EFFECT OF EXCHANGE OF PHOSPHOLIPIDS UPON THE CATALYTIC ACTIVITY OF CYTOCHROME P-450 IN RAT LIVER MICROSOMES AND IN RECONSTITUTED VESICULAR SYSTEMS

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ABSTRACT

The effect of changes in the phospholipid composition of rat liver microsomes with the phospholipid transfer protein, on the cytochrome P-450 dependent monooxygenation of scoparone, has been examined. With different microsomal fractions of phenobarbital-treated rats, an exchange of up to 74 % of the phospholipids with egg phospholipid vesicles, had no detectable effect on the specific activities of the scoparone O-demethylation (0.97-1.37 nmol scoparone O-demethylated x min^{-1} x nmol $P\text{-}450^{-1}$). Furthermore the specific activities of the 7-ethoxycoumarin O-deethylase were examined by incorporating the Cytochrome $P\text{-}450_{PB-B}$ and the NADPH-P-450 reductase into phospholipid vesicles by cholate dialysis with different mixtures of phospholipid. The molar ratio of the two proteins to the lipids was 1:2:200 for the P-450, NADPH-P-450 reductase and the lipid, respectively. In no case examined the specific activity (30-34 nmol 7-hydroxycoumarin x min^{-1} x nmol $P\text{-}450_{PB-B}{}^{-1}$) changed markedly with the nature and composition of the phospholipids in the vesicles.

Key-words: P-450, phospholipids, microsomes, vesicular system

INTRODUCTION

The microsomal mixed function monooxygenase system consisted of the NADPH-cytochrome P-450 reductase and a family of cytochrome P-450 enzymes. In the mammalian liver, these monooxygenase systems are found tightly associated with the endoplasmic reticulum. Therefore it was not surprising, that the lipid matrix should have a markedly influence on the catalytic activity of this system. In numerous studies phospholipid has been reported to be necessary of optimal catalytic activity of a number of mammalian cytochrome P-450 reconstituted systems (1,2). It has been argued that the lipid composition of the membrane is essential in stimulating P-450 dependent activities and is a function of the type of lipid (3,4).

In this study we examined the influence of the type of lipid by incorporating the cytochrome $P\text{-}450_{PB\text{-}B}$ and the NADPH-P-450-reductase into phospholipid vesicles consisted of a single species of PC, a binary mixture PC and PE, or a ternary mixture of PC, PE, and PP, on the catalytic activity of the 7-ethoxycoumarin O-deethylase. And, we describe the influence of the manipulation of the phospholipid composition of rat liver microsomes with the purified phospholipid exchange protein, on the catalytical activity of the scoparone O-demethylation.

MATERIAL AND METHODS

Chemicals: Scopoletin, isoscopoletin and 7-hydroxycoumarin were purchased from Fluka AG (Buchs, Switzerland). Scoparone (5) and 7-ethoxycoumarin (6) were synthesized as described previously.

Phospholipids: PC $(18:1)_2$ was purchased from Avanti Biochemicals Inc. PE $(18:1)_2$ was prepared using phospholipase D to transesterify dioleoyl phosphatidylcholine. Other individual molecular species of mixed fatty acyl phospholipids used in this study were synthesized according to Eibl (7) and Eibl and Nicksch (8).

Enzymes: All materials used for the purification procedure of P-450 (apparent monomeric Mr = 50,000) and NADPH-P-450 reductase (Mr = 74,000) are described by Guengerich et al. (9). The nonspecific phospholipid exchange protein was purified from beef liver as described by Crain and Zilversmit (10). The activity was monitored by measuring the transfer of labeled phospholipid from sonicated egg PC liposomes to heat-treated mitochondria. The specific activity of the preparation was 643 nmol of PC transferred per h/mg of protein.

Abbreviations: P-450, liver microsomal cytochrome P-450; PB, phenobarbital; PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; PP, phosphatidylpropane-1,3-diol (the acyl substituents of specific phospholipids are indicated in parantheses with the carbon chain length followed by the number of unsaturated bonds after a colon.

Reconstitution of P-450 and NADPH-P-450-Reductase into Phospholipid Vesicles by Cholate Dialysis: The procedure utilized was a modification of the method of Taniguchi et al. (11). The synthetic phospholipids PC $(18:1)_2$, PE $(18:1)_2$ and PP (1-16:0, 2-18:1) were dissolved in organic solvent and placed in a small tube at a molar ratio of 2:1:0.06, and the solvent was evaporated under a stream of dry nitrogen gas. To 1.0 µmol of PL was added 0.4 ml of solubilization buffer which contained 50 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol, and 1 % (w/v) sodium cholate. This mixture was placed in a bath sonicator for 1-2 min until completely clear. Then, P-450 and NADPH-P-450 reductase, at a molar ratio of 1:2 were added to give a final molar ratio of PL to protein of 100-200. In another experiment P-450 was reconstituted in the absence of reductase as described. Following the addition of enzymes, an equal volume of solubilization buffer without sodium cholate was added to lower the concentration of detergent in the mixture to 0.5 % (w/w). This mixture was dialyzed against a 50 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA, and 0.1 mM DTT over a period of 60 h and changed every 12 h.

Phospholipid Exchange: Liver microsomal fractions (0.52 mg of protein and 16 µg of lipid phosphorus) prepared from phenobarbital-treated rats were incubated (in a total volume of 1.0 ml) with 5 mM Hepes (pH 7.4), 5 mM DTT, egg PC liposomes containing 158 µg of lipid phosphorus/ml, and an aliquot of the purified exchange protein (spez. act. 643 nmol of PC transferred per h/mg of protein) for varying amounts of time at 30°C. After incubation, samples were chilled to 4°C and layered over 1 ml of 18 % (w/v) sucrose in 5 mM Hepes (pH 7.4) and centrifuged at 105,000 x g for 60 min at 4°C. The resulting microsomal pellets were homogenized in 0.5 ml aliquots of 0.25 M sucrose and portions were used for the analysis of phospholipid, and protein. Phospholipid transfer from vesicles to microsomes was determined from radioactivity measurements, using choline, methyl-^{14}C-labeled PC (60 mCi/mmol, New England Nuclear).

Determination of the enzyme activities: Scoparone O-demethylation activity was assayed using the continuous fluorimetric assay described by Müller-Enoch et al. (12). The 7-ethoxycoumarin O-deethylase activity was performed by the method of Ullrich and Weber (6). The NADPH-cytochrome P-450 reductase activity was measured by the method of Phillips and Langdon (13).

RESULTS

The results of the catalytic activities of reconstituted monooxygenase systems with $P\text{-}450_{PB\text{-}B}$ and NADPH-P-450 reductase into phospholipid vesicles with different phospholipid compositions are summarized in Table I. In all cases regardless of the incorporation of the $P\text{-}450_{PB\text{-}B}$ and the NADPH-P-450 reductase into phospholipid vesicles reconstituted by the cholate dialysis method composed of a single species of PC, a binary mixture of PC and PE, or a ternary mixture of PC, PE, and PP the specific activity of the 7-ethoxycouma-

TABLE I

The $P\text{-}450_{PB\text{-}B}$-dependent 7-ethoxycoumarin O-deethylase activity in reconstituted phospholipid vesicles of various compositions[a]

Phospholipids	7-Ethoxycoumarin O-deethylase activity (nmol 7-hydroxycoumarin x min^{-1} x nmol $P\text{-}450^{-1}$) — vesicle composition: $P\text{-}450_{PB\text{-}B}$ + NADPH-P-450 reductase	$P\text{-}450_{PB\text{-}B}$[b]
PC $(18{:}1)_2$	34	32
PC $(18{:}1)_2$ and PE $(18{:}1)_2$	30	29
PC $(18{:}1)_2$, PE $(18{:}1)_2$ and PP (1-16:0, 2-18:1)	33	32

[a]The vesicle contained cytochrome $P\text{-}450_{PB\text{-}B}$, NADPH-cytochrome P-450 reductase and phospholipid in a molar ratio of 1:2:200. The incubations were performed as described under Material and Methods. [b]A preparation of $P\text{-}450_{PB\text{-}B}$ incorporated into PL-vesicles by cholate dialysis was utilized. NADPH-P-450 reductase was added to this phospholipid-P-450-complex at a ratio of P-450 to reductase of 1:1.1 for 2 h at 25°C prior to assay.

rin O-deethylase was almost the same (34-30 nmol 7-hydroxycoumarin x min^{-1} x nmol $P\text{-}450^{-1}$). Similar results were obtained when only $P\text{-}450_{PB\text{-}B}$ was incorporated into those phospholipid vesicles by cholate dialysis and then NADPH-P-450 reductase was added to the $P\text{-}450_{PB\text{-}B}$ containing phospholipid vesicles (32-29 nmol 7-hydroxycoumarin x min^{-1} x nmol $P\text{-}450^{-1}$). These results indicate, that the nature of the phospholipids in vesicular systems have no or only a very small effect on the 7-ethoxycoumarin O-deethylase activity.

In order to further examine the role of phospholipid in cytochrome P-450 monooxygenase systems we studied the effect of exchanging phospholipids in microsomal fractions from phenobarbital-treated rats. The phospholipids of the natural microsomal membrane were replaced with egg PC, which is more saturated than microsomal PC, using the purified nonspecific phospholipid exchange protein (Table II).

TABLE II

Scoparone O-demethylation activities of rat liver microsomes before and after exchanging phospholipids by the general phospholipid exchange protein.[a]

Experiment	Time of phospholipid transfer	phospholipid exchange	Specific catalytic activity
	min	%	nmol scoparone demethylated x min^{-1} x nmol $P\text{-}450^{-1}$
1	0	0	0.97
	60	66	0.98
	120	74	0.95
2	0	0	1.11
	60	69	1.08
	120	67	1.16
3	0	0	1.36
	30	71	1.35
	60	70	1.37

[a]The exchange of phospholipids of liver microsomes prepared from phenobarbital-treated rats were carried out as described under "Material and Methods" using three different preparations of rat liver microsomes. The reactions contained 5 units of the general phospholipid exchange protein.

The results of three different microsomal fractions with a 66-74 % exchange of the phospholipids with egg PC had no detectable effect on the scoparone O-demethylation activity of the P-450 in the microsomes.

DISCUSSION

The microsomal mixed function monooxygenase system consisting of NADPH-cytochrome P-450-reductase and P-450, was first dissoziated and reconstituted using different phospholipids by Lu and Coon (14). This and other studies indicated, that phospholipids were necessary for optimal catalytic activity (1,2). Since these enzymes are membrane bound, it was not surprising, that individual lipids in which the isolated enzymes have been studied (in a water soluble system) could markedly influence the catalytic activity of the reconstituted systems.

For optimal catalytic activity (calculated on the basis of the P-450 concentration) it is necessary that all P-450 molecules are complexed with the NADPH-P-450 reductase, because the isolated P-450 enzyme and the reductase enzyme alone, have no monooxygenase activity. Miwa and Lu (15) showed, that the catalytic activity depends on the apparent dissoziation constant of the cytochrome P-450:NADPH-P-450 reductase complex and that dilaurylphosphatidylcholine caused an increase in the steady-state concentration of this catalytically active complex.

Therefore we study the effect of different phospholipid compositions in reconstituted vesicular systems in which the dissoziation constant of the P-450:reductase complex is undoubted lower than in any water containing reconstituted system.

Examining the catalytical activity of the $P\text{-}450_{PB\text{-}B}$ dependent deethylation of 7-ethoxycoumarin in reconstituted phospholipid vesicles of different phospholipid compositions, we found no markedly differences; regardless of the use of phospholipid vesicles in which the $P\text{-}450_{PB\text{-}B}$ and the NADPH-P-450 reductase were incorporated by the cholate dialysis method, or by insertion of the reductase into phospholipid vesicles, containing only $P\text{-}450_{PB\text{-}B}$, prior to assay. The specific activities optained by us were higher (34 nmol 7-hydroxycoumarin x min^{-1} x nmol $P\text{-}450_{PB\text{-}B}{}^{-1}$) than the results reported under similar conditions by others, using the cholate dialysis technique.

To exclude many of the drawbacks and limitations of all of the reconstitution methods, we study the effect of modification of the lipid environment <u>in situ</u> under mild condition in biological membranes. The manipulation of the phospholipid composition of rat liver microsomes with purified phospholipid transfer protein and unilamellar lipid vesicles results in a 74 % exchange. A detectable effect of an alteration of the scoparone O-demethylation activity could not be observed. In all cases examined the exchange of the phospholipid has no detectable influence on the scoparone O-demethylation activity.

The conclusions are, that the exchangable microsomal phospholipids have no effect on the catalytical activity of the P-450 dependent scoparone O-demethylation reaction in the microsomes, and, that the 7-ethoxycoumarin O-deethylase activity of the $P\text{-}450_{PB\text{-}B}$ did not change with the nature and composition of the phospholipids in the vesicles.

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