THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 257, No. 12, Issue of June 25, pp. 7030-7036, 1982
Printed in U.S.A.

Rotation of Cytochrome P-450

II. SPECIFIC INTERACTIONS OF CYTOCHROME P-450 WITH NADPH-CYTOCHROME P-450 REDUCTASE IN PHOSPHOLIPID VESICLES*

(Received for publication, August 28, 1981)

Josef Gut, Christoph Richter, Richard J. Cherry, Kaspar H. Winterhalter, and Suguru Kawato

From the Laboratorium für Biochemie, Eidgenössische Technische Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland

Purified rat liver microsomal cytochrome P-450 and NADPH-cytochrome P-450 reductase were co-reconstituted in phosphatidylcholine-phosphatidylethanolamine-phosphatidylserine vesicles using a cholate dialysis technique. The co-reconstitution of the enzymes was demonstrated in proteoliposomes fractionated by centrifugation in a glycerol gradient. The proteoliposomes catalyzed the *N*-demethylation of a variety of substrates.

Rotational diffusion of cytochrome P-450 was measured by detecting the decay of absorption anisotropy $r(t)$, after photolysis of the heme•CO complex by a vertically polarized laser flash. The rotational mobility of cytochrome P-450, when reconstituted alone, was found to be dependent on the lipid to protein ratio by weight (L/P_{450}) (Kawato, S., Gut, J., Cherry, R. J., Winterhalter, K. H., and Richter, C. (1982) *J. Biol. Chem.* 257, 7023-7029). About 35% of cytochrome P-450 was immobilized and the rest was rotating with a mean rotational relaxation time ϕ_1 of about 95 μs in L/P_{450} = 1 vesicle. In L/P_{450} = 10 vesicles, about 10% of P-450 was immobile and the rest was rotating with $\phi_1 \simeq 55$ μs. Co-reconstitution of equimolar amounts of NADPH-cytochrome P-450 reductase into the above vesicles results in completely mobile cytochrome P-450 with a $\phi_1 \simeq 40$ μs. Only a small decrease in the immobile fraction of cytochrome P-450 is observed when the molar ratio of cytochrome P-450 to the reductase is 5. The results suggest the formation of a monomolecular 1:1 complex between cytochrome P-450 and NADPH-cytochrome P-450 reductase in the liposomes.

Cytochrome P-450 and NADPH-cytochrome P-450 reductase are key enzymes of the hepatic microsomal monooxygenase system catalyzing the oxidative metabolism of endogenous substrates and many xenobiotics. The enzymes also catalyze the biotransformation of physiologically important lipids such as fatty acids, prostaglandins, and steroids, and of foreign compounds such as drugs, petroleum products, anesthetics, insecticides, and carcinogens (1-3). NADPH-cytochrome P-450 reductase ($M_r \sim 78{,}000$) is anchored to the membrane of the endoplasmatic reticulum via a small ($M_r \sim 6{,}000$-$10{,}000$) hydrophobic segment (4, 5). The large hydrophilic part, which contains 1 molecule of FMN and FAD and accepts electrons from NADPH, protrudes from the membrane into the cytoplasmic space. Cytochrome P-450 ($M_r \sim 50{,}000$), on the other hand, is deeply imbedded into the membrane (6). No detailed structural picture has so far emerged from the studies concerning the substrate-binding and catalytic site of the cytochrome. The arrangement of the two enzymes in the membrane and their odd stoichiometry (there can be 20-30 cytochromes per reductase) has raised questions as to the mechanism of electron transfer from the reductase to the cytochrome and the functional interactions of the proteins in the monooxygenase system. The situation is further complicated by the fact that the second electron can also be donated at least to some species of cytochrome P-450 via NADH-cytochrom b_5 reductase and cytochrome b_5 (7-9).

Two types of mechanism can be envisaged for the electron transfer between NADPH-cytochrome P-450 reductase and cytochrome P-450: one is transfer by random collisions between rotationally and laterally diffusing proteins, the other is transfer by electron tunneling within a stable complex. To gain insight into the mode of interaction, a number of experiments, leading to different conclusions, have been performed with hepatic microsomal membranes. Based on the kinetics and extent of cytochrome reduction in microsomes, in which the reductase activity had been partially inhibited or stimulated, Franklin and Estabrook (10) postulated a rigid arrangement of reductase and cytochrome ("cluster") in the membrane, whereas the group of Yang (11) proposed an interaction by random collisions. The temperature dependence of the biphasic reduction kinetics led Peterson *et al.* (12) to postulate a model where a number of cytochrome P-450 molecules are envisaged as being grouped around a centrally located reductase molecule. Rapid phase reduction, according to this model, was thought to reflect reduction of cytochrome molecules within the "cluster," whereas the slow phase reduction was thought to result from reduction of satellite cytochrome molecules, which were postulated to exist in unaggregated form, and could be reduced only after lateral diffusion and collision. Based on the analysis of the temperature dependence of hydroxylation reactions catalyzed by the microsomal monooxygenase system, Duppel and Ullrich (13) concluded that lateral diffusion of the proteins is required for their function.

Lately, the mechanism of interaction has been investigated after isolation and reconstitution of the monooxygenase components. Reductase and cytochrome can be incorporated into single-shelled liposomes in an enzymatically active form (14-16). Measurements by Sato and co-workers (15) of cytochrome reduction kinetics and substrate hydroxylation suggested that the interaction between the two proteins is effected by their random collisions caused by their lateral mobility in the plane of the membrane. This view was supported by Ingelmann-Sundberg and Johansson (16).

To date, an inadequate amount of studies has been performed for direct measurements of protein-protein interac-

* This work was supported by Schweizerischer Nationalfonds Grant 3.699.80, the very generous financial help of Solco Basel AG., and a kind donation of the Robert Gnehm Stiftung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

tions of the components of the hepatic microsomal monooxygenase system. A promising approach to this problem is to investigate protein rotational diffusion, which is particularly sensitive to protein-protein interactions. Richter *et al.* (17) reported the rotational mobility of cytochrome P-450 in rat liver microsomal membranes by detecting the decay of absorption anisotropy after photolysis of the heme·CO complex of cytochrome P-450. The mobility of the cytochrome was abolished when the membrane was in the gel phase. With delayed fluorescence of a triplet probe, Stier and co-workers (18) demonstrated rotation of cytochrome P-450 in phospholipid vesicles.

Here we report the influence of NADPH-cytochrome P-450 reductase on the rotational mobility of cytochrome P-450 in vesicles of phosphatidylcholine-phosphatidylethanolamine-phosphatidylserine, which correspond to the major phospholipid components in liver microsomes. A specific interaction between cytochrome P-450 and reductase is demonstrated, and the formation of large aggregates between reductase and cytochromes ("cluster") in liposomes is excluded.

EXPERIMENTAL PROCEDURES

Purification of Microsomal Enzymes

Cytochrome P-450 was purified from liver microsomes of phenobarbital-induced male rats (200–300 g) of the Sprague-Dawley strain according to the method of West *et al.* (19) except that a shorter column (2.4 × 8 cm) was used in the second chromatography step on DE52 cellulose to minimize loss of heme. The preparations had a specific content of 14.5–18 nmol of cytochrome P-450/mg of protein as determined by the heme·CO difference spectroscopy, were homogeneous on SDS[1] slab gels, and showed no conversion to cytochrome P-420. NADPH-cytochrome P-450 reductase was purified according to Yasukochi and Masters (20). The procedure was followed up to the Sephadex G-25 step and then the reductase was stored in 10 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol, 0.1% Renex 690, 0.02 mM EDTA, and 0.2 mM dithiothreitol at −80 °C. The preparations had a specific activity of 50,000–60,000 units/mg of protein and were homogeneous on SDS slab gels.

Preparation of Proteoliposomes

(*a*) Reconstitution of cytochrome P-450 and NADPH-cytochrome P-450 reductase in PC/PE/PS = 10:5:1 (w/w) vesicles was performed by the cholate dialysis procedure outlined by Bösterling *et al.* (14) with some modifications. Ten mg of PC, 5 mg of PE, and 1 mg of PS were freed of the solvent chloroform/methanol under a stream of N_2, further dried under vacuum at 4 °C for 5 h, and suspended in 1.6 ml of 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol. 0.16 ml of a 20% (w/w) sodium cholate solution was added and the mixture was gently agitated until clear. Cytochrome P-450 and reductase were then added and the resulting lipid-protein-cholate mixture was incubated for 15 h at 4 °C. The dispersion was dialyzed at room temperature for 3 h against a 200-fold volume of buffer (20 mM Hepes, pH 8.0, 0.1 mM EDTA, 20% glycerol) containing 1 g of Bio-Beads SM-2 (Bio-Rad Inc.) per 100 ml. The dialysis buffer was changed once. (*b*) For reconstitution of cytochrome P-450 in DPPC vesicles, 1 mg of DPPC was dissolved in 1 ml of chloroform, dried under a stream of N_2, and kept under vacuum at 4 °C for 2 h. The lipid was suspended in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.7) containing 20% glycerol. After addition of 50 μl of 20% sodium cholate, the mixture was incubated at 50 °C for 1 h and then cooled to room temperature. After addition of cytochrome P-450, the suspension was kept for 15 h at 4 °C and subsequently dialyzed as described in (*a*).

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; L/P_{450}, lipid to cytochrome P-450 ratio (w/w); L/P, lipid to total protein ratio (w/w); P-450/reductase, cytochrome P-450 to reductase ratio (moles/mol); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DPPC, L-α-phosphatidylcholine dipalmitoyl; [^{14}C]PC, [*choline-methyl*-^{14}C]Phosphatidylcholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; cytochrome $P\text{-}450_{LM_2}$, $P\text{-}450_{LM_3}$, $P\text{-}450_{LM_4}$, rabbit liver cytochrome purified from phenobarbital-induced microsomes. These isozymes are designated according to their electrophoretic properties.

Characterization of Proteoliposomes

Density Gradient Centrifugation—0.5 ml of the vesicle preparation containing a trace of [^{14}C]PC was applied on top of 4.5 ml of a linear 5–50% (w/w) glycerol density gradient prepared in 20 mM Hepes, pH 8.0, 0.1 mM EDTA, and centrifuged for 15 h at 105,000 × *g*. Subsequently, 0.25-ml fractions were collected and analyzed for radioactivity, cytochrome P-450, and NADPH-cytochrome P-450 reductase activity.

Gel Filtration—The size of proteoliposomes used in flash photolysis experiments was examined by agarose gel filtration on Bio-Gel A-150m (Bio-Rad) in 20 mM Hepes, pH 8.0, 20% glycerol, and 0.1 mM EDTA (21). The column (23 × 0.9 cm) was calibrated with sonicated and centrifuged egg PC liposomes of uniform size distribution (mean diameter ~ 280 Å) (22) and Ca^{2+}-fused bovine PS liposomes, which has been pelleted by centrifugation at 48,000 × *g* for 20 min (diameter ~ 1000 to 2000 Å) (23). Proteoliposomes were prepared as described above. In every case, 500-μl aliquots of liposomal suspension were applied to the column and eluted with a flow rate of 4 ml/h at 4 °C. Fractions of 440 μl were collected and analyzed for their content of [^{14}C]PC. Elution was in 20 mM Hepes, pH 8.0, 20% glycerol, and 0.1 mM EDTA, except for the sonicated egg PC liposomes, where 100 mM KCl was included.

Freeze-Fracture Electron Microscopy

Samples were jet-frozen from room temperature in liquid propane at a temperature of about −190 °C (24). Freeze-fracture was carried out in Balzer's 300 apparatus. Samples were replicated with Pt/C and examined in a Philips EM 301 transmission electron microscope. For negative stain electron micrography, samples were stained with phosphotungstate.

Other Methods

Monooxygenase activity was measured in a final volume of 1 ml containing 20 mM Hepes, pH 7.4, 20% glycerol, and 0.1 mM EDTA at 37 °C for 1 min. The composition of the reconstituted system was L/P_{450} = 1, 0.2 μM P-450, and P-450/reductase = 5:1. 5 mM glucose 6-phosphate and 2.5 units of glucose-6-phosphate dehydrogenase were present as a NADPH-regenerating system. Substrate concentration was 1 mM. The reaction was started by the addition of 0.5 mM NADPH. Formaldehyde was measured according to Nash (25).

NADPH-cytochrome P-450 reductase was assayed according to Strobel and Dignam (28) at room temperature with cytochrome *c* as electron acceptor in 1 ml of 0.3 M potassium phosphate buffer, pH 7.7, 0.1 mM EDTA. One unit is defined as the amount of enzyme that reduces 1 nmol of cytochrome *c* per min. *Cytochrome P-450* was measured spectrophotometrically either in the oxidized form at 417 nm using 100 mM^{-1} cm^{-1} as extinction coefficient or as the heme·CO complex according to Omura and Sato (29) using 91 mM^{-1} cm^{-1} as the extinction coefficient. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis* was performed in the Laemmli system (30). *Protein* concentrations were determined by the method of Lowry (31) with bovine serum albumin as standard; the albumin concentration was determined independently using an extinction coefficient of 6.8 at 280 nm for a 1% solution. Samples containing detergent were analyzed in the presence of 1% sodium dodecyl sulfate in Lowry's reagent to avoid overestimation of the protein content by the presence of the detergent.

Rotational Diffusion Measurements and Analysis

Rotational diffusion measurements of cytochrome P-450 in proteoliposomes and analysis of the data were performed according to the method described in detail in the accompanying paper (26). Briefly, analysis of $r(t)$ is based on a model of rotation of cytochrome P-450 about the membrane normal (27). When there is a single rotating species of cytochrome P-450 with the rotational relaxation time, $\phi_\parallel$, $r(t)$ is given by:

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

where θ_N is the tilt angle of the heme plane from the plane of the membrane. Multiple rotating species of cytochrome P-450 with different $\phi_\parallel$ are considered with analyzing the data by the following equation:

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-4t/\phi_1) + r_3 \quad (2)$$

where ϕ_1 is the mean rotational relaxation time. When samples

contain both mobile and immobile populations of cytochrome P-450, the immobile fraction, f_{im}, can be calculated from the normalized residual anisotropy $r(\infty)/r(0) = r_3/r(0)$ by (26)

$$r_3/r(0) = (1 - f_{im}) \cdot 0.12 + f_{im} \quad (3)$$

Materials

Phosphatidylcholine, phosphatidylethanolamine, both from egg yolk, and phosphatidylserine from bovine spinal cord, all grade I, were purchased from Lipid Products, Nutfield, U.K. [*choline-methyl*-^{14}C]Phosphatidylcholine was from New England Nuclear. L-α-Phosphatidylcholine dipalmitoyl, cytochrome *c* from horse heart, type III, and glucose-6-phosphate dehydrogenase were obtained from Sigma. Benzphetamine was purchased from Applied Science Laboratories, aminopyrine was from Aldrich Europe, and ethylmorphine was obtained from a local pharmacy.

RESULTS AND ANALYSIS

Activity of Enzymes in Phospholipid Vesicles

Catalytic activity of cytochrome P-450 and reductase in PC/PE/PS = 10:5:1 vesicles was assayed by measuring *N*-demethylation of aminopyrine, ethylmorphine, and benzphetamine. The observed turnover numbers (*i.e.* nanomoles of CH_2O formed/nmol of P-450/min) were 4.0 for ethylmorphine, 15.0 for aminopyrine, and around 27 for benzphetamine, therefore indicating a co-reconstitution of cytochrome P-450 with reductase within the same vesicle membrane.

Co-reconstitution of Cytochrome P-450 and NADPH-Cytochrome P-450 Reductase into Phospholipid Vesicles

Since PC, PE, and PS comprise up to 90% of total phospholipid content of the microsomal membrane in a weight ratio of 55:25:10 (32), we chose a composition of the present liposomal system close to that of the natural membrane, *i.e.* PC:PE:PS = 10:5:1. The high percentage of PC proved to be essential for spectral stability of cytochrome P-450. An increase in the amount of PS up to 33% of total liposomal phospholipids caused a strong tendency of converting cytochrome P-450 into cytochrome P-420.

The co-reconstitution of cytochrome P-450 with NADPH-cytochrome P-450 reductase in liposomes can be demonstrated by density gradient centrifugation in glycerol (14, 15). A variety of proteoliposomes with different enzyme compositions was centrifuged in a linear glycerol gradient. In all vesicles examined, a relatively broad single band was observed. After the centrifugation, the distribution of cytochrome P-450 was measured by the absorption at 417 nm, that of reductase was examined spectrophotometrically by cytochrome *c* reductase activity, and that of phospholipids was followed by [^{14}C]PC (see Fig. 1). The good coincidence of distribution of cytochrome P-450 and reductase as well as [^{14}C]PC measured by the above methods clearly demonstrated the co-reconstitution of cytochrome P-450 with reductase. This and the observation that enzymatic hydroxylation activity is present in the reconstituted vesicles strongly suggests that both proteins are present in the same vesicle. When proteoliposome suspensions were incubated with 2% (w/w) sodium cholate, the density gradient separated proteins (found on the bottom of the gradient) from phospholipids (found on the top of the gradient).

Rotational Diffusion of Cytochrome P-450 in Lipid Vesicles

Effect of Lipid to Protein Ratio in Pure Cytochrome P-450 Vesicles—In the preceding paper (26), we have shown the following results. All cytochrome P-450 was mobile in $L/P_{450} \simeq 30$ vesicles with a mean rotational relaxation time $\phi_l \simeq 95$ μs. Only a small amount of immobile cytochrome P-450 (≃ 7%) was observed in $L/P_{450} = 10$ vesicles. A large population of cytochrome P-450 (≃ 35%) was immobile in lipid poor vesicles with $L/P_{450} = 1$, and the rest was rotating with $\phi_l \simeq 95$ μs. The immobilization of cytochrome P-450 upon decreasing L/P_{450} indicates the formation of cytochrome P-450 aggregates in these vesicles.

Effect of Reductase on r(t) in $L/P_{450} = 1$ Vesicles—Co-reconstitution of equimolar amounts of NADPH-cytochrome P-450 reductase and cytochrome P-450 in $L/P_{450} = 1$ vesicles resulted in a significant change in decay parameters of $r(t)$ (Fig. 2 and Table I). Within 500 μs, $r(t)$ decayed to a time-independent value $r(\infty)$, leading to an r_3/r (0) of ≃ 0.16. This pronounced decrease in r_3/r (0) from ≃ 0.43 to ~0.16 by the addition of reductase indicated a dramatic disaggregation of the large cytochrome P-450 aggregates which were present in the absence of reductase. According to Equation 3, nearly no

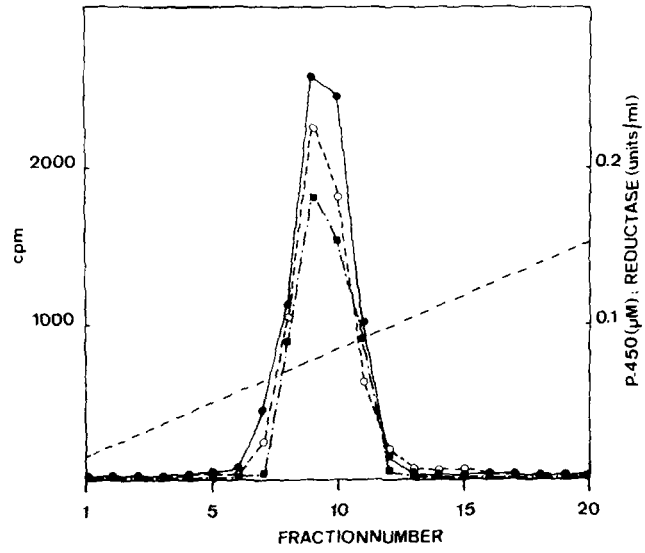

FIG. 1. **Analysis of reconstituted PC/PE/PS = 10:5:1 vesicles by centrifugation in a glycerol density gradient.** Typical distribution profile of the vesicle preparation ($L/P_{450} = 1$, P-450/reductase = 5:1) loaded onto a 5–50% glycerol density gradient (- - -). Fractions were analyzed for radioactivity of [^{14}C]PC (●——●), P-450 content with absorbance at 417 nm (○- - -○), and NADPH-cytochrome P-450 reductase activity (■—·—■) as described under "Experimental Procedures."

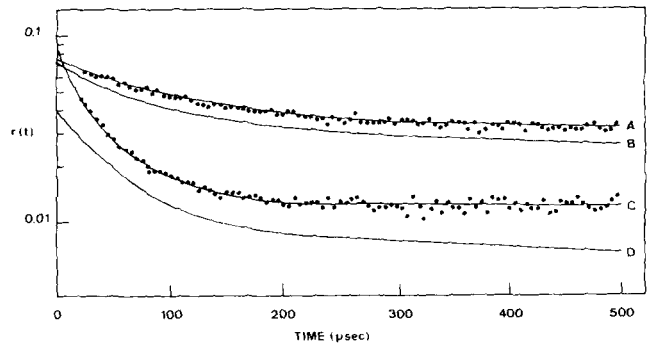

FIG. 2. **Time-dependent absorption anisotropy of cytochrome P-450 in PC/PE/PS = 10:5:1 vesicles.** Photolysis of samples (5–13 μM in cytochrome P-450) by a vertically polarized laser flash at 540 nm, and $r(t)$ recording at 450 nm. All measurements were performed in 60% sucrose solution at 20 °C (0.6 poise). *A*, $L/P_{450} = 1$ without reductase; *B*, $L/P_{450} = 1$, P-450/reductase = 5; *C*, $L/P_{450} = 1$, P-450/reductase = 1; *D*, $L/P_{450} = 30$ without reductase. *Solid lines* were obtained by fitting the data to Equation 2. Data points of *curves B* and *D* were omitted for clarity. The initial anisotropy of *curve D* is arbitrarily displaced to $r(0) = 0.04$ for illustrative purposes in order to avoid overlapping with *curve C*.

TABLE I

Decay parameters of time -dependent absorption anisotropy of cytochrome P-450 in a variety of proteoliposomes analyzed according to Equation 2

Composition of vesicles preparation	L/P_{450} (w/w)	P-450/reductase	ϕ	$r(\infty)/r(o)$	% immobile P-450[a]	Temperature
		mol/mol	*μs*			*°C*
PC/PE/PS = 10:5:1	30		96 ± 50[b]	0.12 ± 0.04	0	20
	10		56 ± 4	0.18 ± 0.05	7 ± 2	20
Cytochrome P-450 alone	1		94 ± 24	0.43 ± 0.05	35 ± 5	20
PC/PE/PS = 10:5:1	10	5:1	57 ± 15	0.17 ± 0.01	6 ± 1	20
		1:1	71 ± 5	0.12 ± 0.03	0 ± 3	20
Cytochrome + P-450 + reductase	1	5:1	156 ± 20	0.35 ± 0.04	26 ± 5	20
		1:1	38 ± 10	0.16 ± 0.02	5 ± 3	20

[a] Calculated according to Equation 3.
[b] Standard deviation; n = 2–4.

(~5%) immobile population of cytochrome P-450 was present in the above P-450/reductase = 1 vesicles. ϕ_1 was also decreased from ≃ 95 μs to ≃ 40 μs. When the amount of reductase was one-fifth of that of cytochrome P-450, r_3/r (0) was ≃ 0.35, implying that about 25% of cytochrome P-450 is still immobile. ϕ_1 was not decreased by reductase when P-450/reductase was 5:1.

Effect of Reductase on r(t) in L/P_{450} = 10 Vesicles—A small but similar mobilization effect was also observed in L/P_{450} = 10 vesicles by the presence of reductase. A complete mobilization of cytochrome P-450 was achieved by the presence of equimolar reductase to cytochrome P-450 without changing ϕ_1 significantly. Almost no change in r_3/r (0) and ϕ_1 was observed when P-450/reductase was 5.

Effect of Renex 690—The stock solution of reductase contained 0.1% (v/w) Renex 690. Since Renex 690 is a nonionic detergent and may be difficult to remove completely by the present cholate dialysis procedures, the remaining Renex 690 might be a cause of mobilization of cytochrome P-450 in P-450 + reductase proteoliposomes. Therefore, rotation of cytochrome P-450 was measured in cytochrome P-450 vesicles with L/P_{450} = 1 in the presence of 0.04% Renex which is a little more than the calculated maximum of Renex (0.02%) in the measured P-450 + reductase vesicles. No significant difference in $r(t)$ was observed in the presence and absence of 0.04% Renex 690, indicating that Renex 690 did not contribute to the observed mobilization of cytochrome P-450 by the addition of reductase.

Vesicle Size Distribution

Fig. 4 illustrates a typical elution pattern of proteoliposomes from a Bio-Gel A-150m column. No significant difference in size distribution was observed between L/P_{450} = 1 and L/P_{450} = 10 vesicles regardless of the presence of reductase. Freeze-fracture electron micrographs showed that PC/PE/PS = 10:5:1 vesicles are 400–1000 Å in diameter relatively independent of L/P_{450} = 1 to 10.

Absence of Vesicle Tumbling

Although rotational diffusion of cytochrome P-450 in vesicles was measured in a viscous medium of 60% sucrose (~0.6 poise at 20 °C), vesicle tumbling might contribute to the observed decay in $r(t)$. The possibility of vesicle tumbling was examined below 35 °C with cytochrome P-450 incorporated in DPPC vesicles. Since at this temperature DPPC is in the gel phase, protein rotation should be inhibited and hence any effect of vesicle tumbling on $r(t)$ would be clearly revealed. Fig. 3 illustrates that no decay in $r(t)$ for the above DPPC vesicles is observed, implying that neither vesicle tumbling nor rotation of cytochrome P-450 occur. Since there was no significant difference in vesicle size distribution between

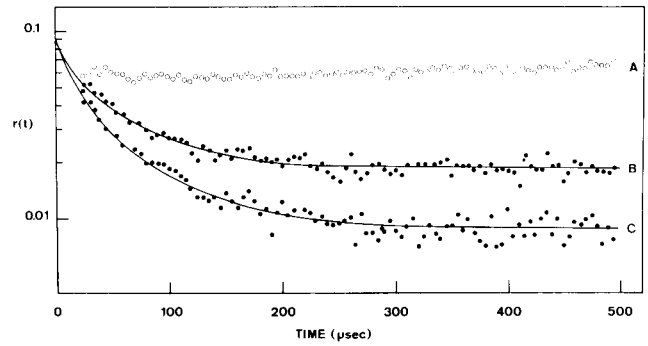

FIG. 3. **Time-dependent absorption anisotropy of cytochrome P-450 in DPPC and PC/PE/PS = 10:5:1 vesicles.** Experimental conditions are the same as in Fig. 2. *A*, DPPC/P_{450} = 10, measured at 35 °C, a very similar result was obtained at 20 °C; *B*, L/P_{450} = 10 without reductase; *C*, L/P_{450} = 10, P-450/reductase = 1. *Solid lines* were obtained by fitting the data to Equation 2. $r(0)$ of *curve C* was displaced to the same value as that of *curve B* in order to facilitate the comparison of these curves.

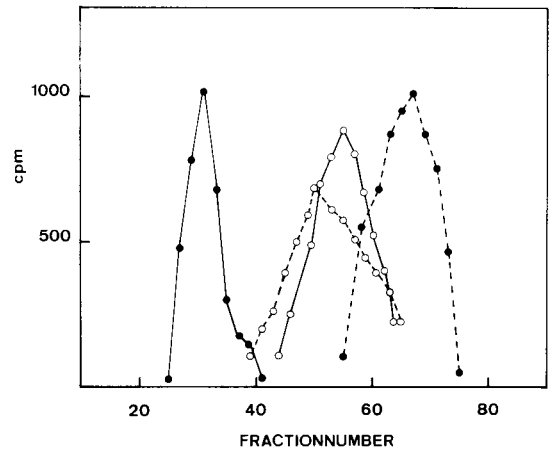

FIG. 4. **Vesicle size distribution of proteoliposomes used in rotational diffusion measurements.** Elution profiles on a Bio-Gel A-150m column of PC/PE/PS = 10:5:1 vesicles (L/P_{450} = 1, P-450/reductase = 1) (○——○), DPPC vesicles (L/P_{450} = 10) (○- - -○), egg PC liposomes (diameter ≃ 280 Å) (●- - -●), and Ca^{2+}-fused PS liposomes (diameter = 1000–2000 Å) (●——●).

DPPC vesicles and PC/PE/PS = 10:5:1 vesicles (see Fig. 4), the above results indicated the absence of vesicle tumbling within the time range of 0–500 μs. Furthermore, no decay of $r(t)$ was observed in eosin-labeled cytochrome P-450 vesicles after cross-linking of the enzymes by a tungsten lamp irradiation, supporting the above conclusion (see the accompanying paper, Ref. 26).

Rebinding Kinetics of CO to Reduced Cytochrome P-450 in the Presence of Reductase

The present experiments were performed with samples saturated with CO. The rebinding kinetics of CO to cytochrome P-450 in the presence of reductase after photolysis was monophasic in PC/PE/PS = 10:5:1 vesicles. The time constant, τ, of this reaction was $\tau = 1.7 \pm 0.6$ ms irrespective of $L/P_{450} = 1$ and 10. No significant change was observed for τ in pure cytochrome P-450 vesicles ($\tau = 1.5 \pm 0.7$ ms). Therefore, although cytochrome P-450 was observed to have a specific interaction with reductase (see above), reductase does not appear to interfere with the accessibility of the ligand to the heme cavity.

DISCUSSION

Complex Formation of Cytochrome P-450 with Reductase in Lipid Vesicles—As shown in this and the preceding report (26), rotational mobility of cytochrome P-450 was decreased in pure cytochrome P-450 vesicles with PC/PE/PS = 10:5:1, when the concentration of the enzyme was increased. All cytochrome P-450 molecules were mobile at $L/P_{450} = 30$ and about 35% of the cytochrome was immobile at $L/P_{450} = 1$ within the present experimental time range of 500 μs. The presence of reductase mobilized cytochrome P-450 in phospholipid vesicles regardless of L/P_{450} from 1 to 10. In pure cytochrome P-450 vesicles, the observed immobilization of cytochrome P-450 is probably due to protein aggregation as a result of the high concentration of the cytochrome in the membrane. Although the presence of reductase increased the concentration of proteins in the membrane, mobilization of cytochrome P-450 was observed. One probable interpretation of the present mobilization effect is as follows: complex formation between cytochrome and reductase is favored over the complex formation between the cytochromes themselves leading therefore to a disassembly of cytochrome aggregates. Since the complete mobilization of cytochrome P-450 was achieved in P-450/reductase = 1:1 vesicles and only little mobilization was observed in P-450/reductase = 5:1 vesicles, the stoichiometry in the above complex could be P-450/reductase = 1:1 (see Fig. 5).

The reductase has a bulky hydrophilic head of $M_r \simeq 70{,}000$ and anchors to the membrane with a small hydrophobic polypeptide of $M_r \simeq 6{,}000$ to 10,000. In a hypothetical P-450·reductase 1:1 complex, the above bulky head of reductase could prevent a hydrophobic association between these complexes, supporting the observed very low affinity of proteins in the presence of reductase. Cytochrome P-450 is thought to be almost completely buried in the membrane. Cytochrome P-450 may directly interact with the anchor of reductase, so that the size of the membrane-immersed part of the P-450·reductase 1:1 complex ($M_r \simeq 58{,}000$ to 62,000) may not be very different from cytochrome P-450 ($M_r \simeq 52{,}000$). The rotational relaxation time, $\phi_\parallel$, of the protein only depends on the size of membrane-immersed region according to

$$\phi_\parallel = 4\pi a^2 h\eta/kT \tag{4}$$

where a is the radius of the cylindrical membrane-immersed part, h the length of the membrane-immersed part, η the membrane viscosity, k the Boltzman constant, and T the absolute temperature. The measured ϕ_l is the weighted average of different $\phi_\parallel$ because of the multiple rotating populations as described under "Experimental Procedures." Thus, the measured rapid $\phi_l \simeq 40\mu$s for P-450/reductase = 1:1 vesicles with $L/P_{450} = 1$–10, which is similar to the most rapid $\phi_l \simeq 55$ μs in the absence of reductase in $L/P_{450} = 10$ vesicles, may represent slight difference of the size of membrane-immersed part between cytochrome P-450 and the possible P-450·reductase 1:1 complex (see Fig. 5).

As discussed in more detail in the accompanying paper, lateral diffusion of the cytochrome P-450·reductase complex can be estimated according to the following relationship (33):

$$D_L^{\text{loc}} \simeq (\ln\eta'/\eta - \gamma)a^2/\phi_\parallel \tag{5}$$

In an accompanying paper, we get $D_L^{\text{loc}} \simeq 10^{-9}$ cm^2/s for cytochrome P-450 alone. As mentioned above, a and ϕ_l of the cytochrome P-450·reductase complex are almost the same as those of cytochrome P-450 alone resulting in the same $D_L^{\text{loc}} \simeq 10^{-9}$ cm^2/s for the complex. This result suggests that the present P-450·reductase complex can undergo rapid lateral diffusion and interact with other cytochrome P-450 molecules within the known rate of electron transfer in the monooxygenase system.

Protein aggregation similar to that in pure cytochrome P-450 vesicles has recently been described for cytochrome oxidase reconstituted in PC/PE/cardiolipin vesicles (34). A decrease in rotational mobility was observed for cytochrome oxidase when the concentration of the enzyme was increased in pure cytochrome oxidase vesicles. In contrast to the case of cytochrome P-450 + reductase, the presence of cytochrome bc_1 complex in cytochrome oxidase vesicles further decreased rotational mobility of cytochrome oxidase (34). When cytochrome bc_1 was replaced by the same amount (by weight) of cytochrome oxidase, no change in rotational mobility was observed for the oxidase. Therefore, the interaction between cytochrome oxidase and cytochrome bc_1 complex can be described as a hydrophobic interaction with the formation of random molecular aggregates.

Catalytic Activity, Electron Transfer, and Other Studies of Cytochrome P-450 + Reductase System Related to the Molecular Organization in Monooxygenase Systems—Complex formation between cytochrome P-450 and reductase has been indicated previously. Lu *et al.* (35) investigated steady state kinetics of substrate hydroxylation of PB-induced rat liver cytochrome P-450 and reductase in egg PC vesicles. The change in P-450/reductase ratio affected only the maximal velocity, V_{max}, but the K_m remained unchanged, and maximal activity was achieved with P-450/reductase = 1:1, implying that cytochrome P-450 forms a 1:1 molecular complex with

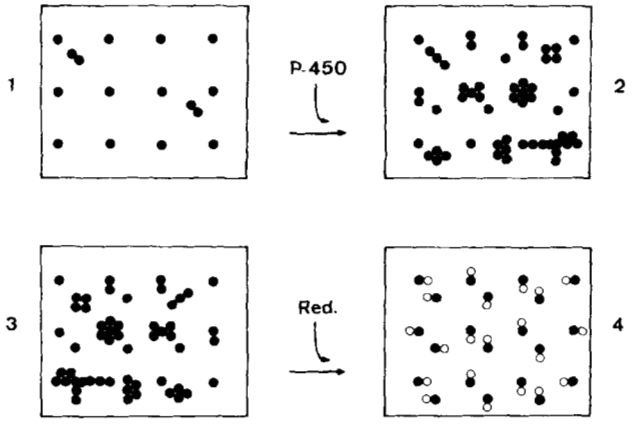

FIG. 5. **Schematic illustration of the mobilization of cytochrome P-450 by reductase in the liposomal membrane.** In $L/P_{450} = 30$ (*1*), all cytochrome P-450 is rotating. Increasing the P-450 concentration to $L/P_{450} = 1$ (*2*) causes the formation of P-450 aggregates which appear immobile within the time range of the present experiments. Adding an equimolar amount of reductase to cytochrome P-450 in the $L/P_{450} = 1$ system (*3*) results in disintegration of cytochrome P-450 aggregates by formation of a monomolecular 1:1 complex of cytochrome P-450 with reductase (*4*) with a rotational mobility of cytochrome P-450 similar to that in $L/P_{450} = 30$ vesicles.

reductase. Very similar steady state kinetic data for substrate hydroxylation were obtained by Miwa *et al.* (36) in a reconstituted nonmembranous rat liver system, supporting a 1:1 molecular complex of cytochrome P-450 with reductase being essential for catalysis. For the nonmembranous reconstituted rabbit liver system, French *et al.* (37) demonstrated a complex formation between six reductase and six cytochrome $P\text{-}450_{LM_2}$ molecules with gel exclusion chromatography. A 1:1 molar ratio in the above complex of solubilized reconstituted system was also indicated with absorption spectrophotometry in the visible region, CD spectrophotometry in the far ultraviolet region, as well as steady state kinetics of substrate hydroxylation. The determination of the dissociation constants suggested that various components of the hydroxylation systems are mutually beneficial in favoring binding reactions which lead to the formation of functional enzyme complexes. However, we should note that molecular interactions in nonmembranous systems may be different from those in membranous systems. For example, although rabbit cytochrome $P\text{-}450_{LM_4}$ forms a 1:1 complex with reductase in nonmembranous reconstituted systems, no such stable complex was observed in phospholipid vesicles as judged from catalytic activities (16).

On the other hand, there are investigations which support the concept of independent lateral mobility of cytochrome P-450 and reductase in the plane of the membrane. Several groups recently described the incorporation of rabbit liver reductase and cytochrome $P\text{-}450_{LM_{1-4}}$ into phospholipid vesicles. At a fixed molar ratio of 1:1 of the two proteins, the reduction rate of cytochrome P-450 and the substrate hydroxylation increased upon decreasing the phospholipid content of vesicles (15). This observation suggests that the interaction between reductase and cytochrome P-450 is effected by their random collisions caused by lateral mobility in the plane of the membrane. Measurements of substrate hydroxylation at various molar ratios of rabbit cytochrome P-450 to reductase in liposomes indicated that a limited number of reductase molecules serve a great number of cytochrome molecules, suggesting a rapid independent lateral diffusion of the proteins (16). The same conclusion was obtained for 3-methylcholanthrene-induced rat liver cytochrome P-448 and reductase in phospholipid vesicles by measuring the rate of substrate hydroxylation with varying protein concentrations (35). Incorporation of purified rat liver cytochrome P-450, P-448, and reductase into microsomal membranes (38, 39) suggests the possibility of lateral collision-controlled electron transfer from reductase to the cytochrome in the natural membrane. Increased kinetics of cytochrome reduction and stimulation of substrate hydroxylation in the above system demonstrated that incorporated proteins became a functional part of the microsomal monooxygenase system, implying that enzymes in the monooxygenase system are translationally mobile.

In the present reconstitution experiments, only one species of cytochrome P-450 has been used. Liver microsomes contain 20–30 times more cytochrome P-450's than reductase, and there are several different species of cytochromes. So far it is unclear whether reconstituted other species of cytochrome P-450, different from that used here, would interact with the reductase in the same manner. In addition, the complex situation in the microsomal membrane does not allow a direct extrapolation of the results obtained with the model membrane to the microsomal membrane. Nevertheless, we feel that these and previous data justify the following speculations as to the situation in microsomes. (i) Microsomal P-450 and reductase may form a monomolecular 1:1 complex. (ii) Excess cytochrome P-450 exists as a mixture of mobile monomers and small aggregates. (iii) Electron transfer takes place in a monomolecular 1:1 complex and/or by collision with mobile cytochrome P-450.

Conclusion—Determination of flash-induced absorption anisotrophy of the cytochrome P-450·CO complex is a nondestructive technique which yields direct information about the mobility and interaction of the cytochrome with neighboring molecules in the membrane. In this report, we show a specific complex formation between cytochrome and reductase in artificial liposomes of a phospholipid composition and protein content closely resembling that of the natural microsomal membrane. The complex has a small size and probably consists of 1 reductase and 1 cytochrome molecule as shown by the very similar rotational relaxation time of freely mobile cytochrome and cytochrome complexed with reductase.

Acknowledgment—We wish to thank Dr. M. Müller for performing the electron microscopy.

REFERENCES

1. Estabrook, R. W., Werringloer, J., and Peterson, J. A. (1979) in *Xenobiotic Metabolism: In Vitro Methods* (Paulson, G. D., Frear, D. S., and Marks, E. P., eds) Symposium Series No. 97, pp. 149–179, American Chemical Society, Washington D. C.
2. White, R. E., and Coon, M. J. (1980) *Annu. Rev. Biochem.* **49,** 315–356
3. Omura, T. (1978) in *Cytochrome P-450* (Sato, R., and Omura, T., eds) Chap. 1, Kodansha, Tokyo
4. Gum, J. R., and Strobel, H. W. (1979) *J. Biol. Chem.* **254,** 4177–4185
5. Black, S. D., French, J. S., Williams, C. H., Jr., and Coon, M. J. (1979) *Biochem. Biophys. Res. Commun.* **91,** 1528–1535
6. De Pierre, J. W., and Ernster, L. (1977) *Annu. Rev. Biochem.* **46,** 201–262
7. Cohen, B. S., and Estabrook, R. W. (1971) *Arch. Biochem. Biophys.* **143,** 54–65
8. Werringloer, J., and Kawano, S. (1980) in *Microsomes, Drug Oxidations, and Chemical Carcinogenesis* (Coon, M. J., Conney, A. H., Estabrook, R. W., Gelboin, H. V., Gillette, J. R., and O'Brien, P. J., eds) pp. 469–476, Academic Press, New York
9. Brunström, A., and Ingelmann-Sundberg, M. (1980) *Biochem. Biophys. Res. Commun.* **95,** 431–439
10. Franklin, M. R., and Estabrook, R. W. (1971) *Arch. Biochem. Biophys.* **143,** 318–329
11. Yang, C. S. (1975) *FEBS Lett.* **54,** 61–64
12. Peterson, J. A., Ebel, R. E., O'Keeffe, D. H., Matsubara, T., and Estabrook, R. W. (1976) *J. Biol. Chem.* **251,** 4010–4016
13. Duppel, W., and Ullrich, V. (1976) *Biochim. Biophys. Acta* **426,** 399–407
14. Bösterling, B., Stier, A., Hildebrandt, A. G., Dawson, J. H., and Trudell, J. R. (1979) *Mol. Pharmacol.* **16,** 332–342
15. Taniguchi, H., Imai, Y., Iyanagi, T., and Sato, R. (1979) *Biochim. Biophys. Acta* **550,** 341–356
16. Ingelmann-Sundberg, M., and Johannson, I. (1980) *Biochemistry* **19,** 4004–4011
17. Richter, C., Winterhalter, K. H., and Cherry, R. J. (1979) *FEBS Lett.* **102,** 151–154
18. Greinert, R., Staerk, H., Stier, A., and Weller, A. (1979) *J. Biochem. Biophys. Meth.* **1,** 77–83
19. West, S. B., Huang, M.-T., Miwa, G. T., and Lu, A. Y. H. (1979) *Arch. Biochem. Biophys.* **193,** 42–50
20. Yasukochi, Y., and Masters, B. S. S. (1976) *J. Biol. Chem.* **251,** 5337–5344
21. Rhoden, V., and Goldin, S. M. (1979) *Biochemistry* **18,** 4173–4176
22. Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, F. D. (1977) *Biochemistry* **16,** 2806–2810
23. Szoka, F., Jr., and Papahadjopoulos, D. (1980) *Annu. Rev. Biophys. Bioeng.* **9,** 467–508
24. Moor, H., Kistler, J., and Müller, M. (1976) *Experientia* **32,** 805
25. Nash, T. (1953) *Biochem. J.* **55,** 416–421
26. Kawato, S., Gut, J., Cherry, R. J., Winterhalter, K. H., and Richter, C. (1982) **257,** 7023–7029
27. Kawato, S., and Kinosita, K., Jr. (1981) *Biophys. J.,* **36,** 277–296
28. Strobel, H. W., and Dignam, J. D. (1978) *Methods Enzymol.* **52,** 89–96

29. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239,** 2370–2378
30. Laemmli, U. K. (1970) *Nature* **227,** 680–685
31. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
32. De Pierre, J. W., and Dallner, G. (1975) *Biochim. Biophys. Acta* **415,** 411–472
33. Saffman, P. G., and Delbrück, M. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 3111–3113
34. Kawato, S., Sigel, E., Carafoli, E., and Cherry, R. J. (1981) *J. Biol. Chem.* **256,** 7518–7527
35. Lu, A. Y. H., and Miwa, G. T. (1980) in *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (Gustafsson, J.-Å., Carlstedt-Duke, J., Mode, A., and Rafter, J., eds) pp. 125–128, Elsevier/North-Holland, Amsterdam
36. Miwa, G. T., West, S. B., Huang, M.-T., and Lu, A. Y. H. (1979) *J. Biol. Chem.* **254,** 5695–5700
37. French, J. S., Guengerich, F. P., and Coon, M. J. (1980) *J. Biol. Chem.* **255,** 4112–4119
38. Yang, C. S., and Strickhart, F. S. (1975) *J. Biol. Chem.* **250,** 7968–7972
39. Miwa, G. T., West, S. B., and Lu, A. Y. H. (1978) *J. Biol. Chem.* **253,** 1921–1929