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Rotation of Cytochrome P-450

I. INVESTIGATIONS OF PROTEIN-PROTEIN INTERACTIONS OF CYTOCHROME P-450 IN PHOSPHOLIPID VESICLES AND LIVER MICROSOMES*

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Rotation of cytochrome P-450 was examined in both liver microsomes and reconstituted phospholipid vesicles. Purified cytochrome P-450 was incorporated into lipid vesicles composed of phosphatidylcholine-phosphatidylethanolamine-phosphatidylserine. Rotational diffusion was measured by detecting the decay of absorption anisotropy, $r(t)$, after photolysis of the heme·CO complex by a vertically polarized laser flash. No contribution of vesicle tumbling to $r(t)$ was observed over the experimental time range of 0-500 μs for samples in 60% sucrose. Analysis of $r(t)$ was based on a "rotation-about-membrane normal" model. The measurements were used to investigate intermolecular interactions of cytochrome P-450.

In vesicles of a high lipid to protein ratio (=30 by weight), the residual time-independent normalized anisotropy, $r(\infty)/r$ (0), reached a limiting low value, implying that all cytochrome P-450 was rotating. The mean rotational relaxation time, ϕ_1, was about 95 μs. In contrast, about 35% of cytochrome P-450 was immobilized in vesicles of a low lipid to protein ratio (=1), with ϕ_1 of about 95 μs for the mobile fraction. The immobile fraction is presumably due to self-aggregation of cytochrome P-450.

In rat liver microsomes, 0-50% of cytochrome P-450 was mobile with ϕ_1 of about 120 μs at 20 °C, and the rest was immobile. A significant temperature dependence of $r(t)$ was observed in microsomes. All cytochrome P-450 was immobile below 7 °C, and about 50% of the enzyme was mobile at 37 °C with $\phi_1 \sim 60$ μs. From the limiting value of $r(\infty)/r(0) \simeq 0.12$, the tilt angle of the heme plane of cytochrome P-450 from the membrane plane was calculated to be about 40°.

The hepatic microsomal monooxygenase system has been extensively studied due to its important function in the metabolism of xenobiotics and endogenous substrates and also its key role in activation of chemical carcinogens and cytotoxic chemicals (1-3). The monooxygenase system consists of several membrane enzymes such as NADPH-cytochrome P-450 reductase, cytochrome P-450, NADH-cytochrome b_5 reductase, and cytochrome b_5. The topological organization of these enzymes is important for the understanding of the mechanism of electron transfer and oxygenation reactions. Essentially two types of organization can be considered: in one, the enzymes are distributed randomly and their functions are controlled by lateral diffusion and collisions (4-6), and in the other the enzymes form functional "clusters" (7, 8).

Cytochrome P-450, a transmembrane protein, is the terminal and key enzyme of the monooxygenase system. Several different species of cytochrome P-450 with molecular weights between 45,000 and 60,000 have been identified in rat liver microsomes (9, 10). Cytochrome P-450 is believed to accept one electron from NADPH-cytochrome P-450 reductase and another electron from cytochrome b_5 (11, 12). Although the kinetics of electron transfer and hydroxylation of substrates for cytochrome P-450 have been extensively studied in both microsomal membranes and reconstituted lipid vesicles, few direct measurements of protein-protein interactions involving cytochrome P-450 have been reported.

Rotational mobility of cytochrome P-450 was recently demonstrated in microsomal membranes by transient dichroism techniques (13, 14), and in reconstituted lipid vesicles by delayed fluorescence polarization techniques (15). Protein rotation is particularly sensitive to protein-protein interactions (16, 17), and this technique has been successfully applied to investigate intermolecular interactions of cytochrome oxidase with cytochrome oxidase or with cytochrome bc_1 complex in both mitochondrial membranes and reconstituted vesicles (18, 19). The results lead to a better understanding of electron transfer in the mitochondrial respiratory chain.

Here we examine rotational diffusion and intermolecular interactions of rat liver cytochrome P-450 in both microsomal membranes and lipid vesicles. Rotational diffusion measurements of cytochrome P-450 are based on observing the flash-induced absorption ansiotropy of the heme·CO complex (13). Some subsidizing experiments with the triplet probe, eosin maleimide, were also carried out. We demonstrate the coexistence of mobile and immobile populations of cytochrome P-450 in liver microsomes and in lipid vesicles.

EXPERIMENTAL PROCEDURES

Materials—Microsomes were isolated from PB[1]-induced male rats (Wistar, Long-Evans, Sprague-Dawley, 200-300 g) as described by Remmer *et al.* (20) and resuspended in 50 mM Tris-HCl buffer (pH 7.8, 150 mM KCl and 10 mM $MgCl_2$).

Cytochrome P-450 was purified from the above microsomes (Sprague-Dawley) (21). Detailed purification procedures are described in an accompanying paper (22). The purified protein had 14.5-18 nmol heme/mg of protein and was suspended in 10 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.5% sodium

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[1] The abbreviations used are: PB, phenobarbital; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L/P, lipid to protein (w/w) ratio.

cholate, and 20% glycerol, and was stored at −80 °C. The concentration was determined using an extinction coefficient $\Delta\epsilon_{450\text{-}490\ nm}$ = 91 mM^{-1} (reduced + CO-reduced) (23).

Phospholipids (egg PC, egg PE, bovine spinal cord PS, egg PA, all Grade I) were purchased from Lipid Products (South Nutfield, U. K.).

Methods—For negative stain electron micrographs, samples were stained with phosphotungstate.

Preparation of Cytochrome P-450 Vesicles of L/P = 1-30—The procedure described by Bösterling *et al.* (24) was modified as follows: 10 mg of PC, 5 mg of PE, and 1 mg of PS were dissolved in chloroform/methanol, 1:1. The solvent was then removed with a N_2 stream. Lipids were further dried under vacuum for 5 h. Then, 1.6 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.16 ml of 20% (w/w) sodium cholate solution were added. After vortexing the dispersion, appropriate amounts of cytochrome P-450 were added. The lipid/protein/cholate mixture was equilibrated at 4 °C for 15 h and subsequently dialyzed at room temperature for 3 h against a 200-fold volume of 20 mM Hepes (pH 8.0) containing 20% glycerol, 0.1 mM EDTA, and 1 g of Bio-Beads per 100 ml of solution. The outer medium was changed after 90 min.

Other vesicle systems (*i.e.* PC/PE/PS = 1:1:1, PC/PE/PA = 10:5:0.3, and egg PC vesicles) were prepared similarly.

Rotational Diffusion Measurements—For rotational diffusion measurements, 60% (w/w) sucrose was dissolved in microsome suspensions (Tris-HCl, pH 7.8, containing 150 mM KCl and 10 mM $MgCl_2$) or proteoliposome suspensions (20 mM Hepes, pH 8.0, containing 20% glycerol and 0.1 mM EDTA). The final heme concentration was 2–8 μM (microsomes) or 5–13 μM (proteoliposomes). Samples were reduced by a few grains of dithionite and slowly bubbled for 1 min with CO. The sample cuvette was then sealed by a rubber cap to keep cytochrome P-450 saturated with CO.

The flash photolysis apparatus used for rotation measurements is described in detail elsewhere (25). Briefly, the sample was excited at 540 nm by a vertically polarized flash of duration 1–2 μs from a dye laser (Coumarin 6 in methanol). Absorbance changes due to photolysis of the heme·CO complex 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

$$r(t) = [A_V(t) - A_H(t)] / [A_V(t) + 2A_H(t)] \quad (1)$$

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

where $A_V(t)$ and $A_H(t)$ are, respectively, the absorbance changes for vertical and horizontal polarization at time t after the flash.

In each experiment, 128 signals were averaged using a Datalab DL 102A signal averager. A further improvement in the signal to noise ratio resulted from averaging the data of several experiments. The measurements were performed in 60% sucrose solution in order to reduce vesicle tumbling and light scattering. The spectral characteristics of the cytochrome P-450·CO complex excluded degradation of cytochrome P-450 (*e.g.* conversion of cytochrome P-450 into cytochrome P-420) during the experiments.

Rotation of eosin-labeled cytochrome P-450 (5–8 μM in eosin maleimide) was measured by observing flash-induced absorption anisotropy of the eosin probe at 520 nm (25).

Analysis of Absorption Anisotropy—Cytochrome P-450 is a transmembrane protein (26), implying that rotation occurs about the normal to the plane of the membrane. Thus, decays in absorption anisotropy, $r(t)$, were analyzed based on a rotation about membrane normal model (27). A theoretical treatment of this case for the heme·CO complex shows that the expected form of $r(t)$ is given by:

$$r(t)/r(0) = r(t)/0.1 = 3\sin^2\theta_N\cos^2\theta_N\exp(-t/\phi_\parallel) + \tfrac{3}{4}\sin^4\theta_N\exp(-4t/\phi_\parallel) + \tfrac{1}{4}(3\cos^2\theta_N - 1)^2 \quad (3)$$

where $\phi_\parallel$ is the rotational relaxation time, θ_N is the angle between the membrane normal axis and the normal to the heme plane ($\phi_\parallel = 1/D_\parallel$, where $D_\parallel$ is the rotational diffusion coefficient). In the case of 4-fold symmetry, θ_N is also the tilt angle of the heme plane with respect to the plane of the membrane.

Equation 3 expresses rotation of a single rotating species and assumes 4-fold symmetry of the heme plane. With a very weak excitation flash, we obtained $r(0)$ = 0.097. This figure is very close to the theoretical value of $r(0)$ = 0.1 for 4-fold symmetry, which therefore appears to be a valid assumption under our experimental conditions (*i.e.* excitation at 540 nm and measurement at 450 nm). However, assuming a single rotating species is not always correct. For example, different populations of cytochrome oxidase were observed in mitochondrial membranes and reconstituted lipid vesicles (18, 19). In the case of multiple populations, the experimental $r(t)$ is the weighed sum of the individual $r(t)$ curves with different $\phi_\parallel$. Although multiple populations are difficult to resolve, their existence can be inferred by fitting the data by the following generalized equation

$$r(t) = r_1\exp(-t/\phi_1) + r_2\exp(-4t/\phi_1) + r_3 \quad (4)$$

and comparing r_i (i = 1,2,3) with the theoretical values in Equation 3. Here ϕ_1 is an average value over all rotating species with different $\phi_\parallel$. When samples contain both mobile and immobile populations of heme proteins, the normalized residual anisotropy $r(\infty)/r(0) = r_3/r(0)$ is given by

$$r(\infty)/r(0) = (1 - f_{im})\cdot\tfrac{1}{4}(3\cos^2\theta_N - 1)^2 + f_{im}\cdot 1 \quad (5)$$

where f_{im} is the fraction of the immobile cytochromes. It should be noted that all mobile populations with different $\phi_\parallel$ have the same $\frac{1}{4}(3\cos^2\theta_N - 1)^2$. In the case of eosin probes, data were analyzed by Equation 4 according to the theory of linear chromophores (25, 27).

Curve fitting of the data by Equation 4 was accomplished by a Hewlett-Packard 9825A desk top computer. It should be noted that in Equation 4, the normalized anisotropy $r(t)/r(0)$ does not depend on the intensity of the excitation flash, however, $r(0)$ decreases when the intensity of photoselecting flash increases (27). In some figures, small variations in absolute values of $r(t)$ have been compensated by normalizing the curves to the same $r(0)$.

RESULTS AND ANALYSIS

All data in this section were obtained with photolysis of the heme·CO complex.

Rotational Diffusion of Cytochrome P-450 in Lipid Vesicles

Since the activity and conformation of cytochrome P-450 are affected by surrounding lipids (28, 29), we mainly use PC/PE/PS vesicles for reconstitution studies, corresponding to the major phospholipid components in liver microsomal membranes (26).

Effect of Lipid to Protein Ratio and the Tilt Angle of the Heme Plane from the Membrane Plane in PC/PE/PS = 10:5:1 Vesicles—Rotational diffusion of cytochrome P-450 in PC/PE/PS = 10:5:1 vesicles was measured at 20 °C with varying L/P. All curves examined decayed within 500 μs to a time-independent value $r(\infty)$ (see Fig. 1). Data were analyzed by Equation 4 and decay parameters are summarized in Table I. The normalized residual anisotropy $r(\infty)/r(0) = r_3/r(0)$ was dependent on L/P, while the mean rotational relaxation time ϕ_1 was relatively independent of L/P.

Increasing the L/P from 1 to 5 decreased $r_3/r(0)$ but little further decrease was observed from L/P = 5 to L/P = 30. $r_3/$

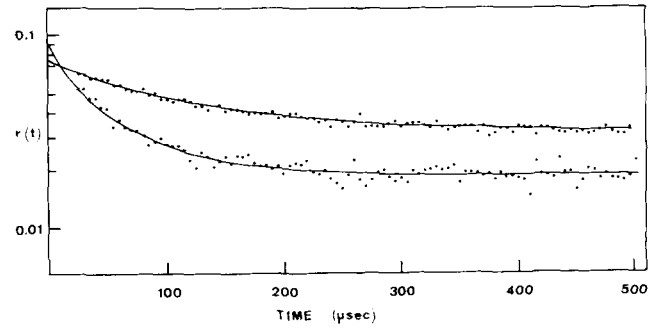

FIG. 1. **Time-dependent absorption anisotropy of the cytochrome P-450·CO complex in PC/PE/PS = 10:5:1 vesicles.** Samples (5–13 μM in heme) were photolyzed by a vertically polarized laser flash at 540 nm, and $r(t)$ was recorded at 450 nm as described under "Experimental Procedures." Measurements were performed in 60% sucrose solution at 20 °C (~0.6 poise). *Upper* and *lower curves* represent L/P = 1 and L/P = 10 vesicles, respectively. *Solid lines* were obtained by fitting the data to Equation 4.

TABLE I

Decay parameters of time-dependent absorption anisotropy of the cytochrome P-450·CO complex in a variety of proteoliposomes analyzed by Equation 4

All measurements were performed in 60% sucrose.

Vesicle preparation	L/P (w/w)	ϕ_1	$r(\infty)/r(0)$	% immobile P-450[a]	Temperature
		μs			°C
PC/PE/PS (10:5:1)	30	96 ± 50[b]	0.12 ± 0.04	0	20
	10	56 ± 4	0.18 ± 0.02	7 ± 2	20
	5	45 ± 4	0.13 ± 0.05	1 ± 6	20
	1	94 ± 24	0.43 ± 0.05	35 ± 5	20
PC/PE/PA (10:5:0.3)	10	56 ± 8	0.21 ± 0.01	10 ± 1	20
Egg PC	10	56 ± 7	0.20 ± 0.02	9 ± 2	20
PB-microsomes	0.4	63 ± 6	0.53 ± 0.09	47 ± 10	37
		120 ± 36	0.65 ± 0.08	60 ± 9	20[c]
			1.00	100	5

[a] The percentage of immobile population of cytochrome P-450 was calculated based on Equation 6.

[b] Standard deviation; n = 2-4.

[c] For PB-microsomes at 20°C, the average was performed for samples which have a mobile population of cytochrome P-450. Several samples showed complete immobilization of cytochrome P-450 at 20 °C.

$r(0)$ and the fraction of immobile cytochrome P-450 in Equation 5 are linearly related. The above change in $r_3/r(0)$ therefore suggests a significant increase in the proportion of mobile cytochrome P-450 upon increasing L/P from 1 to 5. The observation that there is little additional decrease in $r_3/r(0)$ upon further 6-fold dilution of the enzyme in lipid strongly suggests that nearly all cytochrome P-450 is already rotating in L/P = 5 vesicles. Progressive mobilization of cytochrome oxidase was also observed after enriching lipids from both mitochondrial inner membranes (30) and reconstituted vesicles (19). All cytochrome oxidase became mobile at L/P = 30 vesicles. From this we conclude that cytochrome P-450 is completely mobile in lipid-rich vesicles of L/P = 30.

When all cytochrome P-450 molecules are mobile, $r_3/r(0) = 0.12$ should be equal to $\frac{1}{4}(3\cos^2\theta_N - 1)^2$ in Equation 5 since $f_{im} = 0$. We calculated $\theta_N = 41°$ or 71°, which coincides with the angle between the heme plane and the membrane plane. Which of these figures is correct cannot be decided by the present $r(t)$ measurements alone.

In protein-rich vesicles of L/P = 1, a relatively large $r_3/r(0) = 0.43$ was observed, indicating the co-existence of both mobile and immobile populations. It should be noted that any rotating cytochrome P-450 population with $\phi_1 > 5$ ms would be detected as being immobile within the present experimental time range of 500 μs. The fraction of immobile cytochrome P-450 can be calculated by substituting $\frac{1}{4}(3\cos^2\theta_N - 1)^2 = 0.12$ into Equation 5:

$$r_3/r(0) = (1 - f_{im})\cdot 0.12 + f_{im} \quad (6)$$

From this about 35% of cytochrome P-450 appear immobile in L/P = 1 vesicles. Detailed analysis showed that $r_3/r(0)$ in L/P = 5 and L/P = 10 vesicles was slightly larger than 0.12, implying that about 1-7% of cytochrome P-450 is immobile in these vesicles. Over the range L/P = 1-30, ϕ_1 was 50-100 μs and showed no significant dependence on L/P.

Effect of Phospholipid Composition—Rotational diffusion was also examined in vesicles of different composition (see Fig. 2). No significant changes in decay parameters of $r(t)$ were observed between PC/PE/PA = 10:5:0.3 vesicles and PC/PE/PS = 10:5:1 vesicles both with L/P = 5 and 10. Also, the decay parameters of $r(t)$ in egg PC and PC/PE/PS = 10:5:1 vesicles both with L/P = 10 were not significantly different.

In PC/PE/PS = 1:1:1 vesicles (which have a high negative charge density), a partial conversion of cytochrome P-450 into cytochrome P-420 occurred. In this vesicle system, depending on individual preparations, around 20–60% of cytochrome P-450 was found to be immobilized and the rest was rotating with $\phi_1 \simeq 50$ μs.

Effect of Temperature—Lowering the temperature slowed down the rotation of mobile cytochrome P-450 and the immobile fraction became larger. For example, in PC/PE/PS = 10:5:1 vesicles with L/P = 5, the immobile population of cytochrome P-450 increased from ~1% to ~15% upon lowering the temperature from 20 °C to 7 °C, while the ϕ_1 increased from ≃50 μs to ≃200 μs.

Rotation of Cytochrome P-450 in Liver Microsomes

Rotation of cytochrome P-450 was measured in microsomal membranes at different temperatures. $r(t)$ curves decayed within 500 μs to a time-independent value. Data were analyzed by Equation 4 and decay parameters are summarized in Table I. Fig. 3 shows a significant temperature dependence of $r(t)$.

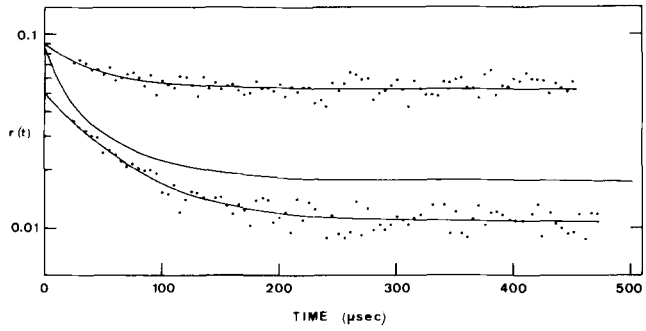

FIG. 2. **Time-dependent absorption anisotropy of the cytochrome P-450·CO complex in a variety of proteoliposomes with L/P = 10.** Proteoliposomes were prepared as described under "Experimental Procedures." Measurements were performed in 60% sucrose solution at 20 °C (~0.6 poise). *Upper curve,* PC/PE/PS = 1:1:1 vesicles. *Middle curve,* PC/PE/PA = 10:5:0.3 vesicles. *Lower curve,* egg PC vesicles. *Solid lines* were obtained by fitting the data to Equation 4. The initial anisotropy of the lower curve is artificially displaced to $r(0) = 0.05$ for illustrative purposes, in order to avoid overlap with the middle curve. Data points of the middle curve are omitted for clarity.

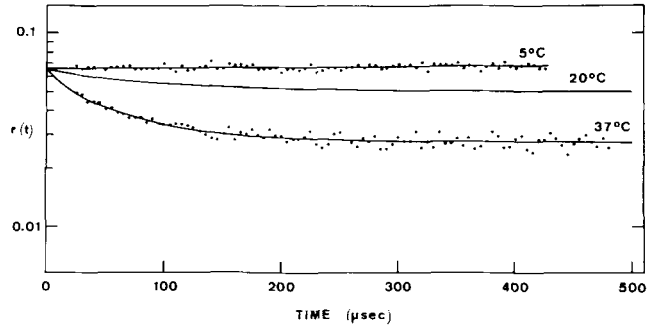

FIG. 3. **Time-dependent absorption anisotropy of the cytochrome P-450·CO complex in PB-microsomes at different temperatures.** Measurements were performed with 2–8 μM cytochrome P-450 in 60% sucrose solution. Temperatures are 5 °C (*upper curve*), 20 °C (*middle curve*), and 37 °C (*lower curve*). *Solid lines* are obtained by fitting the data to Equation 4. The curves have been normalized to the same value of $r(0)$ for illustrative purposes. Data points of the middle curve are omitted for clarity.

Although microsomal membranes are in the liquid-crystalline state above 0 °C as demonstrated by fluorescence polarization measurements with diphenylhexatriene,[2] all cytochrome P-450 was immobile below 5 °C. Co-existence of mobile and immobile populations of cytochrome P-450 was observed at 20 and 37 °C.

Although several different species of cytochrome P-450 exist in rat liver microsomes (9, 10), we calculate the fraction of immobile cytochrome P-450, f_{im}, assuming the same orientation of the heme plane in the membrane (*i.e.* $\frac{1}{4}(3\cos^2\theta_N - 1)^2 = 0.12$) for all species. The immobile fraction of cytochrome P-450, thus estimated by Equation 6, was a function of temperature. We obtained $f_{im} = 0.5$–1 at 20°C and $f_{im} = 0.4$–0.6 at 37 °C. Relatively large variation in the values for f_{im} and $r_3/r(0)$ were seen between different preparations of liver microsomes.

The mean rotational relaxation time ϕ_1 was also dependent on temperature. ϕ_1 was decreased from ~120 μs to ~60 μs by going from 20 °C to 37 °C.

Rotation of Cytochrome P-450 in Isotopic Solution

The absorption anisotropy of cytochrome P-450 was smaller than 0.01 in detergent solution containing 2% sodium cholate, 60% sucrose, and 20% glycerol. In such isotropic solution, $r(t)$ should reach zero, if all enzymes are rotating. We therefore conclude that the rotational correlation time of cytochrome P-450 in the above solution is faster than 10 μs (the time resolution of the present experiments). Hence, the volume of cytochrome P-450 aggregates in this solution should be smaller than 70 Å^3, assuming aggregates to be spherical. The volume 70 Å^3 would correspond to the octamer of cytochrome P-450.

Absence of Vesicle Tumbling

The microsomal size distribution was determined to be 400–2000 Å by negative stain electron micrographs. In an accompanying paper, we show that the diameters of PC/PE/PS = 10:5:1 vesicles are in the range of 400–1000 Å.

The contribution of vesicle tumbling to the decay in $r(t)$ was experimentally ruled out in both liver microsomes and lipid vesicles (Fig. 4). For this, microsomal membranes were labeled by eosin maleimide. Purified cytochrome P-450 was labeled by eosin maleimide and was incorporated into PC/PE/PA = 10:5:0.3 vesicles by cholate dialysis (see above). Eosin-labeled proteins were cross-linked by a 5-min irradiation from a 100-watt tungsten-halide lamp (31). Rotational diffusion of eosin-labeled proteins was measured by observing flash-induced absorption anisotropy of the eosin probe. In 60% sucrose solution, no decay in $r(t)$ curves was observed for both the proteoliposomes at 20 °C and microsomes at 37 °C, ruling out vesicle tumbling and protein rotation.

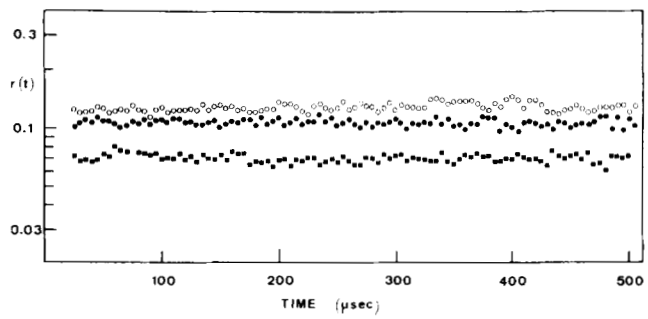

FIG. 4. **Absence of vesicle tumbling demonstrated by lack of decay in $r(t)$ for immobilized cytochrome P-450 in liver microsomes and reconstituted lipid vesicles.** *Upper curve* (○), eosin-labeled cytochrome P-450 in PC/PE/PA = 10:5:0.3 vesicles. The sample contains 8 μM eosin maleimide and 8 μM cytochrome P-450. $r(t)$ was measured with the eosin probe after cross-linking of samples by a tungsten lamp irradiation as described under "Results and Analysis." *Middle curve* (●), liver microsomes (6.6 mg/ml) labeled with 11 μM eosin maleimide. $r(t)$ was measured with the eosin probe after cross-linking by irradiation. *Lower curve* (■), liver microsomes which contain about equimolar cytochrome P-420 to cytochrome P-450 as described under "Results and Analysis." $r(t)$ was measured by flash photolysis of the cytochrome P-450·CO complex. The sample contains 3 μM cytochrome P-450.

Furthermore, no decay in $r(t)$ occurred in microsomes in 60% sucrose at 20 °C in the presence of significant amounts of cytochrome P-420 (about equimolar to cytochrome P-450) which was induced by adding an excess amount of dithionite. The excess dithionite probably induced protein aggregates in membranes in addition to conversion of cytochrome P-450 into cytochrome P-420. Thus, the decay of anisotropy observed in the experiments presented here should be due to rotation of cytochrome P-450 in membranes.

Rebinding Kinetics of CO to Reduced Cytochrome P-450

In PB-microsomes, the rebinding kinetics of CO to reduced cytochrome P-450 after photolysis was at least biphasic. The total absorbance change $A(t)$ was analyzed by a double exponential approximation. Time constants were $\tau_1 \simeq 0.2$ ms (≃20%) and $\tau_2 \simeq 1.6$ ms (≃80%) at 20 °C in 60% sucrose, the parentheses showing the exponential amplitude.

In contrast, rebinding kinetics of CO with purified cytochrome P-450 in liposomes was monophasic. The time constant was $\tau = 1.5 \pm 0.7$ ms in PC/PE/PS = 10:5:1 vesicles over the L/P range 1–30 in 60% sucrose at 20 °C. Similar time constants were observed under these conditions in other vesicles (PC/PE/PA = 10:5:0.3 and egg PC). The double exponential kinetics in liver microsomes is probably due to multiple species of cytochrome P-450 in rat liver microsomes (9, 10).

The rebinding kinetics of other heme proteins was also measured in 60% sucrose solution at 20 °C (19). Monoexponential kinetics were observed for several species of cytochrome oxidase (*i.e.* bovine heart, rat liver, and rat heart) in both phospholipid vesicles and mitochondrial inner membranes with the time constant $\tau \simeq 10$ ms. Double exponential kinetics was observed for human hemoglobin with time constants $\tau_1 \sim 0.4$ ms (~60%) and $\tau_2 \sim 1$ ms (~40%). The reaction of cytochrome P-450 with CO is rapid and similar to that of hemoglobin, implying that the heme pocket of cytochrome P-450 might be relatively easily accessible for ligands.

DISCUSSION

Orientation of Heme Plane of Cytochrome P-450 in Membranes—Recently, the heme *a* and a_3 planes of cytochrome oxidase have been shown by ESR spectroscopy to be approximately perpendicular to the plane of the membrane in both mitochondria and membranous systems (32). Kawato *et al.* (18) have investigated rotation of cytochrome oxidase in phospholipid vesicles by flash photolysis of the heme a_3·CO complex. At a high L/P = 27, they have observed the normalized residual anisotropy of $r(\infty)/r(0) \simeq 0.25$ which corresponds to $\theta_N \simeq 90°$ in Equation 3. Thus, ESR and rotation studies are in good agreement.

Here we determined the tilt of the heme plane of cytochrome P-450 by rotation measurements. We obtained the smallest normalized residual anisotropy $r(\infty)/r(0) = 0.12 \pm 0.04$ at a high L/P = 30 where all cytochrome P-450 rotates. Using Equation 3,41° or 71° ± 5° were obtained for θ_N.

Rich *et al.* (33) investigated the heme orientation of cytochrome P-450 in PB-induced rat liver microsomal membranes by ESR spectroscopy. In contrast with cytochrome oxidase, they observed that the heme plane of cytochrome P-450 was

parallel rather than perpendicular to the membrane plane. A similar orientation of the heme plane was observed for cytochrome P-450 in adrenal cortex mitochondria (34). These results suggest that of the two possible angles obtained from the present protein rotation measurements, the solution $\theta_N \simeq 40°$ is more likely to be correct. Since we treat one special species of cytochrome P-450, we cannot rule out that other species of cytochrome P-450 in microsomes have different heme orientations.

Rotation and Intermolecular Interactions of Cytochrome P-450 in Lipid Vesicles and Liver Microsomes—The fraction of mobile cytochrome P-450 is dependent on L/P in PC/PE/PS = 10:5:1 vesicles. The immobile population of cytochrome P-450 is small ($\lesssim 10\%$) above L/P = 5, but ~35% of the enzyme is immobile in L/P = 1 vesicles. Assuming the same orientation of the heme plane for multiple species of cytochrome P-450, 50–100% (20 °C) and ~50% (37 °C) of cytochrome P-450 were calculated as being immobile in rat liver microsomes. We measured PB-microsomes from more than 40 different rats (Wistar, Long-Evans, and Sprague-Dawley). The immobile population of cytochrome P-450 in microsomes varied in different preparations. Similar results were observed in PB-induced and β-naphthoflavone-induced rabbit liver microsomes (14).

The large amounts of the immobile cytochrome P-450 in microsomal membranes is probably due to the very high concentration of membrane proteins in liver microsomes (L/P ~ 0.5) (22). The immobile population of cytochrome P-450 could be due to either specific interaction with other membrane proteins (*e.g.* NADPH-cytochrome P-450 reductase) or a nonspecific protein aggregation. At the present stage, we cannot decide between these possibilities. However, since the addition of NADPH-cytochrome P-450 reductase to cytochrome P-450 vesicles of L/P = 1 introduced disaggregation of cytochrome P-450 aggregates (see the accompanying paper (22), nonspecific protein aggregates may be more probable. These aggregates are not necessarily stable, more likely there is a slow equilibrium between mobile and immobile cytochrome P-450's. The relatively slow rotational relaxation time $\phi_l \sim 120$ μs for the mobile cytochrome P-450 in liver microsomes suggests that even rotating cytochromes are not monomeric because a more rapid $\phi_l \simeq 50$ μs was observed in proteoliposomes with L/P = 5–10.

Cytochrome oxidase is the terminal enzyme of the electron transfer chain in the inner membrane of mitochondria. Recently, mobility and protein-protein interactions of this protein were studied in bovine heart mitochondria and reconstituted proteoliposomes (18, 19) demonstrating co-existence of mobile and immobile populations. Forty to fifty per cent of cytochrome oxidase in mitochondria rotate with a mean rotational relaxation time of 350 μs. In lipid vesicles, the percentage of immobile cytochrome oxidase increased from ~0% to ~20% upon a decrease in L/P from 27 to 5. The same results were obtained in the presence of cytochrome bc_1 complex, ruling out a molecular complex formation between the two. Since the inner membrane of mitochondria has a very low L/P ≃ 0.39 (35), it was concluded that the high concentration of membrane proteins induces protein aggregates and, therefore, immobilizes about one-half of cytochrome oxidase molecules. This is further supported by the observation of a dramatic increase in the mobile population of cytochrome oxidase from 15% to 75% in rat liver mitochondrial inner membranes upon increasing L/P from 0.3 to 3 by fusion with liposomes (30). These findings parallel the present results which suggest that the immobile fraction of cytochrome P-450 in liver microsomes is caused by nonspecific protein aggregates due to high protein concentration.

Duppel and Dahl (36) proposed cluster formation of microsomal proteins at 4 °C and random distribution of membrane proteins at 30 °C in rat liver microsomes. The present results of complete immobilization of cytochrome P-450 around 5 °C and co-existence of mobile and immobile populations of cytochrome P-450 at 20–37 °C are consistent with their ultrastructural observations. However, it should be emphasized that the above complete immobilization of cytochrome P-450 occurred at around 5 °C, slightly above the lipid phase transition. The microsomal membrane is in the liquid-crystalline state above 0 °C as determined by fluorescence polarization measurements of diphenylhexatriene,[2] in agreement with the earlier observation that the microsomal phase transition occurs between −10 and 0 °C (37).

Lateral Diffusion-controlled Interactions for Cytochrome P-450—There are essentially two possible mechanisms for electron transfer in microsomal membranes: electrons are transferred by lateral collisions between independently diffusing oxidation-reduction component enzymes or electrons are transferred in a relatively long-lived multienzyme complex. Cytochrome b_5 was shown to interact with at least three different enzymes, including cytochrome P-450, with the same catalytic site (5). Morever, careful subfractionation of microsomes yielded two subpopulations; one contains more cytochrome b_5 and NADH-cytochrome b_5 reductase, the other more cytochrome P-450 and NADPH-cytochrome P-450 reductase (38). Therefore, it is more likely that cytochrome b_5 donates electrons to cytochrome P-450 by lateral collisions rather than through a supermolecular complex. In this case, the collision-controlled electron transfer may not need long range lateral diffusion of enzymes but is controlled by local lateral collisions, because microsomal membranes are densely packed with around one-third of the total membrane area occupied by integral membrane proteins (25, 36).

The present rotational relaxation time, $\phi_\parallel$, can be related to the local (free) lateral diffusion coefficient (D_L^{loc}) by the following expression (39):

$$D_L^{loc} \simeq (\ln \eta/\eta' - \gamma)a^2/\phi_\parallel \quad (8)$$

where η is the membrane viscosity, η' is the viscosity of the aqueous phase, and γ is Euler's constant (0.5772). If we assume that the mobile cytochrome P-450 in PC/PE/PS = 10:5:1 vesicles of L/P = 10 is a particle of a ~20 Å with $\phi_\parallel \sim 50$ μs, and if we take $\eta \sim 5$ poise (40) and $\eta' \sim 0.6$ poise at 20 °C in 60% sucrose, we obtain $D_L^{loc} \sim 10^{-9}$ cm²/s. Since the lateral diffusion coefficient is very insensitive to the protein size, the value of $D_L^{loc} \sim 10^{-9}$ cm²/s will also be valid for small aggregates of cytochrome P-450. The above value of D_L^{loc} is at least an order of magnitude larger than the long range lateral diffusion coefficient for many integral proteins examined ($D_L \lesssim 10^{-10}$ cm²/s) (40). These smaller diffusion coefficients were principally obtained with whole cells and may indicate restrictions to long range lateral diffusion by cytoskeletal structures. Cytoplasmic structures in endoplasmic reticulum, if present, could reduce the long range lateral diffusion rate. However, the local lateral diffusion coefficient, which is calculated from rotational diffusion, is probably a more meaningful number for estimating collision rates than the long range lateral diffusion coefficient.

The frequency of possible lateral collisions between cytochrome P-450 and cytochrome b_5 can be estimated as follows. Our rat liver PB-microsomes contain about 2 nmol of cytochrome P-450 per mg of protein[2] and about 0.5 nmol of cytochrome b_5/mg of protein (41) and have L/P ≃ 3/7.[3] Since

[2] C. Richter, unpublished results.

[3] K. Eichenberger and C. Richter, unpublished results.

one phospholipid molecule occupies about 50 Å^2 on the monolayer surface (42), the membrane area per cytochrome P-450 and cytochrome b_5 can be calculated as about 5×10^{-13} cm^2 and 2×10^{-12} cm^2, respectively. According to the treatment of Razi-Naqvi *et al.* (43), the lower limit of the probability of collisions between cytochrome b_5 and cytochrome P-450 may be estimated as follows:

$$P = 16D_L^{loc}\, c\rho/\sigma = 16 \times 10^{-9} \cdot \frac{1}{2 \times 10^{-12}} \cdot \rho/\sigma \geq 8 \times 10^3/\text{s} \quad (9)$$

where c is the concentration of cytochrome b_5 (since the concentration of b_5 is lower than that of P-450), ρ is the sum of radii of cytochromes b_5 and P-450, and σ is the average displacement of cytochromes. For membrane proteins in the lipid bilayer, $\rho/\sigma \geq 1$ (44). Thus, at least several thousand collisions could occur between cytochromes b_5 and P-450 within the known rate of electron transfer, for example, ~1.5 s/e^-/cytochrome P-450 (NADH → NADH-cytochrome b_5 reductase → cytochrome b_5 → cytochrome P-450) (45). In fact, the collision rate could be faster due to the rapid lateral diffusion of cytochrome b_5, which may be in the order of $D_L^{loc} = 5 \times 10^{-8}$ cm^2/s as judged by measurements with other small hydrophobic or amphiphilic proteins (46, 47).

Lipid-Protein Interactions in Cytochrome P-450 Vesicles—In the present study, we used four different phospholipid vesicles, *i.e.* PC/PE/PS = 10:5:1, PC/PE/PA = 10:5:0.3, egg PC, and PC/PE/PS = 1:1:1. Rotational diffusion measurements of cytochrome P-450 revealed that both the relaxation time ϕ_1 and the immobile fraction were virtually independent of the above different lipid compositions, except PC/PE/PS = 1:1:1. The existence of 33% PS in PC/PE/PS = 1:1:1 vesicles led to the conversion of rat cytochrome P-450 into cytochrome P-420 and immobilization of cytochrome P-450. It is of interest to note that no such conversion of cytochrome P-450 from rabbit liver microsomes into cytochrome P-420 is observed even in the presence of negatively charged phospholipids comprising more than one-third of total lipids (48).

The presence of PS and/or PE increased the catalytic activity of rabbit liver cytochrome P-450 (*e.g.* *O*-demethylation of *p*-nitroanisol) (48). The acceleration of electron transfer from NADPH-cytochrome P-450 reductase to cytochrome P-450 by the presence of PS and/or PE was found to be responsible for the above increase in activities. Since changes in rotation and local lateral diffusion of cytochrome P-450 cannot be caused by the addition of PS and/or PE as judged by the absence of change in $r(t)$, PS and/or PE may induce more efficient interactions between the reductase and cytochrome P-450 resulting in an increase in catalytic activities.

Measurements of Rotational Diffusion of Cytochrome P-450 with Triplet Probes—Recently, rotational mobility of cytochrome P-450 was demonstrated by delayed fluorescence of eosin isothiocyanate covalently bound to the enzyme in PC/PE/PA = 10:5:0.3 vesicles with L/P = 5 (15). A rotational time constant of about 110 μs at 25 °C was obtained for the above rabbit liver cytochrome P-450. We also examined rotational diffusion of rat liver cytochrome P-450 covalently labeled with eosin maleimide by observing flash-induced absorption anisotropy of the eosin probe. A rotational relaxation time ϕ_1 = 50–100 μs was obtained in PC/PE/PS = 10:5:1 vesicles with L/P = 10. This is in reasonable agreement with the results using the intrinsic chromophore heme. This agreement is encouraging for studies with probes, because the labeling procedure does not appear to alter the enzyme structure in a way that leads to change in the rotational mobility. The data also show that the rotational mobilities of rabbit and rat cytochrome P-450 are similar in reconstituted lipid vesicles.

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