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An Assay of Flavin-Containing Monooxygenase Activity with Benzydamine N-Oxidation

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An assay system of flavin-containing monooxygenase was developed by fluorometric determination of benzydamine (BZY) N-oxidation with HPLC. The apparent K_m value for the formation of BZY *N*-oxide from BZY by rat liver microsomes was similar to that by purified FMO. The K_m and V_{max} values for the formation of *N*-desmethylbenzydamine (Nor-BZY) by rat liver microsomes were about 50 times greater and 2000 times less, respectively, than those of BZY *N*-oxide. Nor-BZY was not formed upon incubation with purified enzyme. BZY N-oxidation activity was completely inhibited both in the absence of NADPH and by heat inactivation. The reaction was inhibited in the presence of 0.5 mM thiourea, but 2 mM SKF-525A did not affect BZY N-oxidation. Moreover, rabbit antibody raised against the rat enzyme inhibited BZY N-oxidation. These results are in accord with a simple, rapid, and sensitive assay for the enzyme. © 1993 Academic Press, Inc.

Flavin-containing monooxygenase (FMO[2]; EC 1.14.13.8) oxidizes a large number of xenobiotic substrates containing nucleophilic nitrogen, sulfur, and phosphorous atoms (1) and participates in xenobiotic metabolism. Its activity in microsomes or during enzyme purification is generally determined by monitoring substrate-dependent NADPH or oxygen consumption or spectrophotometrically by using *N,N*-dimethylaniline, methimazole N-oxidation, or thiobenzamide S-oxidation (2–5). Some assay systems using radioactive substrates are also reported (6–8). These methods are not necessarily sensitive or simple.

Benzydamine (BZY) is a widely used nonsteroidal anti-inflammatory drug metabolized mainly by N-oxidation to BZY *N*-oxide and N-demethylation to *N*-desmethylbenzydamine (Nor-BZY) *in vivo* (Fig. 1) (9,10). Recently, the pharmacokinetics of BZY and its metabolites were studied at very low levels in biological fluids by HPLC using their strong fluorescence (10–12). Since the N-oxidation of BZY should be catalyzed by FMO, BZY was used as a substrate in the assay of FMO activity.

The present paper describes BZY N-oxidation by microsomes and highly purified FMO preparations from rat liver and the development of a sensitive and simple fluorometric assay for FMO activity.

MATERIALS AND METHODS

Materials. BZY from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan); DEAE-Sepharose CL-6B, Blue-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); NADPH from Oriental Yeast Co. Ltd. (Tokyo, Japan); and bovine serum albumin (BSA, fraction V) from Sigma Chemical Co. (St. Louis, MO) were used. Emulgen 911 was a gift from Kao-Atlas (Tokyo, Japan). Nor-BZY was a gift from F. Angelini Research Institute (Rome, Italy). BZY *N*-oxide was synthesized by the method of Kataoka *et al.* (9). Acetonitrile from Nacalai Tesque Inc. (Kyoto, Japan) was HPLC grade. All other reagents used were of analytical grade.

Preparation of microsomes and purification of FMO. Male Wistar rats (180–200 g) were purchased from Shimizu Laboratory 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. Hepatic microsomes were prepared by differential centrifugation (14). The microsomal pellets obtained were

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[2] Abbreviations used: FMO, flavin-containing monooxygenase; BZY, benzydamine; Nor-BZY, *N*-desmethylbenzydamine; BSA, bovine serum albumin; IgG, immunoglobulin G.

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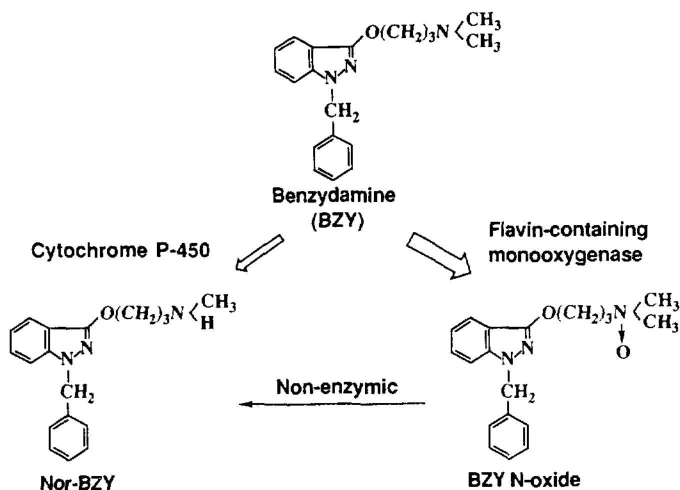

FIG. 1. Metabolism of BZY by microsomal monooxygenases.

suspended in 10 mM potassium phosphate at pH 7.6 containing 20% glycerol and 0.1 mM EDTA and were stored at −70°C.

Rat liver FMO was purified according to the methods reported by Venkatesh *et al.* (14) and Kimura *et al.* (15) with slight modification. 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 (16). All of the procedures described above were carried out at 4°C.

Preparation of polyclonal antibodies. Antisera against purified rat liver FMO were raised in male New Zealand white rabbits (2 kg) purchased from Shimizu by injecting 70 μg of the purified FMO emulsified in Freund's complete adjuvant subcutaneously three times every 2 weeks. The immunoglobulin (IgG) fraction was isolated from the antisera by ammonium sulfate precipitation and DEAE-Sepharose CL-6B chromatography (17).

Metabolic incubations and analyses. The incubation mixtures contained 0.1 M Tricine–KOH at pH 8.5, 0.5 mM NADPH, appropriate amounts of the microsomal protein or the purified enzyme, and the indicated concentrations of 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 the addition of BZY. When appropriate, inhibitors were added to the incubation mixture before addition of the substrate. For kinetic studies, the assay was carried out in the presence of the positive effector, 5 mM *n*-octylamine (18). Thermal inactivation was performed at 50°C for 3 min in the absence of NADPH. For the immunoinhibition assay, the IgG and microsomes were preincubated at room temperature for 20 min prior to the addition of other components. After incubation for 10 min, a twofold volume of methanol was added and 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.* (11). 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 LiCrosorb RP-18 column (4 × 150 mm, Merck, Rahway, NJ). The mobile phase was methanol–acetonitrile–water–25% NH_4OH (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 Pettit and Ziegler (19). The rate of substrate-dependent NADPH oxidation was monitored by the method of Tynes and Hodgson (20) using 1 mM benzydamine or cysteamine as substrate. Aminopyrine N-demethylation was determined by following the formation of formaldehyde by the method of Nash (21). Protein concentration was determined by the method of Lowry *et al.* (22) or for protein in samples containing the detergent, with the DC protein assay kit, both using BSA as a standard.

RESULTS

As shown in Fig. 2, the major metabolite of BZY catalyzed by rat liver microsomes at any pH (pH 7.0 to 10.0) is BZY *N*-oxide. BZY N-oxidation activity is at a maximum near pH 9.0, the optimum pH for liver FMO (23). For the N-oxidation assay, the pH was fixed at 8.5 because BZY was only slightly soluble above pH 9.0.

The N-oxidation of BZY in microsomes followed Michaelis–Menten kinetics in the presence of the positive effector *n*-octylamine. The purified enzyme showed sim-

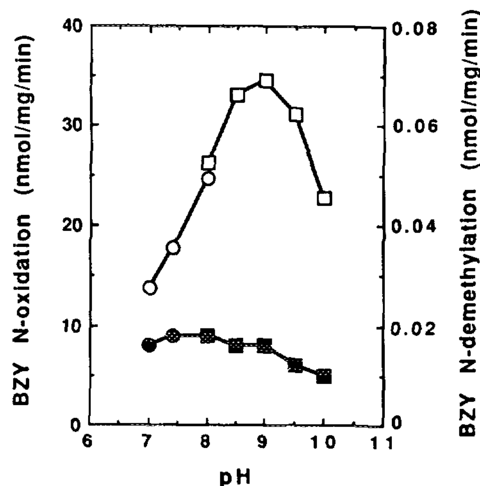

FIG. 2. Effect of pH on BZY N-oxidation (○, □) and BZY N-demethylation (●, ■) by rat liver microsomes. (○, ●) 0.1 M Na, K-phosphate buffer; (□, ■) 0.1 M Tricine–KOH buffer. Results are the means of four separate experiments.

TABLE 1

Kinetic Constants for Benzydamine N-Oxidation or N-Demethylation Catalyzed by Flavin-Containing Monooxygenase and Microsomes from Rat Liver

Enzyme preparation	N-Oxygenation[a] K_m (μM)	N-Oxygenation[a] V_{max} (nmol/mg/min)	N-Demethylation[b] K_m (μM)	N-Demethylation[b] V_{max} (nmol/mg/min)
Microsomes	15 ± 1.4	37 ± 2.9	660 ± 120	0.02 ± 0.01
Purified FMO	15 ± 0.4	1870 ± 360		

[a] Assays were carried out as described under Materials and Methods in the presence of 5 mM n-octylamine, except that BZY was added in variable concentration (from 2.5 μM to 1 mM).

[b] Assays were carried out in potassium phosphate buffer (pH 7.4) containing 1.5 mg of microsomes. Results are the means ± SD of three separate experiments.

TABLE 2

The Minimum Concentration of Microsomal Protein in the Assay Mixture for Detecting Flavin-Containing Monooxygenase Activity

Assay method	Concentration of microsomal protein (μg/ml of assay mixture)
HPLC	
BZY N-oxidation	≧1
Spectrophotometry	
N,N-Dimethylaniline N-oxidation	≧30
Substrate-dependent NADPH oxidation[a]	≧30

Note. Assays were carried out as described under Materials and Methods.

[a] Substrates used are 1 mM cysteamine or benzydamine. Results are the means of three separate experiments.

ilar behavior. In microsomes, the K_m and V_{max} values for BZY N-oxidation were about 50 times less and 2000 times greater than the respective values for BZY demethylation. The K_m for BZY N-oxidation by the purified enzyme was similar; activity with Nor-BZY was not detected with purified enzyme (Table 1).

BZY N-oxidation was proportional to microsomal protein up to 1 mg per milliliter of assay mixture. BZY N-oxidation was linear up to 15 min in the presence of 50 μg of microsomal protein per milliliter of the assay mixture (Fig. 3).

The minimum concentration of microsomal protein for detection of FMO activity with BZY N-oxidation using HPLC was compared with those for other spectrophotometric assay systems. The lowest limit of detection was taken to be twice the signal-to-noise ratio both for BZY N-oxidation using HPLC and for N,N-dimethylaniline N-oxidation, and it was taken to be the rate just over NADPH autooxidation for substrate-dependent NADPH oxidation. For the HPLC assay, BZY N-oxide was detectable at very low concentration of microsomal protein, 1 μg/ml or more; at least 30 μg/ml of microsomal protein was needed for the assay of N,N-dimethylaniline N-oxidation or substrate-dependent NADPH oxidation (Table 2).

Heating microsomes at 50°C for 3 min completely diminished BZY N-oxidation activity as much as omitting NADPH (Table 3); after heating, cytochrome P450 activity, assayed by aminopyrine N-demethylation, was retained. Thiourea, an alternate substrate for FMO, inhibited BZY N-oxidation activity. SKF-525A, a well-known cytochrome P450 inhibitor, slightly inhibited BZY N-oxidation activity, although aminopyrine N-demethylation activity was completely diminished.

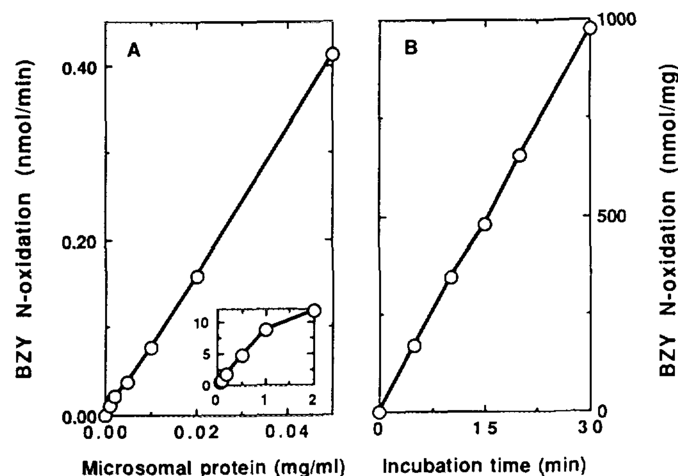

FIG. 3. Dose dependency (A) and time course (B) of BZY N-oxidation by rat liver microsomes. (A) Assays were carried out as described under Materials and Methods, except that microsomes were added at amounts of 1 μg to 2 mg per milliliter of assay mixture. (B) Assays were carried out with 50 μg/ml and the reactions terminated at the times indicated. Results are the means of four separate experiments.

TABLE 3

Effects of Various Inhibitors on Benzydamine N-Oxidation or Aminopyrine N-Demethylation by Rat Liver Microsomes

Inhibitor	BZY N-oxidation (nmol/mg/min)	Aminopyrine N-demethylation (nmol/mg/min)
Complete	31.2 ± 4.3	2.5 ± 0.7
Omit NADPH	n.d.[a]	n.d.[a]
Heat inactivation[b]	n.d.[a]	2.2 ± 0.2
0.5 mM thiourea[c]	13.9 ± 2.2	1.9 ± 0.6
2 mM SKF-525A[c]	25.8 ± 3.4	n.d.[a]

Note. Results are the means ± SD of four separate experiments.

[a] Not detectable.

[b] Microsomes were heated at 50°C for 3 min in the absence of NADPH.

[c] Inhibitors were preincubated with microsomes containing NADPH for 5 min.

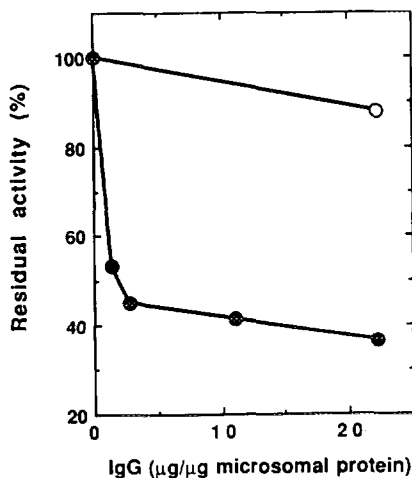

FIG. 4. Effect of anti-FMO IgG on BZY N-oxidation by rat liver microsomes. (○) Control IgG, (●) anti-FMO IgG. IgG and microsomes were preincubated at room temperature for 20 min prior to the addition of other components. Assays included 10 μg of microsomal protein and varying amounts of IgG in 0.1 ml of the reaction mixture. The rate of BZY N-oxidation in the absence of IgG was 32.3 ± 2.5 nmol/mg/min. Results are the means of four separate experiments.

BZY N-oxidation was inhibited by anti-rat liver FMO IgG but about 40% of the activity remained in 22 μg of antibody per microgram of microsomal protein (Fig. 4).

DISCUSSION

BZY *N*-oxide was formed by microsomes and purified rat liver FMO preparation from BZY. The kinetic data for BZY N-oxidation by microsomes and purified FMO from rat liver, shown in Table 1, suggest that BZY is an excellent substrate for FMO.

BZY N-oxidation was dependent upon NADPH but was not inhibited by SKF-525A. The treatment of hepatic microsomes at 50°C for 3 min completely diminished BZY N-oxidation but did not affect aminopyrine N-demethylation activity. Moreover, BZY N-oxidation was depleted by preincubation of microsomes with anti-FMO IgG although about 40% activity of control remained.

In comparison with other spectrophotometric assay systems (Table 2), the BZY N-oxidation assay using HPLC is exceedingly sensitive in detecting FMO activity.

After incubation, the reaction is terminated by the addition of methanol and the supernatant fluid obtained after centrifugation can be applied directly to HPLC. Since BZY and its metabolites are clearly separated within 15 min in HPLC, the assay for FMO using BZY is simple, rapid, and sensitive.

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