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Rotation of Cytochrome P-450

COMPLEX FORMATION OF CYTOCHROME P-450 WITH NADPH-CYTOCHROME P-450 REDUCTASE IN LIPOSOMES DEMONSTRATED BY COMBINING PROTEIN ROTATION WITH ANTIBODY-INDUCED CROSS-LINKING*

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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 by a cholate dialysis technique. 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. All cytochrome P-450 was found to be rotationally mobile when co-reconstituted with equimolar amounts of NADPH-cytochrome P-450 reductase in lipid to cytochrome P-450 ($(L/P_{450}) = 1$ (w/w)) vesicles. Antibodies against NADPH-cytochrome P-450 reductase were raised. Their specificity was demonstrated by Ouchterlony double diffusion analysis. Antireductase Fab fragments were prepared from antireductase IgG by papain digestion. The *N*-demethylation of benzphetamine, catalyzed by the proteoliposomes, was significantly inhibited by antireductase IgG and by antireductase Fab fragments. Cross-linking of NADPH-cytochrome P-450 reductase by antireductase IgG resulted in complete immobilization of cytochrome P-450 in $L/P_{450} = 1$ vesicles. Antireductase IgG also immobilized cytochrome P-450 in $L/P_{450} = 5$ vesicles, although the degree of immobilization was slightly smaller. No immobilization of cytochrome P-450 in $L/P_{450} = 1$ vesicles was detected in the presence of antireductase Fab fragments or preimmune IgG. These results further support the proposal of the formation of monomolecular complexes between cytochrome P-450 and NADPH-cytochrome P-450 reductase in liposomal membranes (Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., and Kawato, S. (1982) *J. Biol. Chem.* 257, 7030-7036).

Cytochrome P-450 and NADPH-cytochrome P-450 reductase are key enzymes of the hepatic microsomal monooxygenase system catalyzing the oxidative metabolism of various xenobiotics and endogenous substrates (1-3). These enzymes are also involved in the biotransformation of physiologically important compounds such as fatty acids, prostaglandins, leukotrienes, and steroids. Cytochrome P-450 is deeply imbedded in the microsomal membrane (4) while NADPH-cytochrome P-450 reductase ($M_r \sim 78{,}000$) is anchored to this membrane by a small hydrophobic segment ($M_r \sim 6{,}000$-$10{,}000$) (5). The hydrophilic part of the reductase molecule protrudes from the membrane into the cytoplasm.

The odd stoichiometry (up to 20-30 cytochrome P-450 molecules per 1 reductase molecule) and the topology of the two enzymes within the membrane both raised questions concerning the mechanism of electron transfer from NADPH via the reductase to the cytochrome and the functional interaction of the two proteins in the monooxygenase system. Essentially two mechanisms of electron transfer between NADPH-cytochrome P-450 reductase and cytochrome P-450 can be envisaged: one is transfer within a stable complex, the other is electron transfer by random collisions of rotationally and laterally diffusing proteins. In recent years, several different models have been proposed for the mode of interaction of NADPH-cytochrome P-450 reductase and cytochrome P-450 in the microsomal membrane. 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 (6) postulated a rigid arrangement of reductase and cytochrome ("cluster") in the membrane, whereas Yang's group (7) proposed an interaction by random collisions. Based on temperature-dependent biphasic reduction kinetics, Peterson *et al.* (8) postulated a modified "cluster model" where a number of cytochrome molecules were grouped around a centrally located reductase molecule. Rapid phase reduction was thought to reflect clustered cytochrome molecules, whereas slow phase reduction was thought to result from reduction of unaggregated satellite cytochrome molecules upon lateral diffusion and collision. Measurements of reduction kinetics and substrate hydroxylation in reconstituted systems led Sato and co-workers (9) to suggest that random collision of cytochrome P-450 and reductase is responsible for their functional interaction. This view was supported by Ingelmann-Sundberg and Johansson (10).

So far, few studies have been performed for direct measurements of protein-protein interactions between enzymes of the hepatic monooxygenase system (11-14). We have recently reported the rotational diffusion of cytochrome P-450 in microsomal and liposomal membranes, detected by measuring the decay of absorption anisotropy after photolysis of the heme·CO complex of cytochrome P-450 (11, 15, 16). The rotational mobility of cytochrome P-450 in proteoliposomes was shown to be dependent on the L/P_{450}[1] ratio. All cyto-

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‡ To whom correspondence and reprint requests should be addressed.

§ Present address, Department of Chemistry, University of Essex, Colchester CO4 3SQ, England.

¶ Present address, Research Development Corp., c/o Maruzen Oil Research Center, Satte, Kitakatsusika-gun, Saitama 340-01, Japan.

[1] The abbreviations used are: L/P_{450}, lipid to cytochrome P-450 (w/w) ratio; P-450/reductase, cytochrome P-450 to reductase (mol/mol)

chrome P-450 was rapidly rotating in lipid-rich vesicles of $L/P_{450} = 30$ having a mean rotational relaxation time ~50 μs within the experimental time range of 500 μs. Decreasing the lipid to protein ratio to $L/P_{450} = 1$ resulted in a significant decrease of the mobile fraction of cytochrome P-450 presumably due to aggregation of the cytochrome molecules in this lipid-poor membrane. On the other hand, co-reconstitution of an equimolar amount of NADPH-cytochrome P-450 reductase with cytochrome P-450 in $L/P_{450} = 1$ proteoliposomes resulted in complete mobilization of cytochrome P-450 with a mean rotational relaxation time of about 50 μs. These findings suggested the formation of a monomolecular[2] 1:1 complex between cytochrome P-450 and reductase in proteoliposomes (16).

Here, we present further proof of the existence of stable cytochrome P-450·reductase complexes. Rotational mobility of cytochrome P-450 in the presence and absence of specific antibodies directed against NADPH-cytochrome P-450 reductase is measured. Immobilization of cytochrome P-450 occurs after cross-linking of reductase with the antireductase antibodies. It is concluded that immobilization of cytochrome P-450 by antireductase antibodies is caused by cross-linking of pre-existing monomolecular cytochrome P-450·reductase complexes in the liposomal membrane.

EXPERIMENTAL PROCEDURES

Purification of Microsomal Enzymes—Cytochrome P-450 and NADPH-cytochrome P-450 reductase were purified from phenobarbital-treated rats according to West *et al.* (17) and Yasukochi and Masters (18). Minor modifications of the original method (17) were according to Gut *et al.* (16). The specific contents were 14.5–17 nmol of cytochrome P-450/mg of protein and 50,000–60,000 units/mg of protein for the reductase.

Preparation of Antibodies—Purified NADPH-cytochrome P-450 reductase was used to immunize female New Zealand white rabbits. Six hundred μg of the immunogen in Freund's complete adjuvant were injected at a few sites along their flanks every 7 days for 3 weeks. One week later, the rabbits were bled and the sera were collected. Every 3 weeks thereafter, the animals were boosted with 100 μg of immunogen without Freund's adjuvant and bled 1 week later. The immunoglobulin fraction G (IgG) was prepared according to Thomas *et al.* (19) and stored at −80 °C in 50 mM potassium phosphate buffer, pH 8.0. Preimmune IgG was prepared in a similar manner.

Preparation of Antireductase Fab Fragments from Antireductase IgG—In order to prepare antireductase Fab fragments, proteolysis of antireductase antibodies was carried out according to Mage (20), using CM-Sephadex C-25 in the ion exchange chromatography of the papain digest. Without further purification, the eluted antireductase Fab material (40 ml, about 300 mg of protein) was dialyzed against a 100-fold volume of 50 mM potassium phosphate, pH 8.0, concentrated by ultrafiltration to about 30 mg of protein/ml, and stored at −80 °C. Prior to use in the experiments, antireductase antibody preparations were dialyzed overnight at 4 °C against the appropriate incubation buffers.

Ouchterlony Double Diffusion Analysis—The immunodiffusion media contained 0.9% agarose A, 80 mM sodium chloride, 15 mM sodium azide, and 1 M glycine; pH was adjusted to 7.4 with sodium hydroxide. The media were poured into Petri dishes and wells were punched with a template resulting in center to center distances of 10 mm. The wells were filled with appropriate amounts of protein and incubated at room temperature in a humid atmosphere for 1–2 days. For protein staining, the plates were washed 3 days with 300 mM sodium chloride in 50 mM potassium phosphate buffer, pH 8.4, then incubated overnight with 0.1% naphthol blue-black in 5% acetic acid and destained in 5% acetic acid.

Incubation of Proteoliposomes with Antibodies—Proteoliposomes were prepared as described previously (16) with $L/P_{450} = 1$ or 5 weight by weight ratios. The molar ratio between cytochrome and reductase was 1. Prior to rotational diffusion measurements, proteoliposomes were incubated with antireductase IgG, antireductase Fab fragments, preimmune IgG, or with equal amounts of buffer for 10 min at room temperature. After incubation of proteoliposomes with antireductase IgG, the proteoliposomes were pelleted at 40,000 × *g* for 20 min, resuspended in 20 mM Hepes, pH 8.0, containing 20% glycerol and 0.1 mM EDTA, washed once, and resuspended again in the above medium. The incubation of proteoliposomes with antireductase Fab fragments, preimmune IgG, or buffer was followed by centrifugation at 160,000 × *g* for 3 h, one washing, and subsequent resuspension in the above medium. No degradation of cytochrome P-450 was observed during this treatment based on the amount of the cytochrome P-450·CO complex determined spectrophotometrically.

Reduction of Cytochrome c by Purified NADPH-Cytochrome P-450 Reductase in the Presence of Antireductase IgG—These measurements were carried out at 25 °C in 50 mM potassium phosphate, pH 7.4. Prior to use, protein preparations were dialyzed against this buffer. The test cuvette contained, in a final volume of 1 ml, 4 μg of NADPH-cytochrome P-450 reductase, 40 μM cytochrome *c*, and appropriate amounts of antireductase IgG, antireductase Fab fragments, or preimmune IgG. After 10 min of preincubation at 25 °C, cytochrome *c* reduction was started with 1 mM NADPH and assayed according to Strobel and Dignam (21).

Hydroxylation Activity of Proteoliposomes in the Presence of Antireductase IgG—The monooxygenase activity of the proteoliposomes was measured in a final volume of 1 ml of 20 mM Hepes, pH 7.4, 20% glycerol, and 0.1 mM EDTA. The composition of the reconstituted system was $L/P_{450} = 1$ (w/w) and P-450/reductase = 5:1 (mol/mol). The cytochrome P-450 concentration was 0.2 μM. Glucose 6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (2.5 units) were included as a NADPH-regenerating system. Benzphetamine at 1 mM was used as the substrate. When antireductase IgG, antireductase Fab fragments, or preimmune IgG was present, the samples were preincubated for 10 min at 37 °C. Hydroxylation was started by the addition of 0.5 mM NADPH and, after 1 min, the formation of formaldehyde was measured according to Nash (22).

Other Methods—NADPH-cytochrome P-450 reductase was assayed according to Strobel and Dignam (21) at room temperature with cytochrome *c* as electron acceptor in 1 ml of 0.3 M potassium phosphate, pH 7.7. Cytochrome P-450 was measured spectrophotometrically according to Omura and Sato (23). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in the Laemmli system (24). Protein concentrations were determined by the method of Lowry *et al.* (25) with bovine serum albumin as standard; samples containing detergent were analyzed in the presence of 1% sodium dodecyl sulfate in Lowry's reagent to avoid overestimation of the protein content due to the presence of the detergent.

Rotational Diffusion Measurements and Analysis—For rotational diffusion measurements, proteoliposomes were suspended in 80% (w/w) glycerol to reduce vesicle tumbling and light scattering. The final heme concentration was 7–10 μM. Samples were reduced by a few grains of dithionite and CO was slowly bubbled through for 1 min. The sample cuvette was then sealed with a rubber cap to keep cytochrome P-450 saturated with CO.

The flash photolysis apparatus used for rotation measurements is described in detail elsewhere (26). Briefly, the sample was excited at 532 nm by a vertically polarized flash of 10-ns duration from a Nd/YAG laser (JK-laser HY 200, second harmonic). 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, 4096 signals were averaged using a Datalab DL 102 A signal averager. A further improvement in the signal to noise ratio was obtained by averaging the data of several experiments. The unchanged amount of the cytochrome P-450·CO complex determined spectrophotometrically excluded degradation of cytochrome P-450 during the experiment.

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ratio; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DPPC, L-α-phosphatidylcholine dipalmitoyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P-450_{LM2}, rabbit liver cytochrome purified from phenobarbital-induced microsomes, designated according to its electrophoretic property.

[2] The term "monomolecular complex" used in this and previous publications (15, 16) is used to designate a heterodimeric complex between one reductase and one cytochrome molecule in membranes.

Analysis of $r(t)$ is based on a model of rotation of cytochrome P-450 about the membrane normal (15, 27). When there is a single rotating species of cytochrome P-450 with the rotational relaxation time, $\phi_{||}$, $r(t)$ is given by:

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

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 $\phi_{||}$ are considered by analyzing the data by the following equation:

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

where ϕ is the average rotational relaxation time. Here it should be noted that $r_3 = \frac{1}{4}(3\cos^2\theta_N - 1)^2$ when all cytochrome P-450 molecules are rotating, even in the presence of multiple rotating species.

Because of a good signal to noise ratio using a Nd/YAG laser, $r(t)$ curves were measured with a 2-ms time range. When all cytochrome P-450 was rotating in $L/P_{450} = 30$ vesicles, the anisotropy decayed to zero within 1 ms (*i.e.* $r_3 = 0$). This indicates that the heme plane is tilted by ≃55° from the membrane plane (see Fig. 5, *curve B*). 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

$$r_3/r(0) = f_{im} \quad (5)$$

Materials—Phosphatidylcholine, phosphatidylethanolamine, both from egg yolk, and phosphatidylserine from bovine spinal cord, all grade I, were purchased from Lipid Products, Nutfield, U. K. Cytochrome *c* from horse heart, type III, mercuripapain, and glucose-6-phosphate dehydrogenase were obtained from Sigma. Benzphetamine was obtained from Applied Science Laboratories.

RESULTS AND ANALYSIS

Specificity of Antireductase IgG as Judged from Ouchterlony Double Diffusion Experiments

In Ouchterlony double diffusion analysis, antireductase IgG precipitated the antigen (*i.e.* purified NADPH-cytochrome P-450 reductase) as a single sharp band. No cross-reactivity was detected against purified cytochrome P-450. A single band formed also when proteoliposomes ($L/P_{450} = 1$ (w/w), P-450/reductase = 1:1 (mol/mol)), solubilized by 2% sodium cholate (w/w) were used in immuno-double diffusion (Fig. 1*A*). No precipitate formed when antireductase IgG was replaced by preimmune IgG. On the other hand, antireductase Fab fragments, prepared from antireductase IgG by papain digestion, significantly inhibited the formation of the precipitate obtained from reacting antireductase IgG with its antigen (Fig. 1*B*), indicating competitive binding of monovalent and divalent antibodies to the antigenic determinations of reductase.

Influence of Antireductase IgG on Cytochrome c Reduction by Purified NADPH-cytochrome P-450 Reductase

Fig. 2*A* shows a significant inhibition of cytochrome *c* reducing activity by NADPH-cytochrome P-450 reductase in the presence of antireductase IgG. A 75-fold excess of antireductase IgG over reductase (w/w) resulted in an inhibition of the initial reductase activity to about 50%. The lowest reductase activity was achieved at a weight ratio of 200. Addition of the same amount (by weight) of antireductase Fab fragments resulted in a similar extent of inhibition. In spite of the absence of detectable precipitation of reductase by preimmune IgG in the Ouchterlony double diffusion analysis, the reductase activity was slightly inhibited.

Inhibition of Hydroxylation Activity in Proteoliposomes by Antibodies

The *N*-demethylation of benzphetamine was measured for 1 min in control proteoliposomes with $L/P_{450} = 1$ (w/w) and P-450/reductase = 5:1 (mol/mol). About 100 nmol of formaldehyde were formed per nmol of P-450 per min. When a 75-fold excess (w/w) of antireductase IgG over reductase was present in the system, only 11% residual activity was observed. No further inhibition was obtained by increasing the amounts of antireductase IgG up to a 200-fold excess (Fig.

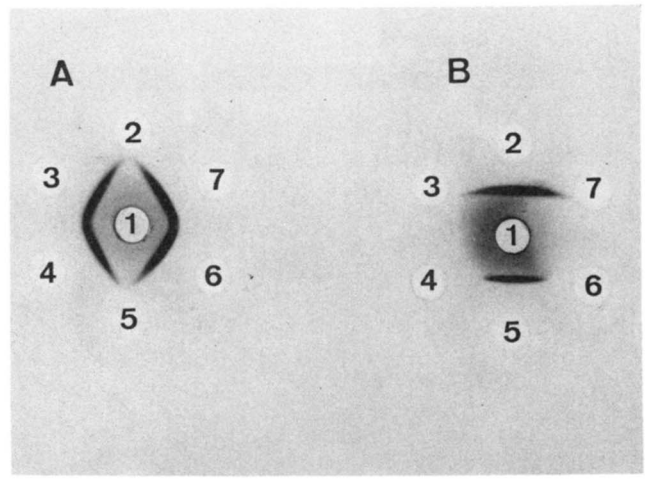

FIG. 1. **Ouchterlony double diffusion analysis.** Preparation of the immunodiffusion plates was as described under "Experimental Procedures." All wells were filled with 10 μl of the indicated protein preparations containing the given amounts of protein. *A:* the *center well* contained antireductase IgG (200 μg) and *wells 2–7* contained, in this sequence, preimmune IgG (200 μg), purified NADPH-cytochrome P-450 reductase (2 μg), solubilized rat liver microsomes (50 μg), purified cytochrome P-450 (10 μg), proteoliposomes (solubilized in 1% sodium cholate solution, P-450/reductase = 1:1, 5 μg of protein total), and reductase (2 μg), respectively. *B:* the *center well* contained antireductase IgG (200 μg), *wells 2* and *5* contained purified NADPH-cytochrome P-450 reductase (2 μg), *wells 4* and *6* contained antireductase Fab fragments (200 μg), and *wells 3* and *7* contained 50 mM potassium phosphate buffer, pH 8.0.

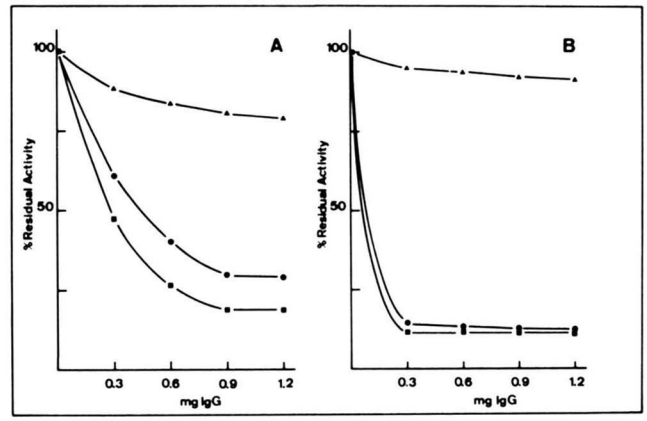

FIG. 2. **Effect of antireductase IgG on the enzymatic activity of NADPH-cytochrome P-450 reductase.** *A*, the reduction of cytochrome *c* by purified NADPH-cytochrome P-450 reductase was measured in presence of antireductase IgG (■——■), antireductase Fab fragments (●——●), and preimmune IgG (▲——▲). Control activity is taken as 100%. The test tube contained, in a final volume of 1 ml, 4 μg of NADPH-cytochrome P-450 reductase and 40 μM cytochrome *c* in 50 mM potassium phosphate, pH 7.4. The reaction was started with 1 mM NADPH. *B*, the *N*-demethylation of benzphetamine was measured in control proteoliposomes ($L/P_{450} = 1:1$, P-450/reductase = 5:1) by determination of the amount of formaldehyde formed per nmol of cytochrome P-450 per min (= 100% activity). Residual activity was determined in the presence of antireductase IgG (■——■), antireductase Fab fragment (●——●), and preimmune IgG (▲——▲). Experimental conditions are given under "Experimental Procedures."

2*B*). Addition of antireductase Fab fragments at the same weight ratio resulted in similar inhibition. In contrast, nearly no inhibition was observed in the presence of an equal amount of preimmune IgG (Fig. 2*B*).

Rotational Diffusion of Cytochrome P-450 Co-reconstituted with Reductase in Proteoliposomes

Effect of Antireductase IgG on Rotational Mobility of Cytochrome P-450—Cytochrome P-450 and NADPH cytochrome P-450 reductase were co-reconstituted in liposomes of a composition closely resembling that of the microsomal membrane (*i.e.* PC/PE/PS = 10:5:1 (w/w)) with varying L/P_{450} ratios. Rotational diffusion measurements were performed at 20 °C. Fig. 3 shows the time-dependent absorption anisotropy $r(t)$ of cytochrome P-450 in proteoliposomes with $L/P_{450} = 1$ and P-450/reductase = 1. As shown previously (16), in the absence of antireductase IgG, the absorption anisotropy decays (Fig. 3, *curve B*) due to rotational motion of cytochrome P-450 about the normal to the membrane plane. The presence of a 100-fold excess (w/w) of antireductase IgG over reductase abolishes the decay in $r(t)$ (Fig. 3, *curve A*), indicating the complete immobilization of cytochrome P-450. Cross-linking of reductase, which is suggested to be the partner in a cytochrome P-450·reductase complex, by antireductase IgG could explain the observed immobilization of cytochrome P-450.

Effect of Antireductase Fab Fragments on Rotational Diffusion of Cytochrome P-450—Monovalent antireductase Fab fragments directed against the reductase inhibited the enzymatic activity of the purified reductase both in solution and in proteoliposomes, but did not decrease the rotational mobility of cytochrome P-450 (Fig. 3, *curve C*). The observed immobilization of cytochrome P-450 by antireductase IgG is therefore not due to a steric hindrance of the P-450 rotation by reductase-antibody complexes and is in keeping with the concept that the immobilization of cytochrome P-450 by the divalent antireductase IgG is a consequence of cross-linking individual reductase molecules.

Similar experiments were performed with preimmune IgG in order to rule out nonspecific effects on cytochrome P-450

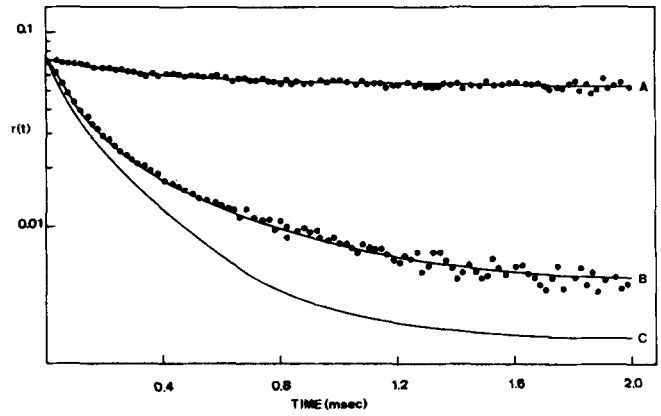

FIG. 3. **Time-dependent absorption anisotropy of cytochrome P-450 in L/P_{450} = 1:1 (w/w) proteoliposomes after binding of antireductase IgG.** Photolysis of samples (7–10 μM cytochrome P-450) was by a vertically polarized laser flash at 532 nm and $r(t)$ recording at 450 nm. All measurements were performed in 80% (w/w) glycerol at 20 °C (~0.6 poise). *Solid lines* were obtained by fitting the data to Equation 4. For clarity, data points of *curve C* are omitted and curves are normalized to the same initial anisotropy $r(0)$. *A*, $L/P_{450} = 1$, P-450/reductase = 1, in the presence of a 100-fold excess (w/w) of antireductase IgG over reductase; *B*, $L/P_{450} = 1$, P-450/reductase = 1, control experiment without any antireductase antibody; *C*, $L/P_{450} = 1$, P-450/reductase = 1, a 100-fold excess (w/w) of antireductase Fab fragments over reductase was present.

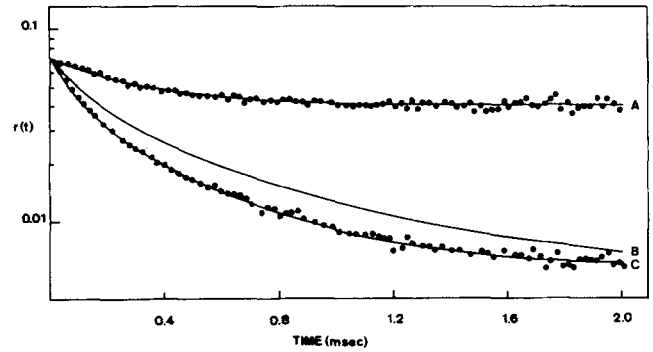

FIG. 4. **Time-dependent absorption anisotropy of cytochrome P-450 in L/P_{450} = 5:1 (w/w) proteoliposomes following antireductase IgG binding.** The following amounts of antibodies were added to $L/P_{450} = 5$, P-450/reductase = 1 proteoliposomes. *A*, a 200-fold excess (w/w) of antireductase IgG over reductase; *B*, a 200-fold excess (w/w) of preimmune IgG over reductase; *C*, no addition. Other experimental conditions are the same as in Fig. 3. Curves are normalized to the same initial anisotropy $r(0)$. Data points of *curve B* are omitted for clarity.

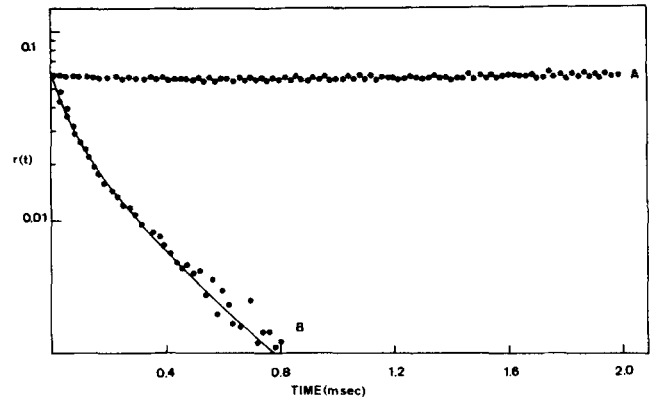

FIG. 5. **Time-dependent absorption anisotropy of cytochrome P-450 in proteoliposomes with different lipid compositions.** Experiments were carried out as described in the legend of Fig. 3. *A*, cytochrome P-450 alone in DPPC liposomes with $DPPC/P_{450} = 1$, measured at 20 °C. Very similar results were obtained with $DPPC/P_{450} = 5$ and 30. *B*, cytochrome P-450 alone in PC/PE/PS = 10:5:1 liposomes with $L/P_{450} = 30$. *Solid lines* were obtained by fitting the data to Equation 4.

rotation. In control proteoliposomes with $L/P_{450} = 5$ and P-450/reductase = 1, the $r(t)$ curve decayed to zero (Fig. 4, *curve C*). The presence of preimmune IgG did not affect the time course of the $r(t)$ curve within experimental error (Fig. 4, *curve B*) indicating no immobilization of cytochrome P-450 by preimmune IgG in contrast to what is observed with antireductase IgG. A similar lack of immobilization by preimmune IgG was also observed in proteoliposomes with $L/P_{450} = 1$ and P-450/reductase = 1. In fact, depending on preparations, not only a slight immobilization but even a slight mobilization of cytochrome P-450 was observed in the presence of preimmune IgG.

Effect of Increased Lipid/Protein Ratio—Fig. 4, *curve A*, shows the time-dependent absorption anisotropy of proteoliposomes with $L/P_{450} = 5$ and P-450/reductase = 1 in the presence of a 200-fold excess (w/w) of antireductase IgG. Not all cytochrome P-450 is immobilized by antireductase IgG in these vesicles. This is probably due to a nonequimolar distribution of cytochrome P-450 and reductase in individual proteoliposomes at higher L/P_{450} ratios.

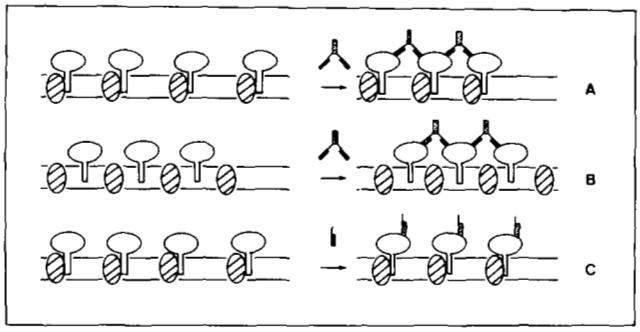

FIG. 6. **Model illustrating the effects of antireductase antibodies on cytochrome P-450 rotational mobility in liposomal membranes.** *A*, cytochrome P-450 (*hatched symbols*) and NADPH-cytochrome P-450 reductase (*open symbols*) form complexes which are cross-linked by antireductase IgG (*dotted symbols*). This aggregation reduces the rotational mobility of cytochrome P-450·reductase complexes. *B*, if cytochrome P-450 and reductase would exist independently in the membrane, cross-linking of reductase by antireductase IgG would not affect the rotational mobility of cytochrome P-450. *C*, monovalent antireductase Fab fragments cannot cross-link reductase molecules. Therefore, cytochrome P-450 rotation is unaffected even when complexed with reductase. Cytochrome P-450 and reductase are shown as monomers, although higher aggregates may also exist in the membrane (16, 39). Antibodies are depicted as interacting exclusively with antigenic sites on the membrane-protruding part of the reductase molecules. One should note, however, that interaction with antigenic sites on the membrane-immersed part of the reductase molecule are also possible.

Absence of Vesicle Tumbling

Rotational diffusion of cytochrome P-450 in vesicles was measured in a viscous medium of 80% glycerol in water (w/w) (~0.6 poise at 20 °C). Nevertheless, vesicle tumbling might contribute to the observed decay in $r(t)$ and the observed immobilization of cytochrome P-450 in antireductase IgG containing vesicles might be due to a simple cross-linking of vesicles. Vesicle tumbling was therefore examined at 37 and 20 °C with cytochrome P-450 incorporated in DPPC vesicles. Since at this temperature DPPC is in the gel phase, protein rotation should be absent and hence any decay in $r(t)$ would reflect vesicle tumbling. No decay in $r(t)$ was observed within the present time range of 2 ms, indicating that neither vesicle tumbling nor cytochrome P-450 rotation occurs (Fig. 5, *curve A*). Since no significant difference in vesicle size distribution between DPPC vesicles and $L/P_{450} = 1$ or 5 proteoliposomes was observed (16), we can exclude any contribution of vesicle tumbling to the observed decay in $r(t)$ in our experiments.

DISCUSSION

Reductase Antibody-induced Immobilization of Cytochrome P-450—In this study, we present direct evidence for the existence of a stable cytochrome P-450·reductase complex in liposomal membranes, as illustrated in Fig. 6*A*. This evidence arises from experiments where the rotational mobility of cytochrome P-450 is largely decreased by cross-linking of reductase by divalent antireductase IgG (see Figs. 3 and 4).

It could be argued that the observed reductase antibody-induced immobilization of P-450 is due to steric hindrance by antireductase IgG bound to reductase or being adsorbed by the lipid bilayer. However, it appears very unlikely that this would be the main cause of the immobilization, because monovalent antireductase Fab fragments and preimmune IgG do not significantly affect the mobility of P-450 (see Figs. 3 and 4), while binding of antireductase Fab fragments to the catalytic site of reductase is confirmed by a strong inhibition of hydroxylation activity in proteoliposomes. Since the rotational mobility depends mainly on the size of the membrane-immersed part of the protein (16), binding of Fab fragments most probably to the membrane-protruding catalytic part of reductase (28) should not significantly affect the rotational mobility of the P-450·reductase complex (see Fig. 6).

As judged from the very slow decay of the $r(t)$ curve on the present time scale, when proteins are cross-linked by antireductase IgG, the P-450·reductase complex should have a lifetime longer than 100 ms.

Therefore, this P-450·reductase complex could exist as a stable association within a known electron transfer rate of about ~85 ms/e^-/P-450 (NADPH→NADPH-cytochrome P-450 reductase→cytochrome P-450) (9, 29). Of course, it would be expected that the above P-450·reductase complex could exchange its partner molecules over a long time range (*e.g.* longer than 100 ms). It should be noted that the present observation alone does not give sufficient information about a stoichiometry of the two proteins in the complex.

With similar experimental techniques, Nigg *et al.* (30) investigated the protein-protein interaction of band 3 with glycophorin A in the human erythrocyte membrane. Rotation of band 3 was measured by observing the flash-induced absorption anisotropy of the probe eosin bound to band 3. Cross-linking of glycophorin A by antiglycophorin A antibodies resulted in complete immobilization of band 3. Thus, the occurrence of pre-existing band 3·glycophorin A complexes in a natural membrane was concluded.

Monomolecular Complex of Cytochrome P-450 with Reductase in Liposomes—A 1:1 complex formation between cytochrome P-450 and reductase, both prepared from phenobarbital-induced rat liver microsomes, has been inferred from steady state kinetics of substrate hydroxylation (31). Changing the P-450/reductase molar ratio in PC liposomes only affected the maximal velocity, V_{max}, whereas K_m remained unchanged. At a given lipid to protein ratio, the maximal activity was achieved at P-450/reductase = 1:1, implying that P-450 forms a binary functional 1:1 complex with reductase. Similar steady state kinetics for substrate hydroxylation (32) were also obtained in a reconstituted nonmembranous system of the proteins, supporting a monomolecular complex of cytochrome P-450 with reductase.

We have observed the mobilization effect of reductase on rotational diffusion of cytochrome P-450 in proteoliposomes (16). The degree of mobilization correlates well with the amount of reductase added and is complete at P-450/reductase = 1:1. It has been concluded from these results that cytochrome P-450 forms a monomolecular complex with reductase (16). An alternative interpretation could be that reductase might have a detergent-like effect, thereby fluidizing the membrane and mobilizing cytochrome P-450. However, if this were the case, antireductase IgG would not be expected to immobilize cytochrome P-450. In addition, the fluorescence polarization measurements of diphenylhexatriene showed a slight decrease of the membrane fluidity in the presence of reductase in the liquid crystalline state of the membrane of cytochrome P-450 proteoliposomes.[3]

Taken together, the above results and the present finding of the stable complex formation between these proteins, we conclude that cytochrome P-450 forms a monomolecular 1:1 complex with reductase in liposomes.

Complications in Intact Microsomal Membranes—In the present experiments, we have used only one species of cytochrome P-450, namely phenobarbital-induced rat liver cytochrome P-450, reconstituted in liposomes of a defined com-

[3] J. Gut and C. Richter, unpublished results.

position. On the other hand, phenobarbital- or methylcholanthrene-induced liver microsomes contain 20–30 times more cytochrome P-450 than reductase. Moreover, multiple species of cytochrome P-450 exist in liver microsomes, be it in respect to molecular weight, substrate specificity, and/or amino acid composition (33–35). Because of the complexity of the microsomal membranes, any extrapolation of our results with model membranes should be treated with caution.

When reductase forms a 1:1 complex with cytochrome P-450 in microsomes as has been observed in our proteoliposome study, not all P-450 can form a complex with reductase, suggesting that the majority of P-450 will independently diffuse and receive electrons by lateral collisions. There are investigations which support this view. Phenobarbital-induced rabbit liver $P\text{-}450_{LM2}$ was suggested to interact with reductase in a manner controlled by lateral collision (10). Decreasing the phospholipid content of liposomes resulted in an increased reduction rate of cytochrome $P\text{-}450_{LM2}$ as well as enhanced hydroxylation activity at a fixed molar ratio of the two proteins. The same conclusion was obtained for methylcholanthrene-induced rat liver cytochrome P-448 and reductase in liposomes by measuring the rate of substrate hydroxylation with varying protein concentrations (31).

On the basis of a different experimental approach, Omura and colleagues showed only about 5% of cytochrome P-450 to be associated with reductase in phenobarbital-induced rat liver microsomes.[4] Microsomes cross-linked by glutaraldehyde were solubilized and applied onto an anti-P-450 antibody column and the amount of trapped cytochrome P-450 was estimated from the reductase activity remaining on the column. This may indicate the existence of cytochrome P-448·reductase complexes in the microsomal membrane but questions about stoichiometry and lifetime of such complexes still remain open. Taken together, these results support the possibility of at least two types of electron transfer mechanisms between cytochrome P-450 and its reductase in microsomal membranes, namely 1) electron transfer within a stable binary complex and 2) lateral collision-controlled electron transport between independently diffusing component proteins.

Orientation of the Heme Plane of Cytochrome P-450—In the present study, we have observed that $r(t)$ decays to zero (*i.e.* $r_3 = 0$) on the time scale of 2 ms in proteoliposomes with $L/P_{450} \geqq 5$ regardless of whether reductase is present or not. Based on the rotation about membrane normal model, this result indicates that the heme plane of cytochrome P-450 is tilted by $\theta_N \simeq 55°$ from the plane of the membrane. The alternative interpretation, that $r_3 = 0$ is due to a possible isotropic rotation of cytochrome P-450 across the membrane, appears unlikely. So far, no integral membrane protein such as cytochrome P-450, cytochrome oxidase, bacteriorhodopsin, band 3 of human erythrocyte membrane, and (Ca^{2+}, Mg^{2+})-ATPase of the saroplasmic reticulum has been observed to rotate isotropically across the membrane within several milliseconds (36–38). Moreover, a bulky hydrophobic head of reductase molecule should prevent the reductase·P-450 complex from rotating across the lipid bilayer when reductase is present. In our previous study (15, 16), we analyzed $r(t)$ curves with Equation 4 and got finite r_3 values for the data with a 0.5-ms time range. Because the decay did not finish within 0.5 ms, we obtained, by curve-fitting procedure artificially, $r_3 > 0$ instead of the true value of $r_3 = 0$. A significant contribution of lateral diffusion-induced depolarization of $r(t)$ was excluded by the finding that in control experiments bacteriorhodopsin showed a finite r_3 when examined in sonicated small L-α-phosphatidylcholine dimyristoyl vesicles, in 80% glycerol solution.

Conclusion—In our experiments, we were able to show not only functional, but also physical interaction between cytochrome P-450 and reductase. Both antireductase IgG and antireductase Fab fragments significantly inhibit the hydroxylation activity of proteoliposomes. The rotational mobility of cytochrome P-450 is abolished after cross-linking of NADPH-cytochrome P-450 reductase via multivalent antireductase-IgG while antireductase Fab fragments against the reductase do not affect the rotational mobility of cytochrome P-450. Taken together, the above results and our previous finding of a specific complex formation between cytochrome P-450 and reductase (16) suggest the formation of a monomolecular complex between cytochrome P-450 and its reductase in the liposomal membrane.

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[4] T. Omura, personal communication.

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. DePierre, J. W., and Ernster, L. (1977) *Annu. Rev. Biochem.* **46,** 210–262
5. Gum, J. R., and Strobel, H. W. (1979) *J. Biol. Chem.* **254,** 4177–4185
6. Franklin, M. R., and Estabrook, R. W. (1971) *Arch. Biochem. Biophys.* **143,** 318–329
7. Yang, C. S. (1975) *FEBS Lett.* **54,** 61–64
8. Peterson, J. A., Ebel, R. E., O'Keeffe, D. H., Matsubara, T., and Estabrook, R. W. (1976) *J. Biol. Chem.* **251,** 4010–4016
9. Taniguchi, H., Imai, Y., Iyanagi, T., and Sato, R. (1979) *Biochim. Biophys. Acta* **550,** 341–356
10. Ingelmann-Sundberg, M., and Johansson, I. (1980) *Biochemistry* **19,** 4004–4011
11. Richter, C., Winterhalter, K. H., and Cherry, R. J. (1979) *FEBS Lett.* **102,** 151–154
12. Greinert, R., Staerk, H., Stier, A., and Weller, A. (1979) *J. Biochem. Biophys. Methods* **1,** 77–83
13. Vaz, W. L. C., Austin, R. H., and Vogel, M. (1979) *Biophys. J.* **26,** 415–426
14. Schwarz, D., Pirrwitz, J., and Ruckpaul, K. (1982) *Arch. Biochem. Biophys.* **216,** 322–328
15. Kawato, S., Gut, J., Cherry, R. J., Winterhalter, K. H., and Richter, C. (1982) *J. Biol. Chem.* **257,** 7023–7029
16. Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., and Kawato, S. (1982) *J. Biol. Chem.* **257,** 7030–7036
17. West, S. B., Huang, M.-T., Miwa, G., and Lu, A. Y. H. (1979) *Arch. Biochem. Biophys.* **193,** 42–50
18. Yasukochi, Y., and Masters, B. S. S. (1976) *J. Biol. Chem.* **251,** 5337–5344
19. Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J., and Levin, W. (1976) *J. Biol. Chem.* **251,** 1385–1391
20. Mage, M. G. (1980) *Methods Enzymol.* **70,** 142–150
21. Strobel, H. W., and Dignam, J. D. (1978) *Methods Enzymol.* **52,** 89–96
22. Nash, T. (1953) *Biochem. J.* **55,** 416–421
23. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239,** 2370–2378
24. Laemmli, U. K. (1970) *Nature (Lond.)* **227,** 680–685
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
26. Cherry, R. J. (1978) *Methods Enzymol.* **54,** 47–61
27. Kawato, S., and Kinosita, K., Jr. (1981) *Biophys. J.* **36,** 277–296
28. Black, S. D., French, J. S., Williams, C. H., and Coon, M. J. (1979) *Biochem. Biophys. Res. Commun.* **91,** 1528–1535
29. Imai, Y., Sato, R., and Iyanagi, T. (1977) *J. Biochem.* **82,** 1237–1246
30. Nigg, E. A., Bron, C., Girardet, M., and Cherry, R. J. (1980)

Biochemistry **19,** 1887–1893
31. Lu, A. Y. H., and Miwa, G. T. (1980) in *Biochemistry, Biophysics and Regulation of Cytochrome P-450* (Gustafsson, J.-A., Carlstedt-Duke, J., Mode, A., and Rafter, J., eds) pp. 125–128, Elsevier/North-Holland, Amsterdam
32. Miwa, G. T., West, S. B., Huang, M.-T., and Lu, A. Y. H. (1979) *J. Biol. Chem.* **521,** 5695–5700
33. Waxman, D. J., and Walsh, C. (1982) *J. Biol. Chem.* **257,** 10446–10457
34. Guengerich, F. P. (1978) *J. Biol. Chem.* **253,** 7931–7939
35. Koop, D. R., Persson, A. V., and Coon, M. J. (1981) *J. Biol. Chem.* **256,** 10704–10711
36. Cherry, R. J. (1979) *Biochim. Biophys. Acta* **559,** 289–327
37. Kawato, S., Sigel, E., Carafoli, E., and Cherry, R. J. (1981) *J. Biol. Chem.* **256,** 7518–7527
38. Bürkli, A., and Cherry, R. J. (1981) *Biochemistry* **20,** 138–145
39. French, J. S., Guengerich, F. P., and Coon, M. J. (1980) *J. Biol. Chem.* **255,** 4112–4119