PURIFICATION OF CYTOCHROME P-450 FROM BOVINE ADRENOCORTICAL MITOCHONDRIA BY AN "ANILINE-SEPHAROSE" AND THE PROPERTIES*

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Received February 7,1975

Summary: Cytochrome P-450 has been purified by an affinity chromatography using an aniline substituted Sepharose column. The preparation is essentially homogeneous as judged by electrophoresis and ultracentrifugation. The absorbance ratio(A394nm/A280nm) is 0.83 and the protein weight per heme is 80 kg. As determined by the sedimentation equilibrium method, the molecular weight is 200 kilodaltons in native protein and 46 kilodaltons in guanidine-treated protein, respectively. The preparation in oxidized state has a high spin type absorption spectrum. When the preparation is treated with the adrenal ferredoxin-dependent electron transfer system, the spectrum is converted into that of a low spin type which is reconverted into the high spin form by the addition of cholesterol.

The steroid hydroxylating system of adrenocortical mitochondria was resolved into three components identified as an flavoprotein(NADPH-adrenal ferredoxin reductase), an iron-sulfur protein(adrenal ferredoxin) and a hemoprotein(cytochrome P-450)(2-5). The reconstitution of these three components could restore the 11β-hydroxylation of deoxycorticosterone as well as the cholesterol side-chain cleavage activity.

In order to elucidate the reaction mechanism of the steroid hydroxylation system, it is valuable that the functionally active three components are available in a pure form. NADPH-adrenal ferredoxin reductase(6,7) and adrenal ferredoxin have been obtained in a pure state and the latter has further been crystallized(8,9). Attempts to purify the cytochrome P-450 have been carried out by a number of group of workers in recent year(10-14).

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* A part of the results was presented at the meeting of International Congress of Biochemistry in Stockholm, 1973(1). This work was supported by the Scientific Research Fund B-947051 from the Ministry of Education of Japan.

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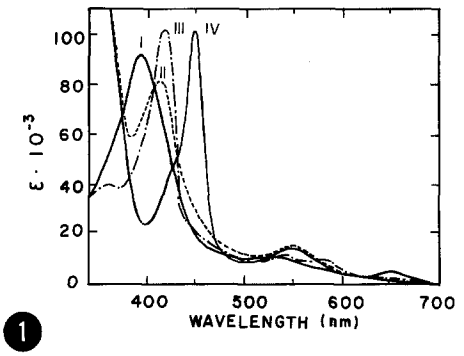
This communication describes the purification of cytochrome P-450 on a column containing Sepharose coupled with aniline and properties of the purified cytochrome P-450.

Materials and methods: Mitochondrial pellets were prepared from bovine adrenal cortex and sonicated as described previously(6,8). The sonicated samples were stored at -80°C until use. Alumina Cγ gel was prepared according to the method described by Colowick(15). Aniline derivatives of Sepharose 4B(Pharmacia) were prepared by a modification of the original method described by Cuatrecasas et al.(16). The side-chain cleavage reaction was assayed by the gas chromatographic method of Ando and Horie (11) using cholesterol, which was solubilized with Tween 80 in the buffer, as substrate. For the preparation of a low spin type cytochrome P-450, the purified preparation(11 nmoles) in a 2-ml solution of 50 mM potassium phosphate buffer(pH 7.0) containing 100 μM EDTA, 100 μM dithiothreitol and 0.01 % cholate was incubated with 6 μmoles of $MgCl_2$, 1.5 μmoles of glucose-6-phosphate, 1.2 units of glucose 6-phosphate dehydrogenase, 0.3 nmole of NADPH-adrenal ferredoxin reductase, 6.8 nmoles of adrenal ferredoxin and 60 nmoles of $NADP^+$. The mixture was then passed through Sephadex G-25 column(0.9 X 12 cm). Polyacrylamide disc gel electrophoresis was carried out according to the methods of both Davis(17) and Weber and Osborn(18). Protein and heme contents were determined by the methods of Gornall et al.(19) and of Appleby and Morton(20), respectively. The endogenous cholesterol and pregnenolone produced, which were found in cytochrome P-450, were extracted with chloroform and assayed by gas chromatography(11). Molecular weight determinations were performed in a Hitachi 282 analytical ultracentrifuge according to the high-speed sedimentation equilibrium technique of Yphantis(21).

Purification of cytochrome P-450

Following purification was carried out at 5°C. Standard buffer is 50 mM potassium phosphate buffer(pH 7.0) containing 100 μM EDTA, 100 μM dithiothreitol and 0.3 % sodium cholate.

The frozen mitochondrial pellets(5 g) were thawed and suspended in 100 mM potassium phosphate buffer(pH 7.0) to a protein concentration of 20 mg/ml. To this suspension 100 μM EDTA, 100 μM dithiothreitol and sodium cholate (0.5 mg/mg of protein) at the final concentration were added. After stirring for 1 hr, the mixture was centrifuged(78,000 X g, 90 min) and the supernatant was obtained(240 ml). The supernatant was diluted with an equal volume of distilled water and applied to Sepharose affinity column(5 X 7 cm) previously equilibrated with the standard buffer. Cytochrome P-450 was adsorbed as a red-brown band near the top of the column. After washing the column with 250 ml of the standard buffer containing 50 mM KCl, the upper part of the column was carefully taken out and suspended in the same buffer. The suspension was slowly poured on the another column containing the fresh aniline-Sepharose(1 cm-height, 2 cm-diameter) equilibrated with the same buffer. The column was washed by passing through 70 ml of the standard buffer containing 150 mM KCl. Cytochrome P-450 was eluted with the buffer(pH 7.5) contain-



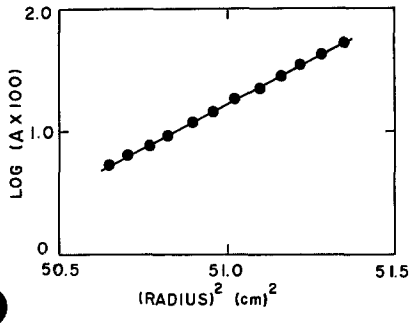


Fig. 1. Ultracentrifugation pattern of cytochrome P-450 at 20°C. Cytochrome P-450 was an 8 mg/ml solution in 50 mM potassium phosphate buffer(pH 7.0) containing 100 μM EDTA, 100 μM dithiothreitol and 0.01 % cholate. Photographs were taken at 8-min interval after attaining a rotor speed of 59,780 rpm.

Fig. 2. High speed sedimentation equilibrium of cytochrome P-450 at 4°C. The buffer system was the same as described in the legend of Fig. 1. A 2.5-mm solution column was sedimented for 17 hrs at a rotor speed of 16,000 rpm. Traces corresponding to absorbance versus radial distance in the cell were recorded at 410 nm.

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ing 1 M KCl. Fractions with the absorbance ratio(A394nm/A280nm) higher than 0.25 were collected. To the combined fractions, alumina Cγ gel, which had been equilibrated with the buffer(pH 7.5) containing 1 M KCl, was suspended in a ratio: 2.0 mg(dry weight)/A280nm = 1.0/ml. After 30 min, the mixture was centrifuged and the supernatant was then dialyzed against the standard buffer for 3 hrs. The dialyzate was fractionated between 27 and 43 % saturation of solid ammonium sulfate. During the addition of ammonium sulfate, the pH of the solution was maintained at about 7.3 by the addition of the aqueous ammonia. The precipitate was dissolved in 1.5 ml of the cholate-free buffer(pH 7.4) containing 200 mM KCl and dialyzed against the buffer (pH 7.4) containing 200 mM KCl for 3 hrs. The absorbance ratio of the preparation was 0.65 and the protein weight per heme was approx. 100 kg.

The dialyzate was again applied to a Sepharose affinity column(1.2 X 7 cm) previously equilibrated with the buffer(pH 7.4) containing 200 mM KCl and cytochrome P-450 was eluted with the same buffer. Fractions with the absorbance ratio higher than 0.8 were collected(30 ml) and then fractionated between 31 and 37 % saturation of ammonium sulfate. The precipitate was dissolved in the cholate-free buffer and dialyzed against the buffer containing 0.01 % cholate. The yield was about 15 mg of the purified cytochrome P-450 from 5 g of the mitochondrial pellets. The absorbance ratio of the final preparation was 0.83 and the protein weight per heme was 80 kg. Preliminary measurements showed that the cholesterol side-chain cleavage activity of the

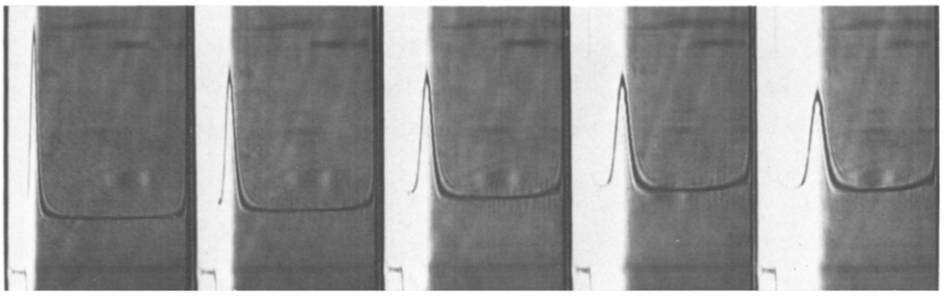

Fig. 3. Absorption spectra of cytochrome P-450. The buffer system was the same as described in the legend of Fig. 1. I, oxidized; II, dithionite-reduced; III, the preparation treated with the electron transfer system; and IV, dithionite-reduced CO complex. The extinction coefficient(ε) is given for a molar concentration of heme.

preparation is 0.7 nmole pregnenolone/min/nmole P-450 at 25°C. The preparation could be stored for a few days at -80°C without substantial loss of activity.

Properties of cytochrome P-450

When subjected to disc electrophoresis on polyacrylamide gel, both native and SDS-treated preparations were essentially homogeneous. The schlieren pattern(Fig. 1) from a sedimentation velocity experiment indicated a single component with the $s_{20,w}$ value of 5.7 S. The plot of log *a* versus r^2 derived from a sedimentation equilibrium experiment is shown in Fig. 2, the molecular weight being 200 kilodaltons. When the preparation was carboxymethylated in the presence of 6 M guanidine-HCl and 100 mM 2-mercaptoethanol, the sedimentation equilibrium data gave a molecular weight of 46 kilodaltons which was in agreement with the value as determined by SDS disc electrophoresis. These findings can be interpreted that the purified cytochrome P-450 exists as a tetramer. Recently, Shikita and Hall(22) have reported that their preparation exists as various polymerized forms.

The absorption spectra of cytochrome P-450 are illustrated in Fig. 3. The preparation in its ferric state showed the high spin type absorption spectrum having maxima at 394 and 645 nm with a shoulder around 540 nm. The CO spectrum in the dithionite reduced form gave maxima at 448 and 550 nm, respectively. The high spin type spectrum could not be changed by extensive dialysis either before or after reduction with dithionite. However, when the cytochrome P-450 was treated with the adrenal ferredoxin-dependent electron transfer system as described under "Methods", the absorption maxima of the preparation were completely shifted to 418, 539 and 570 nm, respectively. On the addition of cholesterol, the peaks were gradually shifted back to those of the high spin type. The rate of complete conversion to the high spin type-

species was accelerated in the presence of the mitochondrial crude extract which contained endogenous cholesterol(half-time: 20 sec). Again the low spin type-species appeared upon incubation of the cholosterol-induced species with the electron transfer system. The original high spin type preparation contained endogenous cholesterol(0.6-1.0 mole/mole of heme) and the conversion to the low spin type spectrum was intimately associated with the appearance of the product, pregnenolone. The substrate for 11β-hydroxylase, deoxycorticosterone, did not cause such interconversion between low and high spin type absorption spectra.

It has been shown that cytochrome P-450$_{cam}$ from Pseudomonas putida has the high spin type spectrum only when complexed with its substrate camphor(23, 24). Judging from results presented above, it seems likely that the cytochrome P-450 purified by an affinity chromatography exists as the cholesterol complex and is specific for the side-chain cleavage reaction.

Acknowledgements: We wish to thank Prof. R. Sato, Osaka University, for supplying the adrenal glands and Dr. E. Itagaki for the performance of sedimentation equilibrium experiments.

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