Determination of Flavin-Containing Monooxygenase Activity in Rat Brain Microsomes with Benzydamine *N*-Oxidation

Akiko KAWAJI,* Kensuke OHARA, and Eigo TAKABATAKE

Department of Toxicology, Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata, Osaka, 573-01, Japan.

Received October 20, 1993; accepted January 19, 1994

The activity of flavin-containing monooxygenase (FMO) in rat brain microsomes was measured by fluorometrical determination of benzydamine (BZY) *N*-oxygenation with HPLC. The apparent K_m value for the formation of BZY *N*-oxide from BZY by brain microsomes was similar to that by hepatic microsomal fraction or purified FMO, but the V_{max} value for brain microsomes was about one-hundredth of that for hepatic microsomes. BZY *N*-oxygenation activity by brain microsomes was at a maximum near pH 8.5, slightly more acidic than the optimum pH for liver FMO. BZY *N*-oxygenation activity was inhibited completely by heat inactivation and markedly in the presence of 1 mM thiourea, but slightly in the presence of 1 mM SKF-525A, and it was only barely activated in the presence of 5 mM *n*-octylamine, a positive effector of liver FMO. The addition of rabbit antisera raised against rat liver FMO resulted in 30% inhibition of BZY *N*-oxygenation by solubilized brain microsomes. Compared with microsomes from five different brain regions, the activity was highest in microsomes of olfactory bulbs. These results show that the activity of FMO in rat brain is distinctly determined by BZY *N*-oxygenation.

Keywords flavin-containing monooxygenase; rat brain microsomes; benzydamine; *N*-oxygenation

Flavin-containing monooxygenase (FMO; EC 1.14.13.-8) oxidizes a large number of xenobiotics containing nucleophilic nitrogen-, sulfur- and phosphorous-atom^{1,2)} and participates in xenobiotic metabolism. FMO has been isolated from microsomes of not only liver but also extrahepatic organs such as kidney and lung.¹⁻⁵⁾

The presence of several drug metabolizing enzymes, especially cytochrome P-450s, have been revealed in brain.⁶⁻⁸⁾ Duffel and Gillespie⁹⁾ demonstrated the presence in corpus striatum of a microsomal monooxygenase with catalytic properties of the hepatic microsomal FMO. Bhamre and Ravindranath¹⁰⁾ reported that the FMO activities in rat brain for several substrates were higher than those in liver. Furthermore, they¹¹⁾ recently showed by immunoblot analysis that rat brain microsomes did not react with antisera to purified porcine liver FMO but reacted with antisera for rabbit lung FMO. On the other hand, Itoh *et al.*¹²⁾ demonstrated that rat liver FMO was expressed in lung and kidney and to a lesser extent in the heart and brain. However, the FMO activity in brain is not still distinctly determined.

We developed a simple and sensitive fluorometric assay of FMO activity for *N*-oxygenation of benzydamine (BZY) to its *N*-oxide.¹³⁾ The present paper describes that the FMO activity was distinctly detected in rat brain microsomes by means of this method.

MATERIALS AND METHODS

Materials BZY from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan); NADPH from Oriental Yeast Co., Ltd. (Tokyo, Japan); DEAE-Sepharose CL-6B, Red-Sepharose CL-6B, and 2',5'-ADP Sepharose from Pharmacia Fine Chemicals (Uppsala, Sweden); hydroxylapatite, and DC protein assay kit from Bio-Rad Laboratories (Richmond, CA, U.S.A.); and bovine serum albumin (BSA, fraction V) from Sigma Chemical Co. (St. Louis, MO, U.S.A.) were used. Emulgen 911 was a gift from Kao-Atlas (Tokyo, Japan). *N*-Desmethylbenzydamine (Nor-BZY) was a gift

from Dr. B. Silvestrini, A.C.R. Angelini (Rome, Italy). BZY *N*-oxide was synthesized by the method of Kataoka *et al.*¹⁴⁾ Acetonitrile from Nacalai Tesque Inc. (Kyoto, Japan) was HPLC grade. All other reagents used were of analytical grade.

Preparation of Brain and Liver 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. Brain and liver microsomes were prepared by differential centrifugation.¹⁵⁾ For regional distribution studies, brains were cut into five regions, namely olfactory bulbs, cortex, cerebellum, midbrain, and medulla oblongata as described by Glowinski and Iversen.¹⁵⁾ The microsomal pellets obtained were resuspended in 10 mM potassium phosphate buffer, pH 7.6 containing 20% glycerol and 0.1 mM EDTA and stored at –70 °C.

Purification of Liver FMO and Preparation of Polyclonal Antibodies FMO was purified according to the methods reported by Venkatesh *et al.*,⁵⁾ except that to FMO-containing fractions which eluted from a 2',5'-ADP Sepharose column were dialyzed against 25 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol and 0.1 mM EDTA and applied to a hydroxylapatite column pre-equilibrated with the dialysis buffer. The column was washed with 45 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol and 0.1 mM EDTA, and detergent-free FMO was eluted with 60 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol and 0.1 mM EDTA, as described by Kimura *et al.*¹⁶⁾ Highly purified FMO appeared as a single band and was homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli.¹⁷⁾ Antisera against purified rat liver FMO were raised as previously described.¹³⁾ All the procedures described above were carried out at 4 °C.

Metabolic Incubations and Analyses The basic incubation mixtures contained 0.1 M Tricine-KOH buffer at

pH 8.5, 0.5 mM NADPH, appropriate amounts of the microsomal protein or the purified enzyme, and 0.3 mM BZY in a final volume of 0.3 ml. The mixtures were incubated at 37 °C for 5 min and the reaction was initiated by an addition of BZY. When appropriate, 0.5 mM of thiourea, 2 mM of SKF-525A or 5 mM of *n*-octylamine was added to the incubation mixture before addition of the substrate. For the kinetic studies, the assay was carried out in the presence of a positive effector, 5 mM of *n*-octylamine.¹⁸⁾ Thermal inactivation of FMO was done at 45 °C for 2 min in the absence of NADPH. For the immunoinhibition assay, the antisera or non-immune sera and solubilized brain microsomes with 0.1% Emulgen 911 were preincubated at 4 °C for 30 min prior to the addition of other components.

After incubation for 20 min, a twofold volume of methanol was added and the mixture was centrifuged at 1000 × *g* for 10 min. The supernatant was subjected to HPLC for determination of BZY and its metabolites according to the method reported by Baldock *et al.*¹⁹⁾ The HPLC equipment consisted of a Shimadzu (Kyoto, Japan) LC-6A system with a fluorescence detector (Shimadzu RF-530, ex. 303 nm, em. 377 nm) and a LiChrosorb RP-18 column (4 × 150 mm, Merck, Rahway, NJ, U.S.A.). The mobile phase was methanol-acetonitrile-water-25% NH₄OH (50:40:10:0.05 (v/v)). The flow rate was 1.5 ml/min (0 to 3 min) and then 3 ml/min (3–20 min). Under the conditions described above, BZY *N*-oxide, BZY, and Nor-BZY were eluted with retention times of 2.1, 5.1, and 15.4 min, respectively.

Other Analytical Methods *N,N*-Dimethylaniline *N*-oxidation was determined by the method of Ziegler and Pettit²⁰⁾ for purification of liver FMO. Protein concentration was determined by the method of Lowry *et al.*²¹⁾ except for samples containing the detergent which were determined by the DC protein assay kit. BSA was used as a standard in both procedures.

RESULTS

The chromatogram shown in Fig. 1 demonstrates distinctly the formation of BZY *N*-oxide from BZY after

incubating with brain microsomes. Nor-BZY, the metabolite of BZY mainly catalyzed by cytochrome P-450s, was slightly detected in brain microsomes.

BZY *N*-oxygenation was proportional to brain microsomal protein at least up to 1.5 mg per milliliter of assay mixture and was linear at least up to 30 min in the presence of 0.5 mg of brain microsomal protein per milliliter of the assay mixture (data not shown).

As shown in Fig. 2, BZY *N*-oxygenation activity in brain microsomes is at a maximum near pH 8.5, which is slightly more acidic than the optimum pH for liver microsomes, pH 9.0. Therefore, the pH was fixed at 8.5 for the *N*-oxygenation assay in brain microsomes.

In brain microsomes, the apparent *K_m* value for BZY *N*-oxygenation was similar to that in microsomes or purified FMO from liver. However, the *V_{max}* value in brain microsomes was about one-hundredth of that in liver microsomes (Talbe I).

Heating microsomes in the absence of NADPH at 45 °C for 2 min completely diminished BZY *N*-oxygenation activity as much as omitting NADPH, although heating microsomes in the presence of NADPH retained the

Fig. 1. HPLC Profiles of Benzydamine and Its Metabolites after Incubation with Brain Microsomes from Wistar Male Rats

Assays and HPLC analysis were carried out as described in Materials and Methods.

Fig. 2. Effect of pH on Benzydamine *N*-Oxygenation by Rat Brain (A) and Liver (B) Microsomes

○, 0.1 M Na, K-phosphate buffer; ●, 0.1 M Tricine-KOH buffer. Results are the mean of three separate experiments.

TABLE I. Kinetic Constant for Benzydamine *N*-Oxygenation by Microsomes from Rat Brain and Liver and Flavin-Containing Monooxygenase Purified from Rat Liver

Enzyme preparation	BZY <i>N</i> -oxygenation	
	K_m (μM)	V_{max} (nmol/mg/min)
Brain		
Microsomes	19 $\pm$ 4.9	0.27 $\pm$ 0.13
Liver		
Microsomes	15 $\pm$ 1.4	37 $\pm$ 2.9
Purified FMO	15 $\pm$ 0.4	1870 $\pm$ 360

Results are the means $\pm$ S.D. of three separate experiments.

TABLE II. Effects of Various Treatments on Benzydamine *N*-Oxygenation by Rat Brain and Liver Microsomes

Treatment	BZY <i>N</i> -oxygenation (nmol/mg/min)	
	Brain	Liver
Complete	0.24 $\pm$ 0.02	31.2 $\pm$ 4.3
Omit NADPH	n.d. ^{d)}	n.d. ^{d)}
0.5 mM thiourea ^{a)}	0.09 $\pm$ 0.02	13.9 $\pm$ 2.2
2 mM SKF-525A ^{a)}	0.18 $\pm$ 0.05	25.8 $\pm$ 3.4
5 mM <i>n</i> -octylamine ^{a)}	0.25 $\pm$ 0.09	40.8 $\pm$ 0.8
Heat inactivation ^{b)}	n.d. ^{d)}	n.d. ^{d)}
Heat with NADPH ^{c)}	0.22 $\pm$ 0.06	— ^{e)}

Results are the means $\pm$ S.D. of four separate experiments. a) Inhibitors were preincubated with microsomes containing NADPH for 5 min. b) Microsomes were heated at 45°C for 2 min in the absence of NADPH. c) Microsomes were heated at 45°C for 2 min in the presence of NADPH. d) Not detectable. e) Not determined.

Fig. 3. Effect of Anti-Rat Liver FMO Antisera on Benzydamine *N*-Oxygenation by Solubilized Rat Brain Microsomes

○, non-immune sera; ●, rabbit anti-rat liver FMO sera.

activity. Thiourea, an alternate substrate for FMO, inhibited BZY *N*-oxygenation activity to 37% of the complete system, but SKF-525A, a well-known cytochrome P-450s inhibitor, slightly inhibited BZY *N*-oxygenation activity to 73% of the complete system. The effects of inhibitors on BZY *N*-oxygenation in brain microsomes were similar to those in liver microsomes. *n*-Octylamine did not activate BZY *N*-oxygenation ac-

TABLE III. Benzydamine *N*-Oxygenation in Microsomes from Five Different Brain Regions from Male Wistar Rats

Region	BZY <i>N</i> -oxygenation (nmol/mg/min)
Olfactory bulbs	1.41
Cortex	0.30
Cerebellum	0.40
Midbrain	0.35
Medulla oblongata	0.44

The microsomes from five different brain regions of ten male Wistar rats were prepared and pooled. Results are the means of three experiments measured on the same microsomal pool.

tivity, although in liver microsomes it activated to 131% of the complete system (Table II).

BZY *N*-oxygenation was inhibited by anti-rat liver FMO antisera but 70% of the activity remained in 20 μ g of antisera per microgram of solubilized brain microsomal protein (Fig. 3).

BZY *N*-oxygenation activities in microsomes from five different brain regions, olfactory bulbs, cortex, cerebellum, midbrain and medulla oblongata, were compared. The activity was highest in microsomes of olfactory bulbs which is fivefold higher than that in cortex. The activities in regions other than olfactory bulbs were almost the same but the activity in medulla oblongata was a little higher (Table III).

DISCUSSION

Liver is the main organ metabolizing xenobiotics, but a wide variety of enzymes that metabolize drugs are located in various extrahepatic organs in experimental animals and human.^{1,2,6-8)} FMO is an enzyme oxidizing many drugs including the psychotropic drugs chlorpromazine, imipramine, trifluoperazine and others.¹⁾

FMO activity has been detected in several organs, namely lung, adrenal, kidney and thymus,^{1,2)} but in brain it has rarely been reported. Itoh *et al.*¹²⁾ demonstrated recently that rat liver FMO was expressed in lung and kidney and to a lesser extent in the heart and brain. Bhamre and Ravindranath¹⁰⁾ reported that the FMO activities for thiobenamide, *N,N*-dimethylaniline, or methimazole were higher in rat brain microsomes than in liver microsomes. We used the same examination conditions but could not confirm the FMO activities for these substrates in rat brain. Although the reason for this discrepancy is unknown, we attempted to identify the FMO activity in rat brain microsomes using a newly developed assay method for *N*-oxygenation of BZY.¹³⁾

BZY *N*-oxide was distinctly detected by HPLC after incubating BZY with rat brain microsomes (Fig. 1). The apparent K_m value was similar to that from liver microsomes, but the V_{max} value for brain microsomes was one-hundredth that for liver microsomes (Table I). These results were in contradiction to the earlier reported findings.^{10,11)} The optimum pH of BZY *N*-oxygenation by brain is slightly more acidic, pH 8.5, than that for liver FMO, pH 9.0 (Fig. 2). The effects of thiourea and SKF-525A or the instability with the heating on BZY *N*-oxygenation by rat brain microsomes were similar to

those of liver microsomes, but *n*-octylamine, a positive effector of liver microsomes, did not activate BZY *N*-oxygenation activity by brain microsomes (Table II).

Moreover, BZY *N*-oxygenation by solubilized brain microsomes was inhibited by anti-rat liver FMO antisera but 70% of the activity remained (Fig. 3). Bhamre *et al.*¹¹⁾ demonstrated by Western immunoblot analysis that rat brain microsomes did not react with antisera to purified porcine liver FMO but reacted with antisera for rabbit lung FMO, and that addition of the antibody raised against rabbit lung FMO resulted in 43% inhibition of the FMO-mediated metabolism of imipramine. Therefore, these facts would indicate the presence of FMO isoform(s) in rat brain microsomes.

Duffel and Gillespie⁹⁾ demonstrated the presence in rat corpus striatum of a microsomal monooxygenase with catalytic properties of the hepatic microsomal FMO. Furthermore, Bhamre *et al.*¹¹⁾ indicated immunohistochemical localization of FMO in hippocampus, brain stem and elsewhere in rat brain. In this study, the FMO activity was highest in microsomes of the olfactory bulbs, about fivefold the activity in other regions. The activities in regions other than olfactory bulbs were almost the same but that in the medulla oblongata was a little higher (Table III). Regional differences of cytochrome P-450s activities were reported for alkoxyresorufin metabolism.⁸⁾ *O*-Deethylation of 7-benzoxoresorufin, a non-specific cytochrome P-450s substrate, was highest in the microsomes of olfactory bulbs similar to the distribution of FMO. On the contrary, *O*-deethylation of 7-pentoxoresorufin, a specific substrate of CYP2B1, was not detected in midbrain microsomes, and *O*-deethylation of 7-ethoxoresorufin, a specific substrate of CYP1A1, was not detected in medulla oblongata. It was recently reported that *N*-oxygenation of *N,N*-dialkylamines was catalyzed by CYP2B1²²⁾ and that *N*-oxygenation of tamoxifen, a therapeutic agent of breast cancer, may be catalyzed by CYP1A1/1A2.²³⁾ It is unclear whether CYP2B1 and/or CYP1A1/1A2 participate in BZY *N*-oxygenation, but the results of the experiments using inhibitors suggested that the contribution of one and/or the other to the *N*-oxygenation were much less than FMO.

The present experiments indicated that BZY was distinctly metabolized to BZY *N*-oxide by rat brain microsomes and that this oxygenation was catalyzed by FMO.

The FMO activities for other substrates than BZY and the physiological or pharmacological roles of FMO in rat brain are still unclear and remain to be determined by purification and characterization of FMO in brain microsomes.

Acknowledgements The skillful technical assistance of Ms. T. Ishikawa and Mr. T. Sawada is gratefully acknowledged.

REFERENCES

- 1) D. M. Ziegler, *Drug Metabol. Rev.*, **19**, 1 (1988).
- 2) E. Heinze, P. Hlavica, M. Kiese, G. Lipowsky, *Biochem. Pharmacol.*, **19**, 641 (1970).
- 3) D. E. Williams, S. E. Hale, A. S. Meurhoff, B. S. S. Masters, *Mol. Pharmacol.*, **28**, 381 (1984).
- 4) R. E. Tynes, P. J. Sabourin, E. Hodgson, *Biochem. Biophys. Res. Commun.*, **126**, 1069 (1985).
- 5) K. Venkatesh, P. E. Levi, E. Hodgson, *Biochem. Pharmacol.*, **42**, 1411 (1991).
- 6) J.-F. Ghersi-Egea, B. Walther, D. Decolin, A. Minn, G. Siest, *Neuropharmacology*, **26**, 367 (1987).
- 7) M. Iscan, K. Reuhl, B. Weiss, M. D. Maines, *Biochem. Biophys. Res. Commun.*, **169**, 858 (1990).
- 8) R. Perrom, A. Minn, J.-F. Ghersi-Egea, M.-C. Grassiot, G. Siest, *Biochem. Pharmacol.*, **40**, 2145 (1990).
- 9) M. W. Duffel, S. G. Gillespie, *J. Neurochem.*, **42**, 1350 (1984).
- 10) S. Bhamre, V. Ravindranath, *Biochem. Pharmacol.*, **42**, 442 (1991).
- 11) S. Bhamre, S. V. Bhagwat, S. K. Shankar, V. Ravindranath, *J. Pharm. Exp. Ther.*, **267**, 555 (1993).
- 12) K. Itoh, T. Kimura, T. Yokoi, S. Itoh, T. Kamataki, *Biochim. Biophys. Acta.*, **1173**, 165 (1993).
- 13) A. Kawaji, K. Ohara, E. Takabatake, *Anal. Biochem.*, **214**, 409 (1993).
- 14) S. Kataoka, K. Taira, T. Ariyoshi, E. Takabatake, *Chem. Pharm. Bull.*, **21**, 358 (1973).
- 15) J. Glowinski, L. L. Iversen, *J. Neurochem.*, **13**, 655 (1966).
- 16) T. Kimura, M. Kodama, C. Nagata, *Biochem. Biophys. Res. Commun.*, **110**, 640 (1983).
- 17) U. K. Laemmli, *Nature (London)*, **227**, 680 (1970).
- 18) D. M. Ziegler, L. L. Poulsen, E. M. McKee, *Xenobiotica*, **1**, 523 (1971).
- 19) G. A. Baldock, R. R. Brodie, L. F. Chasseaud, T. Taylor, *J. Chromatogr.*, **529**, 113 (1990).
- 20) D. M. Ziegler, F. H. Pettit, *Biochem. Biophys. Res. Commun.*, **15**, 188 (1964).
- 21) O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 22) Y. Seto, P. Guengerich, *J. Biol. Chem.*, **268**, 9989 (1993).
- 23) C. Mani, E. Hodgson, D. Kupfer, *Drug Metabol. Disp.*, **21**, 657 (1993).