Studies on the Steroid Hydroxylation System in Adrenal Cortex Microsomes

PURIFICATION AND CHARACTERIZATION OF CYTOCHROME P-450 SPECIFIC FOR STEROID C-21 HYDROXYLATION*

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Cytochrome P-450 was purified to a specific content of 16 nmol/mg of protein from bovine adrenal cortex microsomes by hydrophobic chromatography using an ω-amino-n-octyl Sepharose column in the presence of Emulgen 913, a nonionic detergent. The purified preparation exhibited a single polypeptide band (Mr = 47,000 ± 1,000) when submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The cytochrome P-450 was immunochemically different from mitochondrial cytochrome P-450 catalyzing the side-chain cleavage of cholesterol and cytochrome P-450 catalyzing the 11β and 18 hydroxylation of 11-deoxy-corticosterone. The purified cytochrome P-450 catalyzed the C-21 hydroxylation of 17α-hydroxyprogesterone and progesterone when mixed with NADPH-cytochrome P-450 reductase, which was purified separately from the microsomes. The absorption spectrum of the oxidized cytochrome P-450 had maxima at 419, 535, and 569 nm, characteristic of a low spin form. Upon the addition of 17α-hydroxyprogesterone or progesterone, the cytochrome P-450 showed maxima at 396 and 651 nm, characteristic of a high spin form. The effects of steroid binding were studied optically for the cytochrome P-450 both in the low and the high spin forms. Some steroids caused spectral changes different from the type I spectral changes upon addition to the cytochrome P-450 in the low spin form. These steroids were found to cause the reverse type I spectral changes effectively upon addition to the cytochrome P-450 in the high spin form. A hypothesis to explain the mechanism of these spectral changes was proposed.

The earlier studies of Ryan and Engel (1) showed that C-21 hydroxylation of progesterone and its derivatives was localized in the adrenal microsomes, that such hydroxylation required the presence of NADPH and molecular oxygen in the system, and that carbon monoxide inhibition of the hydroxylation system was light-reversible. Based on the stoichiometry of NADPH and molecular oxygen in the steroid hydroxylation and a characteristic photochemical action spectrum, Cooper et al. (2-5) demonstrated for the first time that cytochrome P-450 served as a mixed function oxidase for steroid C-21 hydroxylation. Since then, steroid C-21 hydroxylation in adrenal microsomes has been further studied by a number of investigators. Bryan et al. (6) have reported that C-21 hydroxylation of progesterone is competitively inhibited by 17α-hydroxyprogesterone. Narasimhulu (7, 8) has studied thermodynamically the role of the microsomal membrane on steroid C-21 hydroxylation and on steroid-cytochrome P-450 binding reaction.

Plager and Samuels (9, 10) observed that bovine adrenal homogenates could catalyze 17α-hydroxylation as well as C-21 hydroxylation of progesterone. Hofmann (11) concluded that steroid 17α hydroxylation of the adrenal cortex required NADPH and molecular oxygen. The activities of 17α and C-21 hydroxylation in the smooth microsomes from porcine adrenals were also reported by Inano et al. (12). The 17α hydroxylation of progesterone in testicular microsomes was found to be inhibited by carbon monoxide (13). Recently, Menard et al. (14, 15) have shown that the destruction of cytochrome P-450's in adrenal microsomes of cortisol-producing animals is caused by the administration of spironolactone although the administration causes no decrease in microsomal C-21-hydroxylase activity in the adrenals of corticosterone-producing animals which contain a low activity of 17α-hydroxylase. These investigations suggest that there might be at least two specific cytochrome P-450's in adrenal cortex microsomes, one catalyzing steroid C-21 hydroxylation and another catalyzing steroid 17α hydroxylation. Earlier investigations on the reaction mechanism of cytochrome P-450 in adrenal cortex microsomes were carried out using microsomal particles or their extract. Some trials of the extraction and purification of cytochrome P-450 were carried out by Narasimhulu (16) and Mackler et al. (17), but they could not succeed in establishing the purification procedure.

The steroid hydroxylation system of adrenal cortex mitochondria is composed of three types of protein: a flavoprotein (NADPH-adrenodoxin reductase), an iron-sulfur protein (adrenodoxin), and a hemoprotein (cytochrome P-450) (18-20). Recently, two different kinds of mitochondrial P-450 cytochromes, catalyzing the cholesterol side-chain cleavage (P-450c), and the steroid 11β and 18 hydroxylation (P-45011p,18), have been separated and highly purified from bovine adrenal cortex (21-23). Furthermore, the characterization of the two mitochondrial cytochromes has been studied in detail (23-26). On the other hand, immunochemical analysis has indicated that the microsomal hydroxylation system is composed of two types of protein, NADPH-cytochrome P-450 reductase and cytochrome P-450, but does not contain an iron-sulfur protein (27). In order to provide more direct information on the mechanism of steroid hydroxylation in adrenal cortex microsomes, it is desirable that the electron transfer components associated with steroid hydroxylation be available in a pure state and that the characterization of the each component be
investigated in detail. We have recently succeeded in purifying cytochrome P-450 from adrenal cortex microsomes and reported briefly some optical properties of this protein (28). We now wish to report the details of the further purification and characterization of adrenal microsomal cytochrome P-450 specific for catalyzing steroid C-21 hydroxylaton.

**EXPERIMENTAL PROCEDURES**

**Procedure for Isolation of P-450**—All the following procedures were performed below 5°C. Tissue homogenates of bovine adrenal cortex were prepared in 0.25 M sucrose by a Nikon Seiki Kogyo Co. "homogenizer." The homogenates were centrifuged for 10 min at 700 x g and for 20 min at 8,000 x g. Subsequently, the 8,000 x g supernatant solutions were centrifuged for 60 min at 105,000 x g. The microsomal pellets were washed once with 0.15 M KCl solution. The washed microsomes were then suspended in 100 mM potassium phosphate buffer, pH 7.2, to give a final protein concentration of approximately 30 mg/ml and were stored at -70°C until used for extraction of P-450. The frozen microsomes (2.4 g of protein) were thawed and diluted with 100 mM potassium phosphate buffer, pH 7.2, to a protein concentration of 15 mg/ml. To that suspension, 0.1 M EDTA, 0.1 M diethyldithithiolethyl, glycerol, and 10% (v/v) sodium cholate were added drop by drop in this sequence to obtain the final concentrations of 0.1 M sodium chloride, 0.1 M EDTA, and the ratio of 3 mg of cholate/l mg of protein, respectively. After stirring for 60 min, the suspension was centrifuged at 105,000 x g for 60 min and the supernatant was dialyzed twice against 3 liters of the basal buffer, which was composed of 50 mM potassium phosphate, pH 7.2, 0.1 mM EDTA, 0.1 M diethyldithithiolethyl, 0.4% sodium cholate, and 20% (v/v) glycerol. The dialyzed solution was applied at a rate of 0.2 ml/min to a column (1.9 x 20 cm) of w-amino-n-octyl Sepharose, previously equilibrated with the basal buffer. The P-450 was eluted with 500 ml of the basal buffer in which Emulgon 913 concentration was increased from 0 to 0.15% (v/v) in a linear gradient. The elution was followed with 300 ml of basal buffer containing 0.16% Emulgon. The amount of P-450 in various fractions was estimated from the difference spectra induced by steroid binding. The fractions, which showed type I spectral changes upon the addition of 17a-hydroxyprogesterone, were collected and pooled. A 0.05 M Tris buffer containing 0.002% Emulgon 913, 0.568 units of glucose-6-phosphate dehydrogenase, 0.04 ml of saturated solution of glucose-6-phosphate, 20% (v/v) glycerol, 20% (v/v) sodium dodecyl sulfate, and 0.02% Emulgon was added to the pooled fractions to bring the final concentration of Emulgon 913 to 0.2%. After incubation, the reaction was arrested by vigorous shaking with 2 ml of chloroform and the corresponding [14C]-labeled product (2 nCi) was added to the mixture as the internal standard. The mixture was then extracted in chloroform and the solvent was evaporated to dryness. The sample was spotted on a Merck 60-F254 silica gel thin layer chromatography plate (20 x 20 cm) and developed using chloroform:methanol:water:methanol:acetic acid (45:45:10) at room temperature for 15 h. The separated steroid products were visualized under UV light and the identity of the product was confirmed by co-chromatography with authentic samples.

**Assay Procedure**—The concentration of P-450 was determined from a CO difference spectrum of a dithionite-reduced sample. The concentration of the protein in the reaction mixture was determined by the method of Lowry et al. (34-36). The sample solution (3 ml) previously mixed with 25% (w/v) trichloroacetic acid. The precipitate was separated by centrifugation at 400 x g for 30 min. After the supernatant was removed carefully, the precipitate was dissolved in 2 ml of 0.3 M sodium benzoate and the absorbance at 660 nm was measured at 4°C to prevent precipitation of the protein. The amount of the product formed in the reaction mixture was calculated using the [14C]-labeled internal standards for correction of the recovery of the whole procedure.

**SDS-Polyacrylamide Gel Electrophoresis—**SDS-gel electrophoresis was performed according to the method of Weber and Osborn (30). The separation gel (10.48 x 7.5 cm) contained 5% (w/v) polyacrylamide and 0.1% (w/v) SDS. P-450 at a final concentration of 72 pg/ml in 20 mM sodium phosphate buffer, pH 7.2, containing 1% SDS, 2 M urea, and 0.1% (v/v) 2-mercaptoethanol, was incubated at 37°C for 2 h. Before application to the gel, hemoglobin blue was added to the incubation mixture to the final concentration of 0.001% of hemoglobin. After electrophoresis, protein bands were stained with 0.075% Coomassie blue in water:methanol:acetic acid (45:45:10) at room temperature for 15 h and subsequently destained at 80°C for 4 h in water:methanol:acetic acid (87.5:5:7.5). Proteins used as molecular weight standards in SDS-gel electrophoresis were: Sigma bovine serum albumin; bovine liver catalase. The gel was prepared by the method of Shihara and Takagi (31). The stripped gel was depolymerized by the treatment of 5 M guanidine HCl and 100 mM 2-mercaptoethanol and used as a monomer preparation; ovalbumin prepared as described by Sunner and Somers (32); Boehringer yeast alcohol dehydrogenase; Sigma chymotrypsinogen and trypsin.

**Ouchterlony Double Diffusion Analysis—**The Ouchterlony double diffusion analysis was performed on Petri dishes covered with 5 mm of the media containing 1.0% agar, 50 mM Tris-Cl buffer, pH 7.2, 0.5% (v/v) NaCl, 0.02% (w/v) NaNO, 0.5% (v/v) Tween 20, and 0.5% sodium cholate. Each well with 4 mm diameter was located with a center-to-center distance of 12 mm in the dish. The antisera used for the experiment was obtained from rabbits which had been immunized with adrenal mitochondrial P-450 (P-450,,) (23). The immunodiffusion was performed at 4°C for at least 3 days.

**Enzyme Assays—**The steroid C-21 hydroxylase activities of P-450 were determined for the reconstituted system with purified NADPH-P-450 oxidoreductase from rat liver and all the isotope-labeled substrates, [7-H]17a-hydroxyprogesterone or [7-3H]progesterone. The assay mixture in 0.4 ml of 50 mM Tris-Cl buffer, pH 7.3, contained 0.002% Emulgon 913, 0.598 units of glucose-6-phosphate dehydrogenase, 4.2 μmol of glucose-6-phosphate, 2 μmol of NADPH, 10−4 M unlabeled substrate (1 μCi), and a quantity of P-450. The mixture was preincubated for 1 min at 37°C and the reaction was initiated by the addition of 200 nmol of NADPH. After incubation, the reaction was arrested by vigorous shaking with 2 ml of chloroform and the corresponding [14C]-labeled product (2 nCi) was added to the mixture as the internal standard. The amount of the product formed in the reaction mixture was calculated using the [14C]-labeled internal standards for correction of the recovery of the whole procedure.

Gas chromatography was applied for the identification of the steroid products. The product, which was extracted and separated by thin layer chromatography in the same way as described in the enzyme assay procedure using nonradioactive steroids, was converted to the corresponding acetate and was separated by gas chromatography equipped with a 1-m column (3% OV-1). Optical spectra measurements were performed in a Union SM-401 split beam recording spectrophotometer equipped with an SM-450 data processor. The data was transferred to a digital computer (Hewlett Packard). The temperature was controlled with a Neslab RTE-8 circulating thermostat. The buffer system was 50 mM Tris-Cl, pH 7.3, containing

![Adrenal Microsomal P-450](image-url)
Purification of P-450 from bovine adrenocortical microsomes: A summary of a typical preparation is given in Table I. The purification procedures are based on a repetition of hydrophobic chromatography of ω-amino-n-octyl Sepharose. In the method previously used in this laboratory (28), P-450 was eluted with a stepwise change of the concentration of Emulgen 913. Considering that a gradient change of the Emulgen eluate resulted in two peaks of P-450 which showed any spectral change and that in the second peak showed the type I spectral change. The latter P-450 fraction was eluted with a linear gradient of Emulgen. P-450 eluted as a single peak which was detected either by the CO difference spectrum. The specific content of P-450 in several fractions around the peak was determined. The concentration of P-450 was estimated from the 17α-hydroxyprogesterone-induced difference spectrum by using $\Delta\varepsilon_{448-422} = 113 \text{ mm}^{-1} \text{ cm}^{-1}$ which was carefully determined in the comparison with the CO difference spectrum. The highest specific content of 18 nmol/mg of protein was obtained at the peak on the chromatogram. The fractions which showed the specific content above 14 nmol/mg of protein were collected and used as the final preparation. As summarized in Table I, the final preparation recovered 6% of the starting microsomal P-450 and had an average specific content of 16 nmol/mg of protein. Starting with 2 g of microsomal protein, about 16 nmol of P-450 could be recovered within 10 days.

Purity of P-450—When the purified preparation was subjected to polyacrylamide gel electrophoresis in the presence of 0.1% SDS, only a single protein band was observed, as shown in Fig. 1. By comparing its mobility in this electrophoresis system with that of marker proteins, P-450 was found to have a molecular weight of 47,000 ± 1,000 (Fig. 2). In contrast, the molecular weights of P-450c and P-450t from adrenocortical mitochondria were estimated to be 50,000 ± 1,000 and 43,000 ± 1,000, respectively, under the same conditions. These results indicate that the P-450 obtained in this experiment is not the mitochondrial contaminant. The possible contamination of the mitochondrial P-450 in the purified preparation was also checked by the Ouchterlony double diffusion test. It has been shown in previous studies that anti-P-450c and anti-P-450t reacted with P-450c, or P-450t, respectively (23). As shown in Fig. 3, either of the anti-mitochondrial P-450's made a precipitation line with corresponding P-450, but did not react with purified microsomal P-450. This shows that purified P-450 does not contain any mitochondrial P-450 and that microsomal P-450 is immunologically different from that of mitochondria. A similar immunodiffusion test was also performed with a constant current of 5 mA/tube for 2 h at room temperature.

**RESULTS**

**Purification of P-450 from Adrenocortical Microsomes**—A summary of a typical preparation is given in Table I. The purification procedures are based on a repetition of hydrophobic chromatography of ω-amino-n-octyl Sepharose. In the method previously used in this laboratory (28), P-450 was eluted with a stepwise change of the concentration of Emulgen 913. Considering that a gradient change of the Emulgen concentration might be used for efficient and reproducible separation of P-450 from other membrane proteins, we have adapted this approach to column chromatography. The first hydrophobic chromatography of the crude extract on ω-amino-n-octyl Sepharose resulted in two peaks of P-450 which were detected by CO difference spectra. Upon the addition of 17α-hydroxyprogesterone, the P-450 in the first peak did not show any spectral change and that in the second peak showed the type I spectral change. The latter P-450 fraction was applied to the second hydrophobic chromatography and eluted with a linear gradient of Emulgen. P-450 eluted as a single peak which was detected either by a 17α-hydroxyprogesterone-induced difference spectrum or a CO difference spectrum. The specific content of P-450 in several fractions around the peak was determined. The concentration of P-450 was estimated from the substrate-induced difference spectrum upon the addition of a sufficient amount of 17α-hydroxyprogesterone by using $\Delta\varepsilon_{448-422} = 113 \text{ mm}^{-1} \text{ cm}^{-1}$ which was carefully determined in the comparison with the CO difference spectrum. The highest specific content of 18 nmol/mg of protein was obtained at the peak on the chromatogram. The fractions which showed the specific content above 14 nmol/mg of protein were collected and used as the final preparation. As summarized in Table I, the final preparation recovered 6% of the starting microsomal P-450 and had an average specific content of 16 nmol/mg of protein. Starting with 2 g of microsomal protein, about 16 nmol of P-450 could be recovered within 10 days.

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**TABLE I**

**Purification of P-450 from bovine adrenocortical microsomes**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Cytchrome P-450</th>
<th>Specific content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>2424</td>
<td>283</td>
<td>0.12%</td>
</tr>
<tr>
<td>Crude extract</td>
<td>987</td>
<td>243</td>
<td>0.25%</td>
</tr>
<tr>
<td>First hydrophobic chromatography eluate</td>
<td>8.85</td>
<td>66.8</td>
<td>7.55%</td>
</tr>
<tr>
<td>Second hydrophobic chromatography eluate</td>
<td>0.98</td>
<td>15.8</td>
<td>6.1%</td>
</tr>
</tbody>
</table>

*We have succeeded in purifying NADPH-P-450 reductase from bovine adrenocortical microsomes. Details of the purification method will be reported elsewhere.*
formed with the original microsomal preparation. The solubilized microsomal preparation showed precipitation lines with the anti-mitochondrial P-450's. These results show that and NADPH generating system. After incubation of the reconstituted system with the P-450, NADPH-P-450 reductase, was reconstituted with the P-450, NADPH-P-450 reductase, and H-labeled progesterone, the radioactive products were analyzed by thin layer chromatography and radiography (Fig. 4). When progesterone was incubated with the reconstituted hydroxylation system (Sample C), two radioactive spots were observed at the position of RF values of 0.95 and 0.75 on the chromatogram. These were attributable to the unreacted progesterone and 11-deoxycorticosterone (which was the C-21-hydroxylated product of progesterone) by comparison with the RF values of the markers. No radioactive spot was observed at the position corresponding to 17α-hydroxyprogesterone. For comparison, the microsomes were incubated with H-labeled progesterone and a NADPH generating system. The steroid products were separated into three main spots on the silica gel thin layer chromatography plate. Their RF values corresponded to progesterone, 17α-hydroxyprogesterone, and 11-deoxycorticosterone. The result indicates that the microsomes have both C-21- and 17α-hydroxylase activities. The reconstituted C-21-hydroxylase activity for 17α-hydroxyprogesterone was 3-fold higher than that of the starting microsomes on the basis of P-450 content. Optical Properties of P-450 — The absolute spectra of purified P-450 are shown in Fig. 6. Purified P-450 in the oxidized state showed a typical low spin spectrum which exhibited absorption maxima at 369 nm (δ-band), 419 nm (Soret band), 535 nm (β-band), and 569 nm (α-band). After the addition of a sufficient amount of 17α-hydroxyprogesterone, the Soret

**FIG. 3.** Ouchterlony immunodiffusion analysis of purified P-450's from bovine adrenocortical mitochondria and microsomes. Antiserum (50 μl) was placed in the center well: A, anti-P-450<sub>m</sub> serum; B, anti-P-450<sub>11β</sub> serum. Each well contained 500 pmol of sample: C, P-450<sub>m</sub>; D, P-450<sub>11β</sub>; E, purified P-450 from bovine adrenocortical microsomes.

**FIG. 4.** Analysis of the products of the reconstituted hydroxylation reaction. The assay mixture in 0.847 ml of 50 mM Tris-Cl buffer, pH 7.3, contained 19.7 pmol of P-450, 0.0375 units of NADPH-P-450 reductase, 0.46 μmol of NADPH, 8.4 μmol of glucose 6-phosphate, 3.32 units of glucose-6-phosphate dehydrogenase, 4 μmol of MgCl₂, 0.0074% Emulgen 913, and 0.24 pmol of H-labeled substrate with 2 μCi. A, authentic sample, 10 nCi of [4-14C]11-deoxycorticosterone; B, control, the reconstituted system containing [7-3H]progesterone without incubation; C, the incubation was carried out for 60 min at 37°C with the same reconstituted system as B; D, control, the reconstituted system containing [7-3H]17α-hydroxyprogesterone without incubation; E, the incubation was carried out for 60 min at 37°C with the same reconstituted system as D; F, authentic sample, 10 nCi of [4-14C]11-deoxycorticosterone. The assay mixtures were extracted and subjected to thin layer chromatography as described under "Experimental Procedures." A radiochromatogram camera (LKB 2105) was used for this photograph.
FIG. 5. Steroid C-21-hydroxylase activity as a function of P-450 concentration. Assays were done under the standard conditions except that the concentration of P-450 was varied. The reaction was run at 37°C for 4.5 min and the hydroxylated products were determined as described under "Experimental Procedures." O, 11-deoxy-cortisol formed from 17α-hydroxyprogesterone; X, 11-deoxycorticosterone formed from progesterone.

Table II
Reconstitution of steroid C-21-hydroxylase activity

<table>
<thead>
<tr>
<th>System</th>
<th>Hydroxylation of progesterone to 11-deoxycorticosterone</th>
<th>Hydroxylation of 17α-hydroxyprogesterone to 11-deoxycorticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>10.3 ± 1.4</td>
<td>19.3 ± 1.7</td>
</tr>
<tr>
<td>-P-450</td>
<td>0.8 ± 0.4</td>
<td>0.8 ± 0.9</td>
</tr>
<tr>
<td>-Reducase</td>
<td>0.0 ± 0.2</td>
<td>0.3 ± 0.4</td>
</tr>
</tbody>
</table>

Fig. 6. Absorption spectra of purified P-450 (2.6 µm) from bovine adrenocortical microsomes at 25°C. ——, oxidized; ——, dithionite-reduced; ——, dithionite-reduced CO-complexed.

band shifted to 396 nm, the α- and β-bands disappeared, and a new band, which was attributable to a charge transfer band in the high spin state, appeared simultaneously at 651 nm. After the subsequent reduction with Na₂S₂O₄, the Soret band shifted to about 413 nm and a new band was observed at 548 nm. Bubbling the reduced sample with CO gas made a typical CO-P-450 spectrum which showed absorption maxima at 450 and 557 nm. No peak was observed at 420 nm, indicating that this preparation was free from the inactive form, P-420. The CO difference spectrum had an extinction coefficient of 91 ± 3 cm⁻¹/mM heme between 450 and 490 nm in which the concentration of protoheme was determined by the pyridine hemochrome method. This value was in good agreement with that of liver microsomal P-450, as reported previously (39). The absorption spectrum in the ultraviolet region was not measurable because, even after hydroxyapatite column chromatography, the Emulgen could not be removed completely from the preparation.

P-450 was quite stable spectroscopically even in the steroid substrate free form and no production of P-420 was observed in the sample kept at 25°C for several hours or in that kept at 0°C for several weeks in 50 mM Tris-Cl buffer, pH 7.2, containing 20% glycerol, 0.15% Emulgen, 0.1 mM dithiothreitol, and 0.1 mM EDTA. The presence of the detergent and glycerol was essential for the stability of P-450. When Emulgen was removed from the buffer system, one-half of the P-450 precipitated in 30 min at 25°C. After incubation of P-450 at 25°C for 2 h in buffer without glycerol, 15% of the original P-450 lost the affinity for 17α-hydroxyprogesterone, and the corresponding conversion of P-450 to P-420 was observed.

The changes in the absolute absorption spectra obtained upon the addition of 17α-hydroxyprogesterone and 11-deoxycorticosterone are shown in Fig. 7. As the concentration of 17α-hydroxyprogesterone was increased, the high spin absorption increased with simultaneous decrease of the low spin absorption (Fig. 7A). There were isosbestic points at 407, 459, and 529 nm. The existence of the isosbestic points indicates that these spectral changes are due to the conversion of a spectral species from one form to another. In contrast, the spectral changes induced by the addition of 11-deoxycorticosterone were different from those induced by 17α-hydroxyprogesterone (Fig. 7B). When the concentration of 11-deoxycorticosterone was

Fig. 7. Titration of purified P-450 in the oxidized state with 17α-hydroxyprogesterone (A) or with 11-deoxycorticosterone (B) at 25°C. A, Curve 1 shows the spectrum of P-450 without steroid. Curves 2 to 5 show the spectra of P-450 after the addition of 6.5, 1.0, 2.0, and 12 µM 17α-hydroxyprogesterone, respectively. B, Curve 1 shows the spectrum of P-450 without steroid. Curves 2 to 5 show the spectra of P-450 after the addition of 5, 15, 55, and 555 µM 11-deoxycorticosterone, respectively. The concentration of P-450 was 2.6 µM.
increased, the Soret band of the original low spin type P-450 shifted gradually towards the shorter wavelength and the \( \alpha \)- and \( \beta \)-bands decreased without showing any new absorptions. The charge transfer band at 650 nm, characteristic of the high spin form, did not appear. There was no isosbestic point in the spectral changes. These results suggest that the spectral change due to 11-deoxycortisol cannot be attributed simply to the spin state conversion. As seen in Fig. 8A, the addition of 17a-hydroxyprogesterone to P-450 exhibited the typical type I difference spectra with an absorption maximum at 389 nm and a minimum at 442 nm. There were isosbestic points at the same positions as in the absolute spectra shown in Fig. 7A. The difference spectra observed upon the addition of 11deoxycortisol were somehow similar to the type I difference spectra (Fig. 8B). The positions for the maximum and the minimum of difference spectra induced by 11-deoxycortisol were, however, shifted to the shorter wavelength by increasing the concentration of the additive; no isosbestic point was observed. The spectral changes, as seen in Fig. 8B, will be termed "modified type I" in this paper. The steroids which clearly cause the type I and the modified type I spectral changes will be referred to hereafter as type I and modified type I steroids, respectively. The spectral changes upon the addition of \( \Delta^* \)-androstenedione did not show a clear isosbestic point at 407 nm, but the increase of the absorbance at 650 nm was observed. It is not easy to classify \( \Delta^* \)-androstenedione as either type I or modified type I steroid. The magnitude of the difference between the maximum and the minimum in the type I and the modified type I difference spectra was different for each steroid. The magnitude of the absorption difference observed in the presence of a sufficient amount of a certain steroid was proportional to the concentration of P-450 in the range from 0.5 to 5 \( \mu \)M and was independent of the concentration of Emulgen in the range of 0.05 to 0.3%. As listed in Table III, the magnitude observed in the presence of a sufficient amount of steroid can be expressed as the value of an extinction coefficient. The different values of the extinction coefficient for type I steroids indicate the different contents of the high spin form in the steroid·P-450 complexes. As seen in Fig. 9, the content of the high spin form in the progesterone·P-450 complex increased with an increase in the temperature of the system and decreased with a reduction of the temperature. This spectral change was eventually due to the spin state conversion. The spin state conversion with temperature was observed for all of the steroid·P-450 complexes and little change was also observed for the steroid-free P-450. This temperature dependence of the spin state conversion was not the effect of temperature on the steroid binding to the P-450 because the steroid was present sufficiently in the system and addition of the steroid did not cause any spectral changes at each temperature. These temperature-dependent spin conversions have also been reported for bacterial P-450, (40) and for hepatic P-450 (41, 42). These phenomena have been explained as being due to the equilibrium between the high and the low spin conformations around heme being quite sensitive to temperature (40). The preliminary experiments on the spin equilibrium of P-450 showed that it was also affected by the composition of the buffer. Detailed analysis of the equilibrium requires further experiments. Modified type I spectral changes have not been reported for any P-450. A possible origin of the modified type I spectral changes will be discussed later in this paper.

The spectral changes caused by the further addition of steroids to the 17a-hydroxyprogesterone·P-450 complex were also investigated. All of the steroids in Table III, except for 17a-hydroxyprogesterone, caused the decrease of the content of the high spin form and the simultaneous increase of the content of the low spin form. The difference spectra induced by the addition of these steroids to the 17a-hydroxyprogesterone·P-450 complexes were the typical reverse type I difference spectra which were the inverse of the spectra seen in Fig. 8A. The magnitude of the reverse type I difference spectrum induced by a certain steroid was dependent on the content of the high spin form in the original sample, and the higher content of the high spin form in the original sample showed the greater magnitude of the reverse type I difference spec-
progesterone in the system were measured after incubating the sample for 20 min at the following temperatures: 1. 0°C; 2. 7.2°C; 3. 15.5°C; 4. 23.5°C; 5. 34°C.

It was difficult, however, to get the maximum magnitude for the sample, which had been converted almost completely to the high spin form by the prior addition of a sufficient amount of 17α-hydroxyprogesterone, because it required a large amount of additive steroid and few steroids can dissolve that much in aqueous solution. Thus, the experiments were performed for the samples containing 81% high spin form, which had been made by the addition of a certain amount of 17α-hydroxyprogesterone. The magnitudes of the reverse type I difference spectra were proportional to the concentration of P-450 and could be expressed by the percentages of high spin form converted to the low spin form by the adequate addition of steroids, as shown in Table III. The final absolute spectrum obtained after the addition of a sufficient amount of modified type I steroid to the 17α-hydroxyprogesterone-P-450 complex was the superimposition of the low spin spectrum on the high spin one and was not the same spectrum as that observed upon the addition of steroid to P-450 in the low spin form. It is quite interesting that modified type I steroids are much more effective for this reverse conversion of the spin states than type I steroids. These reverse type I spectral changes took several minutes to complete after the addition of either the type I or modified type I steroids. The type I and modified type I spectral changes took, however, less than 1 s for completion in the 50 mM Tris-Cl buffer containing 20% glycerol and 0.15% Emulgen.

**DISCUSSION**

The content of P-450 subtypes in adrenal microsomes was about 0.1 nmol/mg of protein. Apparently, adrenal microsomes have lower levels of P-450 than liver microsomes, in which the values of 2.2 and 0.66 nmol/mg of microsomal protein have been reported for the phenobarbital-treated and untreated rats, respectively (43, 44). It is quite surprising that highly purified P-450 with a specific content of 16 nmol/mg of protein can be obtained by a repetition of hydrophobic chromatography of ω-amino-n-octyl Sepharose. Judging from the specific content, SDS gel profile, and immunochemical analysis, P-450 subtypes is quite stable in buffer containing Emulgen and glycerol even without the steroid substrate. It is possible to evaluate the physical and chemical properties both in the substrate-free and substrate-bound forms of P-450 subtypes in detail.

After reconstitution with the NADPH-P-450 reductase, P-450 subtypes was active for C-21 hydroxylation but was not active for 17α hydroxylation. In contrast, the original microsomal preparation catalyzed both C-21 and 17α hydroxylation of progesterone. These results suggest that there are at least two specific P-450's in adrenal microsomes and that one is responsible for C-21 hydroxylation and the other for 17α hydroxylation. Menard et al. (15) have reported that the P-450 for 17α hydroxylation in the testicular and adrenal microsomes showed the type I difference spectrum upon the addition of spironolactone. Purified P-450 subtypes did not show the type I spectral change by the addition of spironolactone (data not shown), suggesting that P-450 subtypes is not the cytochrome for 17α hydroxylation.

It has been reported that the adrenal microsomes from a cortisol-producing animal had higher 17α-hydroxylase activity than those from a corticosterone-producing animal (14). In other words, the level of activity of 17α-hydroxylase appears to determine which steroid hormone is produced in the adrenal. It is of interest that higher C-21 hydroxylation activity was observed for 17α-hydroxypregnerosterone than for progesterone in this study. Higher C-21 hydroxylation activity for 17α-hydroxypregnerosterone can be considered to produce cortisol in the adrenals of the cortisol-producing animals. If higher activity of C-21 hydroxylation for progesterone were the case, 17α-hydroxylase might not be the only enzyme to determine which steroid would be produced in the adrenal and the regulation of hormone biosynthetic pathways would be complicated.

The absolute spectrum of purified P-450 subtypes was a typical low spin form and a sufficient addition of 17α-hydroxyprogesterone converted the spectrum into a typical high spin one. The extinction coefficients of the Soret band of the P-450 subtypes in the steroid-free and the 17α-hydroxyprogesterone-bound forms were 192 mM⁻¹ cm⁻¹ at 419 nm and 100 mM⁻¹ cm⁻¹ at 396 nm, respectively. Gunsalus and Wagner (45) reported the extinction coefficients of 115 mM⁻¹ cm⁻¹ at 418 nm and 102 mM⁻¹ cm⁻¹ at 391 nm for P-450 subtypes in the substrate-free and bound forms, respectively, which were essentially in the low and high spin states. The difference extinction coefficient of 113 mM⁻¹ cm⁻¹ of P-450 subtypes for the wavelength pair 389 nm versus 422 nm in the difference spectrum induced by a sufficient addition of 17α-hydroxyprogesterone was similar to that calculated from the difference between the high spin and low spin spectra of P-450 subtypes (46, 47). These values indicate that purified P-450 subtypes is almost in the complete low spin form and that the 17α-hydroxyprogesterone-P-450 subtypes complex is almost in the complete high spin form. The observed contents of the high spin form in P-450 subtypes complexes with progesterone or with 11β-hydroxyprogesterone in the presence of adequate amounts of these steroids were about 76 and 57% at 25°C, respectively, and were calculated from the magnitudes of the difference spectra. These values do not mean that 76 or 57% of P-450 subtypes is in the steroid-bound form. The temperature dependence of the spectral changes of the progesterone-P-450 subtypes complex was not due to the changes of the binding constant because progesterone was present quite sufficiently in the system. This should be due to the thermally induced interconversion between the high and the low spin forms (40). The low extinction coefficient in the type I difference spectrum must indicate that the spin equilibrium at 25°C has declined to the low spin form in that complex. It is not clear at present what kinds of structural factors of the steroid affect the equilibrium.

The modified type I spectral changes did not show clear isosbestic points, suggesting that the modified type I steroid might bind to more than one site of P-450 subtypes. Binding of modified type I steroid to the 17α-hydroxyprogesterone-P-450 subtypes complex caused typical reverse type I spectral changes, but adequate addition of modified type I steroid could not produce the same spectrum as that which follows a sufficient addition of steroid to low spin P-450 subtypes. Addition of 11-deoxycorticosterol to low spin P-450 subtypes caused almost no increase in the
charge transfer band in the high spin form. On the other hand, a sufficient addition of 11-deoxy cortisol to the 17α-hydroxyprogesterone-P450c21 complex, in which 11-deoxy cortisol was added to about 500 times higher concentration of 17α-hydroxyprogesterone, did not diminish the charge transfer band completely. Similar phenomena were also observed upon sufficient addition of other modified type I steroids (Table III) to the 17α-hydroxyprogesterone-P450c21 complex. These results cannot be explained by a simple exchange of the first steroid into the second one at a single steroid binding site in P450c21, but can be explained by the following hypothesis. There are two binding sites for steroids in the P450c21 molecule. Site I has a higher affinity to the type I steroids than to the modified type I steroids and Site II has a higher affinity to modified type I steroids. The binding of steroids at Site I causes the conformational changes from the low to the high spin form. The effectiveness of the conversion from the low to the high spin form is different for each steroid, as shown in Table III, and the modified type I steroid has little effect at Site I, as seen in Fig. 7B. The binding of steroids to Site II decreases the spin equilibrium to the low spin form. The modified type I steroids have a higher affinity to both Sites I and II and the type I steroids are quite effective at Site I. Cholesterol derivatives by a hypothesis similar to that described by Takigawa et al. (24). Jefcoate (49) explained the temperature dependence of the difference spectra induced by several cholesterol derivatives by a hypothesis similar to that described above. It is of interest that pregnenolone is the product of cholesterol side-chain cleavage by the action of P450c21, and that 11-deoxy cortisol and 11-deoxycorticosterone are the products of the action of P450c21. Since the high spin form can be considered to be a more efficient state than the low spin form for accepting electrons (50, 51), it is possible that binding of modified type I steroid to the substrate P450 complex makes the reduction rate slow down. There might be a regulatory function of the steroid product in the reaction process of P450-dependent hydroxylation.

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