Quaternary structure of the liver microsomal cytochrome P-450

V.L. Tsuprun, K.N. Myasoedova*, P. Berndt+, O.N. Sograf, E.V. Orlova, V.Ya. Chernyak+, A.I. Archakov* and V.P. Skulachev+

*Shubnikov Institute of Crystallography, *Institute of Chemical Physics, USSR Academy of Sciences* and *+Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR*

Received 12 June 1986

Cytochrome P-450$_{LM2}$ was isolated from rabbit liver microsomes in a form which was shown to be homogeneous in AcA-22 Ultrogel and ultracentrifugation studies. The molecular mass determined by sedimentation equilibrium roughly corresponded to hexamer composed of 56 kDa monomers. Hexamer structure of the cytochrome was directly demonstrated by electron microscopic study. In the cytochrome P-450$_{LM2}$ hexamer, monomers seem to be arranged in two layers (three monomers in the layer) in such a way that each monomer occupies a position at the vertices of a triangular antiprism with a 32 point group symmetry.

Cytochrome P-450 *Quaternary structure* *Electron microscopy*

1. INTRODUCTION

Cytochrome P-450, oxidizing a wide range of xenobiotics in liver endoplasmic reticulum was found to exist in several isoforms. Among them isoforms LM2 and LM4 are most studied. The amount of LM2 and LM4 can be greatly increased by in vivo treatment of the animal with phenobarbital or methylcholanthrene [1]. Cytochrome P-450 is tightly bound to the reticular membrane in spite of the fact that the amino acid sequence points to the presence of rather large hydrophilic domain(s) [2]. Solubilized cytochrome P-450 is stable in water solutions supplemented with glycerol. Addition of the NADPH-specific reductase and a small amount of a phospholipid or some detergents to the solubilized cytochrome P-450 results in reconstituting the system competent in oxidation of xenobiotics [1,4,14]. Recently maximal hydroxylating activity of the solubilized cytochrome P-450 was demonstrated in the absence of phospholipids and detergents [3].

Solubilized cytochrome P-450 in glycerol forms oligomers, the molecular mass of which measured in several laboratories varies from 300 to 500 kDa usually being slightly higher than 300 kDa. Under denaturating conditions, ~50 kDa monomers were formed [1]. Maximal enzymatic activity of cytochrome P-450 is observed under conditions when it should be in oligomer form [4]. In some cases it was found that monomers and dimers of LM2 and LM4 isoforms in the presence of high concentrations of detergents cannot oxidize the NADPH-cytochrome P-450 reductase [4,5]. Under other conditions, some oxidizing activity was shown to be inherent in monomers [4]. There are some pieces of indirect evidence that cytochrome P-450 is present in microsomal membrane as an oligomer [6–8].

In this paper, we show that purified cytochrome P-450$_{LM2}$ forms hexamers.

2. MATERIALS AND METHODS

Cytochrome P-450$_{LM2}$ was isolated from liver microsomes of rabbits (males) which had been given 0.1% phenobarbital solution instead of water for a week. Membranes were solubilized by

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50 © 1986 Federation of European Biochemical Societies

sodium cholate and treated with polyethylene glycol as described earlier [9] with some modifications. Fraction containing cytochrome P-450$_{LM2}$ was passed through a column with ω-aminooctyl-Sepharose which was synthesized from 1,8-diaminooctane and Sepharose 4B (Pharmacia) activated by BrCN. For elution of cytochrome P-450, 0.1% non-ionic detergent emulgen 913 (Kao-Atlas) was used. Further purification by means of hydroxyapatite and CM-Sephadex and removal of detergents were carried out as described in [10]. All the solutions contained 20% glycerol. Spectral measurements were done with a Hitachi 557 spectrophotometer. The cytochrome concentration was determined employing a differential scheme.

The following molar extinction coefficients were used: 91 $mM^{-1} \cdot cm^{-1}$ at 450 nm as compared with A_{490} for reduced CO-complex and 100 $mM^{-1} \cdot cm^{-1}$ at 418 nm for oxidized cytochrome.

Electrophoresis of the samples treated with SDS and mercaptoethanol were done in 7–17% polyacrylamide gel in Tris-glycine buffer, pH 8.3–8.4, and 0.1% SDS, using $9 \times 12 \times 0.1$ cm plates and voltage of 220–250 V.

Analytic centrifugation was carried out in Spinco E ultracentrifuge with scanning system, cytochrome chromophore and protein being detected at 418 and 280 nm, respectively. Sedimentation was done at 60000 rpm; equilibrium sedimentation at 8000 rpm. Equilibrium was reached in 30 h.

For electron microscopy, a solution of the cytochrome P-450 in 0.1 M K^+-phosphate buffer, pH 7.5, and 20% glycerol was used. The protein concentration was 3×10^{-5}–1×10^{-4} M. A drop of the protein solution was placed on a thin carbon support film and the grids were rinsed first with distilled water and then negatively stained with 5% potassium silicotungstate. Electron microscopy was carried out with a Philips EM 400 electron microscope at 80 kV (magnification × 50000). The electron micrographs were digitized with a Perkin-Elmer PDS 1010 A flatbed microdensitometer, a sampling distance corresponding to 0.3 nm. Image processing was carried out by a NORD-100 computer. Each image or its part could be displayed on a Tektronics graphic display as a counter map. The first step of processing included masking and low-pass filtration. A circular mask was applied to select the parts of images containing the protein particle. Low-pass filtration eliminated part of the noise of high spatial frequences. For these purposes, the Fourier transform was multiplied by a

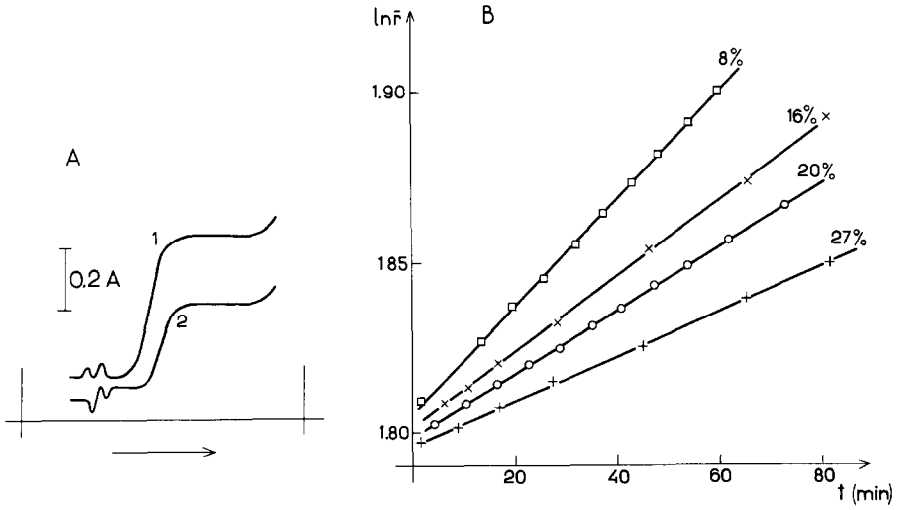

Fig.1. Hydrodynamic characteristics of isolated cytochrome P-560$_{LM2}$. (A) Sedimentograms run at 60000 rpm: (1) 420 nm, 26th min; (2) 280 nm, 32nd min. The mixture contained 4×10^{-6} M cytochrome P-450$_{LM2}$, 0.1 M K^+-phosphate, pH 7.5 and 7% glycerol. (B) Measurement of the sedimentation coefficient of cytochrome P-450$_{LM2}$ in 0.1 M K^+-phosphate at different glycerol concentrations. 60000 rpm, 20°C.

rotational symmetric Gaussian function. The images to be averaged were aligned by a cross-correlation function with one particle selected as a reference [13]. The quality of correlation was controlled by a correlation coefficient. Particles with a correlation coefficient higher than that of the given threshold were taken for averaging.

3. RESULTS AND DISCUSSION

The preparations of cytochrome P-450$_{LM2}$ showed typical spectral characteristics (see [1]), were practically free of detergent and active in the reconstituted monooxyenase system (see [14]). They were homogeneous when passed through a 1.5×95 cm AcA-22 Utrogel column showing a single peak of protein with a 360 kDa apparent molecular mass.

Ultracentrifuge sedimentation experiments (fig.1A) revealed a single protein of 8.5 ± 0.5 S. This value ($s_{20°w}$) did not depend upon protein concentration ($2-14\times10^{-6}$ M) and temperature (4–20°C).

When sedimentation equilibrium was studied, typical distribution of cytochrome in the ultracentrifuge sample showed homogeneity of the protein (fig.2).

Estimation of the molecular mass was carried out as in [12] using a $\bar{V}_p$ value calculated (i) after Coon and Edsal [16] using amino acid composition of cytochrome P-450$_{LM2}$ [17] or (ii) from data of the speed centrifugation in buffers of different density (5–30% glycerol, fig.1B). In cases (i) or (ii), $\bar{V}_p$ and molecular masses were found to be equal to 0.746 $cm^3 \cdot g^{-1}$ and 300–320 kDa or 0.780 $cm^3 \cdot g^{-1}$ and 350–370 kDa, respectively. $\bar{V}_p$ = 0.8 $cm^3 \cdot g^{-1}$ was estimated by Wendel et al. [18].

SDS electrophoresis of cytochrome P-450 (fig.3) did not reveal minor bands. A single protein band was found approximately corresponding to 50 kDa polypeptide. This value is in agreement with data

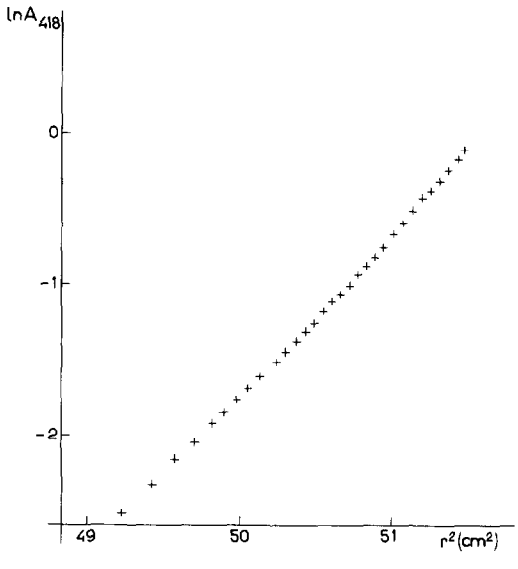

Fig.2. Equilibrium sedimentogram of cytochrome P-450$_{LM2}$. The mixture contained 2×10^{-6} M cytochrome P-450$_{LM2}$, 0.1 M K^+-phosphate, pH 7.5, and 7% glycerol. The all-speed sedimentation equilibrium method was employed to estimate the zero level [12].

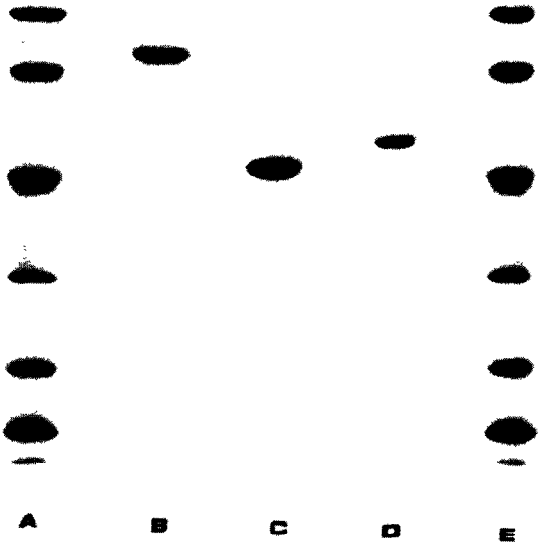

Fig.3. SDS electrophoresis of cytochrome P-450$_{LM2}$. Lanes: A,E, lactalbumin (14.4 kDa), soy-bean trypsin inhibitor (20.1 kDa), carboanhydrase (30 kDa), ovalbumin (43 kDa), beef serum albumin (67 kDa), phosphorylase (94 kDa); B, NADPH-cytochrome *c* reductase; C, cytochrome P-450$_{LM2}$; D, cytochrome P-448$_{LM4}$.

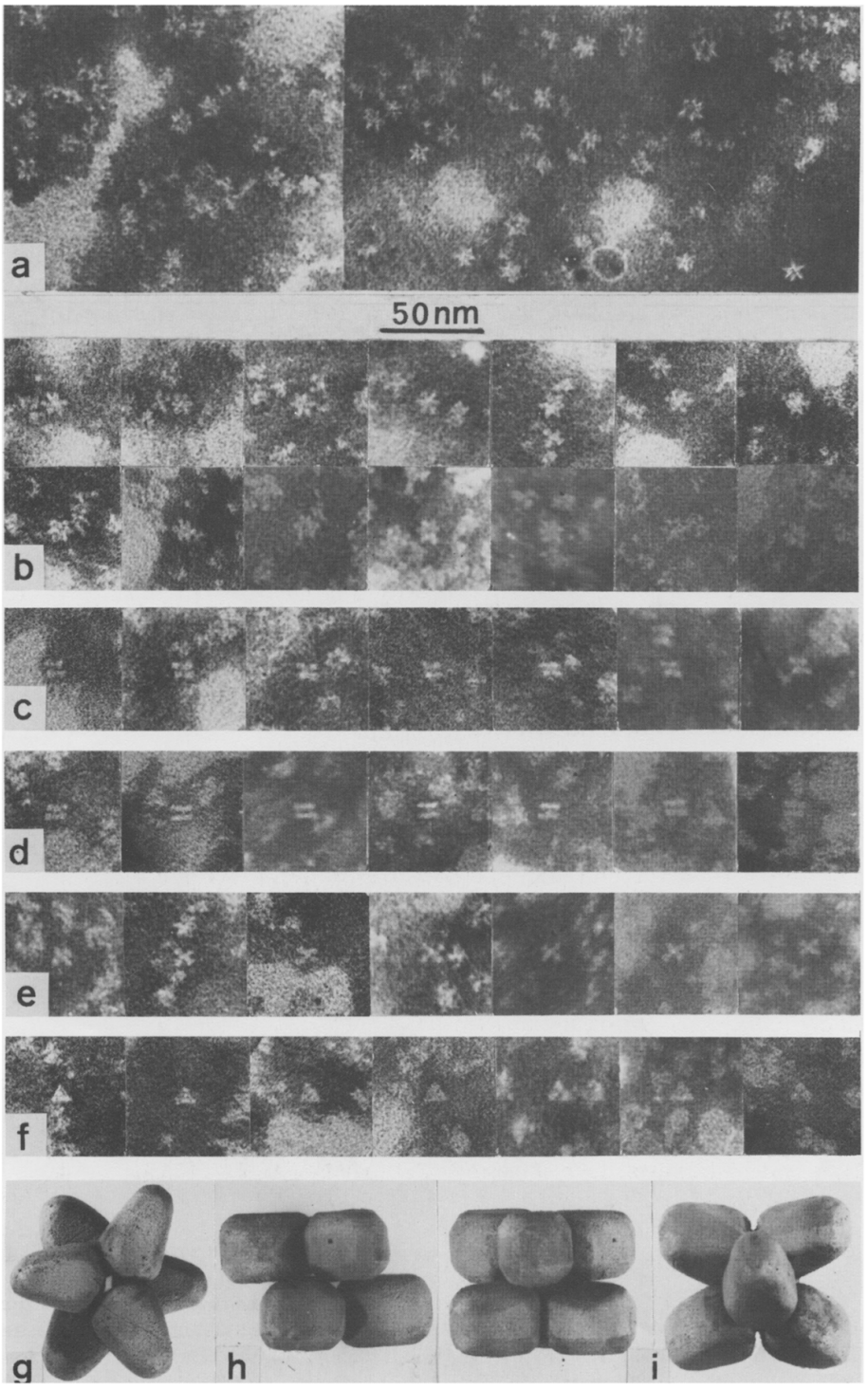

Fig.4. (a) General view of the cytochrome P-450$_{LM2}$ molecules negatively stained with potassium silicotungstate. (b–e) Characteristic images of the first–fourth types, respectively, (f) triangular images and (g–i) different views of the model corresponding to images of the first–fourth type, respectively.

reported previously when the same method was used [1], being slightly lower than that calculated from the amino acid sequence (55.8 kDa) [17,19]. Comparing molecular masses determined in ultracentrifugation and SDS electrophoresis measurements one may assume that cytochrome P-450$_{LM2}$ can exist as oligomer composed of six monomers. Such an assumption was directly proved in the electron microscopic study.

The single particle images of the cytochrome P-450$_{LM2}$ oligomers are shown in fig.4a. The images observed in micrographs can be regarded as projections of one and the same molecule. The first type of images has the form of a six-pointed 'star' 11 ± 1 nm in size (fig.4b). This type is found more frequently than others and most probably corresponds to the stable position of the molecule on the support film. In fig.4c, the second type of images is shown. It looks like a parallelogram of 10.5 ± 0.5 nm length and about 7 nm width. The third type (fig.4d) has a rectangular form about 10×7 nm in size. In the fourth type (fig.4e) cross-like images are observed. When comparing the images of the molecule obtained, it may be suggested that the first type is a front view projection and the second and third types are side views. Analysis of all the projections allowed us to suggest a simplified model for the molecule assuming that six subunits are arranged at the vertices of a triangular antiprism so that the molecule has a bilayer structure. We also observed triangular images having a form and size corresponding to half of the hexameric molecule (fig.4f). The number of such images increased with time of staining. Probably, the staining induced the dissociation of the molecule into two trimers. The projections of the model based on data obtained are shown in fig.4 (g–i). When the model is viewed along the two axes intersecting one another at an angle of about 30° (fig.4h,i, left), it is reminiscent of the second and third type of images (fig.4c,d). The cross-like images (fig.4e) can be also readily explained by this model. The volume of subunits in the proposed model is about 40 nm^3 in agreement with their molecular masses determined by other techniques (49–56 kDa).

We also carried out the computer averaging of the images of the first and second types. The selected images of the molecule were aligned to a reference by rotational and translational shifts using correlation functions. The average of the front view image projection has a form close to a right six-pointed star corresponding to projection of the molecule with six identical or very similar subunits, and can be characterized by 3- or 6-fold rotational symmetry. The average image was additionally 3-fold rotationally symmetrized (fig.5a). In this picture, the protein density of three vertices of a star slightly differs from three others. A possible reason for this phenomenon may be preferential staining of one of the opposing trimers of the molecule, most likely the one in contact with the support film. The results of averaging the images of the second type (side view) are shown in fig.5b. Protein density distribution is in good accordance with the projection of the model shown in fig.4h. Thus computer processing image data indicate that the cytochrome P-450$_{LM2}$ molecule consists of six equal or similar subunits arranged in two layers with 32 (D_3) point group symmetry. A similar picture was revealed when another isoform of the cytochrome, i.e. LM4 was studied (not shown).

In connection with the above data, one may mention that cytochrome H-450, a water-soluble

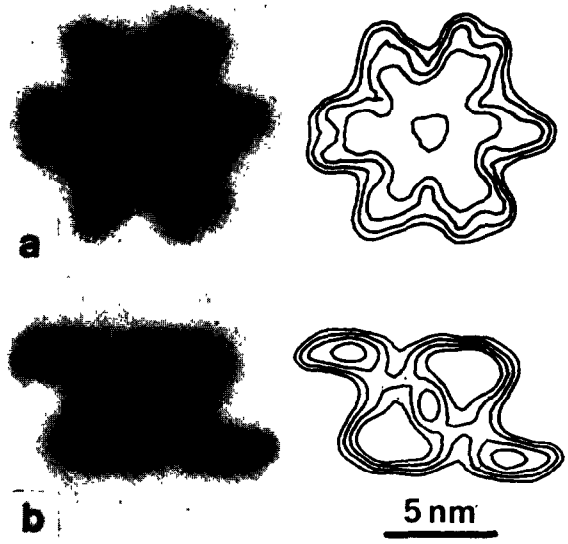

Fig.5. Computer averaging of cytochrome P-450$_{LM2}$ images. (a) 3-fold rotationally symmetrized average of 10 aligned images of the front view (shown in fig.4g). (b) The average of 7 aligned images of the side view (shown in fig.4h) after 180° additional rotational symmetrization. Images are represented as a grey-level picture and contour map.

analog of cytochrome P-450, recently obtained from the liver cytosol was isolated as an oligomer apparently composed of six subunits [15].

REFERENCES

[1] Lu, A.Y.H. and West, S.B. (1980) Pharmacol. Rev. 31, 277-295.
[2] Anzenbasher, P. and Hudecek, J. (1985) Abstr. XIII Intern. Biochem. Congr., FR-420.
[3] Muller-Enoch, D., Churchill, P., Fleisher, S. and Guengerich, F.P. (1984) J. Biol. Chem. 259, 8174-8182.
[4] Wagner, S.L., Dean, W.L. and Gray, R.D. (1984) J. Biol. Chem. 259, 2390-2395.
[5] Wagner, S.L. and Gray, R.D. (1985) Abstr. XIII Intern. Biochem. Congr., TU-498.
[6] McIntosh, P.R., Kawato, S., Freedman, R.B. and Cherry, R.O. (1980) FEBS Lett. 122, 54-58.
[7] Baskin, L.S. and Yang, C.S. (1982) Biochem. Biophys. Res. Commun. 108, 700-707.
[8] Greinert, R., Finch, S.A.E. and Stier, A. (1982) Biosci. Rep. 2, 991-994.
[9] Karusina, I.I., Bachmanova, G.I., Mengazedinov, D.E., Myasoedova, K.N., Zhikhareva, V.O., Kuznetsova, G.P. and Arachakov, A.I. (1979) Biokhimiya 44, 1049-1057.
[10] Imai, Y., Hashimoto-Yutsudo, C., Satake, H. et al. (1980) J. Biochem. 88, 489-507.
[11] Guengerich, F.P. and Holladay, L.A. (1979) Biochemistry 18, 5442-5449.
[12] Chernyak, V.Y. and Magretova, N.N. (1982) Anal. Biochem. 123, 101-109.
[13] Orlova, E.V. (1984) Cyrstallographiya 29, 668-674.
[14] Coon, M.J. (1978) Methods Enzymol. 52, 200-206.
[15] Kim, I.S. (1982) J. Biol. Chem. 257, 1063-1070.
[16] Coon, M.J. and Edsall, J.T. (1973) in: Protein Amino Acids and Peptides, pp. 157-370, Academic Press, New York.
[17] Tarr, G.E., Black, S.D., Fujita, V.S. and Coon, M.J. (1983) Proc. Natl. Acad. Sci. USA 80(21), 6552-6556.
[18] Wendel, J., Behlke, J. and Jäning, G.R. (1983) Biochim. Biophys. Acta 42, 6, 623-631 and 633-640.
[19] Heinemann, F.S. and Ozols, J. (1983) J. Biol. Chem. 258, 4195-4201.