Decreased Lipid Order Induced by Microsomal Cytochrome P-450 and NADPH-Cytochrome P-450 Reductase in Model Membranes: Fluorescence and Electron Spin Resonance Studies†

Barbara C. Kunz,‡ Mike Rehorek,§ Helmut Hauser,‡ Kaspar H. Winterhalter,‡ and Christoph Richter*,‡

Eidgenössische Technische Hochschule, Laboratorium für Biochemie I, ETH-Zentrum, CH-8092 Zürich, Switzerland, and Department of Biophysical Chemistry, Biocenter of the University of Basel, CH-4056 Basel, Switzerland

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ABSTRACT: Cytochrome P-450 and NADPH-cytochrome P-450 reductase were reconstituted in unilamellar lipid vesicles prepared by the cholate dialysis technique from pure dimyristoylphosphatidylcholine (DMPC), pure dipalmitoylphosphatidylcholine (DPPC), pure dioleoylphosphatidylcholine (DOPC), and phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (PC/PE/PS) (10:5:1). As probes for the vesicles' hydrocarbon region, 1,6-diphenyl-1,3,5-hexatriene (DPH) and spin-labeled PC were used. The steady-state and time-resolved fluorescence parameters of DPH were determined as a function of temperature and composition of liposomes. Incorporation of either protein alone or together increased the steady-state fluorescence anisotropy (r_s) of DPH in DOPC and PC/PE/PS (10:5:1) liposomes. In DMPC and DPPC vesicles, the proteins decreased r_s significantly below the transition temperature (T_c) of the gel to liquid-crystalline phase transition. Time-resolved fluorescence measurements of DPH performed in reconstituted PC/PE/PS and DMPC proteoliposomes showed that the proteins disorder the bilayer both in the gel and in the liquid-crystalline phase. Little disordering by the proteins was observed by a spin-label located near the mid-zone of the bilayer 1-palmitoyl-2-(5-doxylstearoyl)-3-*sn*-phosphatidylcholine (8-doxyl-PC), whereas pronounced disordering was detected by 1-palmitoyl-2-(8-doxylpalmitoyl)-3-*sn*-phosphatidylcholine (5-doxyl-PC), which probes the lipid zone closer to the polar part of the membrane. Fluorescence lifetime measurements of DPH indicate an average distance of ≥60 Å between the heme of cytochrome P-450 and DPH.

Cytochrome P-450 and NADPH-cytochrome P-450 reductase are key enzymes of the hepatic microsomal monooxygenase system catalyzing the oxidative and reductive metabolism of endogenous substrates and many xenobiotics (Omura, 1978; Estabrook et al., 1979; White & Coon, 1980). The reductase (M_r ~78 000) is anchored to the membrane of the endoplasmatic reticulum via a small (M_r ~6000–10 000) hydrophobic segment (Gum & Strobel, 1979; Black et al., 1979). The large hydrophilic part, which contains 1 molecule of FMN and FAD, protrudes from the membrane into the cytoplasmic space. The topology of P-450 (M_r ~ 50 000) in membranes, on the other hand, is currently unknown (De Pierre & Ernster, 1977). Limited proteolysis of microsomes does not release a soluble catalytic domain but results in conversion of P-450 to the catalytically inactive form P-420 (Sato et al., 1969). It therefore appears that P-450 is not simply anchored to the membrane by a single hydrophobic domain as its reductase. Recent sequencing studies (cf. Discussion) support this view. P-450 and reductase can be isolated and reconstituted in an enzymatically active form. Successful reconstitution requires the presence of phospholipids (Lu et al., 1969). The arrangement of the two enzymes in the membrane and their odd stoichiometry (there can be 20–30 cytochromes per reductase in the microsomal membrane of rat hepatocytes) have raised questions as to the mechanism of electron transfer from the reductase to the cytochrome and the functional interaction of the proteins.

Rotational mobility of P-450 was recently demonstrated in microsomes and proteoliposomes (Richter et al., 1979; Kawato et al., 1982; McIntosh et al., 1980) by flash-induced absorption anisotropy techniques of the heme–CO complex and in proteoliposomes by the delayed fluorescence polarization technique (Greinert et al., 1979) or by magnetic CD[1] spectroscopy (Bösterling & Trudell, 1982). The absorption anisotropy technique was successfully applied by us to also investigate intermolecular interactions of P-450 with itself or with its reductase in both microsomal membranes and reconstituted vesicles (Kawato et al., 1982; Gut et al., 1982, 1983). These studies indicated the existence of a long-lived heterodimeric complex between one reductase and one cytochrome molecule in membranes. Evidence for significant molecular interactions between the two proteins in a membranous reconstituted system was also recently provided (Nisimoto et al., 1983) by the use of reductase specifically labeled with a fluorescent probe.

While these studies have given information about protein–protein interactions of the enzymes in membranes, little is known about lipid–protein interactions of the monooxygenase system. The study of lipid–protein interactions is of great

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‡ Eidgenössische Technische Hochschule, ETH-Zentum.

§ Department of Biophysical Chemistry, Biocenter of the University of Basel.

[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; T_c, transition temperature; EDTA, ethylenediaminetetraacetic acid; P-450, rat liver cytochrome P-450; reductase, rat liver NADPH-cytochrome P-450 reductase; ESR, electron spin resonance; 5-doxyl-PC, 1-palmitoyl-2-(5-doxylstearoyl)-3-*sn*-phosphatidylcholine; 8-doxyl-PC, 1-palmitoyl-2-(8-doxylpalmitoyl)-3-*sn*-phosphatidylcholine; CD, circular dichroism.

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interest in membrane biology. Such interactions affect the fluidity and order of the bilayer, modulate enzymatic activity of membrane proteins, and are responsible for changes in the melting temperature and cooperativity of the gel to liquid-crystalline phase transition. Biochemical studies give evidence for the importance of acidic phospholipids for reconstitution of the monooxygenase system (Haaparanta et al., 1980; Ingelmann-Sundberg et al., 1981; Kawato et al., 1982). There are also indications that the phospholipid environment can affect the conformation and/or spin state of P-450 (Taniguchi et al., 1980; Gibson et al., 1980; Yang & Tsong, 1980). Much less is known about the perturbation caused by the incorporation of proteins of the monooxygenase system on the dynamics of the lipid motion in the bilayer. By determining the steady-state and time-dependent fluorescence depolarization of the probe diphenylhexatriene (DPH) and the mobility of two PC spin probes, we investigate here the perturbation induced by P-450 and reductase as a function of lipid composition and lipid to protein ratio in reconstituted liposomes. The results show a considerable disordering of phospholipid acyl chains upon incorporation of the proteins into the membrane.

Experimental Procedures

Materials. Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), and bovine spinal cord phosphatidylserine (PS), all grade I, were purchased from Lipid Products (South Nutfield, U.K.) and stored at −20 °C. L-α-Dimyristoylphosphatidylcholine (DMPC), L-α-dipalmitoylphosphatidylcholine (DPPC), and L-α-dioleoylphosphatidylcholine (DOPC) were from Sigma (St. Louis, MO) and used without further purification. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was from Fluka (Buchs). The spin-labeled PC, 1-palmitoyl-2-(5-doxylstearoyl)-3-*sn*-phosphatidylcholine (5-doxyl-PC) and 1-palmitoyl-2-(8-doxylpalmitoyl)-3-*sn*-phosphatidylcholine (8-doxyl-PC), were prepared from 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (Berchtold, Biochemisches Labor, Bern) as described previously (Hauser et al., 1982). All other reagents were of the highest grade available.

Isolation of Proteins. P-450 and its reductase were isolated from phenobarbital-induced rat liver microsomes as described elsewhere (Gut et al., 1982). The specific contents were 14 nmol of P-450/mg of protein and 51 000 units/mg of protein for the reductase.

Preparation of Proteoliposomes. Reconstitution of P-450 and reductase in PC/PE/PS (10:5:1 w/w), DMPC, DPPC, or DOPC vesicles was performed by a cholate dialysis procedure (Gut et al., 1982). The lipid:cytochrome P-450 ratio was 1:1 (w/w) unless indicated otherwise. Reductase was present in amounts equimolar to P-450.

Fluorescence Labeling of Membranes. To a suspension of the vesicles (0.2 mg of lipid/mL), DPH in tetrahydrofuran was added from a 2 mM stock solution to a final concentration of 2 μM. The samples were incubated in the dark for 30 min at room temperature before measurement.

Steady-State Fluorescence Anisotropy. Fluorescence depolarization measurements were carried out with a SPF-500 spectrofluorometer (Aminco). Excitation was at 357 nm. Fluorescence was collected through cutoff filters above 420 nm. Anisotropies were calculated from

$$r_s = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp) \quad (1)$$

No correction for the monochromators' efficiency in the transmission of vertically and horizontally polarized light had to be introduced with our apparatus. The two fluorescence intensities, $I_\parallel$ and $I_\perp$ for each temperature, were alternatively recorded by rotating the analyzer over 90°.

Fluorescence Decay Measurements. Time-dependent fluorescence studies were carried out as will be published in detail elsewhere. Briefly, a single-photon counting instrument with a photomultiplier was used (XP 2020 Q; Philips Electronic Instruments, Inc., Mahwah, NJ, and Ortec electronics, Ortec, Oak Ridge, TN, respectively). The light source was a nanosecond-gated flashlamp (Applied Photophysics Ltd., London, U.K.) modified to reduce the pulsewidth to 1.6 ns full width at half-maximum (FWHM). The lamp was run at 5.0 kV and 30 kHz with H_2 gas at a pressure of 0.75 bar and a spark gap of 0.8 mm. The excitation wavelength was selected with a narrow band-pass filter (362 nm; Balzers Union, Inc., Balzers, Liechtenstein) and the emission wavelength with two low-fluorescence cutoff filters (KV 418; Schott Optical Glass, Inc.). Data analysis was performed on a PDP 11/40 computer (Digital Equipment Corp. Mayard, MA) interfaced to the multichannel analyzer. Exponential decays of the following form were assumed:

$$I_T^\delta(t) = \sum_{i=1}^{N} I_i \exp(-t/\tau_i) \quad (2)$$

$$r^\delta(t) = \sum_{j=1}^{M} r_j \exp(-t/\varphi_j) \quad (3)$$

where δ stands to indicate an infinitely short excitation pulse. The fluorescence intensities for vertical and horizontal polarization at time t after excitation, $I_V(t)$ and $I_H(t)$, are related to $I_T^\delta(t)$ and $r^\delta(t)$ by the equations:

$$I_T(t) = \int_0^t g(t')I_T^\delta(t - t')\,dt' = I_V(t) + 2I_H(t) \quad (4)$$

$$r(t)I(t) = \int_0^t g(t')r^\delta(t - t')I_T^\delta(t - t')\,dt' = I_V(t) - I_H(t) \quad (5)$$

where $g(t')$ is the response function of the apparatus [see Kawato et al. (1977)].

Incorporation of Spin-Labeled PC. Spin-labeled PC was mixed with the other phospholipids prior to evaporating the chloroform/methanol under a N_2 stream. The phospholipid: spin-labeled PC was 200:1 (mol/mol) for all experiments.

ESR spectra were recorded as described before (Hauser et al., 1982). The order parameter S was calculated according to eq 6 provided the inner and outer hyperfine splittings, $T_\perp$

$$S = \frac{I_\parallel - T_\perp}{T_{zz} - T_{xx}} \frac{a_0}{a'} = \frac{1}{2}[3(\cos^2\theta) - 1] \quad (6)$$

and $T_\parallel$ respectively, could be taken directly from the ESR spectrum. $T_{xx} = T_{yy} = 5.8$ G and $T_{zz} = 30.8$ G are the principal components of the hyperfine splitting tensor (Hubbell & McConnell, 1971), a_0/a' is a correction for the polarity dependence of the hyperfine splittings, and θ is the angle of deviation of the nitrogen 2p π orbital from the bilayer normal.

Results

Coreconstitution of P-450 and its reductase into liposomes results in a functionally competent monooxygenase system (Gut et al., 1982). The interaction between the protein and lipid components of this reconstituted system was investigated by the steady-state and time-resolved fluorescence of DPH and the mobility of spin-labeled phospholipids. Degradation of P-450 during the experiments could be excluded by spectrophotometrically monitoring the P-450–CO complex.

Temperature Dependence of Steady-State Fluorescence Anisotropy. The steady-state emission anisotropy, r_s, of DPH incorporated in PC/PE/PS (10:5:1 w/w), DOPC, DMPC, or DPPC vesicles and in proteoliposomes containing P-450 and/or

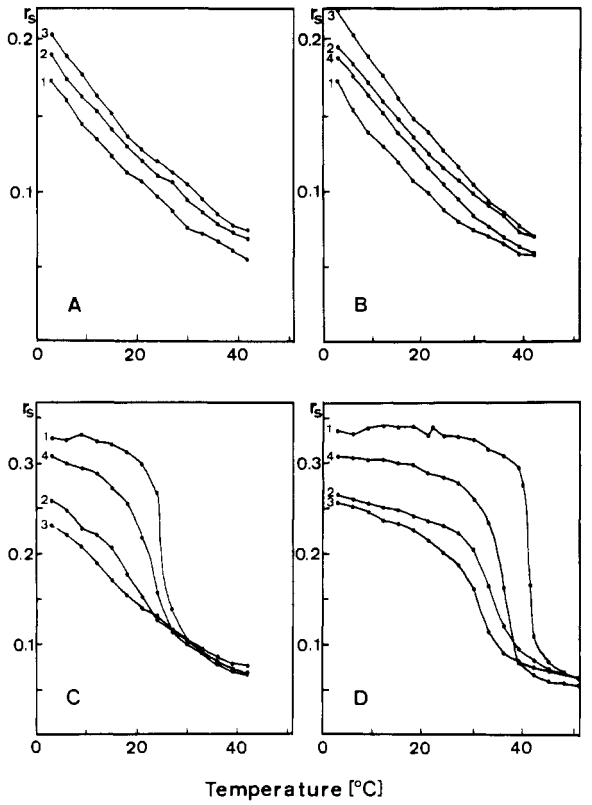

FIGURE 1: Temperature dependence of steady-state fluorescence anisotropy r_s of DPH incorporated into PC/PE/PS (10:5:1 w/w) (panel A), DOPC (B), DMPC (C), and DPPC (D) vesicles. Reconstitution of proteins into vesicles was performed as described under Experimental Procedures. Lipid and cytochrome P-450 were 0.2 mg/mL each. NADPH-cytochrome P-450 reductase was present in amounts equimolar to cytochrome P-450 (2.8 μM). Concentration of DPH was 2 μM. Vesicles were measured in 20 mM HEPES, pH 7.4, with 1.0 mM EDTA and 20% glycerol. (Curve 1) Vesicles without proteins; (curve 2) vesicles containing cytochrome P-450; (curve 3) vesicles containing cytochrome P-450 and NADPH-cytochrome P-450 reductase; (curve 4) vesicles containing NADPH-cytochrome P-450 reductase alone. In PC/PE/PS vesicles (A), NADPH-cytochrome P-450 reductase had no influence on r_s of DPH.

reductase is plotted against temperature in Figure 1. P-450 both in PC/PE/PS and in DOPC vesicles caused an increase in r_s (panels A and B of Figure 1). The value of r_s increased (not presented in the plot) with decreasing lipid to P-450 ratios (lipid:P-450 = 30, 5, 1, and 0.5). Reductase alone had no influence on r_s of DPH in PC/PE/PS vesicles. In DMPC and DPPC vesicles (panels C and D, respectively) the r_s value did not change upon incorporation of P-450 and/or reductase above T_c. Below T_c cytochrome P-450 significantly decreased r_s. In all types of liposomes studied, reductase coreconstituted with P-450 increased the changes in r_s induced by the cytochrome alone. Changes in r_s between 4 and 40 °C were reversible in all experiments.

In other experiments (not shown), proteoliposomes of a lipid to protein ratio of 1 were prepared with P-450 preparations having heme contents ranging from 8 to 14 nmol/mg of protein. The quantum yield of DPH fluorescence was not affected by the variation of the specific heme content.

Time-Resolved Fluorescence Measurements. In contrast to the steady-state technique, time-resolved fluorescence measurements can give unambiguous information about lipid order and dynamics (Heyn, 1979). Time courses of fluorescence intensity and depolarization of DPH in PC/PE/PS (10:5:1) and in DMPC vesicles were measured at 35 and 10 °C. The influence of P-450 and/or reductase on the dynamics and order of the lipid phase was investigated after reconstitution into these two vesicle systems. As an example, fluorescence decay curves of DPH in DMPC and DMPC containing P-450 are shown in Figure 2 (panel A). Panel B shows the corresponding anisotropy decay curves. The fluorescence signals were analyzed according to equations 2–5, using a least-squares algorithm with χ^2 to test the quality of the fits. Mean lifetime $\langle\tau\rangle$, relaxation time $\langle\psi\rangle$, order parameter S, and diffusion constant D obtained in the various systems are listed in Table I. The χ^2 values were all close to 1 (0.9–1.2), indicating the good quality of the fits.

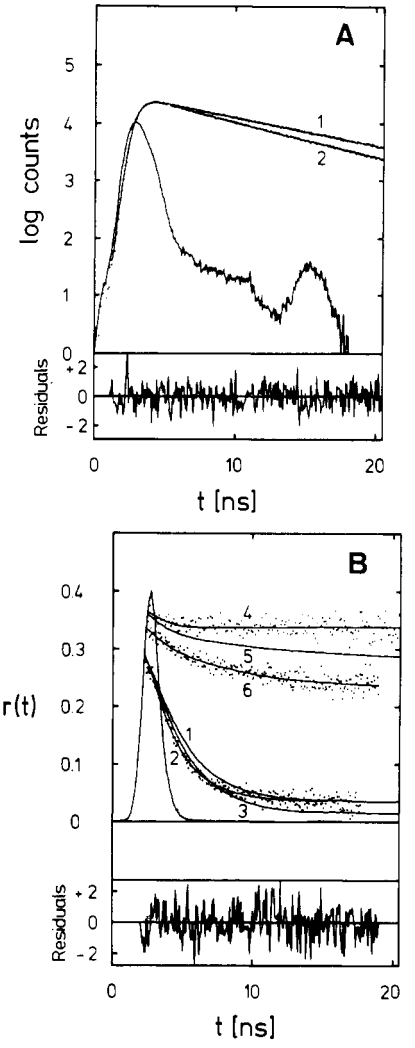

FIGURE 2: (Panel A) Lamp pulse (curve without number) and fluorescence decay curves of DPH, calculated from $I_{\parallel}(t) + 2I_{\perp}(t)$, for DMPC vesicles in the absence (curve 1) and the presence (curve 2) of cytochrome P-450, at 35 °C. (Bottom) Weighted residuals for curve 2. (Panel B) Experimental (···) and calculated (—) anisotropy decay curves: (1) DMPC + P-450 (1:1 w/w), 35 °C; (2) pure DMPC, 35 °C; (3) DMPC + reductase (1:0.65 w/w), 35 °C; (4) pure DMPC, 10 °C; (5) DMPC + reductase (1:0.65 w/w), 10 °C; (6) DMPC + P-450 (1:1 w/w), 10 °C. For clarity, the data points for curves 1, 3, and 5 have been omitted. (Bottom) Weighted residuals for curve 1.

In both vesicle systems either protein lowered the order parameter S, the only exception being P-450 in DMPC proteoliposomes above T_c, where S remained unchanged. The lifetime of DPH fluorescence, τ, was not markedly reduced by P-450 or reductase, indicating little energy transfer between DPH and the prosthetic groups of the proteins. The lifetime measurements were used to determine the position of the heme group in these model membranes. The average distance between the heme and the midplane of the bilayer was estimated to be ≥6.0 nm (see Discussion).

Table I: Fluorescence Anisotropy Decay Parameters of DPH in PC/PE/PS Vesicles Containing Cytochrome P-450 and/or NADPH-Cytochrome P-450 Reductase[a]

		10 °C				35 °C			
lipid	protein	⟨τ⟩ (ns)	⟨φ⟩ (ns)	S	D (ns^{-1})	⟨τ⟩ (ns)	⟨φ⟩ (ns)	S	D (ns^{-1})
PC/PE/PS		7.37 ± 0.06	1.1 ± 0.1	0.41 ± 0.01	0.13 ± 0.01	6.87 ± 0.07	0.48 ± 0.08	0.25 ± 0.01	0.33 ± 0.05
	P-450	6.32 ± 0.05	0.5 ± 0.1	0.33 ± 0.01	0.31 ± 0.06	6.0 ± 0.1	0.68 ± 0.08	0.18 ± 0.01	0.24 ± 0.03
	P-450/Red	6.52 ± 0.05	0.8 ± 0.1	0.32 ± 0.02	0.20 ± 0.04	6.0 ± 0.1	0.82 ± 0.08	0.15 ± 0.01	0.20 ± 0.02
	Red	6.69 ± 0.05	1.1 ± 0.1	0.25 ± 0.02	0.15 ± 0.04	6.3 ± 0.1	0.68 ± 0.08	0.0	0.24 ± 0.02
DMPC	*b*	11		0.9		8.0 ± 0.1	0.44 ± 0.06	0.29 ± 0.01	0.36 ± 0.07
	P-450	7.74 ± 0.08	0.4 ± 0.1	0.76 ± 0.01	0.19 ± 0.04	6.16 ± 0.08	0.55 ± 0.07	0.29 ± 0.01	0.28 ± 0.04
	P-450/Red	8 ± 1	0.6 ± 0.2	0.72 ± 0.01	0.14 ± 0.05	6.20 ± 0.05	0.63 ± 0.09	0.20 ± 0.01	0.26 ± 0.04
	Red	8.20 ± 0.05	0.9 ± 0.1	0.82 ± 0.02	0.06 ± 0.01	6.72 ± 0.06	0.58 ± 0.08	0.17 ± 0.01	0.28 ± 0.04

[a] The vesicles were identical to those used in steady-state anisotropy measurements. Red is reductase. [b] The parameters for pure DMPC vesicles at 35 and 10 °C are taken from M. Rehorek and M. P. Heyn (unpublished data) and Kinosita et al. (1981), respectively.

Table II: Hyperfine Splitting Constants, $2T_{\parallel}$ and $2T_{\perp}$, and Order Parameter S of 5-Doxyl-PC in DMPC Proteoliposomes and of 8-Doxyl-PC in PC/PE/PS Proteoliposomes

		10 °C			35 °C		
lipid	protein	$2T_{\parallel}$ (G)	$2T_{\perp}$ (G)	S	$2T_{\parallel}$ (G)	$2T_{\perp}$ (G)	S
DMPC		65.5	*a*	*a*	50.0	19.6	0.58
	P-450	59.5	16.8	0.78	47.5	20.8	0.51
	P-450/Red	58.5	17.0	0.76	47.8	21.3	0.54
	Red	60.3	16.5	0.79	48.5	20.3	0.50
PC/PE/PS		55.5	18.0	0.68	44.3	22.1	0.42
	P-450	54.5	18.0	0.68	41.3	22.3	0.37
	P-450/Red	49.7	17.8	0.71	42.8	22.0	0.40
	Red	54.8	17.8	0.68	42.5	20.5	0.41

[a] $2T_{\perp}$ and hence the order parameter S cannot be determined at low temperatures.

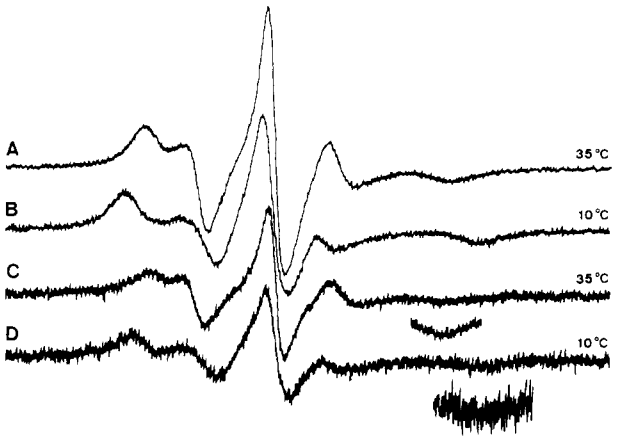

FIGURE 3: ESR spectra of 5-doxyl-PC incorporated into PC/PE/PS vesicles (curves A and B) and PC/PE/PS vesicles containing cytochrome P-450 (1:1 w/w) (curves C and D) in 20 mM HEPES, pH 7.4, containing 0.1 mM EDTA and 20% glycerol. The vesicle concentration was 10 mg of lipid/mL; the lipid:5-doxyl-PC molar ratio was 200:1.

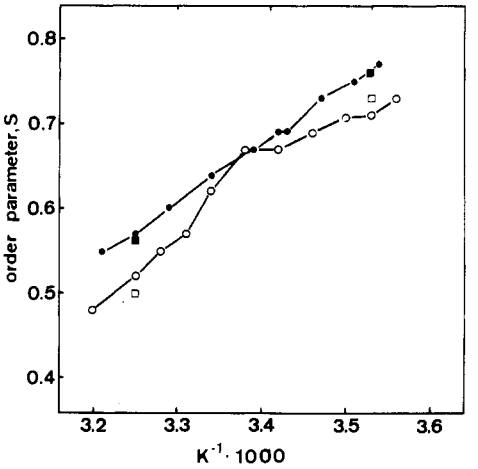

FIGURE 4: Order parameter S as a function of $1/T$ (K^{-1}) for 5-doxyl-PC incorporated into PC/PE/PS (10:5:1) vesicles (●) at 10 mg of lipid/mL, vesicles containing cytochrome P-450 (○), vesicles containing cytochrome P-450 plus reductase (□), and vesicles containing reductase (■). S was calculated according to eq 6 from spectra as shown in Figure 3.

ESR Experiments. To further substantiate the protein-induced disordering of the membrane lipids shown by the fluorescence measurements, ESR spectra of liposomes and proteoliposomes containing spin-labeled PC were recorded at different temperatures. In Figure 3, spectra of 5-doxyl-PC in PC/PE/PS and in PC/PE/PS containing P-450 are shown. Order parameters S calculated from such spectra together with order parameters derived from spectra of 5-doxyl-PC in reductase proteoliposomes are plotted against temperature in Figure 4. P-450 decreased the order parameter in accord with the information obtained by time-resolved fluorescence measurements. 8-Doxyl-PC, whose nitroxide moiety is more deeply imbedded in the membrane than that of 5-doxyl-PC, was also used. Table II shows the hyperfine splitting constants, $2T_{\parallel}$ and $2T_{\perp}$, of 8-doxyl-PC in PC/PE/PS and PC/PE/PS proteoliposomes, together with those of 5-doxyl-PC in DMPC and DMPC proteoliposomes. The dramatic mobilization of the

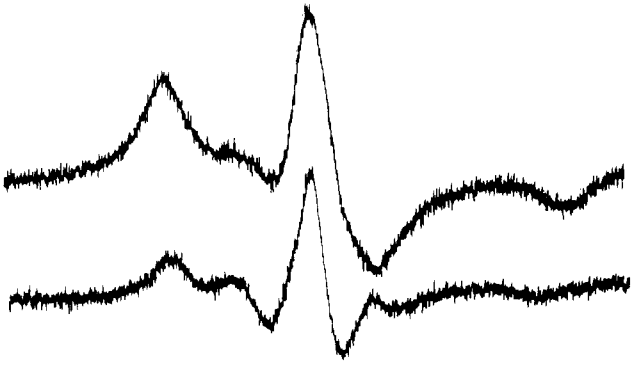

FIGURE 5: ESR spectra of 5-doxyl-PC incorporated into DMPC liposomes (upper spectrum) and into DMPC/cytochrome P-450 proteoliposomes (lower spectrum) both at 10 °C. Protein and lipid concentrations were as described in the legend to Figure 3.

acyl side chains by P-450 is clearly demonstrated below the phase transition of DMPC liposomes (Figure 5). At 10 °C the acyl side chains of the lipids in P-450-free liposomes are immobilized in agreement with the high r_s value of DPH under the same conditions (Figure 1, panel C). In the presence of P-450, the acyl chains are mobilized in a way that an order parameter, S, can be calculated directly from the ESR spectrum since the inner hyperfine splittings, $T_\perp$, are well resolved.

Discussion

Both fluorescence and ESR data conclusively show that P-450 and its reductase reconstituted into liposomes decrease the order of the phospholipid acyl side chains. The effect of P-450 is magnified by coreconstitution of reductase. The decrease is observed in PC/PE/PS liposomes resembling closely the phospholipid composition of the microsomal membrane and in DMPC vesicles above and below T_c. This sets aside the proteins of the microsomal monooxygenase system from other proteins studied by these techniques, e.g., bacteriorhodopsin, cytochrome oxidase, the sarcoplasmic reticulum (Ca^{2+},Mg^{2+})-ATPase, the M13 virus coat protein, and the glycoprotein of vesicular stomatitis virus. The first four proteins increase the order of their surrounding acyl chains, and they do so only above T_c, while the glycoprotein of vesicular stomatitis virus disorders the bilayer in the gel phase and orders it to a small extent in the liquid-crystalline phase. The disordering of surrounding acyl residues by P-450 and its reductase may reflect a rather uneven surface of the proteins' membrane-imbedded part.

NADPH-cytochrome P-450 reductase increases the disordering introduced by P-450 in PC/PE/PS liposomes. We have recently shown that P-450 forms self-aggregates in these vesicles when reconstituted in the absence of reductase. P-450 oligomers dissociate in the presence of equimolar amounts of reductase to form heterodimeric complexes of one reductase and one P-450 molecule (Gut et al., 1982, 1983). A larger surface area of P-450 molecules should therefore become available for interaction with phospholipids in this situation. This may account for the increased disorder of the acyl side chains in the presence of cytochrome and reductase compared to that found with the cytochrome alone.

P-450 is an integral protein of the endoplasmatic reticulum that is synthesized on membrane-bound polysomes and cotranslationally inserted into the membrane (Bar-Nun et al., 1980). Experiments using specific antibodies (Thomas et al., 1977; Matsuura et al., 1981) or proteases (Vlasuk et al., 1982) as probes indicate that P-450 is exposed on the cytoplasmic face of the endoplasmic reticulum and suggest that no immunological determinants within the polypeptide are exposed on the luminal side of the membrane (Matsuura et al., 1981). However, enhancement of the proteolytic susceptibility of P-450 in microsomes by detergent solubilization has been given as evidence for a transmembrane orientation (De Pierre & Dallner, 1975).

Recently, both protein and cDNA sequencing studies (Fujii-Kuriyama et al., 1982; Heinemann & Ozols, 1983; Tarr et al., 1983; Leighton et al., 1984) have allowed new speculations concerning the topology of P-450 in membranes and the location of the heme-binding site in the cytochrome. The main phenobarbital-induced form of rabbit liver microsomal P-450, termed P-450$_{LM2}$, is the first microsomal P-450 to be sequenced completely by protein chemical means (Heinemann & Ozols, 1983, Tarr et al., 1983). The protein has eight hydrophobic regions long enough to span the membrane. They are separated by charged amino acid residues. The COOH terminus contains a large hydrophilic domain (about 115 amino acid residues). This domain contains regions of homology with several other P-450 isozymes and has been proposed by Heinemann and Ozols to contribute the thiolate heme ligand by a cysteinyl residue (Cys-436). The model advanced by Coon's group (Tarr et al., 1983), however, predicts Cys-152, found within a long hydrophilic segment of α helix close to the NH_2 terminus (about 51 amino acid residues), to be the only cysteinyl residue available to function as the fifth ligand to the heme iron atom. Both putative heme-binding domains are hydrophilic, suggesting that they are not located in the membrane's lipid bilayer. Assuming a globular shape of these hydrophilic segments, their diameter can be estimated to be 38 and 29 Å, respectively.

The lifetime of DPH fluorescence in the present liposomes is decreased only very little by cytochrome or reductase and is independent of the specific heme content of P-450 between 8 and 14 nmol/mg. Appreciable energy transfer between the prosthetic groups of the proteins, i.e., heme or flavins, and DPH is therefore ruled out in contrast to the situation in liposomes containing bacteriorhodopsin and cytochrome oxidase for which pronounced energy transfer has been reported (see below). The absence of energy transfer between DPH and cytochrome P-450 allows a rough estimation of the location of the heme in the reconstituted cytochrome.

Fluorescence energy transfer from donors to acceptors, both randomly distributed within the plane of a bilayer, can be described by theories for energy transfer in two-dimensional systems (Wolber & Hudson, 1979; Koppel et al., 1979; Dewey & Hammes, 1980; Fung & Stryer, 1978; Estep & Thompson, 1979). In such systems, where the distances between donors and acceptors can cover an extended range, a large portion of acceptors may be located within R_0, the distance of 50% transfer efficiency. The number of acceptors that are located within an area, R_0^2, around a donor is determined by the concentration of acceptors and by the distance of closest approach, R_c, between the donor and the acceptors (Wolber & Hudson, 1979). The extent of energy transfer can be described by the relative quantum yield, the ratio of the areas under the normalized fluorescence decay curves of the donor in the presence and absence of receptors (Dewey & Hammes, 1980). The relative quantum yield is related to the transfer efficiency by $E = 1 - q_{rel}$. Strong energy transfer from DPH to the chromophore of a protein was observed in lipid vesicles containing bacteriorhodopsin (Rehorek et al., 1983) and cytochrome oxidase (Kinosita et al., 1981). The spectral overlap between the fluorescence emission spectrum of DPH and the absorption spectrum of cytochrome P-450 is large enough to expect strong energy transfer from DPH to the heme group of P-450.

The overlap integral, J, was calculated by numerical integration to be 1.1×10^{-13} cm^3 L mol^{-1}. For random orientations (orientation factor $K^2 = {}^2/_3$) of the two chromophores, this leads to a value for R_0 of 5.0 nm. A similar value for R_0 (5.1 nm) was calculated for DPH and heme a in DMPC vesicles containing cytochrome oxidase (Kinosita et al., 1981). In contrast to the cytochrome oxidase vesicles, where the energy transfer from DPH to heme a leads to a decrease of the average lifetime of DPH fluorescence by a factor of 4, the energy transfer in the P-450 vesicles is very small. The average lifetime of DPH fluorescence in DMPC vesicles at 35 °C decreases from 8.0 to 6.1 ns in the presence of P-450. This corresponds to an efficiency, E, of 0.24. As the protein radius of P-450, determined by rotational diffusion measurements (Gut et al., 1982, 1983), is ~2.0 nm and as the molar protein to lipid ratio is high (1:70, one to two layers of lipid around

one protein), the absence of strong energy transfer can only be explained by (i) a low, i.e., a nonrandom, orientation factor, K^2, or by (ii) a large vertical displacement of the heme group to the outside of the bilayer. The orientation factor for this system was calculated on the basis of eq 21 of Dale et al. (1979), for two different cases:

(1) DPH is located in the middle of the membrane, wobbling around the membrane normal, and the heme group is also located in the middle of the membrane but tilted by an angle of 55° from the normal (Gut et al., 1983). Such a topological arrangement of the two chromophores would be similar to that in bacterorhodopsin vesicles (Rehorek et al., 1981) and that in cytochrome oxidase vesicles (Kinosita et al., 1981).

(2) The same orientation as above but with the heme group vertically displaced within the protein by a distance d.

In case 1, K^2 can be calculated analytically as described in Rehorek et al. (1983). The low order parameter in our vesicles at 35 °C ($S = 0.29$, see Table I) and the 4-fold symmetry of the heme group (Kawato et al., 1981) lead to an orientation factor of 0.56, which corresponds to a R_0 of 4.8 nm. According to the theory of Wolber & Hudson (1979), one can calculate a distance of closest approach between donors and acceptors, R_c, both randomly distributed within the plane of the membrane. The lateral distribution function of the acceptors (P-450) and the donors (DPH) at such a high protein to lipid ratio (1:70) is unknown, since there might be some aggregates of cytochrome P-450 present (~35% Gut et al., 1982). But even if there are about 50% of the proteins aggregated, the concentration of acceptors within the energy transfer area R_0^2 is still high enough to expect strong energy transfer or a large R_c. For the high acceptor concentration in our vesicles and for the low transfer efficiency ($E = 0.24$), we calculate the distance of closest approach, R_c, to be >6.3 nm. As the sum of the protein radius ($R_{P\text{-}450} \sim 2.0$ nm) and the DPH radius ($R_{DPH} \sim 0.8$ nm) is clearly less than 6.3 nm, the low transfer efficiency can only be explained with a large vertical displacement of the heme group.

In case 2, K^2 is slightly dependent on the donor–acceptor distance. R_0 varies between 5.2 and 4.8 nm for all distances between P-450 and DPH from R_c to ∞. The theory of Wolber & Hudson (1979) is only valid for a R_0 value independent of the donor–acceptor distance, but as the variation of R_0 is only small, we apply this theory to our data and find for R_c a value of >6.6 nm (with $R_0 = 5.1$ nm). The distance of vertical displacement, d, can be calculated from R_c and $R_{P\text{-}450} + R_{DPH}$ to be >6.0 nm.

The large vertical displacement of the heme group indicated by the inefficient energy transfer therefore strongly supports the suggestions of Tarr et al. (1983) and Heinemann & Ozols (1983) that one of the large hydrophilic segments of P-450 contains the cytochrome's heme group.

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Determination of the Topography of Cytochrome b_5 in Lipid Vesicles by Fluorescence Quenching†

Tom Markello, Adam Zlotnick,‡ James Everett,§ Joan Tennyson, and Peter W. Holloway*
Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908
Received November 13, 1984

ABSTRACT: Cytochrome b_5, a protein isolated from the endoplasmic reticulum by detergent extraction, interacts spontaneously with small unilamellar phosphatidylcholine vesicles. When the vesicles are made from 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), the tryptophan fluorescence of the cytochrome is enhanced, and when they are made from 1-palmitoyl-2-(dibromostearoyl)phosphatidylcholine (BRPC), the fluorescence is quenched. A series of BRPC were synthesized with bromine atoms at the 6,7, 9,10, 11,12, or 15,16 positions. The vesicles synthesized from each of these lipids were similar in size to those made from POPC. The relative fluorescence intensities of the cytochrome b_5 in POPC and 6,7-, 9,10-, 11,12- and 15,16-BRPC were 100, 19.4, 29.4, 37.1, and 54.0, respectively. These data suggest that the exposed tryptophan(s) is (are) at a depth of 0.7 nm below the surface of the vesicle. Bromine is a collisional quencher; hence, these data may indicate the relative position of the lipid annulus around the protein rather than the depth of the protein below the average vesicle surface. Cytochrome b_5 contains three potentially fluorescent tryptophans, and determinations of fluorescent quantum yield indicate all three are fluorescent with an average quantum yield, when in POPC vesicles, of 0.21. Fluorescence lifetime measurements by the demodulation technique indicated heterogeneity of fluorescence lifetimes in all vesicles. The lifetimes in the BRPC vesicles ranged from 2.0 to 2.4 ns compared to a value of 3.3 ns in POPC. Quenching of fluorescence in vesicles composed of mixtures of POPC and a BRPC indicated that the quenchable tryptophan(s) was (were) well shielded from the bromo lipid with perhaps only a 75° angle of approach. This study suggests that specifically brominated lipid can be used to determine the depth and exposure of tryptophans in membrane binding domains of proteins.

Cytochrome b_5, a membrane protein found in the endoplasmic reticulum, has long been known to be involved in the biosynthesis of unsaturated fatty acids (Holloway, 1983), and it has also been shown to be involved in other aspects of lipid metabolism (Paultauf et al., 1974; Nagai et al., 1983; Grinstead & Gaylor, 1982; Vatsis et al., 1982) and in the metabolism of xenobiotics (Brunstrom & Ingelman-Sundberg, 1980; Waxman & Walsh, 1983). In spite of these important roles, the structure of cytochrome b_5 in the membrane is still the subject of some controversy.

Using photoactivatable phospholipids, Takagaki et al. (1983a,b) showed the orientation of the hydrophobic domain depends upon the reconstitution conditions. The hydrophobic domain can either span the bilayer (in the "nontransferable form") or fold back the carboxyl terminus to the external surface (in the "transferable" form). Enoch et al. (1979)) also made these distinctions, but their more recent reports have suggested that both forms had the carboxyl terminus on the external surface (Dailey & Strittmatter, 1981).

The membrane binding domain of cytochrome b_5 contains the amino acid sequence Pro-Ser-Trp-Trp-Thr-Asn-Trp-Leu (Fleming et al., 1978), and tryptophan fluorescence has been used to explore topology. Fleming et al. (1979) used fluorescence energy transfer from tryptophan residue(s) in the hydrophobic domain to trinitrophenyl and to dansyl groups coupled onto the vesicle surface. The fluorescent tryptophan(s)

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‡ Present address: Du Pont, Central Research and Development, Experimental Station, Wilmington, DE 19898.
§ Present address: Department of Biochemistry, North Carolina Central University, Durham, NC 27707.

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