PURIFICATION AND PROPERTIES OF CYTOCHROME P-450$_{11\beta}$ FROM ADRENOCORTICAL MITOCHONDRIA*

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Summary: A cytochrome P-450, which is functional in the steroid methylene 11β-hydroxylation (P-450$_{11\beta}$), has been purified to a protein weight of 85 kg per heme from bovine adrenocortical mitochondria. The purification is accomplished in the presence of deoxycorticosterone as a substrate stabilizer. The procedure involved solubilization of sonicated mitochondrial pellets, ammonium sulfate fractionation, alumina Cγ gel treatment and aniline-substituted Sepharose 4B chromatography.

The purified preparation when freed from deoxycorticosterone, has a low spin type absorption spectrum which can rapidly be converted into a typical high spin substrate-bound form by the addition of an 11β-hydroxylatable steroid, either deoxycorticosterone or testosterone. The preparation exhibits high 11β-hydroxylase activity and is free from the cholesterol side-chain cleavage cytochrome P-450 (P-450$_{scc}$).

The purified P-450$_{11\beta}$, when submitted to SDS-polyacrylamide gel electrophoresis, exhibits a single protein band (molecular weight of 46 kilodaltons) which is clearly distinguished from P-450$_{scc}$. As determined by the sedimentation equilibrium method, the molecular weight of the guanidine-treated P-450$_{11\beta}$ is estimated to be 43 kilodaltons.

In adrenocortical mitochondria, NADPH-adrenal ferredoxin reductase-adrenal ferredoxin-cytochrome P-450 system has been known to be associated with the steroid hydroxylase activities, viz., cholesterol side-chain cleavage and steroid methylene 11β-hydroxylation. Jefcoate et al. (1), using isooctane pretreated mitochondria, have partially separated these activities into two different ammonium sulfate fractions. Similar attempts have also been carried out in several laboratories (2-6). A technique for the purification of P-450 cytochromes using an aniline-substituted Sepharose chromatography has been developed in our laboratory in 1971 for efficient separation of cytochrome

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P-450 from other hemoproteins such as hemoglobin and cytochrome c (7,8). This communication describes the purification procedure of P-$450_{11\beta}$,* and its characterization.

Materials and methods: Mitochondrial pellets from bovine adrenal cortex, crystalline adrenal ferredoxin, purified NADPH-adrenal ferredoxin reductase and P-450_{scc} were prepared as described previously (9,10,11). Alumina Cγ gel was prepared according to the method described by Colowick (12). Aniline derivatives of Sepharose 4B (Pharmacia), referred to as aniline-Sepharose, were prepared as described previously (11). SDS-polyacrylamide disc gel electrophoresis was carried out according to the method of Weber and Osborn (13). Protein and heme contents were determined by the methods of Gornall et al.(14) and of Appleby and Morton (15), respectively. The endogenous cholesterol in the preparation was extracted with chloroform and analyzed by gas chromatography (16). Molecular weight determination was performed in a Hitachi 282 analytical ultracentrifuge according to the high-speed sedimentation equilibrium technique of Yphantis (17). The activity of the 11β-hydroxylation was assayed fluorimetrically by measuring the rate of corticosterone formation (18). The assay mixture (0.5 ml) contained 8 μmoles of $MgCl_2$, 6 μmoles of glucose-6-phosphate, 1.6 units of glucose 6-phosphate dehydrogenase, 0.05 unit of NADPH-adrenal ferredoxin reductase, 35 nmoles of adrenal ferredoxin, 60 nmoles of $NADP^+$, 100 nmoles of deoxycorticosterone and 30-50 pmoles of P-$450_{11\beta}$. The buffer system was 50 mM potassium phosphate, pH 7.0. The cholesterol side-chain cleavage reaction was assayed as previously described (11).

Purification of P-$450_{11\beta}$

The presence of substrate (deoxycorticosterone) protected P-$450_{11\beta}$ (but not P-450_{scc}) almost completely from inactivation (Fig. 1). Testosterone was equally effective, but cholesterol had no effect. These characterization are very similar to those found with P-450_{cam} markedly stabilized by D-camphor (19). Following purification of P-$450_{11\beta}$ was carried out in the presence of deoxycorticosterone. All steps were at 5°. Standard buffer is 50 mM potassium phosphate buffer, pH 7.3, containing 100 μM EDTA, 100 μM dithiothreitol and 10 μM deoxycorticosterone. The frozen mitochondrial pellets (1.25 g) which had been sonicated as described (10) were thawed and suspended in 100 mM potassium phosphate buffer (pH 7.3) to a protein concentration of 15 mg per ml. To this suspension 100 μM EDTA, 100 μM dithiothreitol, 10 μM deoxycorticosterone and sodium cholate (0.67 mg/mg of protein) at the final concentration were added. After stirring for 1hr, the mixture was centrifuged (136,000 X g,90 min). To the supernatant (83 ml), solid ammonium sulfate (180 g per liter) was added with continuous stirring, the pH being maintained at pH 7.3 by periodic addition of aqueous ammonia. After centrifugation the supernatant fraction con-

* Abbreviations are: P-$450_{11\beta}$, steroid 11β-hydroxylation specific cytochrome P-450; P-450_{scc}, cholesterol side-chain cleavage specific cytochrome P-450; P-450_{cam}, cytochrome P-450 component of a camphor methylene 5-exo hydroxylase system in Pseudomonas putida.

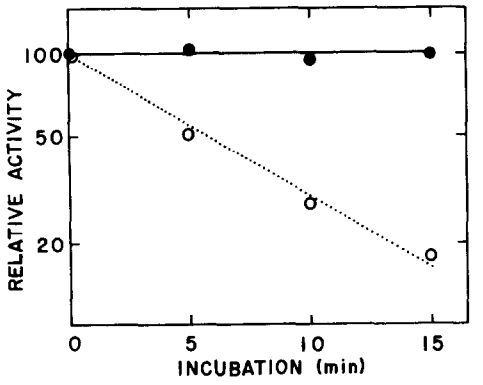

Fig. 1. Effect of deoxycorticosterone on stability of P-450$_{11\beta}$. The crude preparation of cytochrome P-450 (5.2 μM), which had been isolated from the mitochondrial pellets in the absence of deoxycorticosterone, was dissolved in 50 mM phosphate buffer (pH 7.3) containing 100 μM EDTA and 100 μM dithiothreitol and was incubated with (●) or without (O) 50 μM deoxycorticosterone at 30°. At the times indicated, aliquots were transferred to the reaction mixture and assayed for activity as described in "Methods".

tained most of P-450$_{scc}$. This P-450$_{scc}$ fraction could be purified separately using the aniline-Sepharose chromatography according to the procedure described previously (11). The precipitate fraction which contained mainly P-450$_{11\beta}$ was dissolved in 40 ml of the standard buffer (pH 7.5) containing 1 % sodium cholate and 1 M KCl. Alumina Cγ gel (380 mg, dry weight) equilibrated with the same buffer was suspended to the solution. After 30 min, the mixture was centrifuged and the supernatant was then dialyzed overnight against the standard buffer. During dialysis most of P-450$_{11\beta}$ was precipitated. The precipitated protein was collected and dissolved in 20 ml of the standard buffer containing 0.7 % sodium cholate and 200 mM KCl. The solution was applied to an aniline-Sepharose column (1.4 X 2.5 cm) previously equilibrated with the same buffer. After washing with 10 ml of the same buffer, P-450$_{11\beta}$ was eluted with the standard buffer (pH 7.5) containing 0.5 % sodium cholate, 500 mM KCl and 0.5 % Tween 20. Fractions with the absorbance ratio ($A_{394\ nm}/A_{280\ nm}$) higher than 0.80 were collected. The protein was concentrated with ammonium sulfate (180 g/liter) and dissolved in the standard buffer containing 0.3 % sodium cholate and 0.3 % Tween 20. After dialysis against the same buffer, the purified P-450$_{11\beta}$-substrate preparation, could be stored at 4° and at the concentration less than 5 μM for one week without substantial loss of spectral properties and activity. The yield was about 8 mg of the purified P-450$_{11\beta}$ from 1.25 g of the mitochondrial pellets. The absorbance ratio of the final preparation was 0.85 and the protein weight per heme was 85 Kg..

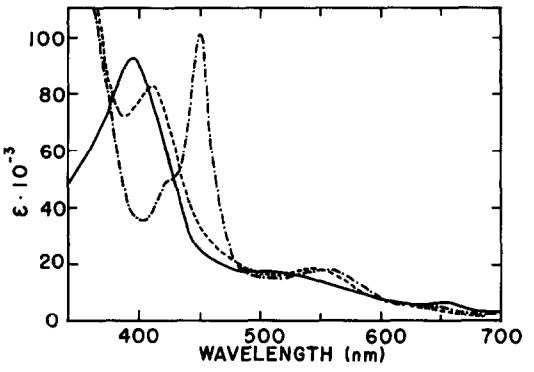

Fig. 2. Absorption spectra of purified P-450$_{11\beta}$. The buffer system (pH 7.3) was 50 mM potassium phosphate containing 100 μM EDTA, 100 μM dithiothreitol 0.3 % sodium cholate and 0.3 % Tween 20. Purified P-450$_{11\beta}$-substrate complex (————); dithionite-reduced (-------); and dithionite-reduced CO complexed (-.-.-.-).

The preliminary sedimentation experiments were carried out under the similar conditions as described for P-450$_{scc}$ (11), except that the concentration of P-450$_{11\beta}$ was 6 μM. Sedimentation velocity showed only one symmetrical peak, with $s_{20,w}$ value of 3.3 S.

Properties of P-450$_{11\beta}$

The absorption spectra of P-450$_{11\beta}$ are illustrated in Fig. 2. Since deoxycorticosterone had been added as a stabilizing agent during preparation and storage, the purified preparation in its ferric state showed the high spin type absorption spectrum having maxima at 394, 510 and 645 nm. The CO spectrum in the dithionite reduced form gave maxima at 448 and 550 nm. The substrate free preparation was obtained after treatment of the P-450$_{11\beta}$- substrate complex with the adrenal ferredoxin-dependent electron transfer system under comparable condition to the method as described previously for P-450$_{scc}$ (11). As in Fig. 3, P-450$_{11\beta}$, in the absence of substrate, showed an absorption spectrum characteristic of a low spin hemoprotein having the maxima at 418, 539 and 570 nm. When an 11β-hydroxylatable substrate, either deoxycorticosterone or testosterone, was added to the oxidized form of P-450$_{11\beta}$, the peaks rapidly and completely shifted to those of the typical high spin or substrate-complexed type spectrum. The purified preparation did not contain significant amount of endogenous cholesterol which is compared with the fact that the purified preparation of P-450$_{scc}$ contained 0.6-1.0 mole cholesterol per mole of protoheme (11).

In the assay mixture, the P-450$_{11\beta}$ preparation could catalyze the conversion of deoxycorticosterone into corticosterone. The turnover number at 30°

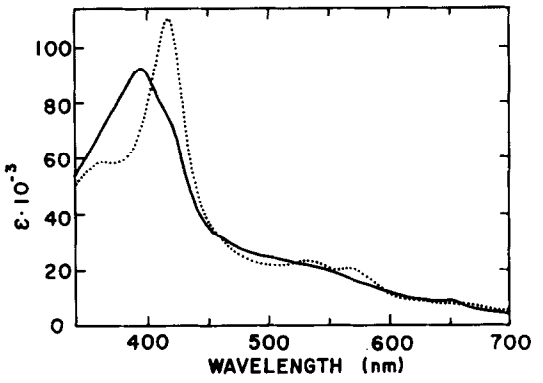

Fig. 3. Spectral changes of P-450$_{11\beta}$ on addition of 10 μM of deoxycorticosterone. Substrate free P-450$_{11\beta}$ (·····); and substrate-complexed in the presence of 10 μM deoxycorticosterone (———). For the buffer system, see the Fig. 2 legend.

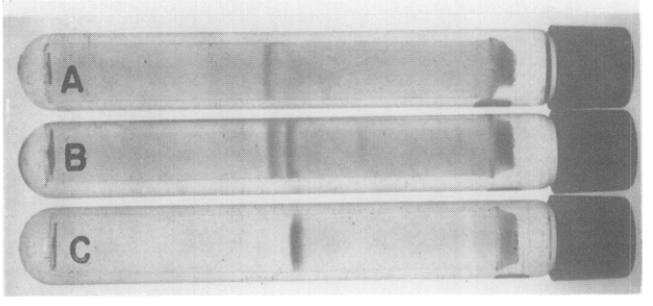

Fig. 4. SDS-polyacrylamide gel electrophoresis of P-450$_{11\beta}$ (A); equiamount mixture of P-450$_{11\beta}$ and P-450$_{scc}$ (B); and P-450$_{scc}$ (C). The preparations (10 ng) which had been treated with SDS and 2-mercaptoethanol were applied to a 7.5 % polyacrylamide gel column (0.7 X 10 cm). The electrophoretic run was performed with a constant current of 5 mA per tube for 10 hrs.

was about 60 moles corticosterone formed per min per mole of P-450$_{11\beta}$. The product was extracted with methylene chloride and analyzed by thin layer chromatography using Eastman 13181 silica gel chromatogram sheet. The spot and Rf values for the authentic corticosterone and the reaction product were identical: 0.53 with chloroform-methanol (97:3), and 0.50 with benzene-acetone-water (75:50:0.2). The P-450$_{11\beta}$ preparation had no significant activity for the cholesterol side-chain cleavage. Conversely, P-450$_{scc}$ has been shown to be free from 11β-hydroxylating activity (11).

When the purified preparation was subjected to SDS-polyacrylamide gel elec-

trophoresis, only a single protein band was observed as shown in Fig. 4 (A). By comparing its mobility with those of marker protein (bovine serum albumin, monomer of bovine liver catalase, ovalbumin and bovine carboxypeptidase A), an apparent molecular weight of about 46 kilodaltons was estimated for P-$450_{11\beta}$. As also in Fig. 4, when a mixture of equal amounts of P-$450_{11\beta}$ and P-450_{scc} was run under the same conditions, two distinct bands were separated clearly. 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 43 kilodaltons which compared with the value of P-450_{scc} (46 kilodaltons).

The present study provides direct evidences that separate cytochrome P-450 species catalyzes the cholesterol side-chain cleavage and steroid 11β-hydroxylation, respectively. P-$450_{11\beta}$ and P-450_{scc} are similar in physico-chemical properties such as in spectral properties and in molecular size per heme, but are different in properties with respect to its specificity either in substrate binding or in catalytic activity. During purification and storage P-$450_{11\beta}$ is less soluble than P-450_{scc} and requires more hydrophobic environment such as in the presence of a non-ionic detergent viz., Tweens, Triton X-100 or Emulgen 220. This evidence may suggest possible difference in hydrophobic nature of the two hemoproteins.

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