Biochemical Pharmacology, Vol. 34, No. 10, pp. 1846–1849, 1985.
Printed in Great Britain.

Separation, partial purification and characterization of cytochrome P-450 having different affinities for NADPH-cytochrome P-450 reductase from rat liver microsomes

(*Received* 5 *July* 1984; *accepted* 23 *November* 1984)

The hepatic microsomal electron transfer system consisting of NADPH-cytochrome P-450 reductase and cytochrome P-450 is responsible for the NADPH-dependent monooxygenation of a variety of endogenous substrates, drugs and toxic compounds. NADPH-cytochrome P-450 reductase functions in the transfer of reducing equivalents from NADPH to cytochrome P-450 during catalysis. It has been recently confirmed that there are multiple forms of cytochrome P-450 in rat liver microsomes [1–3], and that there is about 10 to 25 times more cytochrome P-450 than reductase on a molecular basis in rat liver microsomes [4]. Activities of drug oxidations are increased upon incorporation of the reductase into microsomes suggesting that the reductase rather than the cytochrome P-450 may be the rate limiting component in many drug oxidations catalyzed by the microsomal system [5–7]. The increase in the activity of drug oxidation due to reductase incorporation was dependent on both the substrate and the source of microsomes. This suggests that cytochrome P-450, having different affinities for the reductase, may be present in rat liver microsomes.

To clarify whether or not there are differences in the affinity for the reductase among the isozymes of cytochrome P-450, the separation and partial purification of cytochrome P-450 by reductase immobilized Sepharose 4B column chromatography was investigated.

Sprague–Dawley male and female rats, 10 weeks of age, were maintained on a commercial rat chow, CE-2 Nippon Clea Co., Japan. NADPH-Cytochrome P-450 reductase was purified from phenobartibal-treated male rat liver microsomes by the method of Yasukochi and Masters [8]. The reductase immobilized Sepharose 4B was synthesized by the method of Cuatrecasas [9]. Cytochrome P-450 was solubilized by sodium cholate and applied to a column of octylamino-Sepharose 4B according to the method of Imai and Sato [10]. The column was washed basically the same as described previously [11]. Cytochrome P-450 was eluted into two peaks (I and II) by washing the column with 10 mM potassium phosphate (pH 7.25) containing 20% glycerol, 0.5% sodium cholate, 0.08% Emulgen 913, 1 mM EDTA and 1 mM dithiothreitol, and subsequently with 100 mM potassium phosphate (pH 7.25) containing 20% glycerol, 0.4% sodium cholate, 0.2% Emulgen 913, 1 mM EDTA and 1 mM dithiothreitol. No detectable NADPH-cytochrome *c* reductase activity was found in either the pooled I or II fractions. The pooled I and II fractions were dialyzed against 10 mM potassium phosphate (pH 7.25) containing 20% glycerol and 0.2% of Emulgen 913, and were then applied separately to the reductase immobilized Sepharose 4B column which had been pre-equilibrated with the dialysis buffer. Each cytochrome P-450 fraction was eluted into two peaks (a and b) by washing the column with equilibrating buffer, and subsequently with 10 mM potassium phosphate (pH 7.25) containing 20% glycerol, 0.2% Emulgen 913 and 200 mM KCl. Detergent and/or KCl in each fraction was removed by passage through a hydroxylapatite column.

The incubation mixture (0.5 ml) consisted of the following; 65 mM potassium phosphate (pH 7.25), 0.05 nmole of cytochrome P-450, 0.25 nmole of NADPH-cytochrome P-450 reductase, 25 μg of dilauroyl 3-L-phosphatidylcholine, 25 μg of deoxycholic acid, 0.1 mM of EDTA, an NADPH-generating system (0.33 mM NADP, 8 mM glucose-6-phosphate, 0.1 unit of glucose-6-phosphate dehydrogenase and 6 mM $MgCl_2$) and substrate. The concentration of substrate used was 1 mM except that the biphenyl concentration was 2.5 mM.

Cytochrome P-450 was determined according to the method of Omura and Sato [12]. NADPH-cytochrome *c* reductase activity was measured by the method of Phillips and Langdon [13], and the reductase concentration was determined from the absorbance at 456 nm in the absolute spectrum using an extinction coefficient of 21.4 mM^{-1} cm^{-1} [14]. Protein was assayed by the method of Lowry *et al.* [15] using bovine serum albumin as a standard. Oxidative *N*-demethylation activities of ethylmorphine, codeine and benzphetamine were estimated by determining formaldehyde formation using the method of Nash [16]. 7-Ethoxycoumarin *O*-deethylation activity and aniline hydroxylation activity were measured by the methods of Aitio [17] and Imai *et al.* [18], respectively. The activity of biphenyl hydroxylation was estimated by determining the amount of 2-hydroxybiphenyl or 4-hydroxybiphenyl formed according to the method of Johnson *et al.* [19]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done essentially as previously described [20].

Figure 1 shows the reductase immobilized Sepharose 4B column profiles of I and II fractions obtained from male rats. The amount of cytochrome P-450 recovered from both fractions was about 80%. The amount of cytochrome P-450 eluted into the I-a fraction was greater than that eluted into I-b fraction. However, the cytochrome P-450 content recovered from the II-a fraction was similar to that recovered from the II-b fraction. After dialyzing both fractions I-b and II-b obtained from male rats against 10 mM potassium phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913, the cytochrome P-450s of both fractions were almost completely adsorbed on a second column of reductase immobilized Sepharose 4B. On the other hand, more than 80% of cytochrome P-450 of fraction I-a was not adsorbed on the second column. As seen in Table 1, the recovery of cytochrome P-450 from the I-a and II-a fractions of male rats was slightly less than that observed in female rats, but the recovery of cytochrome P-450 content from fraction I-b of male rats was two times higher than that obtained from fraction I-b of female rats.

Each cytochrome P-450 fraction contained several protein bands on SDS-polyacrylamide gel electrophoresis and the major protein band of each fraction was slightly different in the relative mobility (Fig. 2). The absorption maxima in the carbon monoxide difference spectra were at 450.0, 451.0, 449.0, 449.5, 450.0, 448.5, 448.0, 448.0 nm for cytochrome P-450 contained in fractions I-a, I-b, II-a, II-b (male), I-a, I-b, II-a and II-b (female), respectively. These results agree with those findings of Kahl *et al.* [21] who showed that the absorption maxima of the carbon monoxide difference spectra of cytochrome P-450 of female microsomes occurred at a shorter wavelength than that observed in male microsomes.

The concentration of reductase which was required to give a half maximal ethylmorphine *N*-demethylase activity by each cytochrome P-450 fraction from male rats was

determined. As seen in the double reciprocal plot of ethylmorphine *N*-demethylase activity and the reductase concentration (Fig. 3), the apparent K_m of cytochrome P-450 for reductase of fractions I-b (0.024 μM) and II-b (0.083 μM) was much lower than that of cytochrome P-450 eluted into I-a (0.208 μM) or II-a (0.225 μM) fraction. In addition, the apparent K_m obtained with fractions I-b or II-b were also found to be lower than that obtained with I-a or II-a fraction when the *O*-deethylase activity, using 7-ethoxycoumarin, was studied (not shown). These results suggest that a group of cytochrome P-450 having a relatively high affinity for reductase could be separated from that having a relatively low affinity for reductase using reductase immobilized Sepharose 4B column chromatography.

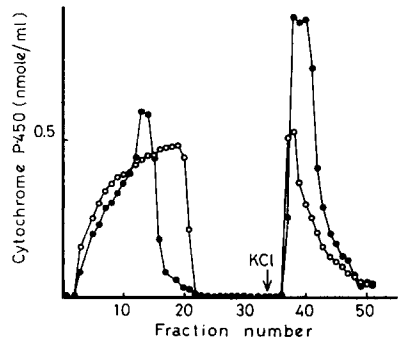

Fig. 1. NADPH-cytochrome P-450 reductase immobilized Sepharose 4B column profiles of fractions I (○) and II (●) obtained from male rats. Experimental details are described in the text.

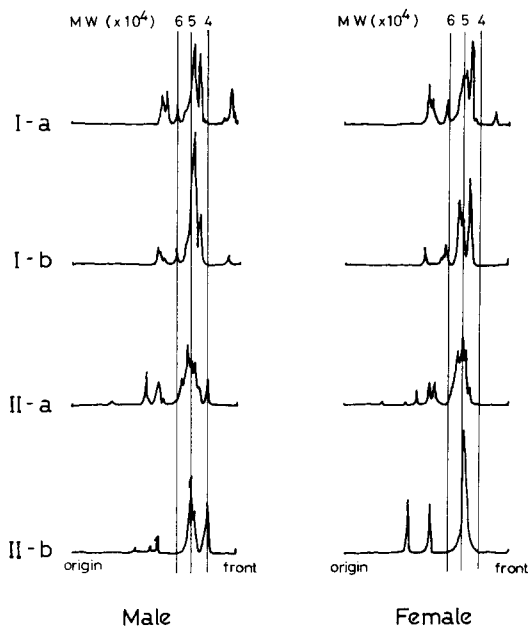

Fig. 2. Densitometric scans (550 nm) of an SDS-polyacrylamide gel after electrophoresis. The concentration of protein applied was about 10 μg each, and the gel was stained with Coomassie brilliant blue.

Table 1. Purification of cytochrome P-450 from rat liver microsomes

		Male				Female			
		Protein (mg)	Total content (nmoles)	Specific content (nmole/mg)	Recovery (%)	Protein (mg)	Total content (nmoles)	Specific content (nmole/mg)	Recovery (%)
Microsomes		1239.8	1285.7	1.04	100.0	530.0	399.7	0.75	100.0
Solubilized supernatant		791.3	885.7	1.12	68.8	386.5	337.6	0.87	84.5
Octylamino-Sepharose 4B	I	115.4	238.9	2.07	18.6	33.7	84.9	2.52	21.2
	II	72.1	125.2	1.74	9.7	30.2	56.1	1.86	14.0
			(I fraction: 97.5 nmoles)				(I fraction: 50.2 nmoles)		
Reductase-Sepharose 4B	I-a	37.7	49.3	1.31	50.7*	18.3	30.9	1.69	61.5*
	I-b	3.2	19.2	6.00	19.7*	0.4	4.3	10.75	8.5*
			(II fraction: 100.4 nmoles)				(II fraction: 44.7 nmoles)		
	II-a	35.2	31.6	0.90	31.5*	19.0	20.2	1.06	45.2*
	II-b	6.5	46.7	7.18	46.5*	1.8	19.9	11.06	44.6*

* Numbers represent per cent of cytochrome P-450 applied to reductase immobilized Sepharose 4B column.

Table 2. Catalytic properties of cytochrome P-450s in reconstituted system

	Male				Female			
	I-a	I-b	II-a	II-b	I-a	I-b	II-a	II-b
	(nmoles product/nmole cytochrome P-450/min)							
Ethylmorphine *N*-demethylation	8.06	15.54	1.52	6.58	8.74	6.36	7.21	2.36
Benzphetamine *N*-demethylation	4.46	9.58	0.65	1.93	3.00	0.46	0.87	1.52
Codein demethylation	2.55	12.84	2.38	3.01	5.36	2.27	3.69	2.53
Aniline 4-hydroxylation	0.61	2.96	2.62	2.60	1.03	1.05	2.02	1.35
Biphenyl 2-hydroxylation	N.D.*	N.D.*	18.26	N.D.*	43.20	24.83	59.54	78.34
4-hydroxylation	0.26	1.24	0.24	0.76	0.06	0.05	0.09	0.11

* N.D., not detectable

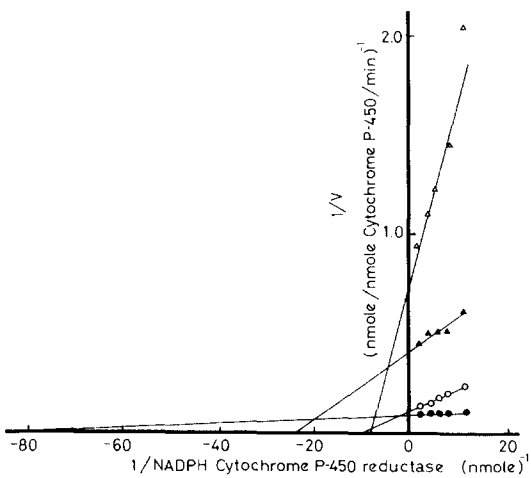

Fig. 3. Double reciprocal plot of ethylmorphine *N*-demethylation and NADPH-cytochrome P-450 reductase concentration. Ethylmorphine *N*-demethylase activity was determined in a reconstituted system containing either fractions I-a (○), I-b (●), II-a (△) or II-b (▲) from male rats as described in the test. Each value represents mean of duplicate determinations.

The cytochrome P-450 of each fraction exhibited different catalytic activities for a variety of oxidations (Table 2). In male rats, the I-b fraction had the highest catalytic activity for ethylmorphine, codeine and benzphetamine *N*-demethylation and for biphenyl 4-hydroxylation but not for aniline hydroxylation. The I-a fraction had higher benzphetamine and codeine *N*-demethylase activity than did the other fractions in female rats. The partially purified cytochrome P-450s of fractions I-b and II-b from male rats had a 2- to 3-fold higher ethylmorphine *N*-demethylase activity than did the corresponding cytochrome P-450 fraction from female rats; however, the cytochrome P-450 of fraction II-a, from male rats, showed a 5-fold lower ethylmorphine *N*-demethylase activity than did the corresponding fraction from female rats. Previous experiments demonstrated that the increase of ethylmorphine *N*-demethylase activity, after incorporation of purified reductase into microsomes, was much greater in female rats than that observed in male rats [21]. Therefore, it seems possible to assume that the cytochrome P-450 of fraction II-a may be, at least in part, responsible for the sex difference observed in the extent of increase of ethylmorphine *N*-demethylase activity due to reductase incorporation.

The results presented in this paper, suggest that there are differences in the affinity of the cytochrome P-450s for the NADPH-cytochrome P-450 reductase in rat liver microsomes. However, it has been shown that the K_D of equilibrium of interaction between the reductase and cytochrome P-450 is affected by substrate [14]. Therefore, the problem still remains unresolved whether the K_D for I-a or II-a is higher than that for I-b or II-b in the absence of ethylmorphine. Furthermore, since each fraction contains several forms of cytochrome P-450 and several proteins other than cytochrome P-450 as judged by SDS-polyacrylamide gel electrophoresis, the possibility that the impurity contained each fraction may affect the interaction between the reductase and cytochrome P-450 cannot be excluded.

Acknowledgement—The authors are indebted to Dr. W. W. Webb, University of Rochester, for his assistance in the preparation of this manuscript.

Faculty of Pharmaceutical Sciences SHIGERU OHMORI
Chiba University NOBUTOMO TSURUZOE
Yayoi-cho MITSUKAZU KITADA
Chiba 260, Japan HARUO KITAGAWA
YOSHIO KANAKUBO

REFERENCES

1. D. E. Ryan, P. E. Thomas and W. Levin, *J. biol. Chem.* **225**, 7941 (1980).
2. P. P. Lau and H. W. Strobel, *J. biol Chem.* **257**, 5257 (1982).
3. F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).
4. R. W. Estabrook, in *Concepts in Biochemical Pharmacology*, (Ed. by B. B. Brodie and J. R. Gillette). Springer, New York (1971).
5. G. T. Miwa and A. K. Cho, *Life Sci.* **18**, 983 (1976).
6. G. T. Miwa, S. B. West and A. Y. H. Lu, *J. biol. Chem.* **253**, 1921 (1978).
7. M. Kitada, H. Kitagawa and T. Kamataki, *Biochem. Pharmac.* **28**, 2678 (1979).
8. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
9. P. Cuatrecasas, *J. biol. Chem.* **245**, 3059 (1970).

10. Y. Imai and R. Sato, *Biochem. biophys. Res. Commun.* **60**, 8 (1974).
11. T. Kamataki, K. Maeda, Y. Yamazoe, N. Matsuda, K. Ishii and R. Kato, *Molec. Pharmac.* **24**, 146 (1983).
12. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
13. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
14. J. S. French, F. P. Guengerich and M. J. Coon, *J. biol. Chem.* **255**, 4112 (1980).
15. O. H. Lowry, N. J. Rosebraugh, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
16. T. Nash, *Biochem. J.* **55**, 416 (1953).
17. A. Aitio, *Analyt. Biochem.* **85**, 488 (1978).
18. Y. Imai, A. Ito and R. Sato, *J. Biochem.* **60**, 417 (1966).
19. E. F. Johnson, G. E. Schwab and U. M. -Eberhard, *Molec. Pharmac.* **15**, 708 (1979).
20. U. K. Laemmli, *Nature* **227**, 680 (1970).
21. R. Kahl, M. Buecker and K. J. Netter, in *Microsomes and Drug Oxidations* (Ed. by V. Ullrich). Pergamon Press, New York (1977).
22. M. Kitada, S. Ohmori, T. Igarashi, Y. Kanakubo and H. Kitagawa, *Biochem. biophys. Res. Commun.* **97**, 1527 (1980).

Biochemical Pharmacology, Vol. 34, No. 10, pp. 1849–1852, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

Identification and quantitation of 1,4-butanediol in mammalian tissues: an alternative biosynthetic pathway for gamma-hydroxybutyric acid

(*Received* 2 *May* 1984; *accepted* 25 *September* 1984)

Gamma-hydroxybutyric acid (GHB) occurs naturally in mammalian brain [1] and has been shown to produce marked central nervous system depression [2, 3] and a trance-like behavioral state, associated with paroxysmal electrical activity, quite similar to that observed in petit mal epilepsy [4–7].

The parent compound for GHB in brain is considered to be gamma-aminobutyric acid (GABA) [8–13]. However, several lines of evidence have been developed that suggest the presence of precursors for GHB other than GABA: (1) GHB in extraneural sites (liver, heart, kidney, muscle) notable for their absence or small concentrations of GABA [14–17], (2) liver and kidney concentrations of $NADP^+$-dependent oxidoreductase (D-glucuronate reductase; EC 1.1.1.10), the primary catabolic enzyme for GHB, ten to twenty times that in brain [15] and (3) the GHB ontogeny significantly different from that of GABA and the enzymes involved in its conversion to GHB [18]. These data suggest that in the periphery, and perhaps in the brain, there is an alternative source for GHB.

In this regard, it has been shown that the compound 1,4-butanediol (BD), administered either i.v. or i.p., is metabolized to GHB, producing increases in both the blood and brain levels of this compound [19, 20]. Thus, BD would appear on the surface to be a likely candidate for an alternative source of GHB in brain and liver.

A method for the extraction, derivatization and gas chromatographic/mass spectrometric (GC/MS) identification and quantification of BD by selected ion monitoring/isotope dilution techniques is presented. The presence of BD as an *in vivo* component of human and rat brain and of rat liver is demonstrated and the level of BD in these tissues is reported.

Deuterated 1,4-butanediol (DBD: 1,1,2,2,3,3,4,4-$^2H_8$-BD, 99.99 atoms% with respect to BD by GC/MS) was obtained from Merck Isotopes, Montreal, Canada. The BD, heptafluorobutyric anhydride (HFBA) organic solvents and other materials were obtained from commercial sources and were of the highest available purity.

Rat brains and livers were obtained from adult male Sprague–Dawley rats (CrL: CS(SD)BR, Charles River; 200–350 g, fed and watered *ad lib.* and maintained on a 12 hr light–dark cycle) that were killed by decapitation; the brain and liver were rapidly removed, dissected, and subjected to the assay procedure outlined below.

Post mortem human brain tissue (frontal cortex and cerebellum) was obtained from patients, ranging in age from 32 to 68 years (mean = 43.7), dying of non-neurologic disease who were autopsied within 12 hr of death (mean = 4.23 hr). The brain tissue samples were then frozen at −76° until the assay procedure was conducted.

The extraction of BD from liver and brain aqueous and lipid fractions was accomplished via modification of the method reported by Bergelson *et al.* [21], who reported the presence of trace amounts of BD in rat liver lipid fractions in 1966 using thin-layer chromatography.

Lipid fraction. To a 15.0-ml conical glass tube in an ice-water bath were added 2.0 ml of water, 0.5 ml of 70% perchloric acid and 0.5 μg of DBD. Tissue was weighed (0.2 to 0.5 g of liver or brain), added to the tube, and thoroughly homogenized. The sample was centrifuged and the supernatant fraction was transferred to a clean 15-ml conical glass-stoppered tube. The supernatant fraction was extracted with 2 × 1.0 ml of isooctane:hexane (15:85, v/v) by shaking for 15 min. The combined organic phases, containing lipids and other neutral compounds, were evaporated to dryness under nitrogen in a 50-ml RB flask; 0.5 μg of DBD and 20.0 ml of 0.4 M methanolic potassium hydroxide were added and the mixture was refluxed for 2 hr. On cooling, the pH was adjusted to 7.0 with 0.25 M sulfuric acid and lyophilized to dryness. The residue was extracted with 6 × 5.0 ml of chloroform:methanol (95:5, v/v), and the combined extracts were evaporated to dryness under nitrogen in 5.0-ml portions in a 15-ml conical glass-stoppered tube. HFBA (250 μl) was added, vortexed, and heated at 50° for 30 min. On cooling, 1.0 ml of methylene chloride was added and then extracted with 3 × 1.0 ml of deionized water. The methylene chloride was transferred to an ampoule, 0.25 g anhydrous sodium sulfate was added and the ampoule was sealed.

Aqueous fraction. The pH of the aqueous phase was adjusted to 12.0 with 45% potassium hydroxide, the mixture was centrifuged, and the supernatant fraction was transferred to a 15-ml conical glass-stoppered tube. The supernatant fraction was extracted with 2 × 1.0 ml of chloroform, and the aqueous phase was adjusted to a pH of 7.0 with perchloric acid. The sample was centrifuged and the supernatant fraction was lyophilized to dryness. The remainder of the procedure was conducted as described above for extraction and derivatization.

GC/MS analyses were conducted using a Hewlett Packard 5985A GC/MS equipped with a RTE-6-VM data system. Gas chromatography was conducted in the splitless mode on a 25 m × 0.25 mm i.d., 0.12 mm coating of BP5 (J & W Scientific Inc., Rancho Cardova, CA) fused silica glass capillary column using helium as the carrier gas. A temperature program was used to effect separation of DBD and BD: 50° for 1.0 min, increasing 10°/min to 300°. The purge function was activated at 0.5 min into the run. The injection port and source temperatures were maintained at 200° while the transfer zone temperature was held at 300°. Analyses were conducted in the positive ion/electron