentheses represent percent similarity with the sequence of P450JM-C.

Isozyme	Animal	1	5	10	15	20	25
P450JM-C	Monkey	M E L S V L L F L A L L T G L L L L L V Q R H P N					
P450CMLa	Monkey	— —					
CYP2B6	Human	— —					
CYP2B4	Rabbit	— — F — L — — L — — F — A — — — — — — F R G — — K					
CYP2B11	Dog	— — — — — — — L — — — — — — — — — — M A R G — — K					
Cyp2b-10	Mouse	— — P — — — — L — — — — V — F — — — — A R G — — K					
CYP2B1	Rat	— — P — I — — L — — — — V — F — — — — — R G — — K					

Isozyme	26 30	35 40 45 50	Homology (%)	Reference
P450JM-C	A H G R L P P G P X P L P L L G N L L Q M D R R G L L			
P450CMLa	— — — — — — — — —		(100)	*20*
CYP2B6	T — D — — — — — —	R —	(96)	*41*
CYP2B4	— — — — — — — — —	S — — — V — — — — — — — — — — K — — —	(78)	*50*
CYP2B11	— Y — H — — — — —	R — — — I — — — F — — — — — — K — — —	(78)	*51*
Cyp2b-10	S R — N F — — — —	R — — — — — — — — — — — — — — — G — — —	(75)	*52*
CYP2B1	S R — N F — — — —	R — — — — — — — — — — — — — — L L L G — — —	(69)	*53*

X, unidentified amino acid. —, the same amino acid as in P450JM-C.

TABLE VIII. **Component requirements and effect of carbon monoxide on 9-ACA-forming activity of P450JM-C.** Enzyme activities were assayed as described in "MATERIALS AND METHODS." The data were expressed as the mean of two experiments. Numbers in parentheses represent the relative activities.

Conditions	9-ACA-forming activity (nmol/min/nmol P450)	
Complete[a]	34.9	(100)
−P450JM-C	ND[b]	(0)
−NADPH-cytochrome *c* (P450) reductase	ND[b]	(0)
−Cytochrome b_5	22.9	(66)
−NADPH	ND[b]	(0)
−NADPH+NADH	3.9	(11)
−DLPC and PS	6.4	(18)
Complete ($CO/O_2=4$)	1.2	(3)

[a]The complete system contained purified P450JM-C (20 pmol), NADPH-cytochrome *c* (P450) reductase (0.3 unit), NADPH (1 mM), dilauroylphosphatidylcholine (5 μg), phosphatidylserine (5 μg), cytochrome b_5 (20 pmol), sodium cholate (50 μg), 9-AA (5.15 μg), and potassium phosphate buffer (100 mM), in a 0.5 ml volume. [b]Not detected.

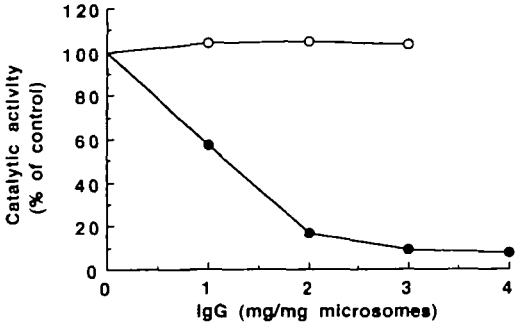

Fig. 3. **Effects of anti-P450JM-A antibody on 9-AA MALDO and coumarin 7-hydroxylase activities in hepatic microsomes of male Japanese monkeys.** Hepatic microsomes were preincubated with various amounts of the IgG fractions for 30 min at 37°C, and then incubated with 9-AA or coumarin in the presence of an NADPH-generating system. The MALDO and coumarin 7-hydroxylase activities without IgG fraction (100% as the control) were 1.91 and 5.62 nmol/min/mg protein, respectively. Open and closed circles indicate the MALDO and coumarin 7-hydroxylase activities, respectively.

TABLE IX. **Catalytic properties of purified P450 isozymes and hepatic microsomes of Japanese monkeys.** Enzyme activities were assayed as described in "MATERIALS AND METHODS." The data were expressed as the mean of two experiments.

Substrate	Metabolite	Forming activities (nmol/min/nmol P450)			
		Male monkey hepatic microsomes	P450JM-A	Female monkey hepatic microsomes	P450JM-C
Benzphetamine	HCHO	8.30	64.4	5.60	72.9
7-Ethoxycoumarin	7-OH coumarin	1.90	34.3	3.41	1.85
Coumarin	7-OH coumarin	0.20	3.4	0.29	ND[a]

[a]Not detected.

incubated in the complete system (data not shown). Table IX shows the catalytic properties of P450JM-A and P450JM-C in the reconstituted system. P450JM-A exhibited high oxidative activities for benzphetamine, 7-ethoxycoumarin, and coumarin. P450JM-C also had high benzphetamine *N*-demethylase activity, but showed only a low activity to metabolize 7-ethoxycoumarin.

Immunoinhibition of 9-AA MALDO Activity—The anti-P450JM-A antibody did not show any inhibitory effect on the activity in male Japanese monkeys, although coumarin 7-hydroxylase activity was significantly inhibited (Fig. 3). Anti-P450JM-C antibody, which almost completely inhibited the oxidative activity towards 9-AA in the reconstituted system of P450JM-C, inhibited the MALDO activity to about 70% of the control value in untreated female Japanese monkeys (Fig. 4). CYP2C11 is responsible for 9-AA MALDO activity in adult male rats (*12*). The effect of antibodies against P450JM-A, P450JM-C, and CYP2C11 on 9-AA MALDO activity of male Japanese monkeys was investigated. As shown in Table X, anti-P450JM-C antibody also inhibited the activity to 66–76% of control, but anti-P450JM-A antibody was ineffective. Anti-CYP2C11

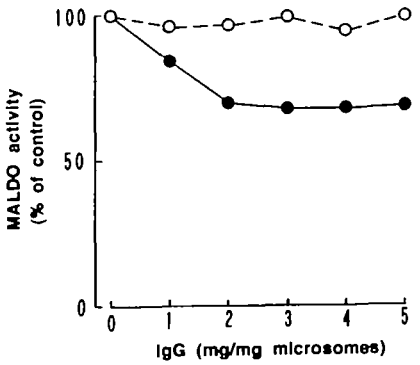

Fig. 4. **Effects of anti-P450JM-C antibody on 9-AA MALDO activity in hepatic microsomes of female Japanese monkeys.** Hepatic microsomes were preincubated with various amounts of the IgG fractions for 30 min at 37°C, and then incubated with 9-AA in the presence of an NADPH-generating system. The MALDO activity without IgG fraction (100% as the control) was 1.91 nmol/min/mg protein. Open and closed circles indicate the addition of IgG fractions from preimmune and anti-P450JM-C sera, respectively.

antibody also significantly inhibited 9-AA MALDO activity.

DISCUSSION

We have shown that mouse hepatic microsomes catalyze the oxidations of 9-AA and 11-oxo-Δ^8-THC to the corresponding carboxylic acids (*1-4*). In the present studies, we determined the NADPH-dependent oxidative activity for 9-AA in order to clarify its subcellular localization in monkey liver. Of the total protein present in the initial homogenate of untreated monkey livers, about 80% was recovered in the individual subcellular fractions. A significant portion of the protein loss probably occurred during the process of washing pellets. These washes were not quantified for protein content or enzyme activity. Protein ratios of cytosol/microsomes were about 5. The ratio was different from that of other animal species, *e.g.* rats (*45*). This may be caused by the relatively high yield of cytosolic protein (42-44% of homogenate) and low yield of microsomal protein (8-9%). However, low levels of succinate-cytochrome *c* reductase and NADPH-cytochrome *c* (P450) reductase activities in cytosolic fraction indicated minimal contamination by mitochondria and endoplasmic reticulum, respectively. The oxidative activity of 9-AA to 9-ACA was mainly located in microsomes. However, about 20% of microsomal specific activity was also found in the mitochondrial and nucleic fractions. The activity in these fractions was probably due to contamination by the microsomal fraction, as indicated by the presence of NADPH-cytochrome *c* (P450) reductase, a marker enzyme for the endoplasmic reticulum. NADPH was an obligatory cofactor to show the maximal activity, while NAD and NADP, cofactors of aldehyde dehydrogenase, were virtually inactive. This requirement indicates that aldehyde dehydrogenase does not significantly contribute to the reaction.

The NADPH-dependent activity for 9-AA in microsomal fraction was markedly inhibited by SKF 525-A, metyrapone and disulfiram, which have been reported to be inhibitors of MALDO in mouse liver (*5, 6*), but not by barbital or pyrazole. The reaction was also significantly

TABLE X. **Effects of anti-P450JM-A, P450JM-C, and CYP2C11 antibodies on 9-AA MALDO activity in hepatic microsomes of male Japanese monkeys.** The data expressed as the mean ± SE of three experiments. Numbers in parentheses represent the relative activities.

Antibodies	69Mf (nmol/min/mg protein)	70Mf (nmol/min/mg protein)
Control	3.92±0.05 (100)	3.13±0.09 (100)
P450JM-A[a]	3.99±0.15 (102)	2.99±0.11 (96)
P450JM-C[a]	2.96±0.16[b] (76)	2.08±0.05[b] (66)
CYP2C11[a]	2.34±0.16[b] (60)	2.22±0.11[b] (71)

[a]3.0 mg IgG/mg microsomal protein. [b]Significantly different from the control ($p < 0.01$).

inhibited by CO. These results indicate that the microsomal formation of 9-ACA in monkey liver is catalyzed by MALDO, as in mice (*5*) and rats (*12*). α-Naphthoflavone did not inhibit the MALDO for 9-AA. Chang *et al.* (*46*) and Newton *et al.* (*47*) recently reported that α-naphthoflavone was a potent inhibitor of CYP1A2, CYP2C9, and a modest inhibitor of CYP3A4 in human hepatic microsomes. The isozymes corresponding to these P450s may make small contributions to the oxidation of 9-AA in monkey liver.

In the present study, we purified P450JM-A and P450JM-C from hepatic microsomes of untreated male and female Japanese monkeys, respectively. The first 20 amino acid residues in the NH_2-terminus of P450JM-A are identical with those of P450FI purified from phenobarbital-treated baboon liver (*19*), which is considered to be a member of the 2A subfamily. P450JM-A showed high coumarin 7-hydroxylase activity, which was catalyzed by P450 belonging to the CYP2A subfamily in monkey (*19*) and human (*42*). The anti-P450JM-A antibody did not show any inhibitory effect on 9-AA MALDO activity in monkey liver, although P450JM-A (14.3 nmol/min/nmol P450) showed relatively high oxidative activity for 9-AA in the reconstituted system. During the purification of P450JM-A, we have also purified P450JM-B from fraction IV eluted from DEAE-5PW column by successive DEAE-5PW column rechromatography and hydroxylapatite column chromatography. The oxidative activities of 9-AA (12.3 nmol/min/nmol P450), benzphetamine (89.2), 7-ethoxycoumarin (37.5), and coumarin (2.3) by P450JM-B were almost the same as those by P450JM-A. The NH_2-terminal amino acid sequence analysis suggested that P450JM-B was a mixture of P450JM-A and epoxide hydrolase.

The specific content of P450JM-C (3.2 nmol/mg protein) was relatively low, although pronounced contamination with other proteins was not observed in analyses by SDS-PAGE and amino acid sequencing. The reason why the specific content of P450JM-C is relatively low is not clear. The overall yield of P450JM-C was about 9 nmol from a starting preparation of 4,682 nmol of microsomal P450. We did not carry out further purification of P450JM-C because of the low yield. P450JM-C showed high oxidative activity for 9-AA in the reconstituted system containing cytochrome b_5, NADPH-cytochrome *c* (P450) reductase, sodium cholate and lipid mixture (DLPC and PS), but did not show any oxidative activity towards 11-oxo-Δ^8-THC (data not shown). Recently, we demonstrated that the major isozymes responsible for MALDO activities towards 9-AA and 11-oxo-Δ^8-THC in mice might be different (*10*).

NADH-cytochrome b_5 reductase-cytochrome b_5 couple and NADPH-cytochrome *c* (P450) reductase can separately donate both the first and second electrons from NADH to P450 for drug oxidation reactions, and that the relative contributions of these two electron pathways vary significantly according to the concentration of NADH, the substrate employed, and the forms of P450 (*48, 49*). NADH alone could sustain the oxidation of 9-AA in the reconstituted system as indicated with the microsomal fraction, but at slower rates than the corresponding NADPH-supported reactions. The activity was not detected in the absence of either NADPH or NADPH-cytochrome *c* (P450) reductase in the reaction mixture. Furthermore, the reaction was almost completely inhibited by CO. These results indicated that the major enzyme responsible for the oxidation of 9-AA was not a minor component contaminating the purified enzyme fractions, including P450JM-C.

The NH_2-terminal amino acid sequence of P450JM-C indicates high homology to the isozymes of the P450 2B subfamily (*20, 41, 50-53*), especially human CYP2B6 (*41*). Ohmori *et al.* have recently purified a P450, P450CMLa, catalyzing testosterone 16β-hydroxylation from hepatic microsomes of untreated male cynomolgus monkeys (*20*). The first 34 amino acid sequence in the NH_2-terminus of this isozyme is identical with that of P450JM-C. However, the molecular mass of P450CMLa (49,500) is somewhat different from that of P450JM-C (51,000) obtained here. It is unclear at present whether these isozymes are identical with each other; definitive identification is not possible with such limited sequence data. Ohmori *et al.* also reported that a P450 isozyme belonging to the 2B subfamily was constitutively expressed in hepatic microsomes of cynomolgus monkeys, Japanese monkeys, baboons, and common marmosets (*20*). We also confirmed that P450JM-C or an immunologically related protein was constitutively expressed in untreated male and female Japanese ($n=5$) and rhesus ($n=3$) monkeys by Western immunoblotting analysis (data not shown). There were no significant differences in the contents of the immunoreactive protein. In the present study, a significant sex difference was not observed in the inhibition of 9-AA MALDO activity by anti-P450JM-C antibody. These results indicate that P450JM-C contributes to the MALDO activity towards 9-AA in the monkeys, although incomplete inhibition by the antibody suggests that other isozymes may also contribute in part to the MALDO activity. These isozymes may be contained in fractions other than the fraction of purified P450JM-C, because the oxidative activity was observed in all the fractions eluted from the DEAE-5PW column. We previously reported that CYP2C11 was the major constitutive catalyst of 9-AA MALDO activity in untreated adult male rat liver (*12*). Anti-CYP2C11 antibody also significantly inhibited the MALDO activity. These results indicate that P450JM-C and some isozyme(s) immunologically related to CYP2C11 are major isozymes contributing to 9-AA MALDO activity in the monkey liver, and there are species differences in the properties of the P450s. Further extensive studies are required to clarify which isozymes other than P450JM-C are responsible for the 9-AA MALDO activity in monkey liver.

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