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PURIFICATION AND PROPERTIES OF CYTOCHROME *P*-450 (11β- AND 18-HYDROXYLASE) FROM BOVINE ADRENOCORTICAL MITOCHONDRIA

MASAAKI WATANUKI, BILL E. TILLEY and PETER F. HALL

Department of Physiology, California College of Medicine, University of California, Irvine, Calif. 92717 (U.S.A.)

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Summary

We describe an improved procedure for the preparation of a cytochrome *P*-450 from bovine adrenocortical mitochondria which catalyzes 11β- and 18-hydroxylation of steroids. The preparation is based upon chromatography on DEAE cellulose which separates the enzyme from the side-chain cleavage *P*-450, which can also be prepared in highly purified form from the same tissue extracts. The enzyme behaves as a single compound in glycerol density gradients. The enzyme aggregates at protein concentrations greater than 1 mg/ml to a series of forms of various molecular weights. On Sepharose 4B the enzyme shows a molecular weight of 185 000, while on glycerol density gradients a molecular weight of $1 \cdot 10^6$ is observed. The subunit molecular weight determined by electrophoresis on polyacrylamide gels with sodium dodecyl sulfate is 47 500 and the protein appears as a single band. The ratio of 11β-/18-hydroxylase activities does not change significantly during purification and is constant through the protein peak on glycerol density gradients. Since there appears to be only one subunit species, it seems likely that the two hydroxylase activities are catalyzed by one protein.

Introduction

The synthesis of corticosteroids by the adrenal cortex requires the activity of a mitochondrial 11β-hydroxylase which is known to be a heme protein of the so-called *P*-450 family [1]. Like other mitochondrial cytochromes *P*-450 the 11β-hydroxylase requires NADPH, a flavoprotein (adrenodoxin reductase) and a non-heme iron protein (adrenodoxin) [1]. 11β-hydroxylase also requires oxy-

Abbreviations: corticosterone: 11β, 21-dihydroxypregn-4-ene-3,20-dione; deoxycorticosterone: 21-hydroxypregn-4-ene-3,20-dione; SDS: sodium dodecyl sulfate.

gen and is believed to be a typical mixed function oxidase or mono-oxygenase [1,2]. The enzyme has been partly purified [3] and a more recent preliminary report indicates that further purification is possible [4] although the enzyme is highly unstable in the more purified form.

Recent studies have shown that a second mitochondrial hydroxylase (18-hydroxylase) copurifies with 11β-hydroxylase through at least one purifying procedure [5]. It was decided to modify the purification procedure in order to improve yields, to prepare both 11β-hydroxylase and side-chain cleavage *P*-450 in the same procedure and to determine whether the two enzyme activities, 11β-hydroxylase and 18-hydroxylase, can be separated.

Experimental Procedure

Preparation of P-450 (11β- and 18-hydroxylase). Bovine adrenocortical mitochondria were prepared and sonicated as described by Shikita and Hall [6]. These and all subsequent steps were performed at 0—4°C. The sonicate was centrifuged at 105 000 × *g* for 120 min and the resulting precipitate was suspended in potassium phosphate buffer (100 mM, pH 7.0) containing EDTA (0.1 mM), dithiothreitol (0.1 mM) and deoxycorticosterone (10 μM) (final concentrations in each case). Sodium cholate (pH 7.0, 0.45 mg/mg protein) was added and the mixture was stirred for 60 min at 4°C. The mixture was centrifuged at 105 000 × *g* for 90 min and the supernate was filtered through glass wool. The filtrate was taken to 30% saturation by ammonium sulfate, stirred for 30 min and then centrifuged at 27 000 × *g* for 20 min. The precipitate from this step was used to prepare 11β-hydroxylase while the supernate was used to prepare side-chain cleavage *P*-450 in order to produce good yields of both enzymes from one batch of adrenal glands. To do this, the supernate from 30% ammonium sulfate precipitation was brought to 60% ammonium sulfate and the precipitate was used as the source of the side-chain cleavage enzyme. This fraction also contains as much as half of the 11β-hydroxylase activity which was however of low specific activity.

The precipitate from 0 to 30% ammonium sulfate was dissolved in approximately 40 ml potassium phosphate buffer (20 mM, pH 7.6) containing EDTA (0.1 mM), dithiothreitol (0.1 mM), deoxycorticosterone (10 μM) and Tween 20 (0.2%) (final concentrations in all cases). The suspension was dialyzed overnight against the same buffer without Tween 20. The dialysate was centrifuged at 105 000 × *g* for 30 min and the resulting precipitate was suspended in the buffer described above (with Tween) using the same volume as that in which it was originally dissolved after ammonium sulfate precipitation. Sodium cholate solution was added to a final concentration of 0.7% (w/v) and the mixture was stirred at 4°C for 1 h. The preparation was centrifuged at 85 000 × *g* for 30 min and the supernate was then ready for column chromatography on DEAE cellulose.

A column of 3.0 cm × 13.0 cm is suitable for extracts of 4 g of mitochondria (30 adrenal glands). The DEAE cellulose is equilibrated with the Tween buffer described above and the same buffer is used to elute proteins after the sample is applied. This buffer elutes two fractions (Fig. 1); the first fraction (breakthrough) is opalescent and contains *P*-450. The second fraction shows side-

chain cleavage but no detectable 11β-hydroxylase activity (<0.1 pmol product/min per mg protein). The side-chain cleavage enzyme was present in small amounts and was discarded. Following the appearance of the second fraction, the buffer is changed to potassium phosphate (100 mM; pH 7.4) with the same additions except that the concentration of dithiothreitol was 1 mM (Fig. 1). A yellow-brown fraction was eluted by means of this buffer; this fraction is found to contain 11β-hydroxylase and 18-hydroxylase activities. The fractions were pooled, pH was adjusted to 7.4 and the preparation was then dialyzed against the eluting buffer (100 mM potassium phosphate pH 7.0 with the three additions described above). This fraction is the 11β- and 18-hydroxylase enzyme with the present studies were performed and which is referred to here by this name.

The 11β-, 18-hydroxylase can be stored at 4°C without significant loss of activity for 1 week. Activity is also maintained for one week when the enzyme is kept in 50% glycerol at −20°C. In either case, activity declines rapidly after one week and is almost absent after two weeks.

The side-chain cleavage fraction (30—60% ammonium sulfate fraction) was purified as described by Shikita and Hall [6]; the enzyme is prepared for chromatography on hydroxyapatite and the remainder of the purification procedure is that described by those authors. This procedure gives the same yields of side-chain cleavage *P*-450 with the same properties as those previously reported [6]. The enzyme was not further examined in connection with the present studies.

Enzyme assay. Side-chain cleavage of cholesterol was measured as described elsewhere using unlabeled cholesterol as substrate [7]. The two hydroxylase reactions (11β- and 18-) were measured by incubating deoxycorticosterone or deoxy[1,2-^{3}H]corticosterone or corticosterone with *P*-450, adrenodoxin and adrenodoxin reductase. Following incubation, the reaction was stopped by boiling and the incubation mixture extracted three times with diethyl ether; throughout the subsequent steps trimethylamine (0.1% v/v) was present in all solvents used (including those used in thin layer chromatography) to prevent formation of hemiketals from the 18-hydroxysteroids. The extracts were taken to dryness and applied to thin layer chromatograms for the isolation and measurement of corticosterone of [^{3}H]corticosterone, 18-hydroxy[^{3}H]corticosterone and 18-hydroxydeoxycorticosterone [8].

The identity and radiochemical purity of the products of 18-hydroxylation and 11β-hydroxylation were demonstrated by incubating deoxy[1,2-^{3}H]corticosterone with the enzyme and isolating [^{3}H]corticosterone and 18-hydroxy-[^{3}H]deoxycorticosterone by thin layer chromatography. The two compounds were added to authentic unlabeled steroids and than recrystallized from three different solvent systems in each case. The specific activities of crystals and mother liquors remained constant within the limits of experimental error with both compounds.

The incubation was performed in potassium phosphate buffer (50 mM; pH 7.0) with cysteine (5 mM) and EDTA (0.5 mM) at 30°C for 10 min in a final volume of 2.0 ml. At this time, the reaction was stopped by plunging the sample in boiling water for a few seconds. It was found that significant loss of 18-hydroxy compounds did not occur under these conditions. It was discovered

that pH 7.0 and temperature of 30°C are important for optimal activity. Controls included flasks with boiled enzyme and others without NADPH; the controls showed negligible activity in all cases. The reaction was started by addition of NADPH. The rates of the two hydroxylase reactions were linear for at least 15 min but declined thereafter. In some studies deoxy[1,2-^{3}H]corticosterone and [1,2,6,7-^{3}H]corticosterone were (separately) incubated with the 11β- and 18-hydroxylase and extracts were examined by paper chromatography [9] which separates the two 18-hydroxy compounds from the corresponding hemiketals. No detectable tritrated hemiketal was found.

The 18-hydroxysteroids were measured by means of a radioimmunoassay [9]. The antibody used reacted with both 18-hydroxysteroids and provided an accurate measurement of each steroid (18-hydroxydeoxycorticosterone and 18-hydroxycorticosterone) after separation on thin layer chromatography. The method used was that described by Chandler et al. [9] with minor modification. The minimal amount of either 18-hydroxysteroid which could be detected under the conditions used was 1.0 pmol. Assays were performed in duplicate and duplicate determinations agreed within 5%.

Assay for 11β-hydroxylation is based upon conversion of deoxycorticosterone to corticosterone which is measured by radioimmunoassay [10]. For most purposes, the additional reaction of 18-hydroxylation yielding 18-hydroxycorticosterone was ignored since the rate of this reaction is approximately one-tenth that of 11β-hydroxylation. For kinetic studies, the additional 11β-hydroxylation represented by the rate of formation of 18-hydroxycorticosterone was included. 11β-hydroxylation of 18-hydroxydeoxycorticosterone was neglected because it proceeded extremely slowly under the conditions used. Recovery of steroids through these procedures was 60—75% as determined by extracting known amounts of steroid from incubation mixtures containing all ingredients except *P*-450. The recovery of steroid was measured by A_{240} and values for enzyme activities are corrected for losses during this isolation procedure.

For both assays, the following components of the system were added to potassium phosphate buffer (50 mM; pH 7.0) containing cysteine (5 mM) and EDTA (0.5 mM), in the following order: *P*-450 0.3—0.4 nmol of heme, adrenodoxin 10 nmol, adrenodoxin reductase 16 units (see reference 6) and substrate. The substrate was either deoxycorticosterone or corticosterone (50 nmol in 10 μl *N*,*N*-dimethylformamide or [1,2-^{3}H]deoxycorticosterone (0.5 μCi : 50 nmol). The reation was started by addition of NADPH (0.2 nmol). In both assays, substrates (25 μM), adrenodoxin and adrenodoxin reductase were present in saturating amounts. The rates of these reactions showed a linear relationship to concentration of enzyme under the conditions used.

Determination of molecular weight. The molecular weight of the enzyme was determined as follows: (i) Analytical ultracentrifugation. Attempts were made to determine molecular weight of the enzyme by ultracentrifugation using a standard method [6]. (ii) Exclusion chromatography. A column of Sepharose 4B (0.9 × 50 cm) was prepared and equilibrated with potassium phosphate buffer (100 mM; pH 7.0), EDTA (0.1 mM) and glycerol (20% w/v). Ethanol dehydrogenase and glutamic dehydrogenase were used as standards. (iii) Glycerol density gradients. Glycerol density gradients (10—30%) were prepared with cholate (0.05% w/v) and potassium phosphate buffer (100 mM, pH

7.0). Enzyme was layered onto the gradient which was then centrifuged at 193 000 × *g* for 19 h in an SW41 rotor. Fractions (0.41 ml) from the gradient were examined for protein content and enzyme activity.

Spectral studies. Absolute spectra of *P*-450 and spectra (absolute and difference) of the CO-*P*-450 complex were performed as described previously [6]. *P*-450 content was calculated using a value for the molar extinction coefficient of 91 000 $M^{-1} \cdot cm^{-1}$ and correction for *P*-420 present in the early stages of the preparation was made by using a value of 213 000 for the molar extinction coefficient for 420 nm in the absolute CO spectrum.

Analytical procedures. Protein determination [11], electrophoresis on polyacrylamide gels [12], isolectric focusing [13], and measurements of cholesterol [6] and heme [6] were performed by established methods which have been reported previously. For determination of phospholipid the enzyme was extracted by the method of Folch et al. [14] and inorganic phosphate was determined by the method of Chalvardjian and Rudnicki [15]. Before Folch extraction the protein was taken to pH 11.0 by addition of ammonium hydroxide. Extraction of purified phospholipids added to the protein after Folch extraction showed a recovery of >95%. A second Folch extraction produced no detectable phospholipid. The minimal amount of phosphate detected by the above method is 5 nmol.

Chemicals. Sources of the chemicals and enzymes used, except those given here, have been reported elsewhere [6]. Deoxycorticosterone and corticosterone were purchased from Sigma Chemical Corporation, [1,2,6,7-^{3}H]corticosterone was purchased from New England Nuclear Corporation (Lot No. 635-232) and 18-hydroxydeoxycorticosterone from Amersham/Searle (Lot No. TRK 458, Batch 4). These steroids were purified in the thin layer chromatography systems described above before use as substrates in these studies.

Results

Purification of 11β-, 18-hydroxylase. The procedure used to purify this enzyme is described above and contains several features which require comment. Firstly, after sonication the enzyme is treated with buffer containing substrate (deoxycorticosterone) which has been reported to stabilize the enzyme [4]. Secondly, precipitation with 30% ammonium sulfate leaves some 11β-hydroxylase in the supernate but precipitates the remainder which is of high specific activity. At this time, it is not possible to explain why the enzyme is partitioned in this way, but the possibility of more than one form of the enzyme must be considered. Table I shows the ratio 11β-/18-hydroxylase for four different enzyme preparations in which the activity was measured in the 30% ammonium sulfate fraction (source of the 11β-hydroxylase) and in the fraction used to prepare the side-chain cleavage enzyme (supernate from 30% ammonium sulfate). Clearly in each preparation the two activities are present in the same ratios in both fractions. Thirdly, we have confirmed the finding of Takemori et al. [4] that the enzyme can be solubilized by detergent, but precipitates when the detergent is removed.

The DEAE cellulose column produces considerable purification of the enzyme which appears as a single band on SDS gels after this step (Fig. 2). The

TABLE I

RATIO 11β-HYDROXYLASE/18-HYDROXYLASE IN TWO AMMONIUM SULFATE FRACTIONS OF MITOCHONDRIAL SONICATE

The fractions were prepared and assayed for the two enzyme activities as described under Experimental Procedure.

Preparation	Ammonium sulfate fraction (30%)	Ratio 11β- /18-hydroxylases
1	<30%	23.7
	30—60%	18.5
2	<30%	19.9
	30—60%	19.6
3	<30%	8.0
	30—60%	5.0
4	<30%	40.9
	30—60%	40.5

breakthrough fraction (Fig. 1) is slightly turbid and shows both 11β-hydroxylase and side-chain cleavage activities resembling in this respect the corresponding fraction in the preparation described by Shikita and Hall [6]. The equilibration buffer (20 mM potassium phosphate with the additions described under Experimental Procedure) also elutes small amounts of a highly purified side-chain cleavage *P*-450 which is usually discarded and should not be confused with the bulk of the side-chain cleavage enzyme which is found in the 30—60% ammonium sulfate fraction (Experimental Procedure). When the concentration of the phosphate is increased to 100 mM, the 11β-hydroxylase is eluted.

Fig. 2 shows that the enzyme appears on SDS gels as a single band, is smaller than and is clearly separated from the side-chain cleavage subunit. The sample of side-chain cleavage *P*-450 shown here was obtained from the same adrenal tissue as the 11β-hydroxylase (see Experimental Procedure). This *P*-450 also appears as a single band in the gels (Fig. 2).

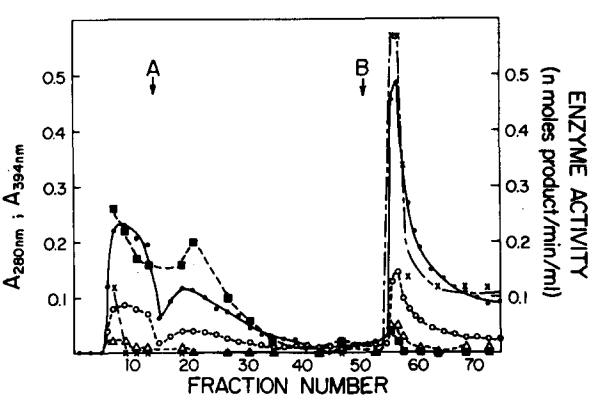

Fig. 1. Elution pattern of adrenal *P*-450 from DEAE cellulose. The details of the procedure are described under Experimental Procedure. After the sample was applied, the initial buffer which contained potassium phosphate 20 mM was started at A and was changed, B, to 100 mM potassium phosphate. ●——●, A_{280}; ○- - - - -○, A_{394}; ■- - - - -■, side-chain cleavage; X- - - - -X, 11β-hydroxylase; △- - - - -△, 18-hydroxylase.

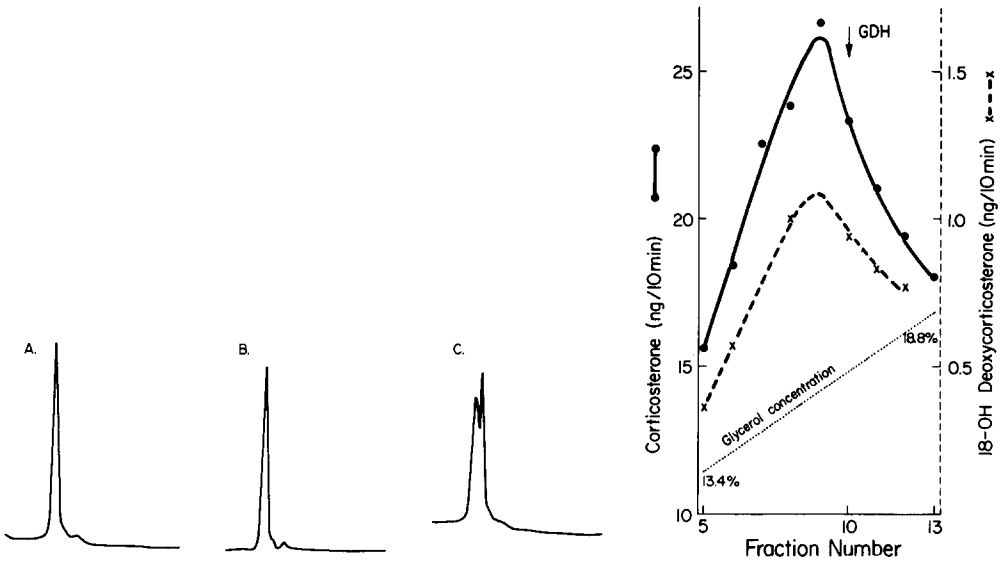

Fig. 2. Densitometric tracings of electrophoretograms on SDS polyacrylamide gels. Electrophoresis was performed for 10 h. Gels were stained with Coomassie Blue and destained [12] A, 11β-hydroxylase; B, side-chain cleavage; C, both enzymes together. Migration of the protein is from left to right in the tracings. The 11β-hydroxylase subunit always moves slightly further than the side-chain cleavage subunit and is seen as the right side of the double peak in C.

Fig. 3. Sedimentation of *P*-450 in glycerol density gradient. The enzyme (700 μg protein) was layered onto a glycerol gradient 10—30% in potassium phosphate buffer (100 mM, pH 7.0) and sedimented for 19 h at 193 000 × *g*. Fractions (9.41 ml) were collected and examined for 11β- and 18-hydroxylase activities. The values shown on the ordinates are for one half of each fraction. GDH indicates position of glutamic dehydrogenase in another gradient centrifuged at the same time in the same rotor. Other standard proteins sedimented nearer to the top of the gradient. The ratio 11β- 18-hydroxylase is constant throughout the peak within the limits of experimental error.

Table II shows the changes in the properties of the enzyme as the purification proceeds. Heme content increases per mg protein as determined by pyridine hemochromogen and by CO-difference spectroscopy. The content of phospholipid and that of cholesterol falls as the enzyme is purified.

Enzymatic activity. It can be seen from Table II that enzymatic specific activity for the three reactions studied increases with cholate extraction and falls after ammonium sulfate precipitation. Thereafter specific activities for 11β- and 18-hydroxylases rise and that of side-chain cleavage *P*-450 falls. The final preparation shows activities for 11β-hydroxylase : 18-hydroxylase : side-chain cleavage in the following ratios 398 : 35.4 : 1.0; the initial sonicate shows the following ratio 2 : 0.12 : 1.0. It is interesting to notice that the ratio of 11β-hydroxylase/18-hydroxylase varied between 6.2 and 29.9. We have encountered some difficulty in measuring enzyme activities with sonicates and in the presence of cholate so that values for absolute activities should not be compared with those using soluble enzyme, but relative values of the two activities can be determined. However, in spite of these limitations, the above ratio is relatively constant and no evidence of separation of the two activities is seen in spite of effective removal of the side-chain cleavage enzyme (Tables I and II). It should be pointed out that a previous report from this laboratory [6] showed

TABLE II

PROPERTIES OF ADRENAL CYTOCHROME *P*-450 DURING PURIFICATION

The various properties shown were measured as described under Experimental Procedure.

Stage of purification	Total protein (mg)	Heme		Phospho lipid nmol/mg protein	Cholest-erol nmol/mg protein	Enzyme activity (nmol/min per mg protein)			
		Pyridine nmol/mg	CO-difference protein			Side-chain cleavage	11β-cleavage	18-cleavage	Ratio 11β- /18-
Sonicated mitochondria	4000	2.53	2.13	388	14.0	0.058	0.115	0.007	16.4
Cholate extract	1720	2.92	3.13	202	1.12	0.892	0.627	0.021	29.9
Ammonia SO_4 (0—30%)	620	3.49	3.61	—	0.044	0.230	0.340	0.033	10.3
Precipitate after dialysis	280	4.55	4.51	99	0.042	0.168	0.560	0.090	6.2
Cholate-Tween extract	210	5.21	5.12	85	0.047	0.309	0.811	0.083	9.8
DEAE-cellulose	30	7.10	7.21	31	0.001	0.030	11.93	1.061	11.2

* The presence of ammonium sulfate interferes with the method used to determine phospholipids.

that a relatively insensitive assay for 18-hydroxylase failed to reveal evidence of this activity in a highly purified side-chain cleavage enzyme which contained traces of 11β-hydroxylase. The present studies used much more sensitive assays for both enzyme activities.

Lineweaver-Burk plots for 11β-hydroxylase and 18-hydroxylase activities of the purified enzyme with deoxycorticosterone as substrate showed the following values: K_m 11β, 20 μM; K_m 18, 20 μM; V 11β, 20 nmol/min per mg protein; V 18, 2 nmol/min per mg protein. It is interesting to notice that the K_m values for the two enzyme activities with the substrate deoxycorticosterone are the same, whereas the V is tenfold greater for the 11β-hydroxylase. The K_m value confirms that reported for a crude preparation of 11β-hydroxylase [1]. It was observed that 18-hydroxylation of corticosterone was too low for accurate determination of kinetic constants.

Molecular weight. Attempts to determine molecular weight of the native enzyme were made by three methods, namely analytical ultracentrifugation, chromatography on sepharose 4B and sedimentation in glycerol density gradients. Analytical ultracentrifugation revealed that under the conditions used the enzyme aggregates to a variety of forms of various molecular weights so that no single value could be determined. Chromatography on sepharose 4B revealed a single peak corresponding to a molecular weight of 185 000 (data not shown). On glycerol density gradients (Fig. 3) a single peak corresponding to a molecular weight of approximately $1 \cdot 10^6$ was observed. It can be seen from Fig. 3 that the ratio of 11β-/18-hydroxylase remains constant in the single peak corresponding to a molecular weight of $1 \cdot 10^6$. In this gradient Tween was present with the enzyme but not in the buffer. Some of the protein was found in a pellet at the bottom of the tube while Tween remained at the top of the gradient. Presumably the enzyme sediments away from the detergent and aggregates to large molecular weight species in the absence of detergent. The standard proteins used for these determinations were as follows: glutamic dehydrogenase (850 000), ethanol dehydrogenase (150 000), bovine serum albumin (67 000) and ovalbumin (45 000). Sucrose density gradients were also used to determine molecular weight of the native form of the enzyme but again aggregation to high molecular weight species was observed.

The molecular weight of the subunit was determined by electrophoresis on SDS gels to be 47 500 (Fig. 2). The following standards were used for this determination: catalase (60 000), glutamate dehydrogenase (53 000), ovalbumin (43 000) and ethanol dehydrogenase (37 000). The subunit of the side-chain cleavage enzyme is 52 000—53 000 and is clearly distinct from the 11β-, 18-hydroxylase subunit (Fig. 2).

Heme content. It can be seen from Table II that the heme content of the enzyme increases during purification to a value of 7.1—7.2 nmol per mg protein. Moreover, values for pyridine hemochromogen and CO-difference spectroscopy are the same. The heme content of the final preparation corresponds to approximately one heme group per 3 subunits (M_r 47 500).

Spectroscopic properties of cytochrome P-450 (11β- and 18-hydroxylase). The preparation of 11β- and 18-hydroxylase presents a problem for spectroscopic examination because when the protein is concentrated it readily aggregates and ceases to be homogeneous with respect to the molecular weight.

Spectra must therefore be examined in dilute solution. The spectrum of oxidized *P*-450 shows peaks at approximately 540, 525, and 417 nm with shoulders at 390 and 335 nm. It is clear that both high and low spin forms are present. The CO-difference spectrum shows a peak at 448 nm and a shoulder at approximately 423 nm. Evidently, the preparation contains significant *P*-420 which we have not succeeded in removing.

Discussion

The present method of preparing the 11β- and 18-hydroxylase is a modification of the procedure reported by Takemori et al. [4]. The affinity column described by these authors uses aniline as the ligand. Presumably this ligand binds non-specifically to hydrophobic regions of the protein; we have experienced difficulty in eluting the enzyme in reasonable yield (<1% of the applied enzyme being recovered). This difficulty has been experienced by another group [16]. The modified procedure can be performed on a large or small scale and is completed within 2 days from frozen mitochondria. It gives reasonable yields (20 mg from 30 adrenal glands) of the enzyme which is homogenous on exclusion chromatography and glycerol density gradient centrifugation. The enzyme shows a molecular weight of 185 000 by exclusion chromatography and appears as a single band on SDS gels corresponding to a molecular weight of 47 500. It would appear that this form of the enzyme consists of 4 subunits which are all of the same molecular weight. Moreover, attempts to concentrate the enzyme beyond 1 mg protein/ml and sedimentation in media not containing detergent result in aggregation to a variety of species including one of molecular weight approximately $1 \cdot 10^6$. Finally, the method described here also yields highly purified side-chain cleavage *P*-450 from the same tissue at the same time.

The heme content of the enzyme is of interest for at least two reasons. Firstly, the final preparation contains one heme group per 3 subunits. Experience with the side-chain cleavage enzyme suggests that there is one heme group per protein subunit and that there is only one species of protein subunit (manuscript in preparation). Moreover, treatment of this enzyme with cholate removes heme (unpublished observation). The 11β-hydroxylase is more difficult to release from membranes and the use of relatively high concentration of two detergents is likely to remove much of the heme. Since it appears that only one species of subunit can be identified in the 11β-hydroxylase by SDS gels, it would not be surprising to find that there is one heme group per subunit in the enzyme as it occurs in mitochondria. Addition of exogenous heme did not alter the enzymatic activity of the present *P*-450.

The second point of interest concerning the heme content of the enzyme is that all the heme measured as pyridine hemochromogen can be accounted for by CO-difference spectroscopy using a value for the molar extinction coefficient of 91 000 $M^{-1} \cdot cm^{-1}$. Evidently no contaminating non-*P*-450 heme proteins are present in the preparation.

The most important finding to emerge from these studies is the evidence suggesting that the two hydroxylase activities occur in a single enzyme. Firstly, the ratio of the two activities in the starting material and the final preparation are

relatively close (16.4 and 11.2 respectively). Furthermore, there is no consistent change in this ratio through the purification procedure to suggest that one or the other activity is being removed (Table II). It should be pointed out that the state of the enzyme is different at these various steps with respect to such factors as solubility and amounts of two detergents in the preparation, so that some variation in the ratio of measured activities is understandable. Secondly, although 30% ammonium sulfate precipitates only half of the total enzyme activity, the ratio 11β-/18-hydroxylase is very similar in both ammonium sulfate fractions (precipitate and supernate) (Table I). Thirdly, the protein appears as a single peak on sedimentation in glycerol density gradients and the ratio of the two activities is the same in the peak fractions. Fourthly, there appears to be only one species of subunit on electrophoresis in polyacrylamide gel (Fig. 2). This would suggest that a single enzyme is present although further studies will be necessary to determine conclusively that the subunits are all identical. The idea that the two activities are associated with a single protein is in keeping with evidence from two other laboratories using different approaches [17,18].

It is also interesting to notice that the present preparation catalyzes 18-hydroxylation of corticosterone rather poorly compared to activity with deoxycorticosterone in spite of the fact that corticosterone is generally believed to be the preferred substrate for 18-hydroxylation in vivo at least in some species [19]. This observation confirms an earlier report [5] and may be explained by the presence of more than one enzyme. The presence of large amounts of 18-hydroxydeoxycorticosterone in the zona fasiculata of the rat adrenal would be consistent with the presence of two different enzymes in glomerulosa and fasiculata. This possibility cannot be excluded by the present studies especially since the enzyme is unstable and significant amounts of enzyme of low specific activity with respect to 11β- and 18-hydroxylase are discarded during purification (see Experimental Procedure). We have not so far been successful in purifying the discarded material and in any case our attempts to separate the glomerulosa from the fasiculata-reticularis by dissection of the cortex with a view to purifying the enzymes from each fraction seem at this stage more likely to succeed (unpublished observations).

The worst difficulty experienced during investigation of this enzyme is the instability seen when it is released from membranes; the enzyme activity is stable at 4°C in sonicated mitochondria. Work is in progress to stabilize the enzyme by re-introducing it into a membrane. Attempts are also being made in this laboratory to demonstrate conclusively whether or not the two enzymatic activities seen with the present enzyme are both attributable to a single species of protein subunit.

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