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ALDOSTERONE BIOSYNTHESIS BY A RECONSTITUTED CYTOCHROME P-450$_{11\beta}$ SYSTEM

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[^{3}H]Corticosterone was incubated with cytochrome P-450$_{11\beta}$ purified to electrophoretic homogeneity from bovine adrenocortical mitochondria, and the reaction products were analyzed by high performance liquid chromatography. The production of aldosterone (21.2 pmol/nmol P-450/min) and 18-hydroxycorticosterone (1.17 nmol/nmol P-450/min) was observed. When lipidic extracts from mitochondria of bovine adrenocortical zona glomerulosa were added to the reaction mixture, the rate of production of aldosterone was increased 28-fold. When [^{3}H]18-hydroxycorticosterone was incubated with cytochrome P-450$_{11\beta}$, the amount of aldosterone produced was 55.7 pmol/nmol P-450/min in the absence of the lipidic extracts and the enhancing effect of the lipidic extracts was 4-fold.

Aldosterone is one of the adrenocortical hormones and possesses the most potent mineralocorticoid activity. Although a number of studies (1-3) have been undertaken on biosynthesis of aldosterone, biochemical characteristics of the final step are not clear. 18-Hydroxycorticosterone seems to be a reasonable candidate for a direct precursor of aldosterone in the biosynthetic pathway. Kojima et al.(4) recently studied the formation of aldosterone from 18-hydroxycorticosterone by bovine adrenocortical mitochondria. They suggested that cytochrome P-450 is involved in the reaction. Some other recent studies (3,5-7) have also shown that the biosynthetic pathway of aldosterone from corticosterone involves steps catalyzed by cytochromes P-450 of adrenocortical mitochondria.

Cytochrome P-450$_{11\beta}$ present in adrenocortical mitochondria is known to catalyze both 11β- and 18-hydroxylations of 11-deoxycorticosterone, both 11β- and 19-hydroxylations of 18-hydroxy-11-deoxycorticosterone, and 18-hydroxylation of corticosterone (8-11). Recognition of the versatile catalytic activity of this cytochrome, especially the 18-hydroxylation of corticosterone, lead us to examine the involvement of this cytochrome in the late pathway of

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aldosterone biosynthesis. In the present communication, we report that cytochrome P-450$_{11\beta}$ catalyzes the formation of aldosterone from corticosterone or 18-hydroxycorticosterone, and that lipidic extracts from mitochondria of adrenal zona glomerulosa enhance this reaction.

MATERIALS AND METHODS

Chemicals Corticosterone was obtained from Sigma. Aldosterone and 18-hydroxycorticosterone were purchased from Makor Chemicals. 18-Hydroxy-11-dehydrocorticosterone was a generous gift from Drs. F. Mitani and Y. Ishimura of Keio University, Tokyo. [1,2,6,7-^{3}H]Corticosterone and [1,2-^{3}H]18-hydroxycorticosterone were purchased from Amersham. [4-^{14}C]Aldosterone was obtained from New England Nuclear.

Enzymes Purification of cytochrome P-450$_{11\beta}$ was performed according to Suhara et al.(12) except that an octyl-Sepharose column was used instead of an aniline-Sepharose column. The purified protein showed a single band on SDS-polyacrylamide gel electrophoresis and the specific content of cytochrome P-450 was 13.2 nmol/mg protein. Adrenodoxin reductase was purified from bovine adrenocortical mitochondria according to the method described by Sugiyama et al.(13). Purification of adrenodoxin was performed as described by Suhara et al.(14).

Lipidic extracts Fresh bovine adrenal glands were obtained from a slaughterhouse. After fat was removed from the glands, the adrenals were bisected and capsular and outer portions (zona glomerulosa) of the adrenal cortex were separated from the inner cortex by the method described by Yagi et al.(15). Then the glomerulosa tissues were homogenized in 0.25 M sucrose solution containing 40 mM Tris-HCl buffer, pH 7.4, and the mitochondrial fraction was prepared by differential centrifugation at 600 x g and 8000 x g. The mitochondrial pellets containing 16 mg protein were suspended in 1 ml of the same buffer. Total lipids were extracted as described by Bligh and Dyer (16). The lipidic extracts were dissolved in 500 µl chloroform and stored at -20 °C. The extracts contained 11.6 µmol phosphorus/ml after perchloric acid treatment.

Enzyme assay Aldosterone synthetic activity was assayed as follows. ^{3}H-Labeled steroids and unlabeled steroids were dissolved in ethanol and placed in incubation tubes. In some tubes, a chloroform solution of the lipidic extracts from mitochondria was added. The organic solution was evaporated to dryness under a nitrogen flow and 20 µl of propyleneglycol was added per tube before incubation. The incubation mixture, in a final volume of 1 ml of 30 mM Tris-HCl buffer (pH 7.4), consisted of 200 nmol (2 µCi) radioactive steroid, 0.86 nmol cytochrome P-450$_{11\beta}$, 16 nmol adrenodoxin, 5 nmol adrenodoxin reductase, 10 µmol glucose-6-phosphate, 0.5 units glucose-6-phosphate dehydrogenase and 4 µmol $MgCl_2$ with, or without, the lipidic extracts. The reaction was started by adding 100 nmol NADPH. As a control, a reaction mixture without cytochrome P-450$_{11\beta}$ was incubated in the same way. The reaction was carried out at 37 °C for 10 min and terminated by adding 1 ml ice-cold methanol. Immediately after the reaction, 0.002 µCi [^{14}C]aldosterone was added to each incubation tube as an internal standard. The steroids were extracted with 2 ml dichloromethane three times. The extract was pretreated with a Sep-Pak cartridge (Waters Associates). The steroid extract was dissolved in 60% methanol and injected into a 300 x 4 mm Chemcosorb ODS (5µm) column in a Hitachi HPLC system, model 635S. The steroids were separated on the column with 60% methanol in water at a flow rate of 0.3 ml/min, and the eluate was monitored for the absorbance at 254 nm. The retention times for standard steroids were 23.8 min for aldosterone, 27.8 min for 18-hydroxycorticosterone and 49.6 min for corticosterone, respectively. Fractions of 0.15 ml each were collected for measurement of radioactivity. The radioactivity was determined in 5 ml ACS II (Amersham) for both ^{3}H- and ^{14}C-activity using an Aloka liquid scintillation counter, model 703.

RESULTS

[^{3}H]Corticosterone was incubated under aerobic conditions with the purified cytochrome P-450$_{11\beta}$ in the presence of adrenodoxin reductase, adrenodoxin and an NADPH-generating system. The reaction products were extracted and subjected to HPLC analysis on a reversed phase column. Fig.1 illustrates the distribution of radioactivity on the chromatogram. As shown in Fig.1B, a small ^{3}H-radioactive peak appeared at 23.8 min after injection. The retention time of the peak coincided with that of [^{14}C]aldosterone which had been added as an internal standard. A big radioactive peak at 27.8 min corresponded to [^{3}H]18-hydroxycorticosterone, an expected product from corticosterone through cytochrome P-450$_{11\beta}$-catalyzed 18-hydroxylation. That neither aldosterone nor 18-hydroxycorticosterone was produced without cytochrome P-450$_{11\beta}$ is shown in Fig.1A.

To confirm the identification of the peak at 23.8 min as that of aldosterone, unlabeled corticosterone was incubated with cytochrome P-450$_{11\beta}$ and the steroid extracts were chromatographed in the same way. The fractions of the UV-peak at 23.8 min were collected and the sample was trimethylsilyl-

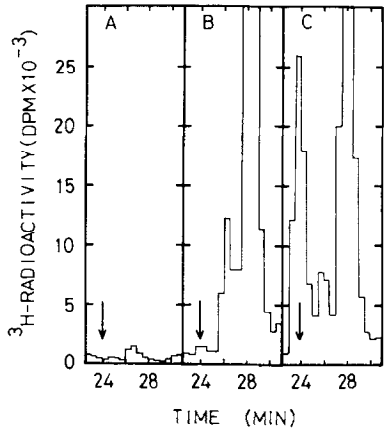

Fig.1 Radiochromatograms of reaction products of [^{3}H]corticosterone with cytochrome P-450$_{11\beta}$. The steroids were separated by HPLC as described under Materials and Methods. (A) Without cytochrome P-450$_{11\beta}$; (B) Cytochrome P-450$_{11\beta}$, 0.86 nmol; (C) Cytochrome P-450$_{11\beta}$, 0.86 nmol plus 100 µl of the lipidic extracts containing 11.6 µmol phosphorus/ml. Arrows in the figure indicate the points of elution of aldosterone which were determined by counting ^{14}C-radioactivity of aldosterone as an internal standard. A peak of substrate, [^{3}H]corticosterone, appeared at 49.6 min, which is not shown in the figure.

ated with N,O-bis-(trimethylsilyl)-trifluoroacetamide-pyridine (1:1) as described by Horning and Maume (17). The derivative was analyzed with a gas chromatography-mass spectrometry system. The mass spectrum of the sample was identical with that of the trimethylsilylated derivative of aldosterone (data not shown).

Raman et al.(2) have reported that 18-hydroxy-11-dehydrocorticosterone was produced from corticosterone by adrenocortical mitochondria and it showed mobilities identical with those of aldosterone in various paper chromatography systems. Authentic 18-hydroxy-11-dehydrocorticosterone was chromatographed with our HPLC system. It was eluted at 20.8 min after injection, indicating that our HPLC system clearly separates aldosterone from 18-hydroxy-11-dehydrocorticosterone.

These results suggest that cytochrome P-$450_{11\beta}$ catalyzes the conversion of corticosterone to aldosterone. However, the rate of production of aldosterone (21.2 pmol/nmol P-450/min) was so low compared to the rate of production of 18-hydroxycorticosterone (1.17 nmol/nmol P-450/min) that a physiological role of the former reaction might be questioned. Therefore we attempted to find a factor stimulating the production of aldosterone by cytochrome P-$450_{11\beta}$. Total lipids were extracted from mitochondrial pellets of zona glomerulosa of bovine adrenocortex. The lipidic extracts were added to the reaction mixture of [^{3}H]corticosterone with cytochrome P-$450_{11\beta}$, and the products were examined by HPLC. As shown in Fig.1C, remarkable enhancement of the production of aldosterone in the presence of the lipidic extracts was observed. Table 1 lists the amounts of steroids produced from corticosterone by cytochrome P-$450_{11\beta}$. The lipidic extracts (1.0 μmol phosphorus) enhanced the production of aldosterone from corticosterone about 28-fold, but not that of 18-hydroxycorticosterone. The ratio of the production of aldosterone to the production of 18-hydroxycorticosterone was increased 35-fold.

To further elucidate the cytochrome P-$450_{11\beta}$-catalyzed production of aldosterone, [^{3}H]18-hydroxycorticosterone was incubated with the purified cytochrome P-$450_{11\beta}$. Fig.2 shows radiochromatograms of the products.

TABLE 1. Effect of mitochondrial extracts on production of aldosterone from corticosterone by purified cytochrome P-450$_{11\beta}$

Extracts (µl)	Products	
	18-Hydroxycorticosterone (nmol/nmol P-450/min)	Aldosterone (pmol/nmol P-450/min)
0	1.55 (1.0)	20.6 (1.0)
50	1.27 (0.82)	429 (21)
100	1.23 (0.79)	568 (28)

Corticosterone (100 nmol) was incubated with purified cytochrome P-450$_{11\beta}$ in the presence of adrenodoxin and adrenodoxin reductase and an NADPH generating system. Lipidic extracts from adrenocortical mitochondria (10.1 µmol phosphorus/ml) were added as indicated. The reaction products were extracted and subjected to reversed phase HPLC analysis. The absorbance at 254 nm was recorded and the amounts of products were calculated from the sums of peak heights. The numbers in parentheses are the ratios of the amounts with the lipidic extracts to those without the extracts.

[^{3}H]Aldosterone was eluted as a single radioactive peak (Fig.2B), and the enhancing effect of the lipidic extracts on the aldosterone production was also observed (Fig.2C). Aldosterone was produced from 18-hydroxycorticosterone by cytochrome P-450$_{11\beta}$ in a somewhat greater amount (55.7 pmol) compared to that produced from corticosterone (20.6 pmol) under these conditions. On the

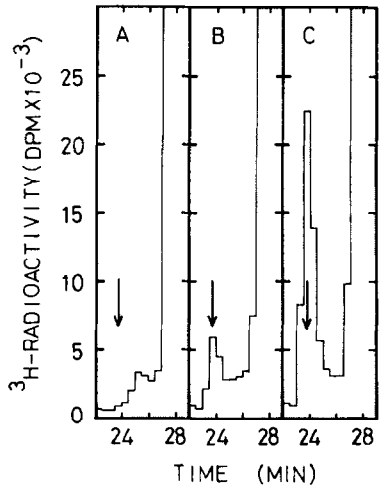

Fig.2 Radiochromatograms of reaction products of [^{3}H]18-hydroxycorticosterone with cytochrome P-450$_{11\beta}$. (A) Without cytochrome P-450$_{11\beta}$; (B) Cytochrome P-450$_{11\beta}$, 0.86 nmol; (C) Cytochrome P-450$_{11\beta}$, 0.86 nmol plus 100 µl of the lipidic extracts. For other details, see the legend to Fig.1 and Materials and Methods.

contrary, the enhancing effect of the lipidic extracts was smaller compared to the production from corticosterone (about 4-fold compared to 28-fold).

DISCUSSION

The results presented in this communication suggest that 18-hydroxycorticosterone is one of the substrates of cytochrome P-$450_{11\beta}$ and that aldosterone is produced from corticosterone via 18-hydroxycorticosterone by cytochrome P-$450_{11\beta}$. The conversion of corticosterone to aldosterone may be possible through two consecutive steps of 18-hydroxylation on the same cytochrome P-$450_{11\beta}$. To our knowledge, this is the first report which clearly shows that cytochrome P-$450_{11\beta}$, in a reconstituted system, catalyzes the production of aldosterone at a physiologically reasonable rate.

Another finding reported in this communication is the remarkable enhancing effect of the lipidic extracts on the aldosterone production catalyzed by cytochrome P-$450_{11\beta}$. Reaction rates of various membrane-bound enzymes such as Ca^{2+}-ATPase of sarcoplasmic reticulum (18), mitochondrial β-hydroxybutyrate dehydrogenase (19), microsomal cytochrome P-450_{LM2} (20) and mitochondrial cytochrome P-450_{scc} (21) were reported to be enhanced by phospholipids and detergents. Pember et al.(22) have reported that cardiolipin and other phospholipids stimulate the side-chain cleavage reaction of cholesterol by lowering the Kd of cholesterol for cytochrome P-450_{scc}. In our experiment, the enhancement by the lipidic extracts may be due to a kinetical change or stabilization of the cytochrome P-$450_{11\beta}$. The chemical nature of the factors involved in the lipidic extracts and effective in enhancing the activity is extremely interesting. We are currently investigating this aspect.

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