J. Biochem. 103, 1039-1044 (1988)

Purification and Characterization of 17β-Hydroxysteroid Dehydrogenase from *Cylindrocarpon radicicola*[1]

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Received for publication, September 14, 1987

An NAD^+-linked 17β-hydroxysteroid dehydrogenase was purified to homogeneity from a fungus, *Cylindrocarpon radicicola* ATCC 11011 by ion exchange, gel filtration, and hydrophobic chromatographies. The purified preparation of the dehydrogenase showed an apparent molecular weight of 58,600 by gel filtration and polyacrylamide gel electrophoresis. SDS-gel electrophoresis gave $M_r = 26{,}000$ for the identical subunits of the protein. The amino-terminal residue of the enzyme protein was determined to be glycine. The enzyme catalyzed the oxidation of 17β-hydroxysteroids to the ketosteroids with the reduction of NAD^+, which was a specific hydrogen acceptor, and also catalyzed the reduction of 17-ketosteroids with the consumption of NADH. The optimum pH of the dehydrogenase reaction was 10 and that of the reductase reaction was 7.0. The enzyme had a high specific activity for the oxidation of testosterone ($V_{max} = 85$ μmol/min/mg; K_m for the steroid = 9.5 μM; K_m for $NAD^+ = 198$ μM at pH 10.0) and for the reduction of androstenedione ($V_{max} = 1.8$ μmol/min/mg; K_m for the steroid = 24 μM; K_m for NADH = 6.8 μM at pH 7.0). In the purified enzyme preparation, no activity of 3α-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase, Δ^5-3-ketosteroid-4,5-isomerase, or steroid ring A-Δ-dehydrogenase was detected. Among several steroids tested, only 17β-hydroxysteroids such as testosterone, estradiol-17β, and 11β-hydroxytestosterone, were oxidized, indicating that the enzyme has a high specificity for the substrate steroid. The stereospecificity of hydrogen transfer by the enzyme in dehydrogenation was examined with [17α-^{3}H]testosterone.

Many fungi are well known to oxidize 17β-hydroxysteroids but the properties of the enzyme catalyzing the reaction are little known. In contrast, enzymes catalyzing dehydrogenation of 17-hydroxysteroids from bacteria [EC 1.1.1.51] and mammals, [EC 1.1.1.63], and [EC 1.1.1.64], have been well studied and characterized. Most of the microbial enzymes are specific for NAD^+ as the hydrogen acceptor and have one or two orders higher activity than mammalian enzymes (*1, 2*). Enzymes isolated from mammalian liver and placenta, however, utilize NAD^+ and/or $NADP^+$ and exhibit dehydrogenase activities toward several kinds of substrates (*3, 4*).

Cylindrocarpon radicicola is a fungus catabolizing progesterone to dehydrotestololactone and further (*5*). In the metabolic pathway, 17β-hydroxysteroid dehydrogenase has been expected to function with steroid monooxygenase and testosterone acetate esterase (*6*). During studies of the steroid monooxygenase (*7, 8*), a large activity of the dehydrogenase was found in the fungal crude extract.

The present paper describes the purification and characterization of the 17β-hydroxysteroid dehydrogenase of this fungus. These studies were undertaken to compare the characteristics of the enzyme with those of other steroid dehydrogenases from microbial and mammalian sources.

MATERIALS AND METHODS

Materials—Cells of *C. radicicola* ATCC 11011 cultured in the presence of progesterone were obtained as previously reported (*7*) and stored at −80°C until use.

[1a,2a(n)-^{3}H]Testosterone, [1a,2a(n)-^{3}H]androstenedione, [4-^{14}C]testosterone, [4-^{14}C]androstenedione, and [1,2,6,7,16,17-^{3}H]progesterone were the products of Amersham International. 17α-^{3}H-labeled testosterone was prepared from [1,2,6,7,16,17-^{3}H]progesterone by the use of purified steroid monooxygenase and testosterone acetate esterase and was purified by thin layer chromatography (*7*). NAD^+, $NADP^+$, NADH, and NADPH were obtained from Oriental Yeast Co. Phenyl-Sepharose 4B was from Pharmacia Co. Non-labeled steroids and other chemicals were of reagent grade and were purchased from commercial sources.

Methods—Assay of 17β-hydroxysteroid dehydrogenase: The enzyme was assayed by spectrophotometric and also spectrofluorometric methods at 25°C. In the dehydrogenase reaction, testosterone was used as a substrate for the routine assay. The assay system in a final volume of 0.53 ml contained 50 mM glycine-sodium hydroxide-sodium chloride buffer, pH 10.0, 190 μM NAD^+, 190 μM testosterone added in 10 μl of dimethyl formamide, and an appropriate amount of the enzyme. The reaction was

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

Abbreviations: testosterone, 4-androsten-17β-ol-3-one; androstenedione, 4-androstene-3,17-dione; 17α-epitestosterone, 4-androsten-17α-ol-3-one; dehydroepiandrosterone, 5-androsten-3β-ol-17-one; dehydroandrosterone, 5-androsten-3α-ol-17-one; estradiol, estra-1,3,5(19)-triene-3,17β-diol; 11β-hydroxytestosterone, 4-androstene-11β,17β-diol-3-one.

started by addition of the steroid. In the reductase reaction, 50 mM potassium phosphate buffer, pH 6.5, 190 μM NADH, and 190 μM androstenedione in 10 μl of dimethyl formamide were added to make a final volume of 0.53 ml. The rate of each reaction was determined from the increase or decrease of absorbance at 340 nm in the initial one minute. In the fluorometric assay, the rate of the enzyme reaction was determined from the increase or decrease of the fluorescence intensity at 460 nm with excitation at 340 nm. In some experiments, 190 μM [1a,2a(n)-^{3}H]testosterone, 10^4 cpm, as a substrate and [4-^{14}C]androstenedione, 10^3 cpm, as the internal standard, in 10 μl of propylene glycol were used. After the incubation for 10 min at 37°C, reaction was terminated by addition of 2 ml of chloroform-methanol mixture (1 : 1, v/v) followed by extraction of the radio-labeled substrate and product, chromatography on a thin layer silica gel HF plate (silica gel 60/Kiesel-guhr F245 precoated TLC plate, Merk) developed with benzene-ethyl acetate-methanol (66 : 33 : 1, v/v), and quantitation by liquid scintillation counting (*7*). In the reductase reaction, 190 μM [1a,2a(n)-^{3}H]androstenedione, 10^4 cpm, as the substrate, and [4-^{14}C]testosterone, 10^3 cpm, as the internal standard, in 10 μl of propylene glycol and 190 μM NADH were used.

Activities of 17α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid dehydrogenase, 3β-hydroxysteroid dehydrogenase, Δ^5-3-ketosteroid-4,5-isomerase, and steroid ring A-Δ-dehydrogenase were assayed under the reported conditions (*9-12*).

One unit of the enzyme activity was tentatively defined as the amount that transfers 1 μmol of the designated substrate per min under the specified assay conditions.

Disc gel electrophoresis: Electrophoresis was performed on polyacrylamide gel at pH 7.8 in 0.4×9.0 cm glass tubes (*13*). Gels of 7-12% acrylamide concentrations were prepared by varying the amount of 30% acrylamide and 0.8% *N,N'*-methylene-bis-acrylamide solution (*14*). The gels were stained either for total protein with Coomassie blue R250 in 10% isopropanol-7% acetic acid and destained with 7% acetic acid, or for enzymic activity with nitroblue tetrazolium-phenazine methosulfate (*1*). Testosterone (10 mM) was dissolved in propylene glycol. After development of a violet color, the reaction was stopped by transferring the gels to 7% acetic acid solution.

Electrophoresis on polyacrylamide gel (8, 10, and 12.5% gel) containing sodium dodecyl sulfate was performed by the method of Fairbanks *et al.* (*15*).

Gel filtration: Proteins were separated according to molecular size on a column (0.8×95 cm) of Sephacryl S-200, eluted at 18 ml/h with 0.1 M KCl-0.03 M Tris buffer, pH 8.4, and detected by measuring absorbance at 280 nm and enzyme activity.

Amino acid analysis and determination of N-terminal residue: Purified enzyme preparation was hydrolyzed with constant-boiling HCl under a nitrogen atmosphere at 120°C for 24 and 48 h. Analysis of the amino acids was performed with the use of an HPLC system by the *o*-phthalaldehyde method (*16*). For determination of the N-terminal residue, the enzyme was dialyzed against water, dried from the frozen state, dansylated, and processed according the method of Gray (*17*). The identity of the dansylated NH_2-terminal amino acid was confirmed on polyamide thin layers by co-chromatography with authentic standards.

Purification: All buffer solutions used contained 1 mM EDTA and 1 mM dithiothreitol. Buffer A is 0.03 M Tris-HCl buffer (pH 8.4) and buffer B is 0.03 M Tris-HCl buffer (pH 7.3). The crude enzyme extract was obtained from the frozen cells as described previously (*7*). The subsequent purification procedures are described in detail in "RESULTS."

Determination of stereospecificity for hydrogen transfer to NAD$^+$: NAD$^+$ (190 μM) was incubated with 17α-^{3}H-labeled testosterone (7×10^5 cpm), carrier testosterone (90 μM), and 17β-hydroxysteroid dehydrogenase (15 units) for 20 min in 0.5 ml of 0.1 M ammonium carbonate (pH 7.9) at 37°C and then beef liver glutamate dehydrogenase (50 μg), Na-α-ketoglutarate (200 nmol), and NADH (200 nmol) were added. The mixture was further incubated for 20 min and then Na-glutamate (6 mg) was added. After the removal of steroids by extraction with ethyl acetate, the residual water was evaporated off, and glutamate was isolated by chromatography on a silica gel plate. Radioactivity and amount of glutamate obtained were determined.

NAD$^+$ (190 μM) and 17α-^{3}H-labeled testosterone (7×10^5 cpm), carrier testosterone (90 μM), and 17β-hydroxysteroid dehydrogenase (15 units) were also incubated for 20 min in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.5) at 37°C. To the incubated mixture, yeast alcohol dehydrogenase (20 μg), acetaldehyde (20 μl), and NADH (200 nmol) were added and incubation was continued for a further 20 min in a closed test tube. Ethyl alcohol (1 ml) was added to the mixture as the carrier followed by distillation at 80°C to recover the produced alcohol into a vial containing 3 ml of liquid scintillator in an ice bath, and the radioactivity was counted.

RESULTS

Purification of 17β-Hydroxysteroid Dehydrogenase from C. radicicola—All of the subsequent manipulations were performed at 4°C unless otherwise noted.

First DEAE-cellulose chromatography: The crude extract from cells of the fungus was mixed with 500 ml of DEAE-cellulose equilibrated with buffer B. The mixture was stirred for 30 min, then the cellulose was collected by filtration and packed into a large column (6.5 cm diameter) which had been prepacked with DEAE-cellulose to a length of 10 cm. The column (6.5×30 cm) was washed with 2 volumes of buffer B and eluted with a 5-liter linear gradient from 0 to 0.5 M KCl in the same buffer at a flow rate of 60 ml/h, and 15 ml fractions were collected. The enzyme was eluted at 0.5-1.0 M KCl in the buffer. Solid ammonium sulfate was added slowly to the pooled fraction while keeping the pH at 7.4. The precipitate between 0 and 75% saturation was collected by centrifugation at 10,000×g for 20 min, dissolved in a minimum volume of buffer A and dialyzed against the same buffer for 18 h to remove ammonium sulfate. The dialyzed material was centrifuged at 10,000×g for 20 min, and the supernatant was obtained.

Second DEAE-cellulose chromatography: The supernatant was applied directly to a DEAE-cellulose column (2.6×36 cm) equilibrated with buffer A. After application of the samples, the column was washed with the buffer and eluted with a 2.5-liter linear gradient from 0 to 0.25 M KCl in the same buffer at a flow rate of 45 ml/h (Fig. 1). The

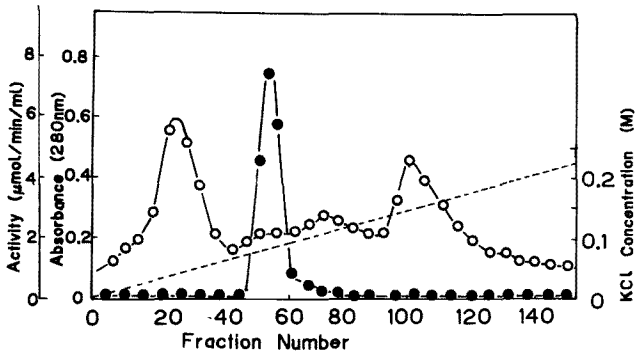

Fig. 1. **Second DEAE-cellulose chromatography of 17β-hydroxysteroid dehydrogenase.** The dialyzed enzyme preparation was applied to a column (2.6×36 cm) equilibrated with buffer A and eluted with a 2.5-liter linear gradient of 0 to 0.25 M KCl in buffer A. The flow rate was 45 ml/h and fraction volumes of 15 ml were collected. The enzyme activity (●), and absorbance at 280 nm (○), and KCl concentration (- - -) of the eluates are indicated.

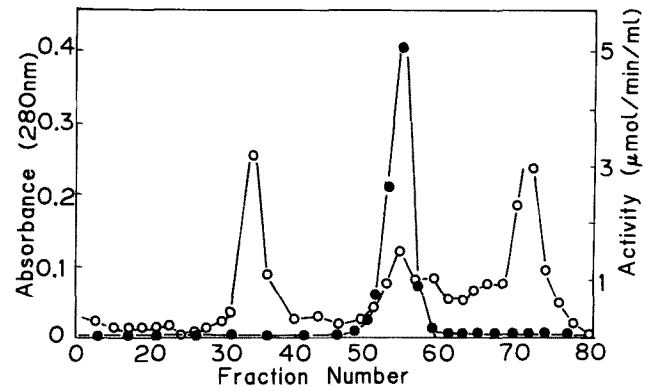

Fig. 2. **Gel filtration of 17β-hydroxysteroid dehydrogenase on Ultrogel ACA-44.** The concentrated enzyme preparation was applied to a column (2.3×96 cm), and eluted with 0.1 M KCl-buffer A. The flow rate was 18 ml/h and the fraction volume was 2.5 ml. The absorbance at 280 nm (○) and the enzyme activity (●) are shown.

fractions (50-67) were pooled, dialyzed against buffer A, then applied to a small column of DEAE-cellulose (1.5×4 cm). The concentrated enzyme was eluted with 0.1 M KCl-buffer A.

Gel filtration: The concentrate was applied to an Ultrogel ACA-44 column (2.3×96 cm) equilibrated with 0.1 M KCl-buffer A and eluted with the same buffer (Fig. 2). The fractions (50-58) were pooled.

Phenyl-Sepharose chromatography: Solid ammonium sulfate was added to the fractions to the concentration of 1.2 M. The pH of the fractions was kept at 8.4 by addition of diluted ammonium hydroxide. The fractions were applied to a phenyl-Sepharose column (1.2×15 cm) equilibrated with 1.2 M ammonium sulfate-buffer A. The column was washed with the same buffer and then buffer A containing 0.5 M ammonium sulfate. A 250-ml reversed linear gradient from 0.5 to 0 M ammonium sulfate in buffer A was then applied (Fig. 3). The fractions were concentrated by ultrafiltration and stored at −80°C.

The five-step procedure described above provides an 846-fold purification of 17β-hydroxysteroid dehydrogenase with an overall 48% recovery from the fungal crude extract (Table I). The specific activity of the purified enzyme preparation was found to be 84.6 units/mg protein under the assay conditions for dehydrogenation of testosterone. Nondenaturing polyacrylamide gel electrophoresis at five different gel concentrations (7-12%) revealed a single protein band. Staining the gel for enzyme activity showed a band which coincided with the protein band (Fig. 4, columns 1 and 2). Electrophoresis of the purified enzyme on SDS-polyacrylamide gels (8, 10, and 12.5% gels) showed a single band corresponding to a molecular weight of 26,000 (Fig. 4, column 3 and Fig. 5).

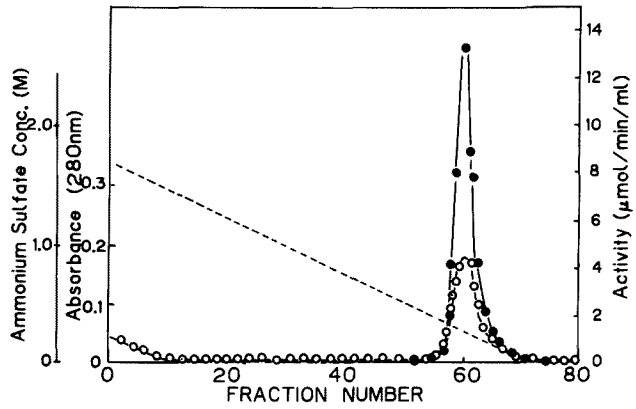

Fig. 3. **Phenyl-Sepharose chromatography of 17β-hydroxysteroid dehydrogenase.** To the enzyme fraction obtained from the Ultrogel column (fractions 50-58), solid ammonium sulfate was added to a concentration of 1.2 M, and the mixture was applied to a phenyl-Sepharose 4B column (1.2×15 cm) equilibrated with 1.2 M ammonium sulfate-buffer A. The column was first washed with 3 column volumes of 1.2- and 0.5-M ammonium sulfate containing buffer A. The column was then eluted with a 0.5-0.0 M ammonium sulfate reversed linear gradient in 250 ml of buffer A at a flow rate of 0.2 ml/min. Fractions of 2.5 ml were collected and assayed for protein (○) and for the dehydrogenase activity (●). Ammonium sulfate concentrations (- - -) are indicated.

The apparent molecular weight of the native enzyme was estimated to be 58,600 by gel filtration on an Ultrogel column (0.8×95 cm) with four molecular weight standard proteins. Ferguson analysis on polyacrylamide gel electrophoresis of the native enzyme at pH 7.4 gave an estimated molecular radius of 2.57 nm and a weight of 58,800 (Fig. 6) (*19*). The native enzyme molecule consists of two subunits of the same size.

The amino acid composition of 17β-hydroxysteroid dehydrogenase is presented in Table II. The amide and cysteine contents were not determined. Dansylation of the native enzyme revealed NH_2-terminal glycine residue.

The purified enzyme showed a typical protein absorption spectrum with a maximum absorption at 278 nm in the ultraviolet region and no absorption in the visible region; suggesting that no prosthetic group molecule was associated with the enzyme molecule.

Enzyme activity: 17β-Hydroxysteroid dehydrogenase from *C. radicicola* is an NAD^+-linked enzyme that promotes oxidoreductions of C_{18}- and C_{19}-steroids. The enzyme catalyzed 17β-dehydrogenation of testosterone to androstenedione with the use of NAD^+ as a hydrogen acceptor (dehydrogenase reaction) and also reduction of the 17-keto group of androstenedione with NADH (reductase

TABLE I. **Purification of 17β-hydroxysteroid dehydrogenase of *C. radicicola*.** Frozen fungal cells (715 g) were used for the purification.

	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	
					Yield (%)	Fold
Crude extract	4,500	4,095	409	0.10	100	1
DEAE-cellulose chromato. I	1,500	374	360	0.96	88	9.6
DEAE-cellulose chromato. II	225	20.5	309	15.1	76	151
Ultrogel ACA-44 gel filtration	65	4.3	288	66.7	70	667
Phenyl Sepharose & concentration	3.4	2.3	198	84.6	48	846

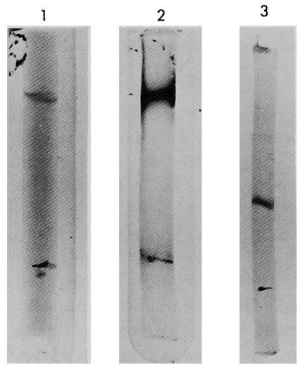

Fig. 4. **Polyacrylamide gel electrophoresis of the purified 17β-hydroxysteroid dehydrogenase.** Native polyacrylamide gel electrophoresis (columns 1 and 2). Purified enzyme (2-10 μg) was subjected to electrophoresis on native gels of 7.5% total acrylamide. Gels were stained for protein with Coomassie blue (column 1) or for the enzyme activity with testosterone, NAD^+, and nitroblue tetrazolium chloride (column 2). SDS-polyacrylamide gel electrophoresis (column 3). Purified enzyme (10-30 μg) was subjected to electrophoresis on 12.5% gel containing 0.1% sodium dodecyl sulfate. The gel was stained with Coomassie blue.

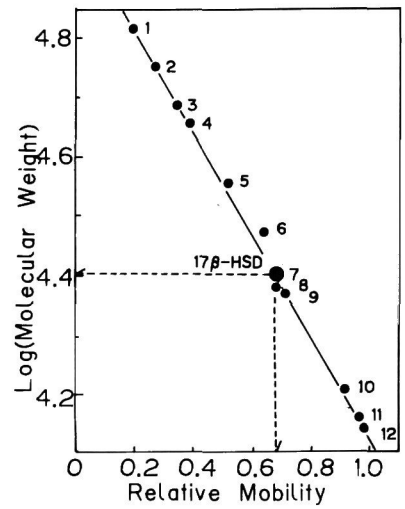

Fig. 5. **Estimation of the apparent molecular weight of the subunit of purified 17β-hydroxysteroid dehydrogenase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** The gel electrophoresis was performed on 12.5% gels as described in "MATERIALS AND METHODS" with standard proteins (10-30 μg). 1, bovine serum albumin; 2, steroid monooxygenase; 3, γ-globulin (heavy chain); 4, ovalbumin; 5, β-lactoglobulin; 6, pancreas deoxyribonuclease; 7, chymotrypsinogen; 8, trypsin; 9, γ-globulin (light chain); 10, bovine hemoglobin; 11, chymotrypsin; 12, lysozyme.

reaction). $NADP^+$ and NADPH could not serve as the electron acceptor or donor in these reactions. Estradiol-17β and 11β-hydroxytestosterone were oxidized at rates as high as 46 and 76%, respectively, of that of testosterone. Activities of 17α-hydroxysteroid dehydrogenase with 17-epitestosterone, 3α-hydroxysteroid dehydrogenase with dehydroepiandrosterone, 3β-hydroxysteroid dehydrogenase with dehydroandrosterone, Δ^5-3-keto-4,5-isomerase with androstenedione, and steroid ring A-Δ-dehydrogenase with androstenedione could not be detected by the respective assay methods in the purified dehydrogenase preparation.

Stability: 17β-Hydroxysteroid dehydrogenase from *C. radicicola* is a stable enzyme. Its stability is enhanced by the substrate steroid, testosterone. The purified enzyme preparation could be stored at -80°C without loss of its activity for several months. In crude extract from the fungal cells, two-thirds of the initial activity remained after 10 days at 0°C and pH 10.0. The activity of the purified enzyme preparation decreased with the half life of 10 min at 43°C and pH 10.0, but the activity did not decrease in the presence of 200 μM testosterone. At neutral pH, 90% of the initial activity was retained after a 30 min incubation at 43°C. The purified enzyme could be lyophilized without loss of the activity.

Effect of pH: Initial velocities of NAD^+ reduction by testosterone or NADH oxidation by androstenedione were studied as a function of pH between 6.5 and 11.6 using two overlapping buffers, 0.05 M potassium phosphate buffer and 0.03 M glycine-NaOH-NaCl buffer (Fig. 7). The value of V_{max} was maximal at a pH of about 10 for the dehydrogenation (Fig. 7A) and the optimum pH of the reverse reaction was about 7 (Fig. 7B). Apparent K_m values for testosterone and androstenedione did not change much in the pH region examined. At each optimum pH, the number of moles of testosterone oxidized was the same as that of NADH formed, and the stoichiometric relation was also confirmed in the reductase reaction. An equal velocity for both reactions was observed at pH 6.5.

Kinetics—Kinetic constants for both substrates in the forward and reverse reactions were determined from secondary plots of the data for the two-substrate system in initial velocity studies (Table III). Double reciprocal plots of the initial velocity against concentration of NAD^+ (40-200 μM) at five fixed concentrations of testosterone (4-13 μM) met at a point on the x-axis (not shown), indicating the presence of a sequential mechanism of the dehydrogenase reaction. The plots for the reverse reaction (25-100 μM androstenedione and 2.7-13.4 μM NADH) showed a similar relationship.

Stereospecific Hydrogen Transfer from Testosterone to NAD^+—17α-^{3}H-labeled testosterone was oxidized with production of ^{3}H-labeled NADH by 17β-hydroxysteroid

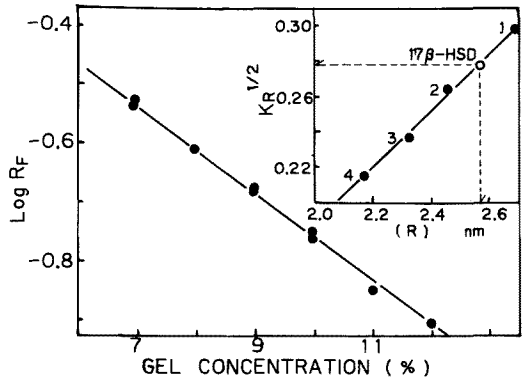

Fig. 6. **Estimation of the molecular weight of 17β-hydroxysteroid dehydrogenase by Ferguson analysis.** Polyacrylamide gel electrophoresis of purified 17β-hydroxysteroid dehydrogenase (9 observations shown) and of standard proteins (6 observations each, not shown) was performed on gels of various concentrations (7-12%) as described in the text. Ferguson plots were made by linear regression of log R_f (relative mobility) of 17β-hydroxysteroid dehydrogenase with respect to gel concentration (%). The slope (K_R) of the line was determined (*18*). The insert shows plots of $K_R^{1/2}$ *versus* the molecular radius values (R) of standard proteins. The standard proteins and their radius values (nm) are as follows: 1, bovine serum albumin (2.69); 2, Taka-amylase (2.46); 3; ovalbumin (2.33); 4, β-lactoglobulin (2.17).

TABLE II. **Amino acid composition of 17β-hydroxysteroid dehydrogenase.** Data were obtained by analysis of 24- and 48-h hydrolysates of the purified enzyme preparation.

Residue	% residue	mol/subunit
Asp	10.20	25
Thr	3.67	9
Ser	10.20	25
Glu	9.80	24
Pro	6.94	17
Gly	11.43	28
Ala	10.20	25
Val	8.16	20
Met	1.63	4
Ile	5.31	13
Leu	8.57	21
Tyr	1.22	3
Phe	3.27	8
His	1.63	4
Lys	4.49	11
Arg	2.45	6
Try[a]	0.82	2
Total	—	245

[a]The value was estimated from the UV-absorption spectrum in 0.1 N NaOH. Cysteine and amide were not analyzed.

dehydrogenase, and it was reoxidized by the yeast alcohol dehydrogenase system or the beef liver glutamate dehydrogenase system as described in the text.

Each reaction product was isolated and counted for radioactivity (Table IV). From the same amount of labeled testosterone (45 nmol, 7×10^5 cpm), the count of radioactivity in glutamate was higher than that in ethyl alcohol. The high specific radioactivity found in glutamate obtained from the incubation mixture containing glutamate dehydrogenase reveals that the tritium transferred by 17β-hydroxysteroid from [17α-^{3}H]testosterone is located at the 4-pro-S position of the nicotinamide moiety of NADH.

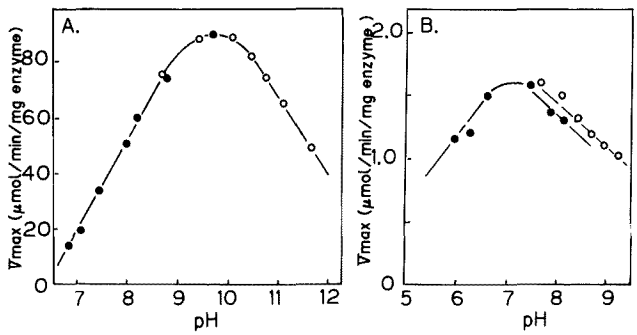

Fig. 7. **Effect of pH on the maximum velocity of interconversion of testosterone and androstenedione by purified 17β-hydroxysteroid dehydrogenase.** (A) Dehydrogenation of testosterone. Each point was obtained by measuring the reaction velocities in various concentrations of testosterone (6-15.2 μM) with a fixed concentration of NAD^+ (190 μM). (●) 0.03 M Tris-HCl buffer and (○) 0.05 M glycine-NaOH-NaCl buffer. (B) Reduction of androstenedione. The plotted values were obtained by measuring the velocities at various concentrations of androstenedione (2.2-11 μM) with a fixed concentration of NADH (128 μM). (○) 0.03 M Tris-HCl buffer and (●) 0.05 M potassium phosphate buffer.

TABLE III. **Kinetic constants for purified 17β-hydroxysteroid dehydrogenase.** Enzymic activities were assayed as described under "MATERIALS AND METHODS." Values for K_m and V_{max} were calculated from secondary plots of double-reciprocal-plotted data.

Dehydrogenase reaction (testosterone + NAD^+ ⟶)	
V_{max}	85 μmol/min/mg protein (pH 10)
(Molecular activity	4,318 mol/min/mol enzyme)
K_m for testosterone	9.5 μM (pH 10)
	14 μM (pH 7.4)
K_m for NAD^+	198 μM (pH 10)
	215 μM (pH 7.4)
Reductase reaction (androstenedione + NADH ⟶)	
V_{max}	1.8 μmol/min/mg protein (pH 7.0)
(Molecular activity	91 mol/min/mol enzyme)
K_m for androstenedione	24 μM (pH 7.0)
K_m for NADH	6.8 μM (pH 7.0)

TABLE IV. **Hydrogen transfer from tritiated testosterone to NAD^+ by 17β-hydroxysteroid dehydrogenase.** Experimental procedures are described in "MATERIALS AND METHODS."

System	Radioactivity	
	Total recovered (dpm)	Specific (dpm/mol)
a. [[17α-^{3}H]testosterone ⟶ Ethanol]		
Complete	525	—
−Alcohol dehydrogenase	0	—
b. [[17α-^{3}H]testosterone ⟶ Glutamate]		
Complete	26,904	343
−Glutamate dehydrogenase	541	7

DISCUSSION

Studies on steroid monooxygenase from *C. radicicola* showed that this enzyme catalyzes Baeyer-Villiger oxidations of both a C_{21}-20-ketosteroid (progesterone) and a C_{19}-17-ketosteroid (androstenedione) (*7, 8*). In the fungal metabolism of steroids (*6*), testosterone is dehydrogenated to androstenedione and then converted to testololactone by the monooxygenase. An enzyme catalyzing the dehydrogenation, *i.e.* 17β-hydroxysteroid dehydrogenase, was

found in the fungal crude extract.

Isolation procedures and properties of (3 and 17)β-hydroxysteroid dehydrogenases from bacteria and mammalian sources have been described in detail (*1-4*). In contrast, characterization of fungal steroid dehydrogenase has not been reported so far. The present study reports for the first time the isolation and characterization of a fungal 17β-hydroxysteroid dehydrogenase. General properties of the fungal enzyme are similar to those of the bacterial enzymes reported by Payne and Talalay (*2*) and Schultz *et al.* (*1*). The purified fungal enzyme reacts specifically with NAD^+ as the hydrogen acceptor and 17β-hydroxysteroids as the steroid substrate with a large turnover number for dehydrogenation.

It should be emphasized that the fungal enzyme catalyzes only the 17β-hydroxysteroid dehydrogenase reaction and not the 3α- or 3β-hydroxysteroid dehydrogenase reaction. Purified 17β-hydroxysteroid dehydrogenase from *Alcaligenes* sp. was reported, however, to catalyze the 3β-hydroxysteroid dehydrogenase reaction for epiandrosterone (3β-hydroxy-5α-androstan-17-one) with a low activity, *i.e.* 0.9% of the activity for dehydrogenation of testosterone (*2*). An enzyme from *Pseudomonus testosteroni*, (3 and 17)β-hydroxysteroid dehydrogenase, which was studied in the greatest detail, catalyzes both the 17β- and 3β-hydroxysteroid dehydrogenase reactions (*1*). Enzymes from rabbit liver (*20*), rat erythrocytes (*21*), human placenta (*22*), and porcine testicular microsomes (*23*) have activities of 17β-hydroxysteroid dehydrogenase and 3α-, 3β-, or 20α-hydroxysteroid dehydrogenase. An enzyme preparation from guinea pig liver exhibits activities of hydroxysteroid dehydrogenase, aldehyde reductase, and carbonyl reductase (*3*).

Most bacterial hydroxysteroid dehydrogenases have very high specific activity, such as 90-355 units/mg of protein (*1, 2, 10*). The fungal enzyme described in this paper also has high specific activity (85 units/mg of protein). In contrast, purified mammalian hydroxysteroid dehydrogenases have specific activities of less than 10 units/mg of protein (*21-24*). Differences of molecular activity among these enzymes may reflect the physiological functions in each organism, *i.e.* one is functioning in anabolic metabolism of steroids for carbon and energy sources and the other in synthesis or breakdown of steroids in the endocrine system and other organs. Variations of substrate specificity and of velocity of the same reaction with these enzymes may be caused by structural differences of the active sites.

As with other pyridine nucleotide-dependent dehydrogenases, the velocities of the forward and reverse reactions of the 17β-hydroxysteroid dehydrogenase are affected strongly by hydrogen ion concentration in the medium. The velocities of the dehydrogenase and reductase reactions are same at pH 6.5, which is more acidic than that of the dehydrogenase from *Alcaligenes* sp. (pH 8.4) (*2*). This implies a difference of kinetic constants of the reactions catalyzed by these enzymes and suggests a structural difference of the catalytic sites.

The K_m values of testosterone and NAD^+ do not change so much at neutral and alkaline pH. The affinity of NADH is likely to be affected by hydrogen ion concentration and seems to determine the direction of the enzyme reactions. Further kinetic studies should be made.

The present study reveals that the molecular weight of the fungal enzyme (58,000), composed of two subunit molecules of a single species, is the smallest so far reported. For example, the enzyme from *Alcaligenes* sp. has a molecular weight of 64,000 (*2*), that from *P. testosteroni*, 98,500 (*1*), and that from human placenta, 68,000 (*4*). Multiple species of the enzyme from *P. testosteroni* (*1*) and also from rabbit liver (*20*) were present in the purified preparations. The significance of these facts is not yet clear.

Studies on stereospecific transfer of hydrogen from substrate steroid to cofactor or from reduced cofactor to ketosteroid were described with several steroid oxidoreductases (*25-28*). Investigation of hydrogen transfer by the 17β-hydroxysteroid dehydrogenase from 17α-^{3}H-labeled testosterone demonstrated that the hydrogen atom on the substrate steroid was directly transferred to the cofactor in the dehydrogenase reaction. The enzyme exhibits a preference for the transfer of a hydrogen atom to the 4-pro-S position of the nicotinamide ring.

Highly specific, active and stable hydroxysteroid dehydrogenases are useful reagents for performing quantitative analysis of a specified steroid in physiological fluids. The properties of the hydroxysteroid dehydrogenase described here are favorable for this purpose.

We are grateful to Dr. Y. Umebachi for performing the amino acid analyses, to Dr. M. Katagiri for helpful advice, and to Miss M. Nakagawa and Mr. T. Komai for technical assistance in this work.

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