JOURNAL OF BACTERIOLOGY, June 1989, p. 2925–2932
0021-9193/89/062925-08$02.00/0

Vol. 171, No. 6

Purification and Characterization of a Novel Form of 20α-Hydroxysteroid Dehydrogenase from *Clostridium scindens*

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Received 9 November 1988/Accepted 27 February 1989

We have purified a steroid-inducible 20α-hydroxysteroid dehydrogenase from *Clostridium scindens* to apparent homogeneity. The final enzyme preparation was purified 252-fold, with a recovery of 14%. Denaturing and nondenaturing polyacrylamide gradient gel electrophoresis showed that the native enzyme (M_r, 162,000) was a tetramer composed of subunits with a molecular weight of 40,000. The isoelectric point was approximately pH 6.1. The purified enzyme was highly specific for adrenocorticosteroid substrates possessing 17α,21-dihydroxy groups. The purified enzyme had high specific activity for the reduction of cortisone (V_{max}, 280 nmol/min per mg of protein; K_m, 22 μM) but was less reactive with cortisol (V_{max}, 120 nmol/min per mg of protein; K_m, 32 μM) at pH 6.3. The apparent K_m for NADH was 8.1 μM with cortisone (50 μM) as the cosubstrate. Substrate inhibition was observed with concentrations of NADH greater than 0.1 mM. The purified enzyme also catalyzed the oxidation of 20α-dihydrocortisol (V_{max}, 200 nmol/min per mg of protein; K_m, 41 μM) at pH 7.9. The apparent K_m for NAD^+ was 526 μM. The initial reaction velocities with NADPH were less than 50% of those with NADH. The amino-terminal sequence was determined to be Ala-Val-Lys-Val-Ala-Ile-Asn-Gly-Phe-Gly-Arg. These results indicate that this enzyme is a novel form of 20α-hydroxysteroid dehydrogenase.

Clostridium scindens is the only bacterium isolated known to synthesize 20α-hydroxysteroid dehydrogenase (20α-HSDH) and steroid-17-20-desmolase activities (3, 51). Preliminary studies with cell extracts indicated that both neutral steroid-transforming activities were coinducible in *C. scindens* cultured in the presence of specific C_{21} steroids. In addition, it was found that both conversions required a pyridine nucleotide coenzyme, bivalent metal cations, and the same adrenocorticosteroid substrates for maximal activity (22). 20α-HSDH (EC 1.1.1.149) is widely distributed in nature. The enzyme has been found previously in bird testes, fungi (8, 11), and a great variety of mammalian tissues. In vertebrate species, 20α-HSDH is thought to be a key enzyme involved in tissue-specific regulation of steroid hormone metabolism (10, 39). Various forms of 20α-HSDH are found in the major steroid-producing tissues of mammals (adrenals, ovaries, testes, and placenta) and in liver, kidney, muscle, lymphatic organs, fibroblasts, and hematopoietic cells (4, 28, 29, 45, 47, 49). Multiple forms of 20α-HSDH have been distinguished in several tissues, largely on the basis of steroid substrate specificity, pyridine nucleotide requirement, and intracellular location (2, 12, 37). A soluble enzyme from human placenta (36), rat ovary (35, 50), and the testes of two species (33, 40) has been purified to homogeneity and well characterized. This report describes the purification and partial characterization of a novel form of 20α-HSDH from *C. scindens*.

(A preliminary report of this work has appeared previously [A. E. Krafft and P. B. Hylemon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, K24, p. 210].)

MATERIALS AND METHODS

Abbreviations and trivial names. The following trivial names and abbreviations are used: Cortisone, 17,21-dihydroxy-4-pregnene-3,11,20-trione; cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20-dione; 11-desoxycortisol, 17,21-dihydroxy-4-pregnene-3,20-dione; cortisol 21-acetate, 11β,17,21-trihydroxy-4-pregnene-3,20-dione 21-acetate; cortisol 21-phosphate, 11β,17,21-trihydroxy-4-pregnene-3,20-dione 21-phosphate; 5β-dihydrocortisol, 11β,17,21-trihydroxy-5β-pregnan-3,20-dione; corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; progesterone, 4-pregnene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; 20α-dihydrocortisol, 11β,17,20α,21-tetrahydroxy-4-pregnene-3-one; 11β-hydroxyandrostenedione, 11β-hydroxyandrost-4-ene-3,17-dione; MES, 4-morpholineethansulfonic acid; MOPS, (3-[*N*-morpholino]propane-sulfonic acid); CHES, (2[*N*-cyclohexylamino]-ethanesulfonic acid); PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Chemicals. The following chemicals were purchased: unlabeled steroids, Steraloids Inc., Wilton, N.H., and Sigma Chemical Co., St. Louis, Mo.; [1,2-^{3}H]cortisol (40.6 Ci/mmol), Amersham Corp; high-pressure liquid chromatography (HPLC) solvents, Burdick and Jackson; brain-heart infusion medium, Difco Laboratories; protease inhibitors, Boehringer Mannheim; pyridine nucleotides, P-L Biochemicals, Inc.; DEAE-cellulose (DE-52), Whatman, Inc.; Cibacron blue agarose, Pierce Chemical Co.; molecular weight standards, Bio-Rad Laboratories. *Escherichia coli* alkaline phosphatase was kindly provided by Jan F. Chlebowski and Nancy Ulbrandt. The Altex Ultrasphere ODS reverse phase column (4.6 mm by 15 cm; 5-μm particle packing) and the Spherogel TM TSK DEAE-3SW (7.5 mm by 7.5 cm) column were obtained from Beckman Instruments, Inc. The Zorbax GF-250 HPLC column was from the Du Pont Co.

Growth of bacteria. Stock cultures of *C. scindens* ATCC 35704 maintained in 33% (vol/vol) glycerol at −70°C were grown in 1 liter of brain-heart infusion-cysteine medium, pH

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7.0 to 7.2, as described previously (22). Cultures were induced to synthesize 20α-HSDH and desmolase by the addition of cortisol dissolved in ethanol at the time of inoculation and at 1.5-h intervals during growth to give a final steroid concentration of 0.2 mM. Growth was monitored with a Klett-Summerson colorimeter (no. 66 red filter). Cells were harvested at 130 Klett units by centrifugation at 8,000 × *g* for 10 min. Cell pellets were washed once with anaerobic 20 mM sodium phosphate buffer (pH 6.8) containing 20% (vol/vol) glycerol and 10 mM 2-mercaptoethanol. Cells were frozen at −20°C until used.

Buffers. The buffers used in the purification protocol were as follows. Buffer A, 20 mM sodium phosphate, pH 7.0, 15% (vol/vol) glycerol; buffer B, buffer A with 0.1 M NaCl; buffer C, buffer A with 0.3 M NaCl; buffer D, 20 mM sodium phosphate, pH 7.0, 20% (vol/vol) glycerol; buffer E, 20 mM sodium phosphate, pH 6.5, 0.1 M NaCl, 15% (vol/vol) glycerol; buffer F, 20 mM sodium phosphate, pH 6.0, 0.1 M NaCl, 10% (vol/vol) glycerol; and buffer G, 20 mM sodium phosphate, pH 6.0, 0.3 M NaCl, 10% (vol/vol) glycerol. All buffers contained 10 mM 2-mercaptoethanol and 0.5 mM PMSF. Buffers were made anaerobic by boiling and then cooling under a nitrogen atmosphere. All procedures, except loading and eluting HPLC columns, were carried out at 4°C.

Enzyme assays. The 20α-HSDH and desmolase activities of each fraction were assayed by using modifications to our previously reported method (22). Throughout the purification procedure, 20α-HSDH was assayed in the reductive direction with the following standard reaction mixture: 80 μmol of sodium MES, pH 6.3, 100 nmol of NADH, 50 nmol of cortisone, 5% (vol/vol) ethanol, 10 mM 2-mercaptoethanol, and enzyme preparation in a total volume of 1 ml. Samples were incubated under an argon atmosphere for 10 min at 37°C. For measurement of desmolase activity, the reaction mixture included 70 μmol of sodium phosphate, pH 7.5, 0.5 μmol of NAD^+, 50 nmol of cortisol, 5% (vol/vol) ethanol, 10 mM 2-mercaptoethanol, and 20% (vol/vol) glycerol in a total volume of 1 ml. Samples were incubated under an argon atmosphere for 30 min at 37°C. The reactions were stopped by the addition of 0.5 ml of 0.1 N HCl. To avoid chemical decomposition of the neutral steroids, assay mixes were immediately extracted once with 5 ml of methylene chloride, evaporated to dryness under an N_2 gas atmosphere at 42°C, and analyzed by C_{18} reverse-phase HPLC. The extraction efficiency of cortisol and the corresponding C_{19} and 20α derivatives from reaction mixtures was typically greater than 95%.

Purified 20α-HSDH activity was also monitored by a continuous spectrophotometric assay following the oxidation or reduction of pyridine nucleotide cofactors with a Shimadzu 160 UV-visible spectrophotometer. The slope of the initial linear change in the A_{340} as a function of time was used to quantitate enzyme activity (ε_{340}, 6,270 M^{-1} cm^{-1}) at 37°C. The assay mixtures (1.0 ml total volume) contained steroids, 5% (vol/vol) ethanol, pyridine nucleotides as indicated, and 80 μmol of MES buffer, pH 6.3, or 80 μmol of Tris hydrochloride buffer, pH 7.9, to monitor pyridine nucleotide oxidation and reduction, respectively. Steroid-dependent NADH oxidation was linear with time and enzyme concentration only with relatively high amounts of enzyme (20 to 25 μg/ml). Stoichiometric analysis revealed that NADH consumption by the purified 20α-HSDH in aerobic assays corresponded to the amount of 20α-dihydrocortisol produced from cortisol in anaerobic assays on an equimolar basis. The sensitivity of the spectrophotometric assay was not sufficient for detection of 20α-HSDH activity until the final step of the purification protocol because of a high background of endogenous NADH oxidation. Least-mean-squares fit of kinetic data was obtained with a Hewlett-Packard 11C programmable calculator. In all cases, the criteria for linearity depended on a correlation coefficient of 0.98 or above.

Enzyme units. The amount of enzyme activity transforming 1 nmol of steroid per min under the assay conditions described above was defined as 1 U of 20α-HSDH activity. Specific activity is expressed as units per milligram of protein.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity was monitored spectrophotometrically at 340 nm under the conditions described by the supplier (Sigma) adapted to 1-ml assay solutions. The substrate DL-glyceraldehyde-3-phosphate was generated from DL-glyceraldehyde-3-phosphate diethylacetal, monobarium salt, according to the instructions of the supplier (Sigma). The assay mix contained 100 μmol of Tris (pH 8.5), 17 μmol of sodium arsenate (pH 8.5), 3.3 μmol of L-cysteine hydrochloride (pH 7), 20 μmol of sodium fluoride, 3.3 μmol of β-NAD, and ca. 300 nmol of DL-glyceraldehyde-3-phosphate.

Preparation of cell extract. Cells (15 g, wet weight) were thawed and suspended in a minimal volume of buffer A. The cell suspension was passed twice through a chilled French pressure cell (14,000 lb/in^2) and centrifuged for 10 min at 12,000 × *g* to remove cell debris. The supernatant fluid was centrifuged at 105,000 × *g* for 2 h.

DEAE-cellulose batch. The 105,000 × *g* supernatant fluid was applied to a DEAE-cellulose column (2.5 by 1.6 cm). The column was washed with 3 bed volumes of buffer C to elute proteins, which were precipitated with ammonium sulfate (45 to 75% saturation), redissolved, and dialyzed against buffer B.

DEAE-cellulose column chromatography. The dialyzed proteins were applied to a column of DEAE-cellulose (13 by 2.6 cm). Proteins were eluted with a 400-ml linear gradient of buffer B and buffer C (0.1 to 0.3 M NaCl, pH 7) at a flow rate of 0.4 ml/min. Fractions (3.2 ml) were collected and assayed for enzyme activities as described above. Fractions 35 to 57, containing the peak of 20α-HSDH activity, were pooled, precipitated by 75% ammonium sulfate, and dialyzed against buffer D.

Cibacron blue affinity chromatography. The dialyzed proteins were stirred with 15 ml of Cibacron blue agarose for 1 h. The gel slurry was poured into a column (2.5 by 1.6 cm), which was washed with buffer D at a flow rate of 0.15 ml/min. 20α-HSDH activity was eluted with 1 mM NAD^+ in 20 ml of buffer C. The NAD^+–0.3 M NaCl eluate was concentrated with an Amicon Centriprep 10 concentrator.

Gel filtration HPLC. Concentrated protein was applied in 1.0-ml portions to a GF-250 column equilibrated with buffer E. The column flow rate was 0.75 ml/min, and fractions were collected at 0.5-min intervals. The peak of 20α-HSDH activity in fractions 22 to 25 was pooled.

DEAE-HPLC. Protein was chromatographed on a DEAE-3SW column equilibrated with buffer F. The column was developed with buffer G at a flow rate of 0.75 ml/min. The gradient was programmed for 0 to 25% buffer G in 5 min and 25 to 100% buffer G over 75 min. The purified enzyme eluting at approx. 42 min (230 mM NaCl) from DEAE-HPLC was concentrated with an Amicon Centricon 10 microconcentrator into 50 mM sodium phosphate, pH 6.8, containing 10 mM 2-mercaptoethanol and 50% (vol/vol) glycerol and stored at −20°C.

Protein determination. Proteins were measured by the spectrophotometric method of Kalb and Bernlohr (21). In

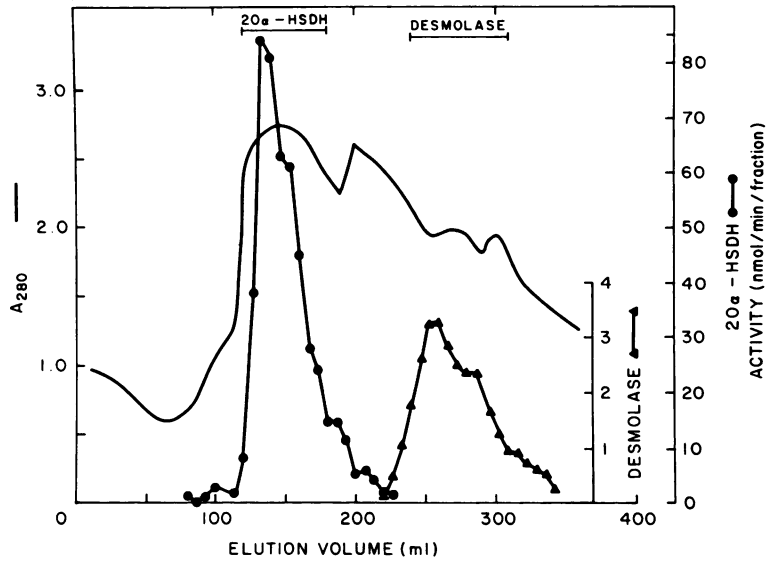

FIG. 1. Separation of 20α-HSDH and 17,20-desmolase by DEAE-cellulose chromatography. Dialyzed proteins from the 45 to 75% ammonium sulfate fractionation step were applied to a column (13 by 2.6 cm) equilibrated in anaerobic 20 mM sodium phosphate containing 15% glycerol, 0.1 M NaCl, 10 mM 2-mercaptoethanol, and 0.5 mM PMSF, pH 7.0. Both enzyme activities were eluted with a linear gradient of 0.1 to 0.3 M NaCl. The A_{280} (——) was monitored, and fractions containing 20α-HSDH (●) and desmolase (▲) activities were determined by a reverse-phase HPLC assay.

monitoring column effluents, the A_{280} value was taken as an estimate of the protein concentration.

PAGE. Proteins present in samples from each step of purification were analyzed by SDS-PAGE as described by Laemmli (24). Proteins were stained with 0.25% Coomassie brilliant blue R-250.

Purified 20α-HSDH was electrophoresed on 7 to 30% polyacrylamide slab gels (pore gradient electrophoresis) in the absence of SDS by an adaptation of the procedure described by the supplier ("Polyacrylamide Gel Electrophoresis Laboratory Techniques," p. 8–10; Pharmacia Fine Chemicals, Uppsala, Sweden). Protein samples were dissolved in or equilibrated with an equal volume of 4× running buffer containing 40% (vol/vol) glycerol and 0.01% bromophenol blue. The standard proteins used for calibration of gels and the molecular weights assumed for them were as follows: egg albumin, 43,000; bovine serum albumin: monomer, 67,000; dimer, 120,000; *E. coli* alkaline phosphatase, 94,000; beef liver catalase, 240,000; jack bean urease: trimer, 272,000; hexamer, 575,000. The gels were electrophoresed for 16 h at 150 V constant voltage at 4°C in 90 mM Tris–8 mM boric acid–2.5 mM EDTA, pH 8.4. Proteins were visualized by Coomassie brilliant blue staining.

Isoelectric focusing was carried out at 25°C with the Bio-Rad Ampholine electrofocusing equipment as described by O'Farrell (32).

Amino acid sequence analysis. A sample of purified protein (0.86 nmol) was prepared for amino acid analysis and N-terminal sequencing by extensive dialysis against 10 mM sodium phosphate, pH 6.8, followed by lyophilization. The amino acid composition was determining by using the following instrumentation: a WISP autosampler, Varian HPLC, SOTA ion-exchange column, Kratos postcolumn reaction system, Shimadzu flowthrough variable-wavelength detector, and Hewlett-Packard HP 3000 integrator.

The amino terminus of *C. scindens* 20α-HSDH (residues 1 to 11) was sequenced by the Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, with an Applied Biosystems model 470A gas-phase sequencer. The phenylthiohydantoin-derivatized amino acids were identified by ODS HPLC on an on-line model 120A phenylthiohydantoin analyzer equipped with the Hewlett-Packard HP 3000 integrator.

RESULTS

Purification of 20α-HSDH. *C. scindens* contains steroid-inducible 20α-HSDH and steroid-17-20-desmolase activities (22). Both enzymes showed maximal specific activities in cells harvested in the early logarithmic phase of growth. 20α-HSDH was separated clearly from steroid-17-20-desmolase by DEAE-cellulose chromatography (Fig. 1). Ste-

TABLE 1. Purification of 20α-HSDH from *C. scindens*

Purification step	Total protein (mg)	Specific activity (U/mg of protein)	Total activity (U)	Yield (%)	Purification (fold)
1. 105,000 × *g* cell extract	2,565	1.12	2,872	100	
2. DEAE-cellulose batch, 45 to 75% $(NH_4)_2SO_4$	1,223	2.11	2,580	90	1.9
3. DEAE-cellulose chromatography, pH 7	155	5.62	871	30	5
4. Cibacron blue NAD–0.3 M NaCl eluate	4.73	146	691	24	130
5. Gel filtration-HPLC	3.04	175	532	19	156
6. DEAE-HPLC, pH 6.0	1.43	282	403	14	252

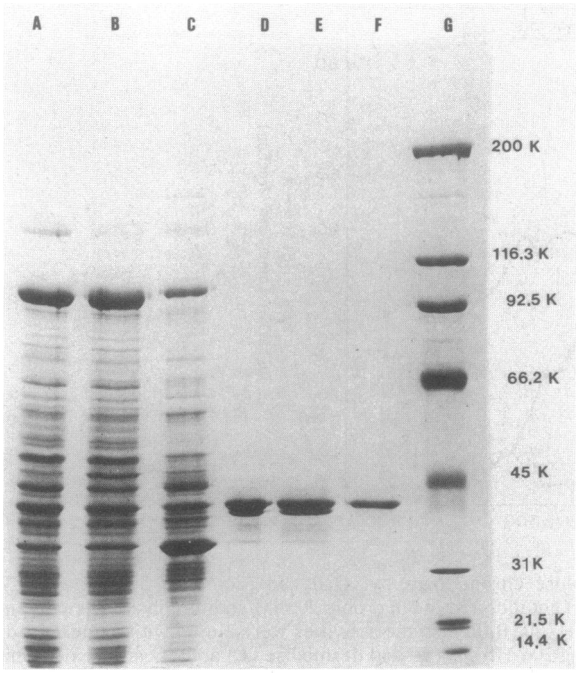

FIG. 2. Protein profile of 20α-HSDH purification analyzed by gradient (10 to 20%) SDS-PAGE. Proteins were stained with Coomassie brilliant blue. Lane A, Soluble cell extract (80 μg); lane B, DEAE-cellulose batch and 45 to 75% ammonium sulfate fractionation (80 μg); lane C, pooled fractions from DEAE-cellulose chromatography, pH 7.0 (80 μg); lane D, protein eluted from Cibacron blue agarose with 1.0 mM NAD^+ (20 μg); lane E, pooled fractions from GF-250 gel filtration column (20 μg); lane F, purified 20α-HSDH from Altex DEAE-3SW DEAE column, pH 6 (5 μg); lane G, molecular weight standards. Molecular weights (in thousands) are shown to the right.

roid-17-20-desmolase eluted immediately following the 20α-HSDH activity but was not further purified.

The procedure for purification of 20α-HSDH is summarized in Table 1. Crude soluble extract protein (2.6 g) was obtained from large-scale anaerobic culture (18 liters), yielding 15 g of cells (wet weight). A 252-fold purification provided 1.4 mg of purified 20α-HSDH with a specific activity of 282 nmol/min per mg of protein (measured with cortisone by reverse-phase HPLC assay) and a 14% recovery.

The key steps in the purification of 20α-HSDH were anion-exchange (see Fig. 3) and affinity chromatography. Initial bulk separation by conventional DEAE-cellulose chromatography (Table 1, step 3) gave a fivefold purification. Despite the considerable loss of enzyme units at this step, it was deemed necessary to remove 260-nm-absorbing materials which interfered with the binding of the enzyme to Cibacron blue agarose. Affinity absorption chromatography proved to be the most effective step, providing a 130-fold purification over the preceding step with an 80% recovery of activity (Table 1, step 4). Subsequent purification to apparent homogeneity was achieved by gel filtration-HPLC and DEAE-HPLC at pH 6 (Table 1, steps 5 and 6).

Molecular weight determination. A sample from each stage of purification was analyzed by SDS-PAGE (Fig. 2). The purified protein resolved as a single band of M_r 40,000 ± 2,000. When the purified protein was subjected to pore gradient gel electrophoresis at pH 8.4 under nondenaturing conditions, a single band was determined with a relative molecular weight of 162,000 when gels were stained for protein (data not shown). The elution position of 20α-HSDH activity on gel filtration-HPLC corresponded to a relative molecular weight of 158,000 (data not shown). The agreement among the molecular weight estimates obtained by SDS-PAGE, nondenaturing gel electrophoresis, and gel filtration is consistent with a native enzyme of molecular weight 160,000 ± 2,000 containing four subunits of identical molecular weights.

N-terminal sequence analysis, amino acid composition, and isoelectric point. The N-terminal sequence (residues 1 to 11) was determined by gas-phase sequencing to be Ala-Val-Lys-Val-Ala-Ile-Asn-Gly-Phe-Gly-Arg. A computer-aided protein sequence homology search (FASTP) revealed striking homology between the N-terminal sequence of 20α-HSDH and that of several previously sequenced GAPDHs from a variety of sources (Table 2). Amino acid analyses of nonalkylated samples revealed that the protein was rich in glycine (22 mol%), serine (20 mol%), aspartate and asparagine (10 mol%), and glutamate and glutamine (8 mol%). No methionine residues were detectable. The isoelectric point was approximately 6.1 at 25°C with 6 M urea.

Stability of the enzyme activity. Throughout the development of a purification protocol, the apparent instability of the 20α-HSDH activity posed considerable difficulties. All buffers were prepared anaerobically and contained 2-mercaptoethanol in order to maintain a reduced oxygen tension during chromatography. Glycerol (10 to 20%) was also included in all buffers to prevent a large loss of enzyme activity (>99%). Concentration of enzyme solutions immediately following each chromatography step was required to retain activity.

TABLE 2. Comparison of N-terminal amino acid sequence of *C. scindens* 20α-HSDH with those of GAPDHs[a]

Enzyme	Beginning residue no.	β-Sheet
20α-HSDH	1	Ala-Val-Lys-Val-Ala-Ile-Asn-Gly-Phe-Gly-Arg
GAPDH		
B. stearothermophilus	1	Ala-Val-Lys-Val-Gly-Ile-Asn-Gly-Phe-Gly-Arg
T. aquatica	1	Met-Lys-Val-Gly-Ile-Asn-Gly-Phe-Gly-Arg
S. cerevisiae 1 + 2	1	Val-Arg-Val-Ala-Ile-Asn-Gly-Phe-Gly-Arg
S. cerevisiae 3	1	Ile-Arg-Ile-Ala-Ile-Asn-Gly-Phe-Gly-Arg
Pig muscle	1	Val-Lys-Val-Gly-Val-Asp-Gly-Phe-Gly-Arg
Chicken	1	Val-Lys-Val-Gly-Val-Asn-Gly-Phe-Gly-Arg
Human muscle	3	Val-Lys-Val-Gly-Val-Asp-Gly-Phe-Gly-Arg
Lobster muscle	1	AcSer-Lys-Ile-Gly-Ile-Asp-Gly-Phe-Gly-Arg

[a] References: *Bacillus stearothermophilus* (1), *Thermus aquatica* (16), *Saccharomyces cerevisiae* 1 (18), *S. cerevisiae* 2 (17), *S. cerevisiae* 3 (19), pig muscle (13), chicken (9), human muscle (31), and lobster muscle (7). The beginning residue number is the number of the first amino acid shown in each row. Established sequence differences are underlined.

TABLE 3. Steroid substrate specificity of purified 20α-HSDH from *C. scindens*[a]

Steroid	Relative velocity
Cortisone	100
Cortisol	42
11-Desoxycortisol	50
5β-Dihydrocortisol	35
Corticosterone	6
Cortisol 21-phosphate	6
Pregnenolone	6
Progesterone	4
17α-Hydroxyprogesterone	4
Cortisol 21-acetate	4
Deoxycorticosterone	1
11β-Hydroxyandrostenedione	<1

[a] The initial velocities of the enzyme-catalyzed oxidation of NADH were determined spectrophotometrically at 340 nm. The reactions were initiated by addition of purified enzyme (25 μg) to 1.0-ml reaction mixtures containing 0.08 M sodium MES (pH 6.3), 20% glycerol, 50 μM steroid, 5% ethanol, and 0.1 mM NADH at 37°C. All values are the means of three to four determinations. Assay detection limit was approx. 1% of the velocity of cortisone reduction.

TABLE 4. Kinetic constants for purified 20α-HSDH

Substrate[a]		K_m (μM)	V_{max} (nmol/min per mg)
Variable	Constant		
NADH	Cortisone	8.1	270
Cortisone	NADH	22	280
Cortisol	NADH	32	110
NAD^+	20α-Dihydrocortisol	526	180
20α-Dihydrocortisol	NAD^+	41	200

[a] Substrate concentrations were varied (1 to 450 μM NADH, 5 to 100 μM steroid, 0.05 to 2.0 mM NAD^+), with a minimum of six concentrations tested, or kept constant (100 μM NADH, 50 μM cortisone, 100 μM cortisol, 1 mM NAD^+, 100 μM 20α-dihydrocortisol).

The enzyme was stable when the pH of solutions was maintained between pH 6 and 7 at 4°C. No enzyme activity was detectable after a single freeze-thaw of the enzyme at any stage. The purified enzyme remained stable for several days when stored at either 4 or −20°C at concentrations of >500 μg of protein per ml in 0.05 M phosphate–50% glycerol–10 mM 2-mercaptoethanol, pH 6.8.

pH optimum. The pH optima of the enzyme were determined in the oxidative and reductive directions by using overlapping buffers between pH 4 and 10 at 0.1 M: citrate, MES, sodium phosphate, MOPS, Tris hydrochloride, and CHES. Steroid reduction, as measured by the formation of 20α-dihydrocortisol with NADH (0.1 mM) as the cosubstrate, was observed within the pH range 5.4 to 7.6, with maximal activity found between pH 6.0 and 6.5 with either MES, MOPS, or sodium phosphate buffer. Steroid oxidation, as measured by the formation of cortisol with NAD^+ (1.0 mM) as the cosubstrate, was observed over a broad pH range of 6.3 to 9.0, with an optimum at ca. pH 8 with Tris hydrochloride.

Steroid substrate specificity. The relative reaction velocity of purified 20α-HSDH for 12 steroids is shown in Table 3. Of the steroids tested, cortisone was reduced most rapidly. The other adrenocorticosteroids tested with 17α,21-dihydroxy groups were all good substrates; however, the activity of the enzyme with steroids differing at the C-11 position was less than that observed with cortisone (compare compound 1 with 2 through 4, Table 3). The enzyme showed comparable activity with 17α,21-dihydroxysteroids having the Δ^4-3-keto configuration (compounds 2 and 3) and with steroids saturated in ring A with 5β-hydrogen (A-B ring junction *cis*). The enzyme was much less reactive with 21-hydroxysteroids lacking the 17α-hydroxy group (compounds 5, 7, 8, and 11). C_{21} steroids with the 17α-hydroxy group which had either a methyl, phosphate, or acetate group at C-21 (compounds 9, 6, and 10, respectively) were also poor substrates. The purified enzyme had no detectable 17-hydroxysteroid oxidoreductase activity towards 4-androstene-11-β-ol-3,17-dione.

Kinetic parameters and pyridine nucleotide specificity. Table 4 shows the kinetic constants and maximal velocities for substrates of 20α-HSDH. Initial velocities were measured by varying the concentration of one substrate at the highest noninhibitory concentration of the cosubstrate. K_m and V_{max} values for each substrate were calculated from the linear portion of each Lineweaver-Burk plot (25). The purified enzyme catalyzed the reduction of cortisol with either NADH or NADPH as the cosubstrate. However, when NADPH was examined as a cosubstrate under optimum conditions for reduction of cortisol by NADH, the reaction rate observed was <50% of that with NADH, indicating that NADH was the preferred pyridine nucleotide. In the oxidative direction, the K_m for 20α-dihydrocortisol and NAD^+ were 41 and 526 μM, respectively.

The K_m for NADH was 8.1 μM with cortisone as the steroid substrate. At concentrations greater than 0.1 mM (Fig. 3), NADH was found to inhibit the rate of cortisone reduction. Although the maximum velocity of the reduction of cortisone was 2.4 times faster than the reduction of cortisol in the presence of NADH (0.1 mM), the enzyme showed similar affinities for each steroid substrate (K_m of 22 and 32 μM, respectively).

DISCUSSION

The purification of a soluble 20α-HSDH from *C. scindens* to apparent homogeneity is described in this article. A comparison of certain properties of the purified clostridial enzyme with those of eucaryotic enzymes indicates that the *C. scindens* enzyme is a novel form of 20α-HSDH.

The steroid substrate specificity and pyridine nucleotide requirement established for the clostridial enzyme differ from those for all previously described 20α-HSDHs. The purified clostridial enzyme is highly specific for 17α,21-dihydroxyadrenocorticosteroids and prefers NADH over NADPH as the cosubstrate. The liver is the major site of adrenocorticosteroid metabolism, and the 20α-HSDHs present in hog and rat liver (5, 20, 38) show a similar steroid specificity; however, these enzymes are microsomal and NADPH dependent (38). With the exception of a soluble mouse liver 21-hydroxysteroid, NADP 20α-steroidoxidoreductase (6, 23), all other 20α-HSDHs studied exhibit selective specificity towards either progesterone, 17α-hydroxyprogesterone, or pregnenolone as a substrate. It has been reported that among the enzymes which are reactive with 21-hydroxysteroids, substitutions at C-11 influence the relative rates of 20-keto reduction. In the current study, a general increasing velocity was observed in the order cortisol <11-desoxycortisol < cortisone, an order of reactivity similar to that of hepatic 20α-HSDH (20, 23).

A comparison of the subunit molecular weight of the clostridial 20α-HSDH (M_r 40,000) with those reported for the mammalian 20α-HSDHs shows that these enzymes are essentially similar in size. However, the active form of the clostridial enzyme, as determined by gel filtration and pore-

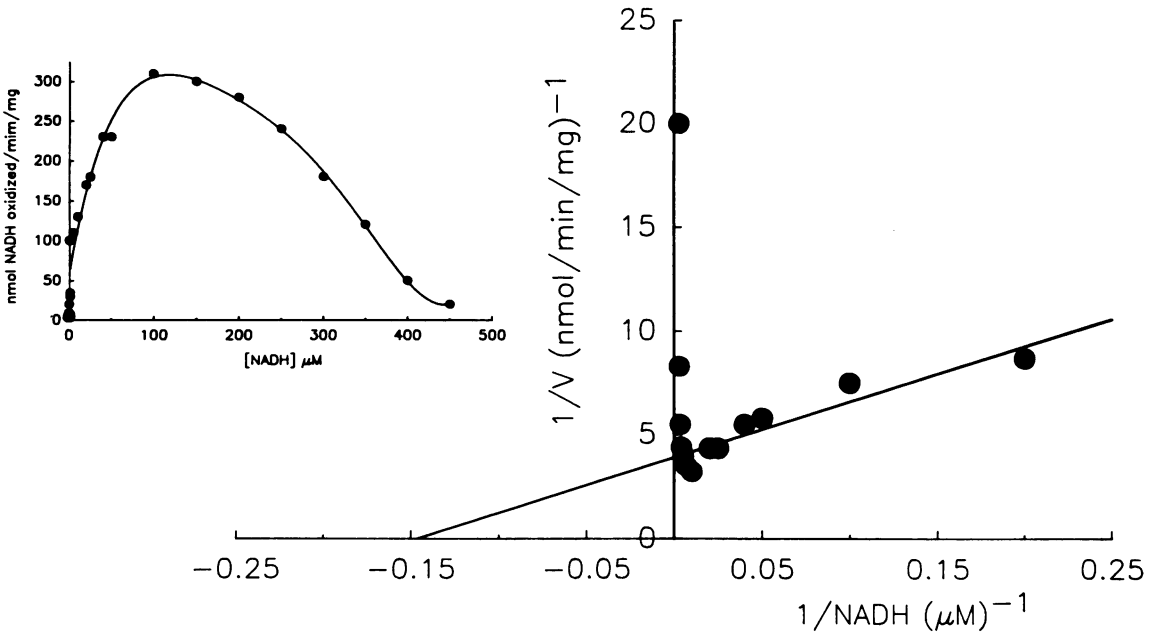

FIG. 3. Determination of apparent K_m for 20α-HSDH. NADH saturation curve (inset) and Lineweaver-Burk plot showing the effect of NADH (1 to 450 μM) on reaction velocities as measured spectrophotometrically at 340 nm. An apparent K_m of 8 μM NADH was obtained from the linear portion of the Lineweaver-Burk plot by linear regression; $r = 1.0$, using data points at 1, 5, 10, 20, 25, 40, 50, and 100 μM NADH. The saturation curve was fitted by using fifth-order polynomial regression analysis with a SigmaPlot program (Jandel Scientific, Sausalito, Calif.).

gradient electrophoresis, is apparently a tetramer with a molecular weight of 160,000. In contrast, the catalytically active form of 20α-HSDH isolated from rat ovary (35), porcine testes (41), and bull testes (33) is reported to be a single polypeptide chain of M_r 36,000, 35,000, and 40,000, respectively; whereas the bifunctional 17β,20α-HSDH from human placenta is a dimer of M_r 68,000 (43, 44).

The determination of the kinetic properties of clostridial 20α-HSDH was complicated by substrate inhibition at relatively low concentrations with each steroid and pyridine nucleotide tested. The inhibition was observed in both the oxidative and reductive reactions. Substrate inhibition by steroid substrates has been observed with an HSDH from *Pseudomonas testosteroni* (26). In many cases, this inhibition is probably a result of the limitations of steroid solubility in aqueous media (50 to 100 μM) with the nonpolar progestational compounds. However, with the highly water-soluble corticosteroids used in this study, no evidence of insolubility was found. Substrate inhibition by NADH has been documented for a great variety of dehydrogenation systems (15, 42, 48).

The N-terminus (residues 1 to 11) of 20α-HSDH was compared with those of GAPDHs from several species (Table 2), with the sequence aligned to illustrate the highest homology. No GAPDH activity was demonstrated with purified 20α-HSDH. It has been proposed that the β-sheet structure formed by residues 3 to 7 of GAPDH is involved in pyridine nucleotide coenzyme binding (see reference 14 for a review). The conservation of the amino acid sequence in that region suggests that it plays an analogous function in these enzymes. To the best of our knowledge, no sequence data are available for any mammalian 20α-HSDH.

Clostridial 20α-HSDH, like many other bacterial HSDHs, is inducible (22, 27, 30, 46). However, the mechanism of steroid induction for any of these enzymes has not been elucidated.

In summary, the 20α-HSDH from *C. scindens* was purified to homogeneity, the N-terminal amino acid sequence was determined, and certain physical and kinetic properties were determined. These results should now allow molecular cloning and studies of steroid regulation of the gene for this enzyme.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grants AM26262 from the National Institute of Arthritis, Metabolism and Digestive Diseases and PO1DK38030 from the National Institutes of Health.

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