Branched Phosphatidylcholines Stimulate Activity of Cytochrome P450SCC (CYP11A1) in Phospholipid Vesicles by Enhancing Cholesterol Binding, Membrane Incorporation, and Protein Exchange*

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Phosphatidylcholines (PCs) with branched fatty acyl chains substituted in the two positions of the main chains (branched PCs) have been shown to be potent activators of the side chain cleavage activity of cytochrome P450SCC (CYP11A1) (Schwarz, D., Kisselev, P., Wessel, R., Jueptner, O., and Schmid, R. D. (1996) J. Biol. Chem. 271, 12840–12846). The present study reports on the effect of a series of branched PC on cholesterol binding, membrane integration, and protein exchange in large unilamellar vesicles prepared by an extrusion technique. Enzyme kinetics using vesicles as well as optical titration using a micelle system with the detergent Tween 20 demonstrate that activation is correlated with the fraction of P450SCC in the high spin form. The potency of branched PCs both to activate the enzyme and to induce spin state changes increases with increasing length of both the branched and main fatty acyl chains. We found that the extent as well as the rate of integration of P450SCC into vesicle membranes studied by gel chromatography and stopped flow kinetics were increased by branched PC. Finally, it is demonstrated by measurement of the enzymatic activity in primary and secondary vesicles that branched PCs are potent in retaining a very rapid exchange of P450SCC between vesicles, in contrast to cardiolipin, that partially inhibits this exchange process. The data suggest that different properties of P450SCC in membrane systems including cholesterol binding, membrane integration, and protein exchange are affected by branched PCs and probably by other phospholipids, too, and therefore must be considered in an explanation of the observed high stimulation of activity.

Cytochrome P450SCC (CYP11A1) is an integral mitochondrial enzyme that is located on the matrix face of the inner membrane of mitochondria. It catalyzes the side chain cleavage of cholesterol to yield pregnenolone, the common precursor of steroid hormones. Enzymatic studies employing both solution (micelle) and membrane (vesicle) systems demonstrated stimulation of activity to varying extent by phospholipids depending on the phospholipid used, the kind of reconstitution, size of vesicles, and small amounts of impurities (for a review see Ref. 1 and references therein and Refs. 2–4). Under the lipids used in reconstitution CL1 played a special role mainly because (i) it is a typical mitochondrial lipid and (ii) it represents the most potent activator of P450SCC activity (5–7). With regard to the mechanism of activation, CL is of further interest because of the exceptional structure of its hydrophobic part, i.e. consisting of four fatty acyl chains instead of two of most other phospholipids.

It has been recently reported by us that certain branched PCs that in the structure of their hydrophobic part are similar to CL but have fully saturated acyl chains cause potent activation of P450SCC comparable only with those by CL (8). By systematic variation of the lengths of both the branched and main chains of these lipids and systematic introduction of unsaturation, we found a relationship between the potency of activation of these PCs and their capacity to increase the propensity of the membrane to form nonbilayer phases. As a major determinant of the potency of activation, we suggested the hydrophobic volume and/or headgroup spacing rather than a specific P450SCC-lipid interaction (9).

A pending question remained regarding the way the branched PCs exert their stimulatory role on the enzymatic activity. It has been shown for CL and other activator lipids that their influence is realized via binding to P450SCC followed by an increase of CHL binding to the enzyme (5–7). Based on preliminary optical measurements in DOPC vesicles, we have suggested (8) that the presence of the branched PCs is related to a significant enhanced content of the high spin form of P450SCC, which is connected with a higher portion of P450SCC to be complexed with its substrate CHL, too. In addition, there are other properties of P450SCC in reconstituted systems that may be affected by branched PCs and lead to subsequent activity increase, e.g. membrane integration into and exchange between vesicles and interaction with its redox partners adrenodoxin and adrenodoxin reductase.

In this paper we justify the conclusion on the importance of

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§ The abbreviations used are: CL, cardiolipin; P450SCC, cytochrome P450SCC (CYP11A1); PC, phosphatidylcholine; DMPC, dimyristoyl-PC; DOPC, dioleoyl-PC; CHL, cholesterol; PC(n,m), branched 1,2-diacyl PC; PC(10,6), 1,2-di-(2-hexyl)decanoyl-PC; PC(12,8), 1,2-di-(2-oc-tyl)decanoyl-PC; PC(14,10), 1,2-di-(2-decy)tetradecanoyl-PC; LU-VET, large unilamellar vesicles prepared by extrusion techniques.
increased CHL binding to P450SCC caused by the branched PCs by performing CHL binding experiments to determine the kinetic parameters $K_m$ and $V_{\text{max}}$ for the side chain cleavage reaction as well as by direct spectral titration of the effect of branched PCs on the CHL binding to P450SCC. Further, we show by stationary (gel chromatography) and time-resolved (stopped flow kinetics) experiments that the P450SCC membrane incorporation efficiency is greatly enhanced in the presence of branched PCs with regard to both the rate and the total extent of membrane association. Finally, we demonstrate by measurements of the P450SCC activity in primary and secondary vesicles that the branched PCs are stimulators of or at least retain rapid P450SCC exchange between vesicles (in contrast to CL, which partially inhibits this exchange). It is known that vesicles prepared by detergent removal and sonication often give nonreproducible results because residual detergent and vesicle size heterogeneity have profound influence on the properties of P450SCC mentioned above (2–4). Thus, we used throughout the study large unilamellar vesicles prepared by the extrusion technique (LUVET), thereby taking the additional advantage of the very slow CHL exchange in these vesicles (3) and a fairly homogeneous size distribution of vesicles prepared this way (10).

### EXPERIMENTAL PROCEDURES

#### Materials—
- $[^{14}C]CHL$ and $[^{3}C]DOPC$ were from Amersham Corp.
- DOPC was from Sigma, and bovine heart cardiolipin, DMPC, Tween 20, and CHL from Serva. The $\alpha$-branched fatty acids for the PC$(m,n)$ synthesis were provided by Condea-Chemie (Hamburg, Germany).
- P450SCC, adrenodoxin, and adrenodoxin reductase were purified from bovine adrenocortical mitochondria to electrophoretic purity using specific affinity adsorbents (11, 12). They were stored until use at $-80 \, ^\circ\text{C}$ in relatively high concentrated form in 50 mM phosphate buffer containing 1 mM NaCl, 0.5% sodium cholate, 1 mM EDTA, and 20% (v/v) glycerol. Before use proteins were dialyzed into side chain cleavage assay buffer, aliquoted into small vials, and frozen at $-80 \, ^\circ\text{C}$ (standard buffer, 20 mM Hepes, pH 7.3, 50 mM KCl, 0.1 mM dithiothreitol).

The three dicar-YLCs containing branched fatty acids were synthesized and purified according to the method as described in Ref. 13. The compounds were analyzed with fast atom bombardment-mass spectrometry and $^{1}H$ and $^{13}C$ NMR and steroid emissions were then further studied through Blodgett monolayer investigations. NMR revealed a different headgroup orientation as compared with PC with only two straight carbon atoms. NMR revealed a different headgroup orientation with regard to both the rate and the total extent of membrane association. Finally, we demonstrate by measurements of the P450SCC activity in primary and secondary vesicles that the branched PCs are stimulators of or at least retain rapid P450SCC exchange between vesicles (in contrast to CL, which partially inhibits this exchange). It is known that vesicles prepared by detergent removal and sonication often give nonreproducible results because residual detergent and vesicle size heterogeneity have profound influence on the properties of P450SCC mentioned above (2–4). Thus, we used throughout the study large unilamellar vesicles prepared by the extrusion technique (LUVET), thereby taking the additional advantage of the very slow CHL exchange in these vesicles (3) and a fairly homogeneous size distribution of vesicles prepared this way (10).

#### Preparation and Characterization of P450SCC-containing Vesicles—
Large unilamellar vesicles were prepared by extrusion of lipid suspensions through filters of convenient size according to the procedure described in Ref. 10. Briefly, 12 mg of the phospholipids (in organic solvent) were mixed with usually 6 mol % of (total lipid) CHL including a small amount of $[^{14}C]CHL$ in absolute ethanol in a test tube, and solvents were evaporated under $N_2$ and kept under vacuum for 4 h. After complete removal of the solvent 3 ml of standard buffer were added and vortexed. Then the suspensions were taken through five freeze/thaw cycles and extruded 10 times through two stacked polycarbonate filters having pore size of 100 nm (Nuclepore) using a thermo-mir-Blodgett monolayer investigations. NMR revealed a different headgroup orientation as compared with PC with only two straight carbon atoms. NMR revealed a different headgroup orientation with regard to both the rate and the total extent of membrane association. Finally, we demonstrate by measurements of the P450SCC activity in primary and secondary vesicles that the branched PCs are stimulators of or at least retain rapid P450SCC exchange between vesicles (in contrast to CL, which partially inhibits this exchange). It is known that vesicles prepared by detergent removal and sonication often give nonreproducible results because residual detergent and vesicle size heterogeneity have profound influence on the properties of P450SCC mentioned above (2–4). Thus, we used throughout the study large unilamellar vesicles prepared by the extrusion technique (LUVET), thereby taking the additional advantage of the very slow CHL exchange in these vesicles (3) and a fairly homogeneous size distribution of vesicles prepared this way (10).

#### Stopped Flow Kinetics of Incorporation of P450SCC into Vesicles—
Stopped flow measurements were conducted at 21 °C using a computer-controlled Sequential Stopped Flow ASVD Spectrofluorimeter DX-17MV (Applied Photophysics). According to Ref. 4 we measured the spectral changes caused by the high spin to low spin spectral transition following association of the enzyme with the membrane of vesicles to monitor the rate of incorporation of P450SCC into the vesicle. Usually kinetic runs were performed at two wavelengths, 393 (high spin peak) and 415 nm (low spin peak), followed by analysis of the difference absorption change $A_{393} - A_{415}$. Thus the net spin change was measured. Typically, two to five runs were performed and accumulated to improve the signal to noise ratio. Mixing of P450SCC with (bound CHL) with LUVETs was conducted with syringes A containing 1.4 μM P450SCC in standard buffer. Syringe B contained 1.7 ml total phospholipid (including 6 mol % CHL) in the same buffer. Data analysis was done using the Kinetic Spectrometer Workstation Software package provided by the manufacturer.

#### Optical Titrations—
All optical spectra were recorded on a double-beam UV2101-PC spectrophotometer (Shimadzu, Japan). Complexation of the substrate CHL with P450SCC was monitored by following changes in the Soret maximum at 415 nm (low spin, substrate-free) and at 393 nm (high spin, substrate-bound) according to Ref. 17. Usually difference spectra were followed with P450SCC (2 μM) in 0.1% Tween 20, 0.05 M Hepes, pH 7.3, 0.1 mM dithiothreitol, 50 mM KCl, and the appropriate concentration of the branched PCs in the sample cuvette and protein in the reference cuvette.

#### Enzymatic Activity and P450SCC Exchange Experiments—
Activity of P450SCC was determined as side chain cleavage activity of CHL to produce pregnenolone according to the following assay: 0.25 μM P450SCC and 7 μM adrenodoxin in standard buffer were incubated at 37 °C for 10 min with vesicles consisting of 400 μM phospholipid with 6 mol % CHL including $[^{14}C]CHL$ in a total volume of 0.5 ml. 0.25 μM adrenodoxin reductase was added, and the reaction was initiated by the addition of 2.5 mM NADPH (to a final concentration of 250 μM). After 5 min the reaction was terminated by the addition of 0.1 ml of 0.5 M HCl. The residual substrate and the product were extracted with 2 × 2 ml of methylene chloride, and pregnenolone was separated from unreacted CHL by thin layer chromatography on silica gel 0.25 mm, 20 × 20 cm (Merck) using a solvent mixture of n-hexane/petroleum ether/acetic acid (15:15:1, by volume). Analysis was done by counting the radioactivity of CHL and pregnenolone using a Linear Analyzer LB284 (Berthold). Unless stated otherwise, each analysis was done three times to ensure reproducibility within a standard error of less than 10%.

The P450SCC exchange between vesicles was monitored by an analytic assay according to the following procedure: Primary DOPC-LUVETs were prepared by dialysis of convenient size according to the procedure described in Ref. 10. Briefly, 12 mg of the phospholipids (in organic solvent) were mixed with usually 6 mol % of (total lipid) CHL including a small amount of $[^{14}C]CHL$ in absolute ethanol in a test tube, and solvents were evaporated under $N_2$ and kept under vacuum for 4 h. After complete removal of the solvent 3 ml of standard buffer were added and vortexed. Then the suspensions were taken through five freeze/thaw cycles and extruded 10 times through two stacked polycarbonate filters having pore size of 100 nm (Nuclepore) using a thermostable exchanger (Lipex Biomembranes Inc., Vancouver, BC, Canada). The extruder was thermostated at 30 °C for DMPC vesicles.

Size and homogeneity of the vesicle preparations were quantitated by light scattering using the methods of cumulants as described earlier (14). An average size could be determined to be about 97 nm in diameter similar to values determined previously (10). The parameter Q characterizing the variance of the size distribution is around 0.25. The particle size and size distribution as well as possible changes upon storage were checked by gel chromatography using Sephacryl S-1000. The vesicles were stable in relation to their size and size distribution within the same day when they were used after preparation.

Reconstitution of P450SCC into the preformed vesicles was done according to Refs. 15 and 16 by incubation of the LUVETs (final concentration, 600 μM) with P450SCC (final concentration, 1 μM) or any other desired lipid/protein ratio, except that the incubation was done for 5 min at 37 °C. Incorporation of the P450SCC could be evidenced by gel chromatography using Sepharose 4B (Pharmacia Biotech Inc.).

#### Results

**Effect of Branched PCs on the Activity of P450SCC in DMPC- and DOPC-LUVETs—**Fig. 1 shows the activity of P450SCC in vesicle preparations containing either pure DMPC or DOPC and mixtures of both lipids with PC$(m,n)$ and CL. Structural formulas for the PC$(m,n)$ can be found in Refs. 8 and 9. The data demonstrate the effect of increasing membrane content of the branched PC$(m,n)$ and CL, respectively. In each case the total phospholipid concentrations (in weight%) remained the same. To facilitate direct comparison of the results with previ-
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Figure 1: Effect of branched PC(m,n) on the rate of cholesterol side chain activity of P450SCC in DMPC- and DOPC-LUVETs—The CHL dependence of the activity in DMPC and DOPC vesicles without and with different membrane content of PC(14,10) showed normal Michaelis-Menten saturation behavior. Kinetic parameters determined from the Lineweaver-Burk plots of Fig. 2 are summarized in Table I. According to Ref. 16 we plotted the (inverse) activities versus the lipid/CHL ratio because CHL is present only in the membrane. In pure DOPC we found two phases, a slow phase and a fast phase at higher content of CHL. In DMPC vesicles containing CL, activity also increases with the proportion of CL reaching a maximum corresponding to 15-fold stimulation at about 19 mol % CL. The latter result is consistent with previous findings for CL in sonicated DMPC and DOPC vesicles (5–7).

Effect of PC(14,10) on Binding of CHL to P450SCC in DMPC- and DOPC-LUVETs—The CHL dependence of the activity in DMPC and DOPC vesicles without and with different membrane content of PC(14,10) showed normal Michaelis-Menten saturation behavior. Kinetic parameters determined from the Lineweaver-Burk plots of Fig. 2 are summarized in Table I. According to Ref. 16 we plotted the (inverse) activities versus the lipid/CHL ratio because CHL is present only in the membrane. In pure DOPC we found two phases, a slow phase and a fast phase at higher content of CHL. The latter is characterized by a nearly 7-fold enhanced $V_{\text{max}}$. However, the fast phase is characterized by an increased $K_m$ value characterizing less CHL binding. As evident from the Fig. 2 for both vesicle systems the effect of increasing the amount of PC(14,10) results in diminishing of the slow phase. The stimulatory effect then resulted from a decrease in $K_m$ rather than from a change in $V_{\text{max}}$ which remains at the high value observed for the fast phase. However, the binding of CHL is characterized by an intermediate $K_m$ value. It is interesting that a sufficiently high membrane content of PC(14,10) in DMPC leads to identical kinetic parameters as measured for the DOPC vesicle systems. Pure DMPC vesicles were not used because activities are relatively low. Because of the high $K_m$ values for CHL in this lipid, near saturating levels of CHL cannot be achieved, and determination of $V_{\text{max}}$ is almost impossible.

Effect of Branched PCs and CL on CHL Binding to P450SCC in Tween 20 Solution—Optical Titration Experiments—Preliminary results obtained in DOPC vesicle systems suggested that the stimulation of P450SCC activity by branched PCs is correlated with the fraction of the enzyme in the high spin form (8) as has been discussed earlier for CL and other activator lipids (5–7). The complexation of CHL with P450SCC causes a conversion of heme iron from the low spin to the high spin form, which can be monitored in the Soret region of the absorption spectrum (17, 6). However, the vesicle-reconstituted system was not ideal for quantitative analysis based on optical binding experiments because of the varying turbidities in the different vesicle preparations. Thus we used a mixed detergent (Tween 20)-lipid system in the optical titration experiments with the branched PC(m,n) and CL. Due to the dilution of CHL in the detergent, the addition of detergent results in partial conversion of P450SCC to the high spin form (Fig. 3, inset, solid line), whereas addition of a branched PC results in partial conversion of P450SCC to the high spin form (Fig. 3, inset, dotted line). At constant CHL and Tween 20 concentrations, increasing quantities of the branched PCs in the micellar system produced increasing fractions of P450SCC in the high spin form. Titrations of the enzyme with branched PC(m,n) and CL were performed; Fig. 3 demonstrates the saturation behavior for PC(12,8), which is typical for all other branched PCs and CL, too. To estimate the potency of a certain branched PC for
induction of the high spin state of P450SCC, we calculated parameters from curves like that of Fig. 3 corresponding to the lipid concentration causing half-maximal spin shift. They are 30, 25, and 18 μM for PC(10,6), PC(12,8), and PC(14,10), respectively. Thus, the potency is increasing in the order PC(14,10) > PC(12,8) > PC(10,6). These changes in the high spin shift potency were paralleled by a similar order in the potential of the branched PCs to activate P450SCC, as can be seen from a comparison with the curves of Fig. 1.

Effect of PC(14,10) on the Incorporation of P450SCC into DOPC- and DMPC-LUVETs—The association of P450SCC with the membrane of phospholipid vesicles during ultracentrifugation and gel chromatography is a well known fact indicating a high affinity for the membrane (e.g. Refs. 7, 15, 20). We analyzed the extent of P450SCC incorporation into LUVETs by gel chromatography using Sepharose 4B. Free, unbound P450SCC was retained at more than twice the elution volume of LUVETs of about 100 nm in diameter that elute near \( v_o \) of the Sepharose column (Fig. 4). When incorporated into LUVETs P450SCC coelutes with the vesicles. The curves of Fig. 4 demonstrate the effect of increasing membrane content of PC(14,10) on the incorporation of the protein. Increasing membrane content of PC(14,10) caused increasing amounts of P450SCC to be incorporated into the vesicles. For DOPC above a concentration of PC(14,10) of 20 mol % (and for DMPC above 30 mol %) almost all P450SCC is associated with the membrane, and no P450SCC elutes at a position where free, unbound protein was expected. Usually about 70% protein and almost all lipid were recovered from the column. The shown profile of vesicle elution monitored with \(^{14}\text{C}\)DOPC as marker for lipid was typical for all preaparations independent of the membrane content of PC(14,10). The data were summarized and represented for both DOPC and DMPC in Fig. 5 to facilitate direct comparison with the data from the activity measurements in Fig. 1. Clearly, the increased membrane incorporation efficiency of P450SCC induced by PC(14,10) is paralleled by a similar enhancement in its activity.

Effect of Branched PCs and CL on the Kinetics of P450SCC Incorporation into DOPC-LUVETs—The rate of incorporation of P450SCC into the membrane of vesicles can be directly monitored by measuring the spectral changes caused by the CHL transfer off from P450SCC to the membrane (4, 7). This transfer of CHL is accompanied by the high to low spin spectral change.Stopped flow experiments were performed to measure the kinetics of incorporation of P450SCC into DOPC-LUVETs containing any of the branched PC(\(m,n\)) and CL. In Fig. 6 the kinetic traces resulting from such an experiment for pure DOPC and DOPC containing 30 mol % PC(10,6) are shown as typical examples. Rates of association of P450SCC with the vesicles and the extent of the spectral spin shift were determined from fits of the experimental curve by one or two exponential functions (Table II). The rate of incorporation of P450SCC as well as the extent were found dependent on the phospholipid composition of the vesicles. Pure DOPC vesicles incorporated P450SCC in a single phase process exhibiting first order kinetics at a relatively slow rate. It can be seen from the lower total extent compared with the other systems that P450SCC was not completely incorporated into the membrane.
When the vesicle systems contained PC(m,n) a biphasic incorporation reaction and complete incorporation were found at higher mole fractions of the nonblayer lipids. For all three branched PCs, fits with two exponentials were significantly better, indicating a two phase reaction of the incorporation process. However, in the case of CL-containing membranes the analysis could not be performed in a unique manner; analysis with two exponentials gave no apparently better fit, as it was in the case of the branched PCs. In general, fast and slow reaction phases differ in their rates by approximately 1 order of magnitude. The extent of the fast phase was between 55 and 70% and was dependent on the membrane content of the branched PC. This dependence was studied further for PC(14,10). It can be seen clearly from the data that increasing the PC(14,10) in the membrane did not change either the biphasicity nor the rates of the processes. On the other hand, increasing PC(14,10) content of the membrane produced a higher portion of P450SCC to be incorporated into the membrane in the fast phase. This does not occur at the expense of the slow phase, it rather seems that an increasing content of branched PC(14,10) causes an approximately 30% higher portion of total P450SCC to be associated with the membrane. Note that a similar observation was made in the (stationary) incorporation experiments based on gel chromatography.

Effect of branched PC(14,10) and CL on the Metabolism of CHL in Secondary DOPC-LUVET and P450SCC Exchange—To analyze the effect of branched PCs and CL on the P450SCC exchange between vesicles, the metabolism of CHL was measured in primary and secondary vesicles. According to Ref. 3 CHL exchange between vesicles is very slow with regard to the time scale of the enzymatic activity assay, particularly in the LUVETs used in our experiments (21). Thus, P450SCC incorporated in one class of vesicles (e.g. primary) can only metabolize CHL in the other (e.g. secondary vesicles) after exchange between the vesicles. Fig. 7A clearly shows an almost concomitant increase of the CHL metabolism in primary and secondary vesicles with the rate decreasing a little after 3 min, demonstrating very rapid exchange of P450SCC between DOPC-LUVETs. When the phospholipid composition in both primary and secondary vesicles was changed by inclusion of 30 mol % PC(14,10) we observed a rapid exchange similar to that in pure DOPC and a pronounced enhancement of the CHL metabolism caused by the stimulating potency of the branched PC (Fig. 7B). After 1–2 min the metabolic rate in secondary vesicles seems a little bit decreased. However, the absence of any delay time in the metabolism of the secondary vesicles indicates a rapid P450SCC exchange between primary and secondary vesicles. Inclusion of CL in primary and secondary vesicles, however, results in completely different metabolic behavior (Fig. 7C). The metabolism in secondary vesicles only slowly increases with time, exhibiting a lag time of about 3 min. Nevertheless, total metabolism is high and is realized almost completely in primary vesicles. Even after 5 min, the pregnenolone produced is almost five times less than in primary vesicles, indicating at least partial inhibition of P450SCC exchange in DOPC/CL-LUVETs.

DISCUSSION

Data represented here for LUVETs and in our preliminary previous report for sonicated vesicles (8) show that an exceptionally high stimulation of the side chain activity of P450SCC can be obtained in phospholipid vesicles containing branched PCs as a membrane constituent. The observed activation was found to increase with the mole percent of branched PC and the lengths of both the branched and main fatty acyl chains, i.e. in the order PC(14,10) > PC(12,8) > PC(10,6). The observed rates (V_max > 30 min⁻¹) are only comparable with the highest rates reported so far for micellar systems using Tween 20 as detergent (17, 19) and for DOPC vesicle systems prepared by octylglucoside dialysis (2). In the latter case the high activity results from residual octylglucoside. In the paper presented we studied three of several ways in which the membrane can modulate the activity of P450SCC: (i) direct stimulation of the activity by

![Fig. 6. Stopped flow kinetic run of the incorporation of P450SCC into the membrane of DOPC-LUVETs. Lower trace, pure DOPC; upper trace, with 30 mol % PC(10,6). The data represent the difference of absorbance at 393 and 415 nm in dependence on the time in seconds. The curves are averages of three runs each. Records were performed as described under “Experimental Procedures.” Experimental data were best fitted by a sum of two exponential functions for DOPC/PC(10,6) (upper trace) and a single exponential for DOPC (lower trace) as described in the legend of Table II. Fits are shown by the smooth curves. For clarity the curves were displaced with regard to the y axis. The lower part of the figure represents residuals of the fitted minus experimental data for DOPC/PC(10,6) at an enlarged scale.](image)

### Table II

| Lipid membrane | mol % | Rate a | Amplitude b |
|----------------|-------|--------|-------------|
|                |       | k1     | k2          | A1  | A2   | Total ΔA |
| DOPC           | 100   | 0.13 ± 0.01 | single exp | 90 ± 2 | 90 |
| DOPC/CL        | 15    | 0.10 ± 0.01 | single exp | 190 ± 10 | 190 |
| DOPC(10,6)     | 36    | 0.32 ± 0.02 | 0.030 ± 0.005 | 130 ± 3 | 100 ± 2 | 230 |
| DOPC(12,8)     | 32    | 0.40 ± 0.02 | 0.050 ± 0.005 | 140 ± 4 | 96 ± 3 | 236 |
| DOPC(14,10)    | 10    | 0.30 ± 0.05 | 0.062 ± 0.010 | 95 ± 12 | 78 ± 12 | 173 |
|                | 20    | 0.29 ± 0.04 | 0.042 ± 0.010 | 125 ± 10 | 77 ± 6 | 202 |
|                | 30    | 0.36 ± 0.02 | 0.018 ± 0.004 | 164 ± 4 | 72 ± 7 | 236 |

a The difference of the kinetic runs at 393 and 415 nm (i.e. high spin minus low spin absorption) was fitted to two exponentials: A1×exp(−k1·t) + A2×exp(−k2·t), or one single exponential: A1×exp(−k1·t), total ΔA = A1 + A2. The mean and standard deviation as determined from the fit are shown.

b Fraction of CL or PC(10,6) in mol % of total lipid of the membrane of DOPC-LUVETs.

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P450SCC-CHL interaction, (ii) changes in the integration of P450SCC with the lipid bilayer, and (iii) exchange of P450SCC between vesicles as additional factor controlling the rate of CHL substrate accessibility to P450SCC. Because very small impurities and vesicle size heterogeneity affect these properties, all studies were performed with extruder-reconstituted vesicles providing large unilamellar vesicles relatively uniform in size and free of any impurity. The general conclusion is that all three properties of P450SCC, i.e., interaction with CHL substrate, membrane integration, and protein exchange, were enhanced by branched PCs and therefore may contribute to the observed high activation of P450SCC by branched PCs.

Branched PCs stimulate in a qualitatively similar manner the rate of P450SCC-CHL complex formation in both DOPC and DMPC vesicles. In general, activities in DMPC are very low, a finding reflecting the well known decreased $K_m$ for CHL in DOPC (7, 16). However, DMPC vesicles containing a low portion of branched PC behave similar to pure DOPC vesicles. In both systems activity is characterized by a biphasic nature. At lower CHL content (phase 1, lipid/CHL ratio > 5–7) the activity in both systems is characterized by a $V_{max}$ of ~5–8 min$^{-1}$ and a small $K_m$ of ~0.05. At higher CHL content phase 2 occurred (lipid/CHL > 5–7) with a significantly enhanced $V_{max}$ of ≥30 min$^{-1}$, but a high $K_m$ of ~0.9. Activities are nearly equal in both vesicle systems at higher portions of branched PC in the membrane. Considerable membrane content of branched PC results in diminishing of the phase characterized by low $V_{max}$ value. A new type of fast activity phase 2 occurred indicated by the same very high $V_{max}$ of the fast phase 2 and corresponding to a relatively low $K_m$ (~0.2). The similarity in the activity dependence of pure DOPC and DMPC vesicles containing a low portion of branched PC tempted us to conclude that CHL and branched PCs in some way act similarly and additively. Both compounds seem to impact the membrane with the same structural property leading to activation of P450SCC via enhancement of CHL binding to P450SCC. Biphasicity in activity is not new; it has been already reported earlier for octylglucoside-reconstituted DOPC vesicles and interpreted as resulting from residual octylglucoside (2). An additional second phase of complex formation also has been reported for DMPC/CL vesicles (7). In contrast to the latter observation, in Ref. 5 it has been reported that phospholipids including CL exert their effect by a monophasic process decreasing $K_m$ for CHL leaving $V_{max}$ unchanged. The above mentioned sensitivity of P450SCC properties to very small impurities and vesicle size heterogeneity may in part explain the diversity of previous studies obtained using vesicles prepared by sonication and/or detergent dialysis.

Direct evidence for branched PC-induced enhancement in the P450SCC-CHL complex formation could be provided by the optical titration experiments. Branched PCs cause an increase in the fraction of P450SCC in high spin conformation reflecting an increased P450SCC-CHL complex formation. The potency of the distinct branched PCs in the induction of the P450SCC-CHL complex formation parallels the same order in which the three branched PCs stimulate activity, i.e. in the order PC14,10 > PC12,8 > PC10,6. Taking together these results and the data from enzymatic assays, it is evident that branched PCs and CL stimulate both the CHL binding to and the activity of P450SCC, i.e. the potency to stimulate the activity of P450SCC is correlated with the enhancement in the fraction of the enzyme in the high spin form. The results suggest our previous conclusion that the hydrophobic volume of the branched PCs is an important determinant of the P450SCC-lipid interaction (8, 9).

An important aspect of the reported studies is the finding that branched PCs increase the membrane incorporation efficiency of P450SCC. Our results demonstrate that an increase in the enzymatic activity of P450SCC caused by branched PCs as membrane constituents is paralleled by an increase in the fraction of membrane-bound enzyme. This demonstrates that the changes in P450SCC activity reflect at least partially an altered degree of the membrane incorporation of P450SCC. On the other side, this cannot account for all activation because even after P450SCC incorporation is complete further stimulation in the activity was found, i.e. increase in the activity of the membrane-bound form of the enzyme. Contrary to our data, occasionally complete membrane incorporation of P450SCC has been reported for DOPC vesicles. This is probably due to
heterogeneity with regard to size vesicle preparations used in most cases. Previous reports and our own preliminary experiments show that the degree of P450SCC integration with the membrane also depends on the vesicle size with the tendency to be higher in smaller vesicles (4, 22, 23).

The kinetics of P450SCC incorporation into DOPC vesicles studied by stopped flow experiments showed a biphasic nature of the process when branched PCs are present in the membrane, whereas incorporation proceeds monophasically in pure DOPC. Moreover, the fast phase occurred with a rate about 3-fold faster than observed with pure DOPC where the reaction is monophasic and relatively slow. The fast phase for all three branched PCs was about double compared with the slow phase. Increasing amounts of branched PCs substantially increased the extent of the fast phase incorporation, whereas the rate was independent of the branched PC content. Monophasicity and first order kinetics for the association of P450SCC with a membrane have been earlier reported for sonicated DOPC vesicles (4, 7). However, for CL-containing DOPC vesicles Kowluru et al. (7) reported an additional fast phase with a significantly greater rate constant and an extent equal to the slow phase. As already mentioned above, on the basis of our data we could not unambiguously conclude on a second phase in the case of DOPC-LUVETs containing CL. The biphasic nature of the incorporation reaction found in the presence of branched PCs may be due to several aspects of the process, such as P450SCC-membrane interaction, the extent of insertion required for optimal P450SCC-CHL complex formation, or the miscibility of the different lipid components of the membrane, i.e., lateral distribution of the membrane components. Branched PCs may both accelerate incorporation and increase CHL transfer off (and on) P450SCC by providing either an optimal integration (penetration) in the membrane or domains more or less enriched in branched PCs and/or CHL, which could result in activation. Particularly the latter point must be considered as a possible explanation because recent calorimetric investigations of the thermotropic behavior of DMPC/PC(14,10) dispersions demonstrated a significantly destabilized bilayer structure of the membrane and existence of a second phase at higher PC(14,10) contents. This might be related to separated membrane domains enriched in PC(14,10) and/or CHL (9).

Lastly, the present study establishes that P450SCC exchanges rapidly between DOPC-LUVETs and that inclusion of branched PCs results in even more rapid exchange, whereas CL significantly inhibits P450SCC exchange. Although earlier reports denied P450SCC exchange in soy PC vesicles (15), later such an exchange was found for DOPC vesicles and explained by the specific capacity of P450SCC to bridge between two vesicle membranes thereby using two distinct domains on the protein (3). One domain must be at least partially overlap with the adrenodoxin binding region, probably involving positively charged regions on P450SCC (3, 24). Note that we never observed a lag time for metabolism in secondary DOPC vesicles as reported for small unilamellar DOPC vesicles (3). Moreover, the P450SCC exchange was rapid enough to make the metabolism in primary and secondary vesicles almost equal (Fig. 7A). This again points onto the role that vesicle size (i.e., membrane curvature) may play in such processes. Our finding that CL inhibits the exchange significantly is in accordance with the previously reported observation that acidic phospholipids such as phosphatidic acid greatly slow down P450SCC exchange (3). Because our stopped flow experiments suggest a different nature for the incorporation process for CL, this latter finding may indicate partial inhibition of P450SCC caused by stronger integration of the enzyme in the DOPC/CL membrane.

In conclusion, our study identified and characterized several properties of P450SCC in vesicle systems that are influenced by branched PCs and/or CL. We believe that the results may contribute to a better understanding of the regulation of the P450SCC activity in mitochondria, because all, or at least some, processes studied in vesicle systems may play a role in mitochondria, too. We would like to note the highly curved vesicle structures of the inner mitochondrial membrane and its induction by enhanced P450SCC synthesis reported long ago (25, 26). The P450SCC-membrane interaction is certainly more complex than the original classical concept has been anticipated of phospholipids being cofactors binding specifically to the enzyme. Beside the negative charge of anionic lipids such as CL, there is probably an important role for physical properties of the membrane including the nonbilayer phase propensity (9, 27, 28) and lateral distribution of nonmiscible membrane constituents, which may control P450SCC conformation, P450SCC membrane incorporation and the accessibility of the CHL substrate. There is increasing evidence that the role of lipids in the regulation of the functions of membrane-bound proteins can be assessed only by taking all of the factors into account (29).

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