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SPECIFICITY IN THE INTERACTION OF PHOSPHOLIPIDS AND FATTY ACIDS WITH VESICLE RECONSTITUTED CYTOCHROME *P*-450

A SPIN LABEL STUDY

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The association of fatty acids, androstane, phosphatidylcholine, phosphatidylethanolamine, and phosphatidic acid with purified and phospholipid-vesicle reconstituted cytochrome *P*-450 was studied by spin labeling. Spin-labeled fatty acids were found to be motionally restricted by cytochrome *P*-450 in both phospholipid vesicles and in microsomes to a much greater extent than spin-labeled phospholipids. The equilibrium of spin-labeled fatty acid between the bulk membrane lipid and the protein interface could be shifted towards an increased amount in the bulk phospholipid phase by the addition of oleic acid or lysophosphatidylcholine, but not by sodium cholate. Microsomes from different animals showed a variable extent of motional restriction of fatty acids, independent of pretreatment of the animals with phenobarbital or β-naphthoflavone, of cytochrome *P*-450 content, of the presence of type I and type II substrates for cytochrome *P*-450. These differences are attributed to the presence of varying amounts of lipid breakdown products in the microsomal membrane such as lysolipids or fatty acids which compete with the externally added spin-labeled fatty acids, or with spin-labeled androstane for the binding to cytochrome *P*-450. The negative charge of the fatty acid was found to be involved in its association with the protein. Cytochrome *P*-450 was shown to interact only with a few spin-labeled phospholipid molecules in such a way that the motional restriction of the spin acyl chains can be detected by electron paramagnetic resonance ($\tau_R > 10^{-8}$ s). The number of associated lipid molecules per protein probably is too small to form a complete shell around the protein. This lipid-protein interaction could be destroyed by the addition of sodium cholate, in contrast to the fatty acid-protein interaction.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DPPA, dipalmitoylphosphatidic acid; DMPE, dimyristoylphosphatidylethanolamine.
Spin labels (see also Fig. 1): spin-labeled androstane; (m,n)-FASL, spin-labeled fatty acid, unbranched carbon chain of $m+n+3$ carbon atoms, spin-labeled at position C-$(m+2)$; (m,n)-SASL, spin-labeled stearic acid $(m+n=15)$; (m,n)-SAASL, spin-labeled aminoethylstearamide $(m+n=15)$; (m,n)-PASL, (m,n)-PESL, and (m,n)-PCSL: DPPA, PE, and PC, respectively, spin-labeled in the 2-acyl group.

Introduction

Liver cytochromes *P*-450, which are integral membrane proteins found mainly in the endoplasmic reticulum, could potentially interact with lipids in a more specific way than simply as a means of defining the localization and sidedness of a protein in the membrane. One argument for this suggestion is the necessity to interact effectively in

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the membrane with cytochrome b_5 and NADPH-cytochrome *P*-450 reductase [1–4]. Another reason is the ability of cytochromes *P*-450 to used membrane components as substrates and targets [5–10]. Both functions are expected to require a high mobility of the protein in the membrane, which could be mediated through lipid-protein interactions of rather short lifetimes compared to the overall rate of enzymatic activity. It follows therefore that the formation of a rigid and tight lipid halo around cytochromes *P*-450 might be an unfavorable structure for drug and lipid metabolism.

The high monooxygenase activity obtained by using the short-chained lipid dilauroylphosphatidylcholine [11,12] in a micellar reconstituted system as well as the possible existence of some of the phospholipids in microsomes being in an isotropic structure [13], favor a very dynamic model for the membrane. Lipids associated with cytochrome *P*-450, if existing at all, could be more rapidly exchanging at the lipid-protein interface as appears to be the case for rhodopsin [14] or cytochrome oxidase [15]. However, the interaction of a small number of lipid molecules with cytochrome *P*-450 seems to be necessary for maintaining an active protein conformation, its ability to interact with cytochrome *P*-450 reductase and for efficient electron transfer [16].

A further argument that stimulates a study of cytochrome *P*-450-lipid interactions would be an understanding of some of the molecular mechanisms of the monooxygenase system. Many substrates are hydrophobic: polyaromatic or halocarbon compounds [17], as well as sterols [8,18], or long chain fatty acids are essentially membrane-contained substrates. Metabolic activity might depend on the membrane by determining the accessibility of these substrates. The demonstrated oxidation of fatty acids [6], especially arachidonic acid to potentially highly active products [19,20], as well as the reduction of spin-labeled lipids (this paper), suggest that cytochrome *P*-450 plays a role in lipid metabolism. In the liver, cytochrome *P*-450 is involved in cholesterol biosynthesis, the synthesis of bile acids, and the conversion of cholesterol to steriod hormones [5]. In this study we present data on the interaction of fatty acids and other lipids with cytochrome *P*-450 LM2 as detected by EPR spectroscopy.

Materials and Methods

Purification of cytochrome P-450 and cytochrome P-450 reductase. Cytochrome *P*-450 was purified from liver microsomes of phenobarbital-treated rabbits [21] to a purity of 16–19 nmol per mg protein. Cytochrome *P*-450 reductase was purified from the same microsomes by affinity chromatography [22] to a specific activity towards cytochrome *c* of 40 μmol/mg per min measured at 30°C in 0.3 M potassium phosphate buffer pH 7.5 with 20% glycerol.

Preparation of spin-labeled lipids. Spin-labeled fatty acids were purchased from Syva. The spin-labeled aminoethylstearamide, (*m*,*n*)-SAASL, was prepared by refluxing 34 μmol spin-labeled fatty acid in the presence of an equimolar amount of dicyclohexylcarbodiimide with a 100-fold excess of ethylenediamine in 2 ml CH_2Cl_2 for 20 min. After evaporation of the solvent and removal of excess ethylendiamine by washing with water, the resulting amide was purified by preparative thin-layer chromatography on silicic acid plates with am-

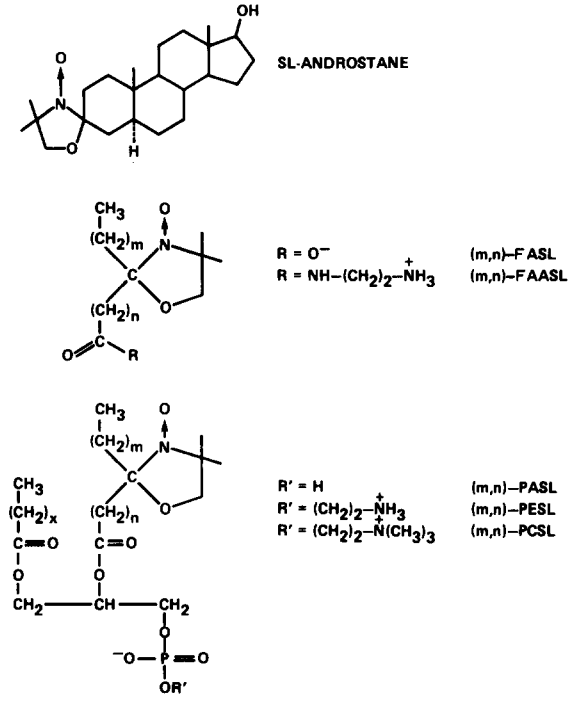

Fig. 1. Structures and abbrevations of spin labels. Note: (*m*,*n*)-FASL is identical to (*m*,*n*)-SASL in all cases where $m + n = 15$.

moniacal chloroform/methanol (3 : 1, v/v). The amide was identified by its positive reaction with ninhydrin and by its paramagnetism. It was eluted from the silicic acid and stored at −20°C in chloroform/methanol (1 : 1, v/v). The yield was about 50%.

Spin-labeled phosphatidylcholines were prepared from egg lysophosphatidylcholine (Lipid Products, U.K.) and stearic acid spin label [23] carrying the nitroxide group at C-5 or C-16. Spin-labeled phosphatidylethanolamine and spin-labeled phosphatidic acid were obtained by enzymatic headgroup exchange of phosphatidylcholine by phospholipase D [24]. Chromatographic purification was carried out by preparative thin layer chromatography on silicic acid plates developed in chloroform/methanol/water (65 : 25 : 4, v/v). The spin-labeled lipids were stored in chloroform/methanol (1 :, v/v) at −20°C.

Preparation of vesicle reconstituted systems and microsomes with spin-labeled fatty acids or lipids. Cytochrome *P*-450 was reconstituted in phospholipid vesicles, consisting of PC or PC/PE (2 : 1) or PC/PE/DPPA (16 : 8 : 1) by the previously described slow cholate dialysis procedure [21]. Spin-labeled stearic acid, (*m*,*n*)-SASL, as well as the amide, (*m*,*n*)-SAASL, and other spin-labeled fatty acids (*m*,*n*)-FASL were incorporated in the membrane by stirring a suspension of vesicles or microsomes for 5 min at 20°C with glass beads coated with the spin label. The amount of glass beads was adjusted to give 1 mol% or less of spin-labeled fatty acid to total lipid. Spin-labeled lipids were incorporated in vesicles by mixing the lipids used for reconstitution with spin-labeled lipid in chloroform/methanol (1 : 1, v/v) to give a molar ratio of 100 : 1. Size of the resulting vesicles, lipid to protein ratio and activity of cytochrome *P*-450 and cytochrome *P*-450 reductase were the same with or without spin labeled lipid. The yield of spin-labeled lipid was 60–80% when the total lipid/protein ratio was 180 : 1 or more. At a ratio of 20 : 1 the yield of spin-labeled lipid was about 30% only. In order to obtain final preparations at low lipid to protein ratios with approx. 1% spin-labeled lipid reconstitutions were started with 3% spin-labeled lipid. Spin-labeled phosphatidylcholines, (*m*,*n*)-PCSL, were incorporated in microsomes (5 mg microsomal protein per ml 0.1 M potassium phosphate buffer pH 7.5 with 20% glycerol by the addition of sonicated vesicles of (*m*,*n*)-PCSL for 1 h at 20°C or for 30 min at 30°C. The initial ratio of spin-labeled lipid to microsomal lipid was 1 : 20. Microsomes were separated from excessive unincorporated spin-labeled lipid vesicles by diluting the suspension 1 : 50 with buffer and pelleting the microsomes at 100 000 × *g*. A residueal amount of (*m*,*n*)-PCSL vesicles, as detected by a broad component in a spectrum of a suspension of these pelleted microsomes, was removed by homogenization of the microsomes in buffer, followed by centrifugation. About 20% of the spin-labeled lipid was incorporated in the microsomal membrane.

Electron paramagnetic resonance (EPR) measurements. A suspension of microsomes or reconstituted vesicles in 1 mm capillaries was placed in a 4 mm quartz tube containing silicon oil for thermal stability. Temperature was adjusted by a thermostatically controlled stream of nitrogen and measured with a thermocouple in the silicon oil. Some spectra of spin labels incorporated in the membrane of vesicles or microsomes were analyzed in terms of the order parameter *S* [25]. In order to estimate the amount of motionally restricted spin label in cytochrome *P*-450 containing vesicles and microsomes, we assumed that the spectra of fatty acid spin label (1,14)-SASL motionally restricted by cytochrome *P*-450 in the membrane and by detergent solubilized cytochrome *P*-450 are identical. The intensity of the broad spectrum of (1,14)-SASL immobilized by solubilized cytochrome *P*-450 was subtracted from the two component spectra. The total amount of (1,14)-SASL present in the preparations was determined by completely extracting the lipid from samples into chloroform/methanol (2 : 1, v/v) from the (1,14)-SASL containing solutions of cytochrome *P*-450, and from the suspensions of vesicles with and without cytochrome *P*-450. Measurements of the EPR spectra of these extracts were used to quantitate the total (1,14)-SASL. EPR spectra were recorded on a Varian E-12 9-GHz spectrometer.

Reduction kinetics of the nitroxides. Molecular oxygen was removed by passing a stream of water-saturated nitrogen over the stirred solution for 15 min on ice. 10 μl of a 10 mM solution of NADPH were mixed with 90 μl of a suspension of vesicles on ice and transferred to an EPR tube.

The reduction reaction was started by quickly heating (1–2 min) the solution in the cavity of the EPR apparatus and the decrease of the EPR intensity monitored continuously at 20°C. The field position was automatically adjusted to remain at maximum EPR intensity using a field-frequency lock.

Rates of NADPH consumption were measured at 340 nm ($\epsilon = 5682\ M^{-1} \cdot cm^{-1}$). All measurements were carried out in 20 mM Hepes buffer pH 7.5 containing 20% glycerol (v/v) and 0.1 mM EDTA.

Results

Motional restriction of spin-labeled fatty acids by cytochrome P-450

In Fig. 2A EPR spectra are compared using the same stearic acid spin label (1,14)-SASL, incorporated in vesicles with and without protein. The lipid mixture was PC/PE/DPPA (16 : 8 : 1, molar ratio). The dashed line shows for comparison, a spectrum of protein-free lipid vesicles, that were also obtained by cholate dialysis. The solid line represents the spectrum when cytochrome *P*-450 was present in the bilayer of the same vesicles at a molar ratio of lipids to protein of 120 : 1. The spectrum obtained with cytochrome *P*-450 containing vesicles appears to be a two component spectrum, whereas the lipid vesicles give a spectrum of only one component which is characteristic of (1,14)-SASL undergoing fast anisotropic motion in lipid bilayers. This difference is best seen at the positions of the outer hyperfine extrema (under the arrows) of the broad spectral component. A percentage of the fatty acid chains in the membrane is motionally restricted on the EPR time scale ($\tau_R > 3 \cdot 10^{-9}$ s) and the remainder is mobile, similarly to the fatty acid spin-label molecules in the PC/PE/DPPA-vesicle preparation without cytochrome *P*-450.

As the only difference between these two vesicle preparations in the presences or absence of cytochrome *P*-450, it seems reasonable to assume that the broad spin-label component in the spectrum results from the interaction of fatty acids with cytochrome *P*-450. In order to determine if this immobilization is a special feature of the reconstituted cytochrome *P*-450 vesicles, the same fatty

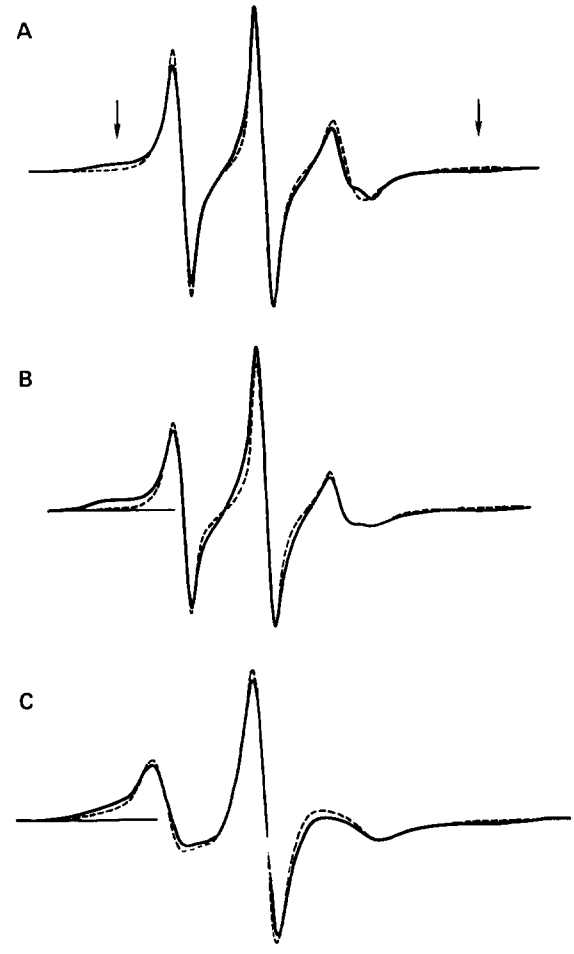

Fig. 2. (A) EPR spectra of spin-labeled stearic acid, (1,14)-SASL, in reconstituted vesicles of PC/PE/DPPA (16:8:1) (dashed line), and the same vesicles with cytochrome *P*-450 at a lipid to protein ratio of 120:1 (mol/mol) (solid line). Arrows indicate the positions of the outer hyperfine extreme due to the spin-labeled stearic acid immobilized by the protein. (B) EPR spectra of (1,14)-SASL in liver microsomes from two different rabbits. (C) EPR spectra of spin-labeled androstane in the same microsome preparations as in (B). All spectra were measured at 10°C.

acid spin label was incorporated into some of those microsomes from phenobarbital-treated rabbits that were also used for the preparative purification of cytochrome *P*-450. Microsomes from eight different animals were compared. The ratio of the broad component (motionally restricted) to bilayer-containing ('free' component) of the fatty acid spectrum was different in each preparation and varied by a factor of seven in peak height of

the low-field peak. The two extreme spectra are shown in Fig. 2B: the dotted line showing little motional restriction (similar to protein-free PC/PE/DPPA or microsomal lipid vesicles) and the solid-line spectrum revealing an even higher percentage of broad component than the reconstituted cytochrome *P*-450 vesicles.

The most likely compounds to compete with those spin-labeled fatty acid molecules motionally restricted by cytochrome *P*-450 are other fatty acids. A suspension of oleic acid was added to the suspension of fatty acid spin label containing microsomes or vesicles. The broad spectral component disappeared linearly with increasing concentration of oleic acid and at 5 mM oleic acid the broad component was unmeasurably small. Lysophosphatidylcholine derived from egg yolk was also found to displace the protein-interacting fatty acid, but up to 1% sodium cholate had no effect. Spin-labeled androstane was incorporated in those microsomes that previously gave spectra of a low and a high percentage of a broadened stearic acid spin-label spectrum. The spectra in Fig. 2C show that these microsomes also restricted the motion of androstane to a similar extent as it was found for (1,14)-SASL. This might suggest that fatty acids, lysophosphatidylcholine and androstane are all motionally restricted on a similar site.

It seemed possible that products of lipid peroxidation compete with the spin-labeled fatty acid for the same binding site of cytochrome *P*-450. On the other hand, the presence of lipid peroxides increases the rate of NADPH-consumption by microsomes. Therefore, one would expect an inverse relationship between the amount of immobilized spin-labeled fatty acid and the rate of NADPH consumption. The results of these measurements are shown on Fig. 3. The data seem to indicate the existence of a linear relationship over a wide range.

In order to obtain some information about the effect of the charge of the fatty acid on the degree of immobilization, the spin-labeled stearic acid (5,10)-SASL was converted to the amide (5,10)-SAASL which carries a positive charge at pH 7.5. There was no difference between the spectra of the spin labels (5,10)-SASL and (5,10)-SAASL when motionally restricted by detergent-solubilized cytochrome *P*-450 or when they were incorporated in

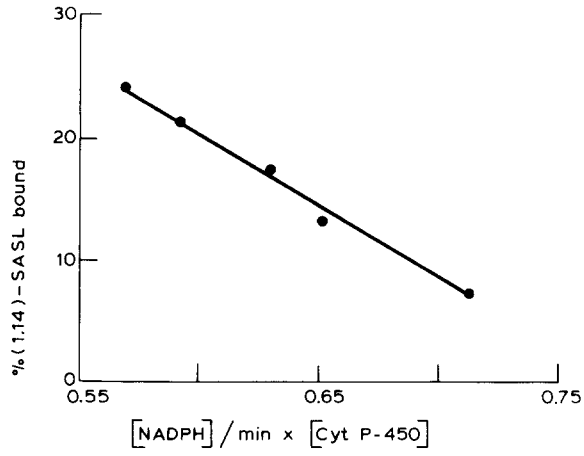

Fig. 3. Dependence of the extent of motional restriction by cytochrome *P*-450 of added spin-labeled fatty acid ((1,14)-SASL 'bound') on the rate of NADPH consumption at 22°C. Each datum point was obtained from a suspension of microsomes from a different rabbit liver. It is concluded that products of lipid peroxidation compete with the added spin-labeled fatty acid for the same binding site on cytochrome *P*-450.

PC/PE/DPPA vesicles. However, when (5,10)-SAASL was incorporated in cytochrome *P*-450 containing PC/PE/DPPA (molar ratio protein: lipid = 1 : 120) there was a difference in the percentage of the motionally restricted spin label.

Fig. 4 shows the spectra of (5,10)-SASL (solid line) and (5,10)-SAASL (dashed line) in vesicles with cytochrome *P*-450. The identical spectra of the two spin labels in lipid vesicles without protein (dotted line) are given for comparison. The difference between the spectra at the low-field hyperfine maxima (arrow) reflects a decrease of the percentage of the motionally restricted component to about one half.

Motional restriction of spin-labeled phospholipids by cytochrome P-450

When spin-labeled phosphatidylcholine (1,14)-PCSL or (12,3)-PCSL, spin-labeled phosphatidylethanolamine (1,14)-PESL or spin-labeled phosphatidic acid (1,14)-PASL were added to detergent-solubilized cytochrome *P*-450, at about equimolar amounts or less, the spin label was completely immobilized and rotated with the protein (Fig. 5). The spectrum of the immobilized fatty acid (1,14)-SASL (trace a) has an additional isotropic component which is due to an equi-

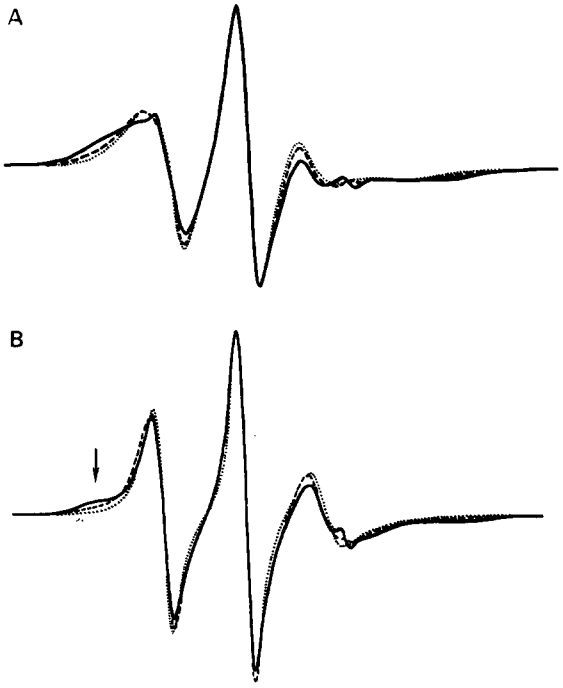

Fig. 4. (A) EPR spectra at 10°C of (5,10)-SASL (solid line) and (5,10)-SAASL (dashed line) in preparations of cytochrome *P*-450 with PC/PE/DPPA (16 : 8 : 1) at a lipid to protein ratio of 120 : 1 (mol/mol) show a dependence of the extent of immobilization of the charge of the spin-labeled fatty acid. The identical spectrum of either one of the spin labels in protein-free vesicles is given for comparison. (B) Same at 30°C.

librium between bound fatty acid and isotropic fatty acid in the water phase.

To examine to what extent acyl chain restriction occurs in cytochrome *P*-450 containing membranes, spin-labeled phospholipids were incorporated into microsomes and vesicles. When the molar ratio of protein to lipid was 1 : 20 or 1 : 40, the broad spectral component in the spectra of (1,14)-PCSL or (1,14)-PESL or (1,14)-PASL was easily observable (as shown in Fig. 6, trace a) in preparations of cytochrome *P*-450 in PC/PE/DPPA (16 : 8 : 1). The relative amounts of motionally restricted PCSL, PESL or PASL were comparable, presumably reflecting the nonspecific type of phospholipid-protein interaction shown in Fig. 5.

When the molar lipid to protein ratio was increased to 120 : 1 to reconstitute cytochrome *P*-450 in PC/PE/DPPA (16 : 8 : 1) vesicles of 400–700 Å diameter [21] could be seen by electron microscopy. Spectra of (1,14)-PCSL were identical to spectra of (1,14)-PESL. Both showed a very small amount of motionally restricted phospholipid (middle trace in Fig. 6b), barely separated from the spectrum of either one of the spin-labeled lipids in PC/PE/DPPA vesicles without protein (dotted trace in Fig. 6b). A selective interaction with spin-labeled phosphatidic acid was found at 120 : 1 (high intensity trace in Fig. 6b).

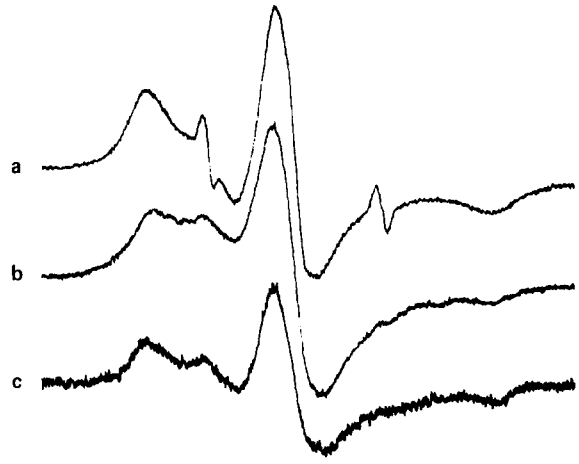

Fig. 5. EPR spectra at 10°C of (1,14)-SASL (a), (1,14)-PCSL (b) and (1,14)-PASL in solutions of purified cytochrome *P*-450 at less than equimolar amounts of spin label to protein.

The addition of sodium cholate (1%) resulted in the complete destruction of these detectable phospholipid-protein interactions in all preparations, whereas the same cholate concentration had no effect on the fatty acid-protein interaction, suggesting a more specific binding of the fatty acid. The preference for fatty acids over PC and PE was as pronounced in microsomes as it was in cytochrome *P*-450/PC/PE vesicles. Spectra obtained from (1,14)-PCSL incorporated in microsomes had a linewidth that was similar to the one from the same spin label in microsomal phospholipid vesicles. When (1,14)-SASL was incorporated in those microsomes which already contained (1,14)-PCSL, motionally restricted fatty acid could be seen in the spectrum even at lower concentrations of (1,14)-SASL than of (1,14)-PCSL.

The spin-labeled lipids were also used in a different way to reveal differences in the interaction of cytochrome *P*-450 with lipids. The nitroxide type spin labels used in this study are also

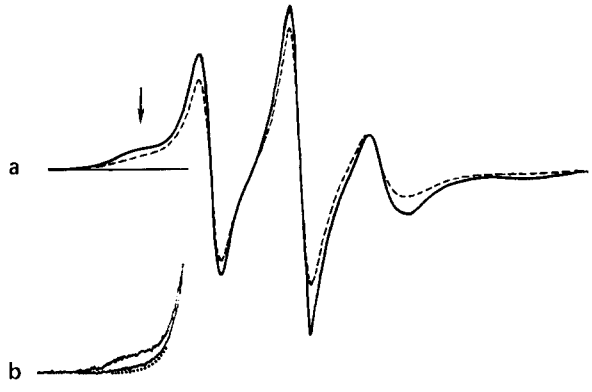

Fig. 6. EPR spectra at 10°C of (1,14)-PCSL preparations of cytochrome *P*-450 with PC/PE/DPPA (16:8:1) at a lipid to protein ratio of 40:1 (mol/mol) (dashed line a), and 20:1 (mol/mol) (solid line a). In vesicles at a lipid to protein ratio of 120:1 (mol/mol) (1,14)-PASL is immobilized to a higher extent (high intensity of low-field hyperfine extremum in the spectrum, trace b) than (1,14)-PCSL (low intensity, trace b). The dotted line shows the identical spectrum of either one of the spin-labeled lipids in the same vesicles without protein.

substrates for cytochrome *P*-450. The nitroxide group is reduced to the hydroxylamine and the rate of loss of EPR intensity can be measured to yield the rate of this reduction reaction [26]. The NADPH-dependent reduction rates by vesicle-reconstituted cytochrome *P*-450 reductase/cytochrome *P*-450 (1:5, mol/mol) at 20°C of the co-reconstituted phospholipid spin labels (1,14)-PCSL, (1,14)-PESL, and (1,14)-PASL were 0.11 ± 0.03, 0.13 ± 0.04, and 0.18 ± 0.03 nmol per nmol cytochrome *P*-450 per min. Spin-labeled stearic acid (1,14)-SASL, having the nitroxide at the same position as the lipids, was reduced in different microsomes 10–30 times faster, and 40 times faster in cytochrome *P*-450/cytochrome *P*-450 reductase PC/PE/DPPA vesicles than the phospholipid spin label (1,14)-PCSL.

Discussion

Spin labeled fatty acid, which was incorporated in the membrane of microsomes from different rabbits, was found to be motionally restricted to a very different extent. The difference in percentage of the broad component in the spectra is not readily explained by the content of cytochrome *P*-450 in the membrane, because all the examined microsomal preparations had consistently a content of 2–3 nmol cytochrome *P*-450 per mg of protein. There was also no correlation between pretreatment of the animals with the cytochrome *P*-450 inducing agent β-naphthoflavone or phenobarbital and the spectral differences did not depend on the presence or absence of EDTA during the preparation of the microsomes. The relative amount of motionally restricted (1,14)-SASL estimated by the procedure described in Methods, as well as a comparison with calculated two-component spectra [14,15] suggests that from less than 5% to over 30% of the total fatty acid spin label was motionally restricted in the eight different preparations. We examined the possibility that there are compounds in the microsomal membranes that bind to cytochrome *P*-450 and affect its interaction with the fatty acid spin label. A spin-labeled fatty acid is expected to bind to the cytochrome *P*-450 in a way similar to other fatty acids, that are known to be hydroxylated [6]. The spin-labeled fatty acid, which is interacting with cytochrome *P*-450, might bind to the binding sites of type I or type II substrates. Therefore, two different substrates were added to a suspension of microsomes displaying a high percentage of broadened spectral component from the fatty acid spin label as well as to the cytochrome *P*-450-containing vesicles. Metyrapone or hexobarbital had no effect at concentrations up to 10 mM. Also no effect was found when substrates were added to fatty acid spin label bound to solubilized cytochrome *P*-450. This suggests that the binding sites for type I or type II substrates are not identical to the one for fatty acids.

The structure of a compound that is able to displace bound fatty acid from cytochrome *P*-450 should give some information on the nature of the interaction. Addition of oleic acid or lysophosphatidylcholine was shown to result in a decrease of the broad component in the spectra of spin-labeled fatty acid that was incorporated in the membrane of microsomes or cytochrome *P*-450 containing vesicles. Therefore it is possible that the different degrees of motional restriction of spin-labeled fatty acid by microsomes reflect a variation in the amount of lipid breakdown products in these microsomal membranes, including lysocompounds, fatty acids, and various intermediate products of

lipid peroxidation reactions. Such compounds could serve as substrates for cytochromes *P*-450 and the higher their content in these membranes, the less added spin-labeled fatty acid is bound. To test if these compounds are 'internal' substrate already present in microsomes, the rate of NADPH-consumption of the various microsomes was measured. There was an inversely proportional relationship between NADPH consumption and percentage of motionally restricted spin-labeled fatty acid. NADPH consumption was also found to be increases if microsomal lipids were allowed to peroxidize and breakdown by aging. It seems therefore that the fatty acid spin-label spectra reflect the relative amount of such forms of membrane-contained (endogenous) substrate.

In order to address the question of whether fatty acids in a membrane bind to cytochrome *P*-450 by an ionic interaction, the carboxylic acid of spin-labeled fatty acid was condensed with ethylenediamine to an amide. A smaller percentage of the resulting amide spin label (5,10)-SAASL was found to be motionally restricted. This spin label carried a positively charged ammonium group at pH 7.5. Therefore, it seems that a negative charge is involved in the interaction with cytochrome *P*-450 and that the contribution of a positive charge to the overall binding is considerably less, if there is a contribution at all. The spectral differences between (5,10)-SASL and (5,10)-SAASL can be interpreted by the existence of a binding site for fatty acids on cytochrome *P*-450 with hydrophobic as well as electrostatic interactions. A specific lysine or arginine residue should be involved with the reversible binding of fatty acids. It has been suggested that different cytochromes *P*-450 hydroxylate at the *w*- and at the $(w-1)$-positions of fatty acids [27]. One might speculate that this electrostatic interaction holds the fatty acid in place. Fatty acid hydroperoxides might act as oxygen donors in peroxide-dependent hydroxylations by cytochrome *P*-450 [28]. Such a mechanism could also result in the destruction of cytochrome *P*-450 [29]. Although we did not observe a displacement of the bound spin-labeled fatty acid by the added substrates for cytochrome *P*-450, it is very likely that there is mutual influence on metabolism. In fact, when oleic acid was present in a micelle-reconstituted system benzphetamine demethylation was inhibited by 99.5% [30]. A similar interference was observed by us when methyloleate was used as a target for the binding of metabolically produced radicals, whereas the use of dioleoylphosphatidylcholine resulted in high rates of halocarbon metabolism [7,9,10].

Another example of preferential binding of fatty acids was shown with receptor-rich membranes for *Torpedo marmorata* and spin-labeled fatty acid [31], which was interpreted [32] as a rigid lipid phase. Because the interaction of cytochrome *P*-450 with spin-labeled lipids is clearly different from the one with fatty acids, one might conclude that the use of fatty acids does not necessarily reveal lipid-protein interactions.

The spin-labeled lipids that were used for the reduction kinetics of the nitroxide, have the nitroxide group at C-16 of the stearic acyl chain and differ therefore in their headgroups only. As the reduction rates were of comparable magnitude, there is no reason to assume the existence of a long-lived arrangement of a special type of lipid around cytochrome *P*-450. This is in agreement with the equally low percentage of motionally restricted PC or PE, with the ease of incorporation of cytochrome *P*-450 in PC/PE vesicles [21] and with the formation of sigmoidal phase transitions in vesicles of PC/DMPE [33]. A further argument for the increased concentration of negatively-charged lipids around cytochrome *P*-450 is the finding [34] that the reducibility of cytochrome *P*-450 and the overall rate of hydroxylation was affected by the presence of the negatively-charged lipid phosphatidylserine.

The fact that spectra of (1,14)-PCSL in microsomes were almost identical to those of (1,14)-PCSL in microsomal lipid is not expected from model studies [14,15,35–37] which suggest that sufficient lipids are seen to interact with intramembraneous proteins to form a single layer of lipid molecules around the protein and that outer layers are also affected. Exchange rates of the protein-interacting phospholipid molecules of 10^6–10^7 s^{-1} could be estimated from our spectra which would be in agreement with other work on model membrane [38].

The part of these membrane which has been shown by ^{31}P-NMR experiments [13] to be different from the lipid bilayer structure might very well

result in the high dynamic in microsomes, although this second structure is not necessarily inverted micelles. Such a high rate of lipid exchange in microsomes and vesicles with cytochrome *P*-450 would favor rather a dynamic model, in which there is an important short-lived interaction of cytochrome *P*-450 with PC as well as with PE and some preferential binding of a few molecules such as negatively-charged lipids or fatty acids.

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