Substrate-Dependent Competition of Different P450 Isozymes for Limiting NADPH−Cytochrome P450 Reductase†

George F. Cawley,‡,§ Christopher J. Batie,‖,§ and Wayne L. Backes*,‡,§

Department of Pharmacology and Experimental Therapeutics, Department of Biochemistry and Molecular Biology, and The Stanley S. Scott Cancer Center, Louisiana State University Medical Center, 1901 Perdido Street, New Orleans, Louisiana 70112

Received August 30, 1994; Revised Manuscript Received October 5, 1994⊗

ABSTRACT: The goal of these studies was to demonstrate that one P450 isozyme can influence the function of another isozyme when combined in a reconstituted system. Benzphetamine and 7-pentoxyresorufin were both shown to be preferred substrates for P450 2B4 (LM2) as compared to P450 1A2 (LM4). However, these substrates exhibited different characteristics when examined in reconstituted systems containing reductase and both P450 isozymes. Whereas benzphetamine demethylation showed a small increase in catalytic activity when both P450 1A2 and 2B4 were present over the activities obtained in simple reconstituted systems, 7-pentoxyresorufin *O*-dealkylation (PROD) was dramatically inhibited when both isozymes were present. These results indicate that the functional interactions between P450s in complex reconstituted systems are dependent on the substrate present. Inhibition of PROD was also dependent on reductase levels, with the inhibitory effect being more pronounced at subsaturating reductase. Finally, these protein−protein interactions were shown to be dependent on the reductase concentration in the reconstituted system rather the P450 concentration, supporting the view that P450 1A2 is inhibiting the reaction by competing with P450 2B4 for reductase molecules.

Microsomal cytochrome P450 is involved in the oxidative metabolism of a wide variety of drugs and other foreign compounds, with the broad substrate selectivity of this enzyme system being due to the existence of multiple P450 isozymes. Although the components responsible for P450-dependent monooxygenase reactions are well known (Lu *et al.*, 1968), basic features of the interactions of these isozymes with the other components of this electron-transfer chain are not well understood. For efficient catalysis to occur, cytochrome P450 must receive electrons from NADPH−cytochrome P450 reductase. These proteins have been reported to interact by forming a 1:1 functional complex (Miwa *et al.*, 1979; Miwa & Lu, 1984) for transfer of at least the first electron (the second electron can also come from cytochrome b_5). However, cytochrome P450 has been reported to exist in a large excess over the reductase, ranging from 10:1 to 25:1 depending on treatment with inducers (Peterson *et al.*, 1976; Estabrook *et al.*, 1971). Since NADPH−cytochrome P450 reductase is the limiting component in microsomes, different P450 isozymes must compete for the available reductase molecules. In previous reports, substrate addition has been shown to increase not only the rate of first electron transfer to several cytochrome P450 isozymes but also the rate at which P450 forms a functional complex with the reductase (Backes & Eyer, 1989; Eyer & Backes, 1992). In light of these findings, substrate binding has been suggested as a potential mechanism regulating which P450 isozymes most effectively bind to the reductase. According to this model, the presence of substrate would increase both the rate (Backes & Eyer, 1989; Eyer & Backes, 1992) and the affinity (French *et al.*, 1980) of the interaction of a particular P450 isozyme with reductase. These results suggest that the relative ability of a particular P450 to compete for reductase can be influenced by the presence of substrate. The goal of the present study is to determine whether one P450 isozyme can affect the metabolism of another isozyme when coexisting in a reconstituted system, and to determine whether these P450 isozymes interact directly with each other or exert their effects by competing for NADPH−cytochrome P450 reductase.

MATERIALS AND METHODS

Cytochrome P450 1A2 (LM4)[1] and 2B4 (LM2) were purified from rabbit liver after treatment with β-naphthoflavone and phenobarbital, respectively (Coon *et al.*, 1979; Backes & Eyer, 1989). NADPH−cytochrome P450 reductase was purified from phenobarbital-treated rabbits (Yasukochi & Masters, 1976; Backes & Reker-Backes, 1988).

Reconstituted systems of reductase and P450 were prepared by mixing the proteins in a sonicated suspension of dilauroylphosphatidylcholine (DLPC) for 2 h prior to assay (Causey *et al.*, 1990). DLPC was first suspended by sonication to a concentration of 8 mM in 50 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.1 M NaCl, and 5 mM EDTA. Reductase, P450, and DLPC were then mixed at the appropriate ratios and allowed to incubate at room temperature for 2 h. In general, the proteins were mixed at a DLPC to P450 ratio ranging from 50:1 to 160:1.

† This work was supported by United States Public Health Service Grant ES04344 from the National Institute of Environmental Health Sciences (W.L.B.), and National Institutes of Health Grant GM48252 (C.J.B.).

‡ Department of Pharmacology and Experimental Therapeutics.

§ The Stanley S. Scott Cancer Center.

‖ Department of Biochemistry and Molecular Biology.

⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1995.

[1] Abbreviations: DLPC, dilauroylphosphatidylcholine; PROD, 7-pentoxyresorufin *O*-dealkylation; P450 1A2, P450 isolated from β-naphthoflavone-treated rabbits (P450 LM4); P450 2B4, P450 isolated from phenobarbital-treated rabbits (P450 LM2); reductase, NADPH−cytochrome P450 reductase.

0006-2960/95/0434-1244$09.00/0

These ratios of phospholipid to reductase have been determined previously to result in optimal monooxygenase activities (Causey *et al.*, 1990). In the preliminary incubation, DLPC, reductase, and P450 were combined and allowed to incubate at high concentrations. For example, the P450 concentrations in each of these preliminary incubations were in excess of 7 μM. After the preincubation step, the reconstituted system was diluted with buffer and other assay components to approximately 0.1 μM P450 as indicated in Results and Discussion.

Benzphetamine demethylation was measured by monitoring the production of the fluorescent derivative of formaldehyde after reaction with Nash reagent (Nash, 1953; Udenfriend, 1969; Causey *et al.*, 1990). Dealkylation of 7-pentoxyresorufin was measured by a direct assay following the production of the fluorescent product resorufin (Perrin *et al.*, 1990). The final concentrations for benzphetamine demethylation were the reconstituted system (containing P450, reductase and DLPC at the indicated concentrations), 1 mM benzphetamine, 5 mM glucose 6-phosphate, 2 units/mL glucose-6-phosphate dehydrogenase, and 10 mM magnesium chloride in 100 mM potassium phosphate, pH 7.25. The final component concentrations for PROD were the reconstituted system (containing P450, reductase, and DLPC at the indicated concentrations), 1.3 μM 7-pentoxyresorufin, 0.1 mM EDTA, and 15 mM magnesium chloride in 50 mM Hepes buffer, pH 7.5. The reaction was initiated by the addition of NADPH to a final concentration of 250 μM (500 μM for benzphetamine demethylation), and the samples were incubated at 37 °C.

RESULTS AND DISCUSSION

The goal of these studies was to determine whether the presence of one P450 isozyme will influence the metabolism of another isozyme. Cytochrome P450 2B4- and 1A2-dependent metabolism was examined in complex reconstituted systems containing reductase and both P450 isozymes and compared to simple reconstituted systems that contain only a single isozyme. The use of reconstituted systems for such studies permits the examination of potential interactions of these isozymes with P450 reductase under defined conditions where the concentrations of individual components could be controlled independently. Benzphetamine and 7-pentoxyresorufin were substrates chosen for this study because both substrates are selective for P450 2B4 (Table 1).

When examined in simple reconstituted systems, benzphetamine demethylation was catalyzed about 35 times more effectively by P450 2B4 than by P450 1A2. This selectivity was observed at both saturating (3:1 and 1.5:1) and subsaturating (0.5:1) reductase:P450 ratios. In order to determine whether the function of a P450 isozyme is modulated by the presence of a second isozyme, benzphetamine metabolism was examined in a mixed reconstituted system containing reductase and P450 1A2 and 2B4. If the interactions between reductase and the P450s were unaffected by their presence in a complex reconstituted system, then the rate of benzphetamine metabolism in the mixed reconstituted system would be expected to be the sum of the rates from the separate reconstituted systems. These results show a slight increase (approximately 20%) from that expected rate, suggesting only minimal changes in the interactions of these proteins. A similar pattern was observed at subsaturating reductase concentrations, with the rate of the mixed reconstituted system being increased 10% from the sum of the rates of the simple reconstituted systems (Table 1).

Table 1: Demonstration of the Interaction between Different P450 Isozymes and NADPH−Cytochrome P450 Reductase in Complex Reconstituted Systems[a]

system components	[reductase]:[total P450]	benzphetamine (nmol/min)	PROD (pmol/min)
2B4 and reductase	3:1	7.7	140
1A2 and reductase	3:1	0.22	18
2B4, 1A2, and reductase	3:1	9.5	116
2B4 and reductase	1.5:1	5.2	76
1A2 and reductase	1.5:1	0.17	1.4
2B4, 1A2, and reductase	1.5:1	6.2	69
2B4 and reductase	0.5:1	2.7	58
1A2 and reductase	0.5:1	0.09	0
2B4, 1A2, and reductase	0.5:1	3.1	12.7

[a] Purified P450 2B4 and 1A2 were combined with NADPH−cytochrome P450 reductase in dilauroylphosphatidylcholine as a reconstituted system containing either one or both P450 isozymes. The reconstituted systems contained P450 2B4 or P450 1A2 (0.1 μM) and reductase at concentrations ranging from 0.05 to 0.3 μM. The metabolism of benzphetamine and 7-pentoxyresorufin was examined in complex reconstituted systems containing both P450 2B4 and 1A2 by comparing the results to those found in simple reconstituted systems containing only a single P450 isozyme. The reductase concentration in the complex reconstituted system was twice the concentration in the systems containing only a single isozyme in order to maintain the [reductase]:[total P450] ratio.

Interestingly, pentoxyresorufin dealkylation produced a completely different pattern. This substrate has also been shown to be more effectively dealkylated by P450 2B4 than by P450 1A2, exhibiting 8- and 54-fold differences at 3:1 and 1.5:1 [reductase]:[P450], ratios, respectively. When examined at a 3:1 reductase to P450 ratio, pentoxyresorufin *O*-dealkylation (PROD) was inhibited in the mixed reconstituted system (containing both P450 1A2 and P450 2B4), being diminished to 75% of the value expected in the absence of protein−protein interactions. However, the magnitude of the inhibitory action of P450 1A2 was dependent on the relative levels of P450 reductase. A greater degree of inhibition was observed when reductase levels were limited (0.5:1 reductase:P450), being decreased to only 22% of the value expected in the absence of an altered interaction between P450 and reductase (Table 1). These results clearly demonstrate that P450 isozymes in complex reconstituted systems interact differently when compared to systems containing only a single P450 isozyme, and that the effect is dependent on the substrate examined. Furthermore, the increased magnitude of the inhibitory response at limiting reductase strongly suggests that P450 2B4 and 1A2 are competing for reductase molecules in the presence of 7-pentoxyresorufin.

Although the studies strongly suggest that P450 1A2 and 2B4 are modulating each other's function in complex reconstituted systems, it is not certain whether P450 1A2 is modulating the activity of P450 2B4 (by direct interaction with the hemoprotein) or whether P450 1A2 is drawing reductase molecules away from P450 2B4 (by more effectively binding reductase). In order to distinguish between these possibilities, the effect of variation of the concentration of 1A2 on reconstituted systems containing fixed concentrations of 2B4 and reductase was examined. When benzphetamine demethylation was examined, addition of small

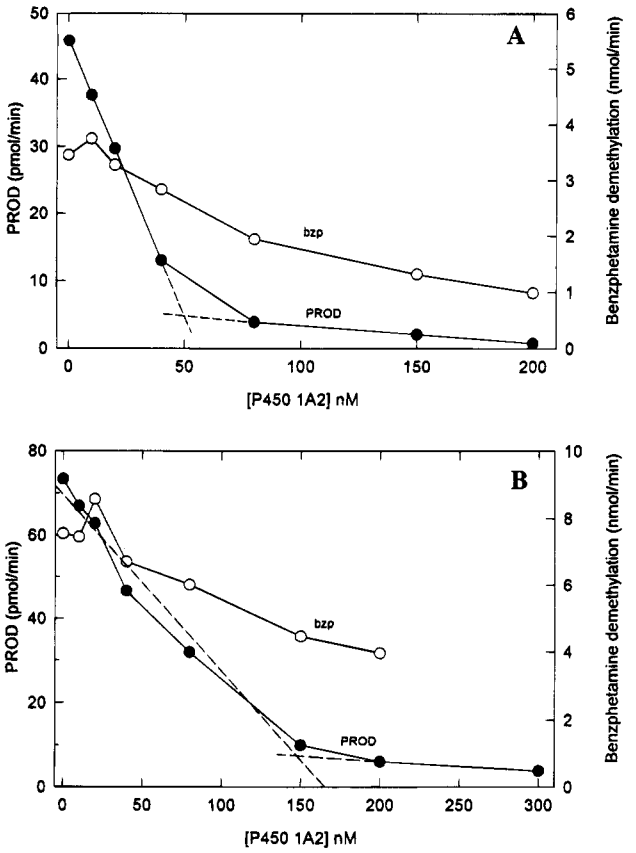

FIGURE 1: Effect of addition of P450 1A2 on P450 2B4-mediated dealkylation of 7-pentoxyresorufin and benzphetamine. (A) Effects at subsaturating reductase. Purified P450 2B4 (0.1 μM) was reconstituted with NADPH–cytochrome P450 reductase (0.05 μM) and P450 1A2 (ranging from 0 to 0.2 μM). (B) Effects at a higher reductase concentration. Conditions were the same as in panel A except that the reductase concentration was 0.15 μM ([reductase]: [P450 2B4] was 1.5:1) and the P450 1A2 concentration ranged from 0 to 0.3 μM.

amounts of P450 1A2 produced only a small decrease, reaching 45% inhibition after addition of 80 nM P450 1A2 (Figure 1A). In contrast, the addition of even small amounts of P450 1A2 had a dramatic effect on 7-pentoxyresorufin *O*-dealkylation, leading to 91% inhibition at 80 nM P450 1A2 (Figure 1A). A biphasic response was observed, showing a steep linear decrease in PROD activity followed by a region that was relatively unresponsive to further increases in P450 1A2 concentration. Extrapolation of these phases resulted in an intersection at a concentration of 1A2 equal to the concentration of reductase in the sample. This is interesting in that direct binding of P450 1A2 to P450 2B4 would be expected to result in an intersection at a concentration equal to the P450 2B4 concentration (0.1 μM, assuming a 1:1 interaction). On the other hand, if P450 1A2 were drawing reductase away from P450 2B4 and the reductase–P450 1A2 interaction had a significantly higher affinity than the reductase–P450 2B4 association, then the intersection would be dependent on the reductase concentration. In order to further test this hypothesis, the effect of P450 1A2 was repeated at a higher reductase concentration. Under these conditions, the intersection was again observed at a concentration equal to the reductase concentration of the reconstituted system (Figure 1B). Again, benzphetamine metabolism was decreased more gradually with increasing P450 1A2 (Figure 1B). These results provide further evidence that P450 1A2 preferentially binds reductase at the expense of P450 2B4 when in the presence of 7-pentoxyresorufin. Furthermore, changes in these interactions occur rapidly, since pentoxyresorufin was added to the reconstituted system less than 1 min prior to initiation of the reaction with NADPH.

Several investigators have attempted to examine functional interactions between different P450 isozymes when placed in complex reconstituted systems. West and Lu (1972) originally reported that P450 and P448 competed for reductase when catalyzing 3,4-benzopyrene hydroxylation. However, if these isozymes were involved in a simple competition for reductase, the activity of the complex reconstituted systems should have approached additivity at saturating reductase. Such results were not obtained in that study. In addition, the fact that both isozymes were capable of catalyzing 3,4-benzopyrene metabolism at similar rates made data analysis more difficult and obscured the conclusions.

More recently, Kaminsky and Guengerich (1985) examined the interactions of eight different isozymes in binary reconstituted systems. These investigators demonstrated that mixtures of different P450 isozymes in reconstituted systems (with reductase) produced a generalized inhibition of P450-dependent warfarin metabolism. The investigators concluded that there was a direct interaction between isozymes rather than a competition between isozymes for reductase. In contrast, Dutton *et al.* (1987) reported that testosterone metabolism catalyzed by P450s 2A1, 2B1, and 1A1 [isozymes also used by Kaminsky and Guengerich (1985)] was not inhibited by the presence of the other isozymes in complex reconstituted systems. The differences in the results obtained by these investigators were attributed to the different substrates used in the respective studies. However, the ability of different substrates to alter these protein–protein interactions was not demonstrated.

Although the isozymes used in the present study are from rabbit rather than rat liver, the results clearly demonstrate that these isozymes function differently in a complex mixture when compared to simple reconstituted systems. The inhibition of pentoxyresorufin dealkylation (PROD) in the complex reconstituted system at limiting reductase is consistent with P450 1A2 competing with P450 2B4 for reductase. Furthermore, when P450 2B4-dependent PROD was examined in reconstituted systems containing varying amounts of P450 1A2, the break in the titration curve occurred when a 1:1 P450 1A2–reductase complex was obtained. The position of the break was then altered by varying the reductase to P450 ratio (Figure 1). The inflection still occurred at a 1:1 reductase to P450 1A2 ratio. The results also clearly demonstrate that the degree of interaction is dependent on the substrate added, with some substrates (e.g., 7-pentoxyresorufin) more effectively enhancing the affinity of P450 1A2 to compete for the reductase. Finally, these studies demonstrate that ligand characteristics that can substantially alter the affinity of a particular P450 isozyme for reductase are different from those which are favorable for catalysis. 7-Pentoxyresorufin is not a good substrate for P450 1A2, although it is capable of causing a dramatic increase in the reductase–P450 1A2 binding affinity (Table 1 and Figure 1). Taken together, these results are consistent with a ligand-dependent alteration in the relative abilities of different P450 isozymes to compete for reductase when in complex reconstituted systems.

In each of the previously reported studies where potential interactions between P450 isozymes in complex reconstituted systems were examined, different conclusions were obtained (West & Lu, 1972; Kaminsky & Guengerich, 1985; Dutton *et al.*, 1987). There are several possible reasons for these discrepancies. First, the conditions used for preparation of reconstituted systems in each of these studies were different and not clearly delineated. In the study described by West and Lu (1972), partially purified P450 and P448 were used as well as partially purified preparations of reductase and "lipid fraction". Furthermore, the [lipid]:[P450] ratio varied considerably in these experiments, ranging from approximately 0.1:1 to 8:1 in their reconstituted systems. Other details such as the preincubation time for preparation of the reconstituted systems were not reported. In the study of Kaminsky and Guengerich (1985), the DLPC to P450 ratio was 15:1, although they reported that higher lipid to P450 ratios did not affect their results. The duration of the preincubation periods used in their studies was unclear. Finally, Dutton *et al.* (1987) used higher DLPC to P450 ratios, but they only preincubated their proteins for 5 min prior to measurement of metabolic activities.

In a previous report, our laboratory examined the effect of preincubation time and DLPC:P450 ratio and defined optimal conditions for the reconstitution of P450 2B4 with reductase (Causey *et al.*, 1990), which is one of the isozymes used in the present study. In these studies, both the rate of catalysis and the rate of electron transfer from reductase to P450 were used as indicators of alterations in the functional interaction between these proteins. The proteins were preincubated with DLPC (at different DLPC:P450 2B4 ratios) for times varying from 5 min to 20 h. During the preincubation step, the proteins were mixed at high concentrations, where the P450 concentration was always greater than 10 μM. After preincubation, the reconstituted systems were diluted with buffer and other assay components for measurement of enzyme activities. Changes in both preincubation time and DLPC:P450 ratio had dramatic effects on the activity of this simple reconstituted system. At a 160:1 [DLPC]:[2B4] ratio, samples preincubated for 5 min had lower rates of electron transfer and monooxygenation than samples preincubated for 2 h. These results demonstrate that stable reconstituted systems are not found after preincubation for short time periods (e.g., 5 min) but require more prolonged preincubations. Furthermore, using the optimal preincubation time (2 h), the rates of monoxygenation and electron transfer were increased almost 2-fold as the [DLPC]:[2B4] ratio was increased from 16:1 to 160:1, demonstrating that the characteristics of the reconstituted system are directly dependent on the preincubation conditions. Although the isozymes used in our present study differ from those used in reports by other authors (West & Lu, 1972; Kaminsky & Guengerich, 1985; Dutton *et al.*, 1987), it is likely that the discrepancies between these studies are due to differences in reconstitution conditions.

These results are consistent with previous data demonstrating a substrate-dependent increase in both the rate of electron transfer through a preformed complex of P450 and reductase and an increase in the rate of interaction between these protein components (Backes & Eyer, 1989; Eyer & Backes, 1992). However, there was no obvious correlation between the relative catalytic efficiency of the substrate and its ability to extract reductase from another P450 isozyme, since both benzphetamine and 7-pentoxyresorufin were selective for P450 2B4. These results indicate that factors other than whether a substrate is effectively catalyzed by a particular isozyme are probably responsible for altering the ability of an isozyme to bind (and therefore compete) for reductase. Despite uncertainties of some of these mechanistic details, these results clearly demonstrate that P450 isozymes can influence each other's metabolism. The results also show that P450 1A2 appears to be modulating 7-pentoxyresorufin metabolism by more effectively competing for P450 reductase rather than by directly interacting with P450 2B4.

REFERENCES

Backes, W. L., & Reker-Backes, C. E. (1988) *J. Biol. Chem. 263*, 247−253.

Backes, W. L., & Eyer, C. S. (1989) *J. Biol. Chem. 264*, 6252−6259.

Causey, K. M., Eyer, C. S., & Backes, W. L. (1990) *Mol. Pharmacol. 38*, 134−142.

Coon, M. J., Van der Hoeven, T. A., Dahl, S. B., & Haugen, D. A. (1979) *Methods Enzymol. 52*, 109−117.

Dutton, D. R., McMillen, S. K., Sonderfan, A. J., Thomas, P. E., & Parkinson, A. (1987) *Arch. Biochem. Biophys. 255*, 316−328.

Estabrook, R. W., Franklin, M. R., Cohen, B., Shigamatzu, A., & Hildebrandt, A. G. (1971) *Metabolism 20*, 187−199.

Eyer, C. S., & Backes, W. L. (1992) *Arch. Biochem. Biophys. 293*, 231−240.

French, J. S., Guengerich, F. P., & Coon, M. J. (1980) *J. Biol. Chem. 255*, 4112−4119.

Kaminsky, L. S., & Guengerich, F. P. (1985) *Eur. J. Biochem. 149*, 479−489.

Lu, A. Y. H., Junk, K. W., & Coon, M. J. (1968) *J. Biol. Chem. 243*, 3714−3721.

Miwa, G. T., & Lu, A. Y. H. (1984) *Arch. Biochem. Biophys. 234*, 161−166.

Miwa, G. T., West, S. B., Huang, M. T., & Lu, A. Y. H. (1979) *J. Biol. Chem. 254*, 5695−5700.

Nash, T. (1953) *Biochem. J. 55*, 416−421.

Perrin, R., Minn, A., Ghersi-Egea, J.-F., Grassiot, M. C., & Siest, G. (1990) *Biochem. Pharmacol. 40*, 2145−2151.

Peterson, J. A., Ebel, R. E., O'Keefe, D. H., Matubara, T., & Estabrook, R. W. (1976) *J. Biol. Chem. 251*, 4010−4016.

Udenfriend, S. (1969) *Fluorescence Assay in Biology and Medicine* (Horecker, B., Kaplan, N. O., Marmur, J., & Scheraga, H. A., Eds.) Vol. II, pp 352−353, Academic Press, New York.

West, S. B. & Lu, A. Y. H. (1972) *Arch. Biochem. Biophys. 153*, 298−303.

Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337−5344.

BI9420471