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P-450$_{11\beta}$-DEPENDENT CONVERSION OF CORTISOL TO CORTISONE, AND 19-HYDROXYANDROSTENEDIONE TO 19-OXOANDROSTENEDIONE

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SUMMARY: Purified bovine adrenal P-450$_{11\beta}$ has been shown to catalyze conversions of cortisol to cortisone (11-oxidase activity), and 19-hydroxyandrostenedione to 19-oxoandrostenedione (19-oxidase activity), in the reconstituted system consisting of NADPH, NADPH:adrenodoxin reductase, and adrenodoxin. The turnover numbers (mol of product formed/min/mol of P-450) were 1.2 for the 11-oxidase activity and 1.4 for the 19-oxidase activity. No reactions took place when any one of the electron-donating components were omitted either in the presence or in the absence of added $NADP^+$. Likewise, rabbit antibody prepared against P-450$_{11\beta}$ immunoprecipitated the 11-oxidase activity with concomitant loss of deoxycorticosterone 11β-hydroxylase activity.

Following the demonstration by this laboratory that purified P-450$_{11\beta}$ obtained from mitochondria of adrenal cortex catalyzes hydroxylation of corticosteroids at the 11β- and 18-positions [1, 2], and androgens at the 11β- and 19-positions [2], recent research by Yamano and his group has revealed that conversions of 18-hydroxycorticosterone to aldosterone [3], and 19-hydroxydeoxycorticosterone to 19-oxodeoxycorticosterone [4] are also catalyzed by the same P-450. In the present work we have investigated the activities of P-450$_{11\beta}$ on cortisol and 19-hydroxyandrostenedione in order to obtain additional information concerning the reactivity of P-450$_{11\beta}$ and its role on the steroid metabolism in adrenocortical mitochondria.

MATERIALS AND METHODS

Materials: The following were perchased: corticosterone, deoxycorticosterone, DLPC, 19-oxoandrostenedione and progesterone, from Sigma; cortisol and cortisone, from Ikapharm; 19-norandrostenedione, from Steraloids; 19-hydroxyand-

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Abbreviations: DLPC, dilauroylphosphatidylcholine; GC-MS, combined gas chromatography-mass spectrometry; and HPLC, high performance liquid chromatography.

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rostenedione, from Makor Chemicals; and prednisone, from Sanko Junyaku. Cortisol was further purified before use by HPLC in a system as described below under analytical procedures. Organic solvents were HPLC grade and all other chemicals used were of the best commercially available grade.

Preparative procedures: P-450$_{11\beta}$ was purified from mitochondria of bovine adrenal cortex according to the previously published method [5]. Purified adrenodoxin reductase [6] and crystalline adrenodoxin [7] were prepared also from bovine adrenal cortex. Rabbit antibody against P-450$_{11\beta}$ was the preparation as described previously [5].

Analytical procedures: The basal buffer used for all of the assay was 30 mM potassium phosphate, pH 7.4. The 11β-hydroxylase activity of P-450$_{11\beta}$ was assayed by the method as previously described [2]. For measurement of P-450$_{11\beta}$-dependent conversions of cortisol and 19-hydroxyandrostenedione, the reaction mixture contained, in 0.3 ml of the basal buffer, 69 nmol of cortisol or 100 nmol of 19-hydroxyandrostenedione, 120 nmol of NADPH, 0.3 unit of NADPH:adrenodoxin reductase, 1.2 nmol of adrenodoxin and 0.2% Tween 20. After 2-min preincubation of the mixture at 37°C, the reaction was started with the addition of a quantity of P-450$_{11\beta}$, and the medium was incubated for an additional 2 min at 37°C. At the end of the incubation, the reaction was terminated by the addition of 1.6 ml of chloroform:methanol (1:1, v/v). After 1 nmol of authentic prednisone or progesterone was added as an internal standard, the mixture was extracted three times with 0.8 ml of chloroform each time. The extracts were combined and the solvent was evaporated under a tream of N_2 at a temperature of 60°C or lower. In case in which cortisol was the substrate, the products were extracted as follows: The residue was dissolved in methanol:water (5:95, v/v), and treated with a Sep-Pak$_{C18}$ cartridge (Waters Associates), using 4 ml of methanol:water (60:40, v/v) as the eluting solvent. The methanol was evaporated and the aqueous residue was extracted three times with 0.5 ml of ethylacetate. The combined solvent was evaporated again, the residue dissolved in 0.1 ml of methanol:water (50:50, v/v) and an aliquot was injected into a Chemcosorb 5-ODS-H (Chemco) column (2.1 × 150 mm), attached with a precolumn (Chemcosorb 5-ODS-H, 1.46 × 30 mm) and a prefilter (Irika Kogyo, 9917-03) in a Hitachi HPLC system, model 655A-12. The C_{21}-steroids were separated with methanol:water (50:50, v/v), and the C_{19}-steroids with acetonitrile:water (50:50, v/v) both at a flow rate of 0.2 ml/min. The elution was monitored at 240 nm with a Hitachi 655A-23 semi-micro UV monitor. The retention times for standard prednisone, cortisone and cortisol were 22.0 min, 24.5 min, and 30.5 min, respectively.

In order to obtain a sufficient quantity of the product for GC-MS analysis, the main HPLC fractions from several experiments were combined. The product from cortisol as well as authentic cortisone was further converted to their bismethylenedioxy derivatives according to the mthod of Kirschner and Fales [8] prior to be subjected to analysis. The GC-MS was carried out on a Jeol MS-GCG Gas Chromatograph-06/JMX-DX 300 Mass Spectrometer/JMA-DA-5000 Mass Data System. The column (2.5 mm × 1 m) was packed with Gaschrome Q (80-100 mesh) coated with Silicone OV-1 (1%). The column temperature was 250°C, injection temperature 280°C, electron energy 70 eV, accelerating voltage 3 kV, and ionizing current 300 μA.

Total protein was determined by micro-biuret method of Gornall et al. [9] using crystalline bovine serum albumin (Sigma) as the standard.

RESULTS AND DISCUSSION

In order to study the reactivity of P-450$_{11\beta}$ on various steroids, we examined as substrates cortisol and 19-hydroxyandrostenedione, the products of P-450$_{11\beta}$-dependent 11β- and 19-hydroxylase reactions, respectively [1, 2]. After cortisol was allowed to react in a reconstituted P-450$_{11\beta}$-monooxygenase system,

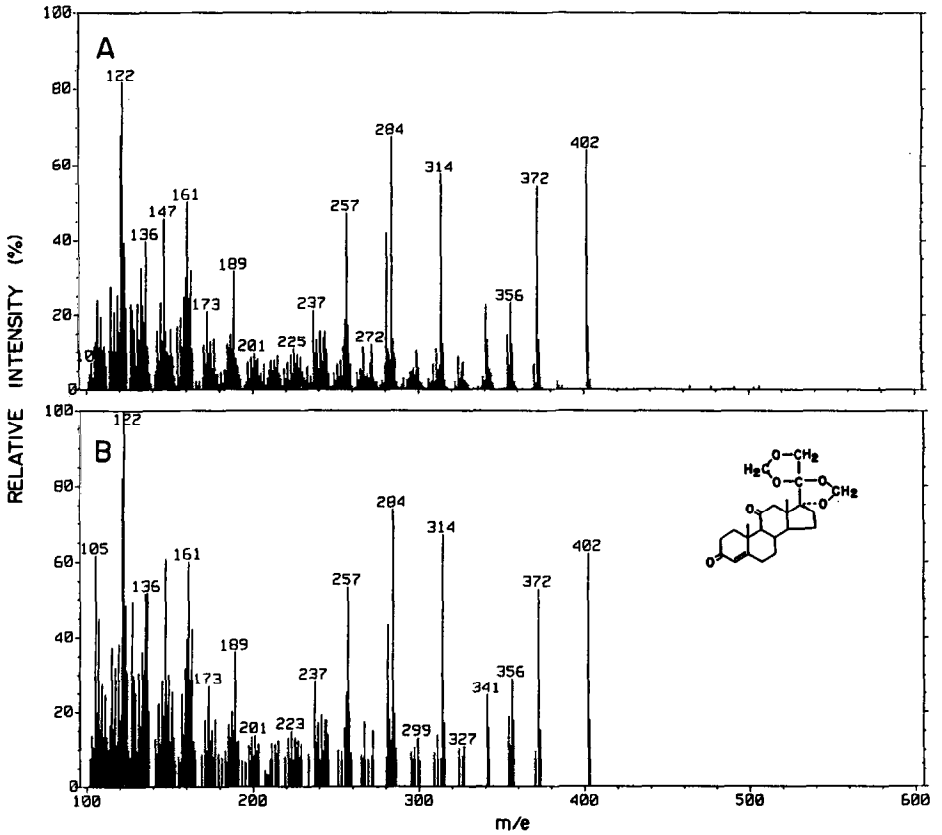

Fig. 1. GC-MS analyses of the bismethylenedioxy derivatives of an 11-oxidase product (A) and authentic cortisone (B). Conditions are as described under MATERIALS AND METHODS.

the products were extracted and analyzed on HPLC. The elution profile revealed several bands with respective retention times of 13.0 min, 14.8 min, 16.5 min, and 24.5 min. Since it was found that the major peak, the 24.5-min fraction, corresponded to that of the authentic cortisone on the HPLC profile, the identity of the reaction product was further confirmed by GC-MS in a form of bismethylenedioxy derivatives (Fig. 1, A and B). Similar experiment with 19-hydroxyandrostenedione in place of cortisol as the substrate indicated conversion of 19-oxoandrostenedione with a single HPLC peak of absorption at 6.0 min. No significant presence of minor peaks was detected in this case. A comparative GC-MS for the authentic 19-oxoandrostenedione was shown in Fig. 2, A and B. The results in Table I show that both of these reactions, referred to as "11-oxidase" and "19-oxidase" reactions, respectively, absolutely require the presence of all of the components of the monooxygenase system, which are also essential for the P-450$_{11\beta}$-

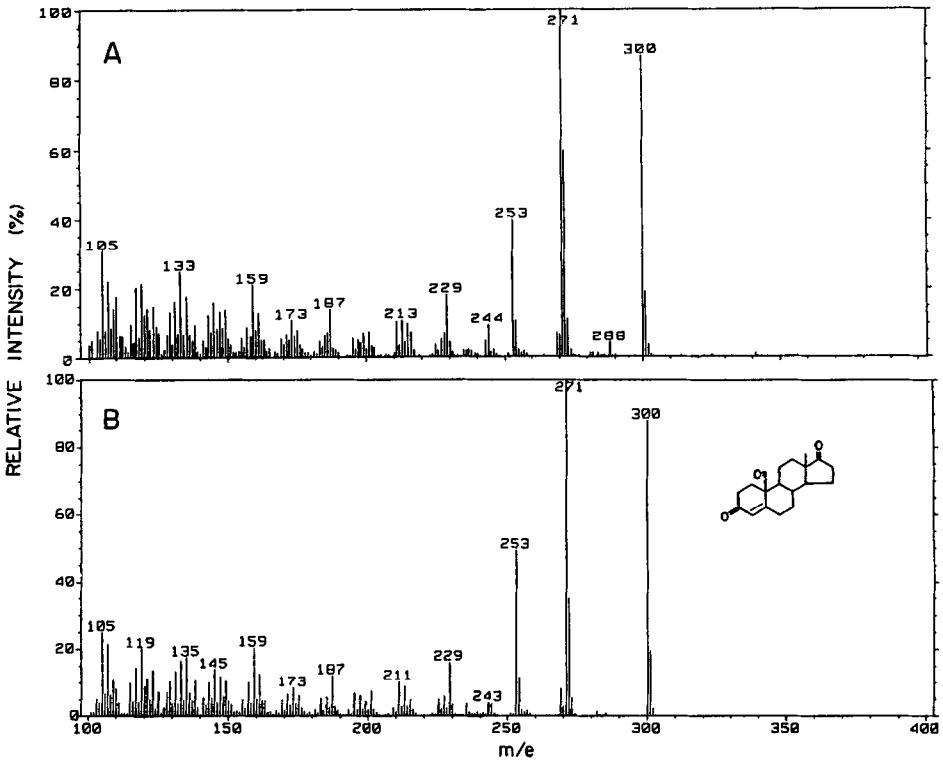

Fig. 2. GC-MS analyses of the 19-oxidase product (A) and authentic 19-oxo-androstenedione (B). Conditions are as described under MATERIALS AND METHODS.

Table I. Effect of various factors on the P-450$_{11\beta}$-dependent conversion of cortisol to cortisone and 19-hydroxyandrostenedione to 19-oxoandrostenedione

Omittion	Relative activity (%)	
	11-oxidase[1]	19-oxidase[2]
None	100	100
Incubation[3]	1	2
P-450$_{11\beta}$	0	1
Adrenodoxin reductase	0	1
Adrenodoxin	1	2
NADPH	0	3
Substrate	0	0

The conditions are as indicated under MATERIALS AND METHODS except otherwise specified below. 1: The substrate was 69 nmol of cortisol and the concentration of P-450$_{11\beta}$ was 380 pmol. 2: The substrate was 100 nmol of 19-hydroxyandrostenedione and the concentration of P-450$_{11\beta}$ was 600 pmol. 3: The reaction was run similarly but without P-450$_{11\beta}$, which was added immediately after the reaction was terminated by chloroform:methanol.

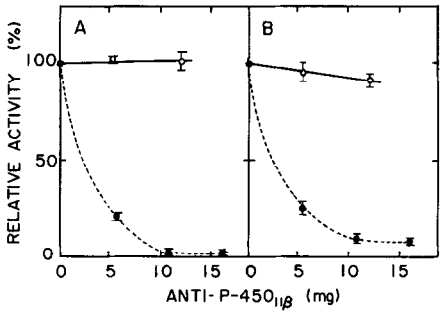

Fig. 3. Immunotitration of the P-450$_{11\beta}$-dependent 11β-hydroxylase activity and 11-oxidase activity. P-450$_{11\beta}$ (800 pmol) and indicated amounts of anti-P-450$_{11\beta}$ (•) or control globulin (o) in 500 µl of the standard buffer supplemented with 10 µM deoxycorticosterone were incubated in an ice bath for 6.5 hours. After centrifugation of the mixture, samples corresponding to 32 pmol and 320 pmol of P-450 were assayed for deoxycorticosterone 11β-hydroxylase activity (A) and for cortisol 11-oxidase activity (B), respectively, under conditions as described in MATERIALS AND METHODS.

dependent hydroxylase reactions such as hydroxylations of various steroids at the 11β-, 18-, and 19-positions [2]. The fact that $NADP^+$ in place of NADPH had no effect on the 11-oxidase activity rules out any contamination of 11β-hydroxysteroid dehydrogenase activity [10] in the present system. Kinetic experiments (data not shown) indicated that apparent Km values of the 11-oxidase reaction and 19-oxidase reaction for cortisol and 19-hydroxyandrostenedione were 170 µM and 190 µM, and V values 2.7 and 2.0 mol/min/mol of P-450$_{11\beta}$, respectively. For comparison an apparent Km and turnover number of P-450$_{11\beta}$ for 18-hydroxycorticosterone are reported to be 325 µM and 5.3 mol/min/mol of P-450 [3]. It is also reported that phospholipids stimulate the rate of P-450$_{11\beta}$-dependent formation of aldosterone and 18-hydroxycorticosterone from corticosterone [11]. However, in our system, varying concentrations of sonicated DLPC, up to 100 µM, caused no stimulating effects on the rate of either 11-oxidase or 19-oxidase reaction.

In order to examine further the cortisol 11-oxidase activity might specifically be immunoprecipitated by the antibody against P-450$_{11\beta}$ the present preparation of P-450$_{11\beta}$ was incubated in the presence of varying concentrations of anti-P-450$_{11\beta}$ (Fig. 3). As expected, anti-P-450$_{11\beta}$ parallely immunoprecipitated the deoxycorticosterone 11β-hydroxylase activity and cortisol 11-oxidase activity.

It is well known that not only liver microsomes [12] but also adrenocortical microsomes [10] contain a 11β-hydroxysteroid-dehydrogenase which catalyzes inter-

conversion between cortisol and cortisone. Thus, the results presented in this report provide an alternative mechanism of cortisone formation, which is catalyzed by P-450$_{11\beta}$-dependent systems in mitochondria. Although exact role of such oxidase reactions in physiology is presently uncertain, there is a good reason to assume that P-450$_{11\beta}$ does convert part of the nascent cortisol to cortisone prior to its circulation, since the substrate of the oxidase reaction, cortisol, is produced also in mitochondria and by the same P-450.

Our interest in a C_{19}-steroid 19-oxidase activity of P-450$_{11\beta}$ is also derived from the known fact that a 19-hydroxysteroid is involved in aromatization of androgens [13]. However, attempts to detect C_{18}-steroids such as 19-norandrostenedione and estrone in the metabolites of 19-hydroxyandrostenedione in the reconstituted P-450$_{11\beta}$ system were negative.

We have already shown that P-450$_{sccII}$ (P-450$_{17\alpha}$), which is a steroidogenic P-450 in microsomes that functions as a 17α-hydroxylase/17-20 lyase, is also capable of catalyzing oxidation of 17β-hydroxy C_{19}- and C_{18}-(aromatic) steroids, such as testosterone and estradiol, to form corresponding 17-ketosteroids [14]. Although such oxidase activities of P-450 are not unique in liver microsomes [15-17], our studies provide evidence that steroidogenic P-450, either in mitochondria or in microsomes, acts as a steroid oxidase which might have physiological significance.

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