Partial Purification and Substrate Specificity of Flavin-Containing Monooxygenase from Rat Brain Microsomes

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Flavin-containing monooxygenase (FMO) was partially purified from rat brain microsomes through two successive chromatographies on columns of DEAE Sepharose and 2′,5′-ADP Sepharose. The specific activity, benzydamine *N*-oxidation of partially purified brain FMO, was 122-fold higher than that of microsomes. A single band of 60 kDa was recognized by Western blotting analysis with anti-rat liver FMO. The K_m value of brain FMO for thiourea was 4-fold lower, but that for cysteamine was 10-fold higher than that of liver FMO. The enzymatic activity for *n*-octylamine was detected in neither brain nor liver FMO. Kinetic analysis for neurotoxins also revealed that K_m values of brain FMO for 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 1,2,3,4-tetrahydroisoquinoline (TIQ) and *N*-methyl TIQ (NMTIQ) were lower than those of liver FMO. These results indicate that rat brain FMO catalyzes several substrates of liver FMO involving neurotoxins, but it seems likely that the kinetic properties of brain FMO are somewhat different from those of liver FMO.

Key words flavin-containing monooxygenase; rat brain; neurotoxin

Flavin-containing monooxygenase (FMO; EC 1.14.13.8) oxidizes a large number of xenobiotics containing nucleophilic nitrogen-, sulfur-, phosphorous- and selenium-atoms, and participates in xenobiotic metabolism. FMO has been isolated from the microsomes not only of liver but also of extrahepatic organs such as the kidney and lung.[1,2] Brain FMO has not yet been completely elucidated, though there are several reports on it.[3-6]

We developed a novel, simple and sensitive fluorometric assay method of FMO activity for *N*-oxidation of benzydamine (BZY) to its *N*-oxide,[7] and described that FMO activity was distinctly detected in rat brain microsomes by means of this method.[8]

In this study, we attempted to purify FMO in rat brain mirosomes and to characterize it.

MATERIALS AND METHODS

Materials BZY from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan); NADPH from Oriental Yeast Co., Ltd. (Tokyo, Japan); DEAE-Sepharose CL-6B and 2′,5′-ADP Sepharose from Pharmacia Fine Chemicals (Uppsala, Sweden); BCA Protein Assay Reagent from Pierce (Rockford, IL, U.S.A.); 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1,2,3,4-tetrahydroisoquinoline (TIQ) from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.); and bovine serum albumin (BSA, fraction V) from Sigma Chemical Co. (St. Louis, MO, U.S.A.) were used.

Part of the *N*-methyl TIQ (NMTIQ) was a gift from Dr. T. Nagatsu, School of Medicine, Fujita Health University (Aichi, Japan); we also synthesized it ourselves as follows: a solution of 4.0 g of TIQ in 10 ml of MeOH was added dropwise to 4.0 ml of 37% aqueous formaldehyde under cooling below 10 °C. To this mixture, 1.0 g of $NaBH_4$ was added with stirring over a period of 1 h at the same temperature. The reaction mixture was diluted with water and extracted with EtOAc, and the extract was dried over $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in 10 ml of EtOAc and refluxed with 2.0 g of succinic anhydride for 30 min. This solution was washed with aqueous $NaHCO_3$, dried over $MgSO_4$, and evaporated *in vacuo* to yield 3.2 g of colorless oil, bp 135—140 °C (50 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 2.45 (s, N-CH_3), 2.71 (m, 4-CH_2), 3.07 (m, 3-CH_2), 3.59 (s, 1-CH_2), 7.11 (m, Ar-H_4) on JEOL-FX90Q.

1-Methyl TIQ (1-MTIQ) was synthesized as follows: to a stirred solution of 3.0 g of 1-methyl-3,4-dihydroisoquinoline[9] in 40 ml of MeOH was added 400 mg of $NaBH_4$ over a period of 30 min. After 30 ml of MeOH was distilled off under reduced pressure, the residue was diluted with water and the solution was extracted with ether three times. The combined ether extract was dried over $MgSO_4$ and evaporated *in vacuo* to give 2.5 g of colorless base, bp 119 °C (18 mmHg). ^{1}H-NMR ($CDCl_3$) δ: 1.44 (d, J=6.9 Hz, CH_3), 1.60 (s, NH), 2.80 (m, 4-CH_2), 3.18 (m, 3-CH_2), 4.08 (q, J=6.9 Hz, 1-CH), 7.13 (br s, Ar-H_4) on JEOL-FX90Q.

Emulgen 911 was a gift from Kao-Atlas (Tokyo, Japan). All other reagents used were of analytical grade.

Partial Purification of FMO from Rat Brain Microsomes Male Wistar rats (5 weeks old, 180—200 g) were purchased from Shimizu Lab. Supplies Co., Ltd. (Kyoto, Japan). They were allowed free access to food (MF, Oriental Yeast, Tokyo, Japan) and water for a week prior to use. All the procedures described below were carried out at 4 °C. Brain microsomes were prepared by differential centrifugation.[10] The microsomal pellets obtained were resuspended in 10 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol and 0.1 mM EDTA (buffer A) and were stored at −70 °C.

Brain microsomes (10 mg protein/ml) were solubilized with 0.5% Emulgen 911 in buffer A for 60 min with gentle stirring, and were centrifuged at 105000 ***g*** for 90 min. The supernant fraction was directly applied to a DEAE-Sepharose CL-6B column (80 ml) pre-equilibrated with buffer A containing 0.1% Emulgen 911 (buffer B). Under these conditions, brain FMO did not bind to the DEAE column. The unbound fraction showing FMO activity

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(DEAE pool) was then applied to a 2′,5′-ADP Sepharose column (15 ml) pre-equilibrated with buffer B. The FMO was eluted from the column with buffer B containing 0.1 mM NADPH. FMO fractions were collected (ADP pool), concentrated with an Ultra Filter Unit (Advantec Toyo, Tokyo, Japan) and stored at −70 °C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on 7.5% gel by the method of Laemmli.[11] The gel was stained using the silver staining method of Oakley *et al.*[12] Moreover, following electrophoresis, the separated proteins were transfered to a PVDF membrane, treated with anti-rat liver FMO antisera (1 : 1000) and peroxidase goat anti-rabbit IgG (1 : 3000), and visualized by a POD Immunostain Set (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Enzyme Assays For purification of brain FMO, BZY *N*-oxidation activity was determined using HPLC as previously described.[7]

The substrate specificity of partially purified brain or purified liver FMO was measured by monitoring NADPH oxidation at 340 nm.[13]

Others Purification of rat liver FMO and the development of antisera against purified rat liver FMO were carried out as described elsewhere.[7,8]

Protein concentration was determined by the method of Lowry *et al.*[14] except for samples containing the detergent, which were determined by BCA Protein Assay Reagent. BSA was used as a standard in both procedures.

RESULTS

FMO was partially purified from rat brain microsomes by DEAE Sepharose CL-6B and 2′,5′-ADP Sepharose column chromatography. Table 1 summarizes the partial purification of rat brain FMO using BZY *N*-oxidation.

Treatment of rat brain microsomes with 0.5% Emulgen 911 solubilized 88% of the microsomal FMO (data not shown), and 80% of the FMO activity was recovered in the 105000 ***g*** supernatant fraction. After a 2′,5′-ADP Sepharose chromatography (Fig. 1), the purity of rat brain FMO increased to 122-fold that of microsomes. Subsequent SDS–PAGE of the ADP pool showed some remaining protein bands stained by silver nitrate (Fig. 2A), but showed a positive band of 60 kDa from Western blot analysis with anti-rat liver FMO antisera (Fig. 2B). While hydroxylapatite chromatography was used to further purify it, FMO activity was not recovered. Thus, the ADP pool was used for the study of substrate specificities.

Table 1. Partial Purification Summary of FMO from Rat Brain Microsomes

	Protein (mg)	BZY *N*-oxidation activity			
		Total (nmol/min)	Specific (nmol/min/mg)	Recovery (%)	Purification fold
Microsomes	210	35.8	0.171	100	1.0
Supernatant	75.8	28.7	0.378	80	2.2
DEAE pool	21.8	12.8	0.588	36	3.4
ADP pool	0.331	6.92	20.9	19	122

FMO-mediated NADPH oxidation of thiourea, *n*-octylamine and cysteamine was studied. *n*-Octylamine was oxidized neither by brain nor liver FMO. The K_m value of brain FMO for thiourea, 27 μM, was one-fourth that of liver FMO. However, the K_m value of brain FMO for cysteamine was 1200 μM, 10-fold higher than that of liver FMO (Table 2).

MPTP and NMTIQ were good substrates for brain

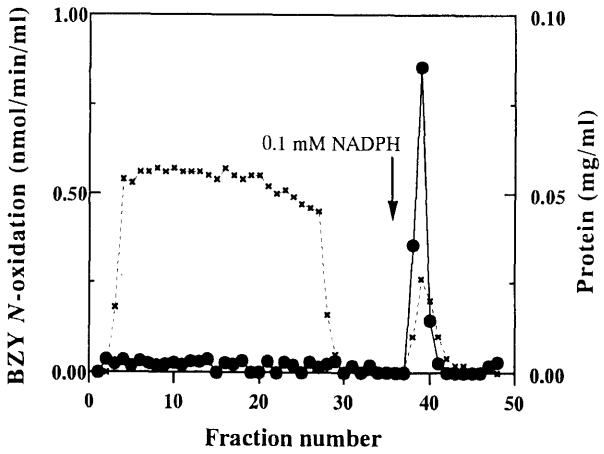

Fig. 1. Elution Profile of Rat Brain FMO from 2′,5′-ADP Sepharose Column Chromatography

—●—, benzydamine *N*-oxidation activity (nmol/min/ml); ···×···, protein concentration (mg/ml).

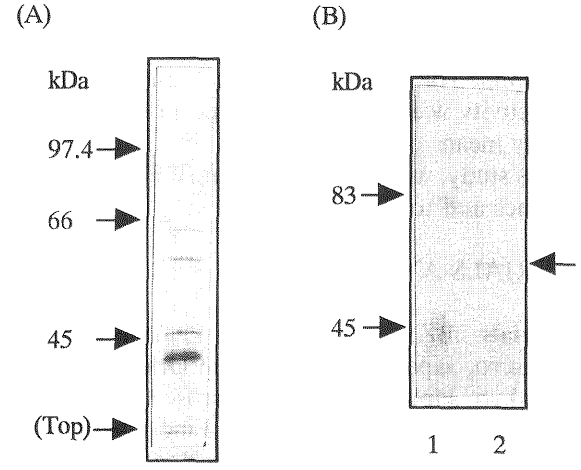

Fig. 2. SDS–PAGE (A) and Western Blot Analysis (B) of Partially Purified FMO from Rat Brain Microsomes

(A) and lane 2 in (B), 20 μg of the ADP pool from rat brain microsomes; lane 1 in (B), molecular weight marker.

Table 2. Kinetic Parameters of Thiourea, *n*-Octylamine and Cysteamine for FMO Purified from Rat Liver Microsomes and Partially Purified from Rat Brain Microsomes

Substrate	Brain FMO		Liver FMO	
	K_m (μM)	V_{max} (nmol/min/mg protein)	K_m (μM)	V_{max} (nmol/min/mg protein)
Thiourea	27± 5	42± 4	91±31	780±74
n-Octylamine	N.D.	N.D.	N.D.	N.D.
Cysteamine	1200±140	34±11	127±31	934±37

N.D.: not detectable. Results are the mean±S.D. of three experiments.

Table 3. Kinetic Parameters of MPTP and TIQs for FMO Purified from Rat Liver Microsomes and Partially Purified from Rat Brain Microsomes

Substrate	Brain FMO		Liver FMO	
	K_m (μM)	V_{max} (nmol/min/mg protein)	K_m (μM)	V_{max} (nmol/min/mg protein)
MPTP	6±0.2	36±0.4	44± 5	1960±130
TIQ	59±7	44±2	348±140	1600±220
NMTIQ	8±2	39±5	32± 7	2030± 95
1-MTIQ	N.D.	N.D.	2870±380	1460±100

N.D.: not detectable. Results are the mean±S.D. of three experiments.

and liver FMO. Especially, the K_m values of brain FMO for MPTP and NMTIQ were very low, 6 μM and 8 μM, respectively. The K_m value of brain FMO for TIQ was 59 μM, about three times lower than that of liver FMO, but seven times higher than that for NMTIQ. The K_m value of liver FMO for 1-MTIQ was very high and 1-MTIQ *N*-oxidation was not detected by brain FMO (Table 3).

DISCUSSION

FMO was partially purified from rat brain microsomes in terms of BZY *N*-oxidation activity, and the kinetic constants for various xenobiotics and endogenous substrates involved neurotoxins which cause Parkinsonism were compared with purified FMO from rat liver.

The K_m value of brain FMO for thiourea, a representative substrate for FMO,[1] was lower than that of liver FMO. However, the K_m value of brain FMO for cysteamine, a known endogenous substrate,[1] was 10-fold higher than that of liver FMO. *n*-Octylamine, a substrate for FMO2,[1] was oxidized neither by brain nor liver FMO.

It is well known that MPTP, an artificial neurotoxic agent causing Parkinsonism, is activated to its pyridinium ion (MPP^+) by monoamine oxidase (MAO) and is metabolized to the non-toxic metabolite, MPTP *N*-oxide, by porcine liver FMO.[2] Brain FMO also catalyzed the *N*-oxidation of MPTP with a very high affinity.

The TIQ and NMTIQ levels in human brain rose in the Parkinsonian brain. NMTIQ is produced from TIQ by *N*-methyltransferase, subsequently activated to its isoquinolinium ion ($NMIQ^+$) by MAO, a potent neurotoxin causing Parkinsonism.[15-17] NMTIQ was an excellent substrate for both brain and liver FMO as well as MPTP. Especially in the brain, the affinity was very high. It was described that porcine liver FMO catalyzed the *N*-oxidation of TIQ and 1-methyl-6,7-dihydroxytetrahydroisoquinoline (MDTIQ) with low affinity.[18] The K_m value of brain FMO for TIQ was 6-fold lower than that of liver FMO. It is still unknown whether *N*-oxidation of TIQ or NMTIQ is a pathway for detoxication, but it has been demonstrated that brain FMO was involved in the metabolism of these compounds.

1-MTIQ, an endogenous amine in human and rat brain, has a protective effect on Parkinsonism, and its content decreased in the brain of Parkinsonian models or aged animals.[19,20] Interestingly, the K_m value of liver FMO for 1-MTIQ was very high compared with other TIQs, and the activity of 1-MTIQ *N*-oxidation by brain FMO was not detected. It seems probable that FMO hardly contributes to the metabolism of 1-MTIQ.

These results suggest that the catalytic properties of partially purified rat brain FMO for various xenobiotics and endogenous substrates resemble those of rat liver FMO, but some differences were observed. This study showed the presence of FMO of 60 kDa in rat brain microsomes which recognized anti-rat liver FMO antibody. By contrast, it was demonstrated that FMO in rat brain microsomes reacted with anti-rabbit lung FMO antibody.[4,5] These reports suggest the presence of FMO1 and FMO2 or other isozyme(s) in rat brain FMO. Further purification is in progress and the role of brain FMO is an important problem to be solved in future.

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