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20β-HYDROXYSTEROID DEHYDROGENASE OF NEONATAL PIG TESTIS: 3α/β-HYDROXYSTEROID DEHYDROGENASE ACTIVITIES CATALYZED BY HIGHLY PURIFIED ENZYME

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Summary—Pig testicular 20β-hydroxysteroid dehydrogenase (20β-HSD) has also 3α- and 3β-HSD (3α/β-HSD) activities. The purified 20β-HSD preparation from neonatal pig testes could catalyze the conversion of 5α-dihydrotestosterone (5α-DHT) in the presence of β-NADPH to 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol at the ratio of 4:3, and the specific 3α/β-HSD activity of 20β-HSD for 5α-DHT was about 10 or 15 times larger than the 20β-HSD activities for 17α-hydroxypregn-4-ene-3,20-dione (17α-hydroxyprogesterone) or progesterone, respectively. The result indicates that the testicular 20β-HSD has high 3α(axial, 3*R*)- and 3β(equatorial, 3*S*)-HSD activity. The testicular 20β-HSD could catalyze the reversible conversion of various 5α- or 5β-dihydrosteroids which have a 3-carbonyl or 3-hydroxyl group with β-NADP(H) as the preferred cofactor. The enzyme transferred the 4-*proS* hydrogen of NADPH to the 5α-DHT for both 3α- and 3β-hydroxylation and it was the same as the 20β-hydroxylation of 17α-hydroxyprogesterone. Although the 3α/β-HSD activity has been known to be present in 3α,20β-HSD of *Streptomyces hydrogenans*, the enzymological properties for 3α/β-HSD activity catalyzed by testicular 20β-HSD were different from the properties for 3α/β-HSD activity catalyzed by prokaryotic 3α,20β-HSD with respect to the specificity of the catalytic reaction and the cofactor requirement.

INTRODUCTION

Among the enzymes concerned with steroidogenesis, it is known that the pyridine nucleotide dependent 20β-hydroxysteroid dehydrogenase [20β-HSD; (EC 1.1.1.53)] is present, which catalyzes the interconversion of oxidation and reduction between the 20-carbonyl and 20β-hydroxyl groups of the C_{21}-steroid. The enzyme occurs in one of the mycelium of *Streptomyces hydrogenans* as a cortisone reductase. It was purified and crystallized and its enzymological properties were investigated [1, 2]. In further studies, investigations have found other catalytic activity at the C_3-position of steroids, such as 3α-HSD and 3β-HSD activity in crystalline 20β-HSD preparations from *S. hydrogenans* [3, 4].

On the other hand, the smallest amounts of 20β-HSD activity in animal species are reported to occur in the human ovary [5], human testis [6], young bull testis [7] and in ovarian tissues of the teleosts, ayu [8] and amago salmon [9]. Recently, we reported that a large quantity of 20β-HSD activity specifically existed in pig testis during the neonatal stage [10]. Then the enzyme was purified for the first time to homogeneity from animal species as demonstrated on several gel electrophoreses (SDS–PAGE, disc–PAGE and isoelectric focusing), and as a result, remarkable differences were observed from prokaryotic 20β-HSD (so-called 3α,20β-HSD) with regard to enzymological properties [11, 12]. However, from the result of substrate specificity, it was considered that testicular 20β-HSD will also have oxidoreductase activities at other positions in addition to the C_{20} position of C_{21}-steroids [13].

In this paper, we show that pig testicular 20β-HSD also has 3α/β-HSD activity as well as

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Abbreviations: 5α-DHT, 17β-hydroxy-5α-androstan-3-one; 17α-hydroxyprogesterone, 17α-hydroxypregn-4-ene-3,20-dione; 20β-HSD, 20β-hydroxysteroid dehydrogenase; KPB, potassium phosphate buffer; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine-5′-triphosphate; TLC, thin layer chromatography; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

prokaryotic $3\alpha,20\beta$-HSD. Therefore, the basic enzymological properties for $3\alpha/\beta$-HSD activity were investigated to compare with those of prokaryotic $3\alpha,20\beta$-HSD.

EXPERIMENTAL

Materials

The following were supplied by Sigma Chemical Co. Ltd (St Louis, MO, U.S.A.): β-nicotinamide adenine dinucleotide phosphate (β-$NADP^+$), reduced form (β-NADPH); α-nicotinamide adenine dinucleotide phosphate (α-$NADP^+$), reduced form (α-NADPH); β-nicotinamide adenine dinucleotide 3′-phosphate (β-3′-$NADP^+$), reduced form (β-3′-NADPH); β-nicotinamide adenine dinucleotide (β-NAD^+), reduced form (β-NADH); α-nicotinamide adenine dinucleotide (α-NAD^+), reduced form (α-NADH); glucose 6-phosphate (G-6-P); isocitric acid; adenosine-5′-triphosphate (ATP); glucose 6-phosphate dehydrogenase (G-6-P-DH); and isocitric dehydrogenase and nicotinamide adenine dinucleotide (NAD^+)-kinase. Trimethyl silyl(TMS)-imidazole was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Radioactive materials. [4-^{14}C]17β-Hydroxy-5α-androstan-3-one (5α-dihydrotestosterone: 5α-DHT, 1.9 GBq/mmol) was purchased from New England Nuclear Corp. (Boston, MA, U.S.A.) and [4-^{3}H]NAD^+ (74 GBq/mmol) was purchased from Amersham International plc (Bucks., England).

Other reagents used were of the best grade available and obtained from Iwai Chemicals (Tokyo, Japan).

Purification of 20β-HSD from neonatal pig testes

The method for preparing purified 20β-HSD from neonatal pig testes has been described in a previous report [11]. 20β-HSD was purified to homogeneity as judged by SDS–PAGE stained with Coomassie blue R-250. In addition, the sharp and single protein peak was observed using HPLC (Waters 650 advanced protein purification system) on a Protein Pack G-DEAE column (1 × 5 cm, Waters), with a gradient from 1 to 100 mM KPB (pH 7.4)–0.1 mM EDTA, as shown in Fig. 1.

Enzyme activity

The assay of $3\alpha/\beta$-HSD activities was carried out spectrophotometrically. This method was based on the production or consumption of NADPH by 20β-HSD catalysis. 5α-DHT (50 nmol/10 μl ethanol) or 5α-androstane-3α,17β-diol (50 nmol/10 μl ethanol; estimated as 3α-HSD activity) was incubated in a quartz cuvette (1 cm path, 1 ml) with 20β-HSD in the presence of NADPH or $NADP^+$, respectively (250 nmol), in 1 ml of 50 mM KPB (pH 7.4) at 37°C. The change in absorbance at 340 nm vs time was continuously monitored. A molecular extinction coefficient of 6200[cm·mol]$^{-1}$ was used. Under these conditions, the 20β-HSD activity was obtained with a high rectilinear relationship to the incubation time from 0 to 10 min along with the amount of 20β-HSD from 0 to 212.8 μg with a high correlation coefficient of 0.9972.

Detection of 3α/β-HSD activity with two-dimensional TLC (2D-TLC)

[4-^{14}C]5α-DHT (300 Bq/50 nmol/10 μl ethanol) was incubated with purified 20β-HSD in the presence of an NADPH-generating system ($NADP^+$ 250 nmol, G-6-P 5 μmol, G-6-P-DH 1 U, $MgCl_2$ 0.5 μmol) in 1 ml of 50 mM KPB (pH 7.4) for 30 min at 37°C. After the incubation, 10 ml of CH_2Cl_2 was added to the

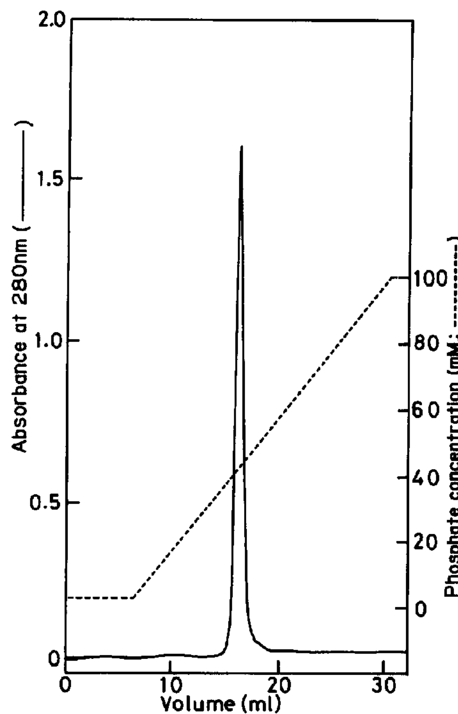

Fig. 1. HPLC chromatogram of purified 20β-HSD on protein pack G-DEAE.

incubation medium and the mixture was shaken vigorously. After removal of the aqueous fluid, the organic solvent extract was dried with anhydrous sodium sulfate and evaporated under a nitrogen stream at 40°C. The residue was applied to a TLC plate (Kodak, 13181 silica gel with fluorescent indicator, 10 × 10 cm), and processed with two-dimensional development with a benzene–acetone mixture (8:2) as the first solvent and benzene–ethylacetate (2:1) as the second one. The spots were detected by the method of radioautography (Fuji X-ray film, RX), and standard steroids were detected in saturated iodine vapor.

Identification of 3α- and 3β-HSD activity by gas chromatography

For identification of the main product, whether the 3α- or 3β-hydroxy form of 5α-DHT, non-radioactive 5α-DHT (2 mg/0.4 ml of ethanol) was incubated with purified 20β-HSD (1 mg) in the presence of an NADPH-generating system in 40 ml of 50 mM KPB (pH 7.4) for 5 h at 37°C. After TLC (Merck Kiesergel 60F254, 10 × 20 cm) with a benzene–acetone mixture (8:2 v/v, twice), the main area was cut out and the steroid was eluted with CH_2Cl_2 and trimethyl silylated with TMS–imidazole at room temperature. Gas chromatography was performed with a Shimadzu GC-4CM PF using a column of OV-1 (1%) on Gaschrom Q (0.3 × 200 cm) at 240°C, flow rate, 40 ml/min, detector, f.i.d. The metabolites were identified by comparing the retention times with standard steroids treated at the same conditions.

Preparation of [4-proR-^{3}H]NADPH and [4-proS-^{3}H]NADPH

The method for preparing the two stereospecific ^{3}H-labeled NADPHs was described in a previous report [12]. A slight modification was employed for preparing the [4-^{3}H]NADP$^+$ step. That is, [4-^{3}H]NAD$^+$ (636 kBq/8.6 nmol) was phosphorylated by incubation with NAD$^+$-kinase (6 U) in the presence of ATP (5 μmol) and NAD$^+$ (150 nmol) in 0.65 ml of 50 mM KPB (pH 7.0)–0.8 mM $MgCl_2$ for 30 min at 37°C and then the mixture was divided into two equal parts. The method for preparation of [4-*proR*-^{3}H]NADPH and [4-*proS*-^{3}H]NADPH from [4-^{3}H]NADP$^+$ as the same as in a previous report [12].

Stereospecificity of hydrogen transfer

The mixture, including [4-*proR*-^{3}H]NADPH or [4-*proS*-^{3}H]NADPH (25 nmol/tube as total NADPH), was incubated with [4-^{14}C]5α-DHT (1000 dpm/50 nmol) and purified 20β-HSD (44.5 μg) in 1 ml of 50 mM KPB (pH 7.0) for 30 min at 37°C. Mixtures of steroids were separated with TLC and the spots were detected by radioautography as previously described. The relevant radioactive areas of spots on the chromatogram were cut out and placed directly in scintillation vials. Methanol (1.0 ml) was added to the portion of the TLC plate in the vial to release the steroid. The ^{14}C and ^{3}H radioactivity of each spot was directly counted with a liquid scintillation counter (Packard Tri-Carb 460) in 10 ml of toluene based on a scintillator containing 2,5-diphenyloxazole (0.4%, w/v) and 2,2′-*p*-phenylene-bis-(5-phenyloxazole) (0.01%, w/v). The 3α/β-HSD activity was determined from the radioactivity of the area corresponding to 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol on the TLC plate. The enzyme activity was corrected for recovery, which was calculated from the ratio of total radioactivity of the areas of the substrate and product on the TLC plate to that of the added radioactive steroid.

RESULTS

Detection and identification of 3α/β-HSD activity of purified 20β-HSD

In the presence of the NADPH-generating system, it was found that the purified 20β-HSD could serve the substrate of [4-^{14}C]5αDHT as at least three kinds of metabolites when the mixture of metabolite steroid was separated by 2D-TLC developed with benzene–acetone (8:2) and benzene–ethylacetate (2:1) (Fig. 2). Although two minor spots were faint, a large main spot showed the same migration with two standard steroids which introduced 3α- or 3β-hydroxy groups to 5α-DHT, 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. On these TLC systems, it was impossible to clearly separate these two 3-hydroxysteroids.

On the other hand, a gas chromatographic method was employed for separate detection of 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol after treatment by trimethysilylation. The main metabolite steroid eluted from the TLC plate was separated into two major peaks and these peaks were identified from the

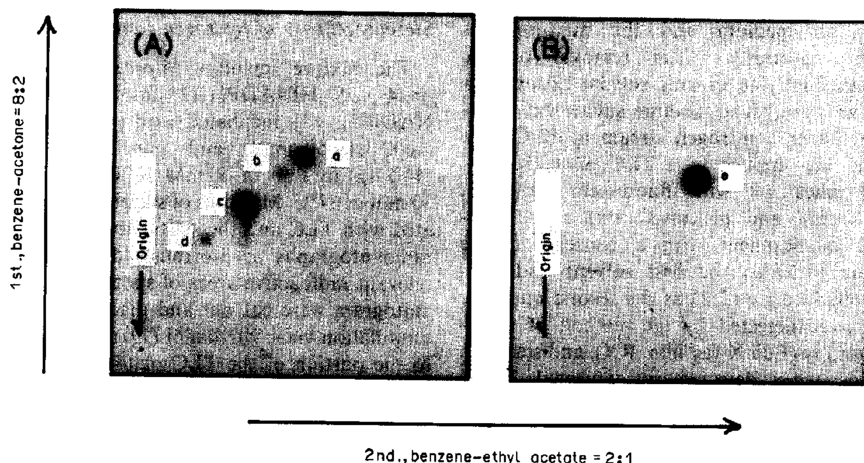

Fig. 2. Radioautograms obtained by TLC on radioactive metabolites from [4-^{14}C]5α-DHT. [4-^{14}C]5α-DHT was incubated in the presence of purified 20β-HSD (A) or in the absence of the enzyme (B) with an NADPH-generating system. Dark spots are (a, e) 5α-DHT, (b) unknown metabolite 1, (c) 3α- or 3β-hydroxy-5α-androstan-17β-ol and (d) unknown metabolite 2, which was obtained by comparison of the migration with non-radioactive standard steroids.

retention times compared with standard steroids of 5α-androstane-3α,17β-diol (8.08 min) and 5α-androstane-3β,17β-diol (10.01 min) (Fig. 3). The ratio of 3α-hydroxysteroid to 3β-hydroxysteroid calculated from the peak areas was about 1.39. This suggested that the pig testicular purified 20β-HSD has both 3α (axial, 3R)- and 3β (equatorial, 3S)-HSD activities when 5α-DHT was employed as the substrate in the presence of β-NADPH, and the ratio of specific activities (3α-HSD/3β-HSD) was estimated at about 4:3.

Substrate specificity of 20β-HSD

As shown in Table 1, when NADPH was used as a cofactor (hydrogen donor), 20β-HSD could catalyze the reduction not only of 5α-DHT but also of various C_{19}-steroids which mainly have a 3-carbonyl group. Among the steroids which usually exist in animal bodies, 5α-DHT, 5α-pregnane-3,20-dione and 5α-androstane-3,17-dione were specifically reduced with a high activity by 20β-HSD. Furthermore, the 20β-HSD showed that it prefers 5α-saturated steroids to 5β-saturated steroids or 4-en-steroids for its substrate. In addition, the specific 3α/β-HSD activity of 20β-HSD obtained from the substrate of 5α-DHT was about 10 and 15 times larger than the original specific 20β-HSD activity obtained at the same conditions from the substrate of 17α-hydroxyprogesterone and progesterone, respectively. In addition the specific activity for 5α-pregnane-3,20-dione was about 17 times larger than for progesterone.

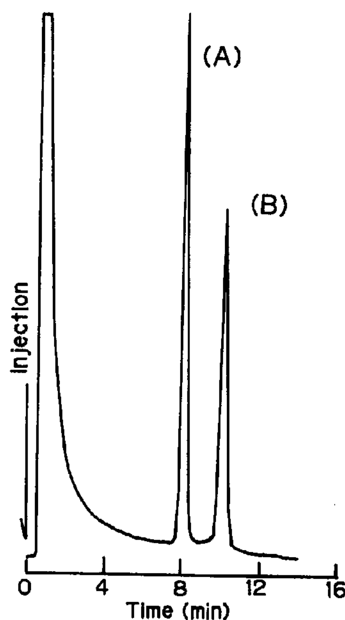

Fig. 3. Gas chromatography of the main metabolite from 5α-DHT. The main metabolite eluted from TLC was silylated with TMS–imidazole at room temperature. Gas chromatography was performed under the following conditions: column, *OV*-1 (1%) on Gaschrom Q (0.3 × 200 cm); temperature, 240°C (column) and 270°C (injector); detector, f.i.d.; carrier gas, N_2 (40 ml/min). Peaks: (A) 5α-androstane-3α,17β-diol and (B) 5α-androstane-3β,17β-diol.

Table 1. Substrate specificity of 20β-HSD on the reaction of steroid reduction

Steroids[a]	Sp. act.[b] (nmol/min/mg)	%	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m
5α-Androstan-17β-ol-3-one (5α-DHT)	46.0	100	1.79	172.6	96.4
5β-Androstan-17β-ol-3-one (5β-DHT)	15.2	33.0	1.90	149.9	78.9
5α-Androstane-3,17-dione (androstanedione)	33.2	72.2	8.45	363.1	43.0
5β-Androstane-3,17-dione	14.7	32.0			NE[c]
5α-Androstan-3α-ol-17-one (androsterone)	27.1	58.9			NE
5α-Androstan-3β-ol-17-one (epiandrosterone)	22.2	48.3			NE
5α-Pregnane-3,20-dione	50.6	110.0	1.60	98.4	61.5
17α-Methyl-5α-androstan-17β-ol-3-one	91.1	198.0			NE
5α-Ol-androstan-17β-ol-3-one	45.5	98.9			NE
4-Androstene-3,17-dione (androstenedione)	7.2	15.7			NE
4-Androsten-17β-ol-3-one (testosterone)	9.7	21.1			NE
5-Androsten-3β-ol-17-one (dehydroisoandrosterone)	23.4	50.9			NE
4-Pregnen-17α-ol-3,20-dione (17α-hydroxyprogesterone)	3.9	8.5	9.42	9.6	1.02
4-Pregnene-3,20-dione (progesterone)	3.0	6.5	1.54	4.4	2.86

Kinetic parameters were determined from Lineweaver–Burk plots.
[a]Trivial names given in parentheses.
[b]Specific activity; assays were carried out with steroid (50 μM), NADPH (250 μM) and 20β-HSD (36.5 μg) in 50 mM KPB (pH 7.4) at 37°C.
[c]Not examined.

On the other hand, when $NADP^+$ was used as a cofactor (hydrogen acceptor), a high specificity was observed against the structure of the substrate steroid if compared with the specificity of the steroid reduction reaction (Table 2). Among the steroids tested, 5α-androstane-3α,17β-diol was converted most rapidly by purified 20β-HSD. The enzyme could also catalyze the conversion of various steroids which have 3α- or 3β-hydroxy-5α- or 5β-androstan structure, but it was seen that a tendency to 3β-hydroxysteroids and 5β-saturated steroids was far less effective than 3α-hydroxysteroids and 5α-saturated steroids, respectively. Furthermore, the oxidation activity of the 5-en-3β-hydroxy group and 17β-hydroxy group were very weak or negligible.

Cofactor requirement

Several pyridine nucleotide cofactors, besides the usual β-NADP(H) and β-NAD(H), including the analogue type in which the phosphate at the 2′ position is transferred to the 3′ position or nicotinamide moiety linked on the α-side, were examined as hydrogen donors for reduction of 5α-DHT or as hydrogen acceptors for oxidation of 5α-androstane-3α,17β-diol. The Michaelis constants (K_m) and maximum velocity (V_{max}) of 20β-HSD for various cofactors are shown in Table 3. Judged from V_{max}/K_m values for each cofactor, β-NADPH for reduction of 5α-DHT and β-$NADP^+$ for oxidation of 5α-androstane-3α,17β-diol were the most preferential cofactors of 20β-HSD. It was

Table 2. Substrate specificity of 20β-HSD on the reaction of steroid oxidation

Steroids[a]	Sp. act.[b] (nmol/min/mg)	%	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m
5α-Androstane-3α,17β-diol	51.8	100	0.72	22.6	31.4
5α-Androstane-3β,17β-diol	1.2	2.3			NE[c]
5β-Androstane-3α,17β-diol	19.0	36.7			NE
5β-Androstane-3β,17β-diol	12.4	24.0			NE
5α-Androstan-3α-ol-17-one (androsterone)	25.2	48.6	3.20	59.4	18.6
5α-Androstan-3β-ol-17-one (epiandrosterone)	9.1	17.6			NE
5β-Androstan-3α-ol-17-one	4.2	8.2			NE
5β-Androstan-3β-ol-17-one	6.4	12.4			NE
Cholic acid	ND[d]				
Deoxycholic acid	ND				
5α-Androstan-17β-ol-3-one (5α-DHT)	ND				
5β-Androstan-17β-ol-3-one (5β-DHT)	ND				
17α-Methyl-5α-androstan-17β-ol-3-one	ND				
5α-Ol-androstan-17β-ol-3-one	ND				
5-Androstene-3β,17β-diol (androstenediol)	ND				
5-Androsten-17β-ol-3-one	ND				
4-Androsten-17β-ol-3-one (testosterone)	0.92	1.8			NE
5-Pregnen-3β-ol-20-one (pregnenolone)	ND				
4-Pregnen-20β-ol-3-one	3.1	6.0			NE

Kinetic parameters were determined from Lineweaver–Burk plots.
[a]Trivial names given in parentheses.
[b]Specific activity; assays were carried out with steroid (50 μM), β-$NADP^+$ (250 μM) and 20β-HSD (36.5 μg) in 50 mM KPB (pH 7.4) at 37°C.
[c]Not examined.
[d]Not detected; enzyme activity was not detected under the assay condition.

Table 3. Cofactor requirement for $3\alpha/\beta$-HSD activity catalyzed by 20β-HSD

Cofactors	Rel. act.[a] (%)	K_m (μM)	V_{max} (nmol/min/mg)	V_{max}/K_m
Reduced form				
β-NADPH	100	7.2	115.9	16.2
α-NADPH	64.8	328.0	141.8	0.43
β-3′-NADPH	79.2	452.8	275.2	0.61
β-NADH	36.5	294.8	79.0	0.28
α-NADH	8.0	126.1	19.7	0.16
Oxidized form				
β-NADP$^+$	100	14.8	46.1	3.11
α-NADP$^+$	ND[b]			NC[c]
β-3′-NADP$^+$	4.5	5.1	3.9	0.76
β-NAD$^+$	103.5	88.2	50.6	0.57
α-NAD$^+$	ND			NC

Kinetic parameters were determined from Lineweaver–Burk plots.
[a]Relative activity; assays were performed in 5α-DHT (50 μM; for reduced form) or 5α-androstane-$3\alpha,17\beta$-diol (50 μM; for oxidized form), 20β-HSD (35.6 μg) and pyridine nucleotide cofactor (240 μM) in 1 ml of 50 mM KPB (pH 7.4) at 37°C.
[b]Not detected; enzyme activity was not detected under the range of cofactor concentration (3.75–240 μM).
[c]Not calculated.

observed that other pyridine nucleotides, except for α-NADP$^+$ and α-NAD$^+$, were also utilized for the $3\alpha/\beta$-HSD activity of 20β-HSD when provided in relatively high concentrations.

Stereospecificity of hydrogen transfer from NADPH

The results of the incubation of [4-^{14}C]5α-DHT with [4-*proR*-^{3}*H*]NADPH or with [4-*proS*-^{3}H]NADPH in the reduction catalyzed by testicular 20β-HSD (for $3\alpha/\beta$-HSD activity) are shown in Table 4. A significant incorporation of tritium into the products, 5α-androstane-$3\alpha,17\beta$-diol and 5α-androstane-$3\beta,17\beta$-diol, were observed when 5α-DHT was incubated with [4-*proS*-^{3}H]NADPH, as compared with the incubation with [4-*proR*-^{3}H]NADPH. Incorporation of ^{3}H into 5α-androstane-$3\alpha,17\beta$-diol and 5α-androstane-$3\beta,17\beta$-diol when [4-*proS*-^{3}H]NADPH was about 13.2 times larger than that for the case of [4-*proR*-^{3}H]NADPH as expressed in terms of dpm of tritium/nmol of product steroid.

These results demonstrated that the hydrogen transfer from β-NADPH to 5α-androstane-$3\alpha,17\beta$-diol and 5α-androstane-$3\beta,17\beta$-diol, catalyzed by both the 3α and 3β-HSD activities of testicular 20β-HSD, is stereospecific and the 4-*proS* hydrogen is transferred to the products.

Optimum pH for $3\alpha/\beta$-HSD activity of testicular 20β-HSD

The effect of pH on $3\alpha/\beta$-HSD activity was examined in K_2HPO_4–H_3PO_4 buffer systems (100 mM, pH 4.5–9.0, data not shown). The maximum enzyme activity was observed at pH 5.0 when 5α-DHT and β-NADPH were used as substrate and cofactor, respectively. The enzyme activity rapidly decreased when the incubation pH was lowered to 5.0, while it only gradually decreased when the pH was increased beyond 5.0. The rate of enzyme activity at the optimum pH of 5.0 was about 11 times higher than that at pH 7.4, which was usually employed for the incubation.

Effect of divalent cations for $3\alpha/\beta$-HSD activity of 20β-HSD

The $3\alpha/\beta$-HSD activity of purified 20β-HSD was strongly inhibited by heavy metal ions such as Hg^{2+}, Cu^{2+}, Fe^{2+} and Cd^{2+} (data not shown). However, other divalent cations did not seem to have any significant effect on enzyme activity.

DISCUSSION

The pig testicular 20β-HSD had been purified to homogeneity and was shown as a single band on SDS–PAGE gel and on some other electrophoreses, and also as a sharp single peak with HPLC. Recently, we have demonstrated the slightly enzymological behavior of this enzyme preparation for catalyzing the oxidation in some types of C_{21}-steroids, which have no 20β-hydroxy group, in the presence of β-NADP$^+$ [13]. Therefore, it was expected that testicular 20β-HSD also have 3α- or 3β-HSD activity like prokaryotic $3\alpha,20\beta$-HSD. In the results, testicular 20β-HSD showed both 3α (axial, $3R$)- and 3β (equatorial, $3S$)-HSD activities for the substrate of 5α (A/B, *trans*)-DHT as the activated form of androgen (testosterone) in animal species in the presence of β-NADPH although prokaryotic $3\alpha,20\beta$-HSD catalyzed only 3α (axial, $3R$)-HSD activity with 5α-DHT as the substrate. Furthermore, although prokaryotic

Table 4. Stereospecificity of hydrogen transfer from NADPH to $3\alpha/\beta$-hydroxy-5α-androstan-17β-ol by 20β-HSD

Cofactor	^{3}H/nmol Product (dpm)	$3\alpha/\beta$-HSD activity (nmol/min/mg)	Ratio of ^{3}H/^{14}C
[4-*proS*-^{3}H]NADPH	438.2 ($\pm$4.34)	32.1 ($\pm$0.12)	22.2 ($\pm$0.49)
[4-*proR*-^{3}H]NADPH	33.3 ($\pm$0.30)	31.9 ($\pm$0.05)	3.0 ($\pm$0.04)

$3\alpha/\beta$-HSD activities were determined by assay using [4-^{14}C]5α-DHT (50 nmol) as the substrate in 50 mM KPB (pH 7.0).

3α,20β-HSD showed the same or lower 3α-HSD activity compared with 20β-HSD activity [16], the 3α/β-HSD activity of testicular enzyme for 5α-DHT was about 10–15 times larger than the 20β-HSD activity. The testicular enzyme catalyzed the reduction not only for 5α-DHT but also for 5β(A/B,*cis*)-DHT, like a prokaryotic enzyme [4]. The catalytic reaction at the 3-position of the 4-en-3-one steroid and 17β-hydroxy group were also obtained as weak signals. However, in the reverse reaction, which employed β-NADP$^+$ as a cofactor, a comparatively strong relationship between steroid structure and enzyme activity has seen, so the enzyme activity was obtained within the hydroxy group at the 3α- and 3β-positions of the 5α- or 5β-saturated steroids. From these phenomena, pig testicular 20β-HSD was regarded as a polyfunctional enzyme of 3α/β,20β-HSD, just like the 3α,20β-HSD from *S. hydrogenans* [4], 3α,3β,17β,20α-HSD in rabbit liver [14] and 3β,20α-HSD from sheep fetal blood [15] etc.

On the other hand, the 3α,20β-HSD from *S. hydrogenans* was reported to catalyze both the 3α-HSD and 20β-HSD activities in the same active site on one protein [16–18]. In the case of testicular 20β-HSD, there is no proof yet which 3α/β- and 20β-HSD activities are catalyzed on the same site of the enzyme molecule. However, comparison of some of the enzymological properties obtained from 3α/β- and 20β-HSD activities is worth consideration. No significant difference was observed in cofactor requirement, stereospecificity of hydrogen transfer from NADPH, optimum pH, and the effect of divalent cations; in particular, the result of hydrogen transfer from NADPH into substrate 5α-DHT was observed as 4-*proS*, and the use of hydrogen at the same position of the nicotinamide moiety for both 3α- and 3β-hydroxylation of 5α-DHT was interesting. From these results, the possibility that pig testicular 20β-HSD also catalyzed the 3α/β- and 20β-HSD activities at the same active site on the enzyme was postulated.

17α,20β-Dihydroxypregn-4-en-3-one, which is supposed to be catalyzed by 20β-HSD from 17α hydroxyprogesterone, was generally recognized for its physiological effect as one of the most potent inducers of oocyte maturation in some kinds of fish [19–21] and is related to the spermiation of salmonids [22]. However, the physiological significance of this steroid in mammalian species is not yet clear. Therefore, the presence of 3α/β-HSD activity on the same protein of 20β-HSD which specifically exists at the neonatal stage in testis as one of the tissues concerning reproduction and as one of the targets of androgens, is very interesting when considering the steroidgenesis in mammals.

Recently, a primary structure of prokaryotic 3α,20β-HSD was deduced [23]. We need to clarify the nucleotide sequence of cDNA and the amino acid sequence of pig testicular 20β-HSD as the basic research for further investigation of this enzyme in animal species. For this purpose, cDNA cloning of pig testicular 20β-HSD is now in progress.

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