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Purification and Characterization of 3α/β-Hydroxysteroid Dehydrogenase from Mature Porcine Testicular Cytosol

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NADPH-dependent 3α/β-hydroxysteroid dehydrogenase (3α/β-HSD) was purified to apparent homogeneity from testicular cytosol of mature pigs. The purified enzyme catalyzes the conversion of 5α-dihydrotestosterone (5α-DHT) to both 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol. The molecular weight of the enzyme was estimated to be 31 kDa by SDS–polyacrylamide gel electrophoresis and 40 kDa by gel filtration chromatography indicating that the native 3α/β-HSD is a monomer. The isoelectric point of the purified enzyme was found to be 6.2 by density gradient isoelectric focusing and 6.4 by chromatofocusing. The enzyme reduced both 5α- and 5β-DHT, 5α- and 5β-dihydroprogesterone, 5α- and 5β-dihydrocortisol, prostaglandin E_2, 13,14-dihydro-15-keto-prostaglandin E_2 and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$. Moreover, the enzyme caused rapid reduction of other carbonyl compounds including aldehydes, ketones and quinones. The rates of reduction of these compounds are fast relative to the rates of reduction of steroids and prostaglandins. The purified enzyme was inhibited by $AgNO_3$, SH-reagent, quercetin, hexesterol, stilbestrol, disulfiram and divalent cation such as Cu^{2+}, Hg^{2+} and Cd^{2+}. The two enzymes show certain similarities (e.g. molecular weight, cross-reactivity to a common antibody) and certain striking differences (e.g. pI, effects of various inhibitors and greater enzyme activity towards steroids (neonatal form) or prostaglandins (mature form). Reasons are give for suggesting that these enzymes are closely related to carbonyl reductase.

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INTRODUCTION

The ambient levels of 5α-dihydrotestosterone (5α-DHT) in target organs for androgens are regulated by the rate of synthesis of this androgen from testosterone and the rate of its conversion to the 3α and 3β reduced products [1]. We previously reported that a highly active form of 20β-HSD enzyme exists in neonatal pig testis [2]. The relevant enzyme has been purified and characterized in this laboratory [3–5]. Moreover, it was found that pig testicular 20β-HSD shows strong 3α (axial, 3R) and 3β (equatorial, 3S)-HSD (3α/β-HSD) activities. That is, the purified mammalian 20β-HSD is also capable of catalyzing the conversion of 5α-DHT to both 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol in the presence of β-NADPH [6]. We have previously reported that both mature and neonatal pig testes contain a high activity of 3α/β-HSD with 5α-DHT as a substrate [7]. The specific activity of 3α/β-HSD is high in the neonatal testis, low at 60 days and then rises again to high levels in the mature animal.

Consequently, we proposed that two molecular forms of 3α/β-HSD may be present in pig testis—one in the neonatal pig which shows both 3α/β-HSD and 20β-HSD activity and a different enzyme in the

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Abbreviations: 3α-hydroxysteroid dehydrogenase (3α-HSD), 3α-hydroxysteroid oxidoreductase; 3β-hydroxysteroid dehydrogenase (3β-HSD), 3β-hydroxysteroid oxidoreductase; 20β-hydroxysteroid dehydrogenase (20β-HSD), 20β-hydroxysteroid oxidoreductase; KPB, potassium phosphate buffer; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PGE_2, prostaglandin E_2; 15KD-PGE_2, 13,14-dihydro-15-keto-prostaglandin E_2; 15KD-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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mature type animal which shows $3\alpha/\beta$-HSD without 20β-HSD activity.

We have purified and characterized the enzyme from neonatal testis [3]. In the present study we report the purification of the enzyme from mature pig testis ($3\alpha/\beta$-HSD only). We also compare the properties of the two enzymes.

EXPERIMENTAL

Preparation of pig testicular cytosol

Fresh pig testes (1 year of age) were obtained from the slaughter house and were immediately transported to the laboratory in ice-cold 0.15 M KCl–1 mM EDTA solution. The testes were decapsulated and the samples were weighted and dissected into smaller pieces with scissors. The weighed samples were homogenized with 4.5 vol 0.15 M KCl–0.1 mM EDTA–0.01% PMSF using a Waring blender. The homogenate was centrifuged at 10,000 *g* for 20 min. The resulting supernate was further centrifuged at 105,000 *g* for 60 min. The final supernate was used as the cytosol fraction. All of the above procedures were carried out at 4°C or under an ice bath.

Purification of $3\alpha/\beta$-HSD

All purification procedures were carried out in a cold room at 4°C or with an ice bath.

Step 1, ammonium sulfate fractionation. The cytosol fraction was brought to 30% saturation by adding solid ammonium sulfate. During the addition of ammonium sulfate, the pH of solution was maintained at 7.0 by adding aqueous ammonia. The mixture was stirred for 30 min and then centrifuged at 12,000 *g* for 15 min. The resulting supernatant was raised to 80% ammonium sulfate saturation, stirred for 30 min and similarly centrifuged. The resulting pellet was dissolved in 3 mM KPB (pH 7.4)–0.1 mM EDTA–0.01% PMSF (KPB basal buffer) and the solution was applied to a Sephadex G-25 column (2.8 × 51 cm) equilibrated with 3 mM KPB basal buffer to remove the ammonium sulfate.

Step 2, chromatography on DEAE–cellulose. The precipitate obtained from 30–80% ammonium sulfate saturation was then applied to a DE-52 column (3.4 × 47 cm) which had been equilibrated with 3 mM KPB basal buffer. Elution was carried out with a linear concentration gradient obtained from 3–100 mM KPB basal buffer (1200 ml each). The fractions eluted at a concentration of 20 mM KPB basal buffer were pooled to give pool I.

Step 3, chromatography on Matrex gel blue A. The DE-52 fraction (pool I) was applied to a column (1.9 × 6.8 cm) of Matrex gel blue A which had been equilibrated with 20 mM KPB basal buffer. The elution was carried out with a linear concentration gradient from 0 to 3 M NaCl in 20 mM KPB basal buffer (100 ml each). The enzyme activity was eluted at a concentration of approx. 0.75 M.

Step 4, chromatofocusing. To exchange the buffer, the Matrex gel blue A fraction was applied to a Sephadex G-25 column (2.5 × 51 cm), which had been equilibrated with 25 mM Tris–acetate buffer (pH 8.3)–0.1 mM DTT–0.01% PMSF, and then applied to a chromatofocusing column (1 × 32 cm) packed with the Polybuffer exchanger PBE 94 and equilibrated with the same buffer. A linear pH gradient from 8.0 to 5.0 was generated through the column by application of 15 bed volumes of Polybuffer 74/acetic acid, pH 5.0 containing 0.1 mM DTT and 0.01% PMSF. The main peak of $3\alpha/\beta$-HSD activity was eluted at approx. pH 6.4.

Step 5, HPLC on protein pack G-DEAE. The fraction containing enzyme activity after chromatofocusing was applied to a Sephadex G-75 column (1.9 × 76 cm) equilibrated with 3 mM KPB basal buffer, and the Polybuffer was removed. The enzyme fraction was then further purified by HPLC (Waters 650 advanced protein purification system) on a Protein Pack G-DEAE column (0.8 × 7.5 cm, waters) with a linear concentration gradient from 3 to 100 mM KPB basal buffer. $3\alpha/\beta$-HSD activity was eluted as a sharp peak at around 60 mM KPB basal buffer. The relevant fractions were pooled to serve as the final preparation of the enzyme. The preparation was stored at −20°C.

Enzyme assay

The assay of $3\alpha/\beta$-HSD activity was carried out in a spectrophotometer cuvette. This method is based on the consumption of NADPH by an enzyme reaction. 5α-DHT (50 nmol/10 μl ethanol) was incubated in a cuvette (1 cm path, 1 ml) with the enzymes in the presence of 180 μM NADPH in 1 ml of 50 mM KPB (pH 7.4) at 37°C. For examination of substrate specificity, substrates at various concentrations (dissolved in 10 μl ethanol) were incubated with the enzyme in place of DHT and in the presence of 80 μM NADPH in 1.0 ml of 60 mM sodium phosphate (pH 6.5) at 37°C. To examine the inhibitory effects of various agents on enzyme activity, 9,10-phenanthrenequinone was used as a substrate instead of 5α-DHT, and 5 μl of ethanol solution or aqueous solution of each agent was added to the incubation medium. The reaction was started by addition of NADPH to the assay mixture. The change in absorbance at 340 nm with time was monitored continuously in a Hitachi 228 spectrophotometer. Cuvettes without substrate or enzyme were routinely included to serve as background. A molecular extinction coefficient of 6200 $[\mathrm{cm} \cdot \mathrm{mol}]^{-1}$ was used.

For identification of the product, either the 3α- or 3β-hydroxy steroid corresponding to 5α-DHT (100 nmol/10 μl ethanol) was incubated with the enzyme in the presence of an NADPH-generating system in 2 ml of 50 mM KPB (pH 7.4) for 2 h at 37°C. Following incubation the steroid including the product was extracted with methylene dichloride and subjected to trimethylsilylation with *N,O*-bis-trimethylsilyl-acetoamide at room temperature. The trimethylsilylated derivatives were then analyzed by

gas-liquid chromatography. Gas-liquid chromatography was performed with a Shimadzu GC-4CM PF using a column of OV-17 on Chromosorb W. The metabolites were identified by comparing the retention times with a standard steroid treated under the same conditions.

Purification of 3α/β (20β)-HSD

3α/β (20β)-HSD was purified from the testes obtained from neonatal pig (2 weeks of age) after castration according to a method reported previously [3].

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to the method of Laemmli [8]. The molecular weight markers (molecular weight in parentheses) used for SDS–PAGE were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Isoelectric focusing

Density gradient-isoelectric focusing for the preparation of enzyme was done at 4°C in a 110-ml glass column according to the method reported by Vesterberg [9].

Western blot analysis

Western blot analysis of 3α/β-HSDs was carried out as described previously [7].

Peptide mapping

The purified 3α/β-HSD was subjected to proteolytic digestion at 37°C for 15 min in 50 mM KPB (pH 7.4) with a lysyl endopeptidase (mole ratio of enzyme/substrate = 1/50). The peptide fragments resulting from this procedure were separated by SDS–PAGE (15% acrylamide gel).

Protein determination

Protein concentrations were estimated by the method of Lowry *et al.* [10] with crystalline bovine serum albumin (Armour Pharmaceutical Co., Fraction V) as a standard. During column chromatography, the concentration of protein was estimated by measuring the absorbance at 280 nm.

Chemicals

Polybuffer exchanger PBE 96 and polybuffer 96 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), and Matrex gel blue A from the Amicon division of W. R. Grace & Co., Conn. (Beverly, MA, U.S.A.). PGs were purchased from Funakoshi Chemical (Tokyo, Japan), and lysyl endopeptidase (Achromobactor protease I) was obtained from Wako Chemicals (Tokyo, Japan). The various materials were obtained from sources already report [2–7, 12] except for the above materials. Other reagents were of the best grade available and obtained from Iwai Chemicals Co. (Tokyo, Japan).

RESULTS

Steroid reductase activity of testicular cytosol of mature pigs

Cytosol was dialyzed, applied to DEAE–cellulose and eluted with a linear concentration gradient obtained from 3–100 mM KPB basal buffer. Oxidation of NADPH, with 5α-DHT as substrate, by eluted

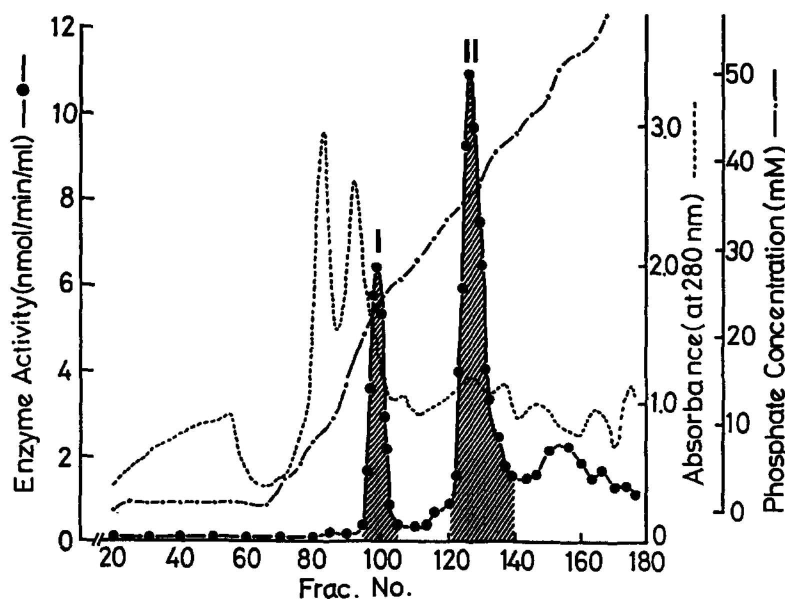

Fig. 1. Column chromatography on DEAE–cellulose. The cytosol prepared from mature porcine testis (1.9 g) was dialyzed with 3 mM KPB (pH 7.4) and applied to column (2.5 × 45 cm) of DEAE–cellulose (DE-52) which had been equilibrated with 3 mM KPB (pH 7.4)–0.1 mM EDTA–0.1 mM DTT. The column was then eluted with a linear concentration gradient of KPB from 3 to 100 mM. Fractions of 7.5 ml each were collected. Proteins were monitored by measuring absorption at 280 nm, and aliquots were assayed for enzyme activity.

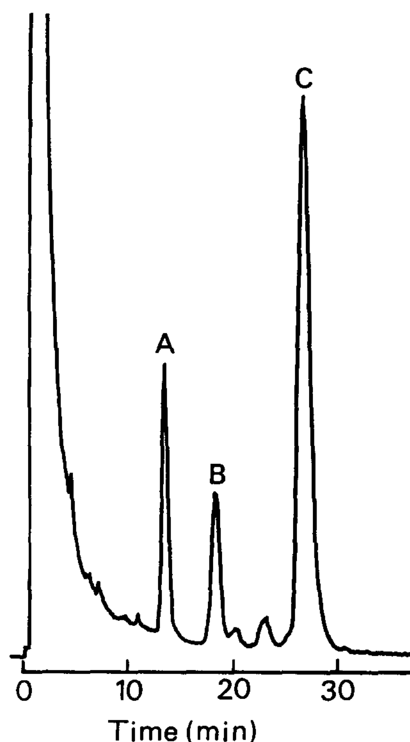

Fig. 2. Gas-liquid chromatography of steroid metabolites of 5α-DHT. Methylene dichloride extract was silylated with TMS-imidazole at room temperature. Operating conditions are described in the Experimental section. Peak assignments: (A), 5α-androstane-3α,17β-diol; (B), 5α-androstane-3β,17β-diol; (C), 5α-DHT.

fraction was found to occur in two major peaks (at approx. 20 and 37 mM KPB) and in a broad unresolved peak (Fig. 1). These peaks are referred to as pools I (20 mM) and II (37 mM), respectively. The products of reduction of 5α-DHT were found by gas-liquid chromatography to be 5α-androstane-3α,17β-diol and 5α-androstane-3β,17β-diol for pool I thereby suggesting the presence of 3α/β-HSD in this pool.

Purification of 3α/β-HSD from testicular cytosol of mature pig

Cytosol was subjected to ammonium sulfate precipitation and the precipitate from 30 to 80% saturation was applied to a column of DEAE–cellulose. The 5α-DHT reductase activity was eluted in two major peaks as before (Fig. 1). Pool I was further purified by the three steps detailed under the Experimental section. Progressive purification through Matrex gel blue A chromatography, chromatofocusing and HPLC are shown in Fig. 3(A–C). Progressive increase in specific activity and fold-purification of the enzyme are shown in Table 1. A Purification of 55-fold for the entire procedure with a yield of 3.7% was obtained.

Properties of 3α/β-HSD

Figure 4 shows that the enzyme appears as a single band (M_W 31 kDa) on SDS–PAGE after preceding purification. Gel filtration chromatography on Sephadex G-100 gave a value of 40 kDa for the molecular weight of the nature enzyme (data not shown). The isoelectric point of the purified enzyme was determined by chromatofocusing and density gradient-isoelectric focusing to be 6.4 and 6.2, respectively.

Substrate specificity of 3α/β-HSD

The substrate specificity of the enzyme is shown in Table 2 in which it can be seen that the purified enzyme reduces both 5α- and 5β-DHT so that the enzyme acts on both *trans* and *cis* configurations of the A and B rings. Moreover both DHP (5α and 5β) and DHC (5α and 5β) were reduced by the enzyme showing that it acts on pregnan steroids. However the following steroids were not reduced: testosterone, androsterone, progesterone and 17α-hydroxyprogesterone. These finding also indicate that the enzyme from mature pigs has no detectable 20β-reductase activity. Moreover the enzyme possesses strong activity with PGE_2,

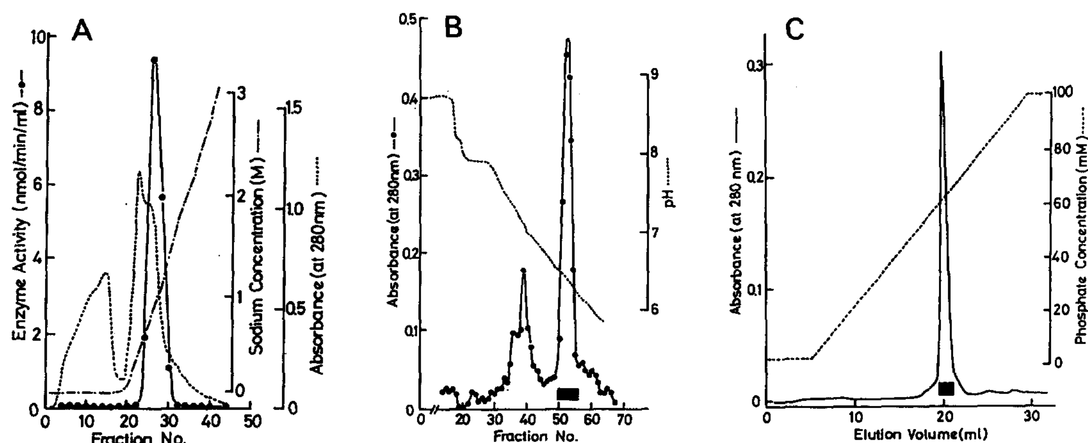

Fig. 3. Elution patterns at various stages of purification. (A) Matrex gel blue A chromatography, (B) chromatofocusing, (C) HPLC on protein pack G-DEAE. Details of chromatography procedures are described in the Experimental section.

Table 1. Purification of $3\alpha/\beta$-HSD from testicular cytosol of mature pig

Purification step	Protein (mg)	Protein (%)	$3\alpha/\beta$-HSD activity sp.act.[a] (nmol/min/mg)	$3\alpha/\beta$-HSD activity T.A.[b] (nmol/min)	Purification (fold)
Cytosol	5444	100	1.33	7240	1.0
$(NH_4)_2SO_4$ Ppt.	4252	78.1	2.24	9525	1.7
DEAE–cellulose	141	2.6	8.58	1209	6.5
Matrex gel blue A	46.1	0.85	17.9	827	13.5
Chromatofocusing	7.3	0.13	58.0	421	43.6
HPLC	3.7	0.07	72.9	269	54.8

[a]Specific activity, [b]total activity.
Enzyme activity at each step was assayed in 50 mM KPB (pH 7.4) at 37°C using 5α-DHT (25 μM) as a substrate and NADPH (180 μM) as a cofactor.

15KD-PGE_2 and 15KD-$PGF_{2\alpha}$. In addition, purified $3\alpha/\beta$-HSD reduced many carbonyl compounds, such as 9,10-phenanthrenequinone, menadion, hydridantin, methylglyoxal etc.

Inhibition of steroid reductase enzymes by various agents

It was decided to compare the effects of various inhibitors on $3\alpha/\beta$-HSD (mature testis) and $3\alpha/\beta$-(20β)-HSD (neonatal testis) are shown in Table 3. $3\alpha/\beta$-HSD from mature pig testis was strongly inhibited by $AgNO_3$, *p*-chloromercuribenzoic acid, and quercetin which are known to be potent inhibitors of carbonyl reductase. In addition, the synthetic estrogens, hexestrol and stilbestrol, which inhibit dihydrodiol dehydrogenase, also inhibited the enzyme activity of $3\alpha/\beta$-HSD, whereas inhibitors of 3α-HSD, such as medroxyprogesterone and indomethacin, and the specific inhibitor of indanol dehydrogenase, 1,10-phenanthroline, failed to inhibit enzyme activity. Further, divalent cations such as Cu^{2+}, Hg^{2+} and Cd^{2+} strongly inhibited the enzyme activity of $3\alpha/\beta$-HSD. The inhibitory effects of these agents on neonatal type $3\alpha/\beta(20\beta)$-HSD were similar to those seen with mature type $3\alpha/\beta$-HSD. On the other hand, the neonatal type $3\alpha/\beta(20\beta)$-HSD was inhibited by steroids such as progesterone, 17α-hydroxyprogesterone, testosterone and deoxycorticosterone which were without effect on enzyme from mature testes. The degree of inhibition by $AgNO_3$, *p*-chloromercuribenzoic acid, quercetin and hexestrol, followed by the divalent cation such as Zn^{2+}, Sr^{2+} and Co^{2+} for the neonatal type $3\alpha/\beta(20\beta)$-HSD was less than that seen with the mature form.

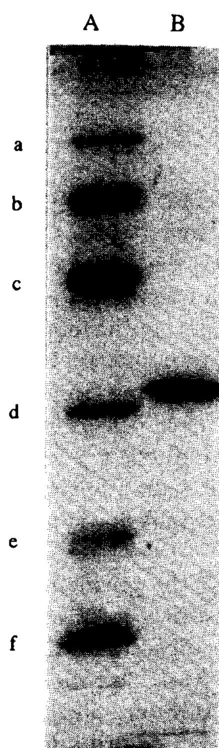

Fig. 4. SDS–PAGE of purified $3\alpha/\beta$-HSD from pig testicular cytosol. Gel electrophoresis was carried out with a slab of 12% polyacrylamide gel (1 mm thick, 6 cm long) as the lower separation gel at 20 mA/slab gel at room temperature. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250. Lane A, molecular weight markers (a, 94 kDa; b, 67 kDa; c, 43 kDa; d, 30 kDa; e, 20.1 kDa; f, 14.4 kDa); lane B, purified enzyme (1.3 μg). The top of the gel represents the cathode.

Peptide maps of steroid reductase enzymes

Peptide maps prepared from digestion with lysyl endopeptidase of $3\alpha/\beta$-HSD from mature pig testis and $3\alpha/\beta(20\beta)$HSD from neonatal pig testis are shown in Fig. 5. The peptide mapping patterns of the two $3\alpha/\beta$-HSD enzymes were significantly different.

Immunochemical properties of steroid reductase enzyme

Polyclonal antibodies were raised in rabbits against the neonatal enzyme. Western blots of the purified enzymes were performed after electrophoresis on SDS–polyacrylamide gels. As shown in Fig. 6, both enzymes cross-reacted antibody raised against the enzyme from neonatal testes.

DISCUSSION

It is generally agreed that in some target organs for androgens, testosterone must be reduced to the active form i.e. DHT before it acts [1]. In such tissues testosterone could be classified as a pro-hormone. In this conversion to DHT, considerable interest attaches to the subsequent metabolism of this product

Table 2. Substrate specificity of 3α/β-HSD from mature pig testis, and the comparison with that of 3α/β-(20β)-HSD from neonatal pig testis

Substrate	Final conc. (mM)	sp.act.[a] 3α/β-HSD (mature type)	sp.act.[a] 3α/β(20β)-HSD (neonatal type)
17β-Hydroxy-5α-androstan-3-one (5α-DHT)	0.05	91.9	235
17β-Hydroxy-5β-androstan-3-one (5β-DHT)	0.05	84.6	74.4
5α-Androstane-3,17-dione (5α-androstanedione)	0.05	75.1	252
5β-Androstane-3,17-dione (5β-androstanedione)	0.05	56.2	123
Testosterone	0.05	5.8	ND
Androstenedione	0.05	2.2	18.4
Dehydroepiandrosterone	0.05	4.5	21.2
5α-Pregnane-3,20-dione (5α-DHP)	0.05	16.2	247
5β-Pregnane-3,20-dione (5β-DHP)	0.05	41.7	64.3
Progesterone	0.05	ND	7.2
17α-Hydroxyprogesterone	0.05	ND	21.5
11β,17α,21-Trihydroxy-5α-pregnane-3,20-dione (5α-DHC)	0.05	144	315
11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione (5β-DHC)	0.05	85.1	264
Cortisol	0.05	1.8	16.6
PGE_1	1.0	61.2	ND
PGE_2	1.0	138	6.6
15KD-PGE_2	1.0	382	35.8
15KD-$PGF_{2\alpha}$	1.0	194	24.2
4-Benzoylpyridine	1.0	3034	59.3
4-Carboxybenzylaldehyde	0.5	34.2	15.8
4-Nitroacetophenone	0.1	93.0	52.8
4-Nitrobenzaldehyde	0.5	888	260
Menadione	0.25	4086	1638
9,10-Phenanthrenequinone	0.01	12458	1056
Phenylglyoxal	1.0	1016	226
Methylglyoxal	50	1441	667
Hydridantine	0.1	3372	1001
Caprinaldehyde	0.2	72.6	7.8
Cyclohexanone	10	572	250
Camphorquinone	0.05	640	269

[a]nmol/min/mg; [b]not detected.

Table 3. Effect of various agents on enzyme activities of 3α/β-HSD from mature pig and 3α/β(20β)-HSD from neonatal pig testis

Addition	Final conc.	Inhibition 3α/β-HSD	Inhibition 3α/β (20β) -HSD
$AgNO_3$	0.001	89	36
p-Chloromercuribenzoic acid	0.01	93	74
Querucetin	0.1	91	16
Hexestrol	0.1	81	31
Stilbestrol	0.1	63	57
Disulfiram	0.1	85	40
Indomethacin	0.1	15	0
1,10-Phenanthroline	0.1	6	17
Medroxyprogesterone acetate	0.1	0	12
Dexamethasone	0.1	0	82
Progesterone	0.01	0	61
17α-Hydroxyprogesterone	0.1	0	72
Deoxycorticosterone	0.1	16	65
Testosterone	0.1	7	68
Dicoumarol	0.01	5	0
$CuSO_4$	0.1	84	68
$HgCl_2$	1.0	98	100
$CdCl_2$	1.0	98	100
$ZnCl_2$	1.0	45	0
$SrCl_2$	1.0	35	0
$CoCl_2$	1.0	33	0

of reduction since metabolism of the active androgen (DHT) would have the effect of limiting androgenic activity in the target tissue in question.

In the present report we have purified the 3α/β-HSD from mature testis to homogeneity as demonstrated by SDS–PAGE. This enzyme occurs in the cytosol and is clearly distinct from the neonatal enzyme which, in addition of 3-reductase activity, also acts as a 20β-reductase. We have shown previously that the enzyme from mature pigs dose not reduce the 20 ketone [7].

The properties of the purified enzyme form mature pigs clearly distinguish it from the neonatal form. The form from mature pigs shows a molecular weight of 31 kDa by SDS–PAGE and 40 kDa by gel filtration. Although SDS–PAGE are likely to provide a more accurate method for determining molecular weight than gel filtration, the importance of the latter method lies in the clear demonstration that the native enzyme is monomeric. In these respects the two forms (neonatal and adult) are similar since the monomeric reductase form the neonatal pig shows a M_w of 30.5 kDa. Again both enzymes cross-react with antibodies raised against the neonatal enzyme. In contrast to these similarities, the isoelectric points of the two proteins are quite

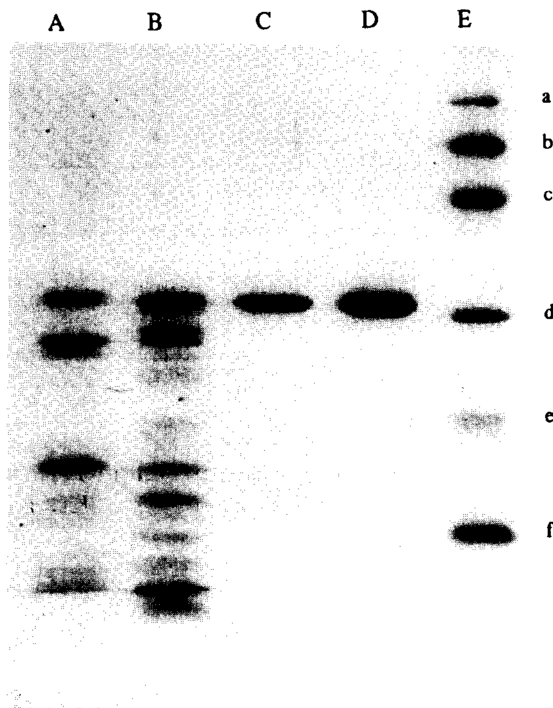

Fig. 5. Peptide mapping of $3\alpha/\beta$-HSD from mature pig testis and $3\alpha/\beta(20\beta)$-HSD from neonatal pig testis was dialyzed at 37°C for 15 min with lysyl endopeptidase (mole ratio of enzyme/substrate = 1/50). Products of proteolysis were separated by SDS–PAGE (15% polyacrylamide gel) and the gel was stained with Coomassie brilliant blue R-250. Lane A, digest of $3\alpha/\beta$-HSD (1.9 μg) from mature pig testis; lane B, digest of $3\alpha/\beta(20\beta)$-HSD (2.9 μg) from neonatal pig testis; lane C, undigested $3\alpha/\beta$-HSD (0.8 μg); lane D, undigested $3\alpha/\beta(20\beta)$-HSD (1.1 μg); lane E, molecular weight markers (a, 94 kDa; b, 67 kDa; c, 43 kDa; d, 30 kDa; e, 20.1 kDa; f, 14.4 kDa). The top of the gel represents the cathode.

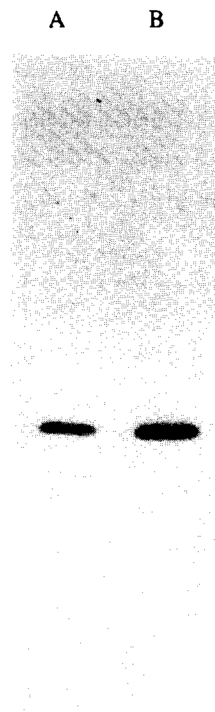

Fig. 6. Western blot analysis with antibody for $3\alpha/\beta(20\beta)$-HSD form neonatal pig testis. The purified enzymes were applied to SDS–PAGE (12% polyacrylamide gel), transferred to nitrocellulose and then immunochemically detected with antiserum raised in rabbit against the $3\alpha/\beta(20\beta)$-HSD as described in the Experimental section. Lane A, $3\alpha/\beta$-HSD (28 ng) from mature pig testis; lane B, $3\alpha/\beta(20\beta)$-HSD (23 ng) from neonatal pig testis. The top of the gel represents the cathode.

different; that of the neonatal enzyme is considerably more acidic than that of the adult form (pH 5.5 as opposed to 6.2 or 6.4 for neonatal and adult forms, respectively). Moreover significant differences are seen between peptide maps for the two proteins.

Presumably these differences account for differences in substrate specificity. It is worthy of note that although steroids and prostaglandins are considered the most likely physiological substrate for these enzymes, both enzymes show higher activities with a variety of chemical substrates that are unlikely to be of physiological importance to the two testicular reductase enzymes e.g. a variety of carbonyl compounds, aldehydes, ketones and quinones. In this connection it is interesting to consider the group of enzymes called carbonyl reductase [EC 1.1.1.184]. These enzymes constitute a family of soluble oxidoreductases that use NADPH to reduce a variety of biologically and pharmacologically active xenobiotic carbonyl compounds. The enzymes differ among themselves in size and charge. They are isolated from a number of sources and more than one form may be found in a given tissue [11–13]. Carbonyl reductase has been isolated from various reproductive tissues including testis [14–16]. The authors of those papers made a careful study of this last group of carbonyl reductase enzymes and classified them into two groups: group one shows high affinity for steroids and group two high affinity for prostaglandins. Both of the $3\alpha/\beta$-HSD enzymes described here show carbonyl reductase activity with the neonatal form showing greater activity with steroids (type one) and the enzyme from mature testis showing greater activity with prostaglandins (type two). Furthermore it has recently been reported that a cDNA clone for the neonatal form of the porcine testicular enzyme [$3\alpha/\beta(20\beta)$-HSD] corresponds to an amino acid sequence that shows 85% homology with human carbonyl reductase [17]. Evidently the two $3\alpha/\beta$-HSD enzymes discussed in this communication belong to or are closely related to the carbonyl reductase family of enzymes.

Although reduction of the ketone at C_3 is commonly thought of as a disposal reaction, the fact that this reaction removes the active form of androgens from the tissue in question suggests that the two forms of $3\alpha/\beta$-HSD could play important regulatory roles by limiting the action of androgens in the tissues concerned. It will be important to understand the role of 3-reductase activity in testes of young and mature animals.

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