Role of Phospholipids in Reconstituted Cytochrome P450 3A Form and Mechanism of Their Activation of Catalytic Activity

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ABSTRACT: Cytochrome P-450 coded for by the 3A gene family requires specific conditions in a reconstituted system, if its catalytic activity is to be efficient. We investigated the mechanism of activation of the catalytic activity of cytochrome P450 3A by phospholipids. Rat P450 PB-1 (3A2), human $P450_{NF}$ (3A4), and rabbit P450 3c (3A6) were used. They had low activity in a reconstituted system (system I) with dilauroylphosphatidylcholine (DLPC) but had high activity with a mixture of phospholipids (DLPC, dioleoylphosphatidylcholine, and phosphatidylserine) and sodium cholate (system II). P450 3A forms are cationic (having a high content of lysine residues) and needed the anionic phospholipid phosphatidylserine to have sufficient activity. Double-reciprocal plots of the metabolic rate of cytochrome P-450 versus the concentration of NADPH–cytochrome P-450 reductase showed that cytochrome P-450 and the reductase interacted more in system II than in system I. P450 PB-1 did not absorb at 450 nm in the presence of reductase, CO, DLPC, and NADPH, although other cytochrome P-450s absorbed at around 450 nm in such a mixture. However, P450 PB-1 was reduced in the presence of the phospholipid mixture and sodium cholate instead of DLPC. These results suggested that the stimulation of catalytic activity by phospholipids involved increased interaction between cytochrome P-450 and the reductase. Studies of proteolytic digestion and chemical cross-linking in systems I and II showed that a P450 3A form needed disaggregation of cytochrome P-450 and/or the reductase, not the formation of an aggregated complex necessary for the catalytic activity of other cytochrome P-450s.

The microsomal monooxygenase system metabolizes a variety of endogenous substrates and xenobiotic compounds. In mammalian liver, the enzyme system is associated with the endoplasmic reticulum and includes cytochrome P-450s and NADPH–cytochrome P-450 reductase, which transfers an electron from NADPH to cytochrome P-450; cytochrome b_5 and NADH–cytochrome b_5 reductase may also contribute to the electron flow (de Montellano, 1986). Cytochrome P-450-dependent activities can be reconstituted by mixture of cytochrome P-450, NADPH–cytochrome P-450 reductase, and sometimes cytochrome b_5 with NADPH and small amounts of a phospholipid such as phosphatidylcholine. However, certain aspects of this reconstituted system are unlike those of the endoplasmic reticulum. Cytochrome P-450 and NADPH–cytochrome P-450 reductase may not have the same range of protein–lipid and protein–protein interactions in a reconstituted system and in the endoplasmic reticulum, because native endoplasmic reticulum has many cytochrome P-450s and other proteins and has phospholipid membranes containing many phospholipids (de Montellano, 1986). Protein–protein and protein–lipid interactions are important for the function of the hepatic microsomal monooxygenase system. To be able to get purified cytochrome P-450s and other component proteins in the monooxygenase system makes possible investigation of interactions between component proteins of multienzyme systems. The organization of constituent proteins in phospholipid membranes and their mechanism of interaction there are not understood.

The hydroxylation of steroid hormones is an interesting biological function of hepatic microsomes. 6β-Hydroxytestosterone is a major metabolite of testosterone generated by the hepatic microsomes of male rats, rabbits, and humans (Orton & Philpot, 1973; Guengerich et al., 1986; Imaoka et al., 1988a). This reaction is catalyzed by one of the cytochrome P-450s (usually P450 3A) in hepatic microsomes (Orton & Philpot, 1973; Guengerich et al., 1986; Imaoka et al., 1988a). However, cytochrome P-450 purified from rats does not have such activity, although its antibody completely inhibits testosterone 6β-hydroxylation activity (Waxman et al., 1985; Guengerich et al., 1986; Imaoka et al., 1988a). It was previously thought that the activity was lost during purification. However, purified P450 PB-1 (P450 3A2) has high testosterone 6β-hydroxylation activity in the presence of sodium cholate, cytochrome b_5, and a mixture of phospholipids, although it has no activity in a reconstituted system containing dilauroylphosphatidylcholine (DLPC), the phospholipid most widely used for the reconstitution of a monooxygenase system containing cytochrome P-450 (Imaoka et al., 1988a). Testosterone hydroxylation activities of other rat hepatic cytochrome P-450s such as P450 UT-2 (2C11) or P450 PB-4 (2B1) are not stimulated under these conditions. Some factors contributed to the stimulation of activity: (1) interaction of cytochrome P-450 and NADPH–cytochrome P-450 reductase (or cytochrome b_5); (2) interaction of cytochrome P-450 and the substrate; (3) aggregation of cytochrome P-450 or NADPH–cytochrome P-450 reductase; and (4) interaction of cytochrome P-450 with lipids. Sodium cholate facilitates the incorporation of cytochrome P-450 or other proteins into

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functional complexes (or phospholipid vesicles), and protein–protein interaction is increased by such incorporation into liposomes (Ingelman–Sundberg & Glaumann, 1977, 1980). Cytochrome b_5 increases the catalytic activity at times by providing the second electron required by cytochrome P-450 for the reduction of the oxyferrous intermediate and later oxygen activation (Bonfils et al., 1981). Cytochrome b_5 may affect the tertiary structure of cytochrome P-450 in a reconstituted system (Imaoka et al., 1988b). DLPC acts as a detergent because of its high critical micellar concentration. In a reconstituted system with DLPC, only artificial complexes of the component proteins without any lipid bilayer structure are formed, and a mixture of phospholipids is required for the formation of phospholipid vesicles more similar to endoplasmic reticulum membrane than DLPC vesicles are (de Montellano, 1986). It is not clear what factors affect the change in testosterone 6β-hydroxylation activity. To identify the mechanism of the increase would help in the understanding of the mechanisms of the electron-transport system in the monooxygenase system of the endoplasmic reticulum.

In this study, we found that the catalytic activity of all cytochrome P-450s in the P450 3A family (rat P450 PB-1, rabbit P450 3c, and human $P450_{NF}$) could be increased by the addition of a phospholipid mixture and sodium cholate to a reconstituted system and that the increase was mainly due to the increased interaction between NADPH–cytochrome P-450 reductase and cytochrome P-450. We also found that the P450 3A form required disaggregation of cytochrome P-450 and/or NADPH–cytochrome P-450 reductase rather than the formation of an aggregated complex, previously thought to be important for cytochrome P-450s to have catalytic activities.

Materials and Methods

Chemicals. Testosterone, DLPC, and dioleoylphosphatidylcholine (DOPC) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylcholine (PS) from bovine was obtained from P-L Biochemicals (Milwaukee, WI). Lidocaine, monoethylglycinexylidide (MEGX), and glycinexylidide (GX) were supplied by Fujisawa Pharmaceuticals Co. (Osaka, Japan). 3-Hydroxylidocaine and methylhydroxylidocaine were kind gifts of Prof. S. Fujita of Hokkaido University. Nifedipine and its metabolite (pyridine derivative) were obtained from Bayer (Leverkusen, Germany). NADPH was obtained from Oriental Yeast Co. (Tokyo). α-Chymotrypsin was obtained from Miles Diagnostics (Kankakee, IL). Cumene hydroperoxide was obtained from Nacalai Tesque (Kyoto, Japan). 7,8-Benzoflavone was obtained from Sigma. Other reagents and organic solvents were from Wako Pure Chemical Industries (Tokyo).

Purification of Cytochrome P-450s. P450 PB-1 (3A2), UT-2 (2C11), and PB-4 (2B1) were purified from hepatic microsomes of rats as previously described (Funae & Imaoka, 1985). $P450_{NF}$ was purified from hepatic microsomes of humans (Guengerich et al., 1986; Imaoka et al., 1990a). Rabbit P450 3c was a kind gift of Dr. M. Komori of University of Osaka Prefecture. A rabbit cytochrome P-450 was purified from hepatic microsomes (Komori et al., 1984), and it was identified with P450 3c by the NH_2-terminal sequence. Purification of NADPH–cytochrome P-450 reductase and cytochrome b_5 was as described elsewhere (Imaoka et al., 1988a). The purified cytochrome P-450s had the specific content of cytochrome P-450 of 12–16 nmol/mg of protein, and gave a single protein-staining band on SDS–polyacrylamide gels.

Assays of Oxidation Activities toward Testosterone, Lidocaine, and Nifedipine. Assay methods for testosterone hydroxylation and lidocaine oxidation activity were as described elsewhere (Imaoka et al., 1988a, 1990a). Nifedipine oxidation activity was assayed by the method of Guengerich et al. (1986). Cumene hydroperoxide-dependent oxidation was assayed by the method of Huang et al. (1981). The reaction mixture contained hepatic microsomes (100 μg) or purified cytochrome P-450 (30 pmol) and phospholipid (10 μg) with sodium cholate (100 μg). The mixture was incubated with substrate and cumene hydroperoxide (75 nmol) in 0.1 M potassium phosphate buffer (pH 7.4). The reaction was started by the addition of 5 μL of cumene hydroperoxide in acetone solution. The reaction mixture (0.5 mL) was incubated at 37 °C for 2 min. Metabolites were analyzed by the same method as for the NADPH-dependent metabolism described above. The modified reconstituted system (Imaoka et al., 1988a) contained purified cytochrome P-450 (30 pmol), NADPH–cytochrome P-450 reductase (0.3 unit), cytochrome b_5 (30 pmol), sodium cholate (100 μg), and a mixture (10 μg) of DLPC, DOPC, and PS (1:1:1). The reconstitution was done by the addition, in this order, of cytochrome P-450, NADPH–cytochrome P-450 reductase, cytochrome b_5, 10% sodium cholate (pH 7.4), the phospholipid mixture, and the reaction buffer. The reaction was done under the same conditions as with the microsomes or the conventional reconstituted system described above.

Other Methods. The amount of cytochrome P-450 was estimated spectrophotometrically by the method of Omura and Sato (1964). The protein concentration was measured by a method of Lowry et al. (1951). The digestion with α-chymotrypsin was done by the method previously described (Imaoka et al., 1990b). Chemical cross-linking was done by the method of Baskin and Yang (1980). Cytochrome P-450 (30 pmol), NADPH–cytochrome P-450 reductase (0.3 unit), and phospholipid (10 μg), with or without sodium cholate (100 μg), were mixed in 0.5 mL of 0.1 M phosphate buffer (pH 7.4). Then, 2 M triethanolamine solution (50 μL) adjusted to pH 8.0 with HCl was added to the reaction mixture. Dimethyl pimelimidate (0.1 M; 25 μL) dissolved in 0.2 M triethanolamine hydrochloride buffer (pH 8.0) was added to the solution, and the mixture was standed at 25 °C for 15 or 60 min. The reaction was stopped by the addition of 1 M ethanolamine (50 μL).

Results

Metabolic Activity of P450 PB-1, P450 3c, and $P450_{NF}$ toward Testosterone, Lidocaine, and Nifedipine in a Reconstituted System. As previously reported, P450 PB-1 (3A2) has low testosterone 6β-hydroxylation activity in a conventional reconstituted system with DLPC (Imaoka et al., 1988a). However, P450 PB-1 had high activity in a modified reconstituted system with cytochrome b_5, the phospholipid mixture, and sodium cholate (Table I). The addition of cytochrome b_5 to a reconstituted system with DLPC did not much increase the catalytic activity of P450 PB-1. The phospholipid mixture and sodium cholate were important for the catalytic activity of P450 PB-1. P450 PB-1 is a cytochrome P-450 derived from the 3A gene family. Therefore, we also assayed rabbit P450 3c (3A6) and human $P450_{NF}$ (3A4). These results are also shown in Table I. As for P450 PB-1, the testosterone 6β-hydroxylation activity of P450 3c and $P450_{NF}$ was increased by modification of the reconstituted system: 48- and 10-fold, respectively. The increase of catalytic activity was not found with other rat or human cytochrome P-450s. We observed the same effect with mouse cytochrome P-450, also in the 3A gene family (data not shown). The effects seem to be common to cytochrome P-450s coded for by the 3A gene family. Lidocaine is metabolized by P450 PB-1 in rat liver and by

Table I: Effect of Phospholipids on the Catalytic Activity of Cytochrome P-450[a]

cytochrome P-450	testosterone 6β	testosterone 2β	lidocaine N-deethylation	nifedipine oxidation
P450 PB-1				
DLPC	<0.1	<0.1	3.14	1.09
+b_5	0.7	<0.1	7.64	1.62
mix	13.9	2.1	15.49	5.71
P450 3c				
DLPC	0.3	<0.1	0.27	0.52
+b_5	0.9	<0.1	0.29	1.12
mix	14.5	2.5	4.50	7.41
$P450_{NF}$				
DLPC	0.9	<0.1	2.44	0.43
+b_5	1.8	0.3	3.91	0.60
mix	8.9	1.3	7.70	3.17

[a] Values are expressed as nanomoles of product per minute per nanomole of cytochrome P-450. "DLPC" shows a reconstituted system containing cytochrome P-450 (30 pmol), rat NADPH–cytochrome P-450 reductase (0.3 unit), dilaurolylphosphatidylcholine (10 μg), NADPH (0.2 μmol), and testosterone (0.5 μmol), lidocaine (0.5 μmol), or nifedipine (0.5 μmol). "+b_5" shows a "DLPC" system to which rat cytochrome b_5 (30 pmol) has been added. "Mix" shows a reconstituted system containing cytochrome P-450, rat cytochrome b_5, rat NADPH–cytochrome P-450 reductase, substrate, NADPH, sodium cholate (100 μg), and 10 μg of a 1:1:1 mixture of the phospholipids dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, and phosphatidylserine.

$P450_{NF}$ in human liver (Imaoka et al., 1990a). Lidocaine N-deethylation activity of P450 PB-1, P450 3c, and $P450_{NF}$ was high in the modified reconstituted system. P450 PB-1 and P450 3c had high nifedipine oxidation activity in the modified reconstituted system. $P450_{NF}$ had high nifedipine oxidation activity, which was enhanced in the modified reconstituted system.

Effects of the Phospholipid Mixture and Sodium Cholate on the Affinity of Cytochrome P-450 for Its Substrate and for NADPH–Cytochrome P-450 Reductase. The catalytic activities toward testosterone, lidocaine, and nifedipine of all cytochrome P-450s used here could be stimulated. One explanation of the higher activity in the modified reconstituted system could be increased affinity of the cytochrome P-450 for its substrate. To find whether the apparent affinity of cytochrome P-450 for the substrate changed in the presence of the phospholipid mixture and sodium cholate, the K_m and V_{max} for lidocaine N-deethylation by P450 PB-1 were calculated in a reconstituted system with DLPC and in the modified reconstituted system. In this study, we used P450 PB-1 and lidocaine, because P450 PB-1 is readily obtained and because the lidocaine N-deethylation activity of P450 PB-1 can be detected in a reconstituted system with DLPC. Testosterone was not suitable for this purpose because its activity in a reconstituted system with DLPC was too low for measurement. The results are shown in Figure 1A. For lidocaine N-deethylation, the apparent K_m was 0.44 mM and the V_{max} was 5.24 nmol min^{-1} $nmol^{-1}$ with DLPC, and these values were 3.92 mM and 50.76 nmol min^{-1} $nmol^{-1}$, respectively, with the phospholipid mixture, suggesting that the difference in activity was not caused by increased affinity of P450 PB-1 for lidocaine in the two reconstituted systems. Both K_m and V_{max} were increased by modification of the reconstituted system. This phenomenon is not explained. The increase in the apparent K_m for lidocaine may be caused by a different solubility of the lidocaine in the phospholipids or may be caused by changes in the tertiary structure of cytochrome P-450.

The rate-limiting step of the overall monooxygenase reaction is the introduction of the electron (Taniguchi et al., 1984). To find if the phospholipid mixture and sodium cholate increased

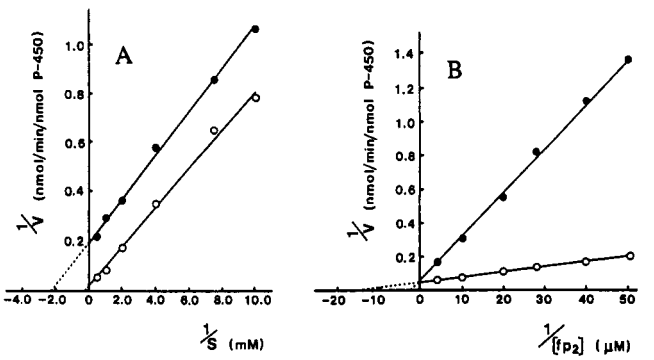

FIGURE 1: Effects of substrate concentration (A) and NADPH–cytochrome P-450 reductase concentration (B) on the lidocaine N-deethylation activity of P450 PB-1 in reconstituted systems with dilauroylphosphatidylcholine or with a phospholipid mixture and sodium cholate. Closed circles, values when P450 PB-1 reacted with lidocaine in the "DLPC" system described in the footnote of Table I. Open circles, values when P450 PB-1 reacted with lidocaine in the "mix" system described in the footnote of Table I. (A) The K_m and V_{max} values for lidocaine N-deethylation were 0.44 mM and 5.24 nmol min^{-1} $nmol^{-1}$, respectively, in the "DLPC" system, and 3.92 mM and 50.76 nmol min^{-1} $nmol^{-1}$ in the "mix" system. The linear regression correlation coefficients were 0.997 in the "DLPC" system and 0.994 in the "mix" system. (B) The K_m and V_{max} value for lidocaine N-deethylation were 0.440 μM and 17.24 nmol min^{-1} $nmol^{-1}$, respectively, in the "DLPC" system, and 0.065 μM and 21.83 nmol min^{-1} $nmol^{-1}$ in the "mix" system. The linear regression correlation coefficients were 0.998 in the "DLPC" system and 0.997 in the "mix" system. fp2, NADPH–cytochrome P-450 reductase.

interactions between cytochrome P-450 and NADPH–cytochrome P-450 reductase, the rate of lidocaine N-deethylation was calculated after the addition of various concentrations of purified NADPH–cytochrome P-450 reductase to the reconstituted system with DLPC or with the phospholipid mixture and sodium cholate. Double-reciprocal plots of the rate of lidocaine N-deethylation versus the total NADPH–cytochrome P-450 reductase concentration are shown in Figure 1B. The K_m and V_{max} in the reconstituted system with DLPC were 0.44 μM and 17.24 nmol min^{-1} $nmol^{-1}$, respectively; with the phospholipid mixture and sodium cholate, these values were 0.065 μM and 21.83 nmol min^{-1} $nmol^{-1}$, respectively. The K_m decreased with modification of the reconstituted system, suggesting that addition of the phospholipid mixture and sodium cholate increased the affinity between cytochrome P-450 and NADPH–cytochrome P-450 reductase and that it may have facilitated electron transfer from the reductase to cytochrome P-450.

To know more about the interaction between the reductase and cytochrome P-450, P450 PB-1 was reduced with NADPH in the presence of NADPH–cytochrome P-450 reductase and CO. P450 UT-2 (2C11, a major constitutive form) and P450 PB-4 (2B1, a major phenobarbital-inducible form) were used for comparison. Results are shown in Figure 2. P450 UT-2, PB-4, and PB-1 were not reduced in the absence of phospholipid. In the presence of DLPC, P450 UT-2 and PB-4 were reduced, absorbing at around 450 nm, but P450 PB-1 was not. P450 PB-1 was reduced in the modified reconstituted system. The extent of reduction was not affected by the addition of a substrate such as testosterone; generally, a substrate reduces the oxidation–reduction potential of cytochrome P-450 (Guengerich et al., 1976; Iyanagi et al., 1978). By the addition of $Na_2S_2O_4$, P450 PB-1 was also completely reduced (Figure 2). These results gave further suggestions that the phospholipid mixture and sodium cholate increased the interactions between cytochrome P-450 and the reductase.

Effects of the Phospholipid Mixture and Sodium Cholate on Protein–Protein Interactions. Protein–protein interactions

Table II: Cumene Hydroperoxide-Dependent Metabolism of Testosterone by Cytochrome P-450[a]

	P450 PB-1		P450 PB-4		P450 UT-2	
	6β	2β	16α	16β	16α	2α
no DLPC	<0.05	<0.05	0.29	0.28	0.28	0.19
DLPC	<0.05	<0.05	1.81	1.87	0.40	0.30
mix	1.97	0.28	0.86	0.83	0.31	0.25

[a] Values are expressed as nanomoles per minute per nanomole of cytochrome P-450. 2α, 2β, 6β, 16α, and 16β refer to testosterone 2α-, 2β-, 6β-, 16α-, and 16β-hydroxylation activities. "no DLPC", only cytochrome P-450 (30 pmol) was reacted with testosterone (0.5 μmol) in the presence of cumene hydroperoxide (75 nmol). "DLPC", dilauroylphosphatidylcholine (10 μg) was added to the "no DLPC" system. "mix", sodium cholate (100 μg) and the phospholipid mixture (10 μg) described in the footnote of Table I were added to the "no DLPC" system.

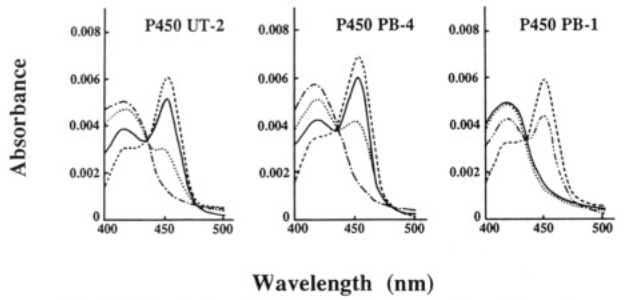

FIGURE 2: Effect of phospholipids on the reduction of cytochrome P-450 with NADPH. P450 PB-1, UT-2, and PB-4 were reduced by NADPH in the presence of CO and NADPH–cytochrome P-450 reductase (fp2) without (···) or with (—) dilauroylphosphatidylcholine (DLPC) or with the phospholipid mixture and sodium cholate (–··–). In one experiment, cytochrome P-450 reduced with $Na_2S_2O_4$ in the presence of the phospholipid mixture and sodium cholate is shown (- - -). Spectra of an oxidized form of cytochrome P-450 before the addition of NADPH are also given (–·–). Cytochrome P-450 (100 pmol) was reduced with NADPH (0.5 μmol) in 1 mL of CO-saturated potassium phosphate buffer (0.1 M, pH 7.4) in the presence of fp2 (0.5 unit) with DLPC (10 μg) or with the phospholipid mixture (10 μg) and sodium cholate (100 μg).

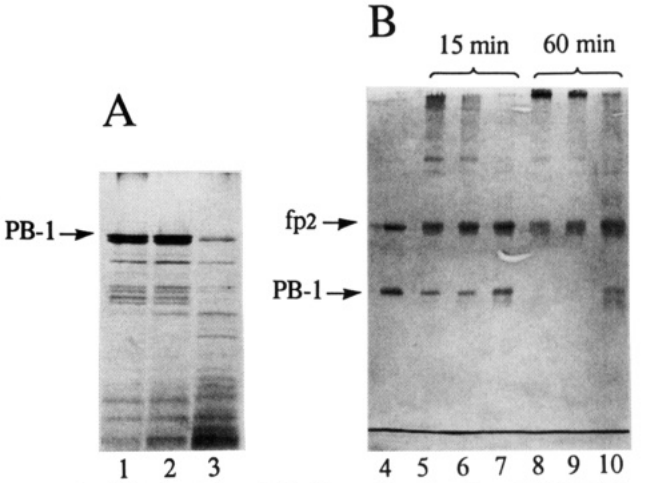

FIGURE 3: Effects of phospholipids on the digestion of P450 PB-1 by α-chymotrypsin (A) and on chemical cross-linking with dimethyl pimelimidate (B). P450 PB-1 (5 μg) was digested with α-chymotrypsin (0.5 μg) in 0.1 M phosphate buffer (lane 1), in the presence of dilauroylphosphatidylcholine (lane 2), or in the presence of the phospholipid mixture and sodium cholate (lane 3) as described under Materials and Methods. The reaction was for 30 min at 37 °C. Reaction mixtures were analyzed by SDS–polyacrylamide gel electrophoresis with a 15% acrylamide gel. Protein and peptide bands were visualized with silver staining. Chemical cross-linking with dimethyl pimelimidate (DMP) was done as described under Materials and Methods. Lane 4 is a mixture of P450 PB-1 and NADPH–cytochrome P-450 reductase (fp2) without DMP. fp2 and P450 PB-1 were reacted with DMP at 25 °C for 15 or 60 min in 0.1 M phosphate buffer (lanes 5 and 8), in the presence of DLPC (lanes 6 and 9), or in the presence of the phospholipid mixture and sodium cholate (lanes 7 and 10). Reaction mixtures were analyzed by the same methods with a 7.5% acrylamide gel.

of cytochrome P-450 and the reductase or of cytochrome P-450 with itself were investigated with proteolytic digestion and with chemical cross-linking. P450 PB-1 in the system with a phospholipid mixture and sodium cholate was more easily digested with α-chymotrypsin than that in the system with DLPC (Figure 3A). These results suggest that P450 PB-1 was disaggregated in the modified reconstituted system, different from the previous report that cytochrome P-450 is in the aggregated form (Ingelman-Sundberg & Glaumann, 1980; Baskin & Yang, 1980; Kaminsky et al., 1987). The chemical cross-linking reagent dimethyl pimelimidate was added to a mixture of reductase and cytochrome P-450 that was in phosphate buffer, in DLPC and phosphate buffer, or in the phospholipid mixture, sodium cholate, and phosphate buffer (Figure 3B). In the absence of a phospholipid or in the presence of DLPC, P450 PB-1 and the reductase aggregated and cross-linked to proteins of high molecular weight. However, the cross-linking reaction was slower in the presence of the phospholipid mixture and sodium cholate than in the presence of DLPC, suggesting that P450 PB-1 or reductase was present in monomer form rather than as an aggregate. DLPC did not affect the degree of aggregation of cytochrome P-450 and reductase, because the addition of DLPC did not affect the cross-linking.

Cumene Hydroperoxide-Dependent Oxidation of Testosterone. As mentioned above, modification of the reconstituted system increased the affinity between cytochrome P-450 and NADPH–cytochrome P-450. Next, we investigated the possibility that the catalytic activity was stimulated after cytochrome P-450 reduction with a second electron (shown as steps 5 and 6 in Figure 4). The cumene hydroperoxide-dependent monooxygenase system requires cytochrome P-450 but not NADPH, NADPH–cytochrome P-450 reductase, and

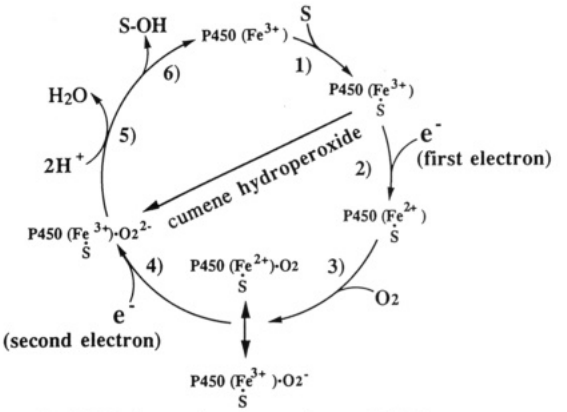

FIGURE 4: Oxidation cycle of cytochrome P-450.

oxygen for oxidation of the substrate (Huang et al., 1981). The results are shown in Table II. In the absence of DLPC or the phospholipid mixture, P450 PB-4 and UT-2 catalyzed testosterone hydroxylation, but P450 PB-1 did not. In the presence of DLPC, P450 PB-4 and UT-2 hydroxylated testosterone but P450 PB-1 did not. In the presence of the phospholipid mixture and sodium cholate, P450 PB-1 catalyzed

Table III: Effect of 7,8-Benzoflavone on the Catalytic Activity of Rat, Rabbit, and Human Hepatic Microsomes[a]

	testosterone				lidocaine		
	2α	2β	6β	16α	MEGX	3-OH LID	Me-OH LID
rat Ms							
7,8-BF, 0 μM	0.41	0.18	1.29	0.66	6.46	0.33	0.09
7,8-BF, 10 μM	0.57	0.57	1.86	0.94	6.22	0.34	0.08
7,8-BF, 100 μM	0.31	0.37	0.85	0.51	2.51	0.38	0.11
rabbit Ms							
7,8-BF, 0 μM	0.02	0.09	0.76	0.17	8.26	0.17	0.09
7,8-BF, 10 μM	0.03	0.18	1.17	0.25	4.07	0.13	0.10
7,8-BF, 100 μM	0.03	0.20	1.05	0.19	2.98	0.14	0.09
human Ms							
7,8-BF, 0 μM	<0.01	0.45	3.17	<0.01	3.43	0.03	<0.01
7,8-BF, 10 μM	<0.01	0.77	3.69	<0.01	3.62	0.01	<0.01
7,8-BF, 100 μM	<0.01	0.59	2.51	<0.01	2.61	<0.01	<0.01

[a] Values are expressed as nanomoles per minute per milligram of protein. MEGX, 3-OH LID, and Me-OH LID show formation of monoethylglycinexylidide (N-deethylation), 3-hydroxylidocaine (hydroxylation of the aromatic ring at the 3-position), and methylhydroxylidocaine (hydroxylation of the methyl group), respectively. Ms, microsomes; 7,8-BF, 7,8-benzoflavone.

testosterone 6β-hydroxylation. The activities of P450 PB-4 and UT-2 were not stimulated by the phospholipid mixture and sodium cholate. The results with cumene hydroperoxide are similar to those with NADPH and the reductase (Table I). The phospholipids affect the aggregation state of P450 3A form. These results also suggest that modification of the reconstituted system increased the cumene hydroperoxide-mediated formation of oxidized cytochrome P450 PB-1 peroxide anion or the next two steps leading to hydroxylated testosterone (Figure 4).

Effect of 7,8-Benzoflavone on Catalytic Activities of P450 PB-1, P450 3c, and P450$_{NF}$. 7,8-Benzoflavone stimulates some catalytic activities of P450$_{NF}$ and P450 3c in microsomes or in a reconstituted system (Huang et al., 1981; Schwab et al., 1988). This stimulation involves increased affinity between cytochrome P-450 and reductase (Huang et al., 1981). This phenomenon resembles the stimulation of the catalytic activity of P450 PB-1, P450 3c, and P450$_{NF}$ by modification of the reconstituted system. Therefore, testosterone and lidocaine were reacted with microsomes and with cytochrome P-450 in a reconstituted system in the presence of 7,8-benzoflavone. Results are shown in Table III. The 2β-hydroxylation activity of rat, rabbit, and human hepatic microsomes toward testosterone was stimulated by the addition of 10 μM 7,8-benzoflavone. 2α-, 6β-, and 16α-hydroxylations were stimulated slightly. 7,8-Benzoflavone did not stimulate the N-deethylation activity of rat, rabbit, and human hepatic microsomes toward lidocaine. In a reconstituted system with the phospholipid mixture and sodium cholate, the testosterone 2β-hydroxylation activity of P450 PB-1, P450 3c, and P450$_{NF}$ was increased by 10 μM 7,8-benzoflavone; 6β-hydroxylation was not increased (Table IV), suggesting that 7,8-benzoflavone affects the tertiary structure of P450 3A forms. In a reconstituted system with DLPC, the lidocaine N-deethylation activity of P450 PB-1, P450 3c, and P450$_{NF}$ was not increased. Lidocaine N-deethylation activity of P450$_{NF}$ only in a reconstituted system with the phospholipid mixture and sodium cholate was increased by 10 μM 7,8-benzoflavone.

Table IV: Effect of 7,8-Benzoflavone on the Catalytic Activity of Cytochrome P-450[a]

	testosterone		lidocaine N-deethylation
	2β	6β	
P450 PB-1 (DLPC)			
7,8-BF, 0 μM	<0.1	<0.1	1.05
7,8-BF, 10 μM	<0.1	<0.1	0.49
P450 PB-1 (mix)			
7,8-BF, 0 μM	2.1	13.9	15.49
7,8-BF, 10 μM	6.7	15.8	15.51
P450 3c (DLPC)			
7,8-BF, 0 μM	<0.1	0.3	0.27
7,8-BF, 10 μM	<0.1	0.3	0.38
P450 3c (mix)			
7,8-BF, 0 μM	2.5	14.5	4.50
7,8-BF, 10 μM	4.2	14.4	5.49
P450$_{NF}$ (DLPC)			
7,8-BF, 0 μM	<0.1	0.9	2.44
7,8-BF, 10 μM	0.2	1.0	2.68
P450$_{NF}$ (mix)			
7,8-BF, 0 μM	1.3	8.9	7.70
7,8-BF, 10 μM	1.9	8.2	12.85

[a] Values are expressed as nanomoles per minute per nanomole of cytochrome P-450. "DLPC" and "mix" described in the footnote of Table I. 7,8-BF, 7,8-benzoflavone.

Discussion

Reconstitution of the microsomal monooxygenase system has made it possible to characterize individual cytochrome P-450s and to investigate protein–protein interactions (for instance, between cytochrome P-450 and NADPH–cytochrome P-450 reductase) and phospholipid–protein interactions in the microsomes. The testosterone 6β-hydroxylation activity of cytochrome P-450s such as P450 PB-1 coded for by the 3A gene family was low or not detected in a reconstituted system with DLPC, but modification of the reconstituted system increased the catalytic activity. These effects were not observed with other rat cytochrome P-450s such as P450 UT-2 (2C11) or PB-4 (2B1), which have high testosterone hydroxylation activity. This increase was characteristic of P450 PB-1. This property was found also in P450 3A forms from other species (rabbit P450 3c and human P450$_{NF}$); not only the testosterone hydroxylation activity but also the lidocaine and nifedipine oxidation activities of P450 PB-1, P450 3c, and P450$_{NF}$ were increased. This stimulation needed three factors: the presence of cytochrome b_5, the presence of sodium cholate, and the presence of phospholipids that contain different charges or a mixture of different fatty acids. These factors stimulate a certain stage of the reaction cycle of cytochrome P-450, which may have six reaction steps (Figure 4): (1) the binding of substrate; (2) the incorporation of the first electron; (3) the incorporation of O_2; (4) the incorporation of the second electron; (5 and 6) the introduction of oxygen into the substrate (in two steps). The results presented here suggest that step 2 (flow of an electron from reductase to cytochrome P-450) was stimulated, because the K_m of lidocaine N-deethylation for the reductase in the modified reconstituted system was lower than that in the conventional reconstituted system. Cytochrome P-450 has different affinities for the reductase in different phospholipid vesicles (Ingelman-Sundberg & Glaumann, 1977, 1980; Rietjens et al., 1989). Probably cy-

tochrome P-450 needs phospholipids for its monooxygenase activity because they increase the interaction of cytochrome P-450 and reductase by incorporating these proteins into lipid vesicles; trypsin-treated reductase, which does not have the peptide region, interacts with lipids and does not reduce cytochrome P-450, although it reduces cytochrome *c* (Coon et al., 1973; Reitjens et al., 1989). The studies of proteolytic digestion and of chemical cross-linking reported here also suggest that P450 PB-1 and reductase were disaggregated or were present in monomer form rather than being an aggregate in the modified reconstituted system. P450 PB-1 needed disaggregation of cytochrome P-450 and/or the reductase to have efficient catalytic activity, rather than an aggregated complex of cytochrome P-450 and reductase, as has been reported before (Ingelman-Sundberg & Glaumann, 1977, 1980). These results are different from those in previous reports that other cytochrome P-450 is in aggregated form in low concentrations of detergent and disintegration of these aggregates of protein multimers caused by detergent inactivates the activity of cytochrome P-450 (Ingelman-Sundberg & Glaumann, 1980; Baskin & Yang, 1980; Kaminsky et al., 1987).

Another effect of phospholipids in a monooxygenase system is stimulation of interactions between the substrate (usually hydrophobic, so readily dissolved in phospholipids) and cytochrome P-450 (Coon et al., 1973; Rietjens et al., 1989). In this study, modification of the reconstituted system did not decrease the K_m for the substrate. Step 1 was not stimulated by modification of the reconstituted system. The effect of the phospholipid mixture in step 3 cannot be estimated from the results of this study. Usually the rate constant in the reaction of cytochrome P-450 and oxygen molecules is much higher than that in the reaction of substrate and cytochrome P-450 or in the reduction of cytochrome P-450 (Guengerich et al., 1976). Therefore, differences in the affinity between cytochrome P-450 and oxygen molecules were negligible in the overall monooxygenase activity. In step 4, the second electron is probably introduced into cytochrome P-450 by cytochrome b_5. Cytochrome b_5 accepts an electron from NADPH–cytochrome P-450 reductase (Bonfils et al., 1981). The affinity between cytochrome P-450 and cytochrome b_5 seemed not to differ much because the effect of cytochrome b_5 on the testosterone 6β-hydroxylation of P450 PB-1 is not different in the conventional and modified reconstituted systems (Imaoka et al., 1988a). P450 PB-1 metabolized testosterone only in the presence of a phospholipid mixture in a cumene hydroperoxide-dependent monooxygenase system, although P450 PB-4 and UT-2 metabolized testosterone both in the absence and in the presence of phospholipids. These results indicate that steps 5 and 6 (the formation of peroxide anion binding oxidized cytochrome P-450 and further substrate oxidation) were also accelerated. When the supply of electrons is sufficient, steps 5 and 6 are rate-limiting (Taniguchi et al., 1984).

Ingelman-Sundberg et al. (1981) reported that the membrane charge is important for the catalytic activity of cytochrome P-450. There is correlation between the net negative charge of the vesicles and the catalytic activity of cytochrome P-450 in a reconstituted system, and phosphatidylserine is efficient for catalytic activity of cytochrome P-450, because it has a high negative charge (Taniguchi et al., 1984). In our modified reconstituted system, phosphatidylserine is again effective for the catalytic activity of cytochrome P-450 (Imaoka et al., 1988a). The effect of a negatively charged membrane on the affinity between cytochrome P-450 and reductase suggests that the structures of these enzymes are altered by ionic interactions with the membrane matrix (Taniguchi et al., 1984). The ϵ-amino group of the lysine residue in cytochrome P-450 is important for interaction with reductase, because modification of the ϵ-amino group of lysine in cytochrome P-450 inhibits its catalytic activity and impairs the rate of electron transfer to cytochrome P-450 from the reductase (Bernhardt et al., 1984). The lysine residue in cytochrome P-450 is important to the catalytic activity or for acceptance of the electron from the reductase. From the results of this study, P450 3A forms seem to need the negative charge of the vesicles more than other cytochrome P-450s. Cytochrome P450 PCN and P450 3c (both in the 3A gene family) have the most lysine residues of the hepatic cytochrome P-450s studied so far (de Montellano, 1986). In addition, the affinity of P450 PB-1 with anion-exchange resins is high (Imaoka et al., 1988a), indicating this cytochrome P-450 is strongly cationic. Lipid peroxidation initiated in hepatic microsomes by NADPH specifically degrades P450 PB-1 (Kidata et al., 1989), and treatment of microsomes with phospholipase A_2 specifically inactivates the testosterone 6β-hydroxylation activity of hepatic microsomes (Yamazoe et al., 1988). These findings indicate that the P450 3A forms are sensitive to changes in chemical structures of lipids or to conditions of the phospholipid membrane and that the P450 3A forms have strong interactions with phospholipids.

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Isomerization of (*R*)- and (*S*)-Glutathiolactaldehydes by Glyoxalase I: The Case for Dichotomous Stereochemical Behavior in a Single Active Site†

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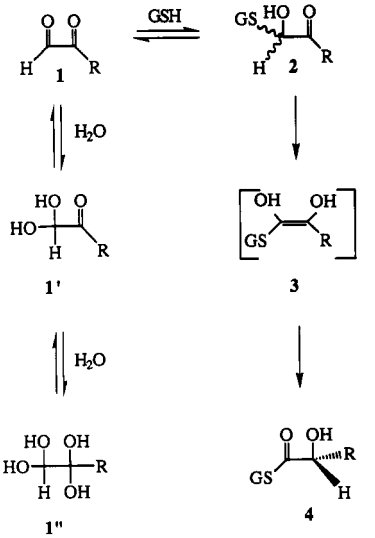
ABSTRACT: The ability of glyoxalase I to isomerize both diastereomeric thiohemiacetals formed between glutathione and α-ketoaldehydes has been probed with stereochemically "locked" substrate analogues. Both (*R*)- and (*S*)-glutathiolactaldehyde (**5** and **5'**) were unambiguously synthesized by employing the Sharpless epoxidation procedure as a key step. In the presence of human erythrocyte glyoxalase I, high-field ^{1}H NMR analysis reveals that the *R* and *S* isomers (~20 mM) are both converted to glutathiohydroxyacetone at rates of 0.8 and 0.4 s^{-1}, respectively. This reaction is characterized by a nonstereospecific proton abstraction followed by a partially shielded proton transfer to the *si* face of the *cis*-enediol intermediate. Glyoxalase I catalyzes the exchange of the *pro-S* proton of glutathiohydroxyacetone with solvent deuterium. Glutathiohydroxyacetone was found to be a good competitive inhibitor of the normal glyoxalase I reaction (K_I = 1.46 mM), suggesting that the slow processing rate of these compounds with respect to the normal thiohemiacetals is not due to poor binding. The results are consistent with a nonstereospecific proton abstraction and a stereospecific reprotonation at contiguous substrate carbons.

The stereochemical course of an enzymatic reaction is an end product of the chemical mechanism and of the spatial organization of the active site functional groups employed by the enzyme to effect catalysis. As a result of the asymmetry inherent in an enzyme active site, enzymes, in general, demonstrate little leniency for changes in substrate stereochemistry, especially at chiral centers which undergo reaction. In addition, the formation of stereochemically ambiguous products is usually not observed. Thus, the absolute stereospecificity with which substrate is converted to product is a hallmark of enzymatic catalysis. However, the selective pressure of a particular physiological condition coupled with the economy of nature may produce an enzyme with broadened substrate stereospecificity. Glyoxalase I has been studied over the years as a paradigm for this type of behavior.

Glyoxalase I [*S*-lactoylglutathione methylglyoxal-lyase (isomerizing) EC 4.4.1.5, GX I[1]] catalyzes the conversion of the thiohemiacetal **2** of α-ketoaldehyde **1** and glutathione [*N*-(*N*-L-γ-glutamyl-L-cysteinyl)glycine, GSH] to thioester

Scheme I: Glyoxalase I-Catalyzed Reaction



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