Zwitterionic Detergent Mediated Interaction of Purified Cytochrome P-450$_{LM4}$ from 5,6-Benzoflavone-Treated Rabbits with NADPH–Cytochrome P-450 Reductase†

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ABSTRACT: Hydroxylation of acetanilide catalyzed by purified cytochrome P-450$_{LM4}$ and NADPH–cytochrome P-450 reductase was reconstituted with the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The optimum rate of production of 4-hydroxyacetanilide was observed between 3 and 7 mM CHAPS and was about half that with 0.05 mM dilauroylglyceryl-3-phosphocholine (di-12-GPC). At higher detergent concentrations, hydroxylase activity decreased until at 15–20 mM CHAPS the system was inactive. The effect of CHAPS on the state of aggregation of P-450$_{LM4}$ and on interaction between the cytochrome and P-450 reductase alone and under turnover conditions was investigated by ultracentrifugation. At 4 mM CHAPS, P-450$_{LM4}$ was hexameric to heptameric (M_r 369 000). Neither reductase nor reductase plus acetanilide and NADPH altered the state of P-450$_{LM4}$ aggregation, suggesting that a stable 1:1 P-450/reductase complex did not form under turnover conditions. Replacing CHAPS with 0.05 mM di-12-GPC resulted in formation of heterogeneous P-450 oligomers (M_r >480 000). At CHAPS concentrations where substrate hydroxylation did not occur (15 and 22 mM), P-450$_{LM4}$ was shown by sedimentation equilibrium measurements to be dimeric and monomeric, respectively. P-450 reductase was shown to reduce monomeric P-450$_{LM4}$ in the presence of NADPH. Thus, the dependence of hydroxylase activity on [CHAPS] may be related to the state of aggregation of the cytochrome. An apparent correlation between P-450 aggregation state and NADPH-supported hydroxylation was also observed with phenobarbital-inducible P-450$_{LM2}$ in the presence of detergents [Dean, W. L., & Gray, R. D. (1982) *J. Biol. Chem. 257*, 14679–14685; Wagner, S. L., Dean, W. L., & Gray, R. D. (1984) *J. Biol. Chem. 259*, 2390–2395].

The ability of liver microsomes to oxidize many xenobiotics depends on interaction between a cytochrome P-450 isozyme and the flavoprotein NADPH–cytochrome P-450 reductase and phospholipid (Strobel et al., 1970). Activity can be reconstituted in solution by mixing the purified proteins in the presence of phospholipid (Coon et al., 1978) or certain detergents (Dean & Gray, 1982a; Wagner et al., 1984). The added amphiphile can be omitted if the proteins are preincubated at high concentration for several minutes before activity measurements are made (Müller-Enoch et al., 1984). The manner in which this system is assembled in the microsomal membrane is not known, although the question continues to be actively investigated (Franklin & Estabrook, 1971; Steir & Sackman, 1973; Peterson et al., 1976; Duppel & Ullrich, 1976; Yang, 1977; Taniguchi et al., 1979, 1984; French et al., 1979; Ingleman-Sundberg & Johansson, 1980; Dean & Gray, 1982a; Gut et al., 1982, 1983; Ingelman-Sundberg et al., 1983; Müller-Enoch et al., 1984; Blanck et al., 1984; Wagner et al., 1984; Tamburini & Schenkman, 1986). One approach to studying microsomal protein organization is to determine the degree of interaction among the components in functional systems containing the purified proteins and chemically defined detergents. Toward this end, Dean and Gray (1982a) and Wagner et al. (1984) showed that certain detergents could replace phospholipid in stimulating catalytic activity of cytochrome P-450$_{LM2}$[1] and NADPH–cytochrome P-450 reductase in dilute solution. For example, relatively low concentrations of the nonionic detergent *n*-octyl glucoside (Baron & Thompson, 1975) or the zwitterionic detergent CHAPS (Hjelmeland, 1980) supported reconstituted catalytic activity by P-450$_{LM2}$ and P-450 reductase. Higher concentrations of either of these detergents caused the catalytically active aggregated cytochrome to undergo progressive subunit dissociation, ultimately to the monomeric state. This detergent-induced disaggregation, while leading to loss of NADPH-supported hydroxylase activity, was not accompanied by formation of cytochrome P-420. Indeed, in the case of P-450$_{LM2}$, organic hydroperoxide mediated hydroxylation was maintained in high detergent (Wagner et al., 1984).

We observed also that catalytic activity by cytochrome P-450$_{LM2}$ and NADPH–cytochrome P-450 reductase in the presence of these detergents did not require formation of a stable complex between the two enzymes. Our solution studies therefore suggest that a long-lived, intermolecular complex

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[1] Abbreviations: P-450$_{LM2}$, cytochrome P-450 isozyme induced in rabbit liver by phenobarbital; P-450$_{LM4}$, cytochrome P-450 isozyme induced in rabbit liver by 5,6-benzoflavone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; *n*-octyl glucoside, *n*-octyl β-D-glucopyranoside; di-12-GPC, dilauroylglyceryl-3-phosphocholine; reductase, NADPH–cytochrome P-450 reductase; HPLC, high-performance liquid chromatography; cmc, critical micelle concentration; EDTA, ethylenediaminetetraacetic acid.

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between P-450$_{LM2}$ and its reductase is not a prerequisite for reconstituted monooxygenase activity. Formation of a transient complex which dissociates after completion of the redox process is apparently sufficient for electron transfer to occur.

The purpose of the present study was to determine whether these observations are generally applicable to other P-450 isozymes. The effect of detergent on reconstituted activity, state of aggregation, and interaction with reductase was therefore examined by using a member of a different family of cytochrome P-450$_{LM}$ isozymes, the polycyclic aromatic hydrocarbon inducible cytochrome P-450$_{LM4}$. The results of these studies are consistent with our earlier observations made with P-450$_{LM2}$ (Dean & Gray, 1982a; Wagner et al., 1984). In addition, two new observations were made: first, the state of aggregation of the reconstituted system did not change under turnover conditions, and second, the reductase could transfer electrons to cytochrome P-450$_{LM4}$ in the presence of high detergent where both proteins are monomers.

Experimental Procedures

Materials. Cytochrome P-450$_{LM4}$ was purified from liver microsomes of 5,6-benzoflavone-pretreated 2-kg male New Zealand rabbits by using a modification of the procedure of Coon et al. (1978). An additional chromatographic step utilizing carboxymethyl-Sephadex was used subsequent to hydroxylapatite chromatography (Imai et al., 1980). The purified cytochrome was washed free of detergent with 0.15 M potassium phosphate, pH 7.5, 20% glycerol, and 0.5 mM EDTA (hereafter referred to as phosphate buffer) while bound to a hydroxylapatite column (Bio-Gel HTP, Bio-Rad Laboratories). The specific content of the resulting preparation was 12.0 nmol of P-450/mg of protein and consisted of a single polypeptide as judged by polyacrylamide gel electrophoresis carried out according to Laemmli (1970). The cytochrome was concentrated by ultrafiltration to 16 μM and stored at −20 °C. In a limited number of the kinetic studies, an electrophoretically homogeneous preparation was used which had a lower specific content (6 nmol/mg).

NADPH–cytochrome P-450 reductase was purified as described previously (van der Hoeven & Coon, 1974; Yasukochi & Masters, 1976) from the same microsomes. Detergent was removed from the purified reductase as described above. The enzyme was concentrated by ultrafiltration and stored at −20 °C. The specific activity of the reductase was 35 μmol of cytochrome *c* reduced min^{-1} mg^{-1}, and the specific content was 11.8 nmol/mg on the basis of the flavin absorbance at 456 nm (Vermilion et al., 1981). CHAPS was purchased from Sigma Chemical Co., and acetanilide was from Eastman. 2-, 3-, and 4-hydroxyacetanilide were from Aldrich Chemical Co.

Assays. Cytochrome P-450$_{LM4}$ concentration was determined by the CO difference spectral method of Omura and Sato (1964). NADPH–cytochrome *c* reductase activity was assayed by the procedure of Strobel and Dignam (1978). Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as standard.

Acetanilide hydroxylase activity of cytochrome P-450$_{LM4}$ reconstituted with P-450 reductase was assessed by using reversed-phase HPLC to separate and quantitate hydroxylated metabolites. In most cases, the assay conditions were 0.05 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 0.4 μM P-450$_{LM4}$, 0.4 μM reductase, CHAPS at the desired concentration (added from a 0.1 M stock solution in water), 5 mM acetanilide (added from a 0.5 M stock solution in methanol), and 0.3 mM NADPH. The temperature was maintained at 30 °C. When di-12-GPC was used in place of CHAPS, it was incubated with the proteins for 1 min at room temperature prior to the addition of the remaining reagents. One set of assays employed conditions identical with those used in the hydrodynamic studies. In both types of experiment, the reaction was quenched with trichloroacetic acid (final concentration of 5%). After centrifugation, the supernatant was diluted with an equal volume of methanol, and an aliquot of 200 μL was applied to a C_{18} reversed-phase column (Varian MCH-10, 4 mm × 30 cm, 10-μm particle size, 2 mm × 4 cm guard column) used in conjunction with a Varian 5000 liquid chromatograph. The products were eluted with 0.1% phosphoric acid and acetonitrile (0–40% linear gradient) at a flow rate of 2 mL/min. Hydroxyacetanilide isomers were detected at 240 nm with a Varian UV-100 detector and quantitated with a Varian 4270 integrator through the use of a standard curve which contained standards along with all other components of the assay mixture. K_m values were estimated by using the weighted least-squares algorithm of Wilkinson (1961).

Rates of NADPH oxidation by cytochrome P-450$_{LM4}$ and NADPH–cytochrome P-450 reductase were also measured under the above conditions at 340 nm with a Varian-Cary 219 spectrophotometer interfaced to an OLIS Model 3820 data acquisition system (On-Line Instruments, Jefferson, GA). The reported rates are those obtained after subtraction of the rate of NADPH oxidation measured with an equivalent volume of methanol without the substrate.

Reduction of cytochrome P-450$_{LM4}$ by P-450 reductase and NADPH was assessed spectrophotometrically with an on-line Cary 219 spectrophotometer as described (Wagner & Gray, 1985). The reaction mixture contained P-450$_{LM4}$ (0.6 μM), reductase (1 μM), amphiphile at the desired concentration, and an oxygen scavenging system (2.5 μg/mL glucose oxidase, 2 μM catalase, and 10 mM glucose). The samples were bubbled with CO for 1 min prior to addition of P-450 and reductase. The absorption spectrum of the oxidized system was recorded at 0.25-nm intervals from 500 to 400 nm and stored on a diskette. NADPH was added to give a final concentration of 0.25 mM, and the spectrum was redetermined until no further change was noted (about 20 min). To determine the degree of reduction, a small amount of sodium dithionite was added prior to recording the final absorption spectrum. Difference spectra (reduced CO − oxidized cytochrome) were then generated by subtracting the appropriate spectra with the computer.

Sedimentation Experiments. Ultracentrifugation was conducted as previously described (Dean & Gray, 1982a,b) at 20 °C in an AN-F rotor with a Beckman L5-75 preparative ultracentrifuge equipped with a Prep UV Scanner attachment operated at 405 nm with an interference filter (Oriel Corp., Stamford, CT). The experimentally determined apparent weight average molecular weight values were corrected for bound detergent as described by Tanford et al. (1974). An attempt was made to measure directly detergent binding to the cytochrome using the gel filtration procedure of Hummel and Dreyer (1962). However, the level of bound detergent was below the limit of detection (1.0 g of detergent/g of P-450) even at the highest CHAPS concentration used (30 mM). It was therefore necessary to assume a value for detergent binding in order to calculate the molecular weight of the protein. These assumptions and the rationalization for their use are summarized in the legend to Table I. Values for $\bar{v}$ of 0.730, 0.732, and 0.731 mL/g were used for NADPH–cytochrome P-450 reductase (Vermilion & Coon, 1978), CHAPS (Wagner et al., 1984), and cytochrome P-450$_{LM4}$ (Haugen & Coon, 1976), respectively.

Table I: Estimation of Cytochrome P-450$_{LM4}$ Molecular Weight by Sedimentation Equilibrium[a]

[CHAPS] (mM)	mol wt[b] bound detergent (assumed) 0	0.3 g/g
4	369 000	
15	131 000	101 000
22	68 000	52 000
30	68 000	53 000

[a] Conditions: 0.15 M potassium phosphate, pH 7.5, 20% glycerol, and 0.5 mM EDTA, 20 °C. [b] Weight-average molecular weight calculated by using the assumed values for detergent binding listed. The actual amount of bound detergent was below the limits of detection (1 g of CHAPS/g of protein). A value of 0.3 g of CHAPS/g of protein was therefore used to calculate molecular weight. This value of bound detergent was chosen because it gave the accepted monomer molecular weight of P-450$_{LM4}$ (Haugen & Coon, 1976). Assuming a larger value of bound detergent gave less than the monomeric molecular weight. Smaller values for detergent binding yielded molecular weights between the monomer and dimer as shown above for 22 and 30 mM CHAPS.

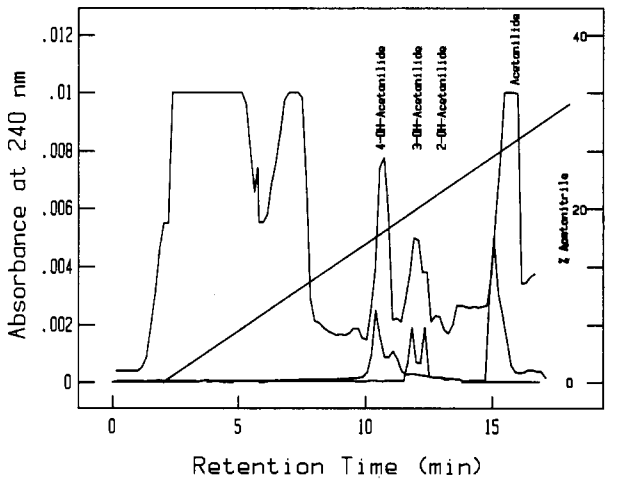

FIGURE 1: Reversed-phase HPLC elution profile of acetanilide metabolites formed by incubation of cytochrome P-450$_{LM4}$ and NADPH–cytochrome P-450 reductase for 30 min in the presence of 4 mM CHAPS. The upper trace was obtained from the reaction mixtures after precipitation of the proteins with trichloroacetic acid, centrifugation, and dilution with methanol. The bottom profile indicates the elution positions of standards. Other conditions of the assay are given in the text.

RESULTS

Activity Studies. To determine whether a relationship exists between hydroxylase activity and detergent concentration, hydroxylation of acetanilide catalyzed by P-450$_{LM4}$ and NADPH–cytochrome P-450 reductase was measured in dilute solution in the presence of the zwitterionic detergent CHAPS. Figure 1 shows an HPLC profile illustrating the hydroxylated products present after a 30-min incubation of P-450$_{LM4}$, reductase, acetanilide, and NADPH in the presence of 4 mM CHAPS. The major hydroxylated metabolite was 4-hydroxyacetanilide along with smaller amounts of the 3-hydroxy and 2-hydroxy isomers. By comparison with a standard curve, approximately 55% of the product was the 4-isomer, and the remaining 45% was the 2- and 3-isomers. Product distribution was similar when 0.05 mM di-12-GPC was the amphiphile.

The dependence of the rate of formation of 4-hydroxyacetanilide on CHAPS concentration is shown in Figure 2. The upper panel (A) shows that the maximum rate of 4-hydroxyacetanilide formation of about 1 nmol (nmol of P-450)$^{-1}$ min^{-1} occurred between 3 and 7 mM CHAPS. Similar

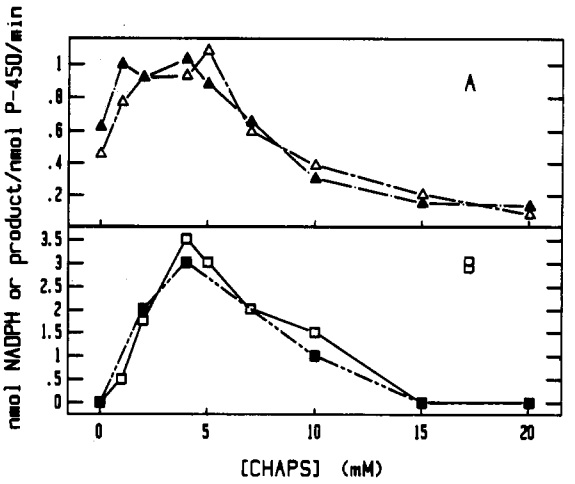

FIGURE 2: (A) Effect of [CHAPS] on the production 4-hydroxyacetanilide by cytochrome P-450$_{LM4}$ and NADPH–cytochrome P-450 reductase. 4-Hydroxyacetanilide was measured by HPLC at 240 nm as shown in Figure 1 either 15 (△) or 30 (▲) min after the addition of NADPH. In independent experiments, the rate of 4-hydroxyacetanilide formed in the presence of 0.05 mM di-12-GPC was approximately 2 nmol of 4-hydroxyacetanilide (nmol of P-450)$^{-1}$ min^{-1}. (B) Effect of [CHAPS] on the rate of NADPH oxidation by cytochrome P-450$_{LM4}$, NADPH–cytochrome P-450 reductase, and acetanilide. The different symbols represent different runs with the same preparation of P-450$_{LM4}$ and reductase. The rates are given after subtracting the rate of NADPH oxidation observed in the absence of acetanilide. The rate of NADPH oxidation observed in the presence of 0.05 mM di-12-GPC and acetanilide was 4 nmol of NADPH min^{-1} (nmol of P-450)$^{-1}$. Conditions are listed under Experimental Procedures.

rates were found for both 15- and 30-min incubations. Above 7 mM CHAPS, substrate hydroxylation decreased and was not detectable from 15 to 20 mM CHAPS. By comparison, the rate of formation of 4-hydroxyacetanilide in the presence of 0.05 M di-12-GPC was about 2 nmol (nmol of P-450)$^{-1}$ min^{-1}. Thus, the detergent system was about half as active as the system reconstituted with phospholipid. The results obtained by measurement of the rate of oxidation of NADPH are shown in Figure 2B. A similar detergent optimum was found. The greater rate of NADPH oxidation compared to the rate of substrate hydroxylation is not unexpected since coenzyme oxidation is generally incompletely coupled to substrate hydroxylation (Nordbloom et al., 1976). The efficiency of hydroxylation does not seem to decrease markedly with increased detergent concentration since the decline in both activities follows a similar course.

To determine whether the decreased hydroxylase activity of P-450$_{LM4}$ and reductase at high detergent concentrations resulted from a decrease in the effective substrate concentration as a result of the possible sequestering of the lipophilic substrate in detergent micelles, the apparent K_m for acetanilide was determined at CHAPS concentrations above and below the cmc of CHAPS (approximately 8 mM; Wagner et al., 1984). The apparent K_m for acetanilide determined by measuring the rate of 4-hydroxyacetanilide production by HPLC was 1.6 ± 0.4 mM in the presence of 4 mM CHAPS and 1.2 ± 0.8 mM in the presence of 10 mM CHAPS (data not shown; the precision of the latter determination is low because of the low activity). When measured by NADPH oxidation, the apparent K_m for acetanilide in 4 mM CHAPS was 1.8 ± 0.2 mM and 3.5 ± 0.8 mM in 10 mM detergent. With 0.05 mM di-12-GPC, the value was 2.9 ± 0.6 mM. These K_m values are comparable to that of 1.5 mM reported for acetanilide with a reconstituted rat liver microsomal P-450 system (Selander et al., 1974). We conclude from these data that the decrease in cytochrome P-450$_{LM4}$/reductase-catalyzed

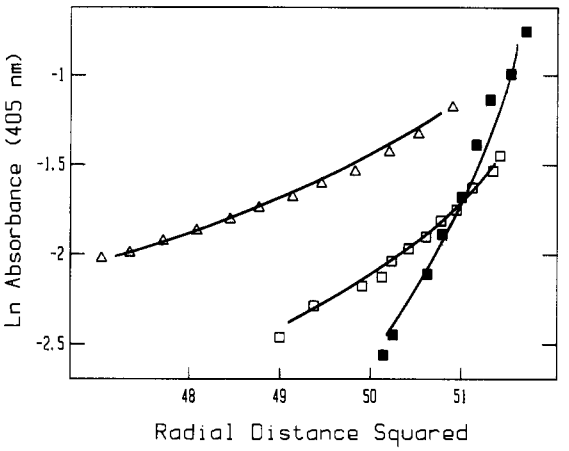

FIGURE 3: Sedimentation equilibrium of cytochrome P-450$_{LM4}$ as a function of CHAPS concentration. The conditions were as follows: initial [P-450$_{LM4}$] = 2.5 μM; 0.15 M potassium phosphate, pH 7.5, 20% glycerol, and 0.5 mM EDTA, 20 °C. The CHAPS concentrations were 4 (■), 15 (□), and 30 mM (△).

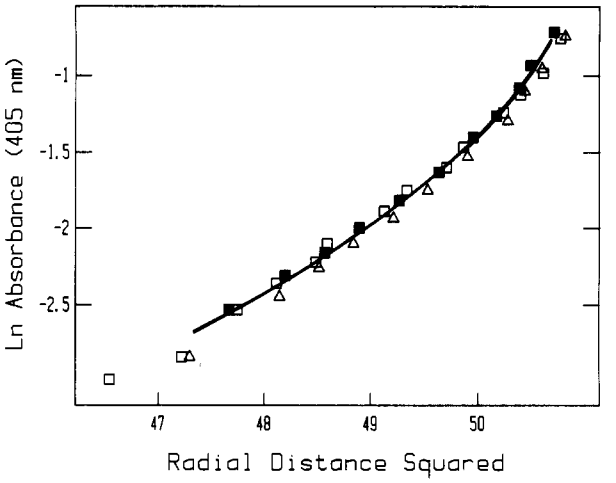

FIGURE 4: Effect of reductase and substrate on the sedimentation equilibrium of cytochrome P-450$_{LM4}$ at 4 mM CHAPS. The data reflect the distribution of P-450 polypeptides in the centrifugation cell in the absence of reductase (△), in the presence of reductase (■), and in the presence of reductase and 5 mM acetanilide (□). In each experiment, the initial concentrations of P-450$_{LM4}$ and reductase were 2.5 μM. The limiting molecular weight of cytochrome P-450$_{LM4}$ in the presence and absence of the reductase was 369 000.

hydroxylase activity at high detergent concentration cannot be attributed to a change in the K_m of P-450$_{LM4}$ for the organic substrate.

Hydrodynamic Studies. The state of aggregation of P-450$_{LM4}$ was determined by sedimentation equilibrium at CHAPS concentrations where the system was catalytically active and where it was inactive. The effect of increasing [CHAPS] on the distribution of P-450 in the centrifuge cell (as indicated by the slope of the plots of ln A_{405} vs. r^2 in Figure 3) was to increase P-450 subunit dissociation. Table I summarizes the weight-average molecular weights of P-450$_{LM4}$ calculated from these centrifugation experiments. When the system was maximally active ([CHAPS] = 4 mM), the state of aggregation of the cytochrome was hexameric to heptameric. When [CHAPS] was raised to 15 or 30 mM, detergent concentrations where activity was not observed, dimers and monomers were the prevailing species.

Sedimentation equilibrium studies of P-450$_{LM4}$ were also conducted in the presence of NADPH–cytochrome P-450 reductase and reductase plus acetanilide in order to assess complex formation between the two proteins. Measurements were made at 405 nm, so only the cytochrome was detected in the centrifuge cell. Since the resulting absorbance profiles shown in Figure 4 are virtually superimposable, it is evident that the state of aggregation of the P-450 was unaffected by reductase or by reductase with acetanilide under these conditions. Therefore, a stable 1:1 complex between P-450$_{LM4}$ and NADPH–P-450 reductase did not form at detergent concentrations where maximal catalytic activity was observed. Had reductase subunits of M_r 76 000 displaced P-450 subunits of M_r 55 000, a change in the equilibrium distribution of the P-450 within the cell would have been observed. When sedimentation equilibrium was performed with P-450$_{LM4}$ and reductase in the presence of 0.05 mM di-12-GPC, conditions known to result in stable complex formation as judged by molecular sieve measurements (French et al., 1979) and by ultracentrifugation (Guengerich & Holladay, 1979), heterogeneous aggregates of M_r >480 000 were observed, indicating that P-450/reductase/lipid complexes can be detected by this method (data not shown).

To assure that the system was active under the conditions of ultracentrifugation, acetanilide hydroxylase assays were carried out under identical conditions of protein concentration, buffer composition, and temperature. In these experiments, the rate of 4-hydroxyacetanilide formation depended on the concentration of detergent. The highest rates of hydroxylation occurred in the presence of 0.05 mM di-12-GPC and 4 mM CHAPS [117 and 42 pmol of 4-hydroxyacetanilide (nmol of P-450)$^{-1}$ min^{-1}, respectively]. At CHAPS concentrations of 15 and 30 mM, there was no measurable 4-hydroxyacetanilide produced up to 60 min. Aside from the lower rates of hydroxylation, these results agree with those of Figure 2 which were carried out under standard assay conditions of higher temperature, lower protein concentration, and in the absence of glycerol. It should be noted that comparatively low rates of substrate hydroxylation have also been reported when reconstitution was carried out at room temperature in the presence of the relatively high glycerol concentrations needed to stabilize the enzymes (Guengerich & Holladay, 1979; Dean & Gray, 1982a; Wagner et al., 1984).

Table II: Effect of Reductase, Substrates, and [CHAPS] on the Sedimentation Coefficient of Cytochrome P-450$_{LM4}$

[CHAPS] (mM)	conditions	$s_{20,w}$[a] (S)
4	5 μM P-450$_{LM4}$	9.1
4	5 μM P-450$_{LM4}$ and 5 μM reductase	9.0
4	5 μM P-450$_{LM4}$, 5 μM reductase, 5 mM acetanilide, and 0.3 mM NADPH	8.7
15	5 μM P-450$_{LM4}$	7.8
30	5 μM P-450$_{LM4}$	6.9

[a] Determined in 0.15 M potassium phosphate, pH 7.5, 20% glycerol, and 0.5 mM EDTA at 20 °C.

In order to determine if P-450/reductase complex formation could be detected in an actively hydroxylating system, sedimentation velocity experiments were conducted with P-450$_{LM4}$, reductase, NADPH, and acetanilide. The production of 4-hydroxyacetanilide at the end of the 60-min centrifugation run was confirmed by HPLC (data not shown). As shown by the results of these experiments (summarized in Table II), there was no effect of reductase and substrates on the sedimentation coefficient of P-450$_{LM4}$. This implies the absence of a stable P-450/reductase complex under conditions where the system is turning over.

Reduction of P-450 by P-450 Reductase. The ability of NADPH and P-450 reductase to reduce cytochrome P-450$_{LM4}$ was assessed in the experiments shown in Figure 5. In the

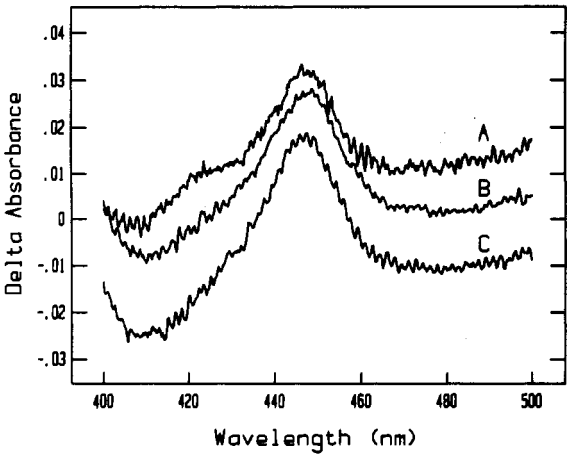

FIGURE 5: Reduction of cytochrome P-450$_{LM4}$ by NADPH–cytochrome P-450 reductase in the presence of CHAPS (A, 20 mM; B, 4 mM) or 0.05 mM di-12-GPC (C). Each spectrum represents the difference in absorption between CO–cytochrome P-450 approximately 20 min after NADPH addition and the spectrum of the oxidized cytochrome prior to coenzyme addition.

three experiments shown, 43%, 40%, and 36% of the P-450 was reduced in the presence of 4 mM CHAPS, 20 mM CHAPS, or 0.05 mM di-12-GPC, respectively. In other experiments not shown, up to 75–80% reduction of the cytochrome could be achieved under these conditions. The reason for the variability in extent of P-450 reduced is not known but may be related to varying degrees of anaerobiosis. In any case, these experiments show that NADPH–cytochrome P-450 reductase can reduce monomeric cytochrome P-450$_{LM4}$ generated by 20 mM CHAPS to the same extent as aggregated P-450$_{LM4}$ in 4 mM CHAPS or phospholipid.

Discussion

A good substrate for the cytochrome P-450 isozyme induced in liver by polycyclic aromatic substrates in mammalian liver (e.g., P-450$_{LM4}$) is acetanilide (Selander et al., 1974; Johnson & Muller-Eberhard, 1977). This substrate was therefore chosen to study the effects of a detergent on mixed-function oxidase activity mediated by P-450$_{LM4}$ and reductase. CHAPS was chosen because of its relatively high cmc and because high levels do not denature the cytochrome (Hjelmeland, 1980). Relatively low levels of this zwitterionic detergent (4–7 mM) were found to stimulate acetanilide hydroxylation by cytochrome P-450$_{LM4}$ and reductase in dilute solution both when 4-hydroxyacetanilide production was assessed or when the substrate-dependent rate of NADPH oxidation was determined. The level of product formation observed with detergent was approximately half that observed when the proteins were preincubated with phospholipid. Higher concentrations of CHAPS (15–20 mM) abolished both substrate-specific NADPH oxidation and 4-hydroxyacetanilide production. These elevated CHAPS concentrations also resulted in disaggregation of P-450$_{LM4}$ as measured both by sedimentation velocity and by sedimentation equilibrium. Oprian et al. (1983) have also shown that high [CHAPS] causes P-450$_{LM4}$ to dissociate to monomers.

A similar effect of detergent on activity and P-450 aggregation state was found previously for phenobarbital-inducible P-450$_{LM2}$ with *n*-octyl glucoside (Dean & Gray, 1982b) and CHAPS (Wagner et al., 1984). High levels of CHAPS or *n*-octyl glucoside had little effect either on the state of aggregation of NADPH–cytochrome P-450 reductase or on its ability to reduce the nonphysiological electron acceptor cytochrome *c* (Dean & Gray, 1982a; Wagner et al., 1984). Thus, the state of aggregation of cytochromes P-450 may be an important determinant of NADPH-dependent catalytic activity.

A possible explanation for inhibition of substrate hydroxylation observed for P-450's at high detergent levels might be an inability of the reductase to reduce monomeric cytochrome P-450. The experiments of Figure 5 argue against this possibility since the degree of P-450$_{LM4}$ reduction by NADPH–cytochrome P-450 reductase was about the same in 4 mM CHAPS or 0.05 mM di-12-GPC (conditions of high catalytic activity) and 20 mM CHAPS (inactive). However, it is possible that the rate of P-450 reduction under the three conditions differed. Indeed, Ingelman-Sundberg et al. (1983) showed in reconstituted vesicular systems that the charge of the phospholipid influences the rate of reduction of P-450 by reductase. Whether high detergent concentrations alter P-450 reduction kinetics in solution remains to be demonstrated.

Our sedimentation experiments carried out with P-450$_{LM4}$ in the presence of NADPH–cytochrome P-450 reductase indicate that at CHAPS levels which supported hydroxylation, a stable 1:1 complex between the cytochrome and reductase was not detectable. Similar results were obtained when a saturating level of acetanilide was included during equilibrium centrifugation, indicating that substrate binding to the cytochrome did not facilitate P-450–reductase association. The relatively long times required to achieve equilibrium in the centrifuge precluded attempts to assess complex formation by this technique under turnover conditions. However, sedimentation velocity measurements could be carried out with an active system; these experiments suggested also that substrate hydroxylation was not dependent on the formation of a bimolecular complex between cytochrome and reductase. Since it has already been established that a 1:1 complex is formed in the presence of phospholipid (French et al., 1979; Miwa et al., 1979), the complexes formed at the optimal level of CHAPS could either be made up of five to six P-450 molecules with an equal number of reductase molecules or be a complex in which some of the P-450 molecules are displaced by the reductase, e.g., a complex of three P-450's with three reductase molecules. The former complex is easily distinguishable by analytical ultracentrifugation since it involves an increase in molecular weight of 2.4-fold. The latter complex would result in a 19% increase in molecular weight, which is twice the usual range of experimental error observed with sedimentation equilibrium measurements of ±10%, and in addition would yield an increase in $s_{20,w}$ of 1.0 S which is a relatively large change in this parameter. We therefore conclude that low levels of detergent can support cytochrome P-450$_{LM4}$-mediated mixed-function oxidase activity without requiring formation of a stable cytochrome–reductase complex. This is the same conclusion we reached earlier with P-450$_{LM2}$ using either *n*-octyl glucoside or CHAPS (Dean & Gray, 1982a; Wagner et al., 1984). Thus, it appears that although a stable complex is formed between cytochrome P-450 and its reductase in the presence of phospholipid (French et al., 1979; Miwa et al., 1979) and also in the absence of any amphiphile (Müller-Enoch et al., 1984), such a complex is not a prerequisite for catalytic activity and may indicate the possibility of diffusion-limited interaction between the two components in the endoplasmic reticulum or liposomes as has been suggested by others (Duppel & Ullrich, 1976; Yang, 1977; Taniguchi et al., 1979, 1984).

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