Dynamic Interactions of Rabbit Liver Cytochromes P450IA2 and P450IIB4 with Cytochrome b_5 and NADPH−Cytochrome P450 Reductase in Proteoliposomes†

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ABSTRACT: Purified liver microsomal cytochrome P450IA2 or P450IIB4 was co-reconstituted with cytochrome b_5 or NADPH−cytochrome P450 reductase in phosphatidylcholine−phosphatidylethanolamine−phosphatidylserine vesicles at a lipid to P450 weight ratio of 2 by cholate dialysis procedures. The proteoliposomes catalyzed drug oxidation. Rotational diffusion of cytochrome P450 was measured by observing the decay of absorption anisotropy, $r(t)$, after photolysis of the heme•CO complex. Analysis of $r(t)$ was based on a "rotation-about-membrane normal" model. The absorption anisotropy decayed within 1 ms to a time-independent value, r_3. Different rotational mobility for the two cytochrome P450s was observed. Though 20% of cytochrome P450IA2 was immobile, all cytochrome P450IIB4 molecules were rotating. The rotational relaxation time, ϕ, of the mobile population was 237 μs for cytochrome P450IA2 and 160 μs for cytochrome P450IIB4. The two cytochrome P450s have shown very different interactions with cytochrome b_5 and NADPH−cytochrome P450 reductase. By the presence of the redox partner, the mobile population of cytochrome P450IA2 was increased significantly from 80% to 96% (plus cytochrome b_5) and to 89% (plus NADPH−cytochrome P450 reductase) due to dissociation of P450 oligomers. On the other hand, the mobility of cytochrome P450IIB4 was not considerably affected by the presence of cytochrome b_5 or NADPH−cytochrome P450 reductase as judged by little difference in ϕ and r_3, keeping the mobile population of 100%. These results imply that cytochrome P450IA2 forms a transient association with cytochrome b_5 and NADPH−cytochrome P450 reductase. Taking together biochemical experiments, it is suggested that cytochrome P450IIB4 would associate transiently with cytochrome b_5 and that cytochrome P450IIB4 would diffuse independently of NADPH−cytochrome P450 reductase. Further analysis showed that the tilt angle of the heme plane from the membrane plane was either 47° or 63° for cytochrome P450IA2 and 55° for cytochrome P450IIB4.

Cytochrome P450 is the terminal enzyme of the hepatic microsomal monooxygenase systems, catalyzing the oxidative metabolism of various drugs, xenobiotics as well as endogenous substrates (Estabrook et al., 1979; White & Coon, 1980; Yang & Lu, 1987). The monooxygenase systems consist of several membrane proteins such as NADPH−cytochrome P450 reductase, NADH−cytochrome b_5 reductase, cytochrome b_5, and cytochrome P450.

Liver microsomal cytochrome P450 is inducible by various drugs. In rabbit liver microsomes, two representative cytochromes, P450IA2[1] and P450IIB4, are induced by methylcholanthrene and phenobarbital, respectively. Such cytochrome P450 induction is known to be a key factor in monooxygenase activity upon administration of various kinds of drugs to animals (Omura, 1978; Harada & Omura, 1981). Cytochrome P450IA2 catalyzes preferably aryl hydrocarbon hydroxylation of benzo[*a*]pyrene, whereas cytochrome P450IIB4 preferably metabolizes N-demethylation of benzphetamine and aminopyrene. Many investigations have shown that there are considerable differences present between these two cytochrome P450s concerning hydrophobicity and physicochemical properties. Cytochrome P450IIB4 is stable and highly soluble in detergent solution, and therefore this cytochrome has been extensively examined in terms of interactions with redox partners, membrane topology, and drug oxidation activity not only in detergent solutions but also in phospholipid vesicles (Davydov et al., 1992; Nishimoto et al., 1983; Sevrukova et al., 1994; Voznesensky & Schenkman, 1992, 1994). Cytochrome P450IA2 is very hydrophobic and was not, so far, successfully incorporated in phospholipid vesicles until the present work. The low solubility of cytochrome P450IA2 in detergent solution prevents quantitative analysis concerning redox interactions, drug oxidation activity, and topology due to forming large aggregates (Bachmanova et al., 1994; Wagner et al., 1987).

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[1] Abbreviations: L/P450, lipid-to-P450 weight ratio; cytochrome P450IA2, major type of rabbit liver cytochrome P450 in 3-methylcholanthrene-induced microsomes; cytochrome P450IIB4, major type of rabbit liver cytochrome P450 in phenobarbital-induced microsomes; cytochrome P450B1, cytochrome P450IIB4 in gene classification, purified from noninduced rabbit liver microsomes; cytochromes P450XVIIA1(17α-lyase) and P450XXIA1(C21), cytochrome P450s in adrenocortical microsomes catalyzing steroid 17α-hydroxylation and steroid 21-hydroxylation, respectively.

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Protein−protein interactions of cytochrome P450 have been extensively examined in microsomes and phospholipid vesicles by observing rotational diffusion of cytochrome P450 (Etter et al., 1991; Gut et al., 1982; Kawato et al., 1982; Ohta et al., 1992). It has been demonstrated that NADPH−cytochrome P450 reductase forms a transient association with rat liver microsomal cytochrome P450IIB1/IIB2 and bovine adrenocortical cytochrome P450XXIA1 in lipid vesicles and with genetically expressed rat liver cytochrome P450IA1 in yeast microsomes (Iwase et al., 1991). On the other hand, P450 reductase did not form such an association with bovine adrenocortical microsomal cytochrome P450XVIIA1 (Ohta et al., 1992). These results imply that the mode of interactions of cytochrome P450 with P450 reductase is significantly different depending on the chemical species of cytochrome P450.

Although many investigations imply that cytochrome b_5 stimulates several drug oxidations of cytochromes P450IIB4 and P450IIB1 (Pompon, 1987; Tamburini et al., 1985), the interactions of cytochrome b_5 with cytochrome P450s have not been much examined physicochemically (Bosterling & Trudell, 1982). Structure and membrane topology of cytochrome b_5 are revealed in detail with crystallography (Mathews et al., 1972) and biochemical investigations (Vergeres & Waskell, 1992). Wobbling motion of cytochrome b_5 was investigated to be at least 10 times more rapid than that of cytochrome P450 rotation in liposomes (Vaz et al., 1979).

In the present study, we have examined different dynamic interactions of cytochromes P450IA2 and P450IIB4 with their redox partners of cytochrome b_5 and NADPH−cytochrome P450 reductase in phospholipid vesicles. Evidence is presented implying that both cytochrome b_5 and cytochrome P450 reductase dissociate cytochrome P450IA2 oligomers, resulting in formation of small rotating complexes in which electron transfer may occur. Present results on cytochrome P450IIB4 rotation are consistent with previous suggestions that in detergent solution cytochrome P450IIB4 forms association with cytochrome b_5 but not with NADPH−cytochrome P450 reductase.

EXPERIMENTAL PROCEDURES

Preparation of Proteins. Cytochrome P450IA2 was prepared from liver microsomes of methylcholanthrene-treated rabbits (New Zealand) according to the method of Alterman and Dowgii (Alterman & Dowgii, 1990). Cytochrome P450IIB4 was purified from liver microsomes of phenobarbital-induced male rabbits (New Zealand) according to the method of Imai et al. (1980). NADPH−cytochrome P450 reductase was purified from liver microsomes of phenobarbital-treated rabbits according to Yasukochi and Masters (1976). Cytochrome b_5 was prepared from liver microsomes of rabbits (New Zealand) with the method of Spatz and Strittmatter (1971).

Other Substances. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were purchased from Lipid Products (U.K.). BCA protein assay reagent was from Pierce Chemical Co. (Rockford, IL). Benzphetamine and benzopyrene were from Sigma. Other chemicals were of the highest grade commercially available.

Preparation of Proteoliposomes. Proteoliposomes were prepared from phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) at a molar ratio of 10:5:1, which is comparable with the lipid composition of liver microsomes (Gut et al., 1982). Phospholipids in chloroform were placed in a glass flask, the solvent was evaporated under a stream of nitrogen, and the mixture was further dried under vacuum for 1 h. The lipids were dispersed by sonication (XL2020 instrument, Heat System-Ultrasonic Inc.) in 50 mM Hepes buffer, pH 7.4, containing 1 mM EDTA, 0.1 mM dithiothreitol, 20% (w/w) glycerol, and 10% (w/v) sodium cholate; thereafter, purified enzymes and buffer were added to give 0.8 mg of cytochrome P450/mL, a lipid to cytochrome P450 weight ratio (L/P450) of 2:1 or 10:1, and 2% sodium cholate. The mixture was incubated overnight at 4 °C. The resulting clear solution was dialyzed for 7 h at 4 °C against a 200-fold volume of 50 mM Hepes buffer, pH 7.4, containing 150 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, and 20% glycerol. The dialysis buffer was changed twice. Then the mixture was dialyzed against the same buffer without 150 mM KCl for 3 h. For co-reconstitution of cytochrome b_5 or NADPH−cytochrome P450 reductase with cytochrome P450, the proteins were incorporated with a molar ratio of 1:1.

Incorporation of proteins in PC/PE/PS vesicles was examined by sucrose density gradient ultracentrifugation followed by spectroscopic measurements and activity assay. Proteoliposomes were layered on to a discontinuous sucrose density gradient (10%, 15%, 20%, 25%, 30%, and 35% sucrose (w/w) in Hepes buffer) in aliquots of 0.6 mL containing 2−3 nmol of heme of cytochrome P450/tube (4.2 mL) and centrifuged at 170000*g* for 19 h at 4 °C.

Drug Oxidation Activity of Cytochrome P450. Benzopyrene hydroxylation activity of cytochrome P450IA2 in phospholipid vesicles was measured according to Yang and Kicha (1978) as follows. The reaction was initiated by the addition of 40 μM NADPH (final concentration) to the mixture containing 100 mM potassium phosphate buffer, pH 7.4, 1.1 μM benzopyrene, and 1.0 μM cytochrome P450 at 37 °C. Benzopyrene fluorescence was measured using 387 nm for excitation and 407 nm for emission wavelengths. NADPH-dependent benzphetamine N-demethylation activity of cytochrome P450IIB4 was determined in proteoliposomes by measuring the formation of formaldehyde according to Kanaeva et al. (1992) and Nash (1953). Proteoliposomes were incubated in 100 mM potassium phosphate buffer, pH 7.4, containing 2 mM benzphetamine and 1 μM cytochrome P450 at 30 °C for 10 min with continuous shaking. The reaction was initiated by the addition of 1 mM (final concentration) NADPH.

NADPH-Dependent Cytochrome c Reduction Activity. Ferricytochrome *c* reduction activity of NADPH−cytochrome P450 reductase was measured according to Phillips and Langdon (1962). Increase in absorbance at 550 nm was measured after the addition of NADPH−cytochrome P450 reductase in proteoliposomes to 50 mM potassium phosphate buffer, pH 7.7, containing 50 μM ferricytochrome *c* as an electron acceptor and 100 μM NADPH.

Rotational Diffusion Measurements and Analysis. For rotational diffusion measurements, 58% (w/w) sucrose was dissolved in proteoliposome suspensions (50 mM Hepes buffer, pH 7.4, 1 mM EDTA, 0.1 mM dithiothreitol, and 20% (v/v) glycerol) in order to reduce light scattering and liposome tumbling. The sample was bubbled with CO for 5 s and then reduced with a few grains of dithionite.

The sample cuvette was then sealed by a rubber cap to keep CO concentration constant.

The time-resolved flash photolysis depolarization measurements were performed as described elsewhere (Cherry, 1978; Kawato et al., 1988; Ohta et al., 1990). The sample (3−5 μM heme) was photolyzed by a vertically polarized flash at 532 nm from a Nd/YAG laser, and absorbance changes were measured at 450 nm. The signals were analyzed by calculating the absorption anisotropy, $r(t)$, and the total absorbance change, $A(t)$, given by

$$r(t) = [A_V(t) - A_H(t)S]/A(t) \quad (1)$$

$$A(t) = A_V(t) + 2A_H(t)S \quad (2)$$

where $A_V(t)$ and $A_H(t)$ are, respectively, absorption changes for vertical and horizontal polarization at time t after laser flash. A slight unbalance of two photomultipliers is corrected using $S = A_{HV}/A_{HH}$, which is the ratio of time-averaged absorption changes of vertical and horizontal components obtained with horizontal flash excitation. In each experiment, 16 384 signals were averaged using a Toyo Technica 2805 transient memory.

Analysis of $r(t)$ is based on a model of the axial rotation of cytochrome P450 about the membrane normal (Kawato & Kinosita, 1981; Kawato et al., 1982). When there is a single rotating species of cytochrome P450 with rotational relaxation time $\phi_{||}$, $r(t)$ is given by

$$r(t)/r(0) = 3 \sin^2 \theta_N \cos^2 \theta_N \exp(-t/\phi_{||}) + {}^3/_4 \sin^4 \theta_N \exp(-4t/\phi_{||}) + {}^1/_4(3 \cos^2 \theta_N - 1)^2 \quad (3)$$

where θ_N is the tilt angle of the heme plane from the membrane plane. Multiple rotating species of cytochrome P450 with different $\phi_{||}$ are considered by analyzing the data via

$$r(t) = r_1 \exp(-t/\phi) + r_2 \exp(-4t/\phi) + r_3 \quad (4)$$

where ϕ is the average rotational relaxation time over multiple rotating species of P450 and r_1, r_2, and r_3 are constants. Curve fitting of the data based on eq 4 was accomplished by a PDP-11/73 minicomputer. It should be noted that in eqs 3 and 4, $r(t)/r(0)$ does not depend on the intensity of the photoselecting flash and $r(0)$ only depends on the laser flash intensity (Kawato & Kinosita, 1981). The population of mobile P450, p_m (%), was calculated with eq 5 on the basis of the experimentally determined minimal anistropy, $[r_3/r(0)]_{min} = {}^1/_4(3 \cos^2 \theta_N - 1)^2$, when all P450s were rotating in proteoliposomes:

$$p_m\,(\%) = 100[1 - r_3/r(0)]/[1 - [r_3/r(0)]_{min}] \quad (5)$$

Other Methods. Cytochrome P450 and cytochrome b_5 were measured spectrophotometrically with a Shimazu MPS-2000 spectrophotometer according to Omura and Sato (1964). NADPH−cytochrome P450 reductase was measured from the absorbance at 456 nm in the absolute spectrum using an extinction coefficient of 21.4 cm^{-1} mM^{-1} (Iyanagi & Mason, 1973). Protein concentration was determined with the BCA protein assay using bovine serum albumin as standard. The diameters of proteoliposomes were determined for negatively stained samples in a JEM-1200EX electron microscope.

RESULTS

Characterization of Proteoliposomes. The formation of PC/PE/PS vesicles and the incorporation of proteins in these vesicles were confirmed by sucrose density gradient centrifugation and negatively stained electron micrographs. On sucrose density gradient, a single band was observed for all proteoliposomes examined. The presence of NADPH−cytochrome P450 reductase or cytochrome b_5, at an equimolar amount to cytochrome P450, displaced the position of the band to ca. 30% sucrose (w/w) from ca. 20% sucrose for pure P450 vesicles of L/P450 = 2. Comigration of phospholipids and cytochrome P450 was determined with the white light scattering by phospholipids and the reddish color of the absorption of the cytochrome. The coincorporation of cytochrome P450 and NADPH−cytochrome P450 reductase in the same vesicles was demonstrated by the high activity values of NADPH-dependent benzphetamine N-demethylation for cytochrome P450IIB4, NADPH-dependent 3,4-benzopyrene hydroxylation for cytochrome P450IA2, and NADPH-dependent cytochrome c reduction for P450 reductase in the proteoliposome band which were obtained from the sucrose density gradient procedures. NADPH-dependent benzphetamine N-demethylation activity of cytochrome P450IIB4 was 8.5 and 7.3 nmol of formaldehyde/min/nmol of P450IIB4 before and after the sucrose density gradient centrifugation, respectively, indicating that cytochrome P450IIB4 and P450 reductase actively interact in the same vesicles. NADPH-dependent benzopyrene hydroxylation activity of cytochrome P450IA2 remained ca. 3.0 relative units of intensity/min/nmol of P450IA2 before and after sucrose density gradient centrifugation, indicating that cytochrome P450IA2 functionally interacts with P450 reductase. The coincorporation of cytochrome P450 and cytochrome b_5 in the same vesicle was analyzed spectrophotometrically. Cytochrome P450 to cytochrome b_5 molar ratio was observed to be 1:1 for the proteoliposome band obtained from the sucrose density gradient procedures for both cytochromes P450IA2 and P450IIB4. The average diameter of proteoliposomes was ca. 80 nm as judged from the negatively stained electron micrographs.

Rotational Diffusion of Cytochrome P450 in Lipid Vesicles. Cytochrome P450 was reconstituted in PC/PE/PS = 10:5:1 (wt ratio) vesicles. Rotational diffusion measurements were performed at 20 °C. In all samples examined, the absorption anisotropy, $r(t)$, decayed within 1 ms to a time-independent value, r_3. The $r(t)$ curves were analyzed by eq 4 on the basis of the rotation about the normal axis of the membrane plane. Decay parameters are summarized in Table 1. The rotational relaxation time, ϕ, (inversely proportional to the rate of rotation) is between 160 and 299 μs.

Orientation of the Heme Plane of Cytochromes P450IA2 and P450IIB4. The tilt angle, θ_N, of cytochromes P450IIB4 and P450IA2 was determined in vesicles containing only one of these P450s. The observed $r_3/r(0)$ of 0.00 for cytochrome P450IIB4 in L/P450 = 2 vesicles implies that all cytochrome P450IIB4 molecules rotate in vesicles, and θ_N is 55° ± 1° from the minimal anisotropy of $[r_3/r(0)]_{min} = {}^1/_4(3 \cos^2 \theta_N - 1)^2 = 0.00 \pm 0.01$ (eq 3). In order to determine the heme angle θ_N for P450IA2, cytochrome P450IA2 incorporated

Table 1: Decay Parameters of the Time-Dependent Absorption Anisotropy of the Cytochrome P450•CO Complex in Proteoliposomes Analyzed by Eq 4 (All measurements were performed in 58% sucrose plus 11% glycerol at 20 °C)

enzyme in vesicles	ϕ (μs)	$r_3/r(0)$	mobile[a] P450 (%)	L/P450
P450IA2	237	0.23	80	2
	(55)[b]	(0.04)	(4)	
P450IA2 + 800 mM KCl[c]	172	0.04	100	10
	(51)	(0.02)	(2)	
P450IA2 + cytochrome b_5	249	0.08	96	2
	(27)	(0.03)	(3)	
P450IA2 + reductase	299	0.15	89	2
	(40)	(0.04)	(4)	
P450IIB4	160	0.00	100	2
	(18)	(0.02)	(2)	
P450IIB4 + cytochrome b_5	196	0.02	98	2
	(29)	(0.02)	(2)	
P450IIB4 + reductase	190	0.02	98	2
	(29)	(0.01)	(1)	

[a] The percentage of mobile cytochrome P450 was calculated from eq 5. [b] Numbers in parentheses are standard deviations in 10−40 experiments. [c] Cytochrome P450IA2 in proteoliposomes was incubated for 30 min at room temperature in the presence of 800 mM KCl. Redox partners (reductase and cytochrome b_5) were incorporated with a molar ratio of 1:1 to P450.

in L/P450 = 10 vesicles was completely mobilized by incubating vesicles with 800 mM KCl for 30 min at room temperature (Ohta et al., 1991; Etter et al., 1991). The final KCl concentration during measurements was 433 mM after sucrose was dissolved in the proteoliposome solution. The observed value of $[r_3/r(0)]_{min} = 0.04 \pm 0.01$ implies that θ_N of P450IA2 is either 47° or 63° ± 1° (see Figure 1A). The percentage of mobile population of cytochrome P450 was calculated on the basis of eq 5. It was observed that 80% of cytochrome P450IA2 was mobile with ϕ = 237 μs, and the rest was immobile (ϕ > 10 ms) in PC/PE/PS vesicles of L/P450 = 2 in the absence of KCl; 20% of immobile population reflects microassociation of cytochrome P450IA2, while all cytochrome P450IIB4 was mobile with ϕ = 160 μs in phospholipid vesicles, even when no KCl was added.

Influence of Cytochrome b_5 and NADPH−Cytochrome P450 Reductase on Rotational Mobility of Cytochrome P450 in Lipid Vesicles. An equimolar amount of either cytochrome b_5 or NADPH−cytochrome P450 reductase to cytochrome P450 (P450IA2 or P450IIB4) was co-reconstituted in PC/PE/PS vesicles at L/P450 = 2 in weight. A significant mobilization of cytochrome P450IA2 was demonstrated by the presence of either cytochrome b_5 or NADPH−cytochrome P450 reductase (see Figure 1B). As calculated from eq 5, the decrease of $r_3/r(0)$ from 0.23 to 0.08 (plus cytochrome b_5) and to 0.15 (plus P450 reductase) corresponds to the increase in the mobile population from 80% to 96% (plus cytochrome b_5) and 89% (plus p450 reductase). The rotational relaxation time, ϕ, was slightly increased from 237 to 249 μs (plus cytochrome b_5) or 299 μs (plus P450 reductase). On the other hand, as judged from little change in $r_3/r(0)$ and the mobile percentage of P450 in Table 1, the mobility of cytochrome P450IIB4 in proteoliposomes was not significantly affected by the presence of cytochrome b_5 or NADPH−cytochrome P450 reductase, except that a slight increase in ϕ was observed from 160 to 190−196 μs (see Figure 2).

Rebinding Kinetics of CO to Reduced P450. The total absorption decay, $A(t)$, of cytochrome P450 was close to

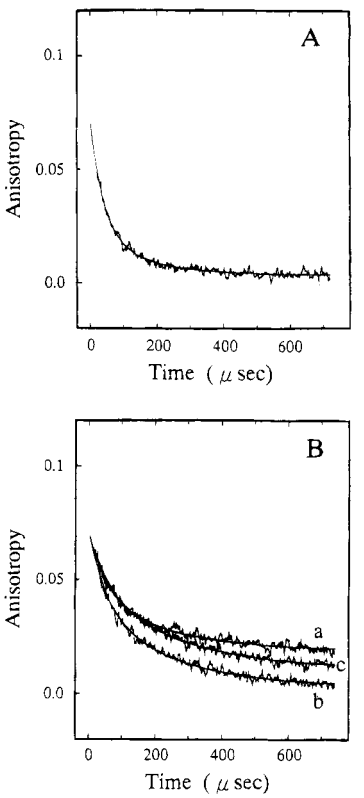

FIGURE 1: Time-dependent absorption anisotropy of cytochrome P450IA2 in phospholipid vesicles. Samples (3−5 μM in heme) were photolyzed by a vertically polarized laser flash at 532 nm, and $r(t)$ was recorded at 450 nm as described under Experimental Procedures. Measurements were performed in 58% sucrose solution at 20 °C (~0.6 poise). A: P450IA2 in L/P450 = 10 vesicles incubated with 800 mM KCl for 30 min at room temperature. B: Curve a, P450IA2; curve b, P450IA2 plus cytochrome b_5; curve c, P450IA2 plus NADPH−cytochrome P450 reductase. A linear scale is chosen for anisotropy. The zigzag lines are experimental data, and the solid curves were obtained by fitting the data to eq 4. The initial anisotropies of curves b and c are slightly normalized to the same $r(0)$ of curve a to facilitate comparison. This is justified by the fact that although $r(0)$ depends on the laser flash intensity, the normalized anisotropy $r(t)/r(0)$ is not affected by the different flash intensity (Kawato & Kinosita, 1981).

monophasic in proteoliposomes under the same conditions as that of the rotational diffusion measurements. $A(t)$ was, therefore, analyzed by a monoexponential approximation. In P450 vesicles, the lifetime of photodissociated P450IA2, obtained from $A(t)$, was τ = 7 ms, and this number was much greater than τ = 4 ms for P450IIB4. These results are consistent with the previous findings that methylcholanthrene-inducible P450IA1/IA2 had a much longer lifetime of τ = 7 ms than the τ = 4 ms of phenobarbital-inducible P450IIB1/IIB2 from rat liver microsomes (Etter et al., 1991). Coexistence of cytochrome b_5 with P450 did not considerably alter the lifetimes for both P450IA2 and P450IIB4. The presence of NADPH−cytochrome P450 reductase also did not alter the CO rebinding kinetics of both cytochrome P450s. These results suggest that there is no direct electronic interaction affecting the CO recombination between cytochrome P450 and cytochrome b_5 and between cytochrome P450 and P450 reductase. It should be noted that the incubation of P450 vesicles with KCl did not affect the CO recombination kinetics.

Absence of Liposome Tumbling. As reported previously (Gut et al., 1983; Etter et al., 1991), no significant anisotropy

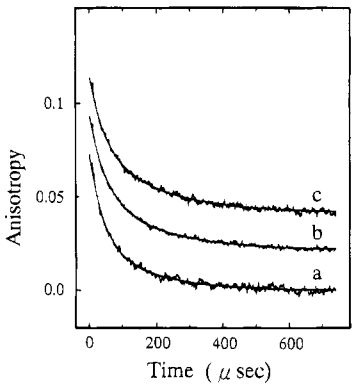

FIGURE 2: Time-dependent absorption anisotropy of cytochrome P450IIB4 in phospholipid vesicles. Measurements were performed under the same conditions as stated in Figure 1. Curve a, P450IIB4; curve b, P450IIB4 plus cytochrome b_5; curve c, P450IIB4 plus NADPH−cytochrome P450 reductase. A linear scale is chosen for anisotropy. The zigzag lines are experimental data, and the solid curves were obtained by fitting the data to eq 4. The initial anisotropies of curves b and c are vertically displaced for illustrative purposes; otherwise, curves b and c are almost completely superimposed on curve a.

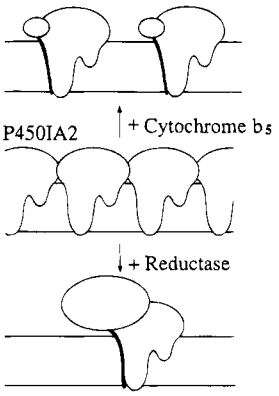

FIGURE 3: Model for electron transfer interactions of cytochrome P450IA2 with redox partners. Schematic model illustrating the protein−protein interactions of cytochrome P450IA2 with cytochrome b_5 and NADPH−cytochrome P450 reductase in proteoliposomes. Cytochrome P450IA2 forms a transient association with cytochrome b_5 and NADPH−cytochrome P450 reductase, resulting in mobilization of P450IA2 by dissociating immobile aggregates which may be larger than 10-fold of monomeric P450 in diameter.

decay was observed for phenobarbital-inducible rat liver P450IIB1/IIB2 incorporated in dipalmitoylphosphatidylcholine liposomes, having a diameter around 50 nm, at 20 °C in the crystalline state of the lipid bilayer. These results exclude the possibility of liposome tumbling contributing to the observed decay in $r(t)$. Since liposome tumbling depends on the diameter of the liposomes, the present liposomes (P450IA2 and P450IIB4) of 80 nm in diameter should tumble slower than immobile dipalmitoylphosphatidylcholine liposomes of 50 nm.

DISCUSSION

Here we discuss transient association and independent diffusion of redox partners, the illustrations of which are deduced from present protein rotation results and previous biochemical and biophysical investigations.

Mobile and Immobile Populations of Cytochrome P450. A total of 20% of cytochrome P450IA2 is immobile, having a rotational relaxation time, ϕ, larger than 10 ms, while all cytochrome P450IIB4 molecules rotate as small rotamers. The immobile molecules of P450IA2 probably form microaggregates because theoretically the population rotating very slowly with a large ϕ should have a large size. The rotational relaxation time $\phi_{||}$ in eq 3 is expressed as $\phi_{||} = 4\pi a^2 h\eta/kT \propto a^2$, where a is the radius of the cross section of cylindrical protein immersed in the membrane, h is the length immersed in the membrane, η is the membrane viscosity, k is the Boltzmann constant, and T is the absolute temperature. We can estimate an approximate radius, a_1, of the immobile oligomer having $\phi_1 \geq 10$ ms to be $a_1/a_2 \geq [10 \times 10^3\ \mu s/40\ \mu s]^{1/2} = 250^{1/2} = 15.8$-fold, where a_2 is the radius of the monomeric P450 with a ϕ value of 40 μs (Gut et al., 1982). Therefore, the immobile P450s are microaggregates having a diameter of more than 16-fold of that of the monomer. However, considering the relatively small size of liposomes with a circumference of ca. 250 nm (80 nm in diameter) and the size of monomeric P450 of 4 nm in diameter, even smaller oligomers of 10−16-fold of monomeric diameter would be already immobile due to steric hindrance. There are many semiquantitative investigations indicating that immobile proteins form microaggregates for cytochrome oxidase and bacteriorhodopsin by comparison of rotation data with pictures of freeze-fracture electron microscopy (Kawato et al., 1981). From similar estimation, the ϕ values of 237 and 160 μs for rotating cytochromes P450IA2 and P450IIB4, respectively, imply the existence of oligomeric rotamers (e.g., 2−3-fold of monomeric P450IA2 in diameter and 2-fold of monomeric P450IIB4 in diameter) in proteoliposomes. It should be noted that ϕ represents the average of rotational relaxation times of several protein associations.

The observed difference in the population of immobile aggregates between cytochromes P450IA2 and P450IIB4 in membranes probably reflects the highly hydrophobic character of cytochrome P450IA2 and the low hydrophobicity of cytochrome P450IIB4. It was observed that cytochrome P450IA2 needed a very high concentration of Emulgen 913 to monomerize, whereas cytochrome P450IIB4 was monomerized by a relatively low concentration of Emulgen (Bachmanova et al., 1994). The average hydrophobicity index of cytochrome P450IA2 calculated from an amino acid sequence is −0.21 which is much lower than −0.09 for cytochrome P450IIB4, indicating the high hydrophobicity of P450IA2 as compared with P450IIB4 (Suzuki & Mitaku, 1993).

Mobility and Redox Interactions of Cytochrome P450 with Cytochrome b_5 and NADPH−Cytochrome P450 Reductase. NADPH−cytochrome P450 reductase and cytochrome b_5 mobilized cytochrome P450IA2 by 9% and 16% in phospholipid vesicles, respectively. This number for NADPH−cytochrome P450 reductase is similar to the 10% mobilization of cytochrome P450IIB1/IIB2 by the addition of NADPH−cytochrome P450 reductase in proteoliposomes (Gut et al., 1982). The mobilization effect on cytochrome P450IA2 was greater by the presence of cytochrome b_5 than by the presence of P450 reductase. These results suggest that cytochrome P450IA2 forms a transient association not only with P450 reductase but also with cytochrome b_5 (see Figure 3). Upon formation of this transient association, probably the immobile aggregates of P450IA2 dissociated into small rotamers, resulting in the mobilization of the cytochrome. These interpretations are derived from our previous observations that immobile aggregates of several

microsomal cytochrome P450s (e.g., P450IIB1/IIB2, P450IA1, and P450XXIA1) were mobilized by the presence of NADPH−cytochrome P450 reductase due to forming a transient association with P450 reductase, as judged from rotation experiments in combination with antireductase antibody-induced cross-linking (Gut et al., 1983; Iwase et al., 1991; Ohta et al., 1992). The associated two proteins (e.g., P450IA2 + cytochrome b_5 and P450IA2 + P450 reductase) could be present in the rotamers in a one to one stoichiometry or, also, in an odd ratio. Although the accuracy of our data and the theoretical models available for analysis of the motion of membrane proteins do not allow an unequivocal decision between the two alternatives, we favor the second alternative. Considerable mobilization due to dissociation of aggregated cytochrome P450IIB4 could not occur, since all cytochrome P450IIB4 molecules rotate in liposomes. There may be two possible explanations for this little mobility change for cytochrome P450IIB4. One explanation is that cytochrome P450IIB4 would diffuse independently of cytochrome b_5 and NADPH−cytochrome P450 reductase, and the other is that the possible increase in the membrane-embedded size of cytochrome P450IIB4 might be very small even after forming association with cytochrome b_5 or P450 reductase.

Archakov and co-workers (Bachmanova et al., 1994; Kanaeva et al., 1992; Sevrukova et al., 1994) performed the carbodiimide-induced cross-linking experiments for cytochrome P450 with NADPH−cytochrome P450 reductase and cytochrome b_5 in Emulgen 913 solutions where all these proteins are in monomeric form. On SDS−PAGE analysis, cytochrome P450IA2 was demonstrated to form significant cross-linked products with both cytochrome b_5 (ca. 40%) and P450 reductase (ca. 20%). On the other hand, it was observed that cytochrome P450IIB4 did not form cross-linked products with P450 reductase, but cytochrome P450IIB4 formed cross-linked products with cytochrome b_5 (ca. 20%). It was also demonstrated by Schenkman and co-workers (Tamburini et al., 1986; Tamburini & Schenkman, 1987; Voznesensky & Schenkman, 1992) that cytochrome P450IIB4 was cross-linked with cytochrome b_5 but not with P450 reductase, when enzymes were mixed with phospholipid vesicles. These results of cross-linking experiments are qualitatively in agreement with the results of rotational diffusion concerning the association between P450 and the redox partners, though experimental conditions are different. In cross-linking experiments, even a 30-fold amount of detergent Emulgen or CHAPS, as compared with the amount used to monomerize P450IIB4, was needed to dissolve cytochrome P450IA2 to small oligomers or monomers, resulting in partial inactivation of P450 reductase and very low drug oxidation activity of the cytochrome (Sevrukova et al., 1994; Bachmanova et al., 1994). Taking together rotation and cross-linking experiments, it can be concluded that cytochrome P450IA2 forms an association with both NADPH−cytochrome P450 reductase and cytochrome b_5 and that cytochrome P450IIB4 does not form such an association with P450 reductase (Figure 3).

As judged from cross-linking experiments, cytochrome b_5 would form a transient association with cytochrome P450IIB4, but this association may not significantly affect the mobility of cytochrome P450IIB4 because of little additional increase in the membrane-embedded part of cytochrome P450IIB4 due to a thin membrane anchor of cytochrome b_5. Electron transfer would be performed within these associations between redox partners and cytochrome P450IA2 and between cytochromes b_5 and P450IIB4 (Figure 3). On the other hand, lateral collision-controlled electron transfer would be performed between NADPH−cytochrome P450 reductase and cytochrome P450IIB4. Significant effect of ionic strength on electron transfer and drug oxidation suggests that the interactions between cytochrome P450IIB4 and P450 reductase are not charge-pairing types but rather hydrophobic ones between hydrophilic head domains protruding in the water phase (Voznesensky & Schenkman, 1994). Participation of Lys residues in these interactions was also reported (Shen & Strobel, 1993). When cytochrome b_5 and NADPH−cytochrome P450 reductase associate with cytochrome P450IA2, hydrophobic domains immersed in the membrane may play an important role (Vergeres & Waskell, 1992), since the carbodiimide-induced cross-linking was not observed with cytochrome P450s for the hydrophilic head domains of cytochrome b_5 and P450 reductase prepared by trypsin digestion (Bachmanova et al., 1994).

The present result is the first demonstration indicating a direct transient association of cytochrome b_5 with cytochrome P450IA2 in the membrane, which sheds a light to illustrate molecular mechanisms of electron donation from cytochrome b_5 to cytochrome P450IA2. Since little is known about the effect of cytochrome b_5 on the activity of cytochrome P450IA2, the present liposome system serves a promising method to investigate these activity problems because of highly homogeneous distribution of cytochrome P450IA2 molecules close to monomeric form. Cytochrome b_5 has been reported to effect a stimulation of the NADH- plus NADPH-supported activity of cytochrome P450IIB4 for deethylation of 7-ethoxycoumarine and *p*-nitroanisole and cytochrome P450IIB1 for *p*-nitrophenetole deethylation (Aoyama et al., 1990; Pompon, 1987; Tamburini et al., 1985). Rabbit liver microsomal cytochrome P450B1 was also stimulated by cytochrome b_5 for deethylation of *p*-nitroanisole (Miki et al., 1980; Sugiyama et al., 1982). Cytochrome P450B1 was demonstrated to be tightly bound to cytochrome b_5 with electron spin resonance spectroscopy, and cytochrome P450B1 can be purified on affinity column chromatography using cytochrome b_5 (Miki et al., 1982).

Several questions remain to be answered concerning interactions among cytochrome P450, cytochrome b_5, and NADPH−cytochrome P450 reductase. One problem is that we measured dynamic interactions of proteins only in the reduced states. The affinity of cytochrome b_5 for cytochrome P450, for example, would probably be dependent on redox states. The affinity may be higher for oxidized P450IA2 than for reduced P450IA2 (also for reduced P450·CO) due to the necessity of binding of cytochrome b_5 to oxidized P450IA2 for electron donation. If so, the cytochrome b_5-induced dissociation effect on P450IA2 microaggregates might be even larger for oxidized P450IA2 than for reduced P450IA2. The reductase-induced dissociation effect on P450IA2 microaggregates might also be larger for oxidized P450IA2 than for reduced P450IA2 due to the same reason. There are so far few reports concerning the affinity between P450 (-IA2 and -IIB4) and its redox partners by changing their redox states, since it is experimentally difficult, for example, to form a stable mixture of reduced cytochrome b_5 and oxidized P450. It should be noted that, concerning adrenocortical mitochondrial P450scc, spectral investigations

demonstrated that adrenodoxin (electron donor for P450scc) has a higher affinity for oxidized P450scc than for reduced P450scc (Lambeth & Pember, 1983). If this relationship is applicable to the present P450 (-IA2 and -IIB4) and redox partners, it is not unrealistic to assume that the electron donor for P450 has a higher affinity for oxidized P450 than for reduced P450. Because of biological significance, dynamic interactions of P450 and redox partners should be extensively investigated under independently controlled redox states. The other questions are as follows. Does cytochrome b_5 associate with cytochrome P450IA2 to transfer an electron after dissociation of reductase from P450? Or do these three proteins form a ternary association to perform sequential electron donation? For this, we need to analyze protein rotation in liposomes containing all these three proteins together (e.g., P450, P450 reductase, and cytochrome b_5).

REFERENCES

Alterman, M. A., & Dowgii, A. J. (1990) *Biomed. Chromatogr. 4*, 221−222.

Aoyama, T., Nagata, K., Yamazoe, Y., Kato, R., Matsunaga, E., Gelboin, H. V., & Gonzalez, F. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5425−5429.

Bachmanova, G. I., Kanaeva, I. P., Sevrukova, I. F., Nikityuk, O. V., Stepanova, N. V., Knushko, T. V., Koen, Y. M., & Archakov, A. I. (1994) in *Cytochrome P450: Biochemistry, Biophysics and Molecular Biology* (Lechner, M. C., Ed.) pp 395−401, John Libbey, Eurotext, Paris, France.

Bosterling, B., & Trudell, I. R. (1982) *J. Biol. Chem. 257*, 4783−4787.

Cherry, R. J. (1978) *Methods Enzymol. 54*, 47−61.

Davidov, D. R., Darovsky, B. V., Dedinsky, I. R., Kanaeva, I. P., Bachmanova, G. I., Blinov, V. M., & Archakov, A. I. (1992) *Arch. Biochem. Biophys. 297*, 304−313.

Estabrook, R. W., Werringloer, J., & Peterson, J. A. (1979) in *Xenobiotic Metabolism: In Vitro Methods* (Paulson, G. D., Frear, D. S., & Marks, E. P., Eds.) Symposium Series No. 97, pp 149−179, American Chemical Society, Washington, DC.

Etter, H. U., Richter, C., Ohta, Y., Winterhalter, K. H., Sasabe, H., & Kawato, S. (1991) *J. Biol. Chem. 266*, 18600−18605.

Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., & Kawato, S. (1982) *J. Biol. Chem. 257*, 7030−7036.

Harada, N., & Omura, T. (1981) *J. Biochem. 89*, 237−248.

Heyn, M. P., Cherry, R. J., & Mueller, U. (1977) *J. Mol. Biol. 117*, 607−620.

Imai, Y., Hashimoto-Yutsudo, C., Satake, H., Girardin, A., & Sato, R., (1980) *J. Biochem. 88*, 489−503.

Iwase, T., Sakaki, T., Yabusaki, Y., Ohkawa, H., Ohta, Y., & Kawato, S. (1991) *Biochemistry 30*, 8347−8351.

Iyanagi, T., & Mason, H. S. (1973) *Biochemistry 12*, 2297−2308.

Kawato, S., Kinosita, K., Jr. (1981) *Biophys. J. 36*, 277−296.

Kawato, S., Sigel, E., Carafori, E., & Cherry, R. J. (1981) *J. Biol. Chem. 256*, 7518−7527.

Kawato, S., Gut, J., Cherry, R. J., Winterhalter, K. H., & Richter, C. (1982) *J. Biol. Chem. 257*, 7023−7029.

Kawato, S., Mitani, F., Iizuka, T., & Ishimura, Y. (1988) *J. Biochem. 104*, 188−191.

Knaeva, I. P., Nikityuk, O. V., Davydov, D. R., Dedinskii, I. R., Koen, Ya. M., Kuznetsova, G. P., Skotselyas, E. D., Bachmanova, G. I., & Archakov, A. I. (1992) *Arch. Biochem. Biophys. 298*, 403−412.

Kominami, S., Tagashira, H., Ohta, Y., Yamada, M., Kawato, S., & Takemori, S. (1993) *Biochemistry 32*, 12935−12940.

Lambeth, J. D., & Pember, S. O. (1983) *J. Biol. Chem. 258*, 5596−5602.

Mathews, F. S., Levine, M., & Argos, P. (1972) *J. Mol. Biol. 64*, 449−464.

Miki, N., Sugiyama, T., & Yamano, T. (1980) *J. Biochem. 88*, 307−316.

Miki, N., Miura, R., Sugiyama, T., Yamano, T., & Miyake, Y. (1982) *Biochem. Int. 5*, 511−517.

Mueller, M., Krebs, J. J. R., Cherry, R. J., & Kawato, S. (1984) *J. Biol. Chem. 259*, 3037−3043.

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

Nigg, E. A., & Cherry, R. J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4702−4706.

Nishimoto, Y., Kinosita, K., Jr., Ikegami, A., Kawai, N., Ichihara, I., & Shibata, Y. (1983) *Biochemistry 22*, 3586−3594.

Ohta, Y., Mitani, F., Ishimura, Y., Yanagibashi, K., Kawamura, M., & Kawato, S. (1990) *J. Biochem. 107*, 97−104.

Ohta, Y., Yanagibashi, K., Hara, T., Kawamura, M., & Kawato, S. (1991) *J. Biochem. 109*, 594−599.

Ohta, Y., Kawato, S., Tagashira, H., Takemori, S., & Kominami, S. (1992) *Biochemistry 31*, 12680−12687.

Ohta, Y., Sakaki, T., Yabusaki, Y., Ohkawa, H., & Kawato, S. (1994) *J. Biol. Chem. 269*, 15597−15600.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370−2378.

Omura, T. (1978) in *Cytochrome P450* (Sato, R., & Omura, T., Eds.) pp 1−22, Kodansha, Tokyo, Japan.

Pernecky, S. J., Fujita, V. S., Bestervelt, L. L., & Coon, M. J. (1994) in *Cytochrome P450: Biochemistry, Biophysics and Molecular Biology* (Lechner, M. C., Ed.) pp 285−292, John Libbey, Eurotext, Paris, France.

Phillips, A. H., & Langdon, R. G. (1962) *J. Biol. Chem. 237*, 2652−2660.

Pompon, D. (1987) *Biochemistry 26*, 6429−6435.

Sevrukova, I. F., Kanaeva, I. P., Koen, Y. M., Samenkova, N. F., Bachmanova, G. I., & Archakov, A. I. (1994) *Arch. Biochem. Biophys. 311*, 133−143.

Shen, S., & Strobel, H. W. (1993) *Arch. Biochem. Biophys. 304*, 257−265.

Spatz, L., & Strittmatter, P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1042−1046.

Sugiyama, T., Miki, N., Miyake, Y., & Yamano, T. (1982) *J. Biochem. 92*, 1793−1803.

Suzuki, K., & Mitaku, S. (1994) Master Thesis, Tokyo University of Agriculture and Technology, Tokyo, Japan.

Tamburini, P. P., & Schenkman, J. B. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 11−15.

Tamburini, P. P., White, R. E., & Schenkman, J. B. (1985) *J. Biol. Chem. 260*, 4007−4015.

Tamburini, P. P., MacFarquhar, S., & Schenkman, J. B. (1986) *Biochem. Biophys. Res. Commun. 134*, 519−526.

Vaz, W. L. C., Austin, R. H., & Vogel, H. (1979) *Biophys. J. 26*, 415−426.

Vergeres, G., & Waskell, L. (1992) *J. Biol. Chem. 267*, 12583−12591.

Vergeres, G., Winterhalter, K. H., & Richter, C. (1989) *Biochemistry 28*, 3650−3655.

Voznesensky, A. I., & Schenkman, J. B. (1992) *J. Biol. Chem. 267*, 14669−14676.

Voznesensky, A. I., & Schenkman, J. B. (1994) *J. Biol. Chem. 269*, 15724−15731.

Yang, C. S., & Kicha, L. P. (1978) *Anal. Biochem. 84*, 154−163.

Yang, C. S., & Lu, A. Y. H. (1987) in *Mammalian Cytochromes P450* (Guengerich, F. P., Ed.) Vol. II, pp 1−17, CRC Press, Boca Raton, FL.

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

Wagner, S. L., Dean, W. L., & Gray, R. D. (1987) *Biochemistry 26*, 2343−2348.

White, R. J., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315−356.

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