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3α-HYDROXYSTEROID DEHYDROGENASE ACTIVITY CATALYZED BY PURIFIED PIG ADRENAL 20α-HYDROXYSTEROID DEHYDROGENASE*

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Summary—In earlier studies, two distinct molecules, 20α-HSD-I and 20α-HSD-II, responsible for 20α-HSD activity of pig adrenal cytosol were purified to homogeneity and characterized [S. Nakajin *et al.*, *J. Steroid Biochem.* **33** (1989) 1181–1189]. We report here that the purified 20α-HSD-I, which mainly catalyzes the reduction of 17α-hydroxyprogesterone to 17α,20α-dihydroxy-4-pregnen-3-one, catalyzes 3α-hydroxysteroid oxidoreductase activity for 5α (or 5β)-androstanes (C_{19}), 5α (or 5β)-pregnanes (C_{21}) in the presence of NADPH as the preferred cofactor. The purified enzyme has a preference for the 5α (or 5β)-androstane substrates rather than 5α (or 5β)-pregnane substrates, and the 5β-isomers rather than 5α-isomers, respectively. Kinetic constants in the reduction for 5α-androstanedione (K_m; 3.3 μM, V_{max}; 69.7 nmol/min/mg) and 5β-androstanedione (K_m; 7.7 μM, V_{max}; 135.7 nmol/min/mg) were demonstrated for comparison with those for 17α-hydroxyprogesterone (K_m; 26.2 μM, V_{max}; 1.3 nmol/min/mg) which is a substrate for 20α-HSD activity. Regarding oxidation, the apparent K_m and V_{max} values for 3α-hydroxy-5α-androstan-17-one were 1.7 μM and 43.2 nmol/min/mg, and 1.2 μM and 32.1 nmol/min/mg for 3α-hydroxy-5β-androstan-17-one, respectively. 20α-HSD activity in the reduction of 17α-hydroxyprogesterone catalyzed by the purified enzyme was inhibited competitively by addition of 5α-DHT with a K_i value of 2.0 μM. Furthermore, 17α-hydroxyprogesterone inhibited competitively 3α-HSD activity with a K_i value of 150 μM.

INTRODUCTION

20α-Hydroxysteroid dehydrogenase [20α-HSD; 20α-hydroxysteroid:NAD(P)$^+$ oxidoreductase, EC 1.1.1.149], which catalyzes the interconversion of the 20-carbonyl group of pregnanes and 20α (20S)-hydroxy group, seems to be widely present in various organs, such as liver [1], ovary [2], testis [3, 4], adrenal [5] and placenta [6, 7] among several mammalian species. In particular, the ovarian [8, 9] and testicular enzymes [10, 11] were purified and characterized. Recently, we purified to homogeneity two distinct enzyme molecules (20α-HSD-I and 20α-HSD-II) responsible for 20α-HSD activity from pig adrenal cytosol and the enzymological properties were established [12]. There were remarkable differences between 20α-HSD-I and 20α-HSD-II on specific activity, isoelectric point, peptide mapping and on the influence of ionic strength, heat treatment and divalent cations, while there were only slight differences in the molecular weight and amino acid composition.

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Trival names and abbreviations: 17α-Hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; 5α-dihydroprogesterone (5α-DHP), 5α-pregnane-3,20-dione; 5β-dihydroprogesterone (5β-DHP), 5β-pregnane-3,20-dione; progesterone, 4-pregnene-3,20-dione; 5α-dihydrocortisol (5α-DHC), 11β,17α,21-trihydroxy-5α-pregnane-3,20-dione; 5β-dihydrocortisol (5β-DHC), 11β,17α,21-trihydroxy-5β-pregnane-3,20-dione; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; 5α-dihydrotestosterone (5α-DHT), 17β-hydroxy-5α-androstan-3-one; 5β-dihydrotestosterone (5β-DHT), 17β-hydroxy-5β-androstan-3-one; testosterone, 17β-hydroxy-4-androsten-3-one; 5α-androstanedione, 5α-androstane-3,17-dione; 5β-androstanedione, 5β-androstane-3,17-dione; androstenedione, 4-androstene-3,17-dione; furazabol, 17β-hydroxy-17α-methyl-5α-androstano[2,3-c]-furazan; cyanoketone, 2α-cyano-17β-hydroxy-4,4,17α-trimethylandrost-5-en-3-one; 20α-hydroxysteroid dehydrogenase (20α-HSD), 20α-hydroxysteroid oxidoreductase; 3α-hydroxysteroid dehydrogenase (3α-HSD), 3α-hydroxysteroid oxidoreductase; KPB, potassium phosphate buffer; DTT, dithiothreitol; HPLC, high performance liquid chromatography; GC, gas chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

In this paper, we describe the steroid substrate specificity of purified adrenal 20α-HSD-I and new findings of 3α-HSD activity catalyzed by purified enzyme.

EXPERIMENTAL

Materials

Radioactive [4-^{14}C]steroids, 17α-hydroxyprogesterone (2.0 GBq/mmol) and 5α-DHT (1.9 GB/mmol), were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). The following were supplied by Sigma Chemical Co. (St Louis, MO, U.S.A.): non-radioactive steroids; β-nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), and oxidized form ($NADP^+$). Other reagents used were from Iwai Chemicals (Tokyo, Japan).

Enzyme assay

Radioisotope method. The enzyme activity of 20α-HSD was examined as described previously [12]. For the assay of 3α-HSD activity, [4-^{14}C]5α-DHT was used as a substrate. This method is based on the conversion of [4-^{14}C]5α-DHT to [4-^{14}C]5α-androstane-3α,17β-diol. The substrate and product were separated by thin layer chromatography using benzene–acetone (8:2, v/v) as a developing solvent. Details were examined according to a previously described method [12].

Spectrophotometric method. The enzyme activity was determined by measurement of NADPH at 340 nm (ε, 6200 $cm^{-1} \cdot M^{-1}$). The purified enzyme was incubated with various steroids (50 nmol each/10 μl ethanol) in 1.0 ml of 50 mM KPB (pH 7.4) in 1.0 cm-path length cuvettes at 37°C, in the presence of cofactor, NADPH or $NADP^+$ (240 nmol each). Initial velocities were determined by measuring the change in absorption at 340 nm. These velocities were corrected for non-enzymic rates. The kinetic parameters were calculated from Lineweaver–Burk plots which were obtained by plotting the reciprocals of the apparent enzyme activity vs the reciprocals of the substrate concentration. K_i values were derived from measurements of the effect of several concentrations of steroids in the presence of five to six fixed substrate concentrations. Enzyme kinetic data were analyzed using a personal computer (NEC PC-9801VX).

GC and HPLC

A product, 5α-androstane-3α,17β-diol or 5β-androstane-3α,17β-diol, from the substrate was identified by GC [Shimadzu GC-4CM PF: column, 3% OV-17 on Chromosorb W (0.3 × 200 cm); injector temperature, 270°C; column temperature, 230°C; detector, FID] as the tri-methylsilyl-derivatives. The identification of 3α-hydroxy-5α-androstan-17-one and 3α-hydroxy-5β-androstan-17-one was performed by HPLC according to the method of Hunter *et al.* [13].

RESULTS

Steroid substrate specificity

The steroid substrate specificity of pig adrenal 20α-HSD-I is summarized in Table 1. The enzyme strongly reduced the 3-keto group of 5α (or 5β)-pregnanes (C_{21}) and 5α (or 5β)-androstanes (C_{19}) compared with the 20-keto group of 17α-hydroxyprogesterone. To assess

Table 1. Substrate specificity of pig adrenal 20α-HSD-I

Substrate	Enzyme activity[a] (nmol/min/mg)
Reduction	
17α-Hydroxyprogesterone[b]	0.85
17α-Hydroxy-5α-pregnane-3,20-dione	3.0
5α-Pregnane-3,20-dione (5α-DHP)	4.7
5β-Pregnane-3,20-dione (5β-DHP)	28.1
Progesterone	ND[c]
11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione (5α-DHC)	4.9
11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione (5β-DHC)	6.1
11β,17α,21-Trihydroxy-4-pregnene-3,20-dione (cortisol)	1.8
17β-Hydroxy-5α-androstan-3-one (5α-DHT)	12.5
17β-Hydroxy-5β-androstan-3-one (5β-DHT)	17.0
17β-Hydroxy-4-androsten-3-one (testosterone)	5.0
5α-Androstane-3,17-dione (5α-androstanedione)	28.2
5β-Androstane-3,17-dione (5β-androstanedione)	68.7
4-Androstene-3,17-dione (androstenedione)	3.1
3β-Hydroxy-5-androsten-17-one	ND
Oxidation	
3α-Hydroxy-5α-pregnan-29-one	ND
3α-Hydroxy-5β-pregnan-20-one	7.7
5α-Androstane-3α,17β-diol	6.6
5α-Androstane-3β,17β-diol	1.2
5β-Androstane-3α,17β-diol	11.5
5β-Androstane-3β,17β-diol	1.5
5-Androstene-3β,17β-diol	ND
3α-Hydroxy-5α-androstan-17-one	31.7
3β-Hydroxy-5α-androstan-17-one	ND
3α-Hydroxy-5β-androstan-17-one	32.2
3β-Hydroxy-5β-androstan-17-one	ND
3β-Hydroxy-5-androstan-17-one	ND
Testosterone	ND
Cholic acid	8.2
Deoxycholic acid	9.1

[a]The enzyme activity was determined spectrophotometrically by measurement of NADPH concentration at 340 nm. The purified enzyme (20α-HSD-I; 72 μg) was incubated with various steroids (50 nmol each/10 μl ethanol and 20 nmol/10 μl ethanol in the case of 17α-hydroxy-5α-pregnane-3,20-dione) in the presence of cofactor, NADPH or $NADP^+$ (240 nmol each), in 1.0 ml of KPB (pH 7.4) at 37°C. Further details are given in the text.

[b]The enzyme activity for 17α-hydroxyprogesterone and progesterone were obtained from work previously done in our laboratory [12].

[c]Not detected.

the substrate specificity of purified adrenal 20α-HSD-I for 5α (A/B *trans*) vs 5β (A/B *cis*) steroids, and C_{19}- vs C_{21}-steroids, the corresponding C_{19}-steroids, 5α (or 5β)-DHT and 5α (5β)-androstenedione, and C_{21}-steroids, 5α (or 5β)-DHP and 5α (or 5β)-DHC served as substrates in the reductive direction. As a result, adrenal purified enzyme was more active on the C_{19}-steroids than on the C_{21}-steroids, and it has a preference for the 5β-isomer steroids over the corresponding 5α-isomer steroids. 5β-Androstanedione was the best substrate. However, the reactivity to the 3-keto group of 4-unsaturated androstane (androstenedione) corresponding 5α (or 5β)-androstanedione was very low. On the other hand, in the oxidative direction, 3α-hydroxy and 3β-hydroxy isomers of 5α (or 5β)-pregnane steroids or various 5α (or 5β)-androstane steroids were tested to assess substrate specificity. It was demonstrated that the enzyme selectively oxidized the 3α-hydroxy groups of 5α (or 5β)-pregnan-20-one, 5α (or 5β)-androstan-17β-ol and 5α (or 5β)-androstan-17-one. In addition, the purified enzyme oxidized at relatively low rates cholic acid and deoxycholic acid which have 3α-hydroxy groups of 5β-cholanic acid. All the products reduced by the enzyme in the presence of NADPH when 5α-DHT, 5β-DHT, 5α-androstanedione or 5β-androstanedione were used as the substrate, were identified to be 5α-androstane-3α,17β-diol, 5β-androstane-3α,17β-diol; 3α-hydroxy-5α-androstan-17-one; and 3α-hydroxy-5β-androstan-17-one, by the method of GC or HPLC, respectively. From the above results, it was clear that 20α-HSD-I purified from pig adrenal cytosol strongly catalyzed the 3α-hydroxysteroid oxidoreductase activity.

Table 2. Kinetic parameters of adrenal 20α-HSD-I against the substrate

Substrate	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m (10^{-2})
Reduction			
5α-Androstanedione	3.3	69.7	2112
5β-Androstanedione	7.7	135.0	1753
5α-DHT	10.2	10.6	104
17α-Hydroxyprogesterone[a]	26.2	1.3	4.9
Oxidation			
3α-Hydroxy-5β-androstan-17-one	1.2	32.1	2675
3α-Hydroxy-5α-androstan-17-one	1.7	43.2	2541

Kinetic parameters for 17α-hydroxyprogesterone and 5α-DHT were determined from enzyme assay using radioactive steroids. Others were determined spectrophotometrically by measurement of NADPH concentration. The concentrations of NADPH or $NADP^+$ as a cofactor were 240 μM each. The Michaelis constant (K_m) and maximum velocity (V_{max}) were obtained by Lineweaver–Burk plots ($r = 0.9421$–0.9998).

[a]The kinetic constants for 17α-hydroxyprogesterone were obtained from work previously done in our laboratory [12].

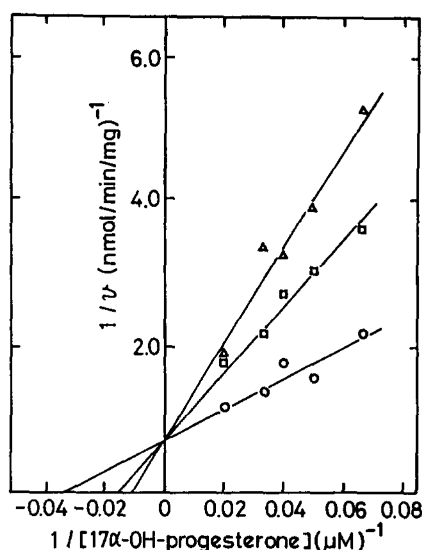

Fig. 1. Inhibition of 20α-HSD activity of adrenal 20α-HSD-I by 5α-DHT. The 20α-HSD-I (39 μg) was incubated with various concentrations of [4-^{14}C]17α-hydroxyprogesterone (15 to 50 μM) and non-radioactive 5α-DHT concentrations were 0 μM (○), 5 μM (□) and 10 μM (△) each. Incubation was carried out in 1 ml of 50 mM KPB (pH 7.4) at 37°C for 60 min.

Kinetic studies

The kinetic studies from some typical steroid substrates were performed and their kinetic parameters are summarized in Table 2. The apparent K_m and V_{max} values determined for the androstane steroid substrates, 5β-androstanedione, 5α-androstanedione and 5α-DHT were compared with that determined for 17α-hydroxyprogesterone which is the substrate for 20α-HSD activity. The K_m values for 5β-androstanedione, 5α-androstanedione and 5α-DHT were 3.4-, 7.9- and 2.5-fold lower than the value determined for 17α-hydroxyprogesterone. By contrast, the V_{max} values for 5β-androstanedione, 5α-androstanedione and 5α-DHT were 104-, 90- and 21-fold higher than the V_{max} value determined for 17α-hydroxyprogesterone. The parameter of substrate utilization efficiency (V_{max}/K_m) indicated that 3α-HSD activity against androgen substrates are more preferential than 20α-HSD activity.

Inhibition of 20α-HSD activity by 5α-DHT

5α-DHT, the substrate for 3α-HSD activity, inhibited strongly and competitively the apparent 20α-HSD activity for 17α-hydroxyprogesterone as a substrate (Fig. 1). From this result, 5α-DHT is a potent alternate substrate against 17α-hydroxyprogesterone with an apparent K_i value of 2.0 μM. On the other hand,

Table 3. Inhibition of 3α-HSD activity by various agents

Agent	Concentration (μM)	Inhibition (%)
Medroxyprogesterone acetate	0.1	0
	1	0.3
Indomethacine	10	7
Dexamethasone	10	7
Hexesterol	1	0
Stilbestrol	10	8
1,10-Phenanthroline	10	7
N-Ethylmaleimide	10	7
p-Chloromercuribenzoic acid	10	2
Frazabol	10	50
Cyanoketone	10	29
Hg^{2+}	1000	99
Cu^{2+}	1000	44

3α-HSD activity was determined with 5α-DHT (20 μM) as a substrate and NADPH (240 μM) in 50 mM KPB (pH 7.4) in the absence or presence of each agent. Further details are given in the text.

17α-hydroxyprogesterone also inhibited competitively the apparent 3α-HSD activity of 5α-DHT with relatively high concentration, more than 50 μM (data not shown). The K_i value was calculated to be 150 μM for 17α-hydroxyprogesterone. These results strongly suggest that the same active site of pig adrenal 20α-HSD-I accounts for both 20α-HSD activity and 3α-HSD activity.

Inhibition of 3α-HSD activity by various agents

The effects of various agents on 3α-HSD activity of pig adrenal 20α-HSD-I are summarized in Table 3. The enzyme activity was not inhibited by 3α-HSD inhibitor such as medroxyprogesterone acetate, indomethacine or dexamethasone. In addition, the synthetic estrogens, hexestrol and stilbestrol which inhibit dihydrodiol dehydrogenase, failed to inhibit the enzyme activity, neither did 1,10-phenanthroline, a specific inhibitor of indanol dehydrogenase. SH-reagents such as β-mercaptoethanol, *p*-chloromercuribenzoic acid and *N*-ethylmaleimide had little effect. On the other hand, cyanoketone as a specific inhibitor of 3β-HSD/isomerase and the anabolic steroid, frazabol inhibited the 3α-HSD activity. Furthermore, divalent cations such as Hg^{2+} and Cu^{2+} inhibited the 3α-HSD activity. Especially, Hg^{2+} strongly inhibited the enzyme activity, but the activity was resistant to inhibition by other divalent cations such as Cd^{2+}, Mg^{2+}, Ni^{2+}, Fe^{2+} and Zn^{2+}.

DISCUSSION

We have demonstrated that 20α-HSD-I purified from pig adrenal cytosol has appreciable 3α-HSD activity in addition to 20α-HSD activity. The enzyme has a preference for the androstane substrates, 5α (or 5β)-DHT and 5α (or 5β)-androstanedione in the reductive direction with NADPH as a cofactor (hydrogen donor), and 3α-hydroxy-5α (or 5β)-androstan-17-one and 5α (or 5β)-androstane-3α,17β-diol in the oxidative direction with $NADP^+$ as hydrogen acceptor. In addition, the enzyme has a slight preference for 5β-isomer steroids in redox reaction. That is, adrenal 20α-HSD-I exhibits 3α (axial, 3R and equatorial, 3R)-HSD activity and has a preferential substrate specificity for 5β-isomer steroids (equatorial, 3R). From these results, pig adrenal 20α-HSD-I was regarded as a poly-functional enzyme catalyzing both activities of 3α-HSD and 20α-HSD, so-called 3α,20α-HSD.

There are numerous cases of polyfunctional enzyme in steroid biosynthesis. For example, there are 3α,20β-HSD from *Streptomyces hydrogenans* [14], 17β,20α-HSD from human placenta [7, 15], 3α,3β,17β,20α-HSD from rabbit liver [16] and 3β,20β-HSD from bovine and sheep erythrocytes [17, 18]. Earlier, the affinity labeling method was applied to some of these enzymes, and it was clearly demonstrated that the same active site accounted for bifunctional enzyme activity on the enzyme in the case of 3α,20β-HSD [19], 17β,20α-HSD [20] and 3β,20α-HSD [21] occurred. Furthermore, a cytochrome *P*-450 enzyme (oxygenase) from neonatal pig testis that converts progesterone to androstenedione, was shown by affinity labeling to catalyze both the 17α-hydroxylation and C_{17}-C_{20} bond cleavage step at the same active site [22].

In the present paper the 20α-HSD activity of adrenal 3α,20α-HSD, in the reductive direction, is competitively inhibited by 5α-DHT with a K_i value of 2.0 μM. These data are considered to show that dual substrates, 17α-hydroxyprogesterone and 5α-DHT compete for the same catalytic active site on the enzyme. That is, 20α-HSD and 3α-HSD activity may be catalyzed at the same active site. Of course, further evidence from affinity labeling experiments is required to prove the bifunctional nature of the same active site on the enzyme.

3α-HSD [EC 1.1.1.50; 3α-hydroxysteroid: NAD(P) oxidoreductase] catalyzing the reversible interconversions of 3α-hydroxy and 3-keto group of steroid substrates is present in numerous animal tissues [23]. Several cytosolic 3α-HSDs have been purified and characterized from rat liver [24, 25], and brain [26], prostate [27, 28], pituitary [29] and mouse liver [30]. The

liver cytosolic 3α-HSD of rat and mouse appears to have a broad substrate specificity, since it also has dihydrodiol dehydrogenase [EC 1.3.1.20] activity [25, 31]. Another notable property of these cytosolic 3α-HSD enzymes is that they are strongly inhibited by a potent synthetic progestational steroid, medroxyprogesterone acetate, and by several anti-inflammatory drugs such as indomethacine and dexamethasone. On the other hand, it was reported that monkey liver indanol dehydrogenase [EC 1.1.1.112] exhibited 3(20)α-HSD activity, and the dehydrogenase activity was inhibited by medroxyprogesterone acetate, hexestrol and 1,10-phenanthroline [32]. The inhibitory effect of these agents on adrenal 3α,20α-HSD was inhibited neither by medroxyprogesterone acetate, indomethacine and dexamethasone used as the anti-inflammatory drugs, nor by hexestrol, stilbestrol and 1,10-phenanthroline. These results suggest that adrenal 3α,20α-HSD is clearly different in some respects from what has been reported for liver 3α-HSD and indalol dehydrogenase with 3(20)α-HSD activity. On the other hand, frazabol, an anabolic steroid with a furazan ring with the basic 17β-hydroxy-17α-methyl-5α-androstane structure, strongly inhibited the 3α-HSD activity of 20α-HSD-I.

The physiological role of this enzyme in the adrenal is not clear at this time. The liver cytosolic 3α-HSD has a broad substrate specificity beyond steroids since it can also use dihydrodiols as the substrate, and this enzyme was indistinguishable for dihydrodiol dehydrogenase [24]. At the first step for clarifying the physiological role of this adrenal enzyme, further investigation is required of substrate specificity for xenobiotics or prostaglandins beyond steroid for this enzyme.

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