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# ADRENAL $P$-450$_{scc}$ CATALYZES DEOXYCORTICOSTERONE 6$\beta$-HYDROXYLASE REACTION

H. MOCHIZUKI, K. SUHARA* and M. KATAGIRI

Department of Chemistry, Faculty of Science, Kanazawa University, Marunouchi 1-1, Kanazawa 920, Japan

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**Summary**—Purified bovine $P$-450$_{scc}$, the cholesterol side-chain cleaving $P$-450 in adrenal cortex mitochondria, was found to catalyze a deoxycorticosterone 6$\beta$-hydroxylase reaction. A turnover number (moles of product formed/min/mol of $P$-450) of 12 was found similar to that for cholesterol side chain cleavage activity. Conversion was dose-dependent in terms of $P$-450$_{scc}$ and no reaction took place when any one of the required electron donating components such as NADPH, NADPH–adrenodoxin reductase, or adrenodoxin was omitted.

These results confirm and extend earlier observations that 21-hydroxypregnenolone is transformed into both deoxycorticosterone and 6$\beta$-hydroxydeoxycorticosterone by incubation of adrenal gland slices.

## INTRODUCTION

$P$-450$_{scc}$ is a mitochondrial heme-thiolate monooxygenase the function of which has generally been considered to be side-chain cleavage of cholesterol to give pregnenolone, the pivotal steroid from which all steroid hormones are produced [1]. Herein, we show that highly purified bovine adrenal $P$-450$_{scc}$ also catalyzes the hydroxylation of deoxycorticosterone at the 6$\beta$-position with activity comparable to its cholesterol side-chain cleavage activity. Therefore, $P$-450$_{scc}$ may play additional roles in the steroid metabolism in the adrenal.

## EXPERIMENTAL

Most of the reagents used in this study have been previously detailed [2]. 6$\beta$-Hydroxydeoxycorticosterone was generously provided by Dr T. Ogishima, Keio University, School of Medicine. All other chemicals were the highest available grade.

$P$-450$_{scc}$ was purified from mitochondria of bovine adrenal cortex according to previously published methods [3]. Hydroxylapatite high-pressure liquid chromatography (HPLC) was as described by Ogishima *et al.* for $P$-450$_{11\beta}$ [4], except that deoxycorticosterone was omitted from the buffer. Purified adrenodoxin reductase [5] and crystalline adrenodoxin [6] were also prepared from bovine adrenal cortex.

*Author to whom correspondence should be addressed.

The basal buffer used for all of the assay was 30 mM potassium phosphate, pH 7.4, and cholesterol side-chain cleavage activity of $P$-450$_{scc}$ was assayed as previously described in a 0.3 ml system [7]. $P$-450$_{scc}$-dependent conversion of steroid was also carried out in the same system, except that Tween 20 was omitted and cholesterol was replaced by 300 nmol of the indicated steroid. Other alterations are as given in the text. At the end of the incubation, the reaction was terminated by the addition of 1.8 ml of chloroform:methanol (1:1, v/v). The organic layer was separated after addition of 1 ml of water and 5 nmol of 19-norandrostenedione, as in internal standard, and extracts were evaporated under a stream of nitrogen gas at 40°C or lower. The residue was then dissolved in 5 $\mu$l of methanol, and injected into a column (Mitsubishi MCI Gel ODS-1HU, 4.6 × 250 mm) with a prefilter (Irika Kogyo, 9917-03) in a Hitachi HPLC system, pump model L-6200 and column oven model 655A-52. Steroids were separated with methanol:water (1:1, v/v) at a flow rate of 0.8 ml/min and a column temperature at 40°C. The absorption was monitored at 240 nm by means of a Hitachi u.v. monitor model L-4200/model D-2500 Chromato-integrator system. The retention times for standard deoxycorticosterone, 6$\beta$-hydroxydeoxycorticosterone, and 19-norandrostenedione were 63.9, 14.7 and 35.9 min, respectively. The amount of 6$\beta$-hydroxydeoxycorticosterone formed was calculated after correction for

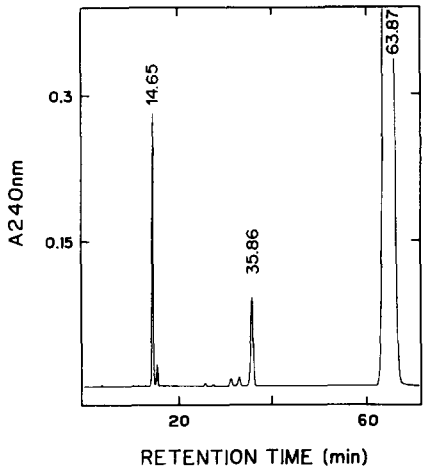


Fig. 1. HPLC elution profile of the reaction product of $P$-450$_{scc}$-dependent conversion of deoxycorticosterone as monitored at 240 nm. After extraction and concentration under $N_2$ gas, steroids were injected into the HPLC column. Other conditions are as described in Experimental.

the recovery of the internal standard, 19-nor-androstenedione.

The combined gas chromatography–mass spectrometry (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 × 1.5 m) was packed with Gaschrome Q (80–100 mesh) coated with Silicone OV-1 (2%). The column temperature was 200–300°C gradient (16°C/min), injection temperature 280°C, and ionizing energy 70 eV.

## RESULTS AND DISCUSSION

For measurement of $P$-450$_{scc}$-dependent conversion of deoxycorticosterone, the reaction was run for 10 min and the extracts subjected to HPLC. Figure 1 shows a typical HPLC profile of the $P$-450$_{scc}$-dependent deoxycorticosterone hydroxylase metabolites. The major peak with a retention time of 14.7 min coincides with that of 6$\beta$-hydroxydeoxycorticosterone, and is quite distinct from that of substrate deoxycorticosterone (retention time: 63.9 min), and of any of the known $P$-450$_{11\beta}$-dependent hydroxylated products of deoxycorticosterone such as corticosterone (retention time: 33.1 min), 18-hydroxy-

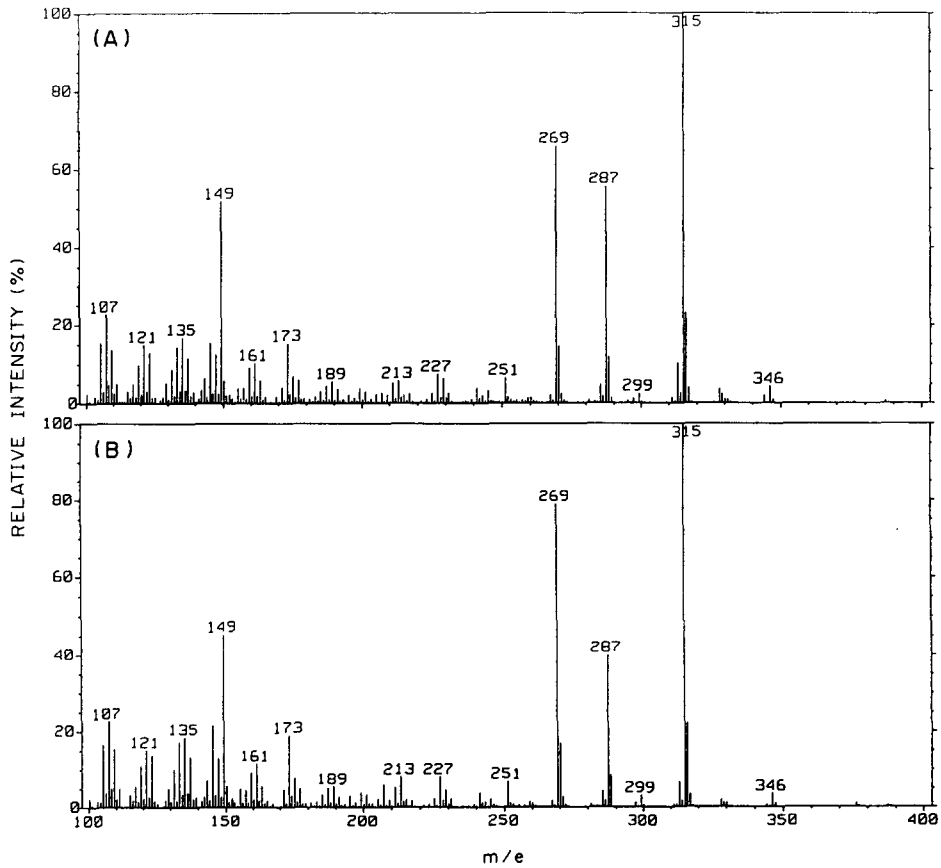


Fig. 2. GC–MS analyses of the $P$-450$_{scc}$-catalyzed product from deoxycorticosterone, with a retention time of 14.7 min on HPLC (A), and of authentic 6$\beta$-hydroxydeoxycorticosterone (B). Conditions are as described in Experimental.

deoxy-corticosterone (retention time: 27.7 min) and 18-hydroxycorticosterone (retention time: 15.6 min). Subsequently, we have definitively identified the 14.7 min product as 6β-hydroxy-deoxycorticosterone by the GC–MS profile which corresponds to that of authentic standard (Fig. 2).

Based on the assumption that the absorption coefficient ($\epsilon$) for the product is $16 \times 10^3$ at 240 nm, the turnover number for the 6β-hydroxylase reaction was calculated to be 12 mol of product formed/min/mol of $P$-450$_{scc}$ (based on 10 experiments) under the standard assay conditions. The hydroxylase reaction was absolutely dependent on each component of the mitochondrial electron donating system i.e. NADPH, NADPH–adrenodoxin reductase and adrenodoxin.

Multiple forms of $P$-450$_{scc}$ in bovine adrenocortical mitochondria have been reported, and no distinct difference has been found in their cholesterol side-chain cleaving activity [8]. We performed HPLC of our purified preparation on a hydroxyl–apatite column. The fractions eluted from the column were collected and assayed for both cholesterol side-chain cleavage and deoxycorticosterone 6β-hydroxylase activities. As Fig. 3 shows, the two activities of $P$-450$_{scc}$ were both detected in the same fractions.

These findings indicate that $P$-450$_{scc}$, the cholesterol side chain cleaving enzyme, is a

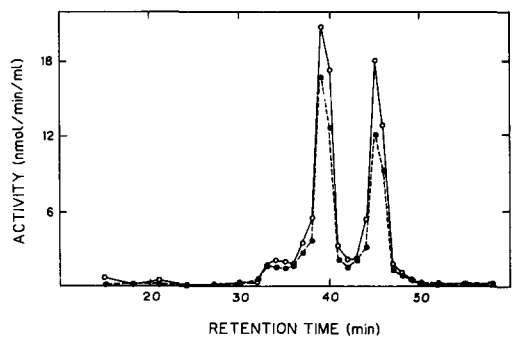


Fig. 3. Comparison between cholesterol side-chain cleavage and deoxycorticosterone 6β-hydroxylase activities of $P$-450$_{scc}$. A purified $P$-450$_{scc}$ preparation was dialyzed against 5 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.3% sodium cholate and 0.2% Tween 20. The dialyzed preparation (10 nmol) was applied to an HA-1000 hydroxylapatite column (7.5 × 75 mm, Tosoh) equilibrated with the same buffer and eluted with the same buffer for 15 min, followed by with a linear gradient of phosphate from 5 to 260 mM over the next 60 min. The flow rate was 0.5 ml/min and fractions were assayed for cholesterol side-chain cleaving activity (—○—) and for deoxycorticosterone 6β-hydroxylase activity (—●—) as described in Experimental.

deoxycorticosterone 6β-hydroxylase. $P$-450$_{scc}$ also converted 11-deoxycortisol and progesterone to metabolites which had the same retention times on HPLC as authentic 6β-hydroxysteroids. However, the estimated turnover number for production of progesterone was less than 1 mol/min/mol of $P$-450 if significant (based on two experiments, data not shown). No equivalent peak was seen when corticosterone and cortisol were used as substrates, suggesting that deoxycorticosterone is the most favored 6β-hydroxylase substrate of $P$-450$_{scc}$.

In the liver, the 6β-hydroxylase reaction is the major route of $P$-450-dependent oxidation in the metabolism of many steroids. These reactions occur in microsomes and various forms of steroids including testosterone, androstenedione, and progesterone are equally good substrates [9]. Previously, we have shown that $P$-450$_{17\alpha}$ ($P$-450$_{sccII}$) from pig testis microsomes, which acted as a steroid 17α-hydroxylase/17–20 lyase, is also capable of catalyzing hydroxylation of various xenobiotics [10]. In the present study, we have examined the 6β-hydroxylase activity of $P$-450$_{17\alpha}$ on deoxycorticosterone. As expected, $P$-450$_{17\alpha}$ catalyzes formation from deoxycorticosterone of a compound with the same retention time as 6β-hydroxydeoxycorticosterone on HPLC. However, the rate of reaction was 2 orders of magnitude lower than the same reaction catalyzed by $P$-450$_{scc}$ (from 2 experiments, data not shown).

3-Oxo-4-ene-steroids such as cholestenone are substrates for $P$-450$_{scc}$ [11, 12]. However, the reaction catalyzed in this case is side chain cleavage of a $C_{27}$-steroid to form the corresponding $C_{21}$-product, progesterone. This is the first demonstration that $P$-450$_{scc}$ catalyzes 6β-hydroxylation of deoxycorticosterone, although the significance of this activity is still unknown. Our findings explain the previously noted formation of 6β-hydroxy-deoxycorticosterone from deoxycorticosterone in rat and rabbit adrenal slices [13], and provide further evidence for possible physiological roles of $P$-450$_{scc}$ in the adrenal cortex. More detailed studies on the 6β-hydroxylase activity of $P$-450$_{scc}$ are under way.

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