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PURIFICATION OF MIXED-FUNCTION AMINE OXIDASE FROM RAT LIVER MICROSOMES

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Summary: To clarify the metabolism of carcinogenic aminoazo dyes in target tissues, mixed function amine oxidase (MFAO) was purified from rat liver. The MFAO was solubilized from microsomes with Triton X in the presence of 20% glycerol and 1 mM EDTA and purified successively with DEAE Sepharose CL-6B, 2',5'-ADP Sepharose 4B and Hydroxyapatite column chromatography. The purified enzyme yielded a single protein band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The apparent molecular weight was about 59,000. When dimethylaniline (DMA) was used as a substrate, the specific activity of the enzyme fortified with NADPH was about 430 nmol DMA N-oxide formed/mg protein/min with a yield of about 15%. N-Demethylation of dimethylaminoazobenzene (DAB) with the enzyme proceeded only when iron was added to the reaction system.

Mixed function amine oxidase (MFAO) is a flavoprotein found in mammalian liver microsomes and nuclear envelopes (1,2). This enzyme catalyzes oxidation of cysteamine to cystamine resulting in the formation of a disulfide bond, thus functioning as a renaturant of proteins (3). MFAO also catalyzes oxidative metabolism of various kinds of carcinogens, drugs and other xenobiotics (4-6).

Special attention has been paid to the fact that MFAO participates in the metabolism of nitrogen-containing carcinogens such as aminoazo dyes, and the role of this enzyme in metabolic activation *in vitro* has been studied in connec-

Abbreviation: MFAO, mixed function amine oxidase; DAB, N,N-dimethyl-4-aminoazobenzene; MAB, N-methyl-4-aminoazobenzene; AB, 4-aminoazobenzene; N-OH-MAB, N-hydroxy-N-methyl-4-aminoazobenzene; N-OH-AB, N-hydroxy-4-aminoazobenzene; 4'-OH-DAB, 4'-hydroxy-N,N-dimethyl-4-aminoazobenzene; 4'-OH-MAB, 4'-hydroxy-N-methyl-4-aminoazobenzene; MC, 3-methylcholanthrene; Cyt. C, cytochrome C; SDS, sodium dodecyl sulfate; h.p.l.c., high pressure liquid chromatography; DTT, dithiothreitol.

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tion with the role of cytochrome p-450 (7,8). For analyzing the N-oxidation of carcinogenic aminoazo dyes, only MFAO purified from porcine liver has been used (7). There have been no studies using MFAO from rat liver microsomes in relation to the metabolism of carcinogenic aminoazo dyes. This is because of the difficulty of purifying rat MFAO due to its instability. Efforts to purify MFAO from rat liver by the Ziegler method commonly used for porcine liver was unsuccessful (8). We improved the method of purification of MFAO from rat liver microsomes by 1) stabilizing the enzyme in glycerol and EDTA during the solubilization process, 2) omitting the steps of ammonium sulfate or polyethylene glycol precipitation which accompanied inactivation of the enzyme and 3) introducing affinity or other additional column chromatography. A preliminary report is presented on the effect of purified MFAO on the metabolic N-oxidation of carcinogenic aminoazo dyes.

Materials and Methods

Chemicals: Triton X-102 and Triton X-45 were obtained from Sigma Chemical Co. Ltd. (St. Louis, Mo.). [^{14}C]-DAB (1.42 mCi/mmol) was purchased from New England Nuclear (Boston, Mass.) and further purified on a silica gel column. NADPH was purchased from Oriental Yeast Co. Ltd. (Tokyo). DEAE-Sepharose CL-6B and 2',5'-ADP Sepharose 4B were purchased from Pharmacia Fine Chem. (Sweden). Hydroxyappatite was obtained from Bio-Rad Labo. (Richmond, Ca). All other chemicals were commercial products of reagent grade.

Preparation of microsomes: Male Sprague-Dawley rats (5 weeks old, 110-120g) treated with 3-MC (i.p. 20 mg/kg) or untreated, were starved for 24 hr prior to sacrifice. The livers were removed and homogenized with 50 mM Tris-HCl buffer containing 1.15% KCl. The postmitochondrial supernatant was prepared by centrifuging the homogenates at 9,000xg for 20 min. The microsomal pellet was precipitated by centrifugation at 105,000xg for 60 min and washed once with the same buffer. For purification of MFAO, microsomes from untreated rats were used, since MFAO was induced with neither PB- nor MC-treatment. For the experiment on the metabolism of DAB, microsomes from MC-treated rats were used, because the metabolic profile was qualitatively the same in microsomes from MC-treated rats as from untreated rats, and the rate of metabolism in the former was 5-6 times higher than in the latter (8,9).

Analytical procedures: N-Oxidation of DMA was assayed by the method of Ziegler and Pettit (10) in a total volume of 5 ml containing 0.1 M glycine-0.025 M pyrophosphate buffer (pH 8.4), 5 μmol NADPH, 15 μmol DMA, 3 mg of microsomal protein or corresponding purified enzyme. After incubation at 37°C for 5 min the DMA N-oxide formed was measured colorimetrically. The metabolism of [^{14}C]-DAB was carried out as follows: The reaction mixture in a total volume of 2 ml containing 1 μmol NADPH, 0.2 μmol [^{14}C]-DAB, 6 μmol $MgCl_2$, 308 μmol KCl, 100 μmol Tris-HCl buffer (pH 7.5) and 0.2 mg of microsomal protein or 1 unit of purified enzyme (1 unit=1 nmol DMA N-oxide formed/min) was incubated at 37°C for 10 min.

After the extraction of metabolites of DAB with benzene, the extract was dried in vacuo. The residue was dissolved in 0.1 ml of methanol and 90 µl of the methanol solution of the metabolites was applied to a reverse phase column. The condition of separation with h.p.l.c. was described previously (11). A one minute collection sample was measured with a scintillation counter. Protein concentration determined by the method of Lowry et al. (12) and SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (13).

Results and Discussion

Purification of MFAO. During solubilization by the Ziegler method, 30-50% of the activity of MFAO from rat liver was lost. This is in sharp contrast to MFAO from porcine liver which is reported to be fairly stable during solubilization (14). The difficulty was overcome by adding 20% glycerol and 1 mM EDTA. Thus, microsomes were solubilized with a mixture of Triton X-102, Triton X-45 and Triton X-45 succinate (5:4:1, final concentration of 1%) in 50 mM Tris-HCl buffer (pH 8.0) with 20% glycerol, 1mM EDTA and 1 mM DTT. After the solution was left standing for 30 min at 4°C, protamine sulfate was added, the solubilized microsomes were centrifuged at 77,000xg for 2 hr and the supernatant fraction was collected. The subsequent steps of ammonium sulfate or polyethylene glycol precipitation were omitted, since much of the MFAO activity was lost during these procedures. The supernatant fraction of the solubilized microsomes was then directly applied to a DEAE-Sepharose CL-6B column (50x290 mm) previously equilibrated with 50 mM of Tris-HCl buffer (pH 8.0) containing 20% glycerol, 1 mM EDTA and 0.2% Triton X-100. The enzyme was eluted with the same equilibrated buffer. Affinity column chromatography was used to increase the purity of the enzyme, since it was successfully applied to the similar NADPH-dependent flavine enzyme, NADPH-Cytochrome P-450 reductase (15). The pooled fraction previously obtained was concentrated by a Diaflo membrane (pM 10) and diluted to the Tris-HCl buffer concentration of 40 mM. The diluted sample was applied to a 2',5'-ADP Sepharose 4B column (16x100 mm) previously equilibrated with 40 mM Tris-HCl buffer (pH 8.0) containing 20% glycerol, 1 mM DTT and 0.2% Triton X-100. After washing the column with 50 mM of buffer, it was eluted by increasing the concentration of the 2'-AMP linearly to 5 mM. The collected MFAO fraction was still contaminated by another protein (M.W.

Table I. Purification of MFAO from rat liver microsomes

	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/mg/min)	Purification (fold)	Recovery (%)
Microsomes	1640	2030	1.24	1	100
DEAE-Sepharose CL-6B column elute	91.5	2037	22.3	18	100
2',5'-ADP-Sepharose column elute	2.1	602	287	131	30
Hydroxyapatite column elute	0.67	289	431	348	14

30,000), which was finally removed by subsequent column chromatography. The pooled fraction was then applied to a Hydroxyapatite column (10x45 mm) previously equilibrated with 25 mM of potassium-phosphate buffer (pH 7.0) with 20% glycerol, 0.2% Triton X-100 and 1 mM DTT. Washing and elution were carried out by 45 mM and 60 mM potassium phosphate buffer, respectively. Table I summarizes the result of the purification of the enzyme. Using DMA as a sub-

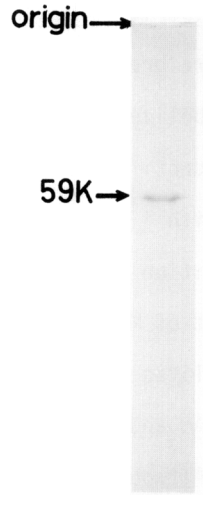

Figure 1. SDS-Polyacrylamide gel electrophoresis of purified MFAO. About 7.5 μg of purified enzyme was electrophoresed in a 10% gel containing 0.1% SDS. The gel was stained overnight with a 0.04% Coomassie Brilliant Blue R-250 in 10% acetone-45% methanol and destained by repeated washing in 10% acetone-10% methanol.

Table II. Metabolism of DAB with purified MFAO compared with microsomes and the effect of iron ions on the MFAO system

Metabolite	Microsomes (nmol/mg/min)	MFAO (nmol/unit/min)			
		control	+$FeCl_2$	+$FeCl_3$	+Cyt. C
N-OH-AB	0.05±0.01[a)]	n.d.[b)]	n.d.	n.d.	n.d.
4'-OH-MAB	0.19±0.03	n.d.	n.d.	n.d.	n.d.
AB	1.52±0.19	n.d.	n.d.	n.d.	n.d.
N-OH-MAB	0.16±0.01	n.d.	0.05±0.003	0.04±0.005	0.04±0.003
4'-OH-DAB	0.68±0.05	n.d.	n.d.	n.d.	n.d.
MAB	7.49±0.49	0.03±0.004	0.47±0.06	0.43±0.08	0.35±0.06

a) means ± S.D. b) n.d. : not detected.

strate, this method gave MFAO with the specific activity of 430 nmol DMA N-oxide formed/mg protein/min in an overall yield of about 15%. This enzyme showed a single protein band on SDS-polyacrylamide gel electrophoresis as shown in Fig. 1. The apparent molecular weight was estimated to be 59,000, nearly the same as that of the porcine liver MFAO obtained by Ziegler and Poulsen (16).

Metabolism of DAB with the purified MFAO. In Table II, the metabolites of DAB with the purified MFAO are presented in comparison with those obtained with microsomes. DAB was metabolized with microsomes to MAB, 4'-OH-DAB, N-OH-MAB, AB, 4'-OH-AB and other unidentified metabolites. In contrast, these metabolites were not detected with 1 unit of MFAO fortified with NADPH, except for a small amount of MAB. Addition of 2 nmol of Fe^{2+}, Fe^{3+} or Cyt. C to this system resulted in the increment of MAB and simultaneous formation of N-OH-MAB. In addition to these two metabolites, two unknown peaks were found to migrate between the retention time of MAB and DAB in both the presence and the absence of iron (data are not shown). Although these peaks are not yet identified, one of them could be ascribed to DAB N-oxide, in view of the fact that MFAO generally metabolizes tertiary amine to tertiary amine N-oxide (10). In the presence of iron, tertiary amine N-oxide was shown to be rapidly rearranged

and degraded to secondary amine (17). This explains why the addition of iron or Cyt. C to the MFAO system considerably increased MAB and a detectable amount of N-OH-MAB appeared.

It is worth noting that with microsomes, the rate of N-demethylation of DAB leading to MAB is extremely high compared with purified MFAO (Table II). Since the content of MFAO in microsomes was estimated to be only about 1.2 unit/mg, participation of MFAO in the N-demethylation of DAB is considered to be fairly small. Thus, N-demethylation of tertiary amine apparently depends mainly on the cytochrome P-450 rather than MFAO.

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