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STEROID 6β-HYDROXYLASE AND 6-DESATURASE REACTIONS CATALYZED BY ADRENOCORTICAL MITOCHONDRIAL *P*-450

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Summary—The minor steroid hydroxylase activity of purified bovine adrenocortical mitochondrial *P*-450 is described. The results indicate that both *P*-450$_{scc}$ and *P*-450$_{11\beta}$ act on deoxycorticosterone and androstenedione to form 6β-hydroxydeoxycorticosterone and 6β-hydroxyandrostenedione (6β-hydroxylase), respectively. Both forms of *P*-450 also catalyze 6-desaturation of androstenedione to form 4,6-androstadiene-3,17-dione (6-desaturase).

INTRODUCTION

Our recent examination of the catalytic properties of bovine *P*-450$_{scc}$, the cholesterol side-chain cleaving *P*-450 in adrenocortical mitochondria [1], indicated that *P*-450$_{scc}$ also acts on deoxycorticosterone to form 6β-hydroxydeoxycorticosterone with a turnover number comparable with that for the cholesterol side-chain cleavage reaction, although its affinity for the substrate is lower [2]. On examining the catalytic activity of bovine *P*-450$_{11\beta}$ with 19-norandrostenedione, a C_{18}-steroid, we also found that this C_{18}-steroid was converted to 6β-hydroxy-19-norandrostenedione, in addition to its conversion to the major products, 11β- and 18-hydroxy-derivatives [3].

In this paper we report the versatile steroid monooxygenase activity of *P*-450$_{scc}$ and *P*-450$_{11\beta}$. The results indicate that both *P*-450$_{scc}$ and *P*-450$_{11\beta}$ show 6β-hydroxylase activity with C_{21}- and C_{19}-steroids, and that, in addition, they catalyze the androstenedione 6-desaturase reaction, which forms 4,6-androstadiene-3,17-dione.

EXPERIMENTAL

Most of the reagents and preparations used in this study have been described previously [2, 4]. 6β-Hydroxydeoxycorticosterone was generously provided by Dr T. Ogishima of the Keio University School of Medicine. Other 6β-hydroxylated steroids and 4,6-androstadiene-3,17-dione were obtained from Steraloids. Metyrapone (2-methyl-1,2-dipyridyl-1-propane) was a gift from Ciba-Geigy Co. (Japan). All other chemicals were of the highest available grade.

The cholesterol side-chain cleaving activity of *P*-450$_{scc}$ and the deoxycorticosterone 11β-hydroxylase activity of *P*-450$_{11\beta}$ were assayed as described previously [5, 6]. Other *P*-450$_{scc}$-mediated monooxygenase reactions were run in a similar way to that described previously [2] for the *P*-450$_{scc}$-mediated 6β-hydroxylase reaction in the presence of 300 nmol of specified steroid in a 0.3 ml system, unless otherwise specified. After preincubation for 2 min at 37°C, the reactions were started with the addition of NADPH, and this was followed by incubation for 10 min at 37°C. *P*-450$_{11\beta}$-mediated 6β-hydroxylase and 6-desaturase activity was assayed in a similar way, but the reaction was started with the addition of *P*-450$_{11\beta}$ and was run for 2.5 min. Reactions were terminated by the addition of 1.8 ml of chloroform–methanol (1:1, v/v). The organic layer was separated after the addition of 1 ml of water and 5 nmol of an internal standard, which was 19-norandrostenedione when the substrate was deoxycorticosterone or androstenedione, and 11β-hydroxyandrostenedione when the substrate was progesterone or 11-deoxycortisol. The extracts were evaporated under a stream of nitrogen gas at 40°C or lower. The residue was then dissolved in 5 μl of methanol and aliquots were injected into a column (Mitsubishi MCI Gel ODS-1HU, 4.6 × 250 mm) equipped with a prefilter (Irika Kogyo, 9917-03) in a Hitachi high-pressure liquid chromatography (HPLC) system, pump model L-6200, and column over model 655A-52,

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except that a column (Chemcosorb 5-ODS-H, 2.1 × 150 mm) with a precolumn (Chemcosorb-5-ODS-H, 1.46 × 30 mm) was used when the presumed product was 4,6-androstadiene-3,17-dione. Steroids were separated with methanol water (1:1, v/v) at a flow rate of 0.8 ml/min and a column temperature of 40°C. Absorptions were monitored at 240 and 290 nm for the 6β-hydroxylase and 6-desaturase assay, respectively, both by means of a Hitachi UV monitor model L-4200/model D-2500 Chromato-Integrator system. 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 a 200–300°C gradient (16°C/min), injection temperature was 280°C, and ionizing energy was 70 eV.

Hydroxylapatite HPLC of $P\text{-}450_{11\beta}$ was performed with an HA-1000 column (Tosoh), as described by Ogishima *et al.* [7].

RESULTS AND DISCUSSION

Our previous work has shown that purified adrenocortical mitochondrial $P\text{-}450_{scc}$ exhibits an unexpected steroid hydroxylase activity which converts deoxycorticosterone to form 6β-hydroxydeoxycorticosterone [2]. In the present study, additional examination of the properties of this deoxycorticosterone 6β-hydroxylase activity indicated that the reaction was dependent on the concentration of $P\text{-}450_{scc}$ (0 to 200 pmol/0.3 ml of reaction mixture), and on

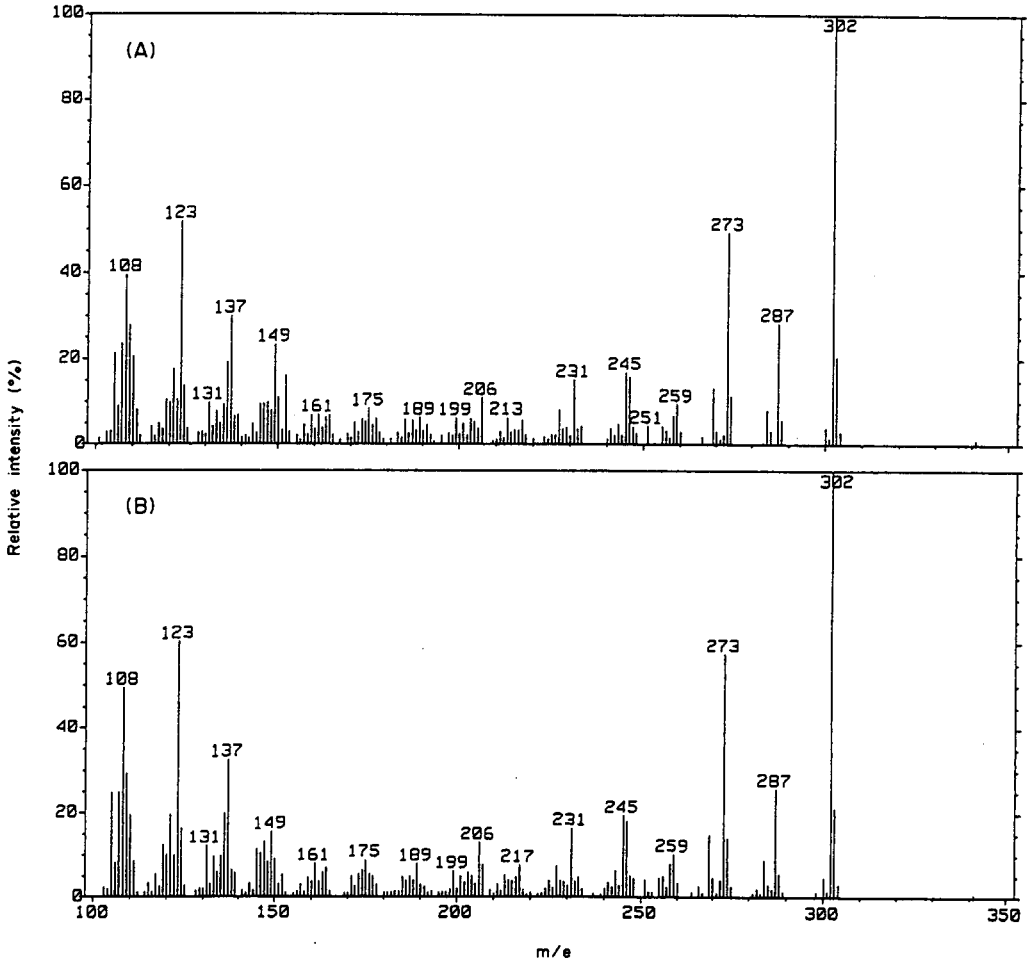

Fig. 1. GC–MS analyses of the $P\text{-}450_{scc}$-catalyzed product from androstenedione with a retention time of 12.8 min on HPLC (A), and of authentic 6β-hydroxyandrostenedione (B). Conditions are as described under Experimental.

time (0 to 20 min), and was optimal at pH 7.0 (data not shown). The activity was also dependent on substrate concentrations, and the K_m and V values were calculated with a Lineweaver–Burk plot of the rate of product formation vs substrate concentrations. The results yielded an apparent K_m value of 1.9 mM and a V value for the conversion of deoxycorticosterone to 6β-hydroxydeoxycorticosterone of 29 mol/min/mol of P-450$_{scc}$ (data not shown). Further examination of the substrate specficity of the 6β-hydroxylase activity of P-450$_{scc}$ revealed that androstenedione was also a 6β-hydroxylatable substrate of P-450$_{scc}$, as evidenced by comparison of the GC–MS profile for the product with a retention time of 12.8 min on HPLC and the GC–MS profile for the authentic 6β-hydroxyandrostenedione (Fig. 1). Progesterone and 11-deoxycortisol were also substrates of P-450$_{scc}$ and formed products with retention times on HPLC corresponding to those of authentic 6β-hydroxyprogesterone and 6β-hydroxy-11-deoxycortisol, with a turnover number of 0.7 mol/min/mol of P-450 and 0.6 mol/min/mol of P-450, respectively. 11β-Hydroxysteroids such as corticosterone and cortisol were not 6β-hydroxylatable substrates of P-450$_{scc}$.

We further examined the activity of another adrenal mitochondrial form of P-450, P-450$_{11\beta}$, to see whether it catalyzed similar hydroxylase reactions. Figure 2 shows the HPLC profile of the P-450$_{11\beta}$-dependent metabolites of deoxycorticosterone as monitored at 240 nm. A retention time of 14.7 min coincided with that of

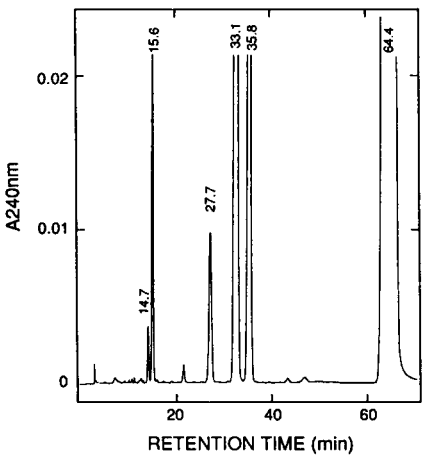

Fig. 2. HPLC elution profiles of the reaction products of P-450$_{11\beta}$-dependent conversion of deoxycorticosterone, as monitored at 240 nm. After the reaction mixture was extracted and concentrated under N_2 gas, steroids were injected into the HPLC column. Other conditions are as described under Experimental.

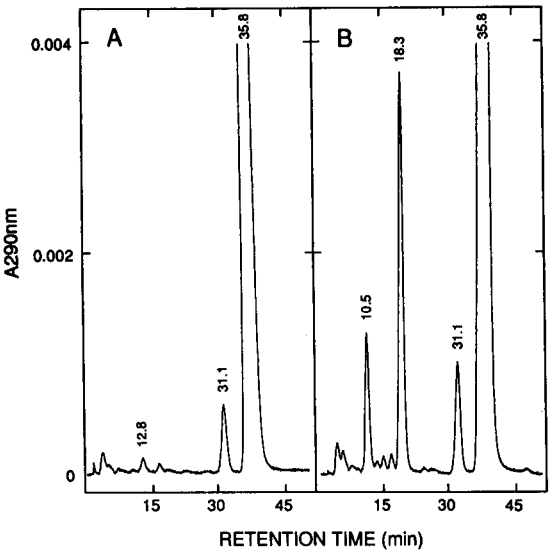

Fig. 3. HPLC elution profile of the reaction products of P-450$_{scc}$-dependent (A) and P-450$_{11\beta}$-dependent (B) conversions of androstenedione, as monitored at 290 nm. After the reaction mixture was extracted and concentrated under N_2 gas, steroids were injected into the HPLC column. Other conditions are as described under Experimental.

6β-hydroxydeoxycorticosterone and was distinct from those of the internal standard 19-norandrostenedione (retention time: 35.8 min), the substrate deoxycorticosterone (retention time: 64.4 min), and any known P-450$_{11\beta}$ metabolites of deoxycorticosterone, such as 18-hydroxycorticosterone (retention time: 15.6 min), 18-hydroxydeoxycorticosterone (retention time: 27.7 min), and the 11β-hydroxylase product, corticosterone (retention time: 33.1 min). We definitely identified the 14.7 min product as 6β-hydroxydeoxycorticosterone by its GC–MS profile, which corresponded to that of the authentic standard (data not shown). From these results, we concluded that P-450$_{11\beta}$ catalyzes the deoxycorticosterone 6β-hydroxylase reaction, with a turnover number of 1.0 mol/min/mol of P-450$_{11\beta}$, which is to be compared with a turnover number of 12 mol/min/mol of P-450 for P-450$_{scc}$ [2], i.e. P-450$_{11\beta}$ catalyzes this reaction less efficiently than P-450$_{scc}$.

To detect other possible activity of P-450$_{scc}$ and P-450$_{11\beta}$, we examined the HPLC profiles of the P-450-dependent hydroxylase products at various wavelengths. The results indicated that both P-450$_{scc}$ and P-450$_{11\beta}$ converted androstenedione, in addition to 6β-hydroxyandrostenedione, to a second metabolite with an absorption peak at 290 nm. Figure 3 shows the HPLC profiles of the P-450$_{scc}$-dependent (panel A) and P-450$_{11\beta}$-dependent (panel B)

androstenedione hydroxylase metabolites as monitored at 290 nm. A retention time of 31.1 min on both panels A and B coincided with that of authentic 4,6-androstadiene-3,17-dione, and was distinct from those of 6β-hydroxyandrostenedione (retention time: 12.8 min), the substrate (retention time: 35.8 min), and any known $P\text{-}450_{11\beta}$ metabolites of androstenedione, such as 19-hydroxyandrostenedione (retention time: 10.5 min) and 11β-hydroxyandrostenedione (retention time: 18.3 min). We definitely identified both of the 31.1 min

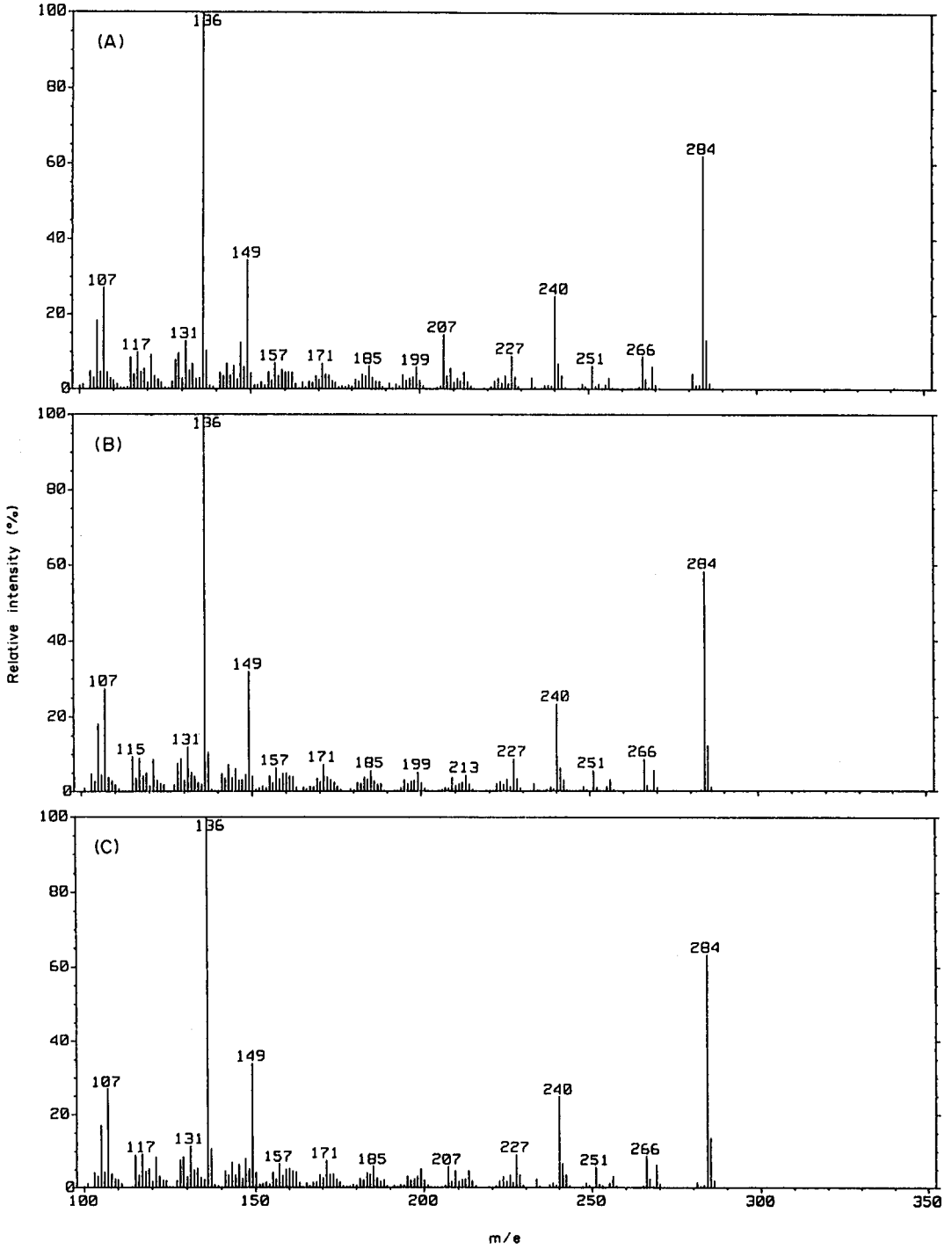

Fig. 4. GC–MS analyses of the $P\text{-}450_{scc}$-dependent (A) and $P\text{-}450_{11\beta}$ -dependent (B) products from androstenedione with a retention time of 31.1 min on HPLC and of authentic 4,6-androstadiene-3,17-dione (C). Conditions are as described under Experimental.

products as 4,6-androstadiene-3,17-dione by the GC–MS profiles, shown in panels A and B of Fig. 4, both of which corresponded to that of the authentic standard [Fig. 4(C)]. These results indicate that both $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$, in addition to catalyzing the 6β-hydroxylase reaction of various steroids, catalyze a 6-desaturase reaction which converts androstenedione to form 4,6-androstadiene-3,17-dione. Figure 5 shows the comparison of the K_m and V values of $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$-mediated 6β-hydroxylase and 6-desaturase activity with androstenedione as substrate. The apparent K_m and V values are calculated and indicated on the graphs.

The presence of multiple forms of $P\text{-}450_{scc}$ has been reported in bovine adrenocortical mitochondria [8]. In a previous paper, we reported that we had detected cholesterol side-chain cleavage and deoxycorticosterone 6β-hydroxylase activity in the two $P\text{-}450_{scc}$ fractions eluted from HPLC chromatography [2]. As is the case for $P\text{-}450_{scc}$, the presence of at least two kinds of $P\text{-}450_{11\beta}$ has been reported in bovine adrenocortical mitochondria [7]. In the present study, we performed HPLC of our purified $P\text{-}450_{11\beta}$ on a hydroxylapatite column. The fractions eluted from the column were collected and assayed for androstenedione 6-desaturase, 6β-hydroxylase, and 11β-hydroxylase reactions. The results, shown in Fig. 6, indicate that $P\text{-}450_{11\beta}$-mediated androstenedione 6-desaturase (panel A), 6β-hydroxylase (panel B), and 11β-hydroxylase activity (panel C) were detected in parallel in the two $P\text{-}450_{11\beta}$ fractions.

Further results confirm that both the $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$-mediated minor reactions were absolutely dependent on each component of the mitochondrial electron donating system, i.e. on NADPH, NADPH-adrenodoxin reductase and adrenodoxin. The $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$-mediated reactions were also dependent on O_2 and were inhibited by 40 and 70%, respectively, by CO–O_2 (4:1, v/v). Metyrapone (50 μM), a known inhibitor of $P\text{-}450_{11\beta}$ [6], inhibited the $P\text{-}450_{scc}$ and $P\text{-}450_{11\beta}$-mediated androstenedione 6-desaturase reaction by 20 and 60%, respectively. We examined 6β-hydroxyandrostenedione and 4,6-androstadiene-3,17-dione as the substrate in place of androstenedione under standard assay conditions. These experiments failed to detect the formation of 4,6-androstadiene-3,20-dione or 6β-hydroxyandrostenedione from each other, indicating that neither of the products is a precursor of the other.

Since the same substrate, androstenedione, was found to be metabolized to two products, 6β-hydroxyandrostenedione and 4,6-androstadiene-3,17-dione, we reexamined the HPLC elution profile of the $P\text{-}450_{scc}$-dependent deoxycorticosterone metabolites at 290 nm. As expected, a second product, with an absorption

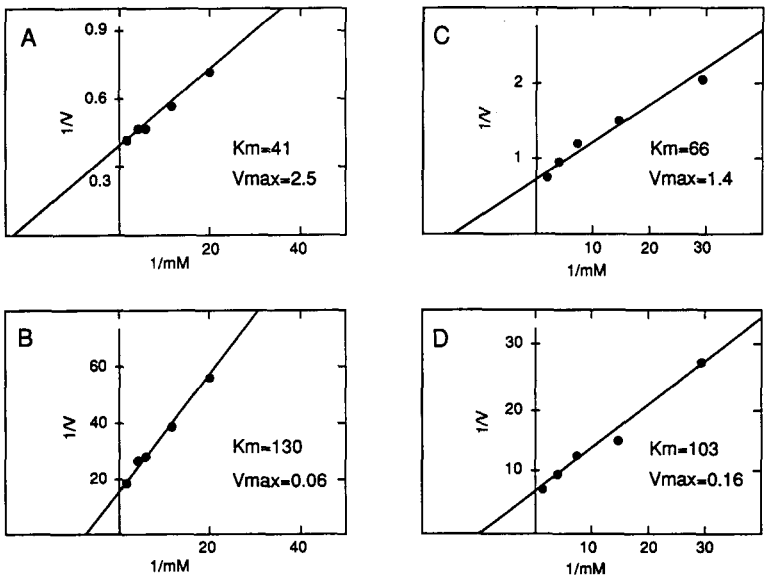

Fig. 5. Double reciprocal plot of the rate of product formation vs substrate concentration in $P\text{-}450_{scc}$ (panels A and B)- and $P\text{-}450_{11\beta}$ (panels C and D)-mediated androstenedione 6β-hydroxylase (panels A and C) and 6-desaturase (panels B and D) activity. K_m and V values are expressed in μM and mol of product formed per min per mol of P-450, respectively, and the calculated values are indicated on the graphs.

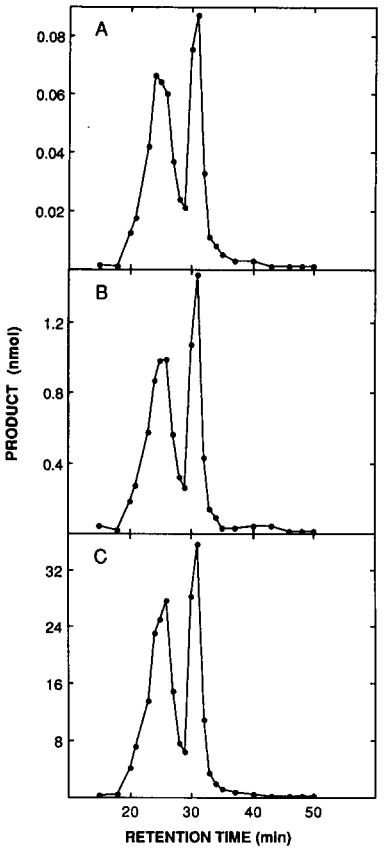

Fig. 6. Androstenedione monooxygenase activity of P-$450_{11\beta}$. A purified P-$450_{11\beta}$ preparation was dialyzed against 5 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.3% sodium cholate, and 0.2% Tween 20. The dialyzed preparation (10 nmol) was applied to a hydroxylapatite column (7.5 × 75 mm), and was equilibrated with and eluted with the same buffer for 15 min, followed by elution 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 6-desaturase (A), 6β-hydroxylase (B), and 11β-hydroxylase (C), as described under Experimental.

peak at 290 nm and a retention time of 52.1 min, was detected in addition to 6β-hydroxydeoxycorticosterone (retention time: 14.6 min) (data not shown). However, owing to the unavailability of an authetic standard such as 21-hydroxypregne-4,6-diene-3,20-dione and to the insufficient quantities of the product, definite identification of this compound was not performed.

Since P-450_{scc} has so far been known to catalyze only side-chain cleavage of C_{27}-steroids to form corresponding products [1, 9, 10], and P-$450_{11\beta}$ has been known to be active only at the 11β- 18-, and 19-positions of steroids to form a variety of products [3, 4, 6, 11–17], our preceding [2, 3] and the present studies are the first demonstration that P-450_{scc} and P-$450_{11\beta}$, in addition to their main steroidogenic activities, convert C_{21}-, C_{19}-, and C_{18}-steroids to two minor products, 6β-hydroxy- and Δ^6-steroids. These findings explain the previously noted formation of 6β-hydroxydeoxycorticosterone in adrenal slices [18]. The observed positional specificities at the 6-position of a steroid are consistent with those of rat liver microsomal forms of P-450, which catalyzes C_{19}-steroid 6β-hydroxylase and 6-desaturase reactions to form the corresponding 6β-hydroxysteroids and Δ^6-steroids, respectively [19, 20].

It is as yet unclear whether the observed minor activities of P-450_{scc} and P-$450_{11\beta}$ are of physiological relevance. However, taken together with our knowledge of the specificity of P-$450_{17\alpha}$, which acts as a steroid 17α-hydroxylase/17,20 side-chain cleaving enzyme on the one hand, and is capable of catalyzing the hydroxylation of various xenobiotics and the synthesis of Δ^{16}-C_{19}-steroids on the other [21, 22], the present study has shown that steroidogenic forms of P-450, including mitochondrial forms, also show versatile catalytic properties such as those seen in the liver microsomal forms of P-450 [20].

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REFERENCES

1. Simpson E. R. and Boyd G. S.: The cholesterol side-chain cleavage system of bovine adrenal cortex. *Eur. J. Biochem.* **2** (1967) 275–285.
2. Mochizuki H., Suhara K. and Katagiri M.: Adrenal P-450_{scc} catalyzes deoxycorticosterone 6β-hydroxylase reactions. *J. Steroid Biochem. Molec. Biol.* **37** (1990) 133–136.
3. Suhara K., Yamamoto M. and Katagiri M.: Hydroxylation of 19-norandrostenedione by adrenal cortex mitochondrial P-$450_{11\beta}$. *J. Steroid Biochem.* **36** (1990) 361–367.
4. Suhara K., Ohashi K., Takahashi K. and Katagiri M.: Aromatase and non-aromatizing 10-demethylase activity of adrenal cortex mitochondrial P-$450_{11\beta}$. *Archs Biochem. Biophys.* **267** (1988) 31–37.
5. Takikawa O., Gomi T., Suhara, K., Itagaki E., Takemori S. and Katagiri M.: Properties of an adrenal cytochrome P-450 (P-450_{scc}) for the side-chain cleavage of cholesterol. *Archs Biochem. Biophys.* **190** (1978) 300–306.
6. Sato H., Ashida N., Suhara K., Itagaki E., Takemori S. and Katagiri M.: Properties of an adrenal cytochrome P-450 (P-$450_{11\beta}$) for the hydroxylation of corticosteroids. *Archs Biochem. Biophys.* **190** (1978) 307–314.
7. Ogishima T., Mitani F. and Ishimura Y.: Isolation of two distinct cytochrome P-$450_{11\beta}$ and aldosterone synthase activity from bovine adrenocortical mitochondria. *J. Biochem. (Tokyo)* **105** (1989) 497–499.

8. Tsubaki M., Ohkubo H., Tsuneoka Y., Tomita S., Hiwatashi A. and Ichikawa Y.: Existence of multiple forms of cytochrome $P\text{-}450_{scc}$ purified form bovine adrenocortical mitochondria. *Biochim. Biophys. Acta* **914** (1987) 246–258.
9. Morisaki M., Duque C., Takane K., Ikekawa N. and Shikita M.: Substrate specificity of adrenocortical-cytochrome $P\text{-}450_{scc}$-II. Effect of structural modification of cholesterol A/B ring on their side-chain cleavage reaction. *J. Steroid Biochem.* **16** (1982) 101–105.
10. Sugano S., Morishima N., Ikeda H. and Horie S.: Sensitive assay of cytochrome $P\text{-}450_{scc}$ activity by high-performance liquid chromatography. *Analyt. Biochem.* **182** (1989) 327–333.
11. Suhara K., Takeda K. and Katagiri M.: $P\text{-}450_{11\beta}$-dependent conversion of cortisol to cortisone, and 19-hydroxy-androstenedione to 19-oxoandrostenedione. *Biochem. Biophys. Res. Commun.* **136** (1986) 369–375.
12. Suhara K., Ohashi K., Takeda K. and Katagiri M.: $P\text{-}450_{11\beta}$-dependent conversion of androgen to estrogen. The aromatase reaction. *Biochem. Biophys. Res. Commun.* **140** (1986) 530–535.
13. Wada A., Ohnishi Y., Nonaka Y., Okamoto M. and Yamano T.: Synthesis of aldosterone by a reconstituted system of cytochrome $P\text{-}450_{11\beta}$ from bovine adrenocortical mitochondria. *J. Biochem. (Tokyo)* **98** (1985) 245–256.
14. Yanagibashi K., Haniu M., Shievely J. E., Shen W. H. and Hall P. F.: The synthesis of aldosterone by the adrenal cortex. *J. Biol. Chem.* **261** (1986) 3556–3562.
15. Ohta M., Fujii S., Wada A., Ohnishi T., Yamano T. and Okamoto M.: Production of 19-hydroxy-11-deoxycorticosterone and 19-oxo-11-deoxycorticosterone from 11-deoxycorticosterone by cytochrome $P\text{-}450_{11\beta}$. *J. Steroid Biochem.* **26** (1987) 73–81.
16. Ohta M., Fujii S., Ohnishi T. and Okamoto M.: Production of 19-oic-11-deoxycorticosterone from 19-oxo-11-deoxycorticosterone by cytochrome $P\text{-}450_{11\beta}$ and nonenzymatic production of 19-nor-11-deoxycorticosterone from 19-oic-11-deoxycorticosterone. *J. Steroid Biochem.* **29** (1988) 699–707.
17. Fujii S., Momoi K., Okamoto M., Yamano T., Okada T. and Terasawa T.: 18,19-Dihydroxydeoxycorticosterone, a new metabolite produced from 18-hydroxydeoxycorticosterone by cytochrome $P\text{-}450_{11\beta}$. Chemical synthesis and structural analysis by ^{1}H NMR. *Biochemistry* **23** (1984) 2558–2564.
18. Pasqualini J. R., Lafoscade G. and Jayle M-F.: Role of 21-hydroxypregnenolone and of 17α,21-dihydroxypregnenolone in the biogenesis of C_{21} and C_{19} steroids by adrenal gland slices. *Steroids* **4**(6) (1964) 739–757.
19. Nagata K., Liberato D. J., Gillette J. R. and Sasame H. A.: An unusual metabolite of testosterone 17β-hydroxy-4,6-androstadien-3-one. *Drug Metab. Dispos.* **14** (1986) 559–565.
20. Halvorson M., Greenway D., Eberhart D., Fitzgerald K. and Parkinson A.: Reconstitution of testosterone oxidation by purified rat cytochrome $P\text{-}450_p$ (IIIA1). *Archs Biochem. Biophys.* **277** (1990) 166–180.
21. Suhara K., Fujimura Y., Shiroo M. and Katagiri M.: Multiple catalytic properties of the purified and reconstituted cytochrome *P*-450 ($P\text{-}450_{scc\,II}$) system of pig testis microsomes. *J. Biol. Chem.* **259** (1984) 8729–8736.
22. Nakajin S., Takahashi M., Shinoda M. and Hall P. F.: Cytochrome b_5 promotes the synthesis of Δ^{16}-C_{19} steroids by homogeneous cytochrome *P*-450 C_{21} side-chain cleavage from pig testis. *Biochem. Biophys. Res. Commun.* **132** (1985) 708–713.