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An Evaluation of Methods for the Reconstitution of Cytochromes P450 and NADPH P450 Reductase into Lipid Vesicles

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Abstract

Two methods (cholate dialysis and cholate gel filtration) used to incorporate cytochromes P450 and reductase into unilamellar phospholipid vesicles were compared to a standard reconstituted system (SRS) where the proteins were reconstituted with preformed liposomes. Both cholate dialysis and gel filtration methods were comparable in their ability to physically incorporate reductase and either CYP2B4 or CYP1A2 into phospholipid, as determined by the elution of enzymes in the void volume using size exclusion chromatography (MW cutoff – 5,000,000 Da). Incorporation of these proteins was more efficient with both cholate methods than when reductase and P450 were mixed with preformed vesicles (SRS). Using either cholate method, greater than 85% of the P450 was physically incorporated into the phospholipids vesicles, whereas less than 40% of the P450 was physically incorporated into the phospholipids vesicles using the SRS. Catalytic activities of the vesicular preparations of reductase and either CYP1A2 or CYP2B4 also were significantly higher than those resulting from the SRS using dilaurylphosphatidylcholine. Although both cholate dialysis and gel filtration methods improved protein incorporation when compared to preincubation of proteins with preformed liposomes, reductase incorporation was dependent on the relative amount of reductase used. Reductase incorporation was complete at a 0.2:1 reductase to P450 ratio; however, the efficiency of incorporation decreased to <50% at equimolar reductase to P450. Interestingly, reductase incorporation was higher in the presence of CYP1A2 than with CYP2B4. Both cholate methods resulted in the loss of a proportion of spectrally detectable carbon monoxyferrous P450, resulting from incubation of the proteins with detergent.

The cytochromes P450 constitute a superfamily of heme-containing enzymes that display enormous diversity with regards to substrate specificity and catalytic activity (Guengerich, 2001). Catalysis by the eukaryotic P450 enzymes involves a complicated, multi-step reaction cycle that includes two steps in which an electron is transferred from a redox partner. The diflavin protein, NADPH cytochrome P450 reductase (reductase) can transfer both electrons needed for the catalytic cycle (White and Coon, 1980). In some P450 reactions, the second electron of the reaction cycle also can be delivered by cytochrome b₅ (Schenkman and Jansson, 2003).

The P450 enzymes and their associated redox partners of the mammalian drugmetabolizing system are embedded in the membrane of the endoplasmic reticulum (Ortiz-De Montellano, 1995). Lipid plays an important role in the reconstitution of P450 dependent activities after protein purification (Lu *et al.*, 1969). Most in vitro studies for the reconstitution of P450 activities use dilaurylphosphatidylcholine (DLPC) as the lipid component. The reconstitution

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of enzymatic activity involves a concentrated incubation of P450, redox partners, and lipid followed by dilution into the final assay components. The reported preincubation conditions vary significantly from one laboratory to another (Causey *et al.*, 1990). For instance, the lipid is typically dispersed by sonication before mixing with the proteins. This step can involve the use of either a probe sonicator inserted directly into the lipid solution or a milder bath sonicator. Furthermore, the duration of the preincubation of proteins and lipid and the temperatures at which the preincubations are performed differ dramatically from one laboratory to the next. In spite of the lab to lab variability, these standard reconstituted systems (SRS) result in catalytically active preparations in which the enzymes are associated with but may not be anchored physically into the phospholipid liposomes (Ingelman-Sundberg and Glaumann, 1980).

In previous studies, our lab has demonstrated that interactions can occur between different P450 enzymes and reductase when combined in a mixed reconstituted system (Backes *et al.*, 1998; Backes and Kelley, 2003). Furthermore, these interactions have been shown to affect metabolism of certain substrates. In order to more fully understand the effects of these protein-protein interactions, we have sought to utilize a reconstitution method that better simulates the lipid environment in the endoplasmic reticulum by having the proteins anchored in a monolamellar bilayer vesicle. The two most common ways of preparing these vesicular reconstituted systems are the cholate gel filtration (Ingelman-Sundberg and Glaumann, 1980) and cholate dialysis (Taniguchi *et al.*, 1979) methods. Both treatments involve the solubilization of the P450, reductase, and lipids with sodium cholate followed by a detergent removal step. With the gel filtration method, the detergent is removed by size exclusion chromatography, whereas in the dialysis method, the concentration of detergent is reduced by dialyzing the detergent with several changes of a large volume of buffer. In the latter treatment, the dialysis steps encompass 1.5 to 2 days of incubation at 4°C. Upon removal of detergent, the lipids form unilamellar bilayer vesicles with the incorporated proteins (Ingelman-Sundberg and Glaumann, 1980; Taniguchi *et al.*, 1979).

In this study, we examined the effectiveness of the cholate dialysis and gel filtration methods to incorporate reductase and either CYP2B4 or CYP1A2 into phospholipid membranes, and compare the characteristics of these vesicular reconstituted systems with those of the SRS. The methods are compared with respect to the proportion of enzymes physically incorporated into the vesicles and the catalytic activities of the preparations.

MATERIALS AND METHODS

Materials

Bovine liver phosphatidylcholine (BPC) was purchased from Avanti polar lipids (Alabaster, AL). Sodium cholate, 7-ethoxyresorufin, and Sephadex G50 were purchased from Sigma (St. Louis, MO). Benzphetamine was a gift from Upjohn (Kalamazoo, MI). Plasmid Mini, Midi, and Maxi kits were purchased from Qiagen Inc (Valencia, CA). C41 cells were purchased from Avidis SA, Biopole (Clermont-Limagne, France).

Enzyme sources

Rabbit NADPH cytochrome P450 reductase was expressed from a recombinant plasmid, containing the wild type cDNA insert in a vector using the T7 promoter, which was provided by Dr. Lucy Waskell (University of Michigan) and has been described previously (Kelley *et al.*, 2005). Recombinant CYP2B4 was expressed and purified from *E. coli* as described previously (Kelley *et al.*, 2005). CYP1A2 was isolated from β NF treated rabbit liver microsomes as previously described (Coon *et al.*, 1978). P450 levels were determined by measuring the carbon monoxy-ferrous complex (Omura and Sato, 1964).

Enzymatic assays

The activity of CYP2B4 was assessed by measuring the rate of *N*-demethylation of benzphetamine. Formaldehyde production was quantified by measuring fluorescence after the reaction with a modified Nash reagent (de Andrade *et al.*, 1999). The spectrofluorometer was set at excitation and emission wavelengths of 410 nm and 510 nm, respectively. The reactions (1 mL) were incubated at 37°C for 10 min with 335 μ M benzphetamine in the presence of 25 mM potassium phosphate (pH 7.4). Reactions were initiated by the addition of NADPH to a final concentration of 0.8 mM and were stopped with 210 μ L of a 1:1:0.1 mixture of zinc sulfate: barium hydroxide: 50 mM semicarbazide as described previously (Hanna *et al.*, 2000).

The activity of CYP1A2 was determined fluorometrically by measuring the rate of formation of 7-hydroxyresorufin upon the *O*-dealkylation of 7-ethoxyresorufin (Lubet *et al.*, 1985). Excitation was set at 522 nm and emission at 586 nm. The samples (1 mL) were incubated at 37°C for 1 min with 7-ethoxyresorufin (2 μ M), 50 mM Hepes (pH 7.5), 0.1 mM EDTA, and 15 mM MgCl₂. Reactions were initiated with NADPH (final concentration of 0.8 mM) and were stopped with 0.75 mL of ice cold methanol. Reactions were shown to be linear over this time range.

Reconstitution of reductase and P450 into preformed lipid vesicles

Standard reconstituted systems (SRS) were prepared by sonicating DLPC in a bath sonicator to clarity at a concentration of 8 mM in 50 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.1 M NaCl, and 5 mM EDTA. Preformed vesicles were also prepared from BPC by drying a chloroform solution containing 2 mg BPC under nitrogen. The dried lipid was mixed with 0.1 mL of 10% sodium cholate and 0.2 mL of 0.5 M potassium phosphate (pH 7.25) and then sonicated as described above. The sonicated lipid was combined with P450 and reductase in the desired amounts, and the volume was taken to 1 mL with water. The mixture was preincubated for 1.5-2 hr at room temperature, and the activities of the reconstituted systems were then measured (Causey *et al.*, 1990). All enzymatic assays were performed with 0.05 nmol P450 with the reductase concentrations indicated.

Preparation of lipid vesicles by the gel filtration method

For both of the reconstitution methods compared in this study (gel filtration (Ingelman-Sundberg and Glaumann, 1980) and cholate dialysis (Taniguchi *et al.*, 1979)), the procedures described in previous publications were followed as closely as possible. Briefly, lipids, dissolved in chloroform and dried extensively (1.5 to 2 hours) under a stream of N₂. The dried lipid sample was suspended in 200 mM potassium phosphate (pH 7.2) containing 2% sodium cholate to a concentration ranging from 1 to 20 mg/ml of BPC. After clarification 0.5 ml of the lipid suspension was combined with 5 nmol of P450 and reductase (ranging from 1 to 10 nmol) and diluted to a final volume of 1 mL. The lipid/protein suspension was preincubated at 4°C for 1-2 h and was then injected on a Sephadex G50 size exclusion column (2.5 $\times$ 30 cm) that was run at a flow rate of 0.8 mL/min on an Akta FPLC system (Amersham Biosciences, Piscataway, NJ). The elution of the P450 heme was monitored by measuring its absorbance at 405 nm. The void volume from this column was collected and used as the vesicular preparation of P450 and reductase.

Preparation of lipid vesicles by the cholate dialysis method

The lipid used in these reconstituted systems was dissolved in chloroform, dried, and sonicated in 200 mM potassium phosphate (pH 7.2) containing 2% sodium cholate solution as described above for the gel filtration method. After solubilization of lipid in 0.5 ml of sodium cholate, P450 (5 nmol) and reductase (ranging from 1-10 nmol) were added and diluted to 1 ml. The

samples were then immediately transferred to 0.5 to 3 mL slide-a-lyzer cassettes (Pierce, Rockville, MD) and were dialyzed at 4°C for 36 hr with three changes (12 hr each) of 3 L of dialysis buffer containing 50 mM potassium phosphate (pH 7.25), 0.1 mM EDTA, and 0.1 mM dithiothreitol.

Characterization of vesicular preparations of P450 and reductase

The percentages of reductase and P450 actually incorporated in the vesicles were determined by injecting a fraction of the vesicular preparations derived from the different methods on a Tricorn Superose 6 column (Amersham Biosciences, Piscataway, NJ). Using this column, sample components with a molecular weight greater than 5,000,000 Da eluted in the void volume. Proteins that were detected in the void volume were considered to be physically incorporated in the lipid vesicle bilayer preparation.

The amounts of P450 reconstituted into the lipid vesicles were detected from the column eluates by monitoring the 405 nm absorbance of the fractions. The percentages of P450 in vesicles were quantified by measuring the P450 concentrations using the CO difference spectra (Omura and Sato, 1964). CO difference spectra were also used to determine the amount of P450 recovered. The recoveries of reductase in lipid vesicles and after cholate treatment were determined by measuring cytochrome c reductase activity (Phillips and Langdon, 1962).

RESULTS

Elution of individual components of the reconstituted system using Superose 6 chromatography

In order to provide a framework with which to evaluate the characteristics of the reconstituted systems on the Superose 6 columns, the individual components of the DLPC reconstituted systems were injected separately (Fig 1), and monitored at 254 nm. DLPC (40 nmol) itself eluted as a broad peak in the void volume of the column (Fig 1A). An injection of 2.5 nmol of CYP2B4 (Figure 1B) eluted after the void volume at approximately 15 mL, which corresponds to a molecular weight of approximately 250,000 Da. Also shown in Figure 1B are chromatograms showing either the 254 nm absorbance or the 417 absorbance of selected fractions (expressed as percentage of P450 eluted) after injection of an SRS with DLPC and CYP2B4. In the presence of DLPC, approximately 35% of the P450 incorporated in the vesicular fraction. The approximately 250,000 Da aggregate observed when P450 was injected without DLPC was also present in addition to a minor proportion of monomeric enzyme, eluting after 20 mL (Fig. 1B). The reductase (Fig 1C) eluted as a broad peak at approximately 12.3 mL, corresponding to a molecular weight of approximately 700,000 Da when absorption was measured at 254 nm. A peak at approximately 20 mL corresponding to monomeric enzyme was also observed. The reconstitution of reductase into DLPC resulted in incorporation of less than 10% of the reductase into lipid vesicles as indicated by cytochrome c reductase activity of selected fractions. The majority of the reductase remained in an aggregate of approximately 700,000 Da. Association with lipid resulted in the loss of the monomeric sized peak and the appearance of a peak (at approximately 16 mL) that corresponds to a molecular weight of approximately 170,000Da.

Elution of the reconstituted systems by Superose 6 chromatography

The standard reconstituted system (SRS), where 2 μ M CYP2B4 and 2 μ M reductase were preincubated with 500 nmol of sonicated DLPC for 2 hours, was also injected on the column (Fig 2). The elution of total lipid and protein were monitored at 254 nm, whereas reductase and P450 elution was monitored by cytochrome c reductase activity and absorbance at 417 nm, respectively. The chromatogram for 254 nm (detecting total protein and lipid) showed five peaks. The proportion of P450 and reductase in each peak are shown in panel B. Some reductase

(40 %) and P450 (27 %) were incorporated into DLPC vesicles. In addition, approximately 37 % of the reductase and 10% of the P450 were incorporated into 1,300 kDa-sized aggregates. Peaks eluting at 14.4 mL and 17.4 mL corresponding to aggregates of 350,000 Da and 115,000 Da, respectively contained P450 with only minor amounts (< 10%) of reductase. The smaller aggregate apparently contained significant amounts of DLPC because of the strong absorbance at 254 nm. Peak 5 was composed of mainly monomeric reductase.

This experiment was repeated with dimyristoylphosphatidylcholine (DMPC), because of a previous report (French *et al.*, 1980) that proteins eluted with the void volume when reconstituted with DMPC, but not DLPC. In our hands, the use of DMPC did not improve incorporation of these proteins, but produced results similar to those observed with DLPC (data not shown); however, we were able to observe some incorporation of protein into the void volume by using the longer chain BPC (Fig 3A).

Next, an equimolar ratio of reductase and either CYP1A2 or CYP2B4 were reconstituted with BPC using the cholate dialysis method as described in Materials and Methods. Representative chromatograms resulting from the elution of these systems from the Superose 6 column were obtained by measuring the absorbance at 405 nm and are shown in Fig 3. A chromatogram of a reconstituted system prepared by adding CYP2B4 and reductase to sonicated BPC (SRS) also is shown. The profiles obtained from the cholate dialysis preparations with either CYP1A2 or CYP2B4 were very similar and showed that most of the absorbance at 405 nm (representing the elution of P450) was present in the void volume of the column. This finding indicates that the P450 is physically associated with the phospholipid vesicles. In contrast, the standard reconstitution method using BPC instead of DLPC showed greater accumulation in the void volume than was observed with DLPC. However, even with phospholipids having longer fatty acid chains, less than a third of the P450 eluted at a size consistent with vesicle incorporation. The three chromatograms in Fig 3 also contain a second peak (indicated as peak 2) that eluted at about 12 mL (approximately 700 kDa). The standard reconstituted system with BPC also contained a third prominent P450 peak, which eluted at approximately 15.5 mL (peak 3, about 180 kDa). This peak was not visible in the chromatograms derived from cholate dialysis preparations. The lower amount of P450 in the void volume of the SRS showed that simply mixing the proteins into preformed vesicles did not lead to efficient physical incorporation of proteins into the liposomes.

Although the 405 nm absorbance indicated that the P450 was successfully incorporated in vesicles using the cholate dialysis method, it was observed that the proportion of reductase incorporated in these liposomes was low when an equimolar ratio of P450 and reductase was used. Figures 3B-3D show the percentages of P450 and reductase, respectively obtained in each of the three peaks shown for the three chromatograms of Fig 3A. Under optimal conditions for both P450 enzymes, only 40% and 65% of the reductase were incorporated in the void volume of the Superose 6 column with CYP2B4 and CYP1A2, respectively. The remaining reductase was recovered mainly in peak 2. It is interesting to note that reductase was more efficiently incorporated into phospholipid in the presence of CYP1A2 than CYP2B4.

We investigated whether the unincorporated reductase is actually the proteolyzed form of the enzyme (Black and Coon, 1982). Samples from peaks 1 and 2 from Fig 3 were subjected to polyacrylamide gel electrophoresis and stained with Coomassie Blue. No proteolytically cleaved reductase was eluted from the void volume of the size exclusion column. Although small amounts of the shortened form of the reductase were found in peak 2 from the column, this was present as a contaminant of the starting sample (data not shown). These results demonstrate that (1) only a portion of the full-length reductase is physically incorporated into the lipid vesicles, and (2) the proteolytically cleaved 71kDa form of the reductase cannot incorporate into the void volume lipid. The eluant collected as peak 2 from the column

contained both forms of the reductase; however, the intensity of the band representing the shortened form of reductase was much less than that of the full-length form contained in this fraction (approximately three times lower). Thus, the incorporation of reductase into the liposomes is not solely a function of proteolysis.

Comparison of the cholate gel filtration and cholate dialysis methods for reconstitution of the P450 monooxygenase system

Because of the incomplete incorporation of reductase in the P450-containing vesicles using the cholate dialysis (CD) method, we also prepared vesicles by the cholate gel filtration (GF) method. Figure 4 compares the two methods using identical conditions on the basis of the percent enzyme eluted in the void volume of the Superose 6 column, the percent recoveries of reductase activity and carbon monoxyferrous P450 after treatment with sodium cholate, and the rate of benzphetamine metabolism by the reconstituted systems.

Both CD and GF methods resulted in indistinguishable preparations with respect to protein incorporation when tested with identical lipid and detergent concentrations. Importantly, the preparations from both methods had comparable proportions of reductase eluting in the void volume at equimolar reductase to P450 ratios. However, as shown in Figure 3, both the CD and GF reconstituted systems were superior to a standard reconstitution with BPC in terms of the P450 recovered in the void volume. One shortcoming of both of the CD and GF methods was that a large proportion of the CO-reactive ferrous heme of P450 was lost upon treatment (Fig 4A). The amount lost in the cholate dialysis procedure was significantly greater than that lost using the gel filtration method. Thus, adjustments to the starting enzyme concentrations are required to achieve a desired P450 concentration in these vesicle preparations.

The rates of benzphetamine metabolism were examined in each of the reconstituted systems (Fig 4B). The catalytic activity of a standard reconstitution system with BPC is also shown for comparative purposes. Physical incorporation of the enzymes into BPC led to much higher rates of benzphetamine metabolism than was observed with the SRS using BPC.

In an effort to determine if the loss of CO-reducible P450 was due to incubation of the proteins in the presence of cholate, experiments were conducted to examine the effect of prolonged cholate incubation with the P450 enzymes. Figure 5 compares the recoveries of ferrous carbon monoxy-CYP2B4 after various durations of incubation at 4°C in the presence and absence of 1% sodium cholate. The loss of the 450 nm-absorbing species was observed upon incubation with the cholate for the shortest time period (1.5 hr). In addition, the loss did not increase significantly with time of incubation with cholate. Thus, it appears a certain proportion of the CYP2B4 is susceptible to damage upon exposure to the cholate. Despite the loss of ferrous carbon-monooxy P450 after incubation with cholate, there did not appear to be a significant loss of heme from the enzyme because the absorbance at 417 nm was not significantly diminished.

Effect of BPC concentration on protein incorporation into the lipid vesicles

Reconstituted systems were prepared using different concentrations of BPC in order to determine if alterations in lipid to protein ratio would influence reductase and P450 incorporation (Fig. 6). Generally, reductase and CYP2B4 incorporation were not greatly affected by increases in BPC, showing small decreases in CYP2B4 incorporation at the lowest and highest BPC to CYP2B4 ratios examined (Fig 6A). In contrast, benzphetamine activity increased as the relative amount of PC was elevated to 500:1 (BPC:P450), but was diminished to less than 25% of the maximal rate at 2500:1 (BPC:P450) (Figure 6B). This decrease in activity is consistent with the 'dilution' of CYP2B4 and reductase into the large volume of phospholipid.

In most studies where the catalytic activities of purified proteins are measured, the proteins are reconstituted with DLPC, generally using some variation of the SRS described in this paper. Figure 6B also compares benzphetamine demethylation rates of the void volume from the Superose 6 FPLC column (i.e. containing physically incorporated proteins) and SRS preparations with DLPC. The SRS preparations with DLPC, which were prepared with identical lipid to P450 ratios as those used in the cholate dialysis preparations, had lower catalytic activities at all lipid to P450 ratios. Furthermore, the catalytic activities of the reconstituted systems with DLPC decreased dramatically with increasing lipid to P450 ratios and no activity was observed at a 2500:1 DLPC:P450 ratio. In other experiments (data not shown), we also compared the rate of benzphetamine N-demethylation by the cholate dialysate to that of the void volume from the Superose 6 column after injection of the dialysate. No significant differences in catalytic activities were observed for these samples. Thus, the presence of unincorporated reductase does not greatly influence metabolism of this substrate by CYP2B4.

The effect of increasing lipid on the incorporation of CYP1A2 and reductase was also examined (Figure 6C). Again, increases in the lipid to P450 ratio had little effect on the incorporation of CYP1A2 or reductase into the vesicles. In contrast to the results shown with CYP2B4, CYP1A2-dependent 7-ER deethylation increased as the lipid:P450 ratio was elevated to 500:1. Further increases in BPC:P450 caused a decline in the EROD activity (Fig. 6D). As observed with SRS preparations containing DLPC, reductase and CYP2B4, the rate of CYP1A2-mediated metabolism of 7-ER was decreased as lipid to P450 ratios increased above 250 to 1. No catalytic activity was detectable in the reconstituted systems containing CYP1A2, reductase and DLPC at a lipid to P450 ratio of 2500 to 1. These results demonstrate that although the fraction of protein incorporated into the vesicles is relatively insensitive to alteration in the BPC:P450 ratio, the catalytic activities vary considerably with elevated lipid. These results are consistent with decreases in reductase:P450 complex formation when the proteins are 'diluted' into larger quantities of lipid, and also show that actual physical incorporation of proteins into the phospholipid (as by the CD and CGF methods) diminishes this dilution effect.

Effect of the reductase to P450 ratio on characteristics of reconstituted systems

CYP2B4 and various concentrations of reductase were reconstituted with PC using the CD method. Interestingly, higher proportions of reductase were incorporated in the vesicles at low reductase to P450 ratios (Fig 7A). Essentially all of the reductase was incorporated when the starting ratio of reductase to P450 was 1 to 5. This proportion decreased steadily up to equimolar concentrations of the reductase and P450 where it leveled off with approximately 40% of the reductase being physically associated with the high molecular weight fraction. Conversely, the percentage of incorporated P450 was greater than 75% at all ratios tested. As expected, benzphetamine demethylation was increased as a function of the reductase concentration using the vesicular reconstituted systems, approaching saturation at a reductase:P450 ratio between 1 and 2. Figure 7B represents the rate of benzphetamine demethylation as a function of reductase after correction for efficiency of incorporation and cholate-dependent degradation. From these data, an apparent $K_m^{\text{Reductase}}$ of 33 nM and V_{max} of 154 nmol/min/nmol CYP2B4 were obtained. Figure 7C shows the rates of benzphetamine N-demethylation by SRS preparations with reductase, CYP2B4, and DLPC at comparable lipid to P450 ratios as those used in the cholate dialysis preparations shown in Figure 7B. The apparent $K_m^{\text{Reductase}}$ and V_{max} of the SRS preparations with DLPC were 42 nM and 87 nmol/min/nmol CYP2B4, respectively. Thus, the catalytic activity of the CYP2B4 monooxygenase system when the enzymes are physically incorporated into BPC vesicles is nearly double that observed with SRS preparations using an equivalent concentration of DLPC.

DISCUSSION

In an effort to simulate a lipid environment that is more representative of the endoplasmic reticulum membrane, we have characterized two methods previously described for the incorporation of P450 and reductase into unilamellar bilayer lipid vesicles, the cholate dialysis method (Taniguchi *et al.*, 1979) and the gel filtration method (Ingelman-Sundberg and Glaumann, 1977). In our hands, both methods produced similar results by forming catalytically active phospholipid vesicles within which the enzymes appeared to be physically anchored. Furthermore, we could adjust conditions to obtain vesicles where we could vary both the lipid:P450 and reductase:P450 ratios.

Although reductase was efficiently incorporated into phospholipid vesicles when low reductase to P450 ratios were used (1 to 5), the proportion of incorporated reductase decreased at higher reductase to P450 ratios. Interestingly, CYP1A2 appears to better facilitate the incorporation of reductase into the lipid membrane than did CYP2B4. Consequently, in order to obtain vesicles with higher reductase to P450 ratios, higher quantities of reductase would need to be added when reconstituting these proteins. Interestingly, the efficient incorporation of the reductase corresponds with the ratio of reductase: P450 observed *in vivo* (1:10-20) (Estabrook *et al.*, 1971; Peterson *et al.*, 1976).

Both cholate methods caused a loss of spectrally detectable carbon monoxy-ferrous heme in the P450 enzyme. The source of the loss in detectable P450 is related to the incubation of P450 in the presence of sodium cholate. Our findings demonstrate that the P450 monooxygenase activities were about 12 fold greater when the enzymes were physically incorporated into the bilayer environment than when they were preincubated with previously sonicated BPC. In fact, the activities of proteins incorporated by the gel filtration and cholate dialysis methods even exceeded those observed with DLPC prepared by the SRS. This finding was consistently displayed with a variety of lipid and reductase concentrations and with both CYP2B4 and CYP1A2. The maximal rate of benzphetamine N-demethylation by CYP2B4 was 154 nmol benzphetamine/min/nmol P450 as determined by the cholate dialysis method. This is significantly greater than the rate with the standard reconstitution system using BPC (Fig. 4B) and even exceeds the activities reported using DLPC, approximately equal to 50 nmol/min/nmol (Fig. 7C) (Harris *et al.*, 2004; Cawley *et al.*, 1995). These results suggest that the physical incorporation of reductase and P450 into phospholipid enhances the catalytic activity by bringing the proteins together and effectively increases their relative concentrations due to their being anchored to the membrane. Thus, the physical incorporation of these enzymes into vesicles may provide a more favorable environment for catalysis.

The cholate dialysis and gel filtration methods are effective techniques for physical incorporation of reductase and P450 enzymes into phospholipid vesicles. However, these methods have several shortcomings. First, both methods are more time consuming than standard reconstitution procedures. Both the gel filtration and cholate dialysis methods require at least 1 day to be completed, as compared with 2 h for the SRS. Second, they lead to a generalized destruction of a fraction of the P450, due primarily to the incubation of the enzymes in cholate. Third, the efficiency of reductase incorporation is dependent on the relative amount of reductase in the incubation, being completely incorporated at 0.2:1 reductase to P450, but decreasing to less than 60% at a 1:1 ratio. The cholate gel filtration method also suffers in that the final preparation is diluted with respect to the enzyme concentration (P450 concentrations are generally less than 1 μ M), limiting the types of studies that can be readily performed. Furthermore, the cholate gel filtration method limits the ability to perform direct comparisons of different reconstitution conditions because samples can only be prepared one at a time. The cholate dialysis method allows for several reconstituted systems to be prepared at once, and the resulting preparations can be made at a relatively high concentration of enzyme (P450

concentrations of approximately 5 μM). Although none of these drawbacks are insurmountable, they do need to be considered when these techniques are used for reconstitution of these membrane proteins.

We are presently investigating whether we can improve the methods for reconstitution of P450 enzymes and reductase by increasing the proportion of reductase physically incorporated into the vesicles and limiting the loss of ferrous carbon monoxide P450 complex while decreasing both the time required for sample preparation and the sample dilution associated with the preparation method.

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Abbreviations

BPC	bovine phosphatidylcholine
CD	cholate dialysis method of reconstitution
DLPC	dilauroylphosphatidylcholine
DMPC	dimyristoylphosphatidylcholine
GF	cholate gel filtration method of reconstitution
SRS	standard reconstitution system

Figure 1. Interactions of reductase or P450 with DLPC in standard reconstituted systems determined by gel filtration

Standard reconstitutions with DLPC were prepared and injected on the Superose 6 column as described in Materials and Methods. (A) Elution of 625 nmol of DLPC as monitored by absorbance at 254 nm. The approximate molecular weights of the peaks are shown at the top of the figure. (B) Elution of either 5 nmol of CYP2B4 monitored by absorbance at 405 nm or 1 nmol of CYP2B4 in an SRS with 250 nmol of DLPC as monitored at 254 nm. The elution of P450 from the preparation with DLPC is expressed as the percentage of total P450 injected and was determined by measuring the absorbance of selected fractions at 417 nm. The points on the chromatogram show where fractions were taken. (C) Elution of either 5 nmol of

reductase monitored at 254 nm or 1 nmol of reductase in an SRS with 250 nmol of DLPC as monitored at 254 nm or by cytochrome c reduction. The elution of reductase in the mixed system with DLPC is expressed as the percentage of total reductase injected as determined from the relative cytochrome c reductase activities of the different fractions. Points on the chromatogram indicate where fractions were taken.

Figure 2. Gel filtration eluate of a standard reconstituted system combining reductase, CYP2B4 and DLPC

CYP2B4 and reductase (1 nmol of each) were reconstituted with previously sonicated DLPC (DLPC:P450 ratio was 250:1) as described in Materials and Methods and injected on the Superose 6 size exclusion column. (A) Elution of the components of the reconstituted system was followed by monitoring absorbance at 254 nm. The molecular weights of the peaks are indicated at the top of the figure. (B) The percentages of the total P450 and reductase eluted from the column in each of the five peaks of panel A are shown in "B". The percentages of P450 and reductase were determined from the absolute absorbance at 417 nm and the relative cytochrome c activities, respectively, of selected fractions of the eluant.

Figure 3. Gel filtration chromatograms of reconstituted systems prepared by different methods
 Reconstituted systems were prepared as described in Materials and Methods. Each system, containing 5 nmol of P450 (CYP1A2 or CYP2B4 as indicated), 5 nmol of reductase, and 2500 nmol BPC, were prepared by the cholate dialysis (CD) or by a standard procedure (SRS) involving sonication of BPC in the absence of detergent prior to addition of the proteins. (A) Elution profile of the reconstituted systems monitored at 405 nm. The molecular weights of the eluted peaks are indicated at the top of the figure. (B) Percentages of the total P450 and reductase in each of the peaks shown in panel A for the CD reconstitution with CYP2B4, reductase, and BPC. P450 and reductase levels were estimated using 417 nm absorbance and cytochrome c reductase activity, respectively, of selected fractions collected after injection of

the reconstitution. Panel (C) The percentages of P450 and reductase in each of the peaks of the chromatogram of the CD reconstitution with CYP1A2. (D) The percentages of the enzymes in the three peaks of the chromatogram for the BPC SRS with CYP2B4.

Figure 4. Comparison of CYP2B4-containing reconstituted systems using the cholate dialysis, gel filtration, and SRS methods

Preparations using 5 nmol each of P450 (either CYP2B4 or CYP1A2 as indicated) and reductase with 1% cholate and 2500 nmol of PC were prepared by the cholate dialysis and gel filtration methods as described in Materials and Methods. (A) Comparison of the methods on the basis of the percentages of P450 and reductase eluting in the void volume from the Superose 6 FPLC column, and the percentages of reductase activity and carbon monoxy-ferrous P450 that were recovered from the original preparation following treatment with sodium cholate. (B) Rates of benzphetamine N-demethylation by the different types of reconstituted systems. Also shown in this panel are results using the “standard” method of reconstitution with 2500 nmol of BPC that was sonicated before adding to the P450 and reductase (BPC SRS). The BPC SRS was assayed for benzphetamine N-demethylation before the sample was run on the Superose 6 FPLC column. * indicates that the average is statistically different from that of the corresponding gel filtration preparation using an unpaired student t-test ($P < 0.001$).

Figure 5. Effect of cholate on the stability of the ferrous carbon-monooxy CYP2B4 complex
CYP2B4 (5 μ M) in 100 mM potassium phosphate (pH 7.4) was incubated at 4°C in the presence or absence of 1 % sodium cholate. The ferrous carbon-monooxy complex was monitored with time as described in Materials and Methods.

Figure 6. Effects of lipid concentration on the incorporation of reductase, P450

Vesicular preparations of reductase and PC with either CYP2B4 (A and B) or CYP1A2 (C and D) were prepared by the cholate dialysis method. (A) The effect of lipid concentration on the elution of CYP2B4 and reductase in the void volume of the Superose 6 column. (B) Benzphetamine demethylation of the reconstituted preparations with CYP2B4. (C) The effect of lipid concentration on the elution of reductase and CYP1A2 in the void volume of the Superose 6 column. (D) 7-Ethoxyresorufin-O-dealkylation of the reconstituted systems containing reductase and CYP1A2. In panels B and D, the catalytic activities of SRS preparations made with the indicated DLPC:P450 ratios are also shown for comparison. The catalytic activities of samples collected from the void volume of the Superose 6 FPLC column

after injection of the reconstitutions prepared by cholate dialysis are indicated as “Superose void”. “N.D.” indicates that activity was not detected at the 1000:1 lipid:P450 ratio. Thus, a DLPC SRS reconstitution was not prepared with CYP1A2, reductase and lipid at the 2500:1 lipid to P450 ratio. * indicates that the average is statistically different from that of the corresponding “Superose void” preparation using an unpaired student t-test ($P<0.001$). ** indicates that the average is statistically different from that of the corresponding “Superose void” preparation using an unpaired student t-test ($P<0.01$)