Dynamic Structures of Adrenocortical Cytochrome P-450 in Proteoliposomes and Microsomes: Protein Rotation Study†

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ABSTRACT: Purified adrenocortical microsomal cytochromes P-450$_{17\alpha,lyase}$ and P-450$_{C21}$ were reconstituted with and without NADPH–cytochrome P-450 reductase in phosphatidylcholine–phosphatidylethanolamine–phosphatidylserine vesicles at a lipid to P-450 ratio of 35 (w/w) by cholate dialysis procedures. Trypsinolysis revealed that a considerable part of each P-450 molecule is deeply embedded in the lipid bilayer, on the basis of the observation of no detectable digestion for P-450$_{17\alpha,lyase}$ and the proteolysis-resistant membrane-bound heavy fragments for P-450$_{C21}$. Rotational diffusion was measured in proteoliposomes and adrenocortical microsomes by observing the decay of absorption anisotropy, $r(t)$, after photolysis of the heme·CO complex. Analysis of $r(t)$ was based on a "rotation-about-membrane normal" model. The absorption anisotropy decayed within 1–2 ms to a time-independent value r_3. Coexistence of a mobile population with an average rotational relaxation time ϕ of 138–577 μs and immobile ($\phi \geq 20$ ms) populations of cytochrome P-450 was observed in both phospholipid vesicles and microsomes. Different tilt angles of the heme plane from the membrane plane were determined in proteoliposomes to be either 47° or 63° for P-450$_{17\alpha,lyase}$ from $[r_3/r(0)]_{min} = 0.04$ and either 38° or 78° for P-450$_{C21}$ from $[r_3/r(0)]_{min} = 0.19$, when these P-450s were completely mobilized by incubation with 730 mM NaCl. Very different interactions with the reductase have been observed for the two P-450s in proteoliposomes. In the presence of the reductase, the mobile population of cytochrome P-450$_{C21}$ was increased significantly from 79% to 96% due to dissociation of P-450 oligomers. Taken together with the observation that a considerable immobilization of the P-450$_{C21}$ has occurred by the further addition of antireductase antibodies to this vesicle system, we conclude that cytochrome P-450$_{C21}$ forms a transient association with the reductase. On the other hand, the mobility of P-450$_{17\alpha,lyase}$ was not affected by the presence or absence of the reductase, keeping the mobile population of about 90%. The values of $r_3/r(0) = 0.38$–0.44 in microsomes are greater than that in vesicles, implying that 30–37% of P-450 is immobile in microsomes. The ϕ value of 558–577 μs in microsomes was much larger than that of 156–221 μs in proteoliposomes. In microsomes, the mobility of P-450 was significantly dependent on the presence of specific substrates. Pregnenolone and progesterone decreased the mobile population from 70% to 63% without affecting ϕ.

The cytochrome P-450-containing monooxygenase systems in adrenocortical microsomes carry out biotransformation of pregnenolone to various steroid hormones (Takemori & Kominami, 1984, 1990). The monooxygenase systems consist of several membrane proteins such as NADPH–cytochrome P-450 reductase (M_r = 77 000), cytochrome P-450$_{17\alpha,lyase}$[1] (M_r = 57 000), and cytochrome P-450$_{C21}$ (M_r = 54 000) (Porter & Kasper, 1985; Yoshioka et al., 1986; Zuber et al., 1986). Cytochrome P-450$_{17\alpha,lyase}$ accepts electrons from NADPH–cytochrome P-450 reductase and also from cytochrome b_5, catalyzing the oxygen-dependent 17α-hydroxylation and C17–C20 bond cleavage of steroids (Kominami et al., 1982; Shinzawa et al., 1985). Most of pregnenolone is metabolized into progesterone by 3β-hydroxysteroid dehydrogenase Δ^4–Δ^5 isomerase (Ishii-Ohba et al., 1986). Cytochrome P-450$_{C21}$ also accepts electrons from the reductase, from cytochrome b_5, catalyzing the oxygen-dependent steroid 21-hydroxylation (Kominami et al., 1980; Katagiri et al., 1982). Progesterone and 17α-hydroxyprogesterone are hydroxylated by cytochrome P-450$_{C21}$ into deoxycorticosterone and deoxycortisol, respectively. The molar ratio of P-450$_{17\alpha,lyase}$ to P-450$_{C21}$ differs in various adrenal microsomes, and the content of the reductase is very low, about one-fourth to one-tenth of the total cytochrome P-450 content (Takemori & Kominami, 1990). On the basis of the rate dependence of reduction of liposomal P-450$_{C21}$ as well as progesterone hydroxylation activity of P-450$_{C21}$ and P-450$_{17\alpha,lyase}$ on the reductase concentration in liposomes, it was shown that electrons were delivered through transient association or random collisions between the reductase and cytochromes on the membrane (Kominami et al., 1989).

Rotational mobility of adrenocortical mitochondrial cytochrome P-450 and that of liver microsomal P-450 have been extensively studied in relation to electron transfer by Kawato and co-workers using transient dichroism techniques (Gut et al., 1983; Ohta et al., 1991). It has been shown that phenobarbital-induced cytochrome P-450IIB1/P-450IIB2 forms a transient heterologous monomolecular association with NADPH–cytochrome P-450 reductase in liposomes and that both mobile and immobile populations of cytochrome P-450 are present in adrenocortical mitochondria and liver microsomes within the experimental time range of 2 ms. In

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[1] Abbreviations: cytochrome P-450$_{17\alpha,lyase}$, cytochrome P-450 having steroid 17α-hydroxylase and C17,C20-lyase activities (P-450XVIIA1); cytochrome P-450$_{C21}$, cytochrome P-450 having steroid 21-hydroxylase activity (P-450XXIA1); L/P(w/w), lipid to protein ratio by weight; L/P-450(w/w), lipid to P-450 ratio by weight; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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adrenocortical mitochondria, the mobility of the cytochrome was significantly dependent on the amount of cholesterol bound P-450$_{scc}$ due to forming a possible transient multienzyme association consisting of P-450$_{scc}$, adrenodoxin, and adrenodoxin reductase during electron transport time scale. This complex is dissociated after the conversion of cholesterol to pregnenolone.

So far, however, little is known about mobility and protein–protein interactions of cytochrome P-450 in adrenocortical microsomes. The present work is the first attempt to investigate the protein dynamics of adrenocortical microsomal cytochrome P-450s related to electron transfer reactions and steroidogenesis. Different heme angles of P-450$_{17\alpha,lyase}$ and P-450$_{C21}$ were determined. These cytochromes showed different interactions with NADPH–cytochrome P-450 reductase in phospholipid vesicles. P-450$_{C21}$ probably associates with the reductase, while P-450$_{17\alpha,lyase}$ would diffuse independently of the reductase. The mobility of cytochrome P-450 in microsomes was significantly dependent on the presence of different substrates of steroid hormone precursors. Furthermore, extensive tryptic digestion demonstrated that a considerable part of microsomal P-450 was embedded in the membrane.

EXPERIMENTAL PROCEDURES

Materials

Preparation of Proteins. Cytochrome P-450$_{C21}$ and NADPH–cytochrome P-450 reductase were purified from bovine adrenal microsomes according to the method previously described (Kominami et al., 1980, 1986; Takemori & Kominami, 1982). Cytochrome P-450$_{17\alpha,lyase}$ was purified from guinea pig adrenal microsomes as reported previously (Kominami et al., 1982). All of these enzymes are homogeneous as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Emulgen 913 used in the purifications of P-450$_{17\alpha,lyase}$ and the reductase was removed by extensive washing of the enzymes before storing at −80 °C. The detergent in the P-450$_{C21}$ preparation was removed just before mixing with the phospholipids, because P-450$_{C21}$ became labile without the detergent during the storage (Kominami et al., 1986). The concentration of P-450 was determined from the CO-reduced difference spectra using a difference extinction coefficient $\Delta\epsilon_{450-490nm}$ = 91 mM^{-1} cm^{-1} (Omura & Sato, 1964). The P-450$_{C21}$ and P-450$_{17\alpha,lyase}$ preparations used in the present study have specific contents of 14–18 nmol and 14.9 nmol of P-450/mg of protein, respectively. The concentration of the reductase was determined from the absorbance at 456 nm using an extinction coefficient of 21.4 mM^{-1} cm^{-1} (French & Coon, 1979). The reductase preparation catalyzed the reduction of 70–80 μmol of cytochrome *c*/(min·mg of protein) at infinite concentrations of both cytochrome *c* and NADPH in 300 mM potassium phosphate buffer, pH 7.7, containing 0.1 mM EDTA.

Antireductase antibodies were prepared by a conventional procedure from male New Zealand White rabbits immunized with purified NADPH–cytochrome P-450 reductase in Freund's adjuvant (Kominami et al., 1983). The high specificity of antireductase IgG was examined with Western blotting assay using bovine adrenocortical microsomes. Antireductase IgG showed a sharp band at the position of reductase but did not show cross-reactivity against cytochromes P-450$_{C21}$ and P-450$_{17\alpha,lyase}$. A significant inhibition of cytochrome *c* reducing activity by NADPH–cytochrome P-450 reductase was observed in the presence of antireductase IgG. A 78-fold excess of antireductase IgG over reductase (w/w) resulted in a inhibition of the initial reductase activity to about 50%.

Adrenal glands of male guinea pigs (Dunkin-Hartley, weighing 300–600 g) were quickly collected after the decapitation and were homogenized in 0.25 M sucrose using a glass–Teflon homogenizer. Microsomes were then prepared by centrifugations at 700*g* for 10 min, 10000*g* for 20 min, and 105000*g* for 60 min. The microsomal pellets were washed with 0.15 M KCl, resuspended in 100 mM potassium phosphate buffer (pH 7.3) containing 0.1 mM EDTA, and stored at −80 °C.

Other Substances. L-α-Phosphatidylserine and L-α-phosphatidylethanolamine were purchased from Lipid Products (U.K.), and L-α-phosphatidylcholine, trypsin from bovine pancrease (type III), and sodium cholate were from Sigma (St. Louis, MO). Phenylmethanesulfonyl fluoride was from Nakalai Tesqu (Japan). BCA protein assay reagent was from Pierce Chemical Company (Rockford, IL), and Emulgen 913 was a gift from Kao Atlas Chemicals (Japan). Other chemicals were of the highest grade commercially available.

Methods

Preparation of Proteoliposomes. Proteoliposomes were prepared from phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) at a molar ratio of 5:3:1, which was comparable with the lipid composition of adrenal microsomes (Kominami et al., 1989). Phospholipids in chloroform were placed in a glass flask, the solvent was evaporated under a stream of nitrogen, and the mixture was further dried under vacuum for 4 h. The lipids were dispersed by sonication (W-225, Heat System-Ultrasonic Inc.) in 500 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM EDTA, 0.1 mM DTT, 10 μM progesterone, 20% glycerol (w/v), and 2% sodium cholate; thereafter, purified enzymes and buffer were added to give 10 mg of phospholipids/mL, a lipid to P-450 molar ratio of 2000, and 1% (w/v) sodium cholate. The mixture was incubated at 0 °C for 1 h. The resulting clear solution was dialyzed for 16 h at 4 °C against a 300-fold volume of deoxygenated 50 mM Tris-HCl buffer (pH 7.2) containing 200 mM NaCl, 0.1 mM EDTA, and 10 μM progesterone.

For rotational diffusion measurements, proteoliposomes were concentrated by ultracentrifugation at 200000*g* for 1.5 h at 4 °C, suspended in the suitable buffer with 20% glycerol, and stored at 77 K. The presence of 20% glycerol prevents the rupture of the vesicles during freeze–thaw treatments. In the experiments where substrate-free cytochromes were required, the dialyzed samples were applied to a TSK-PWH column (Tosoh, 0.75 × 7.5cm), previously equilibrated with the dialysis buffer, which decreased progesterone concentration to less than 5 nM.

The content of P-450 in the liposomes was determined from a CO–dithionite-reduced difference spectra, and the content of the reductase was determined from cytochrome *c* reductase activity after collapse of the liposomes. The content of phospholipids in the sample was estimated from the radioactivity of [^{14}C]dipalmitoylphosphatidylcholine, initially mixed with the phospholipid mixture before dialysis. The liposomes used in these experiments contained 1 mol of cytochrome P-450 per 2500 mol of phospholipids, corresponding to L/P-450 = 35 by weight. Proteoliposomes containing cytochrome P-450IIB1/P-450IIB2 were kindly offered by Drs. H. Etter and C. Richter.

Proteolytic Analysis and SDS–Polyacrylamide Gel Electrophoresis. Proteolysis of P-450 in the liposomal membranes

and also their solubilized forms in the presence of 1% of cholate was carried out in 50 mM Tris-HCl butter, pH 7.4, containing 200 mM NaCl at 37 °C for 2 h in the presence of trypsin at a weight ratio of 1:25 to the cytochrome P-450. The reaction was terminated by the addition of 0.5 mM (final concentration) phenylmethanesulfonyl fluoride or by the heat treatment in the presence of SDS. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of the digested samples was performed using tricine buffer according to the method of Schaegger and von Jagow (1987). The concentration of polyacrylamide of separating (4 cm), spacer (3 cm), and stacking (2 cm) gels was 16.4%, 10%, and 4%, respectively. Electrophoresis was performed at 30 V for 1.5 h and subsequently at 80 V for 5.5 h.

Enzyme Activity Assay. Progesterone hydroxylase activity of the proteoliposomes was measured aerobically at 37 °C, as described previously (Kominami et al., 1989). The steroids extracted from the reaction solution were separated by an HPLC system (CCPM and UV8000, TOSOH Inc.) with a silica gel column (0.46 × 15 cm, Cosmosil 5SL, Nacalai Tesque) using a solvent system of *n*-hexane/isopropanol/acetic acid (93:7:1, v/v) and quantified from their peak areas at 250 nm.

Incubation of Proteoliposomes with Antireductase Antibodies. Prior to rotational diffusion measurements, P-450$_{C21}$ plus reductase vesicles were incubated with antireductase IgG at a 78-fold (w/w) excess over reductase for 30 min at room temperature. The vesicles were then pelleted at 18400*g* for 20 min and resuspended in the 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, 58% (w/w) sucrose was dissolved in proteoliposome suspension in 50 mM Hepes/Tris buffer (pH 7.4) containing 20% glycerol (v/v) for proteoliposomes, and 60% (w/w) sucrose was dissolved in microsome suspension without glycerol. The final concentrations of P-450 and NADPH–cytochrome P-450 reductase were 3.0 μM. Samples were reduced by a few grains of dithionite and gently bubbled for 10 s with CO. The sample cuvette was then sealed by a rubber cap to keep CO concentration constant.

The principle of flash photolysis depolarization apparatus was described in detail elsewhere (Cherry, 1978; Kinosita et al., 1984; Kawato et al., 1988). 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 absorbance changes were divided into vertically and horizontally polarized components with a beam-splitting polarizer and were led to Hamamatsu R567 photomultiplier tubes. The signals were analyzed by calculating the absorption anisotropy, $r(t)$, and the total absorbance change, $A(t)$, given by

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

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

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

In each experiment, 16 384 signals were averaged using a Toyo Technica 2805 transient memory. The measurements were performed in either 58% or 60% sucrose solution in order to reduce light scattering and vesicles tumbling. No denaturation of cytochrome P-450 during the experiment was observed spectrophotometrically.

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

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

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

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

where ϕ is the average rotational relaxation time. Here it should be noted that $[r_3/r(0)]_{min} = 1/4(3\cos^2\theta_N - 1)^2$ when all cytochrome P-450 molecules are rotating, even in the presence of multiple rotating species.

Curve fitting of the data by eq 4 was accomplished by a PDP-11/73 minicomputer. It should be noted that, in eqs 3 and 4, $r(t)/r(0)$ does not depend on the intensity of the photoselecting flash and only $r(0)$ depends on the laser flash intensity (Kawato & Kinosita, 1981). Therefore, $r(t)$ curves obtained at slightly different excitation intensities were normalized to the same $r(0)$ for direct comparison in Figure 2.

Other Methods. The lipid to protein weight ratio (L/P) was determined for microsomes purified by sucrose density gradient ultracentrifugation. Protein concentration was determined with the BCA protein assay using BSA as standard. Lipid phosphorous was measured according to the method of Gerlach and Deuticke (1963). Incorporation of enzymes into lipid vesicles was determined by Ficoll density gradient ultracentrifugation followed by fractionation and optical determination. Proteoliposomes were layered onto a continuous Ficoll density gradient [3–10% (w/v)] in aliquots of about 0.6 mL containing 4 nmol of heme and centrifuged at 190000*g* for 12 h at 4 °C. Electron microscopic observation of the liposome was carried out as described previously (Kominami et al., 1986).

RESULTS AND ANALYSIS

Characterization of Proteoliposomes. The formation of proteoliposomes was confirmed by density gradient centrifugation in Ficoll (Kominami et al., 1988). In all proteoliposomes examined, a single band was observed on the gradient. Comigration of cytochrome P-450, NADPH–cytochrome P-450, and phospholipids was demonstrated by coincident distribution of 417-nm absorption of the cytochrome and cytochrome *c* reductase activity as well as [^{14}C]dipalmitoylphosphatidylcholine on fractionation of the Ficoll density gradient, clearly demonstrating the coincorporation of cytochrome P-450 with the reductase into the liposomal membrane. The high steroid hydroxylase activity of the P-450 plus reductase vesicles strongly suggests that both P-450 and the reductase are present in the same vesicle. More than 80% of enzymes was observed to be located at the external side of unilamellar vesicles of about 50 nm in average diameter. It should be remembered that the cytochromes in membranes were very stable even in the absence of substrates and glycerol, and only 20% of the cytochrome in vesicles was denatured to

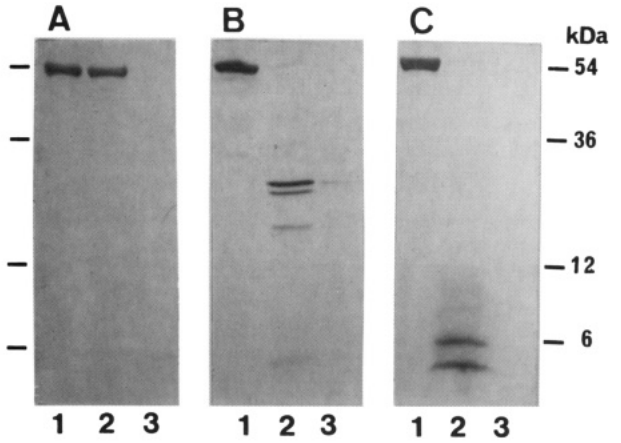

FIGURE 1: Proteolytic analysis of cytochrome P-450 in PC/PE/PS vesicles with SDS–polyacrylamide gel electrophoresis. Enzymes were subjected to trypsin digestion for 2 h at 37 °C as described under Experimental Procedures. (A) Lane 1, control P-450$_{17\alpha,lyase}$ without trypsin digestion; lane 2, P-450$_{17\alpha,lyase}$ in PC/PE/PS vesicles; lane 3, P-450$_{17\alpha,lyase}$ in detergent solution. (B) Lane 1, control P-450$_{C21}$ without trypsin digestion; lane 2, P-450$_{C21}$ in lipid vesicles; lane 3, P-450$_{C21}$ in detergent solution. (C) Lane 1, control P-450IIB1/P-450IIB2 without trypsin digestion; lane 2, P-450IIB1/P-450IIB2 in lipid vesicles; lane 3, P-450IIB1/P-450IIB2 in detergent solution. The bands are silver stained.

P-420 during storage at 25 °C for 24 h, while more than 80% of the cytochrome in the solubilized state with 1% cholate was converted to P-420 under the same conditions.

Proteolytic Analysis. The topological structure of cytochrome P-450 with respect to the membrane was examined by performing trypsinolysis for both proteoliposomes and solubilized cytochromes. All of the P-450s, including P-450IIB1/P-450IIB2 in the solubilized state in the presence of 1% cholate, were cleaved into very small peptides, not observable on SDS–PAGE, as shown in lane 3 of each sample in Figure 1. The SDS–PAGE patterns of digested P-450s in liposomes are greatly different from each other. P-450$_{17\alpha,lyase}$ in the membranes was not digested at all by trypsin at 37 °C for 2 h when trypsin was added in a weight ratio of 1:25 to P-450 molecules. The SDS–PAGE of P-450$_{C21}$ showed four fragments, with approximated molecular masses of 29, 25, 18, and 6 kDa, with a faint band of undigested P-450. A wash of the liposomes after digestion with 500 mM NaCl, 500 mM Na_2CO_3, or 50 mM $Na_4P_2O_7$ did not change the observed patterns, implying that these fragments were embedded in the membrane and not simply bound to the membrane with electrostatic interactions. P-450IIB1/P-450IIB2 vesicles showed the same SDS–PAGE pattern of proteolysis as reported by Vergeres et al. (1989) when they were treated in the same way as other proteoliposomes.

Rotational Diffusion of Cytochrome P-450 in Lipid Vesicles. Cytochrome P-450 alone or together with NADPH–cytochrome P-450 reductase was reconstituted in PC/PE/PS = 5:3:1 (molar ratio) vesicles. Rotational diffusion measurements were performed at 20 °C. In all samples examined, the absorption anisotropy $r(t)$ decayed within 1 ms to a time-independent value r_3. The $r(t)$ curves were analyzed by eq 4 based on rotation of P-450 about the normal of the membrane plane. Decay parameters are summarized in Table I.

Orientation of the Heme Plane of Cytochrome P-450. The tilt angle θ_N of cytochromes P-450$_{17\alpha,lyase}$ and P-450$_{C21}$ was determined in vesicles containing only one of these P-450s. In order to completely mobilize all P-450 molecules, the P-450 vesicles were incubated with 730 mM NaCl for 30 min at room temperature (Ohta et al., 1991; Etter et al., 1991). The

Table I: Decay Parameters of the Time-Dependent Absorption Anisotropy of the Cytochrome P-450·CO Complex in Proteoliposomes with L/P-450 = 35 Analyzed by Equation 4[a]

enzymes in vesicles	ϕ (μs)	$r_3/r(0)$	mobile P-450 (%)[b]
P-450$_{17\alpha,lyase}$	156 (19)[c]	0.15 (0.03)	89 (3)
P-450$_{17\alpha,lyase}$ +730 mM NaCl +progesterone[d]	138 (19)	0.04 (0.01)	100 (1)
P-450$_{17\alpha,lyase}$ +progesterone	165 (29)	0.13 (0.03)	91 (3)
P-450$_{17\alpha,lyase}$ +reductase +progesterone	171 (16)	0.12 (0.03)	92 (3)
P-450$_{C21}$	157 (13)	0.36 (0.02)	79 (2)
P-450$_{C21}$ +730 mM NaCl	167 (8)	0.19 (0.01)	100 (1)
P-450$_{C21}$ +reductase	193 (45)	0.22 (0.01)	96 (1)
P-450$_{C21}$ +reductase +progesterone	221 (34)	0.22 (0.02)	96 (2)
P-450$_{C21}$ +reductase +antireductase IgG	225 (27)	0.59 (0.01)	51 (1)

[a] All measurements were performed in 58% sucrose plus 11% glycerol at 20 °C. [b] The percentage of mobile cytochrome P-450 was calculated from eq 5. [c] Numbers in parentheses are standard deviations in 12–18 experiments. [d] Progesterone is a substrate of both cytochromes P-450$_{17\alpha,lyase}$ and P-450$_{C21}$.

final NaCl concentration during the measurements was 395 mM after sucrose was dissolved in the proteoliposome solution. The observed values of $[r_3/r(0)]_{min} = (1/4)(3\cos^2\theta_N - 1)^2$ were 0.04 for P-450$_{17\alpha,lyase}$ and 0.19 for P-450$_{C21}$, corresponding to θ_N of either 47° or 63° for P-450$_{17\alpha,lyase}$ and either 38° or 78° for P-450$_{C21}$ (Figure 2). In Figure 2, $r(t)$ is best illustrated on a linear scale, since $r(t)$ of P-450$_{17\alpha,lyase}$ looks very noisy on a logarithmic scale when it is smaller than 0.005.

The percentage of mobile population, p_m, can be calculated by

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

where $[r_3/r(0)]_{min}$ is equal to be 0.19 for P-450$_{C21}$ and 0.04 for P-450$_{17\alpha,lyase}$. It was shown that the majority of cytochromes P-450$_{17\alpha,lyase}$ and P-450$_{C21}$ are rotating in phospholipid vesicles with a rotational relaxation time ϕ of about 157 μs. Although we cannot choose a unique value of θ_N from the two possible values for these P-450s, the mobile population is determined uniquely in proteoliposomes which contain only one of these cytochromes. In adrenocortical microsomes, however, p_m cannot be determined straightforwardly. We tentatively have used the average number of $[r_3/r(0)]_{min}$ = 0.11 of these two cytochromes for calculation of the mobile P-450 in microsomes, assuming that both cytochromes have the same population.

Influence of NADPH–Cytochrome P-450 Reductase, Antireductase IgG, and Substrate on Rotational Mobility of Cytochrome P-450 in Lipid Vesicles. Due to the low density of protein in L/P-450 = 35 vesicles, most of cytochrome P-450 is rotating. The small amount of immobile population (with $\phi \geq 20$ ms) reflects nonspecific microaggregates of P-450 (Kawato et al., 1982; Gut et al., 1982). By the presence of an equimolar amount of NADPH–cytochrome P-450 reduc-

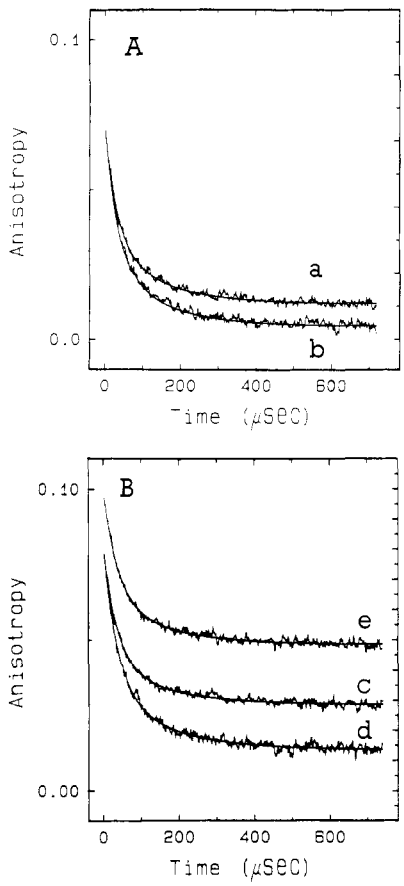

FIGURE 2: Time-dependent absorption anisotropy of cytochrome P-450 in PC/PE/PS vesicles with and without high salt treatment. Samples (2–3 μM in heme) were photolyzed by a vertically polarized laser flash at 532 nm, and $r(t)$ was recorded at 450 nm as described under Experimental Procedures. Measurements were performed in 58% sucrose and 11% glycerol solution at 20 °C (~0.6 poise). (Panel A) Curve a, control P-450$_{17\alpha,lyase}$ vesicles with progesterone; curve b, P-450$_{17\alpha,lyase}$ vesicles containing progesterone incubated with NaCl. (Panel B) Curve c, control P-450$_{C21}$ vesicles; curve d, P-450$_{C21}$ vesicles incubated with NaCl; curve e, P-450$_{C21}$ vesicles with progesterone. The zig-zag lines are experimental data, and the solid curves were obtained by fitting the data to eq 4. The initial anisotropies of curves b and d are normalized to the same $r(0)$ of curves a and c to allow a direct comparison. This is justified because although $r(0)$ depends on the intensity of the photoselecting flash, $r(t)/r(0)$ is independent of the flash intensity. For salt treatment, samples were incubated with 730 mM NaCl and then dissolved in 58% sucrose solution. Curve e is vertically displaced for clarity; otherwise curve e is completely superimposed on curve c. Note that the linear vertical axis is chosen for clarity, because $r(t)$ looks very noisy on a logarithmic scale when it is smaller than 0.005.

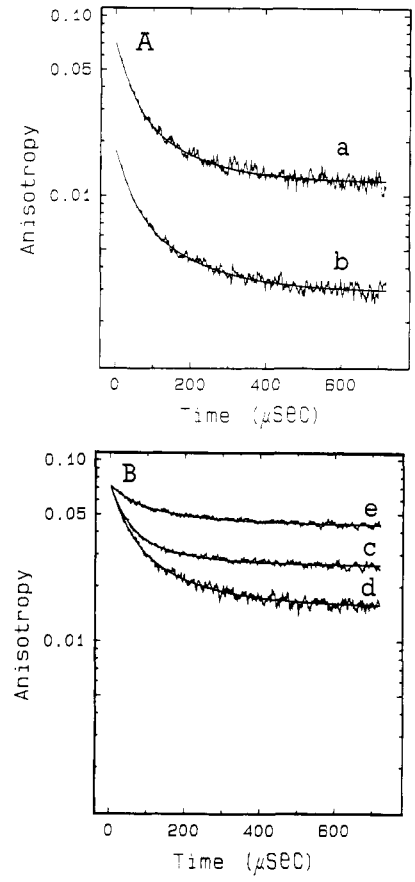

FIGURE 3: Effect of NADPH–cytochrome P-450 reductase and antireductase IgG on the time-dependent absorption anisotropy of cytochrome P-450 in proteoliposomes. (Panel A) Curve a, P-450$_{17\alpha,lyase}$ in vesicles with progesterone; curve b, P-450$_{17\alpha,lyase}$ plus reductase in vesicles with progesterone. (Panel B) Curve c, P-450$_{C21}$ in vesicles; curve d, P-450$_{C21}$ plus reductase in vesicles; curve e, antireductase IgG was added to the same sample shown in curve d. The zig-zag lines are experimental data, and the solid curves were obtained by fitting the data to eq 4. The initial anisotropy of curve b is vertically displaced for illustrative purposes; otherwise curve b is almost completely superimposed on curve a. The initial anisotropies of curves d and e are normalized to the same $r(0)$ of curve c to allow a direct comparison. Note that the $r(t)$ curve of P-450$_{17\alpha,lyase}$ was the same with and without progesterone. When reductase was coreconstituted, P-450/reductase (mol/mol) is 1 and L/P-450 (w/w) is 35.

tase to the cytochrome, a significant mobilization of cytochrome P-450$_{C21}$ was demonstrated (Figure 3). The mobile population was increased to 96%. Though a small increase in ϕ was observed from 157 to 193 μs for P-450$_{C21}$, the difference may not be very significant due to large experimental error. Further addition of a 78-fold excess (w/w) of antireductase antibodies over reductase greatly decreased the mobile population to 51%, while ϕ was not changed significantly (Figure 3). These results imply that cross-linking of reductase by antireductase IgG immobilizes the partner P-450$_{C21}$ in the P-450$_{C21}$·reductase complex. Not all cytochrome molecules were immobilized by the reductase antibody in these vesicles probably due to a nonequimolar distribution of P-450 and reductase in individual vesicles with such a low concentration of proteins. On the other hand, the presence of the reductase did not affect the mobility of P-450$_{17\alpha,lyase}$ for the mobile population. Progesterone, a substrate of cytochrome P-450s, has not significantly affected the rotatinoal mobility of these two cytochromes in vesicles, regardless of the presence or absence of the reductase. The ϕ value ranged from 156 to 221 μs for all samples examined, and the difference in ϕ value may not be very considerable because of large experimental error (see Table I).

Rotational Diffusion of Cytochrome P-450 in Microsomes and Effect of Substrates. Rotational diffusion measurements were performed for adrenal microsomes at 20 °C. Absorption anisotropy $r(t)$ decayed within 2 ms to a time-independent value r_3. Data were analyzed by eq 4, and decay parameters are summarized in Table II. Cytochrome P-450 shows rotation with ϕ = 558 μs and $r_3/r(0)$ = 0.38, which is higher than that of proteoliposomes as shown in Figure 4. The mobile population was calculated to be 70% using eq 5 with $[r_3/r(0)]_{min}$ = 0.11. In contrast to proteoliposomes, progesterone and pregnenolone have significantly affected the rotational mobility of cytochrome P-450 in microsomes. Pregnenolone and progesterone immobilized P-450 to $r_3/r(0)$ = 0.43–0.44 with almost the same ϕ = 565–577 μs.

Recombination Kinetics of CO to Reduced Cytochrome P-450. The total absorption decay $A(t)$ of cytochrome P-450

Table II: Decay Parameters of the Time-Dependent Absorption Anisotropy of the Cytochrome P-450·CO Complex in Adrenocortical Microsomes Analyzed by Equation 4[a]

microsomes	ϕ (μs)	$r_3/r(0)$	mobile P-450 (%)[b]
microsome control	558 (37)[c]	0.38 (0.02)	70 (2)
microsome +progesterone[d]	565 (45)	0.44 (0.01)	63 (1)
microsome +pregnenolone[d]	577 (21)	0.43 (0.03)	64 (3)

[a] All measurements were performed in 60% sucrose at 20 °C. [b] The percentage of mobile cytochrome P-450 was calculated from eq 5. [c] Numbers in parentheses are standard deviations in 15–20 experiments. [d] Pregnenolone is a substrate of cytochrome P-450$_{17\alpha,lyase}$. Progesterone is a substrate of both cytochromes P-450$_{17\alpha,lyase}$ and P-450$_{C21}$.

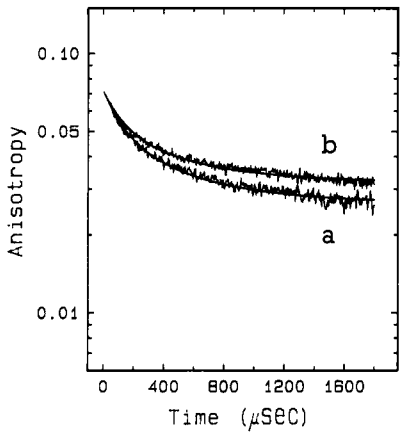

FIGURE 4: Effect of substrates on the time-dependent absorption anisotropy of cytochrome P-450 in adrenocortical microsomes. Measurements were performed with 4–5 μM cytochrome P-450 in 60% sucrose at 20 °C. Curve a, control microsomes; curve b, microsomes with progesterone. The zig-zag lines are experimental data, and the solid curves were obtained by fitting the data to eq 4. The initial anisotropy of curves b is normalized to the same $r(0)$ of curve a to allow a direct comparison.

was close to monophasic in proteoliposomes and microsomes under the same conditions as those of rotational diffusion measurements. $A(t)$ was, therefore, analyzed by a monoexponential approximation. In P-450 vesicles, the lifetime of photodissociated P-450, obtained from $A(t)$, was the same with $\tau \approx 2$ ms for P-450$_{17\alpha,lyase}$ and for P-450$_{C21}$. The presence of progesterone, a substrate for both P-450s, significantly elongated the lifetime to $\tau \approx 8$ ms for P-450$_{17\alpha,lyase}$ and $\tau \approx 10$ ms for P-450$_{C21}$. Coexistence of NADPH–cytochrome P-450 reductase with P-450 did not significantly alter the lifetime for both P-450$_{17\alpha,lyase}$ and P-450$_{C21}$ regardless of the presence or absence of progesterone, suggesting that there is no direct electronic interactions affecting the CO recombination between P-450 and the reductase. It should be noted that the incubation of P-450 vesicles with NaCl did not affect the CO recombination kinetics. In microsomes, the lifetime was about 4 ms, which is slightly longer than that in P-450 vesicles and might be due to the presence of substrate-bearing P-450. The addition of progesterone to microsomes significantly elongated the lifetime to $\tau \approx 8$ ms, a value which is the same as that in proteoliposomes.

Absence of Vesicle Tumbling. As reported previously (Gut et al., 1983; Etter et al., 1991), no significant decay was observed for cytochrome P-450 incorporated in dipalmitoylphosphatidylcholine vesicles, having a diameter around 50 nm, at 20 °C in the crystalline state of the lipid bilayer, excluding the possibility of vesicle tumbling contributing to the observed decay in $r(t)$. Incubation of these vesicles with 500 mM KCl did not induce any decay in $r(t)$, indicating that a high salt treatment does not induce vesicle tumbling or mobilize P-450 in the crystalline state of phospholipids.

DISCUSSION

Different Protein-Protein Interactions and Heme Angle for P-450$_{C21}$ and P-450$_{17\alpha,lyase}$ in Proteoliposomes. The tilt angle of the heme plane from the membrane plane has been demonstrated to be different depending on the molecular species of cytochrome P-450. The tilt angle θ_N of the heme for P-450$_{17\alpha,lyase}$ is either 47° or 63° ± 1° calculated from $[r_3/r(0)]_{min} = r_3/r(0) = (1/4)(3\cos^2\theta - 1)^2 = 0.04 \pm 0.01$. The angle θ_N of P-450$_{C21}$ is either 38° or 78° ± 1° from $[r_3/r(0)]_{min} = 0.19 \pm 0.01$. This is clearly different from P-450IIB1/P-450IIB2 with $\theta_N = 55°$ (Gut et al., 1983) and from P-450IA1/P-450IA2 with θ_N of either 48° or 62° (Etter et al., 1991). These data indicate that different molecular species of P-450s have different tilt angles of the heme from the membrane plane. These values so far observed are distributed around 55° and are clearly far from 0° (parallel to the membrane plane) and 90° (perpendicular to the membrane plane). This calculation is based on the assumption of the axial rotation of P-450 around the membrane normal, and this might be nonsense when the heme-containing part of the P-450 undergoes wobbling or flexible motion in the water phase. Such a motion would occur when P-450 has a cytochrome b_5 type structure, anchoring to the membrane via only one hydrophobic segment (Nelson & Strobel, 1988). Our tryptic digestion analysis demonstrates that a considerable part of both cytochromes P-450$_{17\alpha,lyase}$ and P-450$_{C21}$ is deeply embedded in the lipid bilayer, and these topologies are completely different from those of cytochrome b_5 and NADPH–cytochrome P-450 reductase (Tajima & Sato, 1980; Gum & Strobel, 1979). These results support the proposition that the orientation of P-450 would be fixed with respect to the membrane plane, allowing the axial rotation of the cytochromes. It should be noted that many integral membrane proteins so far examined, having a considerable part deeply embedded in the membrane (e.g., cytochrome oxidase, band 3, bacteriorhodopsin, rhodopsin, and ADP/ATP translocator), have shown axial rotation about the membrane normal without significant rocking motion around the axis lying in the membrane plane (Kawato et al., 1981; Nigg & Cherry, 1980; Heyn et al., 1977; Mueller et al., 1984).

Though the concentration of P-450 is low, L/P-450 = 35 in the present lipid vesicles, around 10–20% of P-450 was immobile (with $\phi \geq 20$ ms) for both P-450$_{17\alpha,lyase}$ and P-450$_{C21}$, probably due to the formation of microassociations. The observed rotational relaxation time $\phi = 157$–221 μs is probably an average number of multiple rotating oligomers (e.g., monomer, dimer, trimer, etc.) as judged from the ϕ of the monomeric cytochrome P-450 whose ϕ is around 40 μs (Kawato et al., 1982). The value of $\phi = 157$–221 μs might correspond to an average-sized rotamer having a diameter of 2–2.5-fold of the monomeric P-450, because ϕ is proportional to the square of the diameter of the rotamer.

By the presence of NADPH–cytochrome P-450 reductase, mobilization occurred for P-450$_{C21}$ by a 17% increase in p_m, while the mobility of P-450$_{17\alpha,lyase}$ was not affected. This implies that P-450$_{C21}$ may form a transient association with the reductase and P-450$_{17\alpha,lyase}$ does not have such observable interactions with the reductase. The P-450$_{C21}$ oligomers would be disintegrated by forming a monomolecular transient

association with the reductase, resulting in mobilization as was observed for cytochrome P-450IIB1/P-450IIB2 (Gut et al., 1982). This explanation is confirmed by the antireductase IgG-induced immobilization of the P-450_{C21}, since the IgG does not cross-react with the cytochrome.

Such a transient association between P-450_{C21} and the reductase would not be in conflict with P-450 reduction kinetics, which show a linear increase in the fast phase reduction with increasing the ratio of reductase/P-450 (Kominami et al., 1989). The lifetime of the transient association is 20–100 ms, which is shorter than the time needed for the P-450_{C21} reduction of 200–1000 ms. The observed reductase + P-450_{C21} association should have a transient nature and might not be stable over the time range of, for example, seconds or minutes. This is because cytochrome P-450_{C21} functionally interacts with several other proteins, including cytochrome b_5. If the association of reductase with P-450 is very stable, cytochrome b_5 would have difficulty to donate electrons to P-450.

Protein–Protein Interactions of Cytochrome P-450 in Microsomes. As judged from the ϕ value of the anisotropy decay, the average size of the rotamer over multiple rotamers with different sizes may be around 1.5–2-fold of that in lipid vesicles. The immobile population of about 30–37% would be due to the higher protein concentrations in microsomes (L/P = 0.7) than that in proteoliposomes. A considerable immobilization was observed with the presence of pregnenolone and progesterone in microsomes. These substrate-induced changes of protein–protein interactions might be responsible for substrate regulation of electron transfer interactions.

A large population (70%) of P-450 was mobile in adrenocortical microsomes, in contrast to adrenocortical mitochondria where only 20% of P-450 was rotating (Kawato et al., 1988; Ohta et al., 1990). This is probably due to the lower concentration of membrane proteins in microsomes (L/P = 0.7) than that of inner membrane of mitochondria (L/P = 0.3). The presence of different substrates (e.g., cholesterol, pregnenolone, and deoxycorticosterone) also significantly affect the mobility of mitochondrial P-450 (Kawato et al., 1988; Ohta et al., 1990, 1991). In microsomes of rat liver (L/P = 0.4), however, no substrates such as aminopyrine, phenobarbital, and octyldiamine significantly affected the mobile population (65%) of P-450, possibly due to the fact that these substrates were exogenous ones (Kawato et al., 1991).

Dynamic Topology of Microsomal Cytochrome P-450. Taking together proteolytic digestion analysis and rotational diffusion measurements, we propose a model for P-$450_{17\alpha,lyase}$ or P-450_{C21} as illustrated in Figure 5, where a large part of cytochrome P-450 is embedded in the lipid bilayer. Even after the extensive tryptic digestions, a considerable part of P-450 polypeptides was bound to liposomal membranes. This is completely different from cytochrome P-450IIB1/P-450IIB2 (Vergeres et al., 1989), NADPH–cytochrome P-450 reductase (Gum & Strobel, 1979), and cytochrome b_5 (Tajima & Sato, 1980), whose major parts were digested, and only one anchor peptide of either carboxy or amino terminal was left in the membrane after the proteolytic digestions. It is also shown in Figure 1 that cytochromes P-$450_{17\alpha,lyase}$ and P-450_{C21} in the liposomal membranes are much less digested by trypsin than their solubilized forms, indicating that these P-450 molecules are protected by the membrane. It should be noted that the trypsin-resistant part of P-450_{C21} in the membrane contained the heme, as demonstrated by absorption spectra. It would be unlikely that the major part of P-450 protrudes into the water phase and that only the sites of trypsinolysis in the solubilized form are protected, being deeply buried in the membranes. The observed slow rotation of P-$450_{17\alpha,lyase}$ and P-450_{C21} with ϕ around 170 μs supports the proposition that these cytochromes have several membrane-embedded segments. These motions are much slower than a rapid wobbling of the water-protruding part with ϕ = 0.16–0.5 μs observed for NADPH–cytochrome P-450 reductase and cytochrome b_5 (Nishimoto et al., 1983; Vaz et al., 1979), having only one peptide embedded in the membrane.

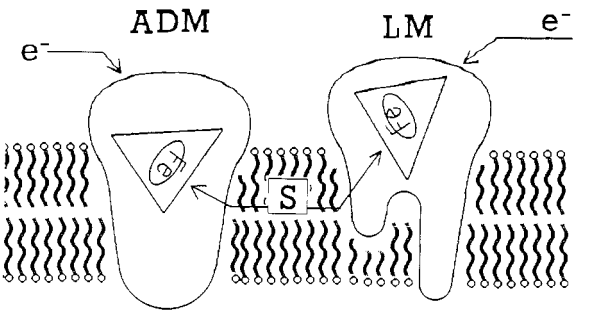

FIGURE 5: Topological structure of cytochrome P-450 in the membrane. A model structure of adrenocortical microsomal cytochrome P-$450_{17\alpha,lyase}$ or P-450_{C21} (ADM, left molecule) is illustrated based on the present mobility measurements and proteolytic digestion analyses. S represents a substrate. The large membrane-embedded part fixes the orientation of P-450 and allows axial rotation about the membrane normal. The heme binds tightly to the membrane-embedded part of the cytochrome and is placed a little bit below the surface of the membrane, being tilted by around 55° from the membrane plane. The triangle represents the active site of P-450 containing I, L, and K helices (Poulos et al., 1985). For comparison, a model of liver microsomal P-450 (LM, right molecule, P-450IIB or P-450IA) is also illustrated. The membrane-embedded part of LM P-450 would be smaller than that of ADM P-450 and would consist of a few segments, as judged from proteolysis and antibody binding studies (De Lemos-Chiarandini et al., 1987). The heme plane is also tilted by around 55° for LM P-450. The domain located on the proximal side of the heme would interact with the reductase with charged groups. (Tamburini et al., 1986; Stayton & Sliger, 1990; Shimizu et al., 1991). The substrate binding site would be located at the distal side of the heme (Poulos et al., 1985). The substrate binding site should face the membrane in order to efficiently react with hydrophobic substrates (S), most of which are hydrophobic and would be concentrated in the membrane.

There are many reports which support the proposal that a major part of rat liver microsomal P-450IIB1/P-450IIB2 is exposed to the water phase and only the N-terminal peptide spans the membrane (De Lemos-Chiarandini et al., 1987; Nelson & Strobel, 1988; Brown & Black, 1989; Vergeres et al., 1989). Although sequences similar to those reported as the tryptic cleavage sites for hepatic microsomal P-450s could be found in P-$450_{17\alpha,lyase}$ and P-450_{C21} (Nelson & Strobel, 1988; Brown & Black, 1989), most of the sites were undigested for these P-450s in liposomal membranes, also supporting the proposition that a much larger part of P-$450_{17\alpha,lyase}$ and P-450_{C21} is incorporated in the membrane than P-450IIB1/P-450IIB2. Since the mobility of P-45011B1/P-450IIB2 with a ϕ of about 200 μs was very similar to that of P-$450_{17\alpha,lyase}$ and P-450_{C21}, other segments besides the N-terminal would be dynamically embedded in the membrane to keep the orientation of P-450IIB with respect to the membrane plane (Monier et al., 1988; Vergeres et al., 1989).

There are interesting reports which imply that other segments besides the N-terminal fragment are also responsible for membrane incorporation of P-$450_{17\alpha,lyase}$, P-450IA1, and P-450IIE1 on the basis of the genetic expression experiments for N-terminal fragment-lacking P-450s which were observed to be bound to the membrane (Clark & Waterman, 1991;

Yabusaki et al., 1988; Larson et al., 1991). The peptide fragment of $P\text{-}450_{C21}$ around Ile 172 might be the membrane anchoring, since the mutation of Ile 172 to Asn resulted in the loss of binding ability to microsomes (Tusie-Luna et al., 1990). Several anchor peptides would therefore fix the orientation of $P\text{-}450_{C21}$ and $P\text{-}450_{17\alpha,lyase}$ with respect to the membrane plane, thereby allowing the axial rotation of P-450 around the membrane normal.

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