Membrane Charge as Effector of Cytochrome P-450$_{LM_2}$ Catalyzed Reactions in Reconstituted Liposomes†

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ABSTRACT: The phospholipid specificity of rabbit liver microsomal cytochrome P-450$_{LM_2}$ catalyzed hydroxylation reactions was examined in reconstituted phospholipid vesicles. An apparent linear relationship between the negative charge of the vesicles and the rate of P-450$_{LM_2}$-catalyzed O-dealkylation of *p*-nitroanisole or 7-ethoxycoumarin was obtained. The membrane charge-mediated increase in hydroxylation activities was found not to be due to (i) an altered lipid/water partition coefficient of the substrate, (ii) a change in the apparent Michaelis constant of P-450$_{LM_2}$ for the substrate, (iii) a different activation energy of the O-demethylation of *p*-nitroanisole, (iv) different spin states of P-450$_{LM_2}$ or (v) an altered secondary structure of this enzyme as monitored by circular dichroism. However, when the formation of the ferrous carbonyl complex of P-450$_{LM_2}$ was followed under aerobic or anaerobic conditions after the addition of NADPH to the vesicles, an increased negative charge of the membrane was accompanied by an increased reducibility of P-450$_{LM_2}$. A similar linear relationship between the reducibility of cytochrome b_5 and the negative charge of the liposomes was also evident in membranes containing NADPH–cytochrome P-450 reductase and cytochrome b_5. It is proposed that the interaction of the reductase with P-450$_{LM_2}$ is inefficient in neutral vesicles and thus rate determining for the overall hydroxylation activities.

The different membrane-bound monooxygenase systems present in the endoplasmic reticulum in liver, which are involved in inactivation and bioactivation of endogenous hormones, drugs, insecticides, and precarcinogens (Gunsalus et al., 1975; DePierre & Ernster, 1978; Coon et al., 1976; Ingelman-Sundberg, 1980), were only recently resolved and the components purified to the extent that detailed kinetic and structure–function relationships could be investigated (Haugen & Coon, 1976; Guengerich, 1978; Thomas et al., 1979; Oprian et al., 1979). Most of the initial studies along these lines were carried out under conditions where the native membrane was missing. Consequently, it cannot be excluded that the properties of the individual monooxygenase components, i.e., the NADPH–cytochrome P-450 reductase and the cytochrome P-450, as well as the recombined functional monooxygenase system, did not reflect those of the corresponding system in the intact membrane. In this context, there are several parameters of the lipid bilayer that may influence a membrane protein partially or fully embedded in the membrane. In general, these include the phospholipid composition which in the case of β-hydroxybutyrate dehydrogenase was found to involve a strict requirement for phosphatidylcholine (Grover et al., 1975; Gazzotti et al., 1975). Another important parameter appears to be the surface charge of the membrane, as shown with the oligomycin-sensitive mitochondrial ATPase (Cunningham & Sinthusek, 1979). In addition, reconstitution in liposomes (Racker et al., 1977) of a number of ion-transporting membrane proteins, e.g., mitochondrial ATPase (Kagawa & Racker, 1971; Serrano et al., 1976), cytochrome oxidase (Racker & Kandrach, 1973; Eytan & Racker, 1977), mitochondrial nicotinamide nucleotide transhydrogenase (Rydström, 1979), Ca^{2+}-ATPase from sarcoplasmic reticulum (Racker, 1972; Warren et al., 1974), and Na^+/K^+-ATPase from electric eel (Goldin & Tong, 1974), has revealed that these proteins also depend on an intact bilayer lipid membrane for maximal activity and function [cf. also Eytan et al. (1975) and Eytan & Kanner (1978)].

The importance of phospholipids for the function of purified monooxygenases was recognized early (Lu et al., 1969; cf. Lu & West, 1978). In these cases, it was likely that the nonmembranous phospholipid used acted partially as a dispersing agent that prevented the protein components from aggregation. A similar dispersing effect was shown to be exerted by Triton X-100 and lysolecithin on purified 11β-hydroxylase from adrenal cortex mitochondria (Ingelman-Sundberg et al., 1978).

In previous papers (Ingleman-Sundberg & Glaumann, 1977, 1980; Ingelman-Sundberg et al., 1979), we have described the properties of different types of reconstituted vesicles containing rabbit liver NADPH–cytochrome P-450 reductase and various forms of cytochrome P-450 in comparison with nonmembranous types of reconstituted systems. Incorporation of the proteins into phosphatidylcholine vesicles led to an inhibition of cytochrome P-450$_{LM_2}$[1] dependent activities and an altered substrate specificity of P-450$_{LM_3}$. Since it was possible to overcome this inhibition of P-450$_{LM_2}$-catalyzed reactions by replacing phosphatidylcholine with microsomal phospholipids (Ingelman-Sundberg & Johansson, 1980b), it appeared that certain acidic phospholipids were of importance for the reconstitution.

In the present paper, we present data indicating that in negatively charged membranes the rate of electron transfer from NADPH to P-450$_{LM_2}$ is about 5 times that in neutral membranes, i.e., in phosphatidylcholine vesicles. Apparently, this may be due to an increased interaction between the protein components of the vesicles.

Materials and Methods

Dioleoylphosphatidylcholine (DOPC, synthetic), dioleoylphosphatidylethanolamine (DOPE, synthetic), and phospha-

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[1] Abbreviations used: P-450$_{LM}$, liver microsomal cytochrome P-450; P-450$_{LM_2}$, P-450$_{LM_3}$, and P-450$_{LM_4}$, forms of P-450$_{LM}$ designated according to their electrophoretic properties; DOPC, dioleoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; PS, phosphatidylserine; CL, cardiolipin; cmc, critical micelle concentration; Tris, tris(hydroxymethyl)aminomethane.

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tidylserine (PS, beef brain) were obtained from Serdary Research Laboratories (Ontario, Canada) and stored for not more than 1 month in sealed tubes under nitrogen at −20 °C. Egg yolk phosphatidylcholine (EYPC, type III) and egg yolk phosphatidylethanolamine (EYPE, type III) were purchased from Sigma and stored as described for DOPC. Microsomal phospholipids were prepared according to Bligh & Dyer (1959) and stored as described above. Dilauroylphosphatidylcholine (DLPC) was obtained from Sigma and stored at −20 °C. [^{14}C]Phosphatidylcholine was purchased from NEN Biochemicals (Dreieichenham, West Germany).

Electrophoretically homogeneous preparations of cytochrome P-450$_{LM_2}$ and NADPH–cytochrome P-450 reductase were prepared from liver microsomes obtained from phenobarbital-treated rabbits as previously described (Ingelman-Sundberg et al., 1979; Ingelman-Sundberg & Glaumann, 1980). The purification methods were similar to those of Haugen & Coon (1976) and Yasukochi & Masters (1976), respectively; the specific contents were 12.5–16.5 nmol of P-450/mg of protein and 12.5 nmol of flavin/mg of protein. Cytochrome b_5 was purified as described elsewhere (Ingelman-Sundberg & Johansson, 1980a). Phospholipid vesicles, prepared by the cholate gel filtration technique (Ingelman-Sundberg & Glaumann, 1977; Halpert et al., 1979), were used within 3 h after preparation. Special attention was taken to work under a nitrogen atmosphere during the initial steps in the preparation. The properties of vesicles prepared from either phosphatidylcholine or microsomal phospholipids have previously been described (Ingelman-Sundberg & Glaumann, 1980; Ingelman-Sundberg & Johansson, 1980b). Distribution of 7-ethoxycoumarin between buffer and liposomes was carried out by preparing DOPC and DOPE/PS (3:1 w/w) vesicles containing P-450$_{LM_2}$, NADPH–cytochrome P-450 reductase, and phospholipid in a molar ratio of 1:1:800. These were then incubated with 50 μM 7-ethoxycoumarin at 37 °C for 10 min. The solutions were subsequently transferred into centrifuge tubes and centrifuged at 200000g for 1 h at 4 °C. The pellet, containing all protein and phospholipid, was collected and solubilized in 1% sodium cholate in 50 mM potassium phosphate buffer, pH 7.4. The concentration of 7-ethoxycoumarin was subsequently determined spectrofluorometrically in the supernatant and in the solubilized pellet. Protein was determined according to Lowry et al. (1951), and cytochrome P-450 was assayed according to Omura & Sato (1964) by using 91 mM^{-1} cm^{-1} as absorption coefficient for the absorbance difference between 450 and 490 nm. Flavin in NADPH–cytochrome P-450 reductase was quantitated by the absorption at 456 nm by using the absorption coefficient 10.7 mM^{-1} cm^{-1} (Iyanagi & Mason, 1973).

Results

Vesicles containing cytochrome P-450$_{LM_2}$, NADPH-cytochrome P-450 reductase, and DOPE or DOPE/PS (3:1 w/w) in a molar ratio of 2:1:800 were subjected to chromatography on precoated Sepharose 4B columns and to electron microscopy. With 200000 dpm of [^{14}C]phosphatidylcholine included in the vesicles, all cytochrome P-450 and NADPH-cytochrome P-450 reductase chromatographed in the void volume together with the radioactivity (not shown in figure), indicating that complete incorporation of the proteins into the vesicles had been achieved. Electron microscopy of the preparations (not shown) revealed unilamellar vesicular structures; DOPE vesicles were heterogeneous in size (35–500 nm in diameter) whereas vesicles prepared from DOPE/PS (3:1 w/w) somewhat irregular in shapes but more homogeneous in size (35–100 nm in diameter).

Table I: Cytochrome P-450$_{LM_2}$ Dependent O-Demethylation of *p*-Nitroanisole, O-Deethylation of 7-Ethoxycoumarin, and NADPH Oxidase Activities in the Presence or Absence of 7-Ethoxycoumarin in Phospholipid Vesicles of Various Compositions[a]

	O-dealkylation act. [nmol (nmol of LM$_2$)$^{-1}$ min^{-1}]		NADPH oxidase act. [nmol (nmol of LM$_2$)$^{-1}$ min^{-1}]	
vesicle composition	7-ethoxy-coumarin	*p*-nitro-anisole	+sub-strate	−sub-strate
MIC PL[d]	0.40	1.39	13.4	27.2
DOPC	0.11	0.24	10.4	9.5
DOPE	0.47	0.89	28.1	31.8
DOPE/PS (3:1)	0.87	1.06	34.1	37.5
DLPC[b]	0.46	1.11	30.2	36.3
EYPC/EYPE (2:1)	c	0.43	c	c

[a] The vesicles contained NADPH–cytochrome P-450 reductase, cytochrome P-450$_{LM_2}$, and phospholipid in a molar ratio of 1:3:800. The incubations were performed at 37 °C. NADP$^+$ was determined spectrophotometrically at 340 nm, 7-hydroxycoumarin spectrofluorometrically as described by Prough et al. (1978), and *p*-nitrophenol spectrophotometrically at 417 nm (Netter & Seidel, 1964). The activities are expressed as mean values from two to four different experiments. [b] This is apparently not a vesicular system; see text. [c] Not determined. [d] Microsomal phospholipids.

Catalytic Properties of Different Types of Vesicles. Cytochrome P-450$_{LM_2}$ and NADPH-cytochrome P-450 reductase were incorporated into vesicles prepared from various phospholipids in a molar ratio of 3:1:800, respectively. The activities for O-demethylation to *p*-nitroanisole, O-deethylation of 7-ethoxycoumarin, and NADPH oxidase in the presence or absence of substrate were determined. As seen from Table I, reconstitution of the P-450$_{LM_2}$-dependent O-demethylation of *p*-nitroanisole was markedly dependent on the lipid composition of the vesicles. Vesicles prepared from phosphatidylcholine (DOPC or EYPC) showed a relatively low activity whereas a 5-fold increased rate of O-demethylation was obtained with vesicles prepared from microsomal phospholipids. When DOPC was diluted with either dioleoylphosphatidylethanolamine or phosphatidylserine, vesicles more efficient in catalyzing the O-demethylation were obtained. DOPE/PS (3:1 w/w) was the most efficient phospholipid mixture for reconstitution of the reaction in the vesicles; this activity was similar to those obtained with microsomal lipids or the nonmembranous DLPC-reconstituted system.

Principally similar results were obtained with 7-ethoxycoumarin as substrate for deethylation. The rate of 7-hydroxycoumarin formation was slow in DOPC vesicles but fast in DOPE/PS (3:1 w/w) vesicles. Vesicles prepared from either DOPE or microsomal phospholipids and the nonmembranous DLPC-reconstituted system showed intermediate rates of deethylation. Interestingly, when the rate of NADPH oxidation was determined in vesicular preparations in the presence or absence of substrate, the rates of NADPH oxidation essentially paralleled those of deethylation, independently of the type of vesicle preparation employed. This indicates that, in the neutral phosphatidylcholine vesicles, an inefficient transfer of electrons from NADPH to cytochrome P-450$_{LM_2}$ may explain the diminished catalytic activities.

Catalytic Activity vs. the Charge of the Vesicles. The cytochrome P-450$_{LM_2}$ catalyzed O-dealkylation of *p*-nitroanisole and 7-ethoxycoumarin was examined in vesicles prepared from mixtures of DOPC and DOPE. As shown in Figure 1, a linear relationship between the amount of DOPE in the vesicles and the rate of O-dealkylation of both substrates was obtained. These results indicate that the rate of the

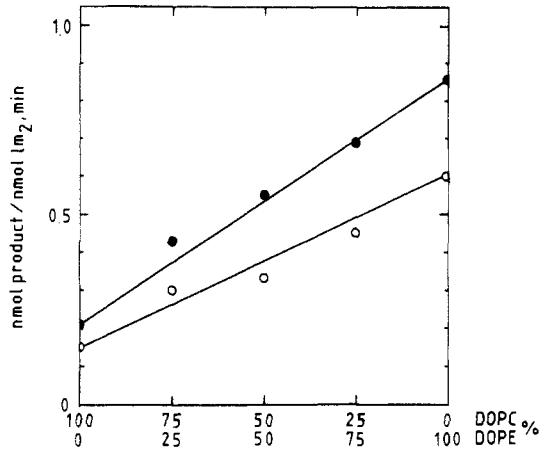

FIGURE 1: Cytochrome P-450_{LM_2} catalyzed O-demethylation of *p*-nitroanisole and O-deethylation of 7-ethoxycoumarin as a function of the relations between DOPC and DOPE in the liposomal membrane. Conditions as described in legend to Table I. (●) O-Demethylation; (○) O-deethylation.

cytochrome P-450_{LM_2} catalyzed reactions in vesicles is strongly dependent on the phospholipid composition. Also, since all active vesicles so far tested contained negative phospholipids, it is apparent that the charge of the phospholipids is essential. Attempts were made to prepare vesicles containing only the proteins and either of the negatively charged phospholipids cardiolipin, phosphatidylserine, or phosphatidylinositol. However, during the preparation, a rapid and irreversible denaturation of cytochrome P-450 occurred; NADPH-cytochrome P-450 reductase remained active. Therefore, negatively charged phospholipids could only be studied in mixtures with neutral PC or slightly negatively charged PE.

Cunningham & Sinthusek (1979) have recently shown that the activity of the oligomycin-sensitive mitochondrial ATPase is directly proportional to the negative charge of the liposomes in which the enzyme was reconstituted. It was therefore of interest to evaluate a possibly similar relationship in the case of cytochrome P-450_{LM_2} catalyzed reactions. As shown in Figure 2, this was indeed the case when the rate of P-450_{LM_2}-catalyzed O-demethylation of *p*-nitroanisole obtained with vesicles containing DOPC and PS or CL was plotted against the calculated electrophoretic mobilities of the various phospholipid vesicles. A similar charge–activity dependence was also found for the O-deethylation of 7-ethoxycoumarin (not shown).

Interactions between 7-Ethoxycoumarin and Liposomes. The altered catalytic properties of the various types of vesicles could apparently be explained by different types of interactions between the substrates and the vesicles due to the charge properties, etc., of the latter. Attempts were therefore made to determine the partition coefficient of 7-ethoxycoumarin between DOPC and DOPE/PS (3:1) liposomes and the buffer (cf. Materials and Methods). Calculations revealed a partition coefficient for 7-ethoxycoumarin between the organic and aqueous phases of 680 in the case of DOPC vesicles and of 820 in the case of DOPE/PS vesicles, i.e., not significantly different.

Experiments were also undertaken to evaluate whether the binding properties of P-450_{LM_2} for 7-ethoxycoumarin were altered upon incorporation into more negatively charged vesicles. The NADPH-dependent formation of 7-hydroxycoumarin was determined at substrate concentrations between 10 and 100 μM (Figure 3). Incorporation of NADPH–cytochrome P-450 reductase and P-450_{LM_2} into DOPE/PS (3:1 w/w) vesicles instead of DOPC vesicles was found to affect

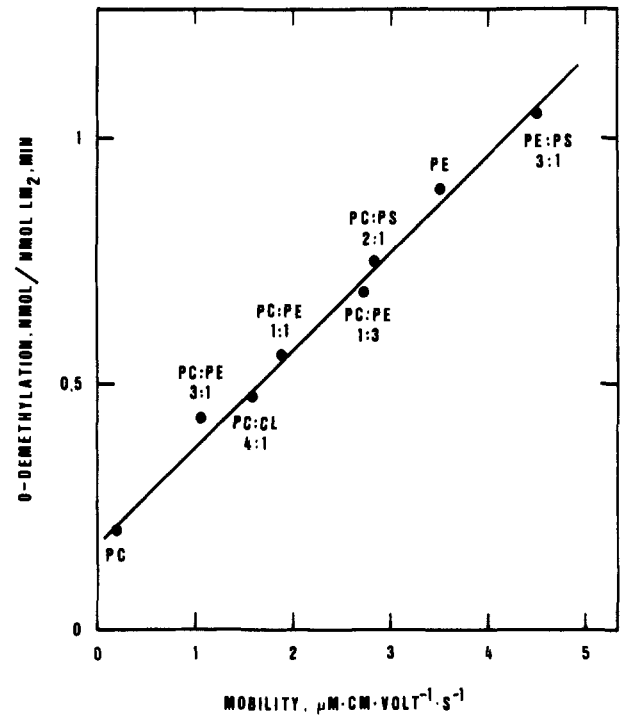

FIGURE 2: Activity of cytochrome P-450_{LM_2} catalyzed O-demethylation of *p*-nitroanisole as a function of the electrophoretic mobility of the reconstituted phospholipid vesicles. Electrophoretic data were taken from Cunningham & Sinthusek (1979). Mobilities of mixed vesicles were calculated proportionally. PS was assumed to have the same electrophoretic mobility as CL. Other conditions are as described in legend to Table I.

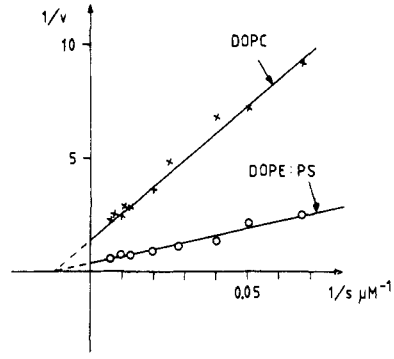

FIGURE 3: Lineweaver–Burk plot of cytochrome P-450_{LM_2} catalyzed O-deethylation of 7-ethoxycoumarin in DOPC vesicles and in mixed DOPE/PS vesicles. v = nanomoles of product per nanomole of P-450_{LM_2} per minute. Conditions are as described in legend to Table I. (×) DOPC vesicles; (○) DOPE/PS (3:1 w/w); s = 7-ethoxycoumarin.

only the V_{max} of the O-deethylation of 7-ethoxycoumarin; the K_m value was constant and determined to be 85 μM, indicating that the substrate binding properties of the enzyme were similar in the two types of vesicles.

Principally similar results were obtained when the rate of iodosobenzene diacetate supported (Gustafsson et al., 1978) O-demethylation of *p*-nitroanisole was examined in different types of vesicles. Liposomes having a molar ratio of P-450_{LM_2}/P-450 reductase/phospholipid of 1:1:800, corresponding to 0.1 nmol of P-450_{LM_2}, were incubated at 37 °C with 8.3 mM iodosobenzene diacetate and 1 mM *p*-nitroanisole. The rate of *p*-nitrophenol formation during the linear phase of the reaction (30 s) was 9.8 nmol/(nmol of LM_2)$^{-1}$ min^{-1} in DOPC vesicles whereas the corresponding values with DOPE and DOPE/PS (3:1 w/w) vesicles were 11.6 nmol and 12.2 (nmol of LM_2)$^{-1}$ min^{-1}, respectively. Thus, no significant

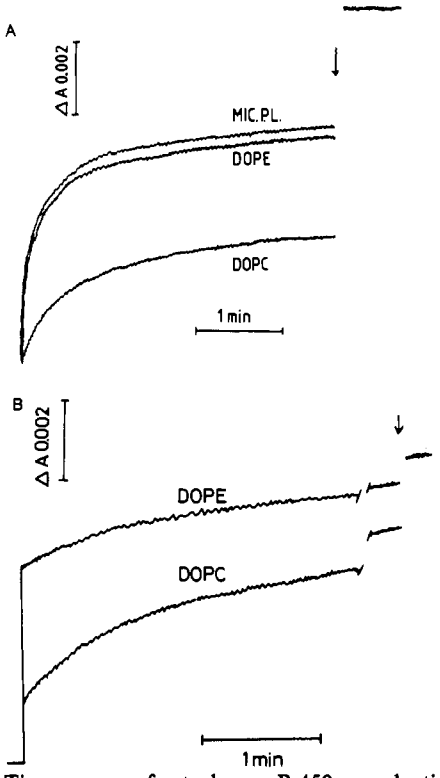

FIGURE 4: Time courses of cytochrome P-450$_{LM_2}$ reduction at 5 °C in vesicles prepared from DOPC, DOPE, or microsomal phospholipids as followed by the formation of the ferrous carbonyl complex at 450 nm. Vesicles corresponding to 0.45 nmol of P-450, having a P-450 reductase/P-450$_{LM_2}$ phospholipid ratio of 1:2:800, were diluted in 50 mM potassium phosphate buffer, pH 7.4, to 1.5 mL, before addition of a 1.5-mL solution of NADPH (100 μM) under aerobic (A) or anaerobic (B) conditions. Arrow indicates the addition of a few grains of sodium dithionite. Anaerobic conditions were conducted by using a 3-mL cuvette having two outlets and a silicon rubber membrane through which an anerobic NADPH solution was injected. The solutions were made anaerobic by alternate evacuation and equilibration with nitrogen or carbon monoxide purified by passage through two flasks of Fiesers solution.

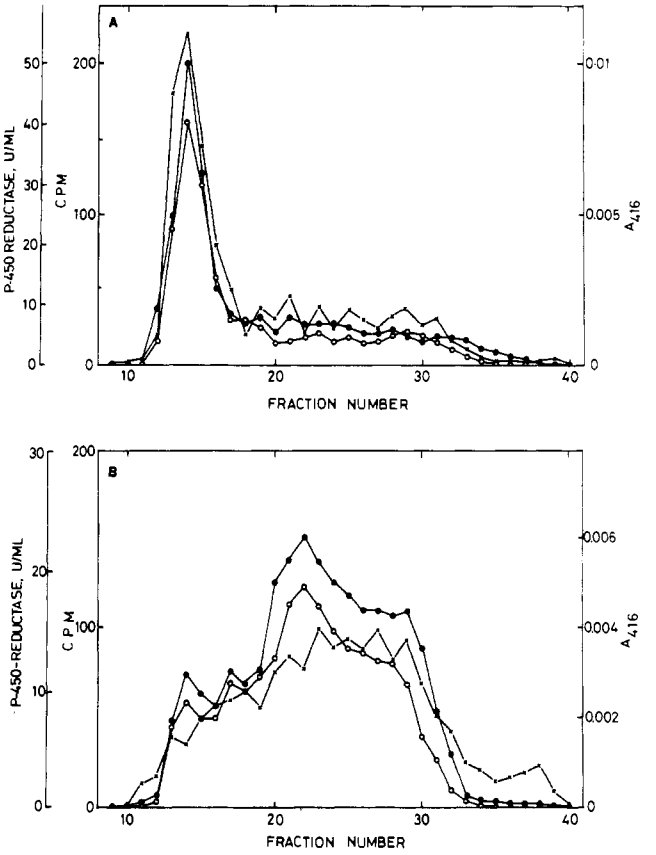

FIGURE 5: Sepharose 2B chromatography of phospholipid vesicles prepared from NADPH–cytochrome P-450 reductase, cytochrome P-450$_{LM_2}$, and either DOPE (A) or DOPC (B). The vesicles were prepared by the cholate gel filtration technique from 5 mg of phospholipids containing 100 000 cpm [^{14}C]phosphatidylcholine, 5 nmol of cytochrome P-450$_{LM_2}$, and 2.5 nmol of P-450 reductase. The vesicle solution was applied onto a precoated Sepharose 2B column (1.3 × 30 cm) equilibrated in 50 mM potassium phosphate buffer, pH 7.4. Elution was performed by using the phosphate buffer at a rate of 6 mL/h. Three-milliliter fractions were collected. (×) [^{14}C]Phosphatidylcholine; (○) cytochrome P-450$_{LM_2}$ as detected by the absorption spectra; (●) NADPH–cytochrome P-450 reductase (U/mL).

differences were seen, possibly indicating that the catalytic differences between the various types of vesicles involve the rate of electron transfer to cytochrome P-450$_{LM_2}$.

Determination of Activation Energies. DOPC and DOPE vesicles containing NADPH–cytochrome P-450 reductase and P-450$_{LM_2}$ were incubated with *p*-nitroanisole at eight different temperatures between 20 and 37 °C. The activation energies were calculated from the Arrhenius plots and were found not to be significantly different between the two types of vesicles, i.e., 54 and 56 kJ/mol, respectively.

Reduction Kinetics. The results presented so far indicate that the differences between the various types of vesicles with respect to catalytic properties are associated with the rate of electron transfer from NADPH to cytochrome P-450$_{LM_2}$. It was therefore of interest to elucidate the reduction kinetics in the various types of vesicles by following the formation of the ferrous carbonyl complex of P-450$_{LM_2}$ upon addition of NADPH. In experiments performed aerobically (Figure 4A), the rate of P-450$_{LM_2}$ reduction in DOPC vesicles was much lower than that in DOPE vesicles or in liposomes prepared from microsomal phospholipids. The steady-state level of the ferrous carbonyl complex, which is an indication of the balance between the rate of reduction and the rate of autooxidation of P-450, was only 25% of the maximal level (after addition of sodium dithionite) in DOPC vesicles but 68% in DOPE vesicles or in vesicles prepared from microsomal phospholipids. Reduction kinetics performed under anaerobic conditions (Figure 4B) revealed an inefficient electron transfer from NADPH to P-450$_{LM_2}$ in DOPC vesicles but, in contrast, a very fast reduction of P-450$_{LM_2}$ present in the DOPE vesicles; 75% of the P-450$_{LM_2}$ present in the DOPC vesicles and 85% of the P-450$_{LM_2}$ present in the other types of vesicles were reducible by NADPH. The reduction kinetics obtained correlates well with the catalytic results presented above; i.e., P-450$_{LM_2}$ is a 5 times more efficient catalyst in vesicles containing either DOPE or microsomal phospholipids than in DOPC vesicles.

Distribution of Cytochrome P-450$_{LM_2}$ and NADPH–Cytochrome P-450 Reductase in the Vesicles. The inefficient reduction of cytochrome P-450$_{LM_2}$ in the neutral phosphatidylcholine vesicles cannot be explained by an inside-out orientation of P-450$_{LM_2}$ in the DOPC membrane, compared to P-450 reductase, since at least 75% of the dithionite-reducible P-450 was reduced by NADPH. However, it is possible that the proteins were nonhomogeneously distributed among the DOPC vesicles, thereby explaining the slow reduction rate of P-450 in these vesicles. [^{14}C]Phosphatidylcholine-labeled DOPC and DOPE vesicles containing cytochrome P-450$_{LM_2}$, NADPH–cytochrome P-450 reductase, and phospholipid in a molar ratio of 2:1:800 were therefore prepared and fractionated on precoated Sepharose 2B columns. As seen from Figure 5, cytochrome P-450$_{LM_2}$ and NADPH–cytochrome P-450 reductase were essentially equally distributed between the DOPC vesicles of different sizes; calculations revealed that each vesicle could contain at least five reductase molecules and

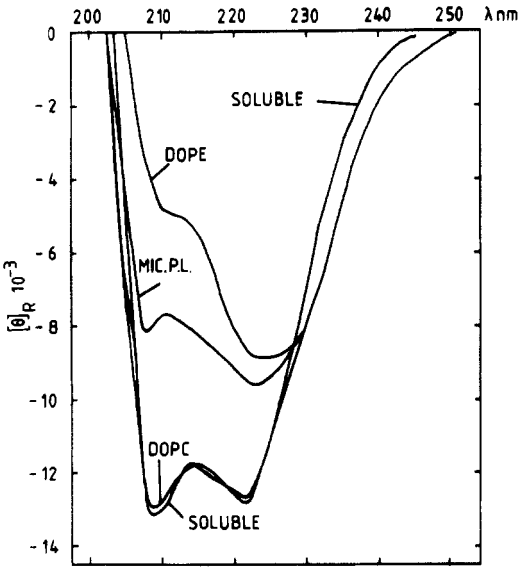

FIGURE 6: Circular dichroism spectra of 2 μM cytochrome P-450$_{LM_2}$ in 50 μM phosphate buffer, pH 7.4, containing 20% glycerol (soluble) and in vesicles prepared from DOPC, DOPE, or microsomal phospholipids. The CD spectra were recorded at 22 °C by using a Model J 41A spectropolarimeter (Japan Spectroscopic Co.) with a path length of 0.1 cm. A mean amino acid residue weight of 113 was used in the computation of mean residue ellipticites $[\theta]_R$. Base lines were determined by using buffer or vesicles devoid of the protein.

eight P-450$_{LM_2}$ molecules. Chromatography of the DOPE vesicles on Sepharose 2B revealed a concomitant elution of P-450, P-450 reductase, and phospholipid from the column (Figure 5).

Circular Dichroism Spectra. The markedly altered properties of cytochrome P-450$_{LM_2}$ when reconstituted in various types of vesicles raised the question whether this was accompanied by a conformational change of the protein to a more reducible and catalytically active species. Liposomes were prepared from P-450$_{LM_2}$ and DOPC, DOPE, or microsomal phospholipids in a molar ratio of phospholipid to P-450$_{LM_2}$ of 800:1 and subjected to spectropolarimetry. In each case, a base line was recorded with the cuvette filled with vesicles devoid of P-450$_{LM_2}$. The circular dichroism spectra in the UV region of P-450$_{LM_2}$ were identical whether the protein was embedded in DOPC vesicles or was present in a soluble state, i.e., was suspended in 50 mM phosphate buffer (pH 7.4) containing 20% glycerol (Figure 6). Calculations according to Greenfield & Fasman (1969) revealed a content of α-helical structure of approximately 33%.

Apparently different circular dichroism spectra were obtained with P-450$_{LM_2}$ incorporated into DOPE or microsomal phospholipids. The bands were remarkedly red shifted and much less intense. However, these samples were, in contrast to the DOPC vesicles and the soluble preparations, quite turbid. Correction for light scattering according to Urry (1972) revealed no significant differences in the circular dichroism spectra produced by cytochrome P-450$_{LM_2}$ in these vesicles compared to the spectra obtained by using the DOPC vesicles.

Apparent Spin State of Membrane-Bound and Soluble Cytochrome P-450$_{LM_2}$. The functional properties of cytochrome P-450 have been correlated to the spin state of the enzyme (cf. White & Coon, 1980), the high-spin form being more easily reduced than the low-spin form. It was therefore considered of importance to evaluate whether the different catalytic and reductive properties of the enzyme in negatively charged compared to neutral vesicles were reflected in different spin states of the enzyme in the various systems. Cytochrome

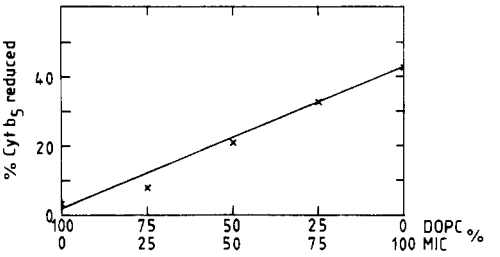

FIGURE 7: Reduction of cytochrome b_5 by NADPH–cytochrome P-450 reductase in vesicles prepared from various proportions between egg yolk phosphatidylcholine and microsomal phospholipids. Vesicles were prepared from cytochrome b_5, NADPH–cytochrome P-450 reductase, and phospholipid in a molar ratio of 8:1:800 by the cholate gel filtration technique. NADPH (100 nmol) was added to vesicles corresponding to 0.4 nmol of cytochrome b_5, and the absorption difference at 423-460 nm was registrated at 22 °C. After 5 min, solid dithionite was added, and the percentage reduction compared to the dithionite-reduced hemoprotein was calculated in the different cases.

P-450$_{LM_2}$ was exclusively in the low-spin form when in the soluble form whereas incorporation of the enzyme into either phosphatidylcholine vesicles or into vesicles prepared from microsomal phospholipids resulted in transformation of a fraction of the enzyme into the high-spin state, as revealed from the absorption at 648 nm of the protein. Thus, the impaired reducibility of P-450$_{LM_2}$ neutral vesicles does not seem to be correlated with the spin state of the enzyme.

Reduction of Cytochrome b_5 in Negatively Charged Vesicles. The results presented above indicate that reduction of cytochrome P-450$_{LM_2}$ in neutral vesicles is inefficient, probably due to hampered interactions between NADPH–cytochrome P-450 reductase and the cytochrome. Since the reductase also reduces the cytochrome b_5 in the microsomal membrane, at even higher rates as compared to cytochrome P-450 (Enoch & Strittmatter, 1979), it was of interest to see whether the same relationship between membrane composition and the reduction efficiency also holds for cytochrome b_5 reduction. From Figure 7 it is evident that by increasing the amount of microsomal phospholipids in vesicles composed of phosphatidylcholine, the steady-state reduction of cytochrome b_5 increases in a similar manner to what was observed for the rate of cytochrome P-450$_{LM_2}$ catalyzed hydroxylation reactions when phosphatidylcholine vesicles were titrated with phosphatidylethanolamine (cf. above).

Discussion

The results presented in this paper demonstrate that the composition of the membrane is essential for the activity of cytochrome P-450$_{LM_2}$ catalyzed hydroxylation reactions. Reconstitution of purified cytochrome P-450$_{LM_2}$ and NADPH–cytochrome P-450 reductase in vesicles of defined lipid compositions led to an enhancement of the *O*-demethylase and *O*-deethylase activities of the recombined monooxygenase system, which was proportional to the amount of negatively charged phospholipids in the membrane. It should be pointed out that, at least in the case where the activities of DOPC and DOPE vesicles were compared, the possible contribution of the structure of the fatty acid moieties could be neglected.

The charge-dependent phospholipid-mediated activations are apparently restricted to the membranous system. It is possible to reconstitute cytochrome P-450$_{LM_2}$ dependent hydroxylase activities at high turnover rates by using small amounts of the neutral dilauroylphosphatidylcholine as phospholipid. This short phospholipid apparently does not form a membrane bilayer but rather small micelles. Use of this phospholipid at concentrations below cmc together with

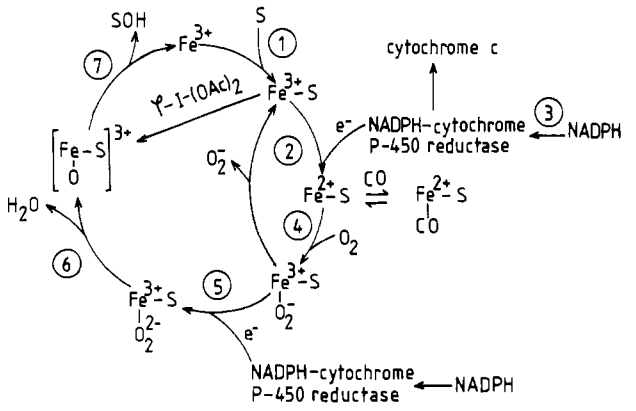

FIGURE 8: Cytochrome P-450 reaction scheme with indications of steps that may be affected by the membrane phospholipid composition.

concentrated solutions of cytochrome P-450$_{LM_2}$ and NADPH–cytochrome P-450 reductase (Coon, 1978) results in catalytically very active P-450–P-450 reductase complexes. Similar complexes are probably not formed in the liposomal membrane due to the excess phospholipid ($\simeq$400 molar excess) and the free lateral diffusion of the proteins. We have previously described some differences between the two types of reconstituted systems (Ingelman-Sundberg & Johansson 1980b).

A fundamental question concerns the mechanism by which a negatively charged membrane influences the cytochrome P-450 activities. The conceivable partial reactions of cytochrome P-450 shown in Figure 8 constitute the possible points of interaction between the enzyme and the membrane. Step 1, i.e., the binding of substrate to cytochrome P-450, is less likely to be affected by the membrane charge since (i) the activation energy, which reflects the thermodynamically limiting step in the overall reaction, usually the binding of substrate, and thus allows the detection of changes in the formation of the binary P-450$_{LM_2}$–substrate complex, was similar for neutral and negatively charged vesicles, (ii) the apparent K_m of the enzyme for the substrate was the same in both types of vesicles, (iii) the apparent water–lipid phase distribution coefficients for the substrate were the same, and (iv) the iodosobenzene diacetate supported hydroxylation reactions proceeded at approximately the same rate in both types of vesicles. Step 2, i.e., the introduction of the first electron to P-450$_{LM_2}$, seems to be affected by the charge of the membrane according to the reduction kinetics experiments performed under anaerobic and aerobic conditions, as judged by the formation of the ferrous carbonyl complex of P-450$_{LM_2}$ after addition of NADPH to the differently charged vesicles.

The interaction of NADPH with NADPH–cytochrome P-450 reductase (step 3) is excluded as being affected by the membrane charge since no differences in the rate of cytochrome *c* reduction were detectable between the various vesicles. Also, the binding of oxygen to cytochrome P-450$_{LM2}$ (step 4) is not likely to be membrane charge dependent since (i) the same charge-dependent increase in reduction efficiency was found by using cytochrome b_5 instead of cytochrome P-450$_{LM_2}$ in the vesicles, (ii) the production of superoxide anions has been found to increase concomitantly with the overall hydroxylase activities and the rate of NADPH oxidation, when neutral PC membranes are titrated with microsomal phospholipids,[2] and (iii) oxygen binding is known to be very fast (cf. White & Coon, 1980).

The introduction of the second electron to P-450$_{LM_2}$ (step 5) is limited by the interaction of NADPH–cytochrome P-450 reductase with the hemoprotein and, in agreement with the present findings, would be expected to be dependent upon the membrane charge. Steps 6 and 7 are known to be very fast and not rate limiting in the cytochrome P-450 catalyzed reactions (cf. White & Coon, 1980). Furthermore, the iodosobenzene diacetate experiments rule out step 7 as being membrane charge dependent since the reactions supported by the oxidizing agent proceeded at about equal rates in the various vesicles. An unfavorable orientation of the cytochrome relative to the reductase in the neutral vesicles is unlikely since at least 75% of the cytochrome was reducible by NADPH. In the presence of cyclohexane, this value increased in PC vesicles to nearly 100% even under aerobic conditions.[3] Furthermore, the gel filtration experiments on Sepharose 2B indicated a homogeneous distribution of both types of proteins among the vesicles of various sizes.

In summary, the available data indicate that the interaction of NADPH–cytochrome P-450 reductase with cytochrome P-450$_{LM_2}$ is dependent upon the surface charge of the liposomal membrane which determines the overall hydroxylation rate in the reconstituted system. More recent results[2] suggest that the surface charge of the membrane is of critical importance for the properties of NADPH–cytochrome P-450 reductase and thus that the effects reported in this paper represent charge-dependent alterations in the functional and perhaps conformational properties of the reductase molecule, rather than in the P-450$_{LM_2}$ molecule. These altered functional properties of the reductase apparently result in impaired interactions with (i) cytochrome P-450$_{LM_2}$, as demonstrated in the present paper, (ii) cytochrome P-450$_{LM_4}$, as will be reported elsewhere,[2] and (iii) cytochrome b_5, as reported in this paper and in a forthcoming paper,[2] but not with cytochrome P-450$_{LM_3}$, as demonstrated earlier (Ingelman-Sundberg et al., 1979) and elsewhere.[3] Hydroxylation reactions catalyzed by the latter cytochrome are apparently not influenced by the membrane charge; the reactions proceed equally well in either neutral phosphatidylcholine membranes or in negatively charged membranes containing microsomal phospholipids or phosphatidylserine, etc. This emphasizes that the results presented in this paper cannot be generalized to all types of NADPH–cytochrome P-450 reductase–cytochrome P-450 interactions.

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