Purification and characterization of a novel 17α-hydroxysteroid dehydrogenase from an intestinal *Eubacterium sp.* VPI 12708

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Abstract A novel steroid-inducible 17α-hydroxysteroid dehydrogenase (17α-HSDH) has been purified over 850-fold from an intestinal *Eubacterium sp.* VPI 12708. The purified protein has a subunit molecular mass of 42,000 daltons and a native molecular weight (M_r) of 160,000 as estimated by gel filtration chromatography. Enzyme activity was induced by growth in the presence of androstenedione or cholic acid, but not deoxycholic acid. Enzymatic activity required anaerobic conditions and was highly specific for $NADP^+$ and the 17α-hydroxy group of C-19 steroids. Estimated K_m values were 31 μM and 70 μM for androstenedione and epitestosterone, respectively. V_{max} values were estimated to be 3250 nmol/min per mg protein and 1800 nmol/min per mg protein for the reductive and oxidative reactions, respectively. The pH optima for both the oxidative and reductive reactions ranged between 5.5 and 7.0. Treatment with EDTA completely inactivated 17α-HSDH activity but activity was partially restored by the addition of either Mg^{2+} (1 mM) or Zn^{2+} (10 mM). The N-terminal amino acid sequence analysis of purified enzyme suggests that 17α-HSDH may belong to a disulfide reductase gene family.—**de Prada, P., K. D. R. Setchell, and P. B. Hylemon.** Purification and characterization of a novel 17α-hydroxysteroid dehydrogenase from an intestinal *Eubacterium* sp. VPI 12708. *J. Lipid Res.* 1994. **35:** 922-929.

Supplementary key words 17α-hydroxysteroid dehydrogenase • intestinal microflora • epitestosterone

The intestinal microflora is capable of the enzymic modification of bile acids and steroid hormones (1, 2). However, because the lumen of the colon is a highly anaerobic environment most of the biotransformations carried out by intestinal bacteria are hydrolytic and reductive in nature. Known modification of bile acids include: deconjugation, oxidation of α-oriented hydroxy groups at carbons 3, 7, and 12, and reduction of resultant oxo groups to either α- or β-hydroxy orientation. Quantitatively, the most important bile acid biotransformation is the 7α-dehydroxylation of cholic acid and chenodeoxycholic acid yielding deoxycholic acid and lithocholic acid, respectively (3). The 7α-dehydroxylation of bile acids has been reported to occur via a multistep pathway (4, 5).

Steroid hormones that undergo enterohepatic circulation can be modified by the intestinal microflora in a variety of ways (1). Microbial biotransformations included hydrolysis of sulfate and glucuronide conjugates of steroids, 16α and 21-dehydroxylation (6, 7), reduction of the 4-ene bond in ring A to either 5α or 5β isomers, reduction of 3-oxo groups to either 3α- or 3β-hydroxy groups, reduction of 17-oxo group to 17β, reduction of the 20-oxo group to either 20α- or 20β-hydroxy groups (8), and side chain cleavage (desmolase) of glucocorticoids containing a 17α-hydroxy group (9). In this paper we report the discovery, purification, and characterization of a novel microbial 17α-hydroxysteroid dehydrogenase from a member of the intestinal microflora. Analysis of the N-terminal amino acid sequence indicates that this hydroxysteroid dehydrogenase may belong to a disulfide oxidoreductase gene family.

MATERIALS AND METHODS

All steroid compounds were purchased from Steraloids (CA). [^{14}C]androstenedione (53.90 mCi/mmol) and [^{14}C]testosterone (57/30 mCi/mmol) were obtained from New England Nuclear (Boston, MA). All organic solvents were obtained from Mallinckrodt (Paris, KY). Blue Agarose affinity media, Centriprep and Centricon dialysis tubes, and Bradford reagent for protein determinations were purchased from Amicon (Beverly, MA). Gel filtration HPLC GF250 Zorbax Bioseries column was ob-

Abbreviations: 17α-HSDH, 17α-hydroxysteroid dehydrogenase; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; GC-MS, gas chromatography-mass spectrometry; TMS, trimethylsilyl; epi-T, epitestosterone.

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tained from Dupont. Phenyl Sepharose CL-4B resin, NADPH, $NADP^+$, dithiothreitol (DTT), and all other chemicals were purchased from Sigma (St. Louis, MO).

Bacteria, media and culture conditions

Eubacterium sp. VPI 12708 was maintained at −70°C as 33% (v/v) glycerol stocks. The bacteria were cultured as previously described (10), except the growth medium was modified by replacing Brain Heart Infusion broth with Tryptic Soy broth (30 g/l). One liter starter cultures were grown overnight and diluted with 7 liters of fresh media. Bacterial growth was monitored at 600 nm, using a Shimadzu UV160U spectrophotometer. Cultures were induced four times, by the individual addition of 0.1 mM of either androstenedione or cholic acid every 90 min. Cells were harvested at late logarithmic phase by centrifugation (6,000 *g*, 4°C, 20 min), and washed once with 500 ml of buffer A (10 mM sodium phosphate, pH 6.8, 2 mM DTT, 0.05 mM $NADP^+$).

Enzyme assay conditions

The 17α-hydroxysteroid dehydrogenase activity was measured under anaerobic conditions, monitoring the conversion of the radiolabeled steroid substrate into the reduced product. The reaction buffer contained 100 mM sodium acetate buffer (pH 5.5), 2 mM DTT, and 50 μM NADPH, 40,000 cpm of [^{14}C]androstenedione and 100 μM cold androstenedione and water, up to a final volume of 1 ml. The mixture was placed in a sealed vial and oxygen was displaced by alternate cycles of vacuum and nitrogen gas flow. The enzyme solution was treated in the same way before being added to start the reaction, which was run for 30 min at 37°C. The reaction was stopped by addition of 1 ml of ethyl acetate. Extraction of both steroid and substrate product was > 90%. The solution was mixed and the aqueous and organic phases were allowed to separate. The ethyl acetate phase was separated and dried down under a nitrogen gas atmosphere. The residue was redissolved in 80 μl of ethyl acetate and spotted onto a silica TLC plate. The plate was developed using dichloromethane–cyclohexane–acetone 80:20:5 (vol/vol/vol). After chromatography, the TLC plate was allowed to air dry and exposed overnight to AXR Kodak film. Enzymatic activity was found to be linear with time (up to 30 min) and protein concentrations (up to 58 μg/ml pure enzyme) using standard assay conditions.

Purification of the 17α-HSDH

The purification protocol is summarized in Table 2. All steps were performed aerobically at room temperature. Protein determinations were performed using the method of Bradford (11). 17α-HSDH activity was measured in the direction of steroid reduction.

Preparation of the cell extract. Harvested bacteria were suspended in buffer A (10 mM sodium phosphate, pH 7.2, 2 mM DTT, 0.5 mM $NADP^+$). Cells were broken by two passages through a French Press (10,000 psi), with addition of DNAse. The soluble cellular fraction was separated from membranes and unbroken cells by ultracentrifugation (105,000 *g*, 4°C, 2 h). The supernatant fluid was collected and kept at −20°C until used.

Ammonium sulfate precipitation. Soluble cell extract (50 ml) was placed in an ice bath and solid ammonium sulfate was slowly added up to a final concentration of 65% saturation. The pH was constantly monitored and kept close to neutrality by addition of 10% ammonium hydroxide. Once dissolved, the mixture was centrifuged for 20 min at 12,000 *g* to pellet insoluble protein. 17α-HSDH activity remained in the supernatant fraction.

Phenyl Sepharose chromatography. The sample collected after the ammonium sulfate treatment was acidified by addition of 10% sulfuric acid until the pH value was 5.5. The mixture was centrifuged for 30 min at 12,000 *g* to remove the insoluble fraction. The supernatant was directly loaded onto a 1 × 10 cm column, previously equilibrated with a buffer B (30% saturation ammonium sulfate in buffer A). The column was washed with 3 bed-volumes of buffer B, followed by 3 bed-volumes of buffer C (10% ammonium sulfate in buffer A). The protein was finally eluted by addition of 2 bed-volumes of buffer A.

Blue Agarose chromatography I. Pooled fractions from the previous step were directly loaded into a Amicon Blue Agarose column (0.5 × 7 cm), previously equilibrated with buffer A. All the activity was found in the flow-through fraction.

Blue Agarose chromatography II. The unbound fraction from the previous column was extensively dialyzed against buffer D (10 mM sodium phosphate, pH 7.2, 2 mM DTT) until NADP concentration was reduced to < 0.5 μM. The sample was then loaded onto the same Blue Agarose column, this time equilibrated with buffer D. Once loaded, the column was washed with 4 bed-volumes of buffer D. Active enzyme was eluted with buffer A.

SDS-PAGE electrophoresis of proteins

SDS gel electrophoresis was performed as described by Laemmli (12), using 5% acrylamide stacking and 12% acrylamide separating gels (8 × 10 cm). The gel was run at room temperature, at a constant current of 1.5 mA/cm. Proteins were stained with Coomassie Brilliant Blue R-250, solubilized in ethanol–water–acetic acid 45:45:10 (by vol).

Native molecular mass determinations

A gel filtration-HPLC column (9.4 × 250 mm) was equilibrated with buffer A, containing 100 mM NaCl at a flow rate of 0.8 ml/min. The column was calibrated using apoferritin (443 kDa), β amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa). A standard curve was generated by

plotting the log of the molecular mass against the retention time. The pure 17α-HSDH was chromatographed under the same conditions and the native molecular weight was estimated from the standard curve.

Optimal pH determination

All assays were performed using 100 μl of partially purified 17α-HSDH (16 μM product/min per mg and 0.060 mg/ml). Reactions were run as described previously, using four different buffer systems: sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–7.5), Tris-HCl (pH 8.0–9.0), and glycine-sodium hydroxide (pH 9.5–10.5). The results are expressed as the percentage of substrate transformed.

Determination of apparent kinetic constants

V_{max} and K_m values were determined under substrate saturating conditions, following the method of Hanes (13). A trace amount of ^{14}C-labeled steroid was used to calculate the percentage of product converted on each reaction. After separating the reaction substrate and product on a silica plate, the radioactivity was determined by scintillation spectrometry, and the percentage of substrate that had undergone oxidation/reduction was calculated. These percentages were used to derive the concentration of total cold substrate and product obtained on each reaction. Reactions were run at 37°C, under anaerobic conditions, for 30 min, in a 100 mM sodium acetate buffer, pH 6.0, using 0.5 μg pure protein.

Metal cofactor study

Partially purified enzyme (3 ml; 16 μmol product/min per mg, 0.060 mg/ml) was dialyzed against 37 ml buffer A containing 10 mM EDTA in a Centriprep-10 tube. EDTA was removed in the same manner, with 80 ml buffer A. The sample was then divided into identical aliquots, each one dialyzed against 15 ml 1 mM solutions of $FeCl_2$, $MgCl_2$, $ZnCl_2$, $CuCl_2$, $CaCl_2$, a mixture of the $ZnCl_2$ and $MgCl_2$, and one kept as a control. The samples were incubated overnight at 4°C before activity determinations were performed as described earlier.

Gas chromatographic–mass spectrometric (GC–MS) analysis

GC–MS analysis of the products was performed after conversion to both the trimethylsilyl (TMS) ether and the methyloxime-trimethylsilyl ether derivatives. The methyloxime derivative was prepared by addition of 50 μl saturated solution of methoxamine hydrochloride in pyridine to the dried extract and heating at 60°C for 30 min. After evaporation of the pyridine by a stream of nitrogen gas, the trimethylsilyl ether derivative was prepared by the addition of 50 μl of a mixture of trimethylchlorosilane–hexamethyldichlorosilane–pyridine (Trisil, Pierce Chemical) and heating at 60°C for 15 min. The derivatizing reagents were removed by passage of the sample through a small column of Lipidex 5000; the derivative was analyzed by GC–MS.

Gas chromatography was performed on a 30M DB-1 fused silica capillary column (0.4 mm i.d., 0.25 μ film thickness) using temperature programmed conditions from 185°C–295°C with increments of 2°C/min. Mass spectrometry was carried out under electron ionization (70 eV) and repetitive acquisition of positive ion spectra (2 secs/cycle) by magnetic scanning over the mass range 50–1000 daltons during elution of the components from the GC column.

N-terminal amino acid sequence

The purified protein (40 μg) was extensively dialyzed against HPLC-grade water, and concentrated using a 2 ml Centricon-10 concentrator. The N-terminal sequences (residues 1–21) were determined by Dr. Bryan White in the Department of Animal Sciences, University of Illinois, Urbana-Champaign, using an Applied Biosystems gas phase amino acid sequencer.

RESULTS

Identification of reaction product

The 17α-HSDH activity was first detected in cultures of *Eubacterium sp.* VPI 12708 induced with cholic acid (100 μM). Soluble cell extracts were assayed under strict anaerobic conditions, using androstenedione and NADPH as substrate and cofactor, respectively. No product was detected under aerobic conditions. Steroid substrate and product were extracted with ethyl acetate and separated by thin-layer chromatography. When observed under ultraviolet light, the chromatogram revealed the presence of two distinct metabolites. Relative mobility for the reaction product was found to be 0.15 in a solvent system consisting of methylene chloride–cyclohexane–acetone 80:20:5 (by vol). R_f values for the 17α- and 17β-hydroxy-4-androsten-3-one were determined to be 0.15 and 0.22, respectively. Gas chromatographic analysis of the TMS ether derivative of the product gave a retention index of 25.75 MU, which was identical to that obtained for the TMS ether of epitestosterone. The electron (70 eV) ionization mass spectrum of the product revealed a molecular ion (M^+) at *m/z* 360 and base peak at *m/z* 129. The presence of one derivatized hydroxyl group was evident from the ion at *m/z* 270 (M-90), and this spectrum was identical to that of the TMS ether derivative of an authentic sample of epitestosterone (**Fig. 1**).

When the methyloxime trimethylsilyl ether derivative was prepared, two partially resolved peaks were observed with retention indices of 26.19 MU and 26.22 MU which represent the ani- and syn-isomers of the 3-methoxime-Δ^4-structure. The electron (70 eV) ionization mass spectra

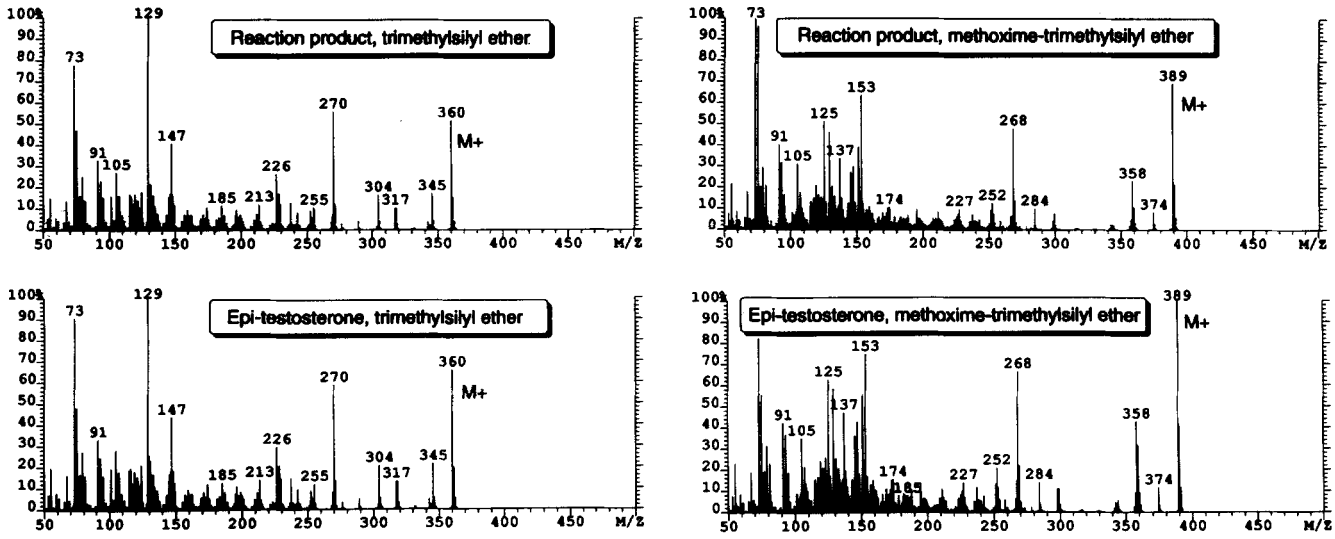

Fig. 1. Mass spectrometric analysis of the 17α-HSDH steroid product. Electron ionization (70 eV) mass spectra of the trimethylsilyl ether derivative of the steroid product (upper left) and epitestosterone standard (lower left). The mass spectra of the methoxime-trimethylsilyl ether derivative of the 17α-HSDH steroid product and epitestosterone, methoxime-trimethylsilyl standard are shown in right panels.

of the two isomers were identical and revealed an intense molecular ion and base peak at *m/z* 389. Fragmentation with loss of the methyloxime group (M-29) yielded the ion at *m/z* 358 from which loss of trimethylsilanol (-90) gave rise to the ion at *m/z* 268. Ions at *m/z* 125, 137 and 153 are characteristic of methyloxime derivatives of 3-oxo-Δ^4-steroids. The mass spectra of the MO-TMS ether derivatives were identical to that obtained from an authentic standard of epitestosterone (Fig. 1).

Steroid inducibility

17α-HSDH activity was first detected in cultures of *Eubacterium sp.* VPI 12708 induced with cholic acid. Optimal expression of the protein was obtained when androstenedione was used as an inducer. Interestingly, no activity was present in cultures induced with deoxycholic acid. This has been found to be the case for the *bai* operon, which encodes proteins involved in the 7α-dehydroxylation pathway for *Eubacterium sp.* VPI 12708 (4, 5). However, none of the activities involved in this pathway could be detected when androstenedione was used as the inducer, suggesting that 17α-HSDH is not included in the *bai* operon (**Table 1**).

Enzyme purification

Purification of the enzyme was achieved using four sequential fractionation steps, as described in **Table 2.** The key step in the purification process was the phenyl-Sepharose (**Fig. 2**) that allowed a 93-fold purification with a 39% recovery. In addition, we took advantage of the high affinity of the enzyme for $NADP^+$ in two consecutive Blue Agarose affinity chromatographic steps. In the first chromatography, the presence of 50 μM $NADP^+$ in the buffer was enough to prevent binding to the column. Removal of $NADP^+$ from the protein solution for the second column, allowed binding and specific elution of the

TABLE 1. Effect of different bile acids and androstenedione on 17α-HSDH induction

Inducer	17α-HSDH Activity[a]	*Bai* Operon Activities[b]
None	< 1	–
Deoxycholic acid	< 1	–
Cholic acid	10	+
Androstenedione	17	–

[a] Activity is expressed in nmol of epitestosterone formed/mg protein in 30 min.

[b] Bai = bile acid inducible operon for 7α-dehydroxylation of primary bile acids.

TABLE 2. Purification of the 17α-hydroxysteroid dehydrogenase

Purification Step	Volume	[Protein]	Activity[a]	Purification	Yield
	ml	*mg*	*units/mg*	*-fold*	*%*
Crude extract	50	9.5	2.7	1.0	100
Ammonium sulfate	65	1.97	9.1	3.4	90
Phenyl Sepharose	40	0.051	250	93	39
Blue Agarose I	14	0.060	393	150	25
Blue Agarose II	3	0.050	2300	850	1

[a] One unit of activity is defined as nmol of epitestosterone produced/min.

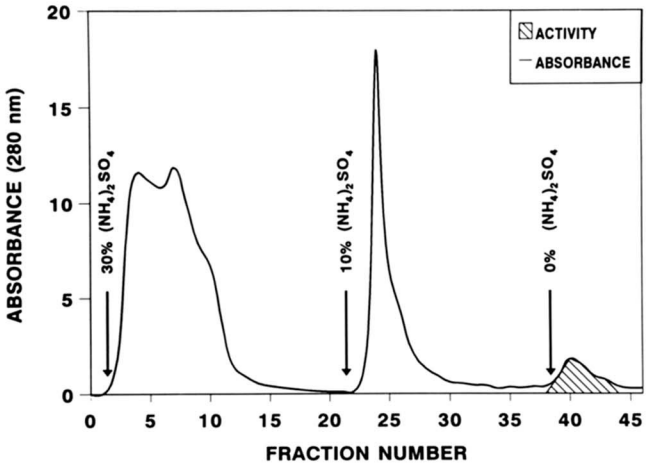

Fig. 2. 17α-HSDH phenyl Sepharose elution profile. The solid graph line represents absorbance at 280 nm. The column was washed with decreasing concentrations of ammonium sulfate, as indicated in the figure by the arrows. 17α-HSDH activity was eluted with a 0% ammonium sulfate buffer, as indicated by the shaded area under the curve.

protein with 50 μM $NADP^+$. The overall process resulted in an 850-fold purification of the enzyme. However, the enzyme rapidly lost activity during the final purification step. Aliquots from each purification step were subjected to SDS-PAGE analysis, to determine the purity of the enzyme (**Fig. 3**).

The size of the subunit, from SDS electrophoretic analysis, was estimated to be 42,000 daltons. The size of the native enzyme was determined using an HPLC GF-250 column. A calibration curve was generated plotting molecular weight logarithms against retention time, using five standards. The pure enzyme elution time indicated a relative molecular weight of 160,000 (data not shown). This suggests that the enzyme exists in the form of a tetramer of identical subunits.

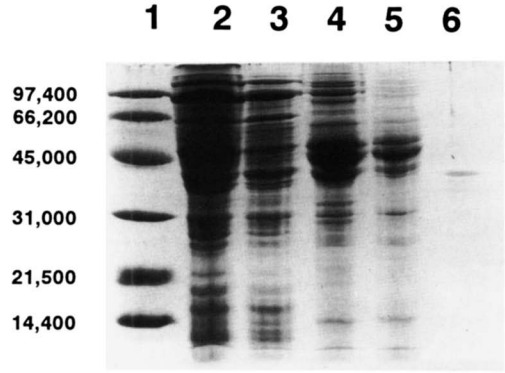

Fig. 3. SDS-PAGE of protein fractions from the purification of the 12α-HSDH. Lanes contain the following: 2, cell-free extract (20 μg), 3, 65% ammonium sulfate supernatant fractions (15 μg); 4, phenyl Sepharose pool fractions (10 μg); 5, Blue Agarose chromatography I fractions (5 μg); and 6, Blue Agarose chromatography II fractions (1 μg). The molecular weight marker sizes are given on the left side of the figure (lane 1).

The effect of pH on enzymatic activity in both the reductive and oxidative directions was studied over a pH range of 4.5–10.5 (**Fig. 4**). In both directions, the enzyme seems to prefer acidic pH values for activity. The pH optimum for the reductive reaction showed a broad peak between pH 5.5–7.5. For the oxidative direction, the pH optimum was found to be pH 6.5.

The enzyme was found to be specific for the 17α-hydroxy group of C-19 steroids. No activity was detected when testosterone (17β-isomer) was used as substrate. Estradiol and estrone were also tested as possible substrates, at pH 6.0 and 8.0, but the enzyme was unable to utilize either C-18 compound. As 17α-HSDH expression was induced by cholic acid, other bile acids were examined as potential substrates, but with negative results.

Determination of kinetic constants

The kinetic constants of the oxidative and reductive reaction were determined using the method of Hanes (13). **Fig. 5** and **Fig. 6** show the results for androstenedione and epitestosterone, respectively. Maximal velocity and affinity constants for each of the substrates in the oxidative and reductive direction of the reaction are shown in **Table 3.** In both directions, the enzyme exhibited higher affinity for the pyridine cofactor than the steroid substrate. In addition, according to the V_{max}/K_m ratios for each substrate, it appears that the reductive reaction would be favored over the oxidative direction. There was no detectable activity when NADH was used as the pyridine nucleotide cofactor.

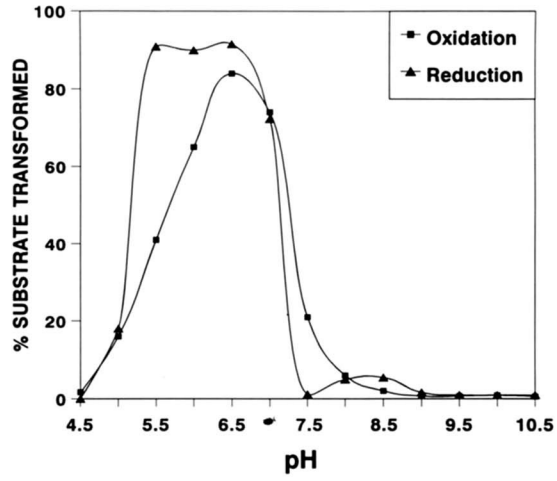

Fig. 4. Effect of pH on 17α-HSDH activity. All buffer ion concentrations were 100 mM. The following buffer systems were used in the pH optima determination: sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–7.5), Tris-chloride (pH 8.0–9.5), and glycine sodium hydroxide (pH 10–10.5). Enzymatic activity is expressed as the percentage of substrate transformed on each reaction, under identical conditions of time and substrate concentration.

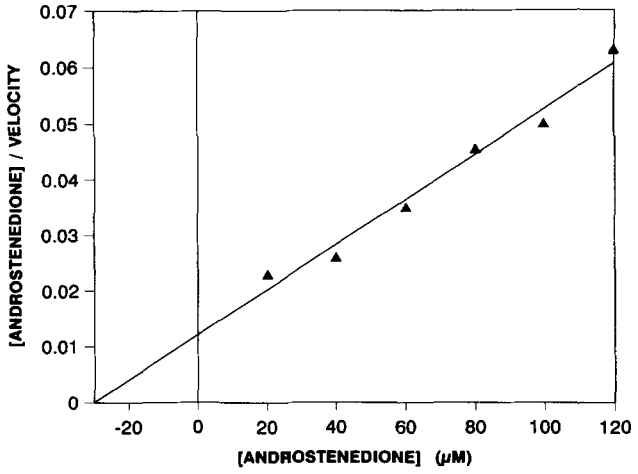

Fig. 5. Hanes plots for 17α-HSDH reductive reaction. Saturation kinetics were performed as described in the Methods section. Primary plot of [androstenedione]/V versus [androstenedione]. NADPH was kept at a constant concentration of 50 μM. Substrate concentrations are expressed in μmols. Velocity is expressed as nmoles of steroid produced/min per mg of protein.

Treatment of the 17α-HSDH with ethylenediaminetetraacetic acid (EDTA) resulted in total loss of enzymatic activity. Five different divalent cations were added individually to the inactive enzyme, to try to restore activity. Iron, calcium, and copper had no effect on the enzyme activity. Zinc and magnesium partially restored the activity, although neither one could return it to its original levels (**Fig. 7**).

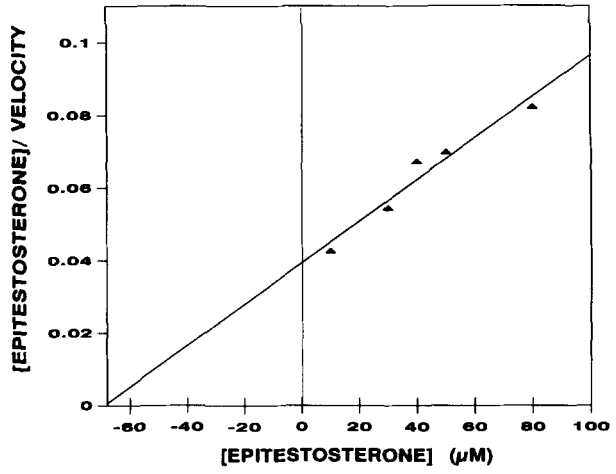

Fig. 6. Hanes plots for 17α-HSDH oxidative reaction. Saturation kinetic determinations were obtained as described in the Methods section. Primary plot of [epitestosterone]/V versus [epitestosterone]. $NADP^+$ was kept at a constant concentration of 50 μM. Substrate concentrations are expressed in μmols. Velocity is expressed as nmols of steroid produced min/mg of protein.

TABLE 3. Kinetic constants for 17α-HSDH

Substrate	V_{max}[a]	K_m	V_{max}/K_m
	units	μM	units/μM
Androstenedione	3257	31	105
NADPH	3236	5	647
Epitestosterone	1757	70	25
$NADP^+$	1851	2	925

[a]One unit of activity is defined as nmol steroid product produced per mg protein per min.

Amino acid sequence analysis

The first 21 N-terminal amino acid residues were determined using a gas-phase protein sequencer (**Fig. 8**). The amino acid sequence showed a significant homology with several disulfide oxidoreductases (14), at the level of the flavin-binding domain (15, 16). No significant N-terminal sequence similarity was found with other hydroxysteroid dehydrogenases.

DISCUSSION

Epitestosterone (17α-hydroxy-4-androsten-3-one) was first isolated and identified from human urine in the early 1960s (17). Both epitestosterone (epi-T) and its sulfate conjugate have been reported to be secreted by the human testis (18). However, the pathway(s) of biosynthesis of epi-T are still controversial. In recent years, there has been

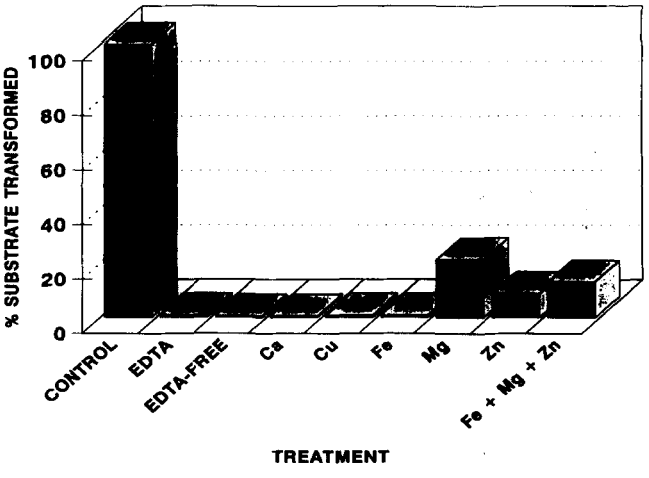

Fig. 7. Effect of divalent cations of 17α-HSDH activity. The experimental conditions are described in the Methods section. All activities are expressed as percentage of substrate transformed on reactions run under identical conditions. All values are normalized by setting the activity of the control to 100%. The cation used is indicated on the x-axis of the figure. In addition, the positive control and two negative controls (EDTA and EDTA-free) are shown.

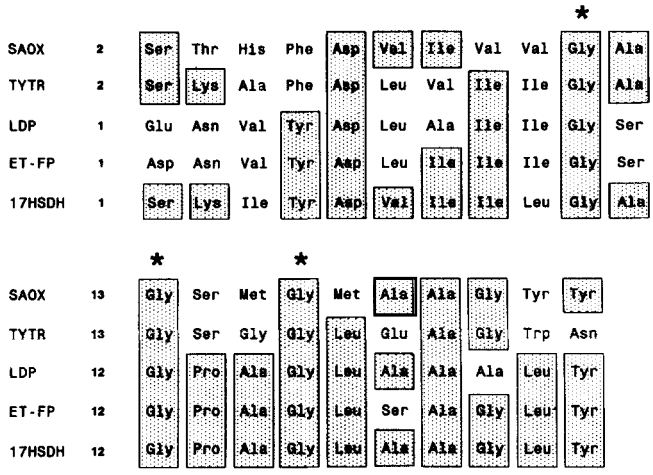

Fig. 8. N-terminal amino acid sequence similarity between 17α-HSDH and other disulfide oxidoreductases. The first 21 residues determined for the 17α-HSDH are illustrated and labeled 17α-HSDH. The other sequences are as follows: SAOX: sarcosine oxidase from *Bacillus sp.* strain ns-129; TYRA: trypanothione reductase from *Trypanosoma congolense;* LPD: dihydrolipoamide dehydrogenase from *Eubacterium acidaminophilum;* ET-FP: electron transferring flavoprotein from *Clostridium sporogenes.* The asterisk at the glycines indicate the consensus motif for the FAD-binding domain (14, 15).

renewed interest in epi-T because of its potential as an antiandrogen (19, 20) and 5α-reductase inhibitor (21). Moreover, the ratio between urinary testosterone and epi-T is accepted by the International Olympic Committee as a marker for testosterone doping in athletes (22, 23).

Little is known about the enzymology of epi-T formation in the body. Animal studies have reported the presence of pyridine nucleotide-linked 17α-oxidoreductase activities in both liver and kidney cytosols of adult hamsters (24). 3(17)α-Hydroxysteroid dehydrogenase has been purified from rabbit kidney and liver (25). Nakagawa et al. (26) purified two forms of mouse kidney dihydrodiol dehydrogenases with 3(17)α-hydroxysteroid dehydrogenase activity. The microbial 17α-hydroxysteroid dehydrogenase described in the present study differs from the mouse and rabbit 17α-HSDH in the following respects: no detectable 3α-hydroxysteroid dehydrogenase activity, sensitivity to molecular oxygen, divalent metal cation requirements and specificity for $NADP^+$, rather than both NAD^+ and $NADP^+$. It is not known whether intestinal microflora plays any role in the formation of epi-T in the body. However, conjugates of testosterone are secreted in bile (27, 28) and may be biotransformed by bacterial enzymes found in the gastrointestinal tract (29, 30). Intestinal microflora have been reported to have 17β-hydroxy-steroid dehydrogenase activity (8) and consequently could epimerize the 17β to the 17α-hydroxy derivative via a 17-oxo intermediate.

17α-Hydroxysteroid dehydrogenase isolated from *Eubacterium sp.* VPI 12708 does not belong to any of the previously described bacterial or animal hydroxysteroid dehydrogenases based on N-terminal amino acid sequence (Fig. 8), molecular weight, EDTA, and molecular oxygen sensitivity. This enzyme appears to be most closely related to the small subfamily of dihydrolipoamide dehydrogenases found in certain species of the genera *Clostridium* and *Eubacterium* (14). These NADPH-dependent electron-transferring flavoproteins in this gene family are capable of reducing a variety of artificial electron acceptors, i.e., menadione, methyviologen. However, 17α-hydroxysteroid dehydrogenase differs from these enzymes in having a tetrameric quaternary structure and EDTA-sensitivity, while the NADPH-dependent electron-transferring flavoproteins have a dimeric quaternary structure and are insensitive to EDTA. Cloning, sequencing, and further characterization of the gene encoding 17α-hydroxysteroid dehydrogenase may provide additional insights into the structure and evolutionary origin of this newly discovered hydroxysteroid dehydrogenase gene family.

17α-Hydroxysteroid dehydrogenase from *Eubacterium sp.* VPI 12708 is induced by growth in the presence of cholic acid and androstenedione, but not by deoxycholic acid. We have previously shown that cholic acid, but not deoxycholic acid, induces a bile acid 7α-dehydroxylation pathway in this bacterium. Androstenedione does not induce the bile acid 7α-dehydroxylation pathway. We hypothesize that a bile acid intermediate (12α-hydroxy-3-oxo-4-cholenoic acid) in the 7α-dehydroxylation pathway is a fortuitous inducer of the 17α-hydroxysteroid dehydrogenase gene. Both androstenedione and this bile acid intermediate have a common steroid A/B ring structure, which may allow for induction of the 17α-hydroxysteroid dehydrogenase gene.

In summary, we have discovered, purified, and characterized a novel 17α-hydroxysteroid dehydrogenase from a human intestinal anaerobic bacterium. Preliminary data indicate that this enzyme belongs to a new gene family separate from other hydroxysteroid dehydrogenases. Our data further suggest that epitestosterone could be generated from testosterone by the intestinal microflora.

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