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HYDROXYSTEROID DEHYDROGENASES OF *PSEUDOMONAS TESTOSTERONI*

SEPARATION OF A 17β-HYDROXYSTEROID DEHYDROGENASE FROM THE 3(17)β-HYDROXYSTEROID DEHYDROGENASE AND COMPARISON OF THE TWO ENZYMES *

ERNEST V. GROMAN ** and LEWIS L. ENGEL

Laboratory of Human Reproduction and Reproductive Biology and the Department of Biological Chemistry, Harvard Medical School, Boston, Mass., 02115 (U.S.A.)

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Summary

When a crude extract of *Pseudomonas testosteroni* induced with testosterone was subjected to polyacrylamide gel electrophoresis, six bands that stained for 17β-hydroxysteroid dehydrogenase activity were observed. A protein fraction containing the enzyme corresponding to the fastest migrating band and devoid of the other hydroxysteroid dehydrogenase activities has been obtained. This preparation appears to be distinct from the previously isolated 3(17)β-hydroxysteroid dehydrogenase (EC 1.1.1.51) in its chromatographic properties on DEAE-cellulose, substrate and cofactor specificity, immunological properties and heat stability. The preparation appears devoid of 3α-, 3β-, 11β-, 17α-, 20α-, and 20β-hydroxysteroid dehydrogenase activities. The enzyme transfers the 4-pro-S-hydrogen of NADH from estradiol-17β (1,3,5(10)estratriene-3,17β-diol) to estrone (3-hydroxy-1,3,5(10)-estratriene-17-one).

Introduction

Pseudomonas testosteroni is a microorganism capable of utilizing C_{19}-steroids as its sole source of carbon and contains many inducible enzymes that act upon steroids. Previously [1] we reported that crude extracts of *Ps. testosteroni* induced with testosterone contain six electrophoretically distinct 17β-hydroxysteroid dehydrogenase activities. The four activities with the lowest mobilities

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** Present address: Clinical Assays, Cambridge, Mass., U.S.A.

in polyacrylamide gels correspond to the well-known 3(17)β-hydroxysteroid dehydrogenase (EC 1.1.1.51) discovered by Marcus and Talalay [2] and isolated in pure form [1,3]. The two activities of greatest mobility possessed no apparent 3β-hydroxysteroid dehydrogenase activity and appear to be different from the 3(17)β-hydroxysteroid dehydrogenase. In this report we describe the partial purification and characterization of one of these two new 17β-hydroxysteroid dehydrogenases.

Materials and Methods

Materials

Unless otherwise noted, all chemicals were obtained from commercial sources. Human placental estradiol-17β dehydrogenase (EC 1.1.1.62) [4] and the 3(17)β-hydroxysteroid dehydrogenase of *Ps. testosteroni* [1] were purified as described by these authors. Antibodies to electrophertically-pure 3(17)β-hydroxysteroid dehydrogenase and estradiol-17β dehydrogenase were raised in rabbits. 4S-[4-^{2}H]NADH [5], and 4R-[4-^{2}H]NADH [6] were prepared by the published procedures. Hydroxyapatite was prepared by the method of Anacker and Stoy [7].

Methods

Enzyme activity was measured by the rate of change of NADH concentration monitored at 340 nm in 50 mM potassium phosphate buffer (pH 7.5) containing 50 μM estradiol-17β and 1 mM NAD. Protein concentration was measured by the method of Warburg and Christian [8]. Analytical polyacrylamide gel electrophoresis was performed in running gels 8 cm long and containing 5% acrylamide using a pH 8.3/8.9 system [9]. Dehydrogenase activity in the gel was detected as described previously [1]. Immunodiffusion in agar was conducted using the conditions of Schultz et al. [1]. Stereochemistry of hydride transfer from NADH to estrone was determined as described by Groman et al. [5].

Purification of 17β-hydroxysteroid dehydrogenase.

All steps were performed at 4° C.

Crude extract. Frozen cells (50 g) were thawed, suspended in five volumes of 10 mM potassium phosphate buffer/1 mM EDTA, pH 7.2, and disrupted by 5 min treatment in a sonicator. Cell debris was removed by centrifugation at 20 000 × *g* for 1 h.

Precipitation of nucleic acids. Streptomycin sulfate (4.2 g/l), adjusted to pH 7 with solid $NaHCO_3$, was added to the supernatant. The suspension was stirred for 2 h, then centrifuged at 20 000 × *g* for 30 min.

Ammonium sulfate precipitation. Ammonium sulfate (209 g/l) was added to the supernatant of the preceding step and the solution was stirred for 4 h. The precipitate was collected by centrifugation at 20 000 × *g* for 30 min and discarded. $(NH_4)_2SO_4$ (129 g/l) was then added and the solution was stirred for 4 h. The precipitate was collected by centrifugation at 20 000 × *g* for 30 min. The pH was maintained at 7.2 with NH_4OH throughout the $(NH_4)_2SO_4$ treatment. The pellet was dissolved in 10—15 ml of 5 mM potassium phosphate

TABLE I

PURIFICATION OF 17β-HYDROXYSTEROID DEHYDROGENASES OF *PSEUDOMONAS TESTOSTERONI*

Step	Volume (ml)	Protein (mg/ml)	Specific activity (μmol · min^{-1} · mg^{-1})	Yield (%)	Purification (-fold)
1. Crude extract	240				
2. Streptomycin sulfate	235	6.6			
3. Ammonium sulfate	25	15.4	0.06	100	1
4a. DEAE-cellulose Peak 1 *	97	0.29	1.05	120	17.5
4b. DEAE-cellulose Peak 2 *	122	0.46	0.04	9.5 **	0.66
5. Hydroxyapatite	17	0.52	0.18	6.7 **	3.0

* Values expressed relative to Step 3.

** See Fig. 1 and text for explanation.

buffer/1 mM EDTA, pH 7.5, and dialyzed against the same buffer.

DEAE-cellulose chromatography. The solution obtained in the preceding step was applied to a Whatman DE52 column (3 × 10 cm) that had been equilibrated with the same buffer and developed with a 200 ml linear gradient (0—0.2 M KCl) formed by supplementing the eluting buffer with 0.2 M KCl. Two peaks of 17β-hydroxysteroid dehydrogenase activity were observed (Fig. 1): one passed directly through the column (Peak 1) and the second was eluted with the KCl gradient (Peak 2).

Hydroxyapatite chromatography. Fractions of DEAE 2 (Fig. 1) were pooled and dialyzed against 5 mM potassium phosphate buffer, pH 7.5. This solution was then applied to a hydroxyapatite column (2 × 7 cm) that had been equilibrated with the dialysis buffer. The 17β-hydroxysteroid dehydrogenase activity was washed from the column with equilibrating buffer. Fractions containing dehydrogenase activity were pooled and concentrated to a protein concentration of 0.5 mg/ml by ultrafiltration with a PM10 membrane (Amicon). This fraction of enzyme was used for all experiments reported here. The purification scheme is summarized in Table I.

Results

Separation of 17β-hydroxysteroid dehydrogenase activities

Electrophoresis on polyacrylamide gels of crude extracts of *Ps. testosteroni* induced with testosterone yield five bands of dehydrogenase activity when testosterone is used as substrate and six when estradiol-17β used [1] (see Fig. 1). The proteins corresponding to Bands 1—4 have both 3β- and 17-c-hydroxysteroid dehydrogenase activity, have the same mobility as pure 3(17)β-hydroxysteroid dehydrogenase, and contain 75% of the total 17β-hydroxysteroid dehydrogenase activity. The proteins corresponding to Bands 5 and 6 have no 3β-hydroxysteroid dehydrogenase activity. All six activities co-purify through the $(NH_4)_2SO_4$ fractionation step. Separation of the fastest migrating 17β-hydroxysteroid dehydrogenase activity (Band 6) from the other 17β-hydroxysteroid dehydrogenase activities was obtained by chromatography on DEAE-cellulose

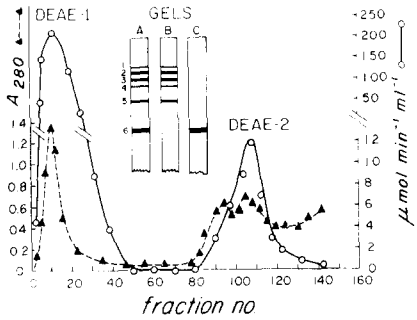

Fig. 1. Chromatography of hydroxysteroid dehydrogenase activities on DEAE-cellulose. The procedure is described in Materials and Methods. The flow rate was 108 ml/h; 3.6-ml fractions were collected. The insert shows the pattern of staining for dehydrogenase activity with estradiol-17β as substrate. Gel A, the mixture before chromatography; Gel B, Peak I; Gel C, Peak II. When testosterone is substituted for estradiol-17β as substrate in the activity staining mixture, Band 5 is absent.

(Fig. 1). This fraction also contained 3α-hydrosteroid dehydrogenase activity that was removed by passage over hydroxyapatite.

Substrate and cofactor specificity

The enzyme preparation obtained after hydroxyapatite chromatography is capable of oxidizing the 17β-hydroxyl group of estradiol-17β and testosterone in the presence of NAD, but does not oxidize 3α-, 3β-, 11β-, or secondary 17α-hydroxyl groups and does not reduce a 20-oxo group. No oxidation of NADH (0.28 mM) was observed when the enzyme preparation (100 μg protein) was incubated (100 mM potassium phosphate, pH 6.5 or 7.5) with 3β-hydroxy-5-pregnen-20-one or cortisol; similarly, no reduction of NAD (0.75 mM) occurred in the presence of estradiol-17α, 3α-hydroxy-5α-androstan-17-one, or 3β-hydroxy-5α-androstan-17-one. The concentration of all steroids was 50 μM. The ability of this preparation to utilize various pyridine nucleotide analogues as co-substrates was examined. The results obtained are contrasted with those obtained with the 3(17)β-hydroxysteroid dehydrogenase (Table II).

TABLE II

ACCEPTABILITY OF NAD AND ITS ANALOGS AS CO-SUBSTRATES BY 17β-HYDROXYSTEROID DEHYDROGENASE AND 3(17)β-HYDROXYSTEROID DEHYDROGENASE

Assays were performed in the presence of 50 μM estradiol-17β in 50 mM potassium phosphate buffer, pH 7.5 at 23°C.

Cofactor **	Concentration (mM)	Relative velocity	
		3(17)β-HSDH **	17β-HSDH
NAD	1.49	100	100
3-Acetylpyridine-adenine dinucleotide	1.11	59	16
3-Pyridinealdehyde-adenine dinucleotide	1.11	75.9	91
Thionicotinamide adenine dinucleotide	0.99	13.3	97

* The following compounds were not accepted as co-substrates by either enzyme: NADP, nicotinamide mononucleotide, nicotinamide-hypoxanthine dinucleotide, 3-pyridinealdehyde-hypoxanthine dinucleotide, 3-acetylpyridine-hypoxanthine dinucleotide, nicotinamide-1-N^6-ethenoadenine dinucleotide.

** HSDH is the abbreviation for hydroxysteroid dehydrogenase.

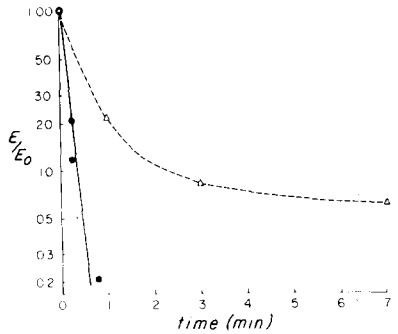

Fig. 2. Thermal inactivation of 17β-hydroxysteroid dehydrogenases. Enzyme solution (0.5 ml) in 5 mM potassium phosphate buffer, pH 7.5, was heated at 58° C. Samples were withdrawn and assayed for 17β-hydroxysteroid dehydrogenase activity with estradiol-17β as substrate. 17β-Hydroxysteroid dehydrogenase, ●———●; 3(17)β-hydroxysteroid dehydrogenase △———△.

Immunological properties and thermal stability

17β-Hydroxysteroid dehydrogenase showed no evidence of reaction with rabbit antisera to placental estradiol-17β dehydrogenase and to 3(17)β-hydroxysteroid dehydrogenase in double diffusion precipitation experiments. A comparison of the thermal inactivation of 17β-hydroxysteroid dehydrogenase and (3(17)β-hydroxysteroid dehydrogenase is shown in Fig. 2.

Discussion

Recently Benson et al. [10] reported a partial resolution of 3β- and 17β-hydroxysteroid dehydrogenase activities by chromatography of crude extracts of *Ps. testosteroni* on a 19-nortestosterone affinity column. These findings are in apparent conflict with the recently reported results of Schultz et al. [1] who showed that the 3(17)β-hydroxysteroid dehydrogenase of the same microorganism consists of a family of five isozymes, each member of which has both 3β- and 17β-hydroxysteroid dehydrogenase activity. The conflict between the two sets of data may be resolved if there are present in crude extracts of *Ps. testosteroni* additional 17β-hydroxysteroid dehydrogenases. Partial resolution of such enzymes from the 3(17)β-hydroxysteroid dehydrogenase by the chromatographic procedure of Benson et al. [10] could generate the profile that they reported. We examined the crude extract of *Ps. testosteroni* for additional hydroxysteroid dehydrogenase activities. Two additional enzymes possessing 17β-hydroxysteroid dehydrogenase activity were found; they co-purified with 3(17)β-hydroxysteroid dehydrogenase through the early stages of purification [1].

In order to determine if the two new activities were unique proteins or were degradation products of the 3(17)β-hydroxysteroid dehydrogenase, we undertook to purify one of them. It was found that the new activity could be separated from the 3(17)β-hydroxysteroid dehydrogenase by chromatography on DEAE-cellulose and from a 3α-hydroxysteroid dehydrogenase by chromatography on hydroxyapatite (Fig. 1). The new enzyme preparation is free from other hydroxysteroid dehydrogenase activities (Fig. 1) and differs from the well-known 3(17)β-hydroxysteroid dehydrogenase in many particulars. It does

not accept 3β-hydroxysteroids and also has different preferences for NAD analogs (Table II). It differs, moreover, in chromatographic and electrophoretic behavior, in thermal stability (Fig. 2), and in immunologic specificity. It is similar to the 3(17)β-hydroxysteroid dehydrogenase in that it also uses the 4-pro-S hydrogen of NADH. The second, new 17β-hydroxysteroid dehydrogenase, which has not yet been purified, has even more restricted substrate preferences than the enzyme presently studied. It accepts 17β-hydroxy-C_{18}- but not C_{19}-steroids and is thus an estradiol-17β dehydrogenase. The three 17β-hydroxysteroid dehydrogenases of *Ps. testosteroni* present an interesting spectrum of substrate preferences. A comparative study of the steroid binding sites of these enzymes in conjunction with studies of the steroid-binding site of the human placental estradiol-17β dehydrogenase should give useful insights into the structural relations that govern the interaction of steroids with proteins.

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