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Cytochrome P-450: hexameric structure of the purified LM4 form

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Purified cytochrome P-450$_{LM4}$ was found to be monodisperse in 20% glycerol by analytical ultracentrifugation. Its $S_{20,w}$ value was quite similar to that of hexameric P-450$_{LM2}$. At lower glycerol concentrations the P-450$_{LM4}$ oligomers showed a tendency to aggregate. The P-450$_{LM4}$ oligomers were immobilized on Ultrogel A4 under conditions allowing only one covalent link to the matrix per oligomer. In the presence of SDS, the oligomers dissociated leaving only 1/6th of the initial amount of bound protein on the matrix, suggesting that the purified P-450$_{LM4}$ is a hexamer. This was confirmed by electron microscopy. The quaternary structure of the P-450$_{LM4}$ was similar to that demonstrated earlier for P-450$_{LM2}$.

Cytochrome P-450$_{LM4}$; Quaternary structure; Hexamer; Immobilized protein

1. INTRODUCTION

Cytochrome P-450, the hepatic microsomal monooxygenase which catalyzes the metabolism of a wide variety of endogenous substrates and xenobiotics, comprises a family of hemoproteins differing in substrate specificities and physicochemical properties [1,2].

Purified cytochrome P-450 forms containing no phospholipids or detergents have been found to be oligomers. Various oligomer molecular masses have been reported for one and the same cytochrome P-450 form [2–6].

Many detergents, including non-ionic detergents used to isolate cytochrome P-450 forms, have been shown to dissociate oligomers [4,6–9]. This suggests that hydrophobic interactions between monomers are involved in the oligomer formation. After removing detergents, oligomers reform in solution [3,8,9]. Variations of oligomer molecular masses may be caused by non-specific association of monomers after removal of phospholipids and detergents. The question is whether the oligomer organization of purified cytochrome P-450 corresponds to its native state in microsomal membranes.

Using purified cytochrome P-450$_{LM2}$ preparations which were monodisperse in ultracentrifugation experiments, we showed that P-450$_{LM2}$ is inherently hexameric [10,11]. Electron microscopy revealed only one type of particles, i.e. hexamers of identical structure [10,11]. The hexamers were stable under a wide variety of conditions and did not dissociate in the presence of phospholipids [8,9]. Rather high concentrations of detergents were required to dissociate the cytochrome hexamers [7–9]. It is known that much lower concentrations of non-ionic detergents can substitute to some extent for phospholipids in stimulating the catalytic activity of P-450 [12,13]. These low detergent levels are not capable of causing P-450 oligomer disaggregation [7,8,13]. In the monooxygenase system reconstituted from NADPH-specific flavoprotein and oligomeric cytochrome P-450$_{LM2}$, maximal hydroxylating activity can be obtained in the absence of phospholipids or detergents if the enzyme concentration is sufficiently high [14].

P-450$_{LM2}$ hexamers have been found in proteoliposomes [15–17] using different phospholipids (e.g. total microsomal phospholipid fraction) and various methods for the proteoliposome reconstitution [16,17]. Comparison of kinetic parameters for the reduction of isolated cytochrome P-450$_{LM2}$ in its oligomeric and dissociated states with those of cytochrome P-450 in microsomes suggested a functional significance for P-450 oligomeric organization [18].

Among cytochrome P-450 forms, cytochrome P-450$_{LM4}$ (other names, 1A2, P-448) shows the most hydrophobic behavior during preparative procedures. It hydroxylates such hydrophobic molecules as aromatic hydrocarbons [1,2,19]. There is evidence that the reduction of P-450 and P-448 in microsomes occurs in different phases, i.e. at the water/membrane interface and in the lipid phase, respectively [19]. If the oligomerization of cytochrome P-450 was non-specific, one would expect a variable number of monomers in oligomers, differing in cytochrome P-450$_{LM4}$ and in the more hydrophilic cytochrome P-450$_{LM2}$. On the contrary, hexameric structures for both P-450$_{LM4}$ and P-450$_{LM2}$ would suggest that this quaternary structure is a natural

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feature of microsomal forms of cytochrome P-450. Evidence for the hexameric structure of the LM4 form is given below.

2. MATERIALS AND METHODS

$P\text{-}450_{LM2}$ was isolated from liver endoplasmic reticulum as described earlier [10,11]. Cytochrome $P\text{-}450_{LM4}$ was purified using a similar procedure It was eluted from ω-aminooctyl-Sepharose with 0.2% Emulgen 913 after removal of other cytochrome P-450 forms with lower concentrations of the same detergent. Further purification and removal of detergent was performed by the method of Imai et al. [20] without any modifications.

$P\text{-}450_{LM4}$ was purified from liver microsomes of rabbits which had been treated with 3-methylcholanthrene (25 mg/kg, 1 injection per day during 3 days). For some experiments, both LM4 and LM2 were isolated from the same microsomes of rabbits which had been treated with phenobarbital. The series of experiments on immobilized LM4 was performed mainly with enzyme preparations of the first type. For electron microscopy, the second type of preparations was also used The identity of cytochrome $P\text{-}450_{LM4}$ in the two types of preparations is beyond doubt [20,21].

SDS electrophoresis of the purified cytochromes was performed in a 7–17% concentration gradient of polyacrylamide gels. Each cytochrome revealed a single band (see our previous publication [11]).

Spectra of the cytochromes were measured with Hitachi-557 and Aminco 2W-a spectrophotometers.

Analytical centrifugation (60,000 rpm) was performed using a Beckman Spinco E ultracentrifuge with a photoelectric scanning system.

The proteins were immobilized using bromocyan-activated Ultrogel A4. Activation was performed in 1 M phosphate buffer, pH 10.7, and various concentrations of bromocyan (3–7 mg/g moist gel). Other conditions were like those described previously in cytochrome $P\text{-}450_{LM}$ study [22]. The concentrations of the cytochrome P-450 forms were measured by a differential scheme using absorbance of the carbon monoxide complex of the dithionite-reduced cytochrome at $\lambda = 450$ nm [23]. The amount of immobilized cytochrome was estimated by the same method and by measuring the amount of Coomassie G 250-protein adduct [22,24]. The protein remaining on the matrix after treatment with SDS was assayed by the latter method. The validity of the methods for quantitative assay of the immobilized cytochromes was established in previous studies using magnetic circular dichroism spectra [25].

For electron microscopy, we diluted the purified cytochrome with 10 mM phosphate buffer (pH 7.4) containing the detergent Emulgen 913, the final detergent concentration being no more than 0.004%. The solution was applied to a thin carbon support film and negatively stained with 5% potassium silicotungstate, pH 7.0. Electron microscopy was performed with a Philips EM 400 electron microscope at 80 kV and 50,000 × magnification.

3. RESULTS AND DISCUSSION

The purified cytochrome $P\text{-}450_{LM4}$ was found to be free of other P-450 forms and showed its typical spectral characteristics [1,2,21]. The Soret peak of the reduced CO complex was at 448 nm. Oxidized cytochrome $P\text{-}450_{LM4}$ showed an absorption maximum at 393 nm and a weak band at 646 nm (the attribute of the high spin state [21]). The α- and β-bands were not well resolved. The A_{393}/A_{276} ratio, reporting on the extent of cytochrome purification from Emulgen 913, averaged about 1. The purified hemoprotein was active in benzo(α)pyrene hydroxylation in the reconstituted monooxygenase system.

The boundary of the $P\text{-}450_{LM4}$ sedimentation area remained symmetrical during analytical ultracentrifugation in 20% glycerol (Fig. 1). The $S_{20,w}$ values calculated for different preparations varied from 8.46 to 8.6 S. This is close to the values found for hexameric cytochrome $P\text{-}450_{LM2}$ (8.5 ± 0.5 S) determined in our previous experiments [10,11].

Because cytochrome $P\text{-}450_{LM2}$ and $P\text{-}450_{LM4}$ monomers have similar molecular masses [1,2], the ultracentrifugation data suggest that cytochrome $P\text{-}450_{LM4}$, like $P\text{-}450_{LM2}$, is hexameric. It should be noted, however, that cytochrome $P\text{-}450_{LM4}$ oligomers, unlike those of the more hydrophilic $P\text{-}450_{LM2}$, had a tendency to aggregate at lower glycerol concentrations producing some heavy fractions. The same effect was observed in the electron microscopic experiments when glycerol was removed from the solution.

In the next series of experiments, the cytochrome $P\text{-}450_{LM4}$ oligomers were immobilized on Ultrogel A4 under conditions such that only one covalent link between an oligomer and the matrix could be formed due to the low density of active groups on the matrix. Immobilization of cytochrome $P\text{-}450_{LM2}$ on Sepharose 4B was described earlier [22]. In that study, SDS treatment of the immobilized $P\text{-}450_{LM2}$ removed 5/6ths of the initially bound protein, i.e. all of the non-covalently bound monomers of each hexamer.

In the present work, Ultrogel A4 activated with bromocyan was used as the matrix (see section 2). The non-specifically absorbed protein was removed as described previously [8,9] using a low concentration of sodium cholate which did not cause dissociation of the cytochrome P-450 oligomers. The immobilized cytochrome $P\text{-}450_{LM4}$ retained the spectral parameters of the original preparation. It preserved the ability to transform into the low-spin state in the presence of substrates. Differential spectrum of the CO complex of immobilized cytochrome did not reveal any absorption band at 420 nm, which indicates the P-450 conversion to an inactive derivative.

The results of SDS treatment of immobilized forms LM2 and LM4 are listed in Table I. The data suggest

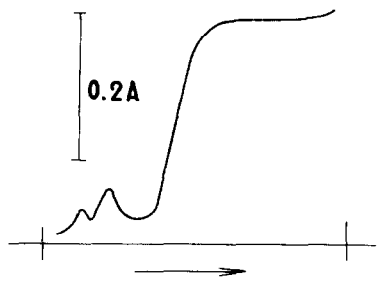

Fig. 1. Analytical ultracentrifugation sedimentogram of cytochrome $P\text{-}450_{LM4}$ at 26th min of the sedimentation experiment (60,000 rpm, 20°C, $\lambda = 393$ nm). The samples contained 0.1 M phosphate buffer (pH 7.4), 20% glycerol, and $P\text{-}450_{LM4}$ 3.5×10^{-6} M heme).

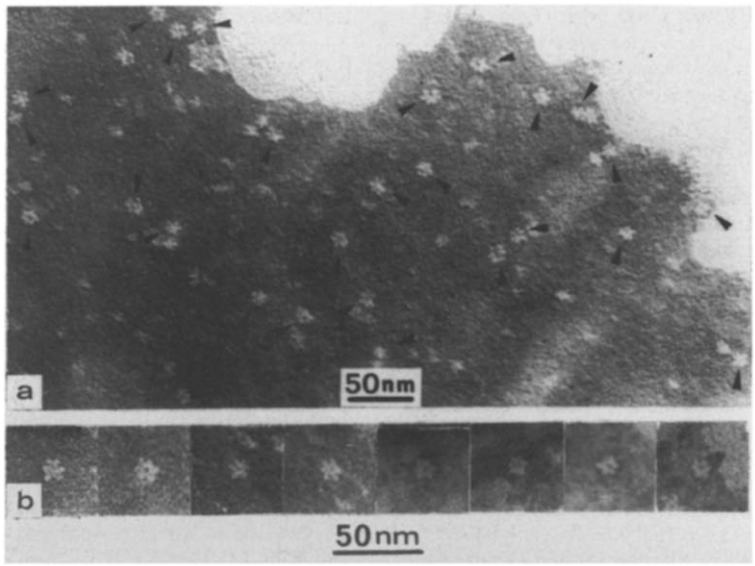

Fig. 2. Micrographs of purified cytochrome P-450 negatively stained with potassium silicotungstate. (a) General view; (b) characteristic images of particles.

the purified LM4, like LM2, is hexameric. After removal of the non-covalently bound monomers, the residually bound cytochrome was approximately one-sixth of the initial quantity of protein immobilized, irrespective of the total amount immobilized.

For electron microscopy of P-450$_{LM4}$, Emulgen 913 was substituted for glycerol to prevent aggregation of the protein. The concentrations of this non-ionic detergent was always less than the critical micelle-forming concentration. In our previous experiments, such low detergent levels did not induce dissociation of the cytochrome oligomers [7].

Micrographs of samples are presented in Fig. 2. Taking into account that P-450$_{LM4}$ preparations are monodisperse, the images observed on the micrographs can be regarded as various projections of a single type of particle.

The most frequently occurring image is a six-pointed 'star' 12 ± 1 nm in diameter. This probably corresponds to the most stable position of the molecule on the support film.

The shape and linear dimensions of the P-450$_{LM4}$ particles are similar to those of P-450$_{LM2}$ [10,11]. Thus the electron microscopic data also suggests that the purified cytochrome P-450$_{LM4}$ has hexameric quaternary structure.

The particle projections shown in the micrographs (Fig. 2a,b) allow for two possible models for the hexamer structure of cytochrome P-450$_{LM4}$: six subunits can be arranged either in one layer or in two layers at the vertices of triangular antiprism. The second model is that proposed previously for cytochrome P-450$_{LM2}$ [10,11].

In this context, cytochrome H-450 should be mentioned. This non-membrane analog of cytochrome P-450 purified from liver cytosol was found to be not identical to any membrane form of cytochrome P-450 [26,27]. Cytochrome H-450, isolated without the use of surface-active compounds is an oligomer of about 400 kDa molecular mass, corresponding to that of a hexamer (64 kDa × 6).

Thus, hexameric quaternary structure is inherent in all the studied members of the family of liver P-450 cytochromes.

Table I

Dissociation of immobilized oligomers of cytochromes P-450$_{LM2}$ and P-450$_{LM4}$ in the presence of SDS

Cytochrome form	Initial amount of immobilized protein (μg/g moist gel)	Protein remaining on matrix after SDS treatment	
		μg	%
LM2	325	54	16.6
LM4	310	52.5	16.9
LM2	220	37	16.8
LM4	220	36	16.6
LM2	160	26	16.2
LM4	175	30	17.1

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