J. steroid Biochem. Vol. 23, No. 6A, pp. 1001–1006, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

PURIFICATION AND CHARACTERIZATION OF 20α-HYDROXYSTEROID DEHYDROGENASE FROM BULL TESTIS

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(*Received* 25 *April* 1985)

Summary—20α-Hydroxysteroid dehydrogenase (20α-HSD) from bull testis has been purified to homogeneity and characterized in terms of apparent molecular weight, lack of subunit composition, substrate and cofactor specificity and certain kinetic parameters. The enzyme activity is localized in the 105,000 *g* supernatant and is stable at 4°C in the presence of glycerol and dithiothreitol. Purification was achieved by ammonium sulfate precipitation followed by affinity chromatography on reactive red 120-agarose and subsequent gel filtration. The apparent molecular weight of the homogeneous enzyme, as determined by gel filtration on Sephacryl S-300 is 34,000. The mobility of the enzyme in sodium dodecyl sulfate (SDS) gel electrophoresis corresponds to a mol. wt of 40,000. These observations indicate that the enzyme is a single-stranded, monomeric polypeptide. The enzyme catalyzes the reduction of the 17-hydroxyprogesterone to 17,20α-dihydroxy-4-pregnene-3-one in the presence of NADPH, the preferred cofactor. Homogeneous 20α-HSD has an SA of 115 mIU/mg, and has been purified 14,000-fold with an overall 68% recovery. It exhibits a pH optimum at 5.6 and appears to be highly specific for 17-hydroxyprogesterone with an apparent K_m-value of 7.3×10^{-5} M. Androstenedione and corticosterone do not serve as substrates under the described experimental conditions. The enzyme does not possess 17α- or 17β-HSD activity.

INTRODUCTION

20α-Hydroxysteroid dehydrogenase (20α-HSD) activity has been reported in various organs (liver, ovary, testis, adrenal, placenta) among several mammalian species. It has been purified from rat ovary [1], rat testis [2] and boar testis [3, 4]. In the rat the substrate specificity of 20α-HSD varies as a function of the organ of source. Progesterone is the preferred substrate for the ovarian enzyme [1], while 17-hydroxyprogesterone is preferred by the testicular enzyme [2]. Further, 17-hydroxyprogesterone is also the preferred substrate for the porcine testicular enzyme [4], as well as for the bull testicular enzyme (as we have confirmed herein). We are interested in obtaining mammalian steroid interconverting enzymes for comparative studies with human placental estradiol 17β-dehydrogenase (EC 1.1.1.62), which has been crystallized [6] and studied extensively [6–8], and the equine placental epimeric estradiol dehydrogenases (17α- and 17β-), which have been purified and characterized [9] in our laboratory. While porcine testicular 20α-HSD has been purified [4], adult boar testes are difficult to obtain and we found little activity in the prepubertal porcine testis (J. A. Pineda and J. C. Warren, unpublished observations). Therefore, we have turned our attention to the enzyme from bull testis. This report describes purification and partial characterization of bovine testicular 20α-HSD.

EXPERIMENTAL

Reagents, organic solvents, Scintiverse™, reagent-grade salts and Eastman silica gel plates (No. 6060) were obtained from Fisher. Pyridine nucleotide cofactors (NADH, NADPH), dithiothreitol, progesterone, 17α-estradiol, 17β-estradiol, estrone, *p*-nitrophenyl acetate, Coomassie blue R250, Sephacryl S-300, nitro blue tetrazolium and phenazine methosulfate were obtained from Sigma. 17-Hydroxyprogesterone, 17,20α-dihydroxy-4-pregnene-3-one and other steroids were purchased from Steraloids Inc. The following were purchased from Bio-Rad Labs: acrylamide; *N,N,N′,N′*-tetramethyethylenediamine; *N,N′*-methylbisacrylamide; ammonium persulfate; sodium dodecylsulfate (SDS); glycine; Tris; and molecular weight standards. The AcA-34 gel filtration media was from LKB. The polyacrylamide gel calibration kit was obtained from Pharmacia. [4-^{14}C]progesterone (57 mCi/mmol) and [4-^{14}C]17-hydroxyprogesterone (50 mCi/mmol) were from New England Nuclear. [4-^{14}C]estrone (55 mCi/mmol), [1α,2α(n)-^{3}H]corticosterone (42 Ci/mmol) and [1,2,6,7,(n)-^{3}H]androstenedione (90 Ci/mmol) were purchased from Amersham. Dialysis tubing (Spectrapor, No. 2) was Fisher. The water used throughout the procedure was double distilled and deionized.

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Trivial names used: androstenedione, 4-androstene-3,17-dione; progesterone, 4-pregnene-3,20-dione; 17-hydroxyprogesterone, 17-hydroxy-4-pregnene-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione.

Enzyme assays

The purity of the radioactive and cold steroids was confirmed by TLC. Steroids were dissolved in absolute ethanol, 7.5 μmol/ml. Unless otherwise noted, enzyme activity was measured by the production of radioactive 17,20α-dihydroxy-4-pregnene-3-one from [4-^{14}C]17-hydroxyprogesterone. Total volume of the assay mixture was 1.0 ml. It contained 0.15 μmol of the appropriate steroid and 0.5 μmol of NADPH in 0.1 M phosphate buffer, pH 7.0, with 10 mM cysteine. Some assays were conducted using NADH as the cofactor. Samples were incubated for 15 min at 37°C after addition of the enzyme. The reaction was terminated by extraction of the mixture with 10 ml of methylene chloride. The organic phase was evaporated under nitrogen and the residue dissolved in 50 μl of ethanol. An aliquot was chromatographed on 2 × 7 cm strips of thin-layer silica gel (Eastman, No. 6060), developed in chloroform–ethylacetate–ethanol (25:2:1). This adequately separates the reaction product from the substrate. The chromatograms were cut in 0.5 cm strips and counted in 5 ml of scintillation fluor each in a Packard Tri-Carb, Model 3320 liquid scintillation spectrometer. Efficiency of the radioactivity determination was 90% for ^{14}C and 50% for ^{3}H. Recovery of the radioactivity was 85%. One unit of 20α-HSD activity is defined as the amount of enzyme reducing 1 μmol of 17-hydroxyprogesterone/min. Protein concentrations were determined by the method of Bradford[10] with crystalline bovine serum albumin as standard.

Purification of 20α-HSD

Ordinarily, two bull testes were collected fresh, transported in ice and decapsulated. All purification steps were carried out at 4°C. The tissue (450 g) is homogenized in 0.01 M potassium phosphate buffer, pH 7.0, containing 20% glycerol and 1 mM dithiothreitol (buffer A) at a tissue–buffer ratio of 1:1 in a Waring blender (five 20 s bursts) and in a Brinkman Polytron (five 30 s bursts). The pH of the homogenate is adjusted to pH 7.0 with dropwise addition of 1.0 N NaOH before centrifugation.

The homogenate is centrifuged at 8000 *g* for 30 min, and the initial supernatant is centrifuged a second time under the same conditions. The resulting supernatant is centrifuged twice at 105,000 *g* for 90 min. The final high-speed supernatant is brought to 40% saturation by addition of solid ammonium sulfate with continuous adjustment of pH to 7.0 using 1.0 N NaOH. The mixture is stirred for 2 h and then centrifuged at 8000 *g* for 30 min. The remaining supernatant is brought up to 80% ammonium sulfate saturation with pH adjustment as above, stirred for 2 h and similarly centrifuged. The resulting pellet is redissolved in a minimal volume of buffer A. At least 70% of the measurable activity is recovered in the 40–80% fraction with an average 10-fold purification. The solubilized material is dialyzed for 48 h against buffer A. The enzyme is stable for up to 8 weeks if maintained at 4°C after this step. The dialysate is applied to a column of reactive red 120-agarose (4.5 μmol ligand/ml of gel, 1.8 × 52 cm column) at a rate of 3 ml/h and washed extensively with buffer A. The enzyme is eluted with buffer A containing 0.1 mM NADPH. The fractions containing 20α-HSD activity are pooled and applied to an AcA-34 gel filtration column (3 × 60 cm) and eluted with buffer A at 10 ml/h.

Polyacrylamide gel electrophoresis

Disc gel electrophoresis was carried out according to the method of Davis[11] at pH 7.0 The acrylamide concentration in the running gels was 7.5%; gels were run at 4 mA/tube in a Hoefer chamber with a ISCO power supply. Gels were stained for protein with Coomassie blue [12] after being fixed in 12.5% (w/v) trichloroacetic acid for 1 h. A duplicate gel was stained for 20α-HSD activity by zymography according to Karavolas *et al.*[13].

Subunit structure and molecular weight of the 20α-HSD from bull testis was determined by SDS polyacrylamide gel electrophoresis as described by Laemmli[14]. The gels were run at 2 mA/tube. The molecular weight standard curve was established by running a separate gel with phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme of mol. wt 92,500, 66,200, 45,000, 31,000, 21,500 and 14,400, respectively.

Molecular weight determination by gel filtration

A column of Sephacryl S-300 (2.1 × 90 cm) was equilibrated with buffer A. A mixture of thyroglobulin, catalase, ovalbumin, ribonuclease A, ferritin, aldolase chymotrypsinogen A and bovine serum albumin was applied separately for molecular weight calibration. The void volume (V_0) was determined by the elution of blue dextran with a flow rate of 14 ml/h. The elution volumes of the standards were determined by monitored absorbance at 280 nm. In separate experiments, 20α-HSD was applied to the same column and eluted with buffer A. The elution volume was determined by measuring the enzyme activity in each fraction.

RESULTS

Subcellular localization and purification of 20α-HSD

The average pair of bull testes weighs 450 g and contains 450 mIU of enzyme activity. The presence of glycerol in the homogenization buffer is essential to stabilize activity. If glycerol is omitted, 80–90% of the 20α-HSD activity is lost within 48 h. Subcellular fractionation of the originally prepared homogenates revealed that 85–90% of the enzyme activity is localized in the supernatant fraction. Similar subcellular localization have been described for the 20α-HSD from rat [2] and pig testes [4].

Table 1. Purification of 20α-HSD from bull testes

Sample	mIU	Protein (mg)	SA (mIU/mg)	Purification (× -fold)	Recovery (%)
8000 *g* Supernatant	424	50,490	0.008		
105,000 *g* Supernatant	343	18,550	0.018	2.3	81
40–80% Ammonium sulfate	1151	4050	0.28	35	272
Reactive red agarose	758	16.4	46.3	5792	178
	(601)[a]	(8.2)	(73)	(9125)	(141)
AcA-34	289	2.5	115	14375	68

[a]Values in parentheses represent the pool of the eluted enzyme peak used in the subsequent step.

Subsequently, decapsulated bull testes were homogenized in buffer A and centrifuged at 8000 *g* and 105,000 *g*, as described. A summary of the purification of the 20α-HSD from two testes with a wet wt of 450 g (decapsulated) is shown in Table 1. After centrifugation at 105,000 *g*, the supernatant was subjected to ammonium sulfate fractionation and the activity recovered in the 40–80% fraction. After dialysis of the resuspended pellet, the consistently noted increase (> 100% yield) of the measurable enzyme activity was found. This situation, not unique to this enzyme [9, 15] suggests the presence of inhibitors in the starting material, which are probably removed during fractionation or dialysis. Therefore, an accurate estimate of overall yield and purification is difficult to assess. Calculations for Table 1 are based on the SA of the starting supernatant material.

Our purification scheme is greatly dependent upon the high affinity of the red agarose for $NADP^+$-dependent dehydrogenases (in contrast with blue 2-agarose which has high affinity for NAD^+-dependent dehydrogenases).

Although more than 80% of the 20α-HSD from bull testes will bind to the reactive red 120-agarose during a 2 h batch incubation (data not shown), the column system was more effective in purifying the enzyme. Optimal purification can be obtained with a sample–gel volume ratio of 2:1 with a protein concentration up to 20–30 mg/ml sample. We loaded and eluted columns at a rate of 3 ml/h under the conditions described above. The best recovery was obtained when enzyme was eluted with 1 mM NADPH in buffer A. A typical result is shown in Fig. 1. Here it can be noted that the great majority of the 20α-HSD activity was retained but eluted sharply when the elution buffer was changed to contain 1 mM NADPH. Analysis of this elution peak is shown in Table 1. If the entire quantity of enzyme activity eluted by NADPH is totalled, it represents 66% of that added to the gel. Elution with NAD^+ in concentrations of 0.5, 0.2 and 0.1 mM yields a lower recovery (30–20%). No measurable 20α-HSD activity could be detected following elution with only 17-hydroxyprogesterone (0.1 mM) in buffer A. However, when 0.1 mM $NADP^+$ and 0.1 mM 17-hydroxyprogesterone in buffer A were used for elution, the enzyme recovered was of slightly higher SA than with 1 mM NADPH alone, but with lower recovery (48%). Use of reactive red 120-agarose allows a 268-fold purification from the previous step. The SA of the enzyme eluted in the best fractions from the red dye gel was 73 mIU/mg (Table 1). Therefore, we have selected elution with 1 mM NADPH, as shown in Fig. 1, for the purification scheme to be permanently employed to obtain the quantities of enzyme necessary for structural studies.

Finally, as shown in Table 1, when the fractions from the red gel which had the highest SA (shown in parentheses) are applied to AcA-34 gel filtration, the final product had an SA of 115 mIU/mg and an overall recovery of 68% of the starting activity. After gel filtration, the eluted enzyme appears to be homogeneous as judged by gel electrophoresis (Fig. 2). A duplicate gel was stained for 20α-HSD activity at pH 8.5. The activity band corresponded to the protein stained band of the homogeneous enzyme preparation.

Molecular weight determination

When subjected to SDS polyacrylamide gel electrophoresis, the enzyme exhibits a single band corresponding to a mol. wt of 40,000 (Fig. 3). This value was an average of three determinations. When applied to a Sephacryl S-300 column calibrated with the appropriate protein standards (Fig. 4), the enzyme had an apparent mol. wt of 34,000 (average of three determinations). The value corresponds well with the molecular weight obtained by SDS electrophoresis and the data suggest that the 20α-HSD has no

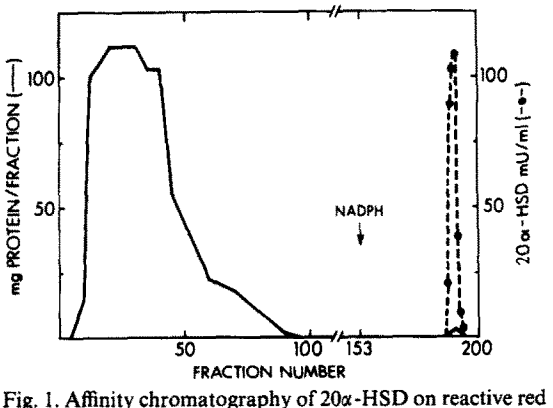

Fig. 1. Affinity chromatography of 20α-HSD on reactive red 120-agarose. The dialyzed, partially purified enzyme (148 ml, 27.2 mg/ml, 7.7 mU/ml) from the 40–80% ammonium sulfate fractionation was applied to the gel and extensively washed (7 ml/fraction) with buffer A and eluted with buffer A containing 1 mM NADPH. Fractions 154–200 are 2 ml. Measurements of protein and enzyme activity are as described in the text.

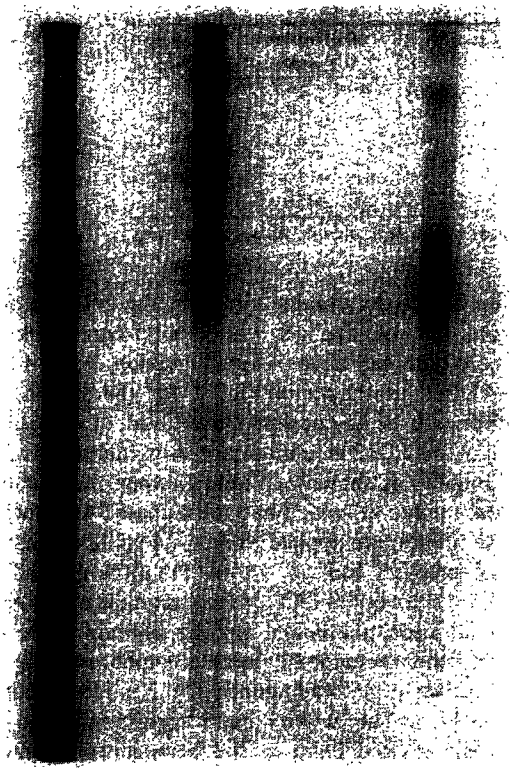

Fig. 2. Polyacrylamide gel electrophoresis of 20α-HSD from bull testis at pH 7.0. Left and center gels represent material eluted from reactive red 120-agarose stained by zymography [13] with 17,20α-dihydroxy-4-pregnene-3-one as substrate (left) and stained for protein with Coomassie blue (center). The gel on the right represents homogeneous enzyme eluted from a gel filtration column AcA-34 and was stained with Coomassie blue.

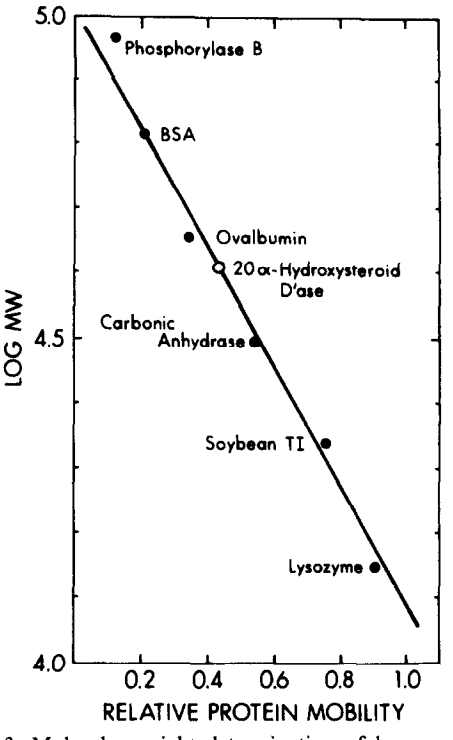

Fig. 3. Molecular weight determination of homogeneous 20α-HSD by SDS polyacrylamide gel electrophoresis at pH 8.3. Each point represents the average value of three determinations, as described in the text.

subunit structure. Similarly, the porcine testicular enzyme [4] has been characterized as a single-stranded polypeptide having a mol. wt of 35,000.

pH optimum

The rate of production of reduced steroid when the pH of the phosphate assay buffer was varied between 4.6 and 9.2 was determined in the presence of NADH and NADPH. With NADPH as cofactor the maximal rate of the enzyme activity was observed at pH 5.6. With NADH the enzyme showed a broad pH optimum between pH 6.8 and 8.4 (Fig. 5).

Cofactor and substrate specificity

The appparent K_m-value of 17-hydroxyprogesterone at pH 7.0 as determined from the double-reciprocal plot (Fig. 6) was 73 μM. Substrate inhibition was consistently found when the steroid concentration was >0.3 mM. When 20α-HSD from bull testis was incubated with 17-hydroxyprogesterone as substrate in the presence of 0.5 mM NADH, the reaction rate was approx. 30% of that seen with 0.5 mM NADPH as cofactor.

Androstenedione, estrone and corticosterone are not substrates for 20α-HSD from bull testis. Assays

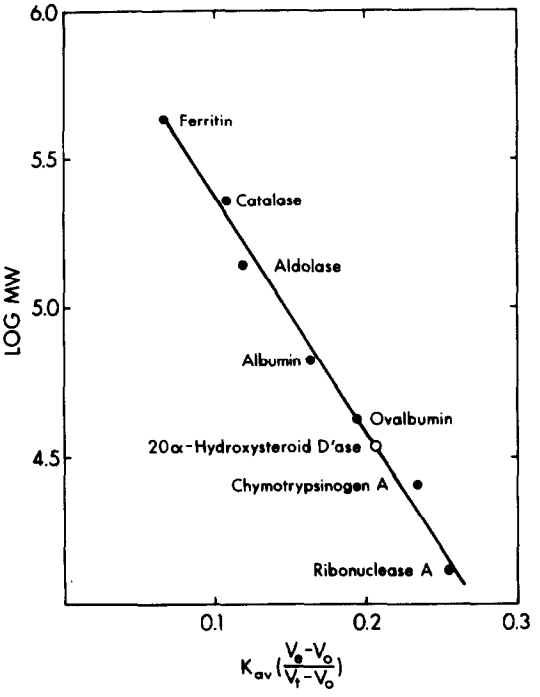

Fig. 4. Molecular weight determination by Sephacryl S-300 chromatography. Homogeneous enzyme was applied to a column of Sephacryl S-300 (2.1 × 90 cm) and eluted with buffer A at a flow rate of 14 ml/h (3 ml/fraction) and monitored at 280 nm. Protein standards were applied to the same column in separate experiments. The void volume (V_0) was determined as the elution volume of blue dextran. Elution volumes (V_e) were determined for each protein as described in the text.

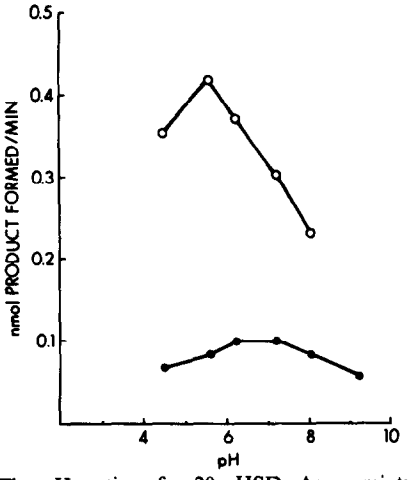

Fig. 5. The pH optima for 20α-HSD. Assay mixtures of 0.01 M potassium phosphate buffer with pH varied from 4.6 to 9.2 were employed with NADPH (○) and NADH (●) as cofactor. The rate of production of 17,20α-dihydroxy-4-pregnene-3-one was measured as described in the text.

were conducted under conditions such that utilization of any of these substrates at 0.5% of the rate of utilization of 17-hydroxyprogesterone by the enzyme would have been detected. The activity of the 20α-HSD with progesterone as substrate was <1% of that observed with 17-hydroxyprogesterone as substrate.

DISCUSSION

20α-HSD from bull testis has been purified to homogeneity. The enzyme activity is localized in the 105,000 *g* supernatant and is stable at 4°C in the presence of glycerol and dithiothreitol. We were unable to demonstrate 20α-HSD activity in washed microsomal fractions, but we made no attempts to inhibit 17–20 desmolase activity. Shikita and Tamaoki [2] reported that with proper inhibition of the desmolase activity no 20α-HSD activity was found in rat testicular microsomes.

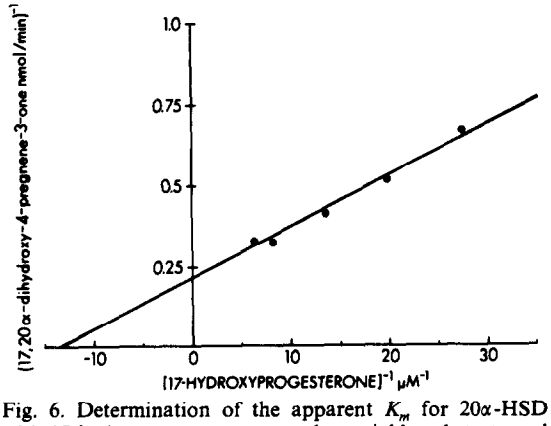

Fig. 6. Determination of the apparent K_m for 20α-HSD with 17-hydroxyprogesterone as the variable substrate and NADPH (0.5 mM) as cofactor. Assay mixtures contained 2.3 mIU of enzyme and reaction velocities were measured by the generation of 17,20α-dihydroxy-4-pregnene-3-one, as described in the text. Each point represents the average of eight determinations.

Purification was achieved by ammonium sulfate precipitation followed by affinity chromatography on reactive red 120-agarose and subsequent gel filtration. The efficacy of affinity chromatography with reactive red 120-agarose is impressive.

Red and blue agarose gels are known to selectively bind $NADP^+$ and NAD^+-dependent dehydrogenases, respectively [16, 17]. Other advantages that have been attributed to these triazine dye matrices is the recovery of highly purified material free from esterases which might interfere with further purification steps, as has been confirmed by Henderson and Warren[9].

Optimal conditions for binding and elution must be determined for each specific enzyme [16, 17]. Inano *et al.*[18] and coworkers purified 17β-HSD from porcine testis using red dye affinity chromatography and 0.5 M NaCl for elution. Henderson and Warren[9] have isolated 17α- and 17β-estradiol dehydrogenases from horse placentae using blue 2-agarose and 1 M KCl for elution. Finally, the appropriate cofactor [9, 15] or a combination of cofactor and substrate can be used for elution as well [19].

Selective removal of the bull testicular 20α-HSD from the reactive red 120-agarose by $NADP^+$ appears to be enhanced by the presence of the 17-hydroxyprogesterone. This is not unexpected as Sweet[9] has suggested that the addition of the steroid to the elution buffer might enhance the enzyme affinity for the cofactor and facilitate its release from the cofactor analogue of the gel matrix. While the originally eluted material is of even higher SA than that eluted with 1 mM NADPH alone, the slower rate of elution, distribution through a larger volume and the poorer recovery make it unattractive for routine use.

Homogeneous 20α-HSD has an SA of 115 mIU/mg, and has been purified 14,000-fold with an overall 68% recovery. It exhibits a pH optimum at 5.6 and appears to be highly specific for 17-hydroxyprogesterone with an apparent K_m-value of 7.3×10^{-5} M. The apparent molecular weight of the homogeneous enzyme, as determined by gel filtration of Sephacryl S-300 is 34,000. The mobility of the enzyme in SDS gel electrophoresis corresponds to a mol. wt of 40,000. These observations indicate that the enzyme is a single-stranded, monomeric polypeptide. The enzyme catalyzes the reduction of the 17-hydroxyprogesterone to 17,20α-dihydroxy-4-pregnene-3-one in the presence of NADPH, the preferred cofactor. The activity of the 20α-HSD with progesterone as substrate was <1% of that observed with 17-hydroxyprogesterone as substrate.

Androstenedione and corticosterone do not serve as substrates under the described experimental conditions. The enzyme does not possess 17α- or 17β-HSD activity. Sato *et al.*[4] performed similar studies with

purified 20α-HSD from boar testis. They found that the reactivity with corticosterone and estrone was less than 3% using spectrophotometric assays. However, their data suggested that the utilization of progesterone was only 0.6% of the reaction rate of 17-hydroxyprogesterone when the radioactive assay was employed, and that no 17β-HSD activity could be detected.

20α-HSD activity has been reported in various organs (liver, ovary, testis, adrenal, placenta) among several mammalian species. In the rat, the substrate specificity of 20α-HSD is dependent upon the organ of source. Progesterone is the preferred substrate for the ovarian enzyme [1], while 17-hydroxyprogesterone is preferred by the testicular enzyme [5]. 17-Hydroxyprogesterone is also the preferrred substrate for the porcine testicular enzyme [4] as well as for the bull testicular enzyme, as we have been able to demonstrate. There are some other similarities: the testicular 20α-HSD in all three species is found in the supernatant and uses NADPH as the preferred cofactor; the porcine and bull testis enzyme have been characterized as monomeric enzymes with mol. wt of 35,000 [3] and 34,000–40,000, respectively. These striking similarities between testicular enzymes from three different mammalian species constitute an interesting observation.

The physiological role of the 20α-HSD and the importance of this reduction step to generate 17,20α-dihydroxy-4-pregnene-3-one is still unknown. Shikita *et al.*[5] attempted to address this subject. In the rat, enzyme activity remained despite absence of the spermatogenesis after irradiation of the testes. They suggested that the enzyme may decrease androgen formation by: (a) reducing the substrate (17-hydroxyprogesterone) for, and (b) producing an inhibitor (the 17,20α-dihydroxy compound) of the 17,20-desmolase.

Whether similar roles can be attributed to the enzyme from bull testis is beyond the scope of the paper. However, with the purification scheme reported here, we can now anticipate sufficient quantities of purified 20α-HSD with which to conduct affinity labeling and cofactor alkylation studies to define the topography of the steroid binding site of the enzyme.

Acknowledgements—This research was supported by Research Grant AM-15708 from the National Institutes of Health. We thank Gary Murdock for valuable suggestions.

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