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HYDRODYNAMIC PROPERTIES OF MONOMERIC CYTOCHROMES P-450$_{LM2}$ AND P-450$_{LM4}$ IN n-OCTYLGLUCOSIDE SOLUTION

William L. Dean and Robert D. Gray

Department of Biochemistry
University of Louisville
Louisville, KY 40292

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Summary. Sedimentation equilibrium and sedimentation velocity measurements were carried out on cytochrome P-450$_{LM2}$ from phenobarbital-treated rabbit liver and on cytochrome P-450$_{LM4}$ from 5,6-benzoflavone-treated rabbit liver in the presence of the nonionic detergent 1-O-n-octyl-β-D-glucopyranoside. P-450$_{LM2}$ was monomeric with a molecular weight of 48,800 and a Stokes radius of 3.1 nm in 7 g/l detergent and P-450$_{LM4}$ was monomeric with a molecular weight of 49,800 and a Stokes radius of 2.6 nm at 5 g/l detergent. Both particles were spherical in shape under these conditions. Neither cytochrome was irreversibly denatured at these detergent concentrations as indicated by the ability to form substantial amounts (>60%) of the CO adduct with an absorption maximum at 451 nm (P-450$_{LM2}$) or 448 nm (P-450$_{LM4}$) when diluted into detergent-free buffer containing CO and sodium dithionite.

Cytochrome P-450$_{LM2}$[1] and cytochrome P-450$_{LM4}$ can be purified in soluble form from liver microsomes of phenobarbital- or 5,6-benzoflavone-pretreated rabbits, respectively (1). The cytochromes, when reduced, bind both dioxygen and an organic substrate, and can be reconstituted into an enzymatically active hydroxylating system by mixing with purified NADPH-cytochrome P-450 reductase and a phospholipid such as dilauroylglyceryl-3-phosphorylcholine (2). While the state of aggregation of the cytochrome within the microsomal membrane is unknown, it has been suggested that multisubunit or multienzyme complexes of cytochrome P-450 may constitute the fundamental functional unit (3-6). Molecular sieve chromatography and sedimentation experiments are consistent with this viewpoint since in the absence of added detergent or phospholipid, or in the presence of phosphatidylcholine or the polyoxyethylene nonyl phenol, Triton

[1] Abbreviations used are: P-450$_{LM2}$, cytochrome P-450 isozyme induced in a rabbit liver by phenobarbital; P-450$_{LM4}$, cytochrome P-450 isozyme induced in rabbit liver by 5,6-benzoflavone; octylglucoside, 1-O-n-octyl-β-D-glucopyranoside.

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N-101, cytochrome P-450$_{LM2}$ and the analogous isozyme from rat liver, cytochrome P-450b, exist as aggregates containing from 6 to 9 monomers (7-10). Similar results have been obtained with P-450$_{LM4}$, although the size of the aggregate may be somewhat larger (8). Previous attempts to produce monomeric P-450$_{LM}$ have been equivocal (7, 11) or unsuccessful except in the presence of a denaturing detergent (7).

The present experiments were undertaken in order to find conditions where monomeric P-450$_{LM}$ could be produced for physico-chemical studies. It was found that the detergent n-octylglucoside at a concentration of 7 g/l produced monomeric P-450$_{LM2}$, and at 5 g/l monomeric P-450$_{LM4}$ was generated without complete denaturation and formation of cytochrome P-420.

MATERIALS AND METHODS

Cytochrome P-450$_{LM}$ - Cytochromes P-450$_{LM2}$ and P-450$_{LM4}$ were purified from hepatic microsomes from phenobarbital- and 5,6-benzoflavone-pretreated immature male rabbits using a modification of the procedure of Coon et al. (1). The specific content of the preparations used here was 12.0 nMol/mg protein for P-450$_{LM2}$ and 14.3 nMol/mg protein for P-450$_{LM4}$. Each preparation exhibited a single major protein band on gel electrophoresis by the method of Laemmli (12).

Other Materials - Octylglucoside was obtained from Sigma Chemical Co. (St. Louis, MO) and [^{14}C]octylglucoside (specific activity, 49 mCi/mMol) was from RPI International, Elk Grove, IL. It was diluted with unlabelled octylglucoside before use to give 100,000-400,000 cpm/ml.

Octylglucoside Binding - The binding of octylglucoside to P-450$_{LM}$ was determined by the gel filtration procedure of Hummel and Dreyer (13). A 7 x 115 mm column of Sephadex G-25 or Sephadex G-100 was equilibrated at room temperature with 0.15 M potassium phosphate, pH 7.5, containing 20% glycerol and 0.5 mM EDTA (phosphate buffer) and the desired [^{14}C]octylglucoside concentration. (All experiments were conducted at octylglucoside concentrations below the critical micelle concentration of the detergent.) The cytochrome sample (50 to 100 µl, approximately 50 µM in hemoprotein in the same octylglucoside-containing buffer) was incubated for 60 to 120 min, loaded on the column, and eluted with the appropriate detergent/buffer solution at a constant flow rate of 0.25 ml/min using a Pharmacia P-3 pump. Fractions of approximately 0.25 ml were collected, weighed, and the detergent concentration determined by counting 50-µl aliquots in 10 ml of Beckman Ready-Solve using a Beckman LS 7500 liquid scintillation counter. A second 50-µl aliquot of each P-450-containing fraction was diluted to 200 µl with phosphate buffer and the cytochrome P-450 content determined by the CO difference spectral method of Omura and Sato (14). A Varian-Cary Model 219 spectrophotometer equipped with a microcuvette accessory was used for spectral measurements. Protein concentration was estimated using the Lowry et al. (15) method with bovine serum albumin as standard.

Sedimentation Experiments - Ultracentrifugation was carried out at 20 °C in an AN-F rotor using a Beckman L5-75 preparative ultracentrifuge equipped with a Prep UV Scanner attachment operated at 405 nm with an interference filter (Oriel Corp., Stamford, CN). Sedimentation velocity measurements were conducted at 50,000 rpm, while sedimentation equilibrium was achieved by overspeeding for 3 hr at 12,000 rpm followed by overnight centrifugation at 8,000 rpm. At the

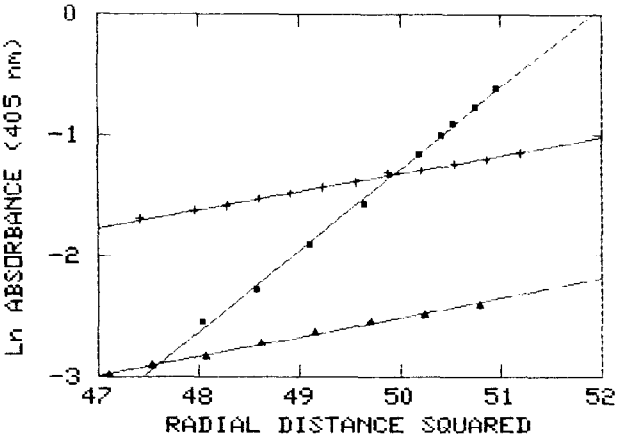

Fig. 1. Radial distribution of cytochrome P-450 isozymes at sedimentation equilibrium. (■): P-450$_{LM4}$ + 3 g/l octylglucoside; (▲): P-450$_{LM4}$ + 5 g/l octylglucoside. (+): P-450$_{LM2}$ + 7 g/l octylglucoside. Buffer was 0.15 M potassium phosphate, pH 7.5, 20% glycerol, 0.5 mM EDTA, 20 °C. Initial P-450 concentration in each case was approximately 5 μM. See text for additional details of the experiment and cytochrome recoveries.

end of each run recovery of P-450 and conversion to P-420 was assessed. The molecular weights were corrected for bound detergent as described by Tanford et al. (16). A $\bar{\nu}$ of 0.742 ml/g was used for P-450$_{LM2}$; values of 0.731 and 0.93 ml/g were calculated for P-450$_{LM4}$ and octylglucoside, respectively (17, 18).

RESULTS

Sedimentation equilibrium experiments conducted with cytochrome P-450$_{LM2}$ at a detergent concentration of 7 g/l and on cytochrome P-450$_{LM4}$ at octylglucoside concentrations of 3 and 5 g/l are shown in Fig. 1. The linear dependence of the natural log of the absorbance at 405 nm (which is proportional to the concentration of the cytochrome) on the square of the distance from the center of rotation indicates a monodisperse system in which only a single sedimenting species is present. When combined with a knowledge of the bound detergent (given in Table I), these experiments allow calculation of the molecular weight in each case (16). These values are summarized in Table I, and may be compared to the values of M_r determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate of 47,800 for P-450$_{LM2}$ and 53,000 for P-450$_{LM4}$ (17). Our results are very close to the latter values, and clearly indicate that octylglucoside at levels of 5 g/l and 7 g/l produces monomeric cytochrome P-450$_{LM4}$ and P-450$_{LM2}$, respectively. Sedimentation equilibrium measurements were also carried out with P-450$_{LM4}$ in the presence of 3 g/l octylglucoside;

TABLE I

Hydrodynamic Properties and Detergent Binding for Cytochromes P-450$_{LM2}$ and P-450$_{LM4}$ in Octylglucoside Solution[a]

Cytochrome Isozyme	[Octyl-glucoside] (g/l)	Bound Octyl-glucoside[d]	$s_{20,w}$	$\bar{M}_w$	R_o[b] (nm)	f/f_{min}	Aggregation State[c]
P-450$_{LM2}$	7	0.61 ± 0.25	4.4	48,800	3.1	1.04	Monomer
P-450$_{LM4}$	5	0.36 ± 0.05	4.7	49,800	2.6	1.00	Monomer
P-450$_{LM4}$	3	0.17 ± 0.02	12.3	220,000	4.1	1.00	Tetramer

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

[b]Stokes radius and frictional ratio determined from sedimentation equilibrium and sedimentation velocity data as described by Tanford et al. (16).

[c]Estimated assuming monomer molecular weight for P-450$_{LM4}$ of 53,000 and 47,800 for P-450$_{LM2}$ (17).

[d]g detergent/g protein.

under these circumstances, the predominant species exhibited a molecular weight of 220,000, which is consistent with a tetramer. As shown in Table I, sedimentation velocity experiments gave sedimentation coefficients consistent with the molecular weights determined by equilibrium centrifugation, and in addition allowed estimation of the frictional ratios and Stokes radii of the cytochromes. These data are also summarized in Table I, where it is evident from the calculated values of f/f_{min} (16) that the particles are spherical with Stokes radii in the range of approximately 2.6 to 4.1 nm.

The stability and recovery of the cytochromes was checked by measuring the CO-reduced difference spectra at the end of the sedimentation and gel filtration experiments. The spectra obtained at the conclusion of the sedimentation equilibrium experiments are reproduced in Fig. 2. These data were chosen for presentation since they represent the most stringent of conditions since the cytochrome preparations were exposed to the detergent for over 24 hrs; thus the P-420 produced represents the maximum amounts encountered. It is evident from the data of Fig. 2 that in all cases, when the samples were removed from the centrifuge cell and diluted 1:1 with detergent-free phosphate buffer, a majority of the cytochrome was capable of forming the characteristic

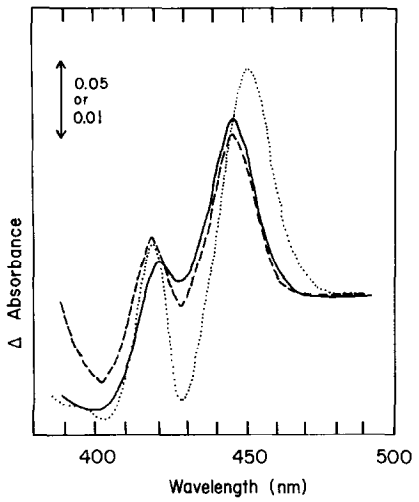

Fig. 2. CO minus reduced difference spectra for cytochrome P-450 isozymes after sedimentation equilibrium experiments of Fig. 1. The spectra were measured after 1:1 dilution of the samples with detergent-free phosphate buffer as described in the text. (·····): P-450$_{LM2}$ + 7 g/l octylglucoside; (----): P-450$_{LM4}$ + 5 g/l octylglucoside; (——) P-450$_{LM4}$ + 3 g/l octylglucoside. The 0.05 scale applies to the solid and dotted curves and the 0.01 scale applies to the dashed curve. Other details are given in the text.

ferrous-CO adduct absorbing near 450 nm, and a lesser portion was converted to the 420 nm absorbing species. Analysis of the spectra according to Sato and Omura (19) assuming a value for P-420 of 111 mM^{-1} cm^{-1} for $\Delta\varepsilon$ (490 nm - 420 nm), gave approximately 60% "native" P-450 for the drug-induced isozyme in 7 g/l octylglucoside, and 65-70% P-450 for the aromatic hydrocarbon-induced isozyme in 3 or 5 g/l detergent. It should be mentioned that in the case of P-450$_{LM2}$, the particular experiment shown in Fig. 2 represents the greatest degree of conversion to P-420 during a centrifuge run which we have observed; generally the conversion was less than 10%. It should also be pointed out that the recovery of P-450$_{LM2}$ at the end of the experiment was always greater than 85%, in contrast to P-450$_{LM4}$ where only about 20% was recovered at the higher detergent concentration, and 85% was recovered at the lower detergent concentration. We noted a tendency for P-450$_{LM4}$ to precipitate from solution at high detergent concentrations, while P-450$_{LM2}$ did not become insoluble even at detergent concentrations as high as 20 g/l. This may indicate that the monomeric P-450$_{LM4}$ produced represents a metastable species and that further aging under these conditions would have resulted in complete precipitation.

DISCUSSION

These experiments demonstrate that the nonionic detergent n-octylglucoside at concentrations of 5 and 7 g/l causes the disaggregation of cytochromes P-450$_{LM4}$ and P-450$_{LM2}$, respectively, with the formation of globular monomers whose molecular weights are close to those expected based on calibrated polyacrylamide gel electrophoresis and on their amino acid compositions (17). Furthermore, the cytochromes are predominately in the "native" conformation under these conditions as evidenced by their ability to form the characteristic CO adduct with an absorption maximum near 450 nm when reduced with sodium dithionite. Although there is some conversion under these conditions to the P-420 form, in the case of P-450$_{LM2}$ this may result from the combination of detergent and dithionite, rather than from the detergent alone, since the conversion was highly time-dependent after the addition of the reducing agent.

In studies to be reported elsewhere (20), we have examined the activity of P-450$_{LM2}$ in a reconstituted system containing soluble NADPH-cytochrome P-450 reductase and octylglucoside. We found that the monomer was unable to catalyze the N-demethylation of benzphetamine, while aggregated P-450$_{LM2}$ in the presence of octylglucoside and reductase did exhibit enzymatic activity.

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