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Physical Incorporation of NADPH-cytochrome P450 Reductase and Cytochrome P450 into Phospholipid Vesicles using Glycocholate and Biobeads*

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Abstract

In a previous study from our lab (*Drug Metab. Disp.* (2006) **34**, 660–666), we found several limitations with published methods (cholate gel filtration and cholate dialysis) for the incorporation of cytochromes P450 and P450 reductase into phospholipid vesicles. We found that a significant proportion of reductase was not incorporated in the vesicles when the amount of reductase was equal to or greater than that of CYP2B4 in the systems reconstituted with phosphatidylcholine. Furthermore, implementation of these methods compromised the ability of the CYP2B4 to form a ferrous carbon monoxy complex. In the current study, a comparison of results using the detergent-dialysis method with five similar detergents having the “bile salt” ring structure finds that glycocholate results in the greatest incorporation of reductase and the least loss in carbon monoxy ferrous CYP2B4 complex. The method is further improved by using Biobeads SM-2 to remove detergent instead of the lengthy dialysis procedure or the size exclusion chromatography which significantly dilutes the protein and lipid concentrations of the preparation. The method is shown to be applicable over a range of lipid to CYP2B4 ratios; and by employing assay methods for total lipid, reductase, and CYP2B4, this improved reconstitution method resulted in increased incorporation efficiencies while minimizing the protein degradation inherent with these procedures.

INTRODUCTION

The cytochromes P450 (P450) constitute a superfamily of heme-containing enzymes that display enormous diversity with regards to substrate specificity and catalytic activity (Guengerich, 2001). The enzymes utilize a complicated reaction mechanism that requires the physical coupling of separate proteins to deliver a single electron at two different steps of the catalytic cycle. The diflavin protein, NADPH cytochrome P450 reductase (reductase) can transfer both electrons needed for the catalytic cycle (White and Coon, 1980), whereas the second electron also can be transferred by cytochrome b₅ (Schenkman and Jansson, 2003). The mammalian, drug-metabolizing P450s and their associated redox partners are anchored in the lipid membrane of the endoplasmic reticulum.

Lipid plays an important role in catalysis by these enzymes as the *in vitro* reconstitution of the active monooxygenase system from the purified proteins requires the addition of a lipid milieu, usually dilauroylphosphatidylcholine (DLPC) (Lu *et al.*, 1969; Strobel *et al.*, 1970). The lipid

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has been postulated to both function as an effector for catalysis (Ingelman-Sundberg, 1977) and provide a framework to allow for the correct orientation and binding of the P450s with the redox partners and substrates (Ingelman-Sundberg *et al.*, 1983; Causey *et al.*, 1990; Taniguchi and Pyerin, 1988). Furthermore, variations in the composition of the lipid milieu that is added to these *in vitro* reconstituted systems have dramatic effects on the stability and the catalytic activity of the P450 enzymes (Ingelman-Sundberg *et al.*, 1996; Ingelman-Sundberg *et al.*, 1981; Ahn *et al.*, 2005).

The composition of lipids used also affects the manner in which proteins of the P450 system are organized in the membrane. Large proportions of the reductase and P450 enzymes in conventional or standard reconstituted systems using sonicated DLPC have been reported to be peripherally associated with micellar and/or vesicular liposomes (Reed *et al.*, 2006; Autor *et al.*, 1973). However, reconstitution methods that use longer chain phospholipids result in higher amounts of reductase and P450 enzymes being physically incorporated into monolamellar, bilayer phospholipid vesicles (Ingelman-Sundberg and Johansson, 1980; Taniguchi *et al.*, 1979; French *et al.*, 1980). The physical incorporation of reductase and P450 into phospholipid bilayers has been shown to result in preparations with different (higher) levels of catalytic activity (Ingelman-Sundberg *et al.*, 1996; Reed *et al.*, 2006). These commonly implemented methods use sodium cholate to disperse lipids and enzymes followed by a detergent removal step (either dialysis or gel filtration).

The existing methods for these types of systems are limited by several practical considerations. The gel filtration method (Ingelman-Sundberg and Johansson, 1980) results in significant dilution of the protein preparation and samples can only be made one at a time which limits direct comparisons of different conditions. The dialysis procedure (Taniguchi *et al.*, 1979) is limited by the fact that samples must be dialyzed for several days with 3 to 4 changes of large volumes of dialysate. Besides being cumbersome and time consuming, this extended incubation has the potential to compromise the stabilities of the enzymes and lipids in the reconstituted system. Previously (Reed *et al.*, 2006), we observed several limitations with implementation of both the cholate dialysis and cholate gel filtration methods for the vesicular incorporation of reductase and P450. For example, the incorporation of reductase was inefficient at higher reductase:P450 ratios. Although at subsaturating reductase ratios, the incorporation was complete. At a reductase:P450 ratio of 1:1, as much as 40% of the reductase resisted incorporation into the vesicles. In addition, the use of sodium cholate to solubilize the protein and lipid constituents prior to either the dialysis or gel filtration steps resulted in a significant loss of the ferrous CYP2B4 carbon-monooxygenase complex (even though total heme measured by 417 nm absorbance was unchanged). Thus, the goal of the present study was to improve these aspects of the published methods by comparing a variety of bile salt-derivative detergents using the dialysis procedure for the reconstitution of reductase and CYP2B4 into vesicles of bovine liver phosphatidylcholine (PC) (Taniguchi *et al.*, 1979). A method is described in which the detergent is removed from the mixture of detergent, lipid, and proteins by batch incubation with Biobeads SM2 (Holloway, 1973; Levy *et al.*, 1990). This method improves several aspects of the commonly used methods for the physical incorporation of reductase and CYP2B4 into phospholipid vesicles. Finally, the method was tested over a range of phospholipid:CYP2B4 ratios in order to determine its practicality when the protein and lipid components are varied.

MATERIALS AND METHODS

Materials

Bovine liver phosphatidylcholine was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Sodium cholate was purchased from Sigma-Aldrich (St. Louis, MO). Benzphetamine was a gift from Upjohn (Kalamazoo, MI). Other detergents used in this study were purchased from

Calbiochem (La Jolla, CA). 7-ethoxy-4-trifluoromethylcoumarin and 7-hydroxy-4-trifluoromethylcoumarin were purchased from Molecular Probes (Eugene, OR). All other reagents were obtained from Sigma-Aldrich at the highest available purity.

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). P450 levels were determined by measuring the carbon monoxy-ferrous complex (Omura and Sato, 1964).

Preparation of lipid vesicles by the detergent-dialysis method

Lipid vesicles were prepared by the detergent-dialysis method according to published methods (Taniguchi *et al.*, 1979; Reed *et al.*, 2006). Briefly, bovine liver phosphatidylcholine (approximately 1250 nmol), was dissolved in chloroform, lyophilized overnight, and bath-sonicated until clarified in a 0.1 mL solution of 250 mM of either Hepes or potassium phosphate buffer (pH 7.2) containing 75 mM MgCl₂ and 5% (w/v) of one of the detergents listed in Figure 1. The solubilized lipid solution was then mixed with a solution containing the enzymes. It was found that less of the “CO-reactive” CYP2B4 was lost if the detergent solution was added gradually. Thus, the detergent solution was added to 0.4 mL of a solution of the CYP2B4 (2.5 nmol) and the reductase (ranging from 0.5 – 5 nmol) in four equal aliquots ($\approx$ 0.025 mL each). Nitrogen was layered over the solution to prevent oxidation of the phospholipids and the samples were gently inverted to mix the solutions after each addition of the lipid-detergent mixture. 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.

Preparation of lipid vesicles using glycocholate and Biobeads SM-2 for detergent removal

The amount of phosphatidylcholine indicated, usually 1 mg, was dissolved in chloroform and lyophilized overnight. The dried lipid was suspended and added to the CYP2B4 and the reductase as described above in the preparation of reconstituted systems using the cholate dialysis method. After the detergent-lipid solution was added to the solution of the CYP2B4 and the reductase, the mixture incubated at 4°C for 1 hr before adding 0.25 g of Biobeads SM-2 (prepared as described previously (Holloway, 1973)). The sample was rocked for 2 hr 4°C, and the solution and a 0.4 mL volume wash of the beads with 0.05 M Hepes buffer (pH 7.25) were drawn up with a 32 gauge needle and then filtered through a 5 micron syringe filter (Osmonics, Minnetonka, MN). In most cases, the final volume of the reconstituted system recovered from the beads was about 0.65 mL.

When different concentrations of lipid were used in the reconstituted systems, the concentration of detergent needed to solubilize the suspension was adjusted according to an approach described previously (Jackson *et al.*, 1982; Bayerl *et al.*, 1986). Briefly, using the CMC of the detergent and the fact that the solubilization of a membrane occurs when the detergent-binding sites in the liposomes are saturated and the monomeric detergent concentration approaches the CMC (Jackson *et al.*, 1982), we estimated the amount of detergent bound to the lipid (as opposed to monomeric detergent in solution) from the amount of detergent needed to optimize the incorporation of 2.5 nmol of enzyme into the vesicles obtained from 1250 nmol of lipid (the lipid-bound detergent concentration = the optimal detergent concentration – the CMC of the detergent). The lipid-bound detergent to lipid ratio determined under the optimized condition could then be used to calculate the amount of detergent that would be bound to any

given amount of lipid, and the amount of detergent needed to solubilize the lipid would be the lipid bound detergent concentration added to the CMC of the detergent. We found that vesicles were not efficiently formed when the concentration of the lipid used in the procedure was less than 1250 μM . The enzymatic activities of the reconstituted systems prepared under these conditions were extremely low (data not shown).

Characterization of vesicular preparations

The percentages of reductase and P450 actually incorporated in the vesicles were determined by injecting a fraction of the vesicular preparations on a Tricorn Superose 6 column run on an Akta FPLC system (Amersham Biosciences). Using this column, sample components with a molecular weight greater than 5,000,000 Da were 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 (French *et al.*, 1980; Reed *et al.*, 2006)

The amounts of P450 reconstituted into the lipid vesicles were detected from the column eluates by manually monitoring the 417 nm absorbance of the fractions using an extinction coefficient of $0.125 \mu\text{M}^{-1}\text{cm}^{-1}$ (Estabrook *et al.*, 1971). The percentages of CO-reducible P450 in vesicles were quantified by measuring the P450 concentrations using the CO difference spectra (Omura and Sato, 1964). The recoveries of reductase in lipid vesicles and after detergent treatment were determined by measuring cytochrome c reductase activity of fractions recovered from the Superose 6 size exclusion column (Phillips and Langdon, 1962).

A colorimetric method using ammonium ferrothiocyanate (Stewart, 1980) was adapted to determine the concentration of lipids in the fractions of Superose 6 column eluant. Lipids were extracted from 0.1 mL aliquots of the aqueous fractions using an established method (Bligh and Dyer, 1959). After taking the 0.1 mL aliquot from the column fraction, 1.68 mL of H_2O and 2 mL each of methanol and chloroform were added. The mixture was vortexed and centrifuged, and 1.5 mL of the lipid/chloroform layer was transferred to a clean test tube. To this layer, 1.5 mL of an aqueous ammonium ferrothiocyanate solution (prepared by dissolving 0.1 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 0.4 M NH_4SCN in water) was added, and the mixture was vortexed for 7 min and centrifuged for another 7 min. The timing of these steps is critical as it was observed that the formation of the colored complex between ammonium ferrothiocyanate and phospholipids was light sensitive in the presence of the residual methanol remaining in the chloroform layer. The lower chloroform layer was removed again, and the absorbance was measured at 488 nm. The concentration of lipid was determined from a standard curve using known amounts of DLPC.

Enzymatic assays

N-demethylation of benzphetamine (BZP) was determined by measuring the fluorescence change from formaldehyde after the reaction with a modified Nash reagent (de Andrade *et al.*, 1999; Hanna *et al.*, 2000). *O*-Dealkylation of 7-ethoxy-4-trifluoromethylcoumarin (7EFC) was determined by monitoring the formation of the product, 7-hydroxy-4-trifluoromethylcoumarin by fluorescence (excitation - 410 nm, emission - 510 nm), and the amount was quantified by reference to a standard curve with 7-hydroxy-4-trifluoromethylcoumarin (Hanna *et al.*, 2000). *O*-Dealkylation of 7-pentoxy-resorufin (7PRF) was measured by following the formation of the fluorescent product, 7-hydroxyresorufin (excitation - 559 nm, emission - 585 nm) using a standard curve for quantification (Hanna *et al.*, 2000; Lubet *et al.*, 1985; Hanna *et al.*, 2000).

RESULTS

Comparison of the bile salt-derived detergents on the physical incorporation of reductase and P450 into PC vesicles

Our study compared a wide range of commercially available detergents with structures related to bile salts by preparing vesicles, using each of the bile salt-derived detergents shown in Fig. 1, that contained 1250 nmol of bovine liver PC and 2.5 nmol each of the reductase and CYP2B4. Each system was then characterized, focusing on the fraction of each protein physically incorporated into the vesicles, the loss of native protein during the reconstitution procedure, and the catalytic activity of the vesicular preparations (Fig. 2).

One characteristic of the vesicular reconstituted systems that was previously reported was that the physical incorporation of reductase into lipid vesicles was incomplete at equimolar reductase to P450 ratios (Reed *et al.*, 2006). As shown in Fig. 2A, only 50% of the reductase was eluted in the void volume after Sepharose 6B chromatography when cholate was used for vesicle preparation. Similar levels of reductase incorporation were observed with all of the detergents except CHAPS and sodium glycocholate. These two detergents resulted in statistically significant increases in the proportion of reductase eluting in the void volume of the Superose 6 chromatography.

The detergent dialysis reconstitution procedure also led to the loss of a portion of active reductase (Reed *et al.*, 2006). About 80% of the reductase activity was recovered when sodium cholate was used in the incorporation of the proteins into the vesicles (Fig. 2A). Each of the tested detergents produced similar fractional recoveries of active protein with the exception of Big CHAPS and deoxycholate, which decreased active reductase recovery to less than 40%.

The incorporation of CYP2B4 into vesicles (as measured by elution of the protein in the void volume of the Superose 6 column) ranged from 88–98% for each of the detergents used with the exception of Big CHAPS, which resulted in only 80% incorporation (Fig. 2A). Despite the uniform response observed with each detergent on the incorporation of CYP2B4 into the PC vesicles, more variability was observed when P450 stability was examined. As reported previously (Reed *et al.*, 2006), the use of cholate for physical incorporation of P450 into PC membranes resulted in a significant loss in the ability of the CYP2B4 to form the ferrous carbon monoxy-complex. This loss was attributable to the denaturation of a fraction of the CYP2B4 by the detergent. As Fig. 2A shows, only 44% of the CYP2B4 was recovered in a CO-reducible form after treatment with sodium cholate. Similar levels of P450 denaturation (or greater with sodium deoxycholate) were seen with all of the other detergents except sodium glycocholate. This was the only detergent that was effective in both facilitating the incorporation of the reductase into the vesicular lipid fraction and minimizing the damage to the CYP2B4. More specifically, the glycocholate procedure generated vesicles in which 85% of the reductase and 98% of the CYP2B4 were physically incorporated into the lipid (at 1:1 reductase:CYP2B4). Furthermore, the recovery of both proteins showed the best profile of the detergents tested – 90% and 75% for reductase and CYP2B4, respectively.

Benzphetamine metabolism was used to assess the catalytic function of the different reconstituted systems (Fig. 2B). Metabolism of this substrate was expressed as a function of the amount of CO-reducible CYP2B4 present in the vesicles. The highest rates of metabolism were attained using CHAPS and deoxycholate. This is due, in part, to the disparate effects of these detergents on the incorporation of reductase into the lipid vesicles and the preservation of CO-reactive ferrous CYP2B4 (discussed above). Given that the binding of reductase and CYP2B4 is characterized as a high affinity complex (Reed and Hollenberg, 2003; French *et al.*, 1980; Reed *et al.*, 2006), the result of these effects is that the reconstituted systems prepared with these two detergents probably have a saturating ratio of reductase to P450 (the

reductase:CYP2B4 was $>1.5:1$). In contrast, the reconstituted systems with cholate and glycocholate have ratios less than 1. The preparations with both Big CHAPS and taurocholate were not catalytically active in the metabolism of benzphetamine.

FPLC profiles comparing reconstituted systems using glycocholate and cholate

The vesicular preparations using dialysis after treatment with cholate and glycocholate were further compared by the Superose 6 elution profiles (Fig. 3). The absorbance at 257 nm shows that the vast majority of each of the components eluted in the void volume of the column (7–8 mL of eluant) with both detergents. The most salient distinction between the two preparations was seen in the large amount of reductase, indicated by a broad peak from 9 to 13 mL that eluted after the void volume in the profile derived from the cholate-derived preparations (Fig. 3A). In contrast, the reconstituted system derived from the preparation with sodium glycocholate did not show the lower molecular weight complex of reductase (Fig. 3B). This is consistent with the results shown in Fig. 2A where the cholate preparation exhibited a much smaller fractional incorporation of reductase into the void volume of the column.

Preparation of vesicular reconstituted systems using Biobeads SM-2 beads

After finding that glycocholate was able to significantly circumvent several of the shortcomings associated with cholate, we tested whether the technical impracticalities of the dialysis and gel filtration methods could be improved by the use of Biobeads SM-2 to remove the detergent. Vesicular preparations in which glycocholate was removed by adsorption to Biobeads SM-2 in an overnight incubation were statistically identical to those prepared by removing the detergent using a two day dialysis incubation involving 3 changes of 3 L of dialysate (Fig. 4). More specifically, the reconstituted systems were indistinguishable with respect to the proportions of reductase and CYP2B4 physically incorporated into the PC vesicles; the proportions of active reductase and CYP2B4 recovered after treatment with glycocholate (Fig. 4A); and catalytic activity of the preparations (Fig. 4B).

Characterization of vesicular reconstituted systems of reductase and CYP2B4 at different PC to CYP2B4 ratios

For many studies, it is desirable to understand how the reconstitution conditions affect CYP2B4 activities. With this in mind, we have examined the effect of the lipid to CYP2B4 ratio on the ability of the proteins to physically incorporate into vesicles and on the enzymatic activities of the vesicular reconstituted systems. Using a method adapted from a previous study (Jackson *et al.*, 1982) as described in the Materials and Methods, the detergent concentration needed to solubilize different amounts of lipid was optimized.

In the process of optimizing our method, we identified a lower limit of lipid concentration that could be used to obtain catalytically efficient preparations of physically incorporated reductase and CYP2B4. When the lipid concentrations in the reconstituted systems were less than 1250 μM , the incorporation of lipids and enzymes into large-sized aggregates (those eluting in the void volume of the Superose 6 column) was limited. More specifically, the percentages of lipid, reductase, and P450 incorporated in the void volume of the column were 33%, 46%, and 54% when this concentration of lipid was used to prepare the vesicles. Furthermore, reconstituted systems of CYP2B4 and reductase prepared using less than 1250 μM lipid resulted in large-sized aggregates with low catalytic activity (rates of benzphetamine N-demethylation less than 20 nmol/min/nmol CYP2B4 which were less than those catalyzed by conventional reconstituted systems using sonicated DLPC at identical lipid to P450 ratios (data not shown)). Conversely, in all cases where the lipid to P450 ratios were greater than or equal to 500 to 1 (2500 μM), the enzymes were physically associated with large vesicles having catalytic activities that were significantly greater than those prepared with sonicated DLPC (data not shown).

The rates of metabolism of three CYP2B4 substrates were examined in reconstituted systems prepared using different ratios of lipid:CYP2B4 (Figure 5). The optimal lipid:CYP2B4 ratio for each enzymatic reaction was about 500:1. A decrease in the rate of metabolism of each substrate was observed at higher lipid:P450 ratios. Interestingly, the rates of 7EFC and 7PRF metabolism were inhibited to a greater degree than that of benzphetamine at the higher lipid:P450 ratios.

Characterization of vesicular reconstituted systems of reductase and CYP2B4 at different reductase to CYP2B4 ratios

The catalytic activities of vesicular reconstituted systems prepared using the method at a variety of reductase:CYP2B4 ratios also were determined. As discussed in our previous study (Reed *et al.*, 2006), the incorporation of reductase into the vesicular fraction was more efficient at the lower reductase:CYP2B4 ratios (ranged from 100% incorporation at the 1:4 ratio to approximately 50% at a 2:1 ratio), whereas the incorporation of CYP2B4 was greater than 90% at all of the reductase:CYP2B4 ratios.

Figure 6A–6C shows the rates of metabolism of BZP, 7EFC, and 7PRF, respectively, as a function of the vesicular-incorporated reductase concentration. The results indicate that the reductase and CYP2B4 form a high affinity catalytic complex as indicated by the low apparent K_m values calculated from the nonlinear regressions (ranging from 14 to 60 nM). The roughly four-fold range in the apparent K_m values measured in the presence of the different substrates is consistent with the findings from studies using conventional methods to reconstitute the enzymes with sonicated dilauroylphosphatidylcholine that have found that the affinity of the enzyme complex is dependent on the substrate used to measure the binding (French *et al.*, 1980; Backes and Eyer, 1989; Reed and Hollenberg, 2003). Metabolism of 7PRF was characterized by the lowest apparent K_m , whereas the highest value was calculated for 7EFC metabolism. We previously reported that the apparent K_m values calculated for vesicular reconstituted systems using PC and standard systems prepared with sonicated dilauroylphosphatidylcholine were similar. However, the catalytic activities for the vesicular systems were significantly higher (Reed *et al.*, 2006).

DISCUSSION

This work describes a significant improvement to the method for preparing reconstituted systems of reductase, CYP2B4, and PC in which the enzymes are physically incorporated into a lipid bilayer. We have demonstrated increases in the proportions of both the reductase that is incorporated into vesicles and the active CYP2B4 recovered when sodium glycocholate is substituted for sodium cholate. In addition, the described method using Biobeads SM-2 also eliminates many of the technical difficulties associated with previously published methods (cholate dialysis and cholate gel filtration) in that several different preparations can be prepared simultaneously without prolonged or laborious handling. We have shown that the characteristics of the vesicular preparations using Biobeads and dialysis are statistically identical with regard to enzyme incorporation and activity.

We have also defined a lower limit of the lipid concentration ($> 1250 \mu\text{M}$) needed for efficient vesicle incorporation of the lipid and proteins using the glycocholate-biobeads method. In our hands, the vesicles prepared under conditions in which the lipid was $\leq 1250 \mu\text{M}$ had significantly lower percentages (only 30–50%) of enzymes and lipid eluting in the void volume of the superpose 6 column (the criterion used to indicate vesicle-incorporation) and had low catalytic activity. Because the reconstituted systems using low PC concentrations were limited in the formation of large-sized liposomes and had low catalytic activities, it seems the enzymes and lipid were organized in a different structure from that studied in vesicular reconstituted systems using higher lipid concentrations. Previously, it has been shown that unilamellar

vesicles merge into multilamellar structures at low lipid concentrations (Akhrem *et al.*, 1982). Thus, it seems likely that the vesicles prepared in our hands at low lipid:protein ratios using either the detergent dialysis or Biobeads methods represent multilamellar vesicles that have low catalytic activity.

Our study has compared a wide range of commercially available detergents with structures related to bile salts. It is possible that deoxycholate and CHAPS may have facilitated the interaction of the reductase and CYP2B4 and in turn stimulated catalysis. The evidence for this assumption resides in the fact that the vesicular preparations derived using these detergents catalyzed extremely high rates of benzphetamine N-demethylation (> 175 nmol/min/nmol). This is significantly higher than the maximal rate of catalysis calculated from reconstituted systems prepared with sodium cholate (Reed *et al.*, 2006). A previous study also has reported that CHAPS is able to activate the CYP2B-related activity in rats (Dutton *et al.*, 1998). However, as mentioned in the results, the higher rates of activity associated with these detergents probably could be attributed to disparate effects on the incorporation of reductase into the phospholipid vesicles and the CYP2B4 stability which resulted in higher reductase:P450 ratios in the reconstituted systems.

Our results showed that the reductase and CYP2B4 formed a high affinity complex during the metabolism of three different substrates, and the catalytic activity of the vesicular reconstituted systems with CYP2B4 decreased at higher lipid:CYP2B4 ratios. The decrease in activity associated with the metabolism of each substrate as a function of the lipid:CYP2B4 is consistent with the reductase and CYP2B4 being incorporated into separate lipid vesicles at the higher ratios, which would hinder the enzymes from physically interacting and transferring the electrons needed for metabolism. Interestingly, the rates of *O*-dealkylation of 7EFC and 7PRF by the CYP2B4 reconstituted systems dropped to very low levels at a 5000:1 PC:CYP2B4 ratio, whereas that of benzphetamine only decreased gradually after the ratio exceeded 700:1. One possible explanation for the difference observed with BZP may be the evidence that a high proportion of the total CYP2B4-mediated metabolism of this substrate occurs by the peroxidative pathway which circumvents the need for the reductase and P450 to physically interact (Estabrook *et al.*, 1984).

In conclusion, the efficiency of incorporation of reductase and CYP2B4 was examined in reconstituted systems prepared by the detergent-dialysis method as a function of the detergent used. Glycocholate was shown to be superior to the other detergents tested not only in the stability of the proteins, but also in their ability to be physically incorporated into the vesicular membrane. Building on this data, a modified procedure was developed using glycocholate for the initial solubilization of components, followed by replacement of the dialysis step with Biobeads treatment. The characteristics of reconstituted systems prepared by this method are comparable to those of the detergent-dialysis method with regard to efficiency of protein incorporation and catalytic activity. Additionally, the detergent-Biobeads method has the advantage of being more rapid to prepare and allows for the simultaneous preparation of several reconstituted systems under widely ranging conditions, such as variable lipid to protein and reductase to P450 ratios.

Abbreviations

PC	phosphatidylcholine
CD	cholate dialysis method for preparation of reconstituted system
DLPC	dilauroylphosphatidylcholine
reductase	NADPH-cytochrome P450 reductase

P450	cytochrome P450
CYP2B4	cytochrome P450 2B4
GC	sodium glycocholate
BZP	benzphetamine
7EFC	7-ethoxy-4-trifluoromethylcoumarin
7PRF	7-pentoxoresorufin

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Bile acid derivative detergents compared in this study.

Detergent	R ₁ ^a	R ₂ ^a	CMC ^b	Aggregation Number ^c
Cholate	-O-Na ⁺	OH	7	4.8
Chaps	-NH-C ₃ H ₆ N ⁺ (CH ₃) ₂ C ₃ H ₆ SO ₃ ⁻	OH	4	15
Glycocholate	-NHCH ₂ CO ₂ -Na ⁺	OH	7	16
Deoxycholate	-O-Na ⁺	H	2.5	12
Glycodeoxycholate	-NHCH ₂ CO ₂ -Na ⁺	H	1.1	19
Taurocholate	-NHCH ₂ CH ₂ SO ₃ -Na ⁺	OH	4.5	4.5
Big Chaps	-N(C ₃ H ₆ NHCOC ₅ (CH ₃) ₄ (OH) ₅) ₂	OH	3.4	10

^aRefers to the sites of the molecule shown in Figure 1.

^bCritical micelle concentration

^cAverage number of detergent molecules making up micelle.

Figure 1. “Bile salt” structural motif common to all of the detergents compared in this study
Chemical structure of the basic structural unit common to the detergents compared for the effects on the vesicular reconstitution of reductase and P450.

Figure 2. Comparison of vesicular preparations of reductase, CYP2B4, and PC using different “bile salt”-derived detergents

(A) The dialysis method (see Materials and Methods) was used to prepare reconstituted systems with reductase and P450 physically incorporated into PC vesicles by using the sodium salt of each of the detergents identified in Fig. 1. The preparations were compared for the proportions of reductase and P450 physically incorporated in the vesicles as indicated by the proportions of enzyme eluting in the void volume of the Superose 6 column. These systems also were characterized by the proportions of active reductase (cytochrome c activity) and P450 (ferrous CO complex) recovered after detergent treatment. The errors associated with measurements for cholate (n=5) and glycocholate (n=4) represent averages $\pm$ standard deviation of the

indicated parameter. All other conditions were measured in triplicate. The significance of the difference of an average relative to that observed with cholate is indicated by the * using an unpaired student t-test ($P < 0.01$). (B) The catalytic activities as determined by the rates of benzphetamine N-demethylation of the vesicular reconstituted systems prepared using the detergents from Fig. 1. Benzphetamine N-demethylation was measured as described in Materials and Methods using $0.05 \mu\text{M}$ of P450 (as determined from the ferrous-CO complex) from each of the corresponding vesicular preparations. The values represent the averages $\pm$ the standard deviation of triplicate determinations.

Figure 3. FPLC elution profiles from the Superose 6 column of reconstituted systems prepared using cholate (A) and glychocholate (B)

Preparations using 5 nmol each of CYP2B4 and reductase with 1% sodium cholate and 2500 nmol of PC were prepared by the dialysis method as described in Materials and Methods. The elution profiles of reductase, P450, and lipid were monitored as described in Materials and Methods. The volumes of elution of the total components, as indicated by the 257 nm absorbances, are also shown in the two profiles.

Figure 4. Comparison of vesicular reconstituted systems prepared using Biobeads SM-2 with dialysis

Vesicular preparations using 2.5 nmol each of reductase and P450, 1250 μ M PC, and 1% sodium glycocholate were prepared using either Biobeads SM-2 or dialysis as described in Materials and Methods. (A) The preparations were compared for the proportions of reductase and P450 physically incorporated in the vesicles as indicated by the proportions of enzyme eluting in the void volume of the Superose 6 column. The systems also were characterized by the proportions of active reductase (cytochrome c activity) and P450 (ferrous CO complex) recovered after detergent treatment. The errors associated with the measurements represent averages $\pm$ standard deviation of the indicated parameter ($n=3$). (B) Benzphetamine N-demethylation was measured as described in Materials and Methods using 0.05 μ M of P450 from each of the corresponding vesicular preparations. The values represent the averages $\pm$ the standard deviation of triplicate determinations.

Figure 5. Enzymatic activities of vesicular reconstituted systems prepared with a 1:3 ratio of reductase:CYP2B4 and variable amounts of phosphatidylcholine

The vesicular reconstituted systems were prepared with reductase, 2.5 nmol of CYP2B4, and the indicated amounts of phosphatidylcholine (PC) using sodium glycocholate and Biobeads SM2 as described in Materials and Methods. The reductase:CYP2B4 ratio in the vesicles of PC for all of the preparations was 1:3 as was determined from the cytochrome c reductase activity and the reduced CO-CYP2B4 complex contained in the void volume after injecting 0.1 mL of the reconstituted system on the Superose 6 size exclusion column. The rates of benzphetamine *N*-demethylation (A); 7EFC *O*-dealkylation (B); and 7PRF *O*-dealkylation (C) were determined as described in Materials and Methods. The error bars represent the standard deviation associated with triplicate determinations.

Figure 6. Enzymatic activities of vesicular reconstituted systems prepared with a 500:1 ratio of PC:CYP2B4 and variable amounts of the reductase

The vesicular reconstituted systems were prepared with 1 mg (~1250 nmol) of phosphatidylcholine (PC), 2.5 nmol of CYP2B4, and variable amounts of the reductase by using sodium glycocholate and Biobeads SM2 as described in Materials and Methods. The concentrations of reductase and CYP2B4 in the bilayer vesicles of PC for all of the preparations were determined by measuring the cytochrome c reductase activity and the reduced CO-CYP2B4 complex, respectively in the void volume of the Superose 6 size exclusion column after injecting 0.1 mL of the vesicular reconstituted systems. The rates of benzphetamine *N*-demethylation (A); 7EFC *O*-dealkylation (B); and 7PRF *O*-dealkylation (C) were determined as described in Materials and Methods. All enzyme assays used 0.1 μM CYP2B4, and the

ordinate of the plots represent the reductase concentration used in the respective assays. The error bars represent the standard deviation associated with triplicate determinations.