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Rotation and Interaction with Epoxide Hydrase of Cytochrome P-450 in Proteoliposomes*

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Purified rat liver cytochrome P-450MC or P-450PB was co-reconstituted with epoxide hydrase in liposomal vesicles made of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine at a lipid to protein weight ratio of 5 by the cholate dialysis procedure. Rotational diffusion of the cytochromes was measured by observing the decay of absorption anisotropy, r(t), after photolysis of the heme·CO complex by a vertically polarized laser flash. Analysis of $r(t)$ was based on a "rotation-about-membrane-normal" model. The measurements were used to investigate interactions of cytochrome P-450MC or P-450PB with epoxide hydrase.

Different rotational mobilities of the two cytochromes were observed. The amount of mobile molecules was 78% for cytochrome P-450MC and 91% for P-450PB, and the rest was immobile within the experimental time range of 1 ms. In the presence of epoxide hydrase 85% of cytochrome P-450MC and 96% of P-450PB were mobile. Cross-linking of epoxide hydrase by anti-epoxide hydrase antibodies resulted in a drastic immobilization of the cytochromes, reducing the mobile population to 49% for P-450MC and to 60% for P-450PB. The rotational relaxation times ϕ of the mobile populations ranged from 210 to 283 μs. These results imply that both cytochromes P-450MC and P-450PB transiently associate with epoxide hydrase in liposomal membranes.

Further analysis of the data showed that the angle between the heme plane of P-450MC and the membrane is 48° or 62°, different from the value of 55° reported previously for P-450PB (Gut, J., Richter, C., Cherry, R. J., Winterhalter, K. H., and Kawato, S. (1983) *J. Biol. Chem.* 258, 8588–8594).

The hepatic microsomal monooxygenase system catalyzes the oxidative metabolism of drugs and endogenous substrates and also plays a role in the formation of carcinogens and cytotoxins (Omura, 1978; Estabrook *et al.*, 1979; White and Coon, 1980; Guengerich, 1987). It consists of several membrane-bound proteins such as NADPH-cytochrome P-450 reductase, NADH-cytochrome b_5 reductase and cytochrome P-450 (P-450).[1] P-450 receives two electrons, from NADPH through NADPH-cytochrome P-450 reductase and from NADH through cytochrome b_5 reductase and cytochrome b_5, and hydroxylates substrates with activated oxygen (Omura, 1978; Peterson and Prough, 1986; Ortiz de Montellano, 1986).

There are numerous isozymes of P-450, most of which are inducible with various chemicals. Extensive evidence exists that particularly the methylcholanthrene-induced cytochrome, P-450MC, converts polycyclic aromatic hydrocarbons to highly carcinogenic compounds. However, this capacity as well as conversion of arachidonic acid to epoxides has also been ascribed to phenobarbital-induced cytochrome, P-450PB (Harada and Omura, 1981; Hara *et al.*, 1983; Capdevila *et al.*, 1981, 1990).

Epoxide hydrase can be considered a functional partner of P-450, since it catalyzes the hydrolysis of highly reactive and carcinogenic epoxides to less harmfull diols. The enzyme is probably deeply embedded in the membrane lipid bilayer, since it is not sensitive to proteases in microsomes (Seidegård *et al.*, 1978; Ozols, 1988) and proteoliposomes.[2]

Rotational and lateral mobilities of membrane proteins may play a significant role in electron transfer and drug metabolism in the monooxygenase system (Kawato *et al.*, 1982b; Gut *et al.*, 1983; Ingelman-Sundberg, 1986). Rotational mobility of P-450 has been extensively examined in liver microsomal membranes and in reconstituted lipid vesicles by transient dichroism techniques (Richter *et al.*, 1979; Greinert *et al.*, 1979; McIntosh *et al.*, 1980; Kawato *et al.*, 1982b; Gut *et al.*, 1982, 1983, 1985). Protein rotation is particularly sensitive to protein-protein interactions (Kawato *et al.*, 1980, 1981, 1982a, 1988; Gut *et al.*, 1982, 1983; Ohta *et al.*, 1990), and its analysis has been successfully applied to investigate interactions of P-450 molecules with themselves and with NADPH-cytochrome P-450 reductase in both liver microsomes and proteoliposomes (Gut *et al.*, 1982, 1983). It was demonstrated that P-450PB forms a heterodimeric 1:1 complex with NADPH-cytochrome P-450 reductase in liposomes and that both mobile and immobile populations of P-450 within the electron transfer time range are present in liver microsomes of phenobarbital-treated rats. The immobile population reflects microaggregates formed due to the rather high protein content in the microsomal membrane which has a lipid to protein weight ratio of about 0.5 (DePierre and Ernster, 1977; Ingelman-Sundberg, 1986).

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[1] The abbrevations used are: P-450, cytochrome P-450; DPPC, dipalmitoylphosphatidylcholine; Hepes, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; MC, 3-methylcholanthrene; PB, phenobarbital; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate.

[2] H. U. Etter, unpublished results.

Indirect biochemical evidence for an association of the microsomal monooxygenase system with epoxide hydrase was obtained by Oesch and Daly (1972). However, Oesch and coworkers (Bentley *et al.*, 1980) later suggested that the bulk of epoxide hydrase is not firmly associated with P-450. In a first step to clarify the possible association of P-450 and epoxide hydrase we analyzed the rotational mobility of P-450 in proteoliposomes. Here we show the formation of transient complexes between epoxide hydrase and P-450MC (a mixture of P-450IA1 and P-450IA2)[3] or P-450PB (a mixture of P-450IIB1 and P-450IIB2)[3] in these model membranes.

EXPERIMENTAL PROCEDURES

Materials

P-450MC was prepared from liver microsomes of methylcholanthrene-treated rats (Sprague Dawley) similar to Waxman and Walsh (1982) as follows. The microsomes (60 ml in 0.25 M sucrose) were diluted (4 °C) with 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA, and 30% (v/v) glycerol to 200 ml. Sodium cholate was added to 3 g/g of protein. After stirring for 15 min polyethylene glycol 6000 (50%, w/w) was slowly added until 8% (v/v) was reached. After stirring for another 15 min, the mixture was centrifuged for 45 min at 32,000 × *g*. The pellet was discarded. The amount of polyethylene glycol in the supernatant was raised to 14%. After 15 min stirring the centrifugation was repeated. The pellet was dissolved at room temperature to a final volume of 40 ml in 10 mM potassium phosphate (pH 7.5), 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.5% sodium cholate, and 0.2% Emulgen 911 (buffer A). This solution was applied to a DEAE-52 column (2.7 × 58 cm) equilibrated with buffer A. The flow rate was 48 ml/h. A NaCl gradient from 0 to 0.3 M (1.6 liters) in buffer A was used to separate the proteins. Fractions eluting from the column were analyzed by SDS-gel electrophoresis and CO difference spectra. Pooled fractions were dialyzed against buffer identical to buffer A except that sodium cholate was 0.2%. The dialyzed solution was applied to a hydroxyl apatite column (1.8 × 7.5 cm, flow rate 72 ml/h), which was washed with the same buffer for 1 h. Subsequently, for 40 min a buffer with increased salt concentration (30 mM) was used. Thereafter, a buffer free of Emulgen was used to remove this detergent from the column. The protein was eluted with 0.4 M potassium phosphate (pH 7.5), 20% glycerol, and 0.4% sodium cholate and dialyzed against 50 mM potassium phosphate (pH 7.5) and 20% glycerol at 4 °C. This isolation procedure yields a 3:1 mixture of P-450IA1[3] and P-450IA2[3] (Thomas *et al.*, 1983). The specific content was 12.3 nmol of P-450/mg of protein.

P-450PB was prepared from liver microsomes of phenobarbital-treated rats as described for P-450MC with the following modifications: the polyethylene glycol window was 9–16%, the dialysis buffer after the DE-52 column and the buffers for the hydroxyl apatite column were without sodium cholate. The protein was eluted with 140 mM potassium phosphate (pH 7.5), 20% glycerol, and 0.4% sodium cholate. This procedure yields a 1:1 mixture of P-450IIB1[3] and P-450IIB2[3] (Thomas *et al.*, 1983). The specific content was 13.8 nmol of P-450/mg of protein. The cytochromes were enzymatically active as jugded by benzphetamine demethylation (Gut *et al.*, 1982) and 7-ethoxycoumarin dealkylation (Vergères *et al.*, 1989).

Epoxide hydrase was prepared from liver microsomes of phenobarbital-treated rats according to Lu *et al.* (1975) with the following modification: instead of whole microsomes, the first protein peak eluting from the DEAE-52 column during P-450PB isolation was used as the source of epoxide hydrase. The material was applied to a hydroxylapatite column, washed free of Emulgen with the same buffer, eluted at room temperature with 120 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 0.4% sodium cholate, dialyzed against 5 mM Tris (pH 8.4 at 4 °C) containing 0.1% sodium cholate, and subsequently applied to the DEAE-52 column (Lu *et al.*, 1975).

Anti-epoxide hydrase antibodies were prepared by a conventional procedure from female New Zealand White rabbits immunized with purified epoxide hydrase in Freund's complete adjuvant (Thomas *et al.*, 1976). The titer was measured with the enzyme-linked immunoabsorbent assay (peroxidase bound to anti-IgG antibodies and p-nitrophenylphosphate as substrate) and found to be about 2^{-16}, *i.e.* the antibodies when used at dilutions between 1:100 and 1:50,000 were highly specific for epoxide hydrase in enzyme-linked immunoabsorbent assay. At these dilutions no cross-reaction with P-450MC or P-450PB was observed.

Other substances: phospholipids were purchased from Lipid Products (Nutfield, United Kingdom), Amberlite from Fluka (Switzerland), and Orugano (Japan), BCA protein assay reagent was from Pierce Chemical Co., hydroxylapatite from Bio-Rad Laboratories (Richmond, CA), and DEAE-52 from Whatman Biosystem Ltd. (Maidstone, United Kingdom). Emulgen 911 was a gift from Kao Atlas Chemicals (Japan).

Methods

Epoxide Hydrase Activity—Enzymatic hydrolysis of [^{3}H]styrene epoxide was measured essentially as described by Oesch *et al.* (1971). The activity was similar to that reported (Oesch *et al.*, 1971).

Preparation of Proteoliposomes—They were formed with phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) (10:5:1, weight ratio) as follows: phospholipids in chloroform-methanol were placed in a small glass flask, the solvent was evaporated under a stream of nitrogen, and the mixture was further dried under vacuum for 1 h. The lipids were dispersed by adding the purified proteins, 50 mM Hepes buffer (pH 7.4) with 20% glycerol (v/v) and, thereafter, sodium cholate (in a 20% solution) to give 5 mg of phospholipid/ml, a lipid to protein weight ratio of 5:1 (L/P = 5), and 2% sodium cholate. The mixture was incubated over night at 4 °C. The resulting clear solution was dialyzed for 4 h at room temperature against a 200-fold volume of Hepes buffer, same as above, containing 1 g of Amberlite/100 ml. The buffer was changed once. For co-reconstitution of epoxide hydrase and P-450 the proteins were present in equal amounts.

To prepare dipalmitoylphosphatidylcholine (DPPC) vesicles, 4 mg of DPPC dissolved in chloroform was placed in a small glass flask, dried under a stream of nitrogen, and kept under vacuum at 4 °C for 2 h. The lipid was dispersed in 0.4 ml of 50 mM Hepes buffer (pH 7.4) containing 20% glycerol. After addition of 0.1 ml of 20% sodium cholate, the mixture was heated to 50 °C, sonicated until the solution became clear (about 15 min), and then cooled to room temperature. After addition of P-450, the suspension, now with a content of 2% sodium cholate, was stored over night at 4 °C and subsequently dialyzed as described for PC/PE/PS proteoliposomes.

Incubation of Proteoliposomes with Anti-epoxide Hydrase IgG—Prior to rotational diffusion measurements, proteoliposomes were incubated with anti-epoxide hydrase IgG at a 50-fold (w/w) excess over the enzyme for 30 min at room temperature. To avoid tight aggregates of cross-linked proteoliposomes, they were collected by differential centrifugation (18,400 × *g*, 30,500 × *g*, 45,500 × *g*, 20 min each), and the combined pellets were resuspended in Hepes buffer. No denaturation of P-450 to P-420 was observed spectrophotometrically upon antibody treatment.

Rotational Diffusion Measurements and Analysis—For rotational diffusion measurements sucrose was dissolved to give 57% (w/w) in the proteoliposome suspension in 50 mM Hepes buffer (pH 7.4) containing 20% glycerol (v/v). The final concentration of cytochromes (holoenzymes) was 4.5 μM. The amount of epoxide hydrase equaled the total amount of the cytochrome. Samples were reduced with a few grains of dithionite and gently gassed for 20 s with CO. The sample cuvette was then sealed by a rubber cap to keep the P-450·CO concentration.

The principle of the flash photolysis depolarization apparatus is described in detail elsewhere (Cherry, 1978; Kinoshita *et al.*, 1984). Briefly, the sample was excited at 532 nm by a vertically polarized flash of 7 ns duration from a Nd/YAG laser (Quanta-Ray, DCR-2) which was operated at 30 Hz. Absorbance changes due to photolysis of the heme·CO complex were measured at 450 nm selected with a Jovin-Yvon H-20 monochromator. The signal at 450 nm was divided into vertically and horizontally polarized components with a beam-splitting polarizer, which were led to Hamamatsu R567 photomultiplier tubes. To obtain the absorbance changes $A_V(t)$ and $A_H(t)$ the transmission changes $I_V(t)$ and $I_H(t)$, measured with the photomultipliers, were converted using the following expressions:

$$A_V(t) = -\log_{10}[1 + I_V(t)/I_0] \quad (1)$$

$$A_H(t) = -\log_{10}[1 + I_H(t)/I_0] \quad (2)$$

where the subscripts V and H indicate vertical and horizontal polar-

[3] The nomenclature follows the recommandation of Nebert *et al.*, (1989) DNA, 8:1.

ization, I_0 is the steady-state transmission level, and t is the time after the flash. The absorption anisotropy, $r(t)$, and the total absorbance change, $A(t)$, are given by

$$r(t) = [A_V(t) - A_H(t) \cdot S]/A(t) \quad (3)$$

$$A(t) = A_V(t) + 2A_H(t) \cdot S \quad (4)$$

where $S = A_{HV}/A_{HH}$ represents a correction for a small monochromator polarization and a slight unbalance of the two photomultipliers. A_{HV} and A_{HH} are, respectively, the time averaged absorbance changes of vertically and horizontally polarized components, ($A_{HV} = \int A_{HV}(t)dt/\int dt$, $A_{HH} = \int A_{HH}(t)dt/\int dt$), obtained with the horizontal flash excitation. Practically, S was around 1.002. The total absorbance change at time 0 was around 30 milliabsorbance units.

In each experiment, 16,384 signals were averaged using a Toyo Technica 2805 transient memory. The measurements were performed in 57% sucrose solution in order to reduce light scattering and vesicle tumbling. No degradation of P-450 during the experiment was observed spectrophotometrically.

Analysis of $r(t)$ is based on a model of rotation of P-450 about the membrane normal, the axis perpendicular to the membrane plane (Kawato and Kinoshita, 1981). When there is a single rotating species of P-450 with the rotational relaxation time, $\phi_\parallel$, $r(t)$ is given by

$$r(t)/r(0) = 3\sin^2\vartheta_N \cdot \cos^2\vartheta_N \cdot \exp(-t/\phi_\parallel) + \tfrac{3}{4}\sin^4\vartheta_N \cdot \exp(-4t/\phi_\parallel) + \tfrac{1}{4}(3\cos^2\vartheta_N - 1)^2 \quad (5)$$

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

$$r(t) = r_1 \cdot \exp(-t/\phi) + r_2 \cdot \exp(-4t/\phi) + r_3 \quad (6)$$

where ϕ is the rotational time constant. Although Equation 6 is not theoretically accurate for multiple rotating species, Equation 6 agrees with Equation 5 for a single rotating species. Therefore, Equation 6 is used to judge to what an extent the mode of rotation is deviated from the rotation of a single rotating population by comparing the experimental values of ϕ, r_1/r_2, and $r_3/r(0)$ with $\phi_\parallel$, $4\cot^2\vartheta_N$, $\frac{1}{4}(3\cos^2\vartheta_N - 1)^2$ in Equation 5. Here it should be noted that ϑ_N can be determined from the following relationship when all P-450 molecules are rotating, even in the presence of multiple rotating species.

$$[r_3/r(0)]_{min} = \tfrac{1}{4}(3\cos^2\vartheta_N - 1)^2 \quad (7)$$

Curve fitting of the data by Equation 6 was accomplished with a PDP-11/73 minicomputer. It should be noted that in Equations 5 and 6 only $r(0)$ but not $r(t)/r(0)$ depend on the photoselecting laser flash intensity (Kawato and Kinoshita, 1981). Therefore, $r(t)$ curves obtained at slightly different excitation intensities were normalized to the same $r(0)$ for direct comparison in Figs. 3 and 4. The measurements were performed at 20 °C.

Other Methods—P-450 was measured spectrophotometrically according to Omura and Sato (1964) using an extinction coefficent of $\Delta\epsilon_{450-490\ nm} = 91\ mM^{-1} \cdot cm^{-1}$ (reduced·CO − reduced). Proteins were measured with the BCA protein assay using bovine serum albumin as standard.

Incorporation of proteins in liposomes was determined by sucrose density gradient ultracentrifugation followed by SDS-polyacrylamide gel electrophoresis. Proteoliposomes were layered onto a discontinuous sucrose density gradient (10, 15, 20, 25, 30, 35% sucrose (w/w) in Hepes buffer) in aliquots of 0.6 ml containing 2–3 nmol of heme/tube (4.2 ml) and centrifuged at 175,000 × g for 18 h at 4 °C. For SDS-polyacrylamide gel electrophoresis the method of Laemmli was used (Laemmli, 1970).

RESULTS AND ANALYSIS

Reconstitution of Proteoliposomes—The formation of proteoliposomes can be demonstrated by sucrose density gradient centrifugation and negatively stained electron micrographs. A variety of proteoliposomes with different enzyme compositions was analyzed in this way. With all proteoliposomes examined, a single band in the sucrose density gradient was observed. Co-migration of phospholipids and P-450 was judged from white light scattering by phospholipids and the reddish color of the cytochrome. The co-incorporation of epoxide hydrase and P-450 was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1) of the proteoliposome band taken from the sucrose density gradient.

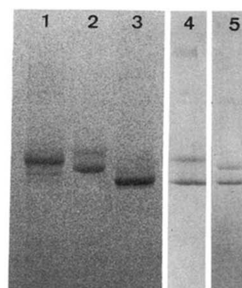

FIG. 1. **Analysis of enzyme incorporation into PC/PE/PS vesicles by ultracentrifugation in a sucrose density gradient and SDS-polyacrylamide gel electrophoresis.** SDS-polyacrylamide gels. *Lane 1*, cytochrome P-450MC; *lane 2*, cytochrome P-450PB; *lane 3*, epoxide hydrase; *lane 4*, cytochrome P-450MC and epoxide hydrase from the band obtained after ultracentrifugation of the co-reconstituted proteoliposomes in a sucrose density gradient; *lane 5*, cytochrome P-450PB and epoxide hydrase from the band obtained after ultracentrifugation of the co-reconstituted proteoliposomes in a sucrose density gradient. The proteins were stained with Coomassie Blue.

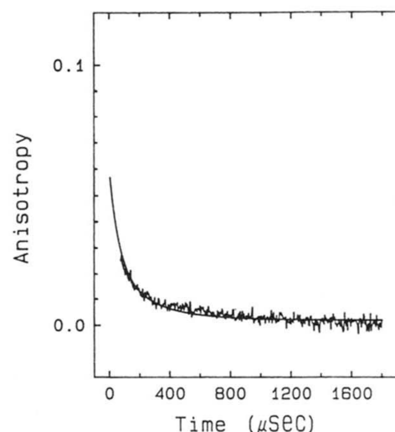

FIG. 2. **Time-dependent absorption anisotropy of cytochrome P-450MC in PC/PE/PS vesicles in the presence of KCl.** Samples were photolyzed by a vertically polarized laser flash at 532 nm, and $r(t)$ was recorded at 450 nm as described under "Experimental Procedures." Measurements were performed in 25 mM Hepes (pH 7.4) containing 57% sucrose, 10% glycerol, and 250 mM KCl at 20 °C (about 0.6 poise). The *zigzag line* represents experimental data; the *solid curve* was obtained by fitting the data to Equation 6. Note that a linear vertical axis was chosen for clarity (in a logarithmic scale $r(t)$ looks very noisy when it is smaller than 0.007).

Rotational Diffusion of P-450 in Lipid Vesicles—P-450 alone and together with epoxide hydrase was reconstituted in proteoliposomes of a composition closely resembling that of the microsomal membrane (*i.e.* PC/PE/PS = 10:5:1 (w/w)) with varying lipid to protein weight ratios. Absorption anisotropy $r(t)$ decays due to rotational motion of P-450 about the normal of the membrane plane. In all samples examined, $r(t)$ curves decayed within 2 ms to a time-independent value r_3 as it is shown in Fig. 2. Data were analyzed by Equation 6. The decay parameters are summarized in Table I.

Orientation of the Heme Plane of P-450—The tilt angle ϑ_N of P-450MC was determined in vesicles containing only this protein by analyzing the $r(t)$ curve of the freely rotating protein. In order to completely mobilize all P-450 molecules, the P-450MC vesicles with L/P = 5 were incubated with 500 mM KCl for 30 min at room temperature (Ohta *et al.*, 1990).

TABLE I

Decay parameters of the time-dependent absorption anisotropy of the cytochrome P-450·CO complex in proteoliposomes with L/P = 5 analyzed by Equation 6

All measurements were performed in 25 mM Hepes (pH 7.4), 57% (w/w) sucrose, and 10% glycerol at 20 °C.

Enzymes in vesicles	ϕ	$r_3/r(0)$	Mobile P-450[a]
	μs		%
P-450MC	283	0.24	78
	(18)[b]	(0.03)	(3)
P-450MC + EH[c]	225	0.18	85
	(11)	(0.02)	(2)
P-450MC + EH + IgG	210	0.52	49
	(41)	(0.03)	(3)
P-450PB	255	0.09	91
	(22)	(0.03)	(3)
P-450PB + EH	213	0.04	96
	(20)	(0.01)	(1)
P-450PB + EH + IgG	243	0.40	60
	(16)	(0.01)	(1)
P-450PB + IgG	266	0.07	93
	(44)	(0.05)	(6)
P-450MC + 0.5 M KCl	264	0.03	100
	(4)	(0.01)	(1)

[a] The percentage of mobile population of cytochrome P-450 is calculated with Equation 8.

[b] Number in parentheses are standard deviations in 8–10 experiments.

[c] EH, epoxide hydrase.

The final KCl concentration during the measurements was 250 mM after dissolving the proteoliposomes in sucrose. The observed $[r_3/r(0)]_{min} = 0.03$ (Fig. 2) corresponds to ϑ_N of either 48 or 62° calculated from Equation 7. In contrast, an angle of 55° was reported previously for P-450PB (Gut *et al.*, 1983).

The percentage of mobile cytochrome P-450, p_m, can be calculated by

$$p_m = (1 - r_3/r(0))/(1 - [r_3/r(0)]_{min}) \times 100 \quad (8)$$

where $[r_3/r(0)]_{min}$ is equal to 0.00 for P-450PB and 0.03 for P-450MC. We found that 78% of P-450MC are rotating in L/P = 5 vesicles, whereas 91% of cytochrome P-450PB are mobile.

Effect of Epoxide Hydrase on Rotational Mobility of P-450—Due to the low protein density in L/P = 5 proteoliposomes, most of P-450 molecules are rotating (Figs. 3 and 4). The immobile population reflects nonspecific microaggregates of P-450 (Kawato *et al.*, 1982b; Gut *et al.*, 1982). With equimolar amounts of epoxide hydrase and P-450 in proteoliposomes, a significant mobilization of both cytochromes is documented in Figs. 3 and 4. A small decrease in ϕ was observed from 283 to 225 μs for P-450MC and from 255 to 213 μs for P-450PB. The mobile population was increased to 85% for P-450MC and to 96% for P-450PB.

Influence of Anti-epoxide Hydrase IgG on Rotational Mobility of P-450—The addition of a 50-fold excess (w/w) of anti-epoxide hydrase antibodies over epoxide hydrase greatly decreased the amount of mobile P-450MC and P-450PB (Figs. 3 and 4). In the presence of IgG only 49% of P-450MC and 60% of P-450PB were still rotating. However, the rotational relaxation time was not increased significantly. These results imply that cross-linking of epoxide hydrase by anti-epoxide hydrase IgG immobilizes the partner P-450 in the P-450·epoxide hydrase complex.

Not all cytochrome molecules were immobilized by anti-epoxide hydrase IgG in these vesicles probably due to a nonequimolar distribution of P-450 and epoxide hydrase in individual proteoliposomes.

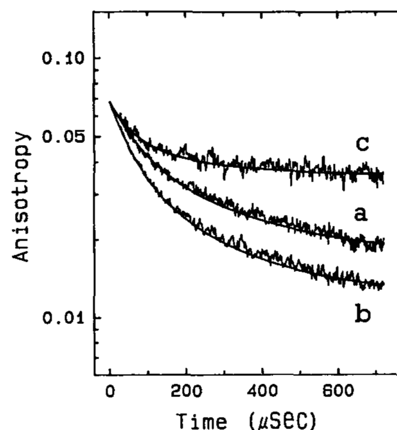

FIG. 3. **Effect of epoxide hydrase and anti-epoxide hydrase IgG on the time-dependent absorption anisotropy of cytochrome P-450MC in proteoliposomes.** *Curve a*, cytochrome P-450MC in proteoliposomes; *curve b*, cytochrome P-450MC plus epoxide hydrase in proteoliposomes; *curve c*, anti-epoxide hydrase IgG was added to the sample shown in *curve b*. Absorption changes were measured and analyzed as in Fig. 2. Measurements were performed in 25 mM Hepes (pH 7.4) containing 57% sucrose and 10% glycerol at 20 °C (about 0.6 poise). The *zigzag lines* represent experimental data; the *solid curves* were obtained by fitting the data to Equation 6. The initial anisotropies of *curves b* and *c* are normalized to the same $r(0)$ of curve a to facilitate comparison.

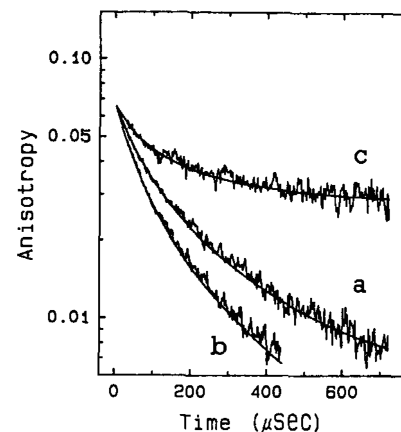

FIG. 4. **Effect of epoxide hydrase and anti-epoxide hydrase IgG on the time-dependent absorption anisotropy of cytochrome P-450PB in proteoliposomes.** *Curve a*, cytochrome P-450PB in proteoliposomes; *curve b*, cytochrome P-450PB plus epoxide hydrase in proteoliposomes; *curve c*, anti-epoxide hydrase IgG was added to the sample shown in *curve b*. Other experimental conditions as in Fig. 3. The *zigzag lines* represent experimental data; the *solid curves* were obtained by fitting the data to Equation 6. The initial anisotropies of *curves b* and *c* are normalized to the same $r(0)$ of curve a to facilitate comparison. It was confirmed in a linear scale that all curves reached small plateau values within 2 ms time range. To illustrate this in a logarithic scale, however, is difficult, because the curve is very noisy when the value is smaller than 0.007.

Recombination Kinetics of CO to Reduced P-450—The recombination kinetics of CO to reduced P-450 was different for P-450MC and P-450PB. The total absorption decay $A(t)$ was close to monophasic in proteoliposomes in 57% sucrose plus 10% glycerol solution. $A(t)$ was analyzed by a monoexponential approximation. The lifetimes of photodissociated P-450, τ, obtained from $A(t)$ were about 7.9 ms for P-450MC and 3.6 ms for P-450PB. No significant difference was observed for τ in the absence and in the presence of epoxide

hydrase for P-450MC and P-450PB, suggesting that there are no direct electronic interactions between P-450 and epoxide hydrase affecting the heme.

Absence of Vesicle Tumbling—As reported previously (Gut *et al.*, 1983), no significant anisotropy decay was observed for P-450 in DPPC vesicles at 20 °C where the lipid bilayer is in the crystalline state. This excludes a contribution of vesicle tumbling to the observed decay in $r(t)$. Incubating these vesicles with 500 mM KCl did not cause a significant decay in $r(t)$, indicating that KCl does not induce vesicle tumbling nor mobilizes P-450 when the phospholipids are in the crystalline state.

DISCUSSION

Epoxide Hydrase-induced Mobilization of P-450—Epoxide hydrase induced a significant mobilization of both P-450MC and P-450PB, which implies that both cytochromes form specific complexes with the enzyme. This conclusion is based on our finding that at a constant lipid to total protein weight ratio (L/P = 5) epoxide hydrase mobilizes the cytochromes from self-aggregates. In contrast, cytochrome oxidase and cytochrome bc_1 do not appear to interact specifically in membranes, since replacement of half of the oxidase by cytochrome bc_1 did not alter $r(t)$ of cytochrome oxidase in L/P = 5 vesicles in the presence of cytochrome *c* (Kawato *et al.*, 1981). Electron transfer between these two proteins is achieved by cytochrome *c*, which shuttles between them (Schneider *et al.*, 1980; Kawato *et al.*, 1981).

Because of the relatively low protein concentration of L/P = 5, only 10–20% of the cytochromes form microaggregates in pure P-450 proteoliposomes. Epoxide hydrase mobilized 5–7% of the cytochromes in L/P = 5 vesicles. This number is similar to the 10% mobilization for P-450PB by the addition of NADPH-cytochrome P-450 reductase in L/P = 5 proteoliposomes (Gut *et al.*, 1982).

A P-450 monomer rotates with a ϕ value of about 40 μs (Gut *et al.*, 1982). The values of 283 and 255 μs reported here for P-450MC and P-450PB, respectively, indicate the existence of oligomeric rotamers in the present proteoliposomes. It should be noted here that ϕ represents the average of rotational relaxation times of several protein complexes. The small decrease in the ϕ values induced by epoxide hydrase suggests a decrease in the average size of such complexes. The two proteins could be present in the rotamers in a one to one stoichiometry, or also in an odd ratio. Although the accuracy of our data and the theoretical models available for an analysis of the motion of membrane proteins do not allow an unequivocal decision between the two alternatives, we favour the second alternative.

Immobilization of P-450 Induced by Anti-Epoxide Hydrase IgG—The rotational mobility of P-450 is largely decreased by cross-linking of epoxide hydrase by anti-epoxide hydrase antibodies. This is direct evidence for the existence of transient complexes between P-450 and epoxide hydrase in liposomal membranes, as illustrated in Fig. 5. This figure shows a possible model for mobilization of cytochrome P-450 by epoxide hydrase and for antibody-induced immobilization of the cytochrome.

Not all mobile P-450 molecules were immobilized by anti-epoxide hydrase IgG in proteoliposomes reconstituted with epoxide hydrase and P-450. A 36% decrease in the mobile population was observed for both P-450MC and P-450PB. This may be due to a heterogeneous distribution of P-450 and epoxide hydrase molecules in the same vesicles (at L/P = 5). Conceivably not all epoxide hydrase molecules may, therefore, associate with the cytochrome as partner. An increase in the

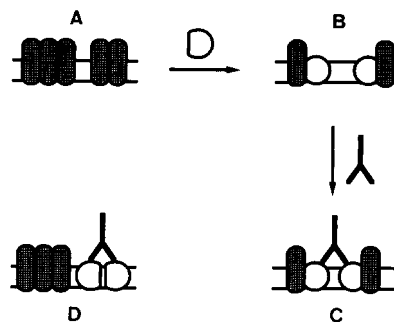

FIG. 5. **Schematic model illustrating the interactions between cytochrome P-450 and epoxide hydrase.** *A*, oligomers of cytochrome P-450 of various sizes (*shaded symbols*) are rotating in L/P = 5 vesicles. *B*, adding equimolar amounts of epoxide hydrase (*open symbols*) to cytochrome P-450 in vesicles results in disintegration of cytochrome P-450 oligomers due to the formation of transient complexes between the cytochrome and epoxide hydrase, which exhibit a higher rotational mobility than those in *A*. *C*, complexes between cytochrome P-450 and epoxide hydrase are cross-linked by anti-epoxide hydrase IgG. This aggregation reduces the rotational mobility of cytochrome P-450·epoxide hydrase complexes. *D*, if cytochrome P-450 and epoxide hydrase would exist independently in the membrane, cross-linking of anti-epoxide hydrase IgG would not affect the rotational mobility of cytochrome P-450. In *A*, although there are also monomeric cytochrome P-450 molecules, only dimers and trimers are illustrated for clear demonstration of mobilization of P-450 induced by epoxide hydrase. In *C*, although only small cross-linked aggregates are shown due to illustrative difficulty in one plane, cross-linked immobile aggregates should be at least two times larger. Typical oligomeric cytochrome P-450·epoxide hydrase complexes are shown, although other types of aggregates may also exist in the membrane.

protein concentration to a L/P = 1 could, in principle, facilitate heterologous complex formation as demonstrated with P-450PB and its reductase (Gut *et al.*, 1983). However, incorporation of cytochromes and epoxide hydrase into vesicles became less efficient and less reproducible when we increased the protein concentration toward L/P = 1. Possible bidirectional incorporation of epoxide hydrase might also inhibit proper association of the enzyme with P-450. Although 90–95% of P-450PB is incorporated right-side-out in proteoliposomes as judged by trypsin digestion experiments (Vergères *et al.*, 1989), none of several proteases so far tested could digest any peptide of membrane-embedded epoxide hydrase.[2] The exact orientation of this enzyme in proteoliposomes is, therefore, presently not clear. It should be noted that the heterogeneous distribution of P-450PB and its reductase was also observed in co-reconstituted vesicles with L/P = 5 as judged from only 34% immobilization of P-450PB by antireductase IgG (Gut *et al.*, 1983).

It could be argued that the immobilization of P-450 observed with anti-epoxide hydrase IgG is due to steric hindrance by the IgG bound to epoxide hydrase or due to interference of IgG adsorbed by the lipid bilayer. However, it appears very unlikely that this would be the main cause of the immobilization, because no antibody-induced immobilization occured in vesicles containing only P-450 and ϕ is in the usual range. The observed changes in $r_3/r(0)$ might be due to a change in the tilt angle ϑ_N of P-450 rather than a change in the mobile population. If this were the case, upon binding of antibody to epoxide hydrase, ϑ_N would have to decrease from 38 to 25° for P-450MC and from 46 to 29° for P-450PB. These numbers of ϑ_N would be unrealistically large for an indirect effect on P-450s due to binding of the antibody to epoxide hydrase.

Transient Complex Formation of P-450 with Epoxide Hydrase (Fig. 5)—As judged from the time-independent phase of

the $r(t)$ curve on the present time scale, when proteins are cross-linked by anti-epoxide hydrase IgG, the P-450·epoxide hydrase complex should have a lifetime longer than 20 ms. Therefore, this complex could exist as a stable association during the time of conversion of substrate to diol (aromatic compound→epoxide→diol) which is possibly longer than several tens of milliseconds. It can be expected that the partner molecules in the P-450·epoxide hydrase complex can be exchanged over a longer time range (*e.g.* longer than 100 ms). This is because P-450MC and P-450PB interact with several other proteins including NADPH-cytochrome P-450 reductase and cytochrome b_5. If the association of P-450 with epoxide hydrase is stable, reductase or cytochrome b_5 may have difficulties to donate electrons to P-450.

Different Protein-Protein Interactions and Heme Angles of P-450MC and P-450PB—P-450MC easily forms microaggregates, which was also observed during purification of the protein. In L/P = 5 vesicles, 22% of P-450MC was immobile while only 9% of P-450PB was immobile. When the protein concentration was decreased to L/P = 20, P-450PB was completly mobile, whereas 15% of P-450MC was still immobile. Complete mobilization of P-450MC could be achieved by the incubation with 500 mM KCl in L/P = 5 vesicles.

The tilt angle ϑ_N of the heme for P-450MC is either 48 or 62° calculated from $[r_3/r(0)]_{min} = r_3/r(0) = \frac{1}{4}(3\cos^2\vartheta - 1)^2 = 0.03$. This is clearly different from $\vartheta_N = 55°$ of P-450PB. Cytochrome P-450_{C21} showed[4] either 38 or 78° from $[r_3/r(0)]_{min} = 0.19$. These data indicate that different isozymes of cytochrome P-450s have different tilt angles.

Although P-450PB preparations consist of roughly equal amounts of the subspecies P-450b and P-450e, the unique value of 55° for P-450PB indicates that the subspecies P-450b and P-450e have identical heme angles.

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