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BOVINE ADRENAL CYTOCHROME *P*-450(11β)-MEDIATED CONVERSION OF 11-DEOXYCORTISOL TO 18- AND 19-HYDROXY DERIVATIVES; STRUCTURAL ANALYSIS BY ^{1}H-NMR

MIHO OHTA, SHIGERU FUJII[1], RETSU MIURA[1], YASUKI NONAKA and MITSUHIRO OKAMOTO*

Departments of Biochemistry and Molecular Physiological Chemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565 and [1]Laboratory of Chemistry, Kansai Medical University, Hirakata, Osaka 573, Japan

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Summary—Incubation of 11-deoxycortisol with a cytochrome *P*-450(11β)-reconstituted system yielded, in addition to cortisol, several new steroid products. In this study, structures of the three steroid products were elucidated. Retention time of the first product (Peak 2 substance) coincided with that of authentic 18-hydroxycortisol on reverse phase HPLC. To further confirm the chemical identity of this product, the purified sample was subjected to ^{1}H-NMR analysis. The spectrum was essentially identical to that of 18-hydroxycortisol. The retention time of the second product (Peak 3 substance) did not coincide with those of commonly occurring steroids. The one- and two-dimension ^{1}H-NMR spectra provided strong evidence for its structure of 19-hydroxy-11-deoxycortisol. The retention time of the third product (Peak 4 substance) did not coincide with those of commonly occurring steroids. The ^{1}H-NMR spectrum showed the presence of signals of 19-CH_3 and 18-CH_2 protons. There was also evidence that this product is not hydroxylated at the 11-position. Further analysis of the COSY spectra identified its structure as 18-hydroxy-11-deoxycortisol. From these results, we conclude that bovine *P*-450(11β) can catalyze the hydroxylation of 11-deoxycortisol at 11β-, 18- and 19-positions and produce cortisol, 18-hydroxy-11-deoxycortisol, 18-hydroxycortisol and 19-hydroxy-11-deoxycortisol.

INTRODUCTION

The formation of cortisol from 11-deoxycortisol involves the reaction catalyzed by cytochrome *P*-450(11β) [1] and thus-produced cortisol acts as a main glucocorticoid *in vivo*. On the other hand, 18-oxidative derivatives of cortisol have been detected in two hypertensive disorders [2, 3]. The first derivative, discovered in primary aldosteronism [2], is 18-hydroxycortisol, which appears in the urine largely in the unmetabolized form [3]. The second derivative, 18-oxocortisol, was detected first in adrenal incubates [4] and subsequently in the urine of patients with primary and genetic aldoseronism [5]. The amount of 18-oxocortisol excreted in the urine of a normal subject was below the limit of detection. Ulick *et al.* have shown that the incubation of bullfrog adrenals with cortisol generated 18-hydroxycortisol and 18-oxocortisol, thus suggesting a possibility of participation of angular methyl oxidase system (corticosterone methyloxidase I and II enzymatic complex) in this 18-oxidation pathway [2, 4]. However, its enzymatic characteristics remain to be elucidated.

P-450(11β) in bovine adrenocortical mitochondria has been shown to catalyze hydroxylation of 11-deoxycorticosterone not only at the C-11 position but also at the 18- and 19-positions [6–13]. We have recently demonstrated that *P*-450(11β) catalyzes hydroxylation of 11-deoxycorticosterone at the C-19 position and confirmed that the products are 19-hydroxy-11-deoxycorticosterone, 19-oxo-11-deoxycorticosterone and 19-oic-11-deoxycorticosterone. 19-Oic-11-deoxycorticosterone was nonenzymatically converted to 19-nor-11-deoxycorticosterone [12, 13], which has been reported to be a potent mineralocorticoid [14–16]. Since

*To whom correspondence should be addressed.

The trivial names used are: 11-deoxycortisol, 17α,21-dihydroxy-pregn-4-ene-3,20-dione; 18-hydroxycortisol, 11β,17α,18,21-tetrahydroxy-pregn-4-ene-3,20-dione; 18-oxocortisol, 11β,17α,21-trihydroxy-pregn-4-ene-3,20-dione-18-al; 19-hydroxy-11-deoxycortisol, 17,19,21-trihydroxy-pregn-4-ene-3,20-dione.

the cytochrome shows such versatile catalytic activities toward steroid substrates, it is possible that it can also catalyze the hydroxylations of 11-deoxycortisol at the C-18 and 19 positions in addition to that at the 11β-position.

In the present study, we examined the possibility of hydroxylation of 11-deoxycortisol at the C-18 and C-19 positions by *P*-450(11β). Several metabolites in the reaction mixture of 11-deoxycortisol and the purified *P*-450(11β)-reconstituted system were purified by repeated HPLC and analyzed by one- and two-dimensional ^{1}H-NMR techniques. We found that 18-hydroxycortisol and 18-hydroxy-11-deoxycortisol were really produced in the *P*-450(11β) catalyzed reaction. Moreover, 19-hydroxy-11-deoxycortisol was also identified as a new metabolite of 11-deoxycortisol.

EXPERIMENTAL

Chemicals

Most of the steroids used were purchased from Sigma (U.S.A.). 18-Hydroxycortisol and 18-oxocortisol were generous gifts from Dr Ulick at Veterans Administration Hospital, Bronx, New York. NADPH glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast Co., Japan. Other chemicals were of the highest grade available from commercial sources.

Preparation of enzymes

P-450(11β) was purified from bovine adrenocortical mitochondria as described previously [8, 12, 17]. The specific cytochrome *P*-450 content of the purified sample was 16 nmol/mg protein. Adrenodoxin and NADPH-adrenodoxin reductase were purified from bovine adrenal mitochondria according to the methods described previously [12].

Analysis of products

Steroid (11-deoxycortisol or cortisol) (100 μM) was incubated with the reconstituted *P*-450(11β) system in the final volume of 0.5 ml in the presence of an NADPH-generating system under aerobic conditions as described previously [12, 13]. Products were extracted with two 5 ml-portions of dichloromethane, and then the solvent was evaporated under nitrogen. The dried extract was analyzed by HPLC. The amounts of formed product were calculated from the data with correction for the recovery of the internal standard.

Large scale preparation of unidentified products

11-Deoxycortisol (3 μmol) was incubated with *P*-450(11β) (18 nmol) for 15 min at 37°C in 20 ml of 10 mM Tris–HCl (pH 7.4) containing NADPH (3 μmol), glucose-6-phosphate (300 μmol), glucose-6-phosphate dehydrogenase (30 units), $MgCl_2$ (60 μmol), adrenodoxin (600 nmol) and adrenodoxin reductase (30 μmol). After the reaction was terminated by the addition of 20 ml of ethanol, the products were extracted three times with 100 ml of dichloromethane, and then the solvent was evaporated. The residue was applied to the reverse phase HPLC system (60% MeOH/H_2O, 0.5 ml/min). Three main peak fractions were collected separately. Each peak substance was further applied to the same column repeatedly until each isolated product gave a single peak on the chromatography. Each preparation was then subjected to ^{1}H-NMR spectrometric analysis.

HPLC

For HPLC, a Shimadzu model LC-6A system equipped with a u.v. monitor was employed. For reverse phase HPLC, a 4.5 × 250 mm ODS column (M&S Packs, 5 μ) and the solvent system of methanol–water = 60:40 (v/v) or 55:45 (v/v), at a flow rate of 0.5 ml/min, were employed.

NMR analysis

The ^{1}H-NMR spectra were obtained at 400 MHz with a Varian Unity-400 spectrometer equipped with a Fourier transform accessory. Chemical shifts (δ) in a $CDCl_3$ solution were measured in ppm, the residual $CHCl_3$ of the solvent system being used as the internal standard (δ = 7.27). 2-Dimensional ^{1}H-homonuclear shift-correlated spectra were obtained with 1024 data points in the f2 dimension and f1 data points were zero-filled to achieve a symmetrical data matrix on Fourier transformation.

Analytical methods

Protein concentrations were determined according to the method of Lowry *et al.* [18] with bovine serum albumin as a standard. The *P*-450 content was estimated as described by Omura and Sato [19] using an extinction coefficient of 91 mM^{-1} cm^{-1} for the absorbance difference between 450 and 490 nm of the CO-bound reduced enzyme. The concentration of adrenodoxin and adrenodoxin reductase were

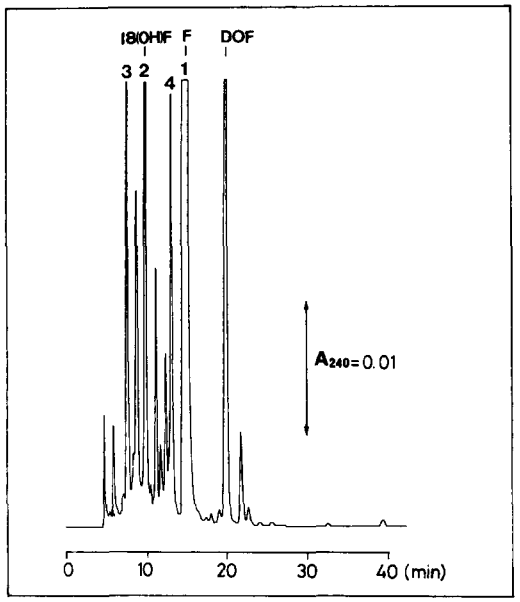

Fig. 1. Chromatogram of reaction products from 11-deoxycortisol. 11-Deoxycortisol (100 μM) was incubated with cytochrome P-450(11β) (600 nM) in the presence of adrenodoxin, adrenodoxin reductase and a NADPH-generating sysem (a final volume of 0.5 ml) at 37°C for 15 min. The products were analyzed by reverse phase HPLC with the solvent system of 60% MeOH–H_2O at a flow rate of 0.5 ml/min. Detailed conditions for this experiment are given under Experimental. The eluate was monitored at 240 nm. DOF, 11-deoxycortisol.

determined using $\epsilon_{414} = 10\ mM^{-1}\ cm^{-1}$ and $\epsilon_{450} = 11\ mM^{-1}\ cm^{-1}$, respectively [20, 21].

RESULTS

An HPLC profile of the reaction products of 11-deoxycortisol with a P-450(11β)-reconstituted system

11-Deoxycortisol was incubated with a P-450(11β)-reconstituted system, and the products were extracted with dichloromethane as described in Experimental. Figure 1 presents a chromatogram of the products on reverse phase HPLC. Retention time of the main peak (Peak 1) was 15 min, which coincided with that of authentic cortisol. A ^{1}H-NMR spectrum of Peak 1 substance was identical to that of authentic cortisol (data not shown). The turnover number of cortisol formation under these conditions was 70 mol/min/mol P-450. Several new product peaks were found on the chromatogram (Peaks 2, 3 and 4). The retention times of Peaks 2, 3 and 4 were 10, 7.5 and 13 min, respectively. Peak 2 substance was also found in the reaction products from cortisol as shown in Fig. 2(B), though the elution conditions in Figs 1 and 2 were slightly different. However, the peaks corresponding to Peaks 3 and 4 in Fig. 1 could not be detected on the chromatogram of the products from cortisol [Fig. 2(B)]. These results suggest that Peak 2 substance has a hydroxyl group at the 11β-position and that both peaks 3 and 4 lack the 11β-hydroxyl group.

Identification of Peak 2 substance as 18-hydroxycortisol

To investigate the chemical structures of these products, their retention times were compared to those of authentic steroids on the reverse phase HPLC. The retention time of Peak 2 substance coincided with that of authentic 18-hydroxycortisol. To further confirm the chemical identity of this product, its ^{1}H-NMR spectrum was measured and compared to that of 18-hydroxycortisol (Fig. 3). The ^{1}H-NMR spectrum of Peak 2 substance [Fig. 3(B)] was essentially identical to that of 18-hydroxycortisol [Fig. 3(A)]. According to the results of Genard *et al.* [22], Fujii *et al.* [11] and Arison *et al.* [23], the resonance peaks at $\delta = 1.42$, $\delta = 4.50$ and $\delta = 5.71$ were assigned to those of

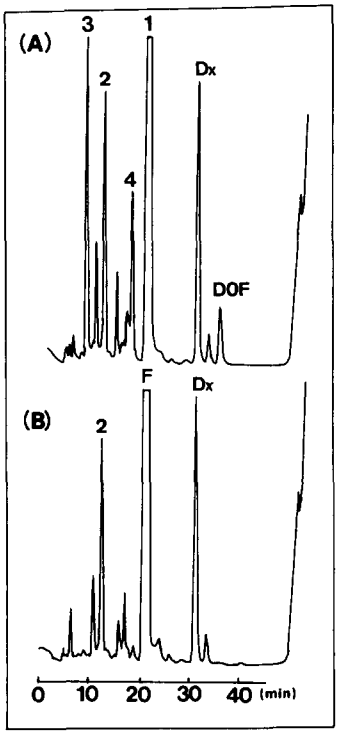

Fig. 2. Chromatograms of reaction products from (A) 11-deoxycortisol and from (B) cortisol. Each substrate was incubated with the reconstituted cytochrome P-450(11β) system in a final volume of 0.5 ml at 37°C for 20 min. The products were analyzed by reverse phase HPLC with the solvent system of 55% MeOH–H_2O at a flow rate of 0.5 ml/min. Other conditions for this experiment are given under Experimental. DOF, 11-deoxycortisol. Dx, dexamethazone (internal standard). F, cortisol.

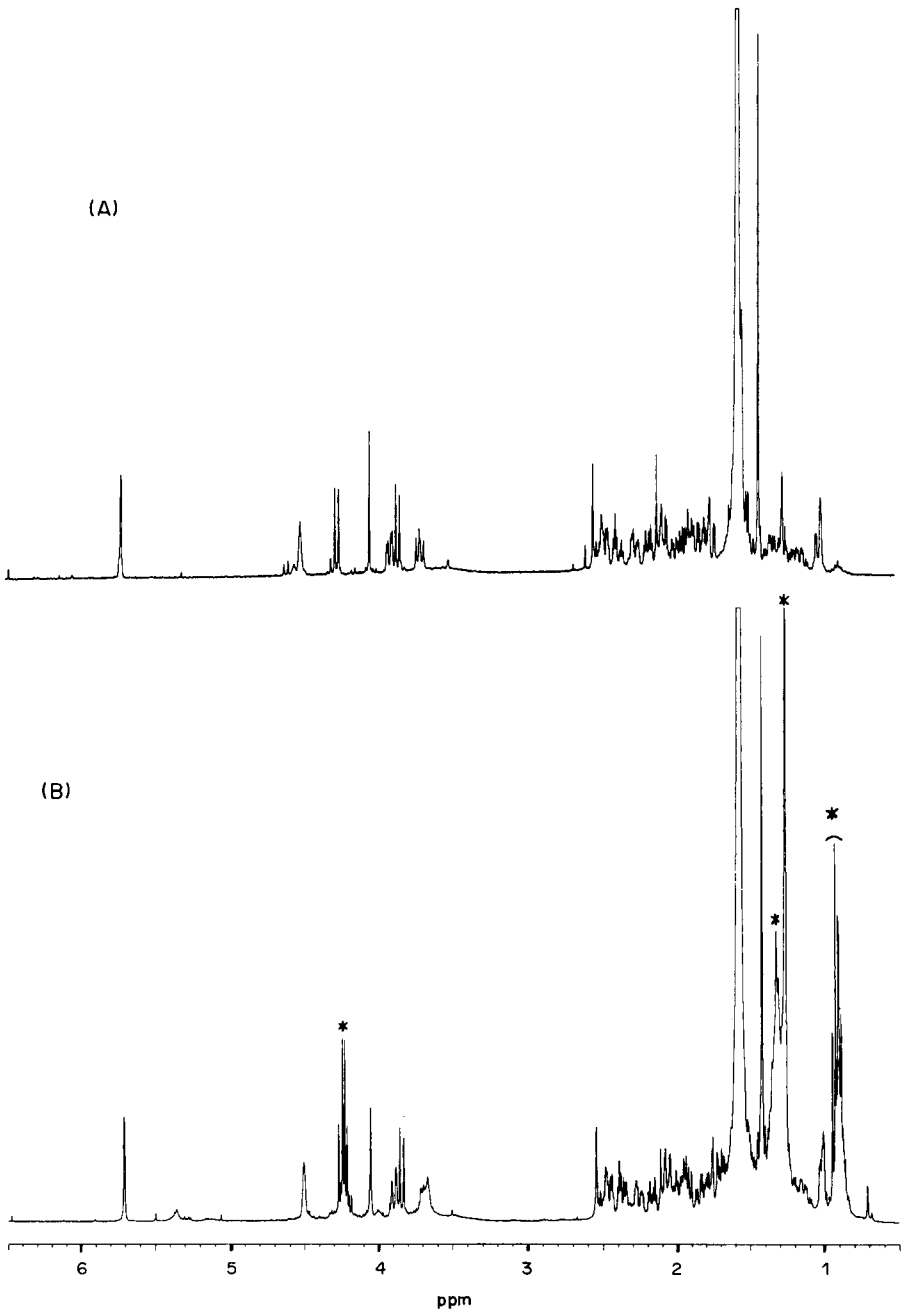

Fig. 3. ^{1}H-NMR spectra of (A) authentic 18-hydroxycortisol and (B) Peak 2 substance in $CDCl_3$. The peaks indicated by asterisks are due to the contaminants in the solvent.

19-methyl-, 11β- and 4-protons, respectively. Signals of methylene proton at C-18 position were observed as two doublets ($\delta = 3.83$ and 3.86; 4.24 and 4.27). They seemed not to couple with any other proton than with each other. Due to formation of hemiketal bridge between C-18(OH) and C-20 in $CDCl_3$ solution, rearrangement of the hydroxyl group from C-18 to C-20 position is expected. Existence of some steroids having this hemiketal form in $CDCl_3$ has been reported [11, 23]. As for 18,19-dihydroxy-11-deoxycorticosterone, resonance peaks at $\delta = 3.24$ and $\delta = 3.08$ could be assigned to those of the hydroxy proton at C-20 position [11], but the assignment of the rearranged hydroxy proton at C-20 position has not been made for 18-hydroxycortisol [23]. To make further solid assignment of chemical shifts to individual protons at particular sites, COSY spectra of 18-hydroxycortisol and cortisol (Fig. 4) were measured. By doing this, a sharp resonance peak at $\delta = 4.03$ could be

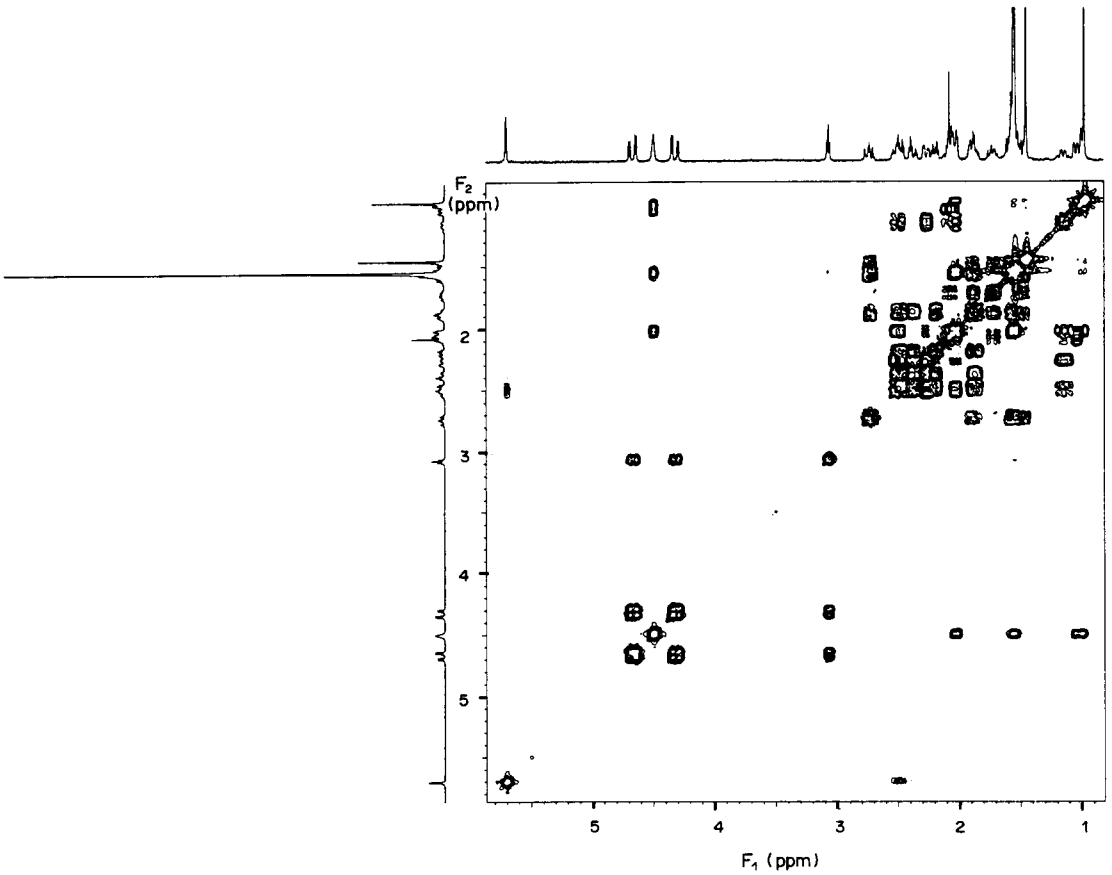

Fig. 4. COSY spectrum of authentic 18-hydroxycortisol in $CDCl_3$.

assigned to a signal of the rearranged hydroxyl group at C-20 position. Peaks in regions from $\delta = 3.67$–3.72 and 3.88–3.92 in Figs 3(A) and (B) could be assigned to signals of the methylene proton at C-21 position. These characteristics of the HPLC elution profiles and the ^{1}H-NMR spectra provided strong evidence for the production of 18-hydroxycortisol by bovine adrenal *P*-450(11β). Turnover number of 18-hydroxycortisol formation was estimated to be 2.4 mol/min/mol *P*-450.

Identification of Peak 3 substance as 19-hydroxy-11-deoxycortisol

On the basis of the result shown in Fig. 2, Peak 3 substance seemed not to have a hydroxyl group at the 11β-position. To further confirm the chemical identity of Peak 3 substance, the purified preparation of Peak 3 substance was subjected to ^{1}H-NMR analysis. Figure 5(A) shows the ^{1}H-NMR spectrum of Peak 3 substance. For reference, ^{1}H-NMR spectrum of 11-deoxycortisol, the substrate in the reaction, is also shown [Fig. 5(B)]. In both spectra, a signal of the resonance peak of methyl proton at C-18 was observed at $\delta = 0.72$. However, a resonance peak of 11β-proton, found at $\delta = 4.50$ in cortisol (Fig. 4) and in 18-hydroxycortisol (Fig. 3), could not be found in either spectrum. A resonance peak of 19-CH_3, found at $\delta = 1.20$ in 11-deoxycortisol, was missing in Peak 3 substance. A signal of the resonance peak of the 4-H appeared at $\delta = 5.97$ in Peak 3 substance, which was considerably shifted downfield as compared with that of 11-deoxycortisol [$\delta = 5.75$ in Fig. 5(B)]. A similar shift of the 4-H peak to lower magnetic field generally occurs in the NMR spectra of 19-CH_2OH-steroids [11]. We have reported previously that the 4-H resonance of 18-hydroxydeoxycorticosterone was found at $\delta = 5.75$ whereas that of 18,19-dihydroxydeoxycorticosterone was found at $\delta = 5.97$ [11]. These observations support that 19-CH_3 of 11-deoxycortisol was converted to 19-CH_2OH in Peak 3 substance. On the basis of the assignment by Fujii *et al.* [11] and comparison of the ^{1}H-NMR spectra (Fig. 5), the resonance peaks in the regions of $\delta = 3.91$–3.93 and 4.05–4.08 were assigned to those of the methylene protons of the hydroxymethyl group at the

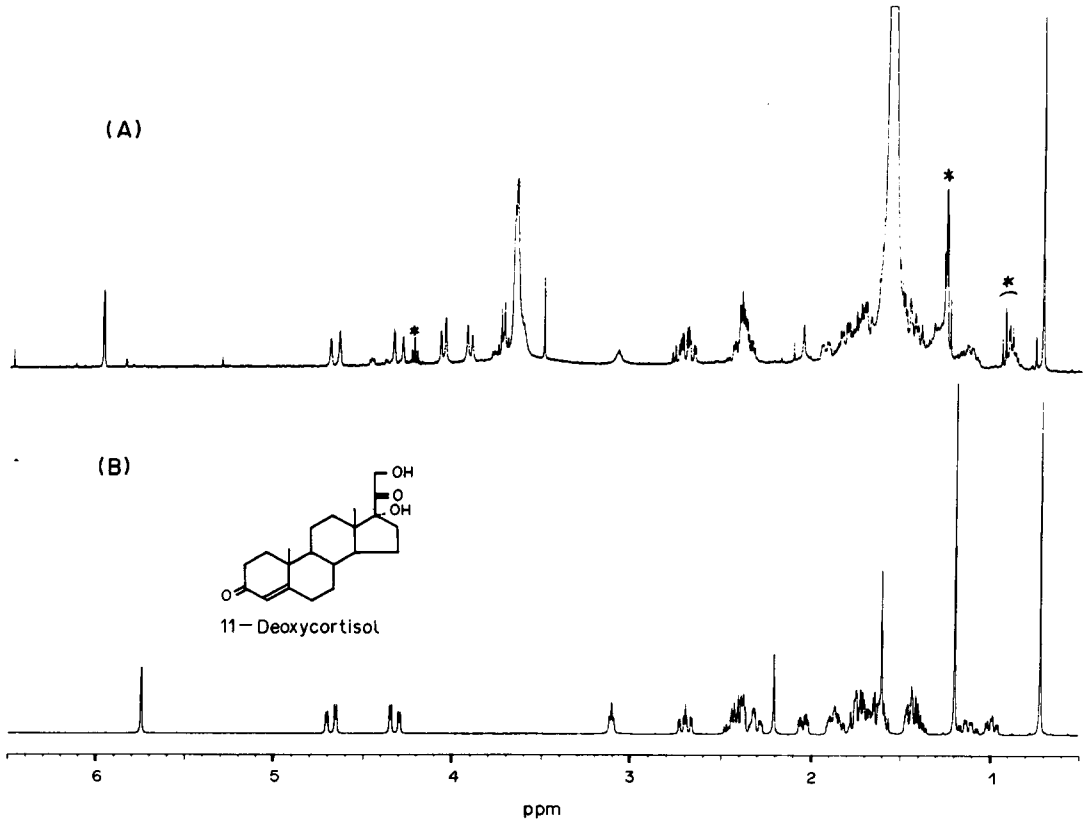

Fig. 5. ^{1}H-NMR spectra of (A) Peak 3 substance and (B) 11-deoxycortisol in $CDCl_3$. The peaks indicated by asterisks are due to the contaminants in the solvent.

C-19 position. The doublet signals at $\delta = 4.29$ and 4.34, and $\delta = 4.65$ and 4.70 were also identified as those of 21-CH_2. These results led us to conclude that the chemical structure of the Peak 3 substance is 19-hydroxy-11-deoxycortisol. Turnover number of 19-hydroxy-11-deoxycortisol formation was estimated to be 1.9 mol/min/mol *P*-450.

Identification of Peak 4 substance as 18-hydroxy-11-deoxycortisol

The retention time of Peak 4 substance did not coincide with any of those of commonly used authentic steroids. In agreement with the result that this unidentified compound does not contain a hydroxyl group at the 11β-position (Fig. 2), a signal of the 11α-proton resonance which was found in the spectra of cortisol and 18-hydroxycortisol (Fig. 3) at $\delta = 4.5$ was not detected in the ^{1}H-NMR spectrum of Peak 4 substance (Fig. 6). Resonance peaks at $\delta = 1.14$, 3.79 and 5.75 were identified as those derived from 19-CH_3-, 18-CH_2- and 4-protons, respectively, on the basis of the results of Fujii *et al.* [11]. A singlet peak at $\delta = 4.07$ was also assigned to one of hydroxy proton at the C-20 position [in 18-hydroxycortisol, the signal was observed at $\delta = 4.03$ (Fig. 3)]. Peaks around $\delta = 3.69$ and 3.89 were also assigned to those of methylene protons at the C-21 position. Resonance peaks that appeared around $\delta = 0.93$, 1.31 and 4.22 (indicated by asterisks in Fig. 6) are considered to be due to a contaminants in the solvent, because similar peaks were observed in the other purified samples [see Figs 3(B) and 5(A)] and further purification of this material by HPLC caused decrease in these signals. These results support that the Peak 4 substance is 18-hydroxy-11-deoxycortisol. Turnover number of 18-hydroxy-11-deoxycortisol formation was estimated to be 1.5 mol/min/mol *P*-450.

DISCUSSION

In the present study, we have demonstrated that 11-deoxycortisol can be hydroxylated at the 18- and 19-positions as well as at the 11β-position by bovine *P*-450(11β). From the results of the ^{1}H-NMR in one- and two-dimensions, the chemical structures of these products were identified as 18-hydroxy-11-deoxycortisol, 18-hydroxycortisol and 19-hydroxy-11-deoxycortisol. Moreover, it was shown that 18-hydroxycortisol could be formed when cortisol was

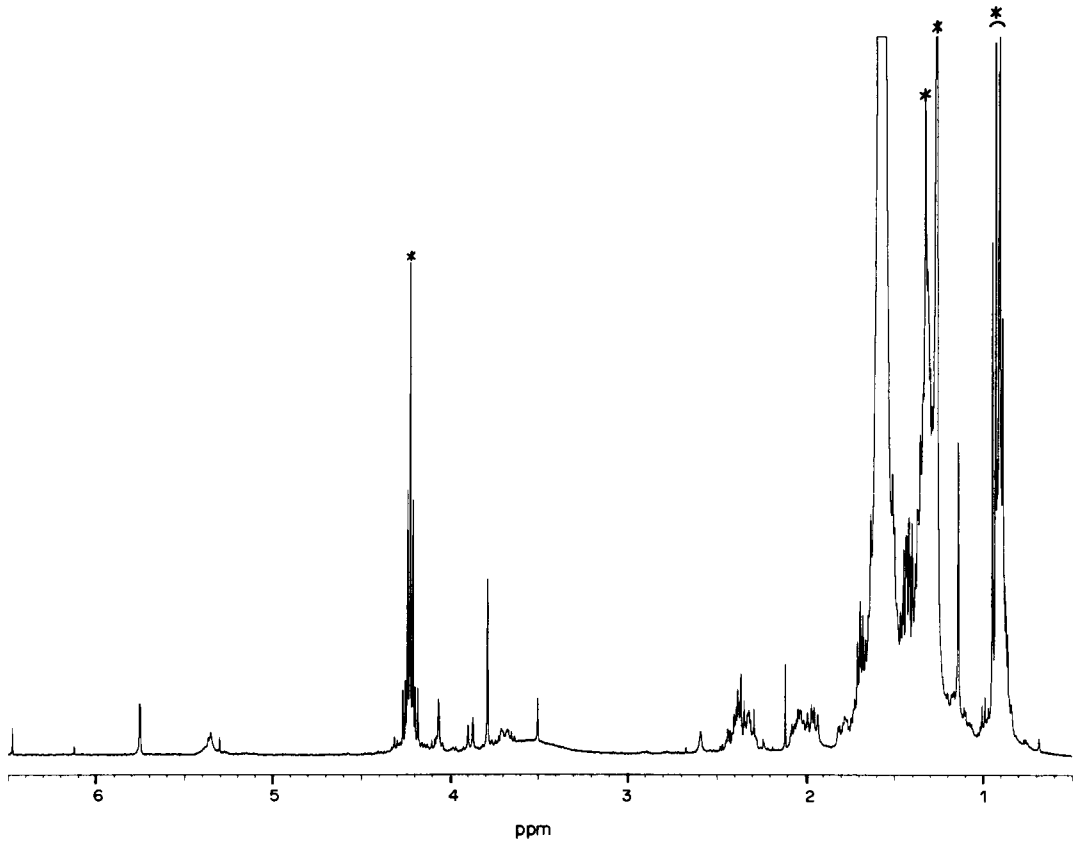

Fig. 6. ^{1}H-NMR spectrum of Peak 4 substance in $CDCl_3$. The peaks indicated by asterisks are due to the contaminants in the solvent.

used as a substrate in the similar reconstituted system of *P*-450(11β), the 18-hydroxylation activity toward cortisol being almost identical to that toward 11-deoxycortisol. These findings suggest that two pathways exist for the conversion of 11-deoxycortisol to 18-hydroxycortisol through the bovine *P*-450(11β)-catalyzed reaction. In the first pathway, 11-deoxycortisol is first hydroxylated at the 18-position to 18-hydroxy-11-deoxycortisol which would then be hydroxylated at the 11-position to yield 18-hydroxycortisol. In the second pathway, 11-deoxycortisol is first hydroxylated at the 11β-position and cortisol so generated is again hydroxylated at its 18-position to yield 18-hydroxycortisol. Such a pathway has been shown to exist for the conversion of 11-deoxycorticosterone to 18-hydroxycorticosterone by bovine adrenocortical mitochondria [24]. Further studies on the kinetical interrelation between the two pathways will be described elsewhere.

As to the 19-oxygenated metabolites of 11-deoxycortisol, Levy *et al.* [25] reported that 19-hydroxy-11-deoxycortisol was produced from 11-deoxycortisol in the perfusate through cow adrenals, but enzymes involved were not explored. Here we have demonstrated that *P*-450(11β) catalyzes the 19-hydroxy-11-deoxycortisol formation from 11-deoxycortisol. It is known that removal of the 19-methyl group on a steroid nucleus affects both the biological and chemical properties of the corresponding methylated parent compound [14–16, 26]. 19-Nor-11-deoxycorticosterone is known as a potent mineralocorticoid with high affinity for a mineralocorticoid receptor [15] and it has higher sodium-retaining activity than 11-deoxycorticosterone [14, 16]. We have previously shown that putative precursors of 19-nor-11-deoxycorticosterone such as 19-hydroxy-11-deoxycorticosterone, 19-oxo-11-deoxycorticosterone and 19-oic-11-deoxycorticosterone were successively produced through the *P*-450(11β)-catalyzed reaction. The bovine *P*-450(11β) may likewise catalyze the successive hydroxylation reaction of 19-hydroxy-11-deoxycortisol, i.e. the 17-hydroxylated analog of 19-hydroxy-11-deoxycorticosterone, to yield 19-oxo-11-deoxycortisol and 19-oic-11-deoxycortisol. Since, as shown in Fig. 1, several peaks on HPLC remain to be identified, further studies to examine this

possibility are of our interest. Our preliminary data of ^{1}H-NMR analyses of the other metabolites (other peak substances in Fig. 1) suggest a possibility of the production of 18- and/or 19-oxo-derivatives of 11-deoxycortisol by the *P*-450(11β)-catalyzed reaction in addition to the formation of the products described herein.

18-Hydroxycortisol and 18-oxocortisol are known to be produced by adrenal tissues of several species [4] and also isolated from the urine of patients with primary and genetic (dexamethasone-suppressible) aldosteronism [2, 5, 27]. In patients with primary aldosteronism due to adrenal adenoma, hypertension or metabolic abnormality are caused by hypersecretion of aldosterone, but sometimes the severity of symptoms is unexplainable solely on the basis of the increased secretory rate of aldosterone [5, 28]. Plasma mineralocorticoid receptor activity increased in patients with dexamethasone-suppressible hyperaldosteronism when compared to normal subjects. However, this fact could not be explained by the data of simultaneous determination of plasma aldosterone, cortisol and 11-deoxycorticosterone [29, 30]. The abnormally stimulated production of 18-oxocortisol in these patients makes it likely that this steroid contributes to the increased plasma mineralocorticoid receptor activity [5]. Furthermore, hypertensinogenic potency of 18-oxocortisol has also been observed in several animals [31, 32]. Thus it is possible that 18-oxocortisol plays a role in the pathogenesis of hypertension in those cases where its production is markedly increased.

Ulick *et al.* [4] suggested the participation of the CMO I and II enzymatic complex in the biosynthesis of the 18-oxygenated compound from cortisol in a study using bullfrog adrenals, a model of zona glomerulosa. Though the relationship between our purified *P*-450(11β) and the CMO I and II enzymatic complex is unknown, we surmize that *P*-450(11β) could be involved in the 18-oxygenation of 11-deoxycortisol to produce 18-hydroxy-11-deoxycortisol, 18-hydroxycortisol and presumably 18-oxo derivatives. This enzyme system might contribute to the clinial manifestation of primary and genetic aldosteronism.

Recently, Morohashi *et al.* isolated two kinds of *P*-450(11β) cDNAs from bovine adrenal cortex [33] and measured several steroidogenic activities of these *P*-450(11β)s expressed in COS-7 cells [34]. The hydroxylation activity at the 11β-position of 11-deoxycortisol by one *P*-450(11β) was almost identical to that by the other. On the other hand, the rates of aldosterone and 18-hydroxycorticosterone production by the two *P*-450(11β)s differ in several fold. The characteristics of the hydroxylation activities at the 18- and 19-positions of 11-deoxycortisol or cortisol by the two *P*-450(11β)s remain to be elucidated.

Gomez-Sanchez *et al.* [35] found that the synthesis of 18-hydroxycortisol and 18-oxocortisol in bovine adrenal slices takes place primarily in the outer slice which mainly consists of the zona glomerulosa. Using an anti-cytochrome *P*-450(11β) monoclonal antibody, Ohnishi *et al.* [36] showed the presence of cytochrome *P*-450(11β) in the mitochondria of the zona glomerulosa as well as the zonae fasciculata-reticularis. They also suggested that the aldosterone synthesizing activity of bovine cytochrome *P*-450(11β) is inhibited in the intact zonae fasciculata-reticularis mitochondria and that the inhibition is abolished by detergent treatment. Based on these results, they proposed a working hypothesis that specific mitochondrial regulators control enzymatic activities related to aldosteronogenesis in the zona fasciculata. It is possible that the similar regulatory mechanism functions in the cortisol 18-oxidation pathway.

Taken together, we showed in this paper that bovine *P*-450(11β) catalyzes the hydroxylations at the 18- and 19-positions of 11-deoxycortisol in addition to that at the 11β-position. Bovine *P*-450(11β) has versatile enzymatic activities; it catalyzes not only the 11β-, 18- and 19-hydroxylations of C-21- and C-19-steroids but also the conversion of corticosterone to 18-hydroxycorticosterone and aldosterone [14–16, 18, 19], and aromatization of androgen [37]. The products identified in this study, 18-hydroxy-11-deoxycortisol, 18-hydroxycortisol and 19-hydroxy-11-deoxycortisol might be mainly involved in the pathogenesis of several disorders. Further studies are in progress to identify the other metabolites of 11-deoxycortisol in this *P*-450(11β)-catalyzed reaction and to elucidate their biological and pathological significance.

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