INELASTIC LASER LIGHT SCATTERING AND PARTICLE SIZE OF DETERGENT-TREATED MICROSOMES

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SUMMARY: Using inelastic laser light scattering we have determined the hydrodynamic diameters of a variety of hepatic microsomal preparations. Whole microsomes have a diameter of 3200 Å. Treatment of microsomes with deoxycholate or cholate and chromatography on DEAE-cellulose give three protein fractions: a "non-absorbed" fraction with particles 2650 Å in diameter, cytochrome P-420 1700 Å in diameter and cytochrome c reductase 760 Å in diameter. Preparation of cytochrome P-450 by $(NH_4)_2SO_4$ precipitation from cholate solution gives particles 640 Å in diameter. All of these sizes are much too large to represent single molecular species, indicating that these fractions are aggregates of membrane proteins with varying concentrations of lipids.

INTRODUCTION

The hepatic microsomes are a complex mixture of molecular species which can be identified by their spectral and enzymatic properties, but which because of their hydrophobic nature have proven difficult to solubilize and purify. Since the biochemical and physical properties of such a mixture are often difficult to study and to interpret, it is not surprising that a great deal of effort has been directed to the development of relatively gentle procedures which will allow some separation of the various components with minimal loss of enzymatic activity. Of these procedures, that of Lu et al. (1) has, with minor modifications (2,3) been the most widely accepted. In this method, microsomal suspensions are clarified by treatment with either cholate or deoxycholate in a buffer containing glycerol and dithiothreitol. After centrifugation and chromatography or precipitation procedures, three major fractions are obtained: a cytochrome P-420 fraction, a cytochrome c reductase

fraction, and a phospholipid fraction. When these are combined some over-all mixed-function oxidase activities can be observed.

In spite of the obvious importance of this method for the study of the microsomal enzyme complexes, the precise physico-chemical structures of the cytochrome and reductase fractions are not known. On the one hand the failure of these fractions to sediment after ultracentrifugation for one to three hours (1) would suggest that they are composed of relatively low molecular weight species. On the other hand we have found that these fractions fail to enter a five percent acrylamide gel suggesting that they are in reality very large particles. Yet because a number of assumptions must be made in interpreting the results of either of these types of experiments, neither method can give more than a crude estimate of the molecular weight or size of the particles in the separated fractions. We have sought to resolve these ambiguities by determining the size of the predominant species in the cytochrome and reductase fractions. For this we have employed inelastic laser light scattering, a technique that yields the translational diffusion coefficient, and thereby the hydrodynamic radius, of macromolecular particles in solution. Our results indicate that even the smallest of these particles is much too large to be considered mono-molecular species or even aggregates of a few molecules. It is more likely that they represent small membrane fragments of very high molecular weight.

METHODS

Hepatic microsomes and microsomal fractions were prepared as previously described from male, Sprague-Dawley rats obtained from Charles River, Inc. (4,5). In the initial series of experiments the animals were used without prior treatment. In subsequent studies they were given phenobarbital (1 mg/ml) in their drinking water for four days prior to sacrifice.

In the initial experiments the microsomal suspension was treated with a buffer containing deoxycholate as the detergent (1), while in later experiments cholate was substituted (3). In both cases, the microsomes were sonicated

prior to treatment with the detergents. After clarification, the suspensions were centrifuged for one hour at 105,000 g and the pellet discarded.

Portions of the supernatant were used for the preparation of cytochrome P-450 by precipitation with ammonium sulfate (42-50% of saturation at 0°) as described by Lu et al. (3). This fraction was further purified by treatment with calcium phosphate gel (3).

The remainder of the supernatant was chromatographed on DEAE-cellulose with a 0-0.5 M NaCl gradient (1) and three fractions were obtained: a "non-absorbed" peak, a cytochrome P-420 peak, and a cytochrome c reductase peak. In some experiments the latter peak was further treated with alumina c γ gel (1).

All the above fractions were concentrated with an Amicon Diaflo pressure ultra filtration apparatus with a UM-10 membrane to give 2-3 mg of protein/ml.

The average hydrodynamic diameter of the particles in each fraction was determined by the method of inelastic laser light scattering, using the apparatus previously described by Lin et al. (6). For a scattering angle of 90°, the diameter D of the particles (in Å) is related to the half-width at half-height Γ (hz) of the scattered light spectrum by the equation.

$$D = 9.19\, n^2 T/\eta\Gamma$$

The solvent viscosity, η, and the refractive index, n, were taken equal to those of water. T is the absolute temperature.

Except where noted, all spectra had Lorentzian line shapes characteristic of monodisperse solutions of spherical particles. Diameters measured by this technique are accurate to ± 3%, although observed reproducibility may be higher.

RESULTS AND DISCUSSION

As can be seen in Table 1, all the microsomal preparations prior to treatment with detergents have identical diameters (ave. = 3290 Å ± 50 Å SEM). Moreover, as can be seen in Figure 1, the scattered light spectrum from these particles can readily be fit to a single Lorentzian curve, suggesting that there is little variation in the particle size within any preparation. It is surprising that these preparations should have such reproducible diameters in

TABLE 1

THE HYDRODYNAMIC DIAMETER OF VARIOUS HEPATIC MICROSOMAL PREPARATIONS FROM MALE RATS

Preparation[a]	Other Manipulations	Diameter[b], Å
Whole microsomes	Fresh	3178
	Frozen[c]	3348
Smooth microsomal fraction	------	3178
Rough microsomal fraction	------	3332
Breakthrough peak	------	
	Rechromatographed	1884, 1108[f]
Cytochrome P-420[d]	Fresh	1710
	Frozen[c]	1760
Cytochrome reductase[d]	Before alumina c γ gel	1040-1880[g]
	After alumina c γ gel	760[g]
Cytochrome P-450[e]	Fresh	600
	Frozen[c]	680

[a] Preparations given in the text.
[b] Determined by inelastic laser light scattering.
[c] Suspension or pellet frozen for 3-12 days at -20°C.
[d] Fractions from DEAE-cellulose chromatography according to the method of Lu et al. (1).
[e] Prepared by $(NH_4)_2SO_4$ precipitation by the method of Lu et al. (3).
[f] Spectral shape indicated polydispersity, fit as sum of two Lorentzians corresponding to particles with diameters shown.
[g] Spectral shape indicated polydispersity.

view of the fact that they were obtained over a period of several months from several groups of animals, some of which had received phenobarbital while others had not. Further, the microsomes, unlike other organelles, do not exist as such in the living cell, but rather are an artifact of the homogenization of the endoplasmic reticulum. These data suggest that the characteristic size of these particles is a result of the composition of the membranes and of a consequent balance between the various forces (hydrophobic, surface tension, etc.) characterizing membrane - membrane and membrane - water interactions.

The "non-absorbed" peak appears by inelastic light scattering to be relatively monodispersed with a particle size near that of whole microsomes and

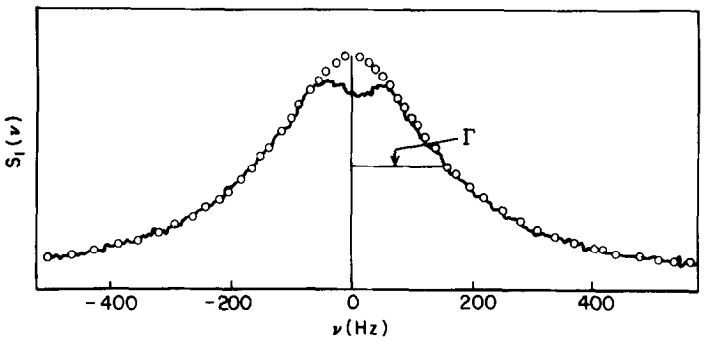

Figure 1. Inelastic laser light scattering spectrum from dilute suspension of rough microsomes. Solid line, experimental; dotted line, best Lorentzian fit, with Γ = 160.2 Hz, corresponding to a hydrodynamic diameter of 3332 Å.

having high concentrations of both cytochrome P-450 and cytochrome c reductase. Rechromatography of this fraction leads to two subfractions, the first of which has no cytochromes or reductases. The second subfraction contains both cytochrome P-450 and cytochrome c reductase and has a spectrum which, at 5kHz sweep width, cannot be fit to a single Lorentzian but appears to be the sum of two Lorentzians, corresponding to particles with diameters of 1884 Å and 1108 Å.

The second peak contains the highest concentration of the cytochromes. It has been our experience that in the preparation of fractions by this method the predominant cytochrome is cytochrome P-420, although some cytochrome P-450 is also observed. This species has an average diameter of 1710 Å ± 20 Å SEM. Clearly this is not a monomolecular species, for if it were a single compact globular protein, it would have a molecular weight of 2.1×10^9 (7). Rather it represents an aggregate of protein molecules which in these studies we have found to contain essentially no phospholipid.

The last chromatographic peak studied, the cytochrome c reductase, as prepared from the column had a highly variable size ranging from 1040-1880 Å. When this preparation was purified with alumina c γ gel the light scattering spectrum still exhibited polydispersity, but the size was now much smaller (~760 Å). These particles again must be aggregates of a number of proteins for this diameter corresponds to a compact, globular protein with a molecular

weight of 1.9 x 10^8. The reductase contained, per mg protein, about 10% of the original phospholipids.

Finally cytochrome P-450 prepared by ammonium sulfate fractionation proved to be considerably smaller (640 ± 40 Å) than the cytochrome P-420 prepared by the column method but still is much too large to represent a single molecular species (globular protein of equivalent size = 1.1 x 10^8).

Neither pretreatment of the animals with phenobarbital nor freezing of the preparations appeared to affect the size of any of the fractions.

CONCLUSIONS

These data clearly indicate that the predominant species in all these fractions are aggregates of membrane proteins with varying concentrations of lipids. It may well be that the detergent in the clarified preparations replaces the phospholipids leading to the formation of large low density particles. These data cannot distinguish whether the particles are vesicles or whether they are more or less "solid" with no water core. The resolution of this question will have to await the results of more extensive studies now in progress on the physical properties of these preparations.

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