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PROPERTIES OF HIGH SPIN TYPE P-450 PREPARATIONS FROM BOVINE ADRENAL CORTEX MITOCHONDRIA

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SUMMARY

A high spin type P-450 preparation (Fraction H) was prepared from bovine adrenocortical mitochondria by extraction with cholate followed by fractionation with ammonium sulfate. Several sub-fractions were further obtained from Fraction H by chromatography and gel filtration.

The ratio of heme/protein (nmol/mg) of Fraction H was 4–5 and that of the purest sub-fraction (Fraction A3B) was 12–13. The ratio of $A_{280\,nm}/A_{393\,nm}$ of Fraction H was about 3 and that of Fraction A3B was 1·2. All sub-fractions were active for side chain cleavage of cholesterol and (20S)-20-hydroxycholesterol (20α-hydroxycholesterol), but the apparent activity decreased as the purification proceeded in spite of the fact that there was no sign of denaturation of P-450. The activity varied depending on the kind of medium in which steroid substrates were dissolved.

The molar ratio of bound cholesterol/heme approached 0·6–0·8 with purification and a small amount of 20α-hydroxycholesterol was also found in the preparations. The bound cholesterol could be metabolized to pregnenolone with a concomitant change of P-450 to a low spin state, and pregnenolone was still bound to P-450 even after re-fractionation. The addition of cholesterol to the low spin form caused a change to a high spin state.

On the basis of these observations, the nature of high spin P-450 and the possible requirement of a steroid carrier factor or a structural factor for effective hydroxylation are discussed.

INTRODUCTION

The spin state of adrenal mitochondrial P-450 changes both on binding to substrate and depending on pH [1]. A high spin type preparation of P-450 from adrenocortical mitochondria was first reported by Jefcoate, Hume, and Boyd[2]. They obtained the preparation by treatment of sonicated mitochondrial particles with iso-octane followed by extraction with cholate and fractionation with ammonium sulfate. Only after the treatment with iso-octane the high spin type could be separated by the fractionation. Similar preparations were prepared by Isaka and Hall[3] using high speed centrifugation to separate the high spin form and also by Ando and Horie[4] using autolyzed mitochondria as the starting material. The high spin type preparations could be precipitated at relatively high concentrations of ammonium sulfate and were readily soluble in the absence of added detergent [2–4]. They were relatively specific for side chain cleavage of cholesterol or (20S)-20-hydroxycholesterol (20α-hydroxycholesterol) and showed only type II substrate-induced spectral change [2–4]. The amount of high spin type in the adrenal gland increases on exposure of rats to stress and is probably intimately related to the regulation of hormonal steroid production by ACTH [5, 6]. Therefore, studies on the nature of high spin type are important for elucidating of the mechanism of ACTH action [5].

It is believed that the high spin type P-450 is in itself a low spin hemoprotein but appears to be high spin by complexing with cholesterol [5, 7] but the unequivocal evidence is not yet available. To establish that high spin type P-450 is a complex with cholesterol, results which meet the following criteria must be presented: (a) Absolutely pure preparations of high spin type contain cholesterol in a constant molar ratio of cholesterol/heme. (b) By removal of cholesterol from the pure preparation, P-450 can be converted to free enzyme and the free enzyme is in a low spin state. (c) Cholesterol added to the free enzyme converts the spin state from low to high.

Unfortunately, such a pure preparation of adrenal mitochondrial P-450 is not available at present, although a preparation has been reported to be nearly homogeneous [8]. Furthermore, we have found it difficult to prepare free enzyme from soluble cholesterol complex. Nevertheless our studies are towards this objective and, in this paper, we report a method of purification which gives a final purity of approximately 70% and describe the properties of the preparation.

EXPERIMENTAL

Purification of high spin type P-450

The outline of the procedure is illustrated in Fig. 1. All manipulators were made out at 4°C unless otherwise stated. The isotonic sucrose and phosphate buffer invariably contained ethylenediamine tetra-acetic acid (EDTA) in a concentration of 0·1 mM.

Step 1. Preparation of cholate extract. Mitochondria were prepared as described previously [9] from 720 g of commercially obtained frozen adrenal cortex tissues. The mitochondria were washed with 0·1 M phosphate buffer, pH 7·0, suspended in 380 ml of

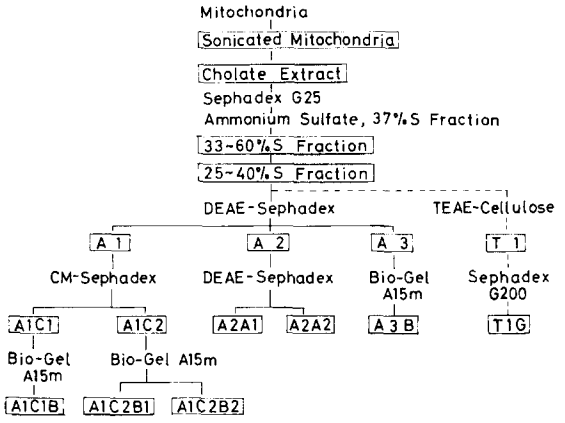

Fig. 1. Outline of fractionation and purification of high spin type P-450.

water, and sonicated at 20 kHz for 10 min at below 10°C. The sonicate was centrifuged at 100,000 *g* for 45 min. The precipitate was suspended in the buffer to a protein concentration of 30 mg/ml. The preparation could be stored at −20°C for three weeks at this stage. Five batches of the suspension were combined (about 800 ml), and 9 ml of 10% Na-cholate, pH 7·0, were added per 100 ml of the suspension (0·3 mg Na-cholate/mg protein). When desired, 20 to 40 mg of steroid were added to the extraction medium by dissolving, in advance, into the 10% Na-cholate solution. The mixture was stirred for one hour and centrifuged at 11,000 *g* for 30 min. The semi-turbid supernatant was concentrated to about 450 ml by membrane filtration using a membrane whose limit of molecular weight exclusion was 30,000. The concentrate was centrifuged at 100,000 *g* for 90 min and the clear supernatant (cholate extract) was collected.

Step 2. Preparation of 37%S Fraction. To remove excess cholate, the extract was passed through a Sephadex G25 column (5 × 90 cm.) equilibrated with 0·1 M phosphate buffer, pH 7·0. To the semi-turbid fraction, 0·861 volume of 80% saturated ammonium sulfate solution, pH 8·0 [9], was added to obtain a final concentration of 37% saturation. The precipitate was collected by centrifugation at 11,000 *g* for 30 min, suspended in 180 ml of the buffer, and the protein concentration was adjusted to 10 mg/ml.

Step 3. Preparation of 33–60%S Fraction. The 10% Na-cholate solution was added to the 37%S Fraction in the amount of 4·5 ml/100 ml. To the mixture 0·455 volume of 80% saturated ammonium sulfate was added to obtain 25% saturation. After centrifugation at 11,000 *g* for 15 min, the supernatant was brought to 33% saturation by addition of 0·170 vol. of 80% saturated ammonium sulfate. The preparation was again centrifuged for 15 min and, to the supernatant, 1·35 volume of 80% saturated ammonium sulfate were added to obtain 60% saturation. The preparation was then centrifuged at 11,000 *g* for 30 min (to 90 min, if necessary) and the precipitate was dissolved in 15–30 ml of the buffer. The protein concentration was adjusted to 10 mg/ml by diluting with the same buffer.

Step 4. Preparation of 25–40%S Fraction. Without addition of cholate, the concentration of ammonium sulfate was brought to 25% saturation by addition of 0·455 vol. of the 80% saturated solution. The preparation was centrifuged for 15 min and 0·375 vol. of 80% saturated ammonium sulfate was added to the supernatant to obtain a final concentration of 40% saturation. The precipitate was collected by centrifugation and dissolved with 2–3 ml of the phosphate buffer. The preparation could be stored at −20°C at this stage.

Step 5. Sub-fractionations. The solution was dialyzed against 2000 ml of 0·05 M phosphate buffer, pH 6·7, for 3 h, applied to a DEAE-Sephadex A25 column (2·6 × 90 cm.) equilibrated with 0·05 M phosphate buffer, pH 6·7, and eluted with the same buffer. Three fractions, A1, A2, and A3 were obtained.

The fraction A1 was concentrated by ammonium sulfate precipitation, dialyzed against 10 mM phosphate buffer, pH 7·0, applied to a CM-Sephadex C25 column (1·8 × 90 cm.) equilibrated with 2 mM phosphate buffer, pH 7·0, and eluted by a gradient from 2 to 200 mM. Two fractions, A1C1 and A1C2, were thus obtained.

The fraction A2 was concentrated, dialyzed, and re-chromatographed on the DEAE column, and fractions A2A1 and A2A2 were obtained.

The fraction A3 was concentrated by both membrane filtration and ammonium sulfate precipitation.

Most sub-fractions were dialyzed against 0·02 M phosphate buffer, pH 7·0, and passed through a Bio-Gel A15 m column (1·8 × 62 or 1·6 × 90 cm.) equilibrated with 0·02 M phosphate buffer, pH 7·0, and thus fractions A1C1B, A1C2B1, A1C2B2, and A3B were obtained.

In some cases the 25–40%S fraction was applied to a TEAE-cellulose column (1·5 × 50 cm.) equilibrated with 0·02 M phosphate buffer, pH 7·0, and fraction T1 was obtained. By passing this fraction through a Sephadex G-200 superfine column (1·8 × 30 cm.), fraction T1G was prepared.

Measurement of absorption spectra

Absorption spectra were measured using a Hitachi 124 recording spectrophotometer. To prepare a carbon monoxide complex of P-450, carbon monoxide was bubbled through the sample for 90 s and after dissolving a small amount of solid sodium dithionite, the sample was allowed to stand at a room temperature for 30 to 60 min until the Soret peak at 448–449 nm reached a maximum. The amount of heme in P-450 preparations was estimated from the difference in absorbance in the carbon monoxide difference spectrum between 448·5 and 490 nm using a millimolar extinction coefficient increment of 85 [4].

Electron spin resonance (ESR) spectroscopy

ESR spectra were measured at the temperature of liquid nitrogen in a Japan Electron Optics ESR Spectrometer, model ME-1X, equipped with 100 kHz field modulation. Manganese ion was used as an external standard in calculating g-values.

Sedimentation analysis

This was carried out in a Beckman Model E analytical ultracentrifuge.

Determination of protein

The concentration of protein was estimated by the biuret method using pure bovine serum albumin as the standard. Heme was destroyed by addition of hydrogen peroxide and a small amount of Tween 20 was added to clarify colored solutions, if necessary.

Method for the assay of enzymatic activity [10]

The reaction mixture contained either 0·05 ml of 10 mM steroid (deoxycorticosterone in propylene glycol, 20α-hydroxycholesterol in propylene glycol, or cholesterol in acetone) or 0·075 ml of 6·67 mM steroid (cholesterol in 10% sodium cholate or 20α-hydroxycholesterol in 10% sodium cholate), 0·30 ml of 20 mM sodium glucose-6-phosphate (G-6-P), 0·05 ml of 14 units/ml G-6-P dehydrogenase, 0·20 ml of 0·1 M $MgCl_2$, 0·10 ml of 970 μM adrenodoxin, 0·30 ml of 114 DCPIP units/ml NADPH-adrenodoxin reductase preparation [10], 0·10 ml of 16·5–23·1 μM P-450 preparation, and 1·20 ml of 0·1 M phosphate buffer, pH 7·0, in a total volume of 2·4 ml. The incubation was carried out at 37°C and the reaction was started by addition of 0·10 ml of 5 mM NADPH.

Adrenodoxin was prepared by the method of Kimura as modifed by Mitani and Horie[10], and NADPH-adrenodoxin reductase was prepared by the method of Omura *et al.*[11] as modified by Mitani and Horie[10].

For the assay of 11β-hydroxylation, the reaction was terminated after 7 min by addition of 1 ml of 1 N H_2SO_4. Corticosterone was estimated by the fluorometric method of Mattingly[12]. For the assay of side chain cleavage reaction, the reaction was terminated after 30 min as above. Steroids were extracted with chloroform and washed once with 5% Na_2CO_3, twice with water, evaporated to dryness, and analyzed gaschromatographically.

Determination of steroids bound to P-450 preparations

To 0·5–1·0 ml of the sample containing 70 to 200 nmol of protoheme of P-450, 100 nmol of testosterone was added as an internal standard, and steroids were extracted with chloroform–methanol (2:1, v/v) according to the method of Folch *et al.*[13]. The extract was evaporated to dryness and analyzed gaschromatographically.

Gaschromatographic estimation of steroids

This was carried out essentially as described by Ando and Horie[4] except that a Shimadzu GC-5A gaschromatograph, equipped with a dual ionization detector, was used. Under the conditions employed the molar correction factor for the detector response was 1·41 for cholesterol, 1·45 for 20α-hydroxycholesterol, 0·81 for pregnenolone, and 1·00 for standard testosterone.

Reagents

20α-hydroxycholesterol was purchased from Ikapharm Co. Deoxycorticosterone, corticosterone, pregnenolone, testosterone, NADPH, G-6-P, and G-6-P dehydrogenase were supplied from Boehringer Mannheim Japan Co. Cholesterol was a product of Wako Chemical Co. Cholesterol and pregnenolone were recrystallized from ethanol.

Table 1. Effect of various pre-treatments of sonicated mitochondria on extraction and fractionation of P-450

Addition or treatment (at 4°C, overnight with stirring)	Extraction with cholate	Fractionation with ammonium sulfate 1st 25–60% Saturation	2nd <35% Saturation	2nd 35–60% Saturation
None	249	156 (1·39)	163 (1·34)	0·1 (1·13)
Cholesterol (0·09 mg/mg protein)	228	134 (1·44)	109 (1·43)	2·1 (1·13)
Pregnenolone (14 nmoles/mg protein)	342	213 (0·80)	196 (0·94)	0·1 (0·86)
Nagarse* (0·05 mg/mg protein)	0†	—	—	—
Steapsin, sigma (0·2 mg/mg protein)	61†	—	—	—
Steapsin, sigma (0·05 mg/mg protein)	92†	42†	6·5†	29·6†
Acid phosphatase, sigma (0·05 mg/mg protein)	363	186 (1·36)	164 (1·35)	22 (1·34)
Lysosomal fraction (0·15 mg protein/mg protein)	294	163 (1·31)	84 (1·21)	63·2 (1·45)

* Bacterial protease supplied from Nagase Sangyo Co.

† P-420 was found in these fractions.

Values are nmoles P-450 obtained/1,000 mg of protein of sonicated mitochondrial fraction. Values in parentheses are the ratio of difference in absorbance, $\Delta A_{393-470\,nm}/\Delta A_{416-470\,nm}$.

RESULTS

Preparation of high spin type P-450

Although the high spin type preparation prepared by Ando and Horie[4] from autolyzed mitochondria was a fraction precipitating between 35 and 60% saturation of ammonium sulfate, Mitani and Horie† later found a larger amount of high spin P-450 in a fraction precipitating between 25 and 35% saturation. Therefore, a re-examination of the effect of autolysis has been carried out to see the relation between these two fractions.

As shown in Table 1, most of the high spin P-450, showing $\Delta A_{393-470\,nm}/\Delta A_{416-470\,nm}$ ratio (high spin/low spin index) of 1·3 or more, precipitated between 25 and 35% saturation. The addition of neither cholesterol nor pregnenolone to the sonicated mitochondria caused any increase in the yield of the fraction precipitating between 35–60% saturation (35–60%S fraction). By treating sonicated mitochondria with a lysosomal fraction from adrenal cortex, however, we could obtain the 35–60%S fraction in a fairly good yield. A commercial sample of acid phosphatase from wheat germ (Sigma) could imitate the effect of lysosomal fraction to some extent. Since we wished to avoid possible alteration of P-450 by lysosomal enzymes the autolysis was not employed in the present study, although the method of Ando and Horie[4] gave a good apparent separation of the side chain cleavage activity from the 11β-hydroxylation activity.

† F. Mitani and S. Horie, unpublished data.

In the *Step 3* of the preparation procedure, when the precipitation by 25% saturation of ammonium sulfate was omitted, almost all P-450 precipitated at 33% saturation. It must be noted that, only after less soluble P-450 had been removed by successive precipitation at 25% and 33% saturations, high spin P-450 could be separated as 33–60%S fraction. As shown in Table 2, about two-thirds of P-450 present in sonicated mitochondria was extracted under the conditions employed (0·3 mg Na-cholate/mg protein), and about a half of the extracted P-450 was recovered as high spin P-450 fraction. In the absence of added cholate the high spin fraction could be precipitated between 25 and 40% saturation.

An example of DEAE-Sephadex chromatography of the dialyzed 25–40%S fraction (Fraction H) is shown in Fig. 2. In some cases a gradient elution from 0·05 to 0·2 M phosphate was adopted to obtain a faster elution of Fraction A3. After completion of the gradient elution, a small amount of P-450 (Fraction A4) could be obtained by a prolonged elution with 0·2 M phosphate but its purity was low.

The Fraction A1 was further chromatographed on CM-Sephadex and two fractions, A1C1 and A1C2, were obtained. By a re-chromatography of Fraction A2 on the DEAE-Sephadex column about a half the P-450 appeared in the same position as A1 and the remainder appeared in the position of A2. The former was named A2A1 and the latter A2A2.

Sub-fractions obtained by ion exchange chromatography could be further purified by gel filtration with Bio-Gel A15 m which has been used by Shikita

Table 2. Yield, purity, and spin state of P-450 fractions

Preparation	Yield %	$A_{280\,nm}/A_{393\,nm}$	P-450/Protein nmoles heme/mg	High spin/low spin index*
Sonicated mitochondria	100	—	0·51	—
Cholate extract	60	—	2·08	1·07
Ammonium sulfate Fractionation				
33–60% Saturation	34	—	4·10	1·39
25–40% Saturation	29	2·87	4·53	1·39
DEAE-sephadex chromatography				
A1	6·5	1·93	—	1·50
A2	2·4	1·87	—	1·45
A3	5·0	1·36	—	1·41
CM-sephadex chromatography				
A1C1	1·8	2·16	—	1·39
A1C2	1·9	1·58	—	1·48
Bio-Gel A15m treatment				
A1C1B	0·9	1·82	6·75	1·35
A1C2B1	0·4	1·47	—	1·36
A1C2B2	0·4	1·47	—	1·28
A3B	4·1	1·20	12·3	1·49
TEAE-cellulose chromatography				
T1	16·3	1·89	6·14	1·32
Sephadex G200 treatment				
T1G	12·5	1·70	7·81	1·40

* The ratio of difference in absorbance, $\Delta A_{393-470\,nm}/\Delta A_{416-470\,nm}$.

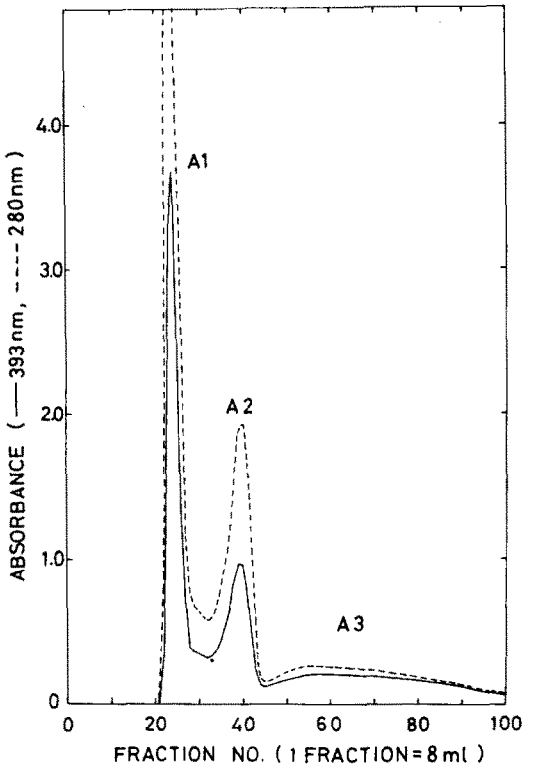

Fig. 2. Chromatography of crude high spin type preparation of P-450 (Fraction H) on DEAE-sephadex.
Eight ml of dialyzed Fraction H, containing about 600 mg of protein, were applied to a DEAE-Sephadex A-25 column (2·6 × 90 cm.) equilibrated with 0·05 M phosphate buffer, pH 6·7, and eluted with the same buffer. The flow rate was 15 to 30 ml/h.

and Hall[8] for the purification of their preparation of P-450. The pattern of the gel filtration of Fraction A3 is shown in Fig. 3.

Another procedure of purification by chromatography on TEAE-cellulose and gel filtration with Sephadex G200 did not give the purity of more than 8 nmoles heme/mg protein. But, as a relatively good yield could be obtained by this procedure, it was used when larger quantities of partially purified P-450 were needed.

So far as we obtained, A3B was the purest fraction and contained 12 to 13 nmoles heme per mg protein. Repeated treatments of A3B with Bio-Gel did not result in any improvement of purity. After addition of 20α-hydroxycholesterol Fraction A3B showed $A_{280\,nm}/A_{416\,nm}$ ratio of 1·0. Our calculation indicated that the crystalline $P\text{-}450_{CAM}$ preparation prepared by Yu *et al.*[14] from *Pseudomonas putida* had the ratio of about 0·7 in its low spin state. Therefore, the absolute purity of Fraction A3B would be about 70% assuming that adrenal P-450 had similar minimum molecular weight and similar aromatic amino acid content to those of $P\text{-}450_{CAM}$.

Physical properties

Absorption spectra of fraction A3B are shown in Fig. 4. The Soret peak of the oxidized form always had a slight shoulder at about 416 nm, indicating some low spin component was present. The ratio of absorbance, $A_{280\,nm}/A_{393\,nm}$ of A3B was 1·20. Except for the height of the peak at 280 nm, most sub-fractions showed essentially the same absorption spectra and were practically free from contamination by P-420. By addition of 20α-hydroxycholesterol the oxidized spectrum changed to a low spin form having maxima at about 570, 535, and 416 nm. The isosbestic point of high spin and low spin and low spin forms was at 402–403 nm. The ratio of the absorbance, $A_{280\,nm}/A_{420\,nm}$, was 1·35. According to our calculation, the corresponding ratio of crystalline $P\text{-}450_{CAM}$ [14] was about 1·05 and that of adrenal mitochondrial preparation reported by Shikita and Hall [8] was about 2·0.

At the temperature of liquid nitrogen, Fraction A3B showed weak ESR signals of low spin hemoprotein (g-values: 1·19, 2·24, and 2·42), and after addition of 20α-hydroxycholesterol the intensity of the signals increased by about 5 times with a slight change in the g-values (1·92, 2·24, and 2·40).

On sedimentation analysis the Fraction A3B showed a main peak having the $s_{20,w}$ value of about 14·2 and also two minor peaks on both sides of the main peak. By comparing the elution volume observed in the step of gel filtration with Bio-Gel A15 m, at least one fraction (A1C2B2) was found to be eluted later than human hemoglobin and most other fractions to be eluted earlier than catalase.

Enzymatic activity

As shown in Table 3, the side chain cleavage activity of the sub-fractions was much lower than that of crude preparations. The 11β-hydroxylation activity was about one-tenth of that of the mixed spin type preparation and about twice to three times of that of the crude high spin type preparation. The side chain cleavage activity was affected by the kind of

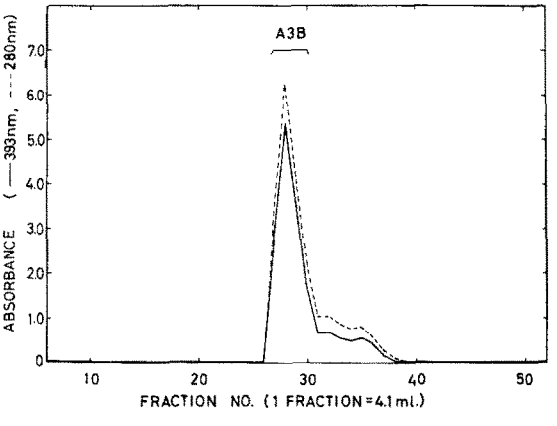

Fig. 3. Gel filtration of fraction A3 with a bio-gel A15m column.
Seven ml of a dialyzed sample of fraction A3 containing about 1200 nmol of P-450 were applied to a Bio-Gel A15m column (1·8 × 62 cm.) equilibrated with 0·02 M phosphate buffer, pH 7·0, and eluted with the same buffer. The flow rate was 8 ml/h. The ratio of elution volume/void volume at the peak was about 2·1.

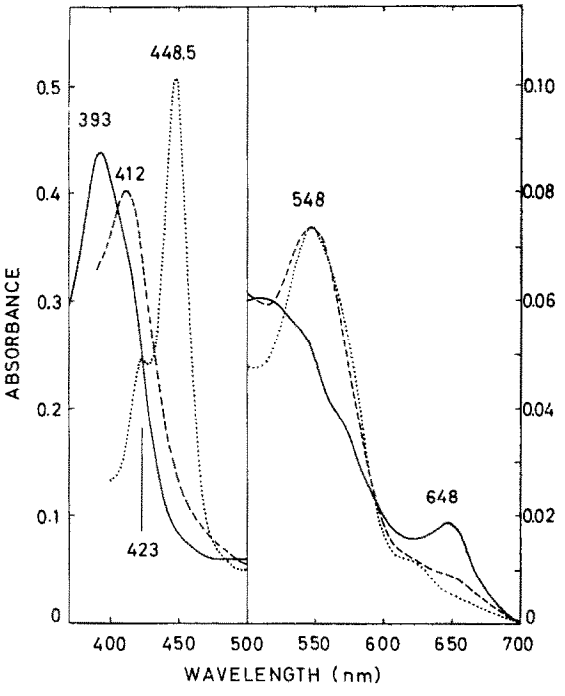

Fig. 4. Absorption spectra of fraction A3B.
Oxidized form ——, dithionite-reduced form – – – –, and carbon monoxide complex ·····. The concentration of P-450 was 4·3 μM with respect to heme.

solvent used for dissolving substrate. Even with Fraction T1G, almost no activity could be detected when cholesterol was dissolved in acetone, but a considerable activity was observed when cholesterol was dissolved in Na-cholate solution. With 20α-hydroxycholesterol as substrate, the activity could also be enhanced by using cholate solution as the solvent, and the addition of cholate separately to the reaction medium was also effective in this case. The addition of either phospholipid or a heated supernatant from adrenal cortex homogenate showed only a little stimulating effect under the conditions employed.

Analysis of bound steroids

A P-450 preparation (T1G) was incubated in the presence of electron carrier system to metabolize bound cholesterol. The result is shown in Table 4. It is clear that bound cholesterol could be metabolized to pregnenolone with a concomitant change of the spin state from high to low and the product, pregnenolone, was still bound to P-450 even after re-fractionation. The resulting low spin form could be converted back to high spin form by the addition of excess cholesterol dissolved in cholate solution. The addition of neither cholesterol dissolved in acetone nor cholate alone caused any change in the absorption spectrum. The addition of 20α-hydroxycholesterol to the low spin form showed no effect but the addition to the high spin form resulted in a change to a low spin form. These results indicate that the binding of cholesterol is closely related to the formation of high spin type P-450. Unfortunately, however, since the removal of pregnenolone from P-450 was not possible, these experiments failed to prove that the free enzyme was in a low spin state.

In Table 5, the relation between the purity and the amount of bound cholesterol is shown. Results with various fractions are listed in a decreasing order of the ratio of $A_{280\,nm}/A_{393\,nm}$. The molar ratio of cholesterol/heme decreased from about 3 to 0·6–0·8 as the preparations were purified. The ratio of bound 20α-hydroxycholesterol/heme, on the other hand, was always around 0·1. The addition of either cholesterol or deoxycorticosterone at the stage of extraction with cholate did not affect the steroid/heme ratio of the sub-fractions. No bound deoxycorticosterone could be detected in the sub-fractions. As the sonicated mitochondria contained as much as 50 mol of cholesterol per mol heme, P-450 in the cholate extract was probably already nearly saturated with cholesterol and, hence, no effect was observed by further addition of cholesterol.

Table 3. Enzymatic activity of P-450 preparations

Preparation	Activity (nmol product formed/nmol heme of P-450) 11β-Hydroxylation /7 min	C.S.C.*/30 min	20α-OH C.S.C.* /30 min
Mixed spin type (Mitani-Horie†)	85 (p)	7 (a)	128 (p)
High spin type (Ando-Horie‡)	3 (p)	13 (a)	181 (p)
T1G	10 (p)	−0 (a)	64 (p)
		30 (c)	
T1G	17 (p)	27 (c)	57 (p)
			117 (c)
A1C1B	6 (p)	18 (c)	50 (c)
A1C2B1	9 (p)	17 (c)	32 (c)
A1C2B2	10 (p)	5 (c)	53 (c)
A3B	9 (p)	10 (c)	48 (c)

* C.S.C.: Cholesterol side chain cleavage.
† Reference (4, 10).
‡ Reference (4).
Solvent for steroids: (p) propylene glycol, (a) acetone, (c) 10% sodium cholate.

Table 4. Enzymatic conversion of high spin type P-450 to low spin form and effect of addition of steroid to the low spin form

	Treatment or additions (Fraction T1G)	High spin/low spin index*	Steroid/Heme (mol/mol) Cholesterol	Pregnenolone
(1)	P-450 before incubation	1·41	1·34	−0
(2)	P-450 after incubation† and re-fractionation‡	0·56	−0	1·30
(3)	(2) 1 μM + 20α-hydroxycholesterol (p), 80 μM	0·56		
(4)	(2) 1 μM + deoxycorticosterone (p), 80 μM	0·56		
(5)	(2) 1 μM + cholesterol (a), 55 μM	0·56		
(6)	(2) 1 μM + Na-cholate, 2 mM, as a control for (7)	0·56		
(7)	(2) 1 μM + cholesterol (c), 55 μM	1·30		
(8)	(7) + 20α-hydroxycholesterol (p), 80 μM	0·67		

* The ratio of difference in absorbance, $\Delta A_{393-470\,nm}/\Delta A_{416-470\,nm}$.

† The incubation mixture contained 257 nmol of P-450, 1630 nmol of adrenodoxin, 1320 DCPIP units of the reductase, and 50 μmol of NADPH in a total volume of 60 ml. The incubation was carried out at 37°C for 30 min.

‡ P-450 was precipitated by ammonium sulfate at 40% saturation, dialyzed, applied to a TEAE-cellulose column, eluted with 1 M NaCl −0·01 M phosphate buffer, pH 7·0, and further purified by gel filtration with Sephadex G200 superfine.

Solvents for steroid: (p) propylene glycol, (a) acetone, (c) 10% sodium cholate.

By extraction in the presence of 20α-hydroxycholesterol, however, the amount of bound 20α-hydroxycholesterol in the purified sub-fractions increased definitely and most sub-fractions contained 0·5 to 0·7 mol of this steroid per mol heme. Although cholesterol was also found in these cases, the purest sub-fraction, A3B, contained less cholesterol than 20α-hydroxycholesterol.

DISCUSSION

On the preparation procedure

As P-450 is a membrane-bound enzyme, it probably exists as a complex with lipids and proteins even after extraction by cholate. By the treatment with the lysosomal fraction a part of these lipids or proteins would be removed and hence the difference in solubility between low spin and high spin type P-450 in ammonium sulfate solution became larger. This was probably the reason why a good separation of side chain cleavage activity from 11β-hydroxylation activity could be obtained by Ando and Horie[4] after autolysis. Because a commercial sample of acid phosphatase having phospholipase activity could imitate the effect of lysosomal fraction (Table 1), the lipolytic action of lysosomes might be the essential part of the effect of autolysis.

In the present study, it is shown that high spin P-450 could be separated without any pre-treatment by careful fractionation with ammonium sulfate. The addition of 20α-hydroxycholesterol, which converted the high spin P-450 to a low spin form, did not affect the yield of 33–60%S Fraction. Therefore, the high spin type P-450 precipitates at higher concentrations of ammonium sulfate not because it is high spin but because it is innately more soluble.

In view of the results obtained by determination of activity (Table 3) it seems that the purification of high spin type P-450 with respect to heme/protein ratio does not always result in a clear separation of side chain cleavage activity from 11β-hydroxylation activity. The reason for this is not known. It must be noted that each sub-fraction of P-450 behaved as a nearly homogenous fraction in spite of the fact that the heme/protein ratio varied from fraction to fraction. Except for a small protein peak which appeared initially in the CM-Sephadex chromatography, all protein peaks obtained by chromatography or gel filtration were associated with the color of P-450. The binding of various amounts of mitochondrial protein to P-450 and the different state of polymerization were probably the reason why we obtained so many sub-fractions having a similar pattern of catalytic activity.

Our Fraction A3B showed the heme/protein ratio of 12–13 nmol/mg and the $A_{280\,nm}/A_{402\,nm}$ ratio of 1·35 (402 nm : isosbestic point). The preparation reported by Shikita and Hall[8] had the heme/protein ratio of 10 or more but the A_{280nm}/A_{402nm} ratio of about 2·0 calculating from their spectral data. These results contradict each other.

On the physical properties

The spectral properties of the sub-fractions were essentially the same as those of high spin type preparation reported by Ando and Horie[4]. The low spin hemoprotein signals observed on ESR measurement were also similar to those described by Ando and Horie except that the uncharacterized signal of g = 1·98–1·99 [1, 4] was faint with the present preparations.

Although we did not carry out a systematic study for the state of polymerization of the preparations, the result reported by Shikita and Hall[8] was supported, at least in part, by our result of gel filtration which showed that the apparent molecular weight of one fraction (A1C2B2) was 60,000 or less and those of many other sub-fractions were 300,000 or more.

Table 5. Purity, bound steroid, and spin state of various P-450 fractions

Fraction	$A_{280\,nm}/A_{393\,nm}$	$A_{280\,nm}/A_{416\,nm}$	Cholesterol/ Heme (mol/mol)	20α-Hydroxycholesterol/ Heme (mol/mol)	High spin/ low spin index*4
25–40%S *1	2·97		2·89		1·51
25–40%S *1	2·89		3·43		1·49
25–40%S *2	2·78		2·00		1·34
25–40%S	2·45		5·96		1·42
A1 *1	2·45		1·56	0·087	1·41
A2 *1	2·07		2·32		1·53
T1G	1·91		1·34		1·33
A1 *2	1·83		1·32	0·101	1·54
A1C1B *1	1·82		1·30	0·096	1·35
T1	1·77		1·52		1·48
A1B *2	1·73		1·11	0·082	1·40
A2 *2	1·69		1·07	0·100	1·55
T1G	1·68		0·59		1·48
T1G *1	1·66		1·41	0·111	1·40
T1G	1·63		1·29	0·099	1·45
T1G	1·63		0·99		1·36
T1G	1·52		0·97		1·48
A1C2B1 *1	1·47		1·39	0·099	1·48
A1C2B2 *1	1·47		0·98	0·087	1·33
T1G	1·44		1·18	0·096	1·41
A3 *2	1·27		0·70	0·100	1·58
A3B *1	1·20		0·60	0·106	1·49
A3BB *1	1·20		0·82	0·057	1·52
25–40%S *3		3·21	2·03	6·99	0·59
A4 *3		1·81	1·36	0·59	0·54
A2 *3		1·66	1·76	0·54	0·74
A1 *3		1·61	1·36	0·48	0·76
A2 *3		1·55	1·12	0·71	0·54
A1 *3		1·55	0·65	0·69	0·54
A1B *3		1·30	0·59	0·59	0·70
A3B *3		1·00	0·36	0·50	0·70

The extraction of P-450 by cholate was carried out in the presence of: *1 added cholesterol, *2 added deoxycorticosterone, and *3 added 20α-hydroxycholesterol.

*4 The ratio of difference in absorbance, $\Delta A_{393-470\,nm}/\Delta A_{416-470\,nm}$.

On the enzymatic activity

Ritter and Dempsy[15] described a steroid carrier factor effective for biological synthesis of cholesterol. Later, this factor was reported to be also effective for steroid hydroxylation [16]. Strobel *et al.*[17] showed that phosphatidyl choline is required for ω-hydroxylation of fatty acids catalyzed by a reconstituted microsomal system of liver. Wang *et al.*[18] also studied the relation between phospholipid content and hydroxylation activity of adrenal preparations. Since steroids are scarcely soluble in aqueous media, some factor which holds steroid in aqueous media must be required for hydroxylation to occur. It is, therefore, not surprising that the apparent activity of P-450 decreased with purification (Table 3). Our results indicate that the sub-fractions were "over purified" as an enzyme preparation up to a stage where the addition of some factor(s), either chemical or structural, became necessary. Cholate was found to be effective for this purpose but this substance could not be considered to have physiological significance. Our observation on the effect of cholate (Table 3) also explained the reason why the high spin type preparation reported by Ando and Horie was apparently much more active for 20α-hydroxycholesterol than for unsubstituted cholesterol.

On the bound steroids

For the determination of bound steroids, the method of extraction is of primary importance. In the earlier stage of this study, we used chloroform for the extraction but later we found that about twice as much cholesterol could be extracted by chloroform—methanol (2:1). To minimize the possible experimental error we added testosterone to the P-450 preparation as an internal standard and then chloroform-methanol with gentle stirring to make fine protein flocks. Even with this procedure, we could not rule out the possibility that a part of cholesterol remained unextracted in the flocks. This possibility may explain the reason why we found (cholesterol + 20α-hydroxycholesterol)/heme (mol/mol) ratio of 0·7–0·9 with purified preparations. One of the many other possibilities was that steroid which was present in the inside of polymerized molecule might be hardly extractable.

We observed that the extraction with dichloromethane caused severe destruction of cholesterol under our experimental conditions.

The lipids present in the chloroform–methanol extracts were also analyzed and a small amount of esterified cholesterol, phospholipids, and free fatty acids were found. In crude preparations a small amount of cholate was often found. On gaschromatographic analysis, cholate appeared very close to 20α-hydroxycholesterol. Therefore, 20α-hydroxycholesterol in crude preparations could not be measured.

Whether added 20α-hydroxycholesterol replaces bound cholesterol or P-450 and these two steroids form a tertiary complex is an interesting problem to be studied [7]. But a more purified preparation is necessary for us to present a final answer to this question. Since the purest fraction (A3B), prepared from 20α-hydroxycholesterol-treated sonicated mitochondria, contained less cholesterol (0·36 mol/mol heme) than 20α-hydroxycholesterol (0·50 mol/mol heme) we tentatively assume that bound cholesterol could be replaced by added 20α-hydroxycholesterol.

It was an unexpected finding that the complex of P-450 with pregnenolone, an enzyme-product complex, was so stable (Table 4). In view of the results shown in Table 4, the bound product (pregnenolone) is probably replaced by substrate (cholesterol) at the catalytic site of P-450 during side chain cleavage reaction.

Further efforts are in progress to obtain purer preparations of high spin type P-450.

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