STIMULATION OF MICROSOMAL DRUG OXIDATION ACTIVITIES BY INCORPORATION INTO MICROSOMES OF PURIFIED NADPH-CYTOCHROME *c* (P-450) REDUCTASE

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Abstract—The effects of addition of purified NADPH-cytochrome *c* (P-450) reductase on microsomal activities of aniline hydroxylation, *p*-phenetidine O-deethylation and ethylmorphine and aminopyrine N-demethylations were investigated utilizing microsomes from untreated, phenobarbital-treated and 3-methylcholanthrene-treated rats. The purified reductase was incorporated into microsomes. The drug oxidation activities were increased by the fortification of microsomes with the reductase while the extent of increase in the activities varied with the substrate and microsomes employed. The most pronounced enhancement was seen in *p*-phenetidine O-deethylation, followed by aniline hydroxylation and aminopyrine and ethylmorphine N-demethylations. The enhancement was more remarkable in microsomes from rats treated with 3-methylcholanthrene or phenobarbital. α -Naphthoflavone inhibited *p*-phenetidine O-deethylation activity markedly when the reductase was incorporated into microsomes, indicating that a larger amount of a species of cytochrome P-450 sensitive to the inhibitor was capable of participating in the oxidation of this substrate in the presence of the added reductase. One of the two K_m values seen with higher concentrations of aniline or aminopyrine was altered by the fortification of microsomes with the purified NADPH-cytochrome *c* (P-450) reductase. From these results, we propose that NADPH-cytochrome *c* (P-450) reductase transfers electrons to the selected one or two of multiple species of cytochrome P-450 more preferentially depending upon the substrate and the concentration of the substrate in microsomal membranes.

The current view of the liver microsomal monooxygenase enzyme system is that in microsomal membranes, cytochrome P-450 catalyzes the biotransformation of a variety of drugs, toxic compounds including carcinogens, and endogeneous substrates such as steroids and fatty acids (1-5). In this enzyme system, the reducing equivalents from NADPH are transferred through NADPH-cytochrome *c* (P-450) reductase to cytochrome P-450 which in turn catalyzes the oxidation of various compounds. Several investigators have almost completely purified cytochrome P-450 and NADPH-cytochrome *c* (P-450) reductase (6-11). The determination of molecular weight and specific activity of these purified enzymes led to the conclusion that cytochrome P-450 is present in 10 to 25 times larger amounts than NADPH-cytochrome *c* (P-450) reductase on the molar basis in microsomal membranes, and that at least six species of cytochrome P-450 exist in microsomes from phenobarbital- and 3-methylcholanthrene-treated rats (12). West and Lu have demonstrated the compe-

tion between cytochrome P-450 and P-448 for NADPH-cytochrome *c* (P-450) reductase in reconstituted 3,4-benzpyrene hydroxylase system (13). In addition, there are few reports suggesting that NADPH-cytochrome *c* (P-450) reductase may be the rate limiting enzyme in drug oxidations in whole microsomes. From results obtained using reconstituted hydroxylase system, Kamataki et al. (10) proposed that in whole microsomes, NADPH-cytochrome *c* (P-450) reductase rather than cytochrome P-450 is the rate limiting enzyme for benzphetamine N-demethylation. Quite recently Miwa and Cho (14) and Miwa et al. (15) demonstrated that a detergent solubilized NADPH-cytochrome *c* (P-450) reductase was incorporated into microsomes and enhanced drug oxidation activities. If cytochrome P-450 and NADPH-cytochrome *c* (P-450) reductase are not rigidly organized in the membranes as mentioned by Yang and Strickhart (16) and Yang (17), then it seems reasonable to propose that multiple species of cytochrome P-450 interfere each other in functional binding to the limited amount of NADPH-cytochrome *c* (P-450) reductase in microsomal membranes.

We report herein data which support the idea that there is an order in the cytochrome P-450 species for receiving electrons from limited NADPH-cytochrome *c* (P-450) reductase in microsomal membranes.

MATERIALS AND METHODS

Male rats of Crl: CD (SD) strain weighing 120 to 150 g used throughout this study were maintained on a commercial rat chow, CE-2 Nippon Clea Co., Japan and then deprived for food for about 20 hr prior to sacrifice. Water was provided *ad libitum*. The animals were given either an i.p. administration of 3-methylcholanthrene (25 mg/kg) dissolved in olive oil once a day for three successive days or sodium phenobarbital (80 mg/kg) dissolved in saline once a day for three days. When necessary, i.p. administration of 3-methylcholanthrene and s.c. administration of phenobarbital were given simultaneously once a day for three days.

DEAE-Sephadex (A-50) and 2',5' ADP-Sepharose 4B were purchased from Pharmacia Fine Chemicals Co., and hydroxylapatite from Bio-Rad. NADP, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and cytochrome *c* (horse heart) were purchased from Boehringer Mannheim. Emulgen 913, a nonionic detergent, was kindly provided by the Kao-Atlas Co. Commercial aniline was redistillate was stored at about -10° under an atmosphere of nitrogen. Other chemicals were of the highest purity commercially available. Microsomes were prepared as described previously (18). Protein was determined by the method of Lowry et al. (19) using bovine serum albumin as a standard. Cytochrome P-450 was measured by the method of Omura and Sato (20) using an Aminco DW-2 recording spectrophotometer, and the content was calculated from the absorbance difference between 450 nm and 490 nm using the extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ (21).

Purification of NADPH-cytochrome c (P-450) reductase from phenobarbital-treated rat liver microsomes: NADPH-Cytochrome *c* (P-450) reductase (EC 1.6.99.2) was purified from phenobarbital-treated rat liver microsomes by a minor modification of the method of

Yasukochi and Masters (11). Eluate from 2',5' ADP-Sepharose 4B column was adsorbed on a hydroxylapatite column equilibrated with 0.01 M K-phosphate (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. The column was washed with 0.01 M K-phosphate (pH 7.25) containing 20% glycerol until the absorbance at 276 nm was less than 0.03 and then NADPH-cytochrome *c* (P-450) reductase was eluted with 0.15 M K-phosphate (pH 7.25) containing 20% glycerol. Cytochrome *c* reduction was measured by the method of Phillips and Langdon (22). The final NADPH-cytochrome *c* (P-450) reductase preparation had a specific activity higher than 50 units per mg of protein. One unit of the reductase activity is defined as the amount of enzyme catalyzing the reduction of cytochrome *c* at an initial rate of 1 μ mole per min at 25°.

Assay of microsomal drug oxidation activities: A typical reaction mixture for drug oxidation assay consisted Na,K-phosphate (0.1 M, pH 7.4), NADP (0.33 mM), glucose 6-phosphate (8 mM), $MgCl_2$ (6 mM), glucose 6-phosphate dehydrogenase (0.045 unit), EDTA (0.1 mM), microsomes (approx. 1.0 mg of protein) and a substrate (5 mM) in a final volume of 1.0 ml. N-Demethylation of ethylmorphine or aminopyrine and hydroxylation of aniline or O-deethylation of *p*-phenetidine were carried out at 37° for 10 min and 20 min, respectively. Drug oxidation reactions were linear during the incubation period under these incubation conditions. N-Demethylation activities of ethylmorphine and aminopyrine were measured by determining formaldehyde formed, by the method of Nash (23). Aniline hydroxylation and *p*-phenetidine O-deethylation were assayed by determining *p*-aminophenol formed according to the method of Imai et al. (24).

*Fortification of rat liver microsomes with purified NADPH-cytochrome *c* (P-450) reductase:* Fortification of rat liver microsomes with purified NADPH-cytochrome *c* (P-450) reductase was carried out essentially by the method described by Miwa and Cho (14). Microsomes, phosphate (0.1 M, pH 7.4) and EDTA (0.1 mM) were incubated at a concentration of about 10 mg of protein per ml for 20 min at 30° with desired amounts of purified NADPH-cytochrome *c* (P-450) reductase. Control samples were incubated with the buffer in place of the reductase. After the incubation period, the mixture were diluted about 5- to 10-fold with ice cold 0.1 M phosphate (pH 7.4) and centrifuged at $105,000 \times g$ for 60 min. The microsomal pellets were resuspended in 0.1 M phosphate (pH 7.4) and centrifuged at $105,000 \times g$ for 30 min. The washed microsomal pellets were resuspended in 0.1 M phosphate (pH 7.4) and used for the assay. Cytochrome P-450 reduction by NADPH was measured essentially by the method of Diehl et al. (25).

RESULTS

The amounts of NADPH-cytochrome *c* (P-450) reductase incorporated into microsomes were determined as cytochrome *c* reduction after addition of varying amounts of purified NADPH-cytochrome *c* (P-450) reductase. As shown in Fig. 1, the amount of reductase incorporated into untreated rat liver microsomes was increased with the amount of purified reductase added; roughly 20 to 25% of the added reductase was incorporated. The reduction rate of cytochrome P-450 by NADPH was also measured after the addition of purified

FIG. 1. Incorporation of purified NADPH-cytochrome *c* (P-450) reductase into microsomes. Liver microsomes from untreated rats were separated and placed in four flasks. Each flask was incubated at 30°C for 20 min after addition of various amounts of purified NADPH-cytochrome *c* (P-450) reductase shown in the figure in the presence of 0.1 M Na,K-phosphate (pH 7.4). Microsomes were incubated with corresponding amounts of buffer as a control. Control activities of NADPH-cytochrome *c* (P-450) reductase of exp. 1 and exp. 2 were 0.175 and 0.214 unit per mg of protein, respectively.

FIG. 2. Effects of NADPH-cytochrome *c* (P-450) reductase incorporation on the rate of cytochrome P-450 reduction by NADPH. Liver microsomes were incubated in the presence of various amounts of NADPH-cytochrome *c* (P-450) reductase. The specific activity of NADPH-cytochrome *c* (P-450) reductase in control (---) and "fortified" microsomes were 0.135, 0.247 (·····) and 0.563 (—) unit per mg of protein, respectively.

NADPH-cytochrome *c* (P-450) reductase (Fig. 2). Cytochrome P-450 reduction rates in control and reductase fortified microsomes were 5.16, 7.75 and 10.80 nmoles cytochrome P-450 reduced per min per mg of protein, respectively. The rate of cytochrome P-450 reduction was increased by about 1.5 to 2.1-fold after the incorporation with the purified reductase indicating that the incorporated NADPH-cytochrome *c* (P-450) reductase was functionally active in the electron transport from NADPH to cytochrome P-450.

The effects of varying amounts of added NADPH-cytochrome *c* (P-450) reductase on *p*-phenetidine O-deethylation were then examined. Since the reductase was assumed to limit the rate of drug oxidations in whole microsomes, double reciprocal plots of the activities were drawn towards the amounts of the reductase. As can be seen in Fig. 3, a non-linear

FIG. 3. Double reciprocal plots of *p*-phenetidine 0-deethylation activity against NADPH-cytochrome *c* (P-450) reductase activity. Liver microsomes from 3-methylcholanthrene-treated rats were separated and placed in five flasks. Each flask was incubated at 30° for 20 min after addition of desired amounts of reductase. *p*-Phenetidine 0-deethylation activities were measured using microsomes fortified with various amounts of reductase.

TABLE 1. Ideal maximum velocities of aniline hydroxylation, *p*-phenetidine 0-deethylation and ethylmorphine and aminopyrine N-demethylations in microsomes from untreated, phenobarbital-treated and 3-methylcholanthrene-treated rat livers

Microsomes	Aniline*	<i>p</i> -Phenetidine*	Ethylmorphine**	Aminopyrine**
Intact				
Actual	0.897	0.587	10.66	7.30
Ideal	1.06	0.83	10.96	8.07
Ideal/actual	1.18	1.41	1.03	1.11
Phenobarbital-treated				
Actual	0.546	0.413	8.10	5.10
Ideal	1.17	1.14	13.09	10.32
Ideal/actual	2.13	2.76	1.62	2.02
3-Methylcholanthrene-treated				
Actual	0.709	0.610	2.64	2.86
Ideal	1.67	1.93	3.86	6.90
Ideal/actual	2.35	3.16	1.46	2.41

The actual activities of drug oxidations represent the activities in control microsomes. The ideal ones were calculated from double reciprocal plots as function of the NADPH-cytochrome *c* (P-450) reductase and represent as the activities in the presence of theoretically large excess amounts of the reductase. Microsomes from untreated, phenobarbital-treated and 3-methylcholanthrene-treated rats were separated and placed in three to four flasks, respectively. The flasks were incubated at 30° for 20 min after addition of desired amounts of NADPH-cytochrome *c* (P-450) reductase in the presence of 0.1 M Na,K-phosphate (pH 7.4). The specific activities of NADPH-cytochrome *c* (P-450) reductase in the microsomes from untreated, phenobarbital-treated and 3-methylcholanthrene-treated rats were 0.132, 0.234 and 0.100 unit per mg of protein, respectively. Other experimental conditions were as described in MATERIALS AND METHODS.

*nmoles *p*-aminophenol formed per nmole cytochrome P-450 per min. **nmoles formaldehyde formed per nmole cytochrome P-450 per min.

curve was obtained in double reciprocal plots of the activity of *p*-phenetidine O-deethylation towards the amounts of NADPH-cytochrome *c* (P-450) reductase using 3-methylcholanthrene-treated rat liver microsomes. From the double reciprocal plots, V_{max} values for drug oxidations were calculated to determine the maximal activities of cytochrome P-450 in the presence of theoretically large excess amounts of NADPH-cytochrome *c* (P-450) reductase and a substrate using microsomes from untreated, phenobarbital- and 3-methylcholanthrene-treated rats (Table 1). The ideal activities were tentatively calculated from the plots having a higher affinity for reductase because of the apparent reliability when two K_m values for reductase were obtained from the double reciprocal plots of the hydroxylation activity to the reductase activity. As shown in Table 1, the ratios of the ideal activities to the actual ones varied with the substrates and microsomes employed. Treatment of rats with phenobarbital or 3-methylcholanthrene increased the ratios in all substrates. Moreover, the highest ratios were observed in *p*-phenetidine O-deethylation activities in untreated, phenobarbital-treated and 3-methylcholanthrene-treated microsomes, and the lowest ones in ethylmorphine N-demethylation activities.

The effects of α -naphthoflavone, a specific inhibitor of one of the cytochrome P-450

TABLE 2. Effects of α -naphthoflavone and tetrahydrofuran on *p*-phenetidine O-deethylation activity in microsomes fortified with purified NADPH-cytochrome *c* (P-450) reductase

Addition/Microsomes	<i>p</i> -Phenetidine O-deethylation*		
	Control	Reductase fortified	Relative activity
Exp. 1			
None	0.732	2.755	3.8
α -Naphthoflavone (0.2 μ M)	0.702	2.604	3.7
α -Naphthoflavone (1.0 μ M)	0.448	1.508	3.4
α -Naphthoflavone (10.0 μ M)	0.230	0.409	1.8
Exp. 2			
None	0.811	3.141	3.9
Tetrahydrofuran (5 mM)	0.767	3.071	4.0
Tetrahydrofuran (10 mM)	0.785	2.879	3.7

Liver microsomes (80 mg of protein) from rats treated with phenobarbital and 3-methylcholanthrene were separated and placed in four flasks. Exp. 1: Microsomes (20 mg of protein) was incubated at 30° for 20 min after addition of 216.4 units of NADPH-cytochrome *c* (P-450) reductase and another group of microsomes was incubated with corresponding amounts of buffer as a control. The specific activities of NADPH-cytochrome *c* (P-450) reductase and the specific content of cytochrome P-450 in the control and the "fortified" microsomes were 0.174 and 0.714 unit per ng of protein and 2.11 and 1.92 nmoles per mg of protein, respectively. Exp. 2: Microsomes (20 mg of protein) were incubated after addition of 144.2 units of NADPH-cytochrome *c* (P-450) reductase and another group of microsomes was incubated with corresponding amounts of buffer as a control. The specific activity of reductase and specific content of cytochrome P-450 in the control and the "fortified" microsomes were 0.187 and 0.518 unit per mg of protein and 2.12 and 1.99 nmoles per mg of protein, respectively.

*nmoles *p*-aminophenol formed per nmole cytochrome P-450 per min.

species, cytochrome P-448, on *p*-phenetidine O-deethylation activity were studied using microsomes from rats treated with phenobarbital and 3-methylcholanthrene in the presence or absence of the incorporated reductase since *p*-phenetidine O-deethylation was largely enhanced by fortification of NADPH-cytochrome *c* (P-450) reductase into microsomes from phenobarbital- or 3-methylcholanthrene-treated rats (Table 2). Fortification of the microsomes resulted in about 3.8-fold increase in *p*-phenetidine O-deethylation activity. The activities seen in both control microsomes and the microsomes fortified with the reductase

FIG. 4. Double reciprocal plots of the activity of aniline hydroxylation against the concentration of aniline. Microsomes (60 mg of protein) from untreated rats were divided into two flasks. The flasks were incubated after addition of 0 or 129.6 units of purified NADPH-cytochrome *c* (P-450) reductase and 0.1 M Na,K-phosphate (pH 7.4). Specific activities of NADPH-cytochrome *c* (P-450) reductase in the control and the "fortified" microsomes were 0.111 and 0.304 unit per mg of protein, respectively. Open and closed circles represent the activities of aniline hydroxylation in the control and the "fortified" microsomes, respectively.

FIG. 5. Double reciprocal plots of the activity of aminopyrine N-demethylation against the concentration of aminopyrine. Microsomes (60 mg of protein) derived from untreated rats were divided into two groups and each group was incubated after addition of 0 or 99.9 units of purified NADPH-cytochrome *c* (P-450) reductase and 0.1 M Na,K-phosphate (pH 7.4). Specific activities of NADPH-cytochrome *c* (P-450) reductase in the control (○) and the "fortified" microsomes (●) were 0.082 and 0.308 unit per mg of protein, respectively.

were inhibited by α -naphthoflavone, however, the enhancement of the activity caused by fortification of reductase into microsomes was diminished with the concentration of α -naphthoflavone added to the reaction mixture. About 3.8-fold increase in *p*-phenetidine O-deethylation activity was observed in the absence of α -naphthoflavone, on the contrary, about only 1.8-fold increase in the activity was observed in the presence of 10 μ M of α -naphthoflavone. Tetrahydrofuran is another example of a specific inhibitor of one of cytochrome P-450 species which is induced by ethanol administration. The effect of addition of the inhibitor on *p*-phenetidine O-deethylation activity was also examined (Table 2). In contrast to the case with α -naphthoflavone, the tetrahydrofuran did not affect *p*-phenetidine O-deethylation activities mediated by both control and the fortified microsomes. These results suggest that *p*-phenetidine O-deethylation catalyzing by cytochrome P-450 species sensitive to α -naphthoflavone may be increased by the fortification of the reductase into microsomes.

Wada et al. (26) first demonstrated the possibility that aniline hydroxylation was capable of being catalyzed by more than one drug metabolizing enzyme since a non-linear curve was found in the double reciprocal plots of aniline hydroxylation activity towards the substrate concentrations. Aust et al. (27, 28) observed essentially the same results using aminopyrine as a substrate. Thus, the effects of fortification of liver microsomes with purified NADPH-cytochrome *c* (P-450) reductase on the reaction kinetics were examined using aniline and aminopyrine. The results are shown in Figs. 4 and 5. As can be seen, two K_m values were observed both in aniline hydroxylation and aminopyrine N-demethylation, depending upon the concentrations of the substrates. The two K_m values were also shown when the microsomes fortified with the reductase were used. The K_m values obtained using higher concentrations of aminopyrine and aniline were altered to higher values whereas those obtained with lower concentrations of substrates remained unchanged.

DISCUSSION

The possibility that not all of the reductase recovered in the microsomes was functionally involved in drug oxidations remains to be elucidated, however, at least a portion of the "fortified" reductase is assumed to be functionally acting in the microsomal membranes since the rate of cytochrome P-450 reduction by NADPH as well as drug oxidation activities was enhanced by the fortification.

It should be noted that treatment of rats with either phenobarbital or 3-methylcholanthrene did not elevate drug oxidation rates when the activities were calculated on the basis of nmole of cytochrome P-450. The decrease in the activities appears to be caused by the increased ratios of nmole of cytochrome P-450 to units of NADPH-cytochrome *c* (P-450) reductase present in these microsomes since the ratios in untreated, phenobarbital-treated and 3-methylcholanthrene-treated rat liver microsomes were 5.1, 11.2 and 24.2, respectively. Consequently, the ideal activities in the presence of theoretically large excess amounts of NADPH-cytochrome *c* (P-450) reductase were about the same or even slightly higher than controls in all cases except in the cases of aminopyrine and ethylmorphine N-demethylation

in 3-methylcholanthrene-treated microsomes. These results suggest that when one wishes to measure the true activity of cytochrome P-450 catalyzing *p*-phenetidine O-deethylation in microsomes of animals treated with inducers, one should fortify the electron transport capacity from NADPH to cytochrome P-450.

As shown in Table 1, *p*-phenetidine O-deethylation activity was enhanced to a greater extent than other oxidation activities, on the other hand, ethylmorphine N-demethylation activity was affected to a extent lesser than other activities. These results suggest that cytochrome P-450 species catalyzing ethylmorphine N-demethylation receives election by limited NADPH-cytochrome *c* (P-450) reductase for accepting electrons after binding with the substrate ethylmorphine, while in the case of *p*-phenetidine, cytochrome P-450 species cannot receive electrons even after binding with the substrate, probably because of the interference by other species of cytochrome P-450. Moreover, the stimulation of *p*-phenetidine O-deethylation activity due to reductase fortification was markedly decreased by the addition of α -naphthoflavone to the reaction mixture indicating that the cytochrome P-450 species sensitive to α -naphthoflavone became more effective in *p*-phenetidine O-deethylation after NADPH-cytochrome *c* (P-450) reductase fortification. Wada et al. (26) and Aust et al. (27, 28) demonstrated that there is more than one drug metabolizing enzyme involved in aniline hydroxylation and aminopyrine N-demethylation since non-linear curves in the double reciprocal plots of the activity to the substrate concentrations were seen and two K_m values were obtained. Thus, it was of interest to know which one of these two K_m values were affected by the fortification of microsomes with the reductase. As shown in Figs. 4 and 5, a K_m value obtained by the use of higher ranges of substrate concentrations were increased. These results indicate that activities obtained in the presence of lower concentrations of aniline and aminopyrine are due to a single species of cytochrome P-450, while in the presence of higher concentrations of the substrates, multiple species of cytochrome P-450 are involved, and that the former cytochrome P-450 is a preferential species for receiving electrons from NADPH-cytochrome *c* (P-450) reductase.

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