Evidence for an 4-Ene-3-oxosteroid-5α-reductase and Δ^4-Δ^5-Ketosteroid Isomerase Activity in Extracts *of Streptomyces hydrogenans*

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A homogenate of cells of *Streptomyces hydrogenans* which have been cultivated in the presence of estradiol-17β, was separated by gel filtration on Sephadex G-200. In the presence of NADH, a few distinct fractions converted about 10% of [^{3}H]testosterone to 5α-dihydrotestosterone (5α-DHT), 5α-androstanedione, 4-androstenedione, and dehydroepiandrosterone (DHEA). The metabolites were separated after two consecutive runs on silica gel and propanediol-1,2-impregnated cellulose plates by thin layer chromatography. Identification was achieved by comparison with known steroid samples, specific staining procedures and by crystallization to constant specific radioactivity. The 5α-reductase activity responsible for the formation of 5α-DHT and 5α-androstanedione *in vitro* required NADH as co-substrate and could only be found after induction of the cells with estradiol-17β *in vivo*. Within the same chromatographic fractions, a Δ^4-Δ^5-ketosteroid isomerase activity can be detected which catalyzes the reverse reaction from the 4-androstene testosterone to the 5-anthrostene DHEA *in vitro*.

Introduction

Cell-free homogenates of the actinomycete *Streptomyces hydrogenans* contain different steroid-metabolizing enzymes, *e. g.* 20β-hydroxysteroid dehydrogenase (EC 1.1.1.53) and 17β-hydroxysteroid dehydrogenase (17β-HSD, EC 1.1.1.63) [1, 2]. The synthesis of both enzymes can be induced by certain steroids. In the case of 17β-HSD the intracellular activity of the enzyme increases up to 10-fold in the presence of testosterone, 5α-dihydrotestosterone (5α-DHT), estradiol-17β, or 17α-methyltestosterone *in vivo* [3]. Thereby, 80% of testosterone is converted to androstenedione within 2 h [2]. But small amounts of 5α-androstanes can be detected as well. Therefore, further steroid-metabolizing enzymes must be active *in vivo*.

In order to investigate the biochemical properties of the enzymatic activities which are responsible for the formation of those minor testosterone metabolites, extracts of the microorganism were tested for 5α-reductase activity *in vitro*.

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Materials and Methods

[7-^{3}H]Testosterone (25 Ci/mmol) was obtained from New England Nuclear Corp., Dreieichenhain, W.-Germany. Non-radioactive steroids, silica gel plates 60 F 254 (layer-thickness 250 μm), cellulose plates without fluorescent indicator (layer-thickness 100 μm), solvents and staining chemicals were obtained from E. Merck AG, Darmstadt. NAD^+, NADH and NADPH were purchased from Boehringer, Mannheim.

Cultivation of the microorganism

Streptomyces hydrogenans (ATCC 19631) was cultivated as described previously [1]. Cells were grown for 3 h in a culture medium supplemented with estradiol-17β to an end-concentration of 0.09 mM, and then the culture was further incubated for 4 h at 30 °C. Cells were harvested by vacuum filtration and wet cell material was homogenized sonically using a Branson Sonifier S 75 at a current of 4.5 A (power setting of 8) [4] in 10 mM Tris buffer, pH 7.4, for 90 sec [5]. Cytosol was obtained by centrifugation of the cell homogenate at 165000 × *g* for 3 h. Protein content was determined turbidimetrically after precipitation with trichloroacetic acid [1].

Gel filtration

The cytosolic fraction was applied to a column of Sephadex G-200 (82 × 2.6 cm), previously equilibrated with 10 mM Tris buffer, pH 7.4, at 4 °C.

Elution was carried out at a flow rate of 8 ml/h, with the same buffer at 4 °C. Optical density was continuously monitored at 254 nm.

Extraction of steroids

Eluates from Sephadex G-200 were tested for 17β-HSD activity with [^{3}H]testosterone and NAD^+ as substrates. The reaction mixture (final volume of 3.5 ml) contained: 0.1 ml = 0.1 mCi of [7-^{3}H] testosterone (25 Ci/mmol, end. conc. 1.1 μM) in benzene/ethanol (the organic solvent was removed under N_2 stream), 3 ml eluate in Tris buffer, pH 7.4, and 0.5 ml of 5 mM NAD^+ in the same buffer. The reaction mixture was incubated at 30 °C for 3 h. Metabolism of testosterone was determined after extraction of the radioactive testosterone metabolites and chromatography of the mixture by thin layer chromatography (TLC) on silica gel F 254 [solvent system chloroform – ethyl acetate – petroleum benzine (60 – 80 °C) (50 : 45 : 5, v/v/v)]. Rechromatography was performed on propanediol-1,2-impregnated cellulose plates [solvent system benzene – cyclohexane (50 : 50, v/v)] [6].

5α-Reductase activity was tested in the Sephadex eluates with [^{3}H]testosterone and NADH as substrates. The reaction mixture (final volume of 3.5 ml) contained: 0.1 ml = 0.1 mCi of [7-^{3}H]testosterone (25 Ci/mmol, end. conc. 1.1 μM) in benzene/ethanol (evaporated to dryness under N_2), 3 ml of Sephadex-eluate in Tris buffer, pH 7.4, and 0.5 ml of 40 mM NADH in the same buffer. The reaction mixture was incubated at 30 °C for 3 h. 5α-Reduction of testosterone was followed after extraction and chromatography of the steroid metabolites [6]. Final identification of the metabolites was achieved by three-fold recrystallization of the metabolites to a constant specific radioactivity [2].

Scintillation counting

After the two consecutive separations of the steroids by TLC, 0.5 cm wide bands of the chromatographic layers were scraped off. To 1/14 of the 7 cm long band (0.5 × 0.5 cm), 5 ml of toluene-based liquid scintillation cocktail was added. Counting was performed in a Packard Tri-Carb, model 3375. Counting efficiency: 40%, background: 16 – 20 cpm/0.5 × 0.5 cm TLC band.

Results

Identification of testosterone metabolites

After gel filtration of the cell homogenate on Sephadex G-200, high and low molecular weight components were separated from the fractions containing 17β-HSD and 5α-reductase activity (Fig. 1).

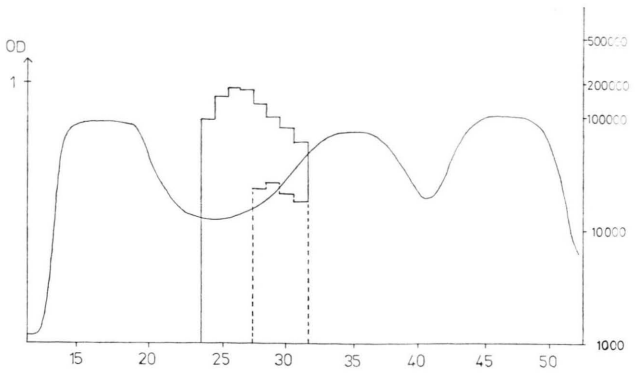

Fig. 1. Gel filtration of 50 ml cytosol (5.9 mg protein/ml) on Sephadex G-200 (82 × 2.6 cm) at 4 °C. Elution with Tris buffer, pH 7.4, flow rate 8 ml/h, fraction volume 8 ml. Optical density was monitored continuously at 254 nm. Relative activities of 17β-HSD and 5α-reductase were estimated as radioactivity of [^{3}H]androstenedione or [^{3}H]5α-androstanedione, respectively, formed by 3 ml of the eluted fractions in the presence of NAD^+ or NADH, respectively (see Methods). Abscissa: fraction No. Left ordinate: optical density at 254 nm. Right ordinate: radioactivity of the metabolites formed by each fraction, logarithmic plot of 1/14 cpm/fraction No. 24 – 31. ——— [^{3}H]androstenedione (17β-HSD); – – – [^{3}H]androstanedione (5α-reductase)

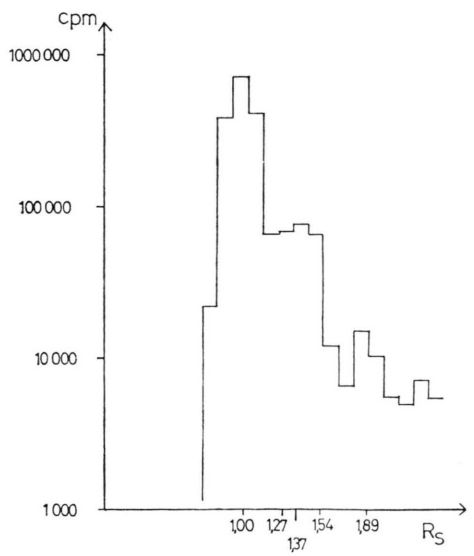

Fig. 2. Thin layer chromatography of radioactive metabolites of [^{3}H]testosterone after incubation with 3 ml Sephadex fraction No. 29 in the presence of 0.1 mCi [7-^{3}H]testosterone and NADH (end concentration 5.7 mM) at 30 °C for 3 h. After ether extraction the radioactive steroids were separated on silica gel F 254 plates [solvent system chloroform – ethyl acetate – petroleum benzine (60 – 80 °C) (50 : 45 : 5, v/v/v)]. Radioactivity was counted after elution of the different metabolites from the 0.5 cm wide layer bands. Abscissa: R_S-values (S = testosterone; R_S testosterone 1; DHEA 1.27; 5α-DHT 1.37; androstenedione 1.54; 5α-androstanedione 1.89). Ordinate: log of total radioactivity cpm/0.25 cm^2.

Enzyme activities were localized by testing the ability of the column eluates to convert [^{3}H]testosterone into androstenedione, 5α-androstanedione, or 5α-dihydrotestosterone (5α-DHT), respectively, in the presence of the appropriate co-substrate *in vitro*. As can be seen in Fig.1, 17β-HSD activity eluted before the 5α-reductase activity which can be found in fractions No. 28 – 31. A typical TLC of the radioactive metabolites of [^{3}H]testosterone is shown in Fig. 2.

For better separation of the testosterone metabolites, the area of the silica gel chromatogram at R_S 1.1 – 2.0 was scraped off, the radioactive material extracted, concentrated and rechromatographed on propanediol-1,2-impregnated cellulose plates (Fig. 3). Surprisingly, DHEA could be detected as an additional testosterone metabolite, which points to a Δ^4-Δ^5-ketosteroid isomerase (EC 5.3.3.1) active within Sephadex fractions No. 28 – 31.

Recrystallization to constant specific radioactivity

For final identification of the testosterone metabolites localized after two consecutive chromatographic separations and by specific staining reactions, the radioactive spots were eluted and supplemented with the pure non-radioactive steroid in question (Table I). The course of the specific radioactivities determined after each recrystallization proved that the assumptions for the identity of the four metabolites were correct.

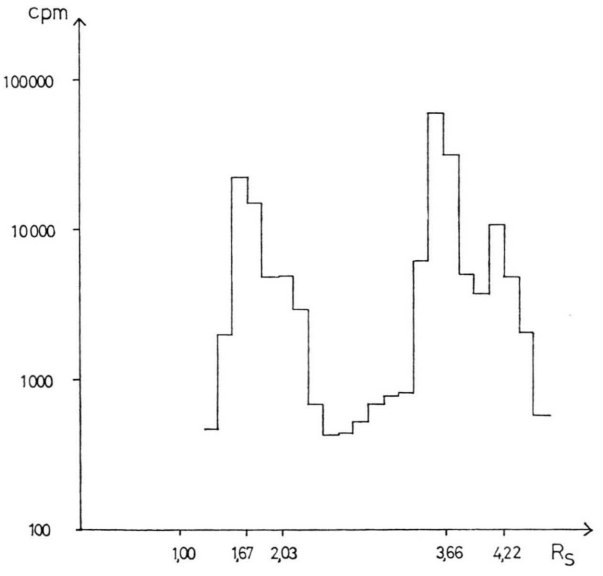

Fig. 3. Rechromatography of steroid metabolites on propanediol-1,2-impregnated cellulose plates in solvent system benzene – cyclohexane (50:50, v/v). After the preceding run on silica gel plates the area corresponding to R_S 1.1 – 2.0 was scrapped off, eluted, the extract concentrated and reseparated on impregnated cellulose plates. Abscissa: R_S-values (S = testosterone; R_S testosterone 1; 5α-DHT 2.03; DHEA 1.67; androstenedione 3.66; 5α-androstanedione 4.22). Ordinate: log of total radioactivity cpm/0.25 cm^2.

Table I. Scheme of specific radioactivities of four different testosterone metabolites after three consecutive recrystallizations (R_S-values are given after TLC on propanediol-1,2-impregnated cellulose plates).

R_S	Metabolite	Specific radioactivity [cpm/mg]			
		Sample	1. Recrystallization	2.	3.
1.67	dehydroepi-androsterone	40 870	25 520	26 450	25 930
2.03	5α-dihydro-testosterone	15 600	8 290	8 130	8 370
3.66	4-androstene dione	93 480	64 540	65 460	65 000
4.22	5α-androstane-dione	20 440	12 360	13 000	12 730

In comparison to the total amount of testosterone (100%) at the start of the incubation, the following average amounts of metabolites were found after 3 h at 30 °C *in vitro*: 0.7% 5α-DHT, 1.3% 5α-androstanedione, 5.2% androstenedione, and 2.1% dehydroepiandrosterone (DHEA).

Cosubstrate requirement, induction and inhibition of the 5α-reductase activity

After replacement of NADH by the same amount of NADPH, no 5α-androstanedione or 5α-DHT could be detected. Therefore, NADPH could not replace NADH as a co-substrate for the bacterial 5α-reductase (EC 1.3.1.4). After addition of 5α-androstanedione to the incubation mixture (0.34 mM end. conc.) an average increase of 15% of the amount of [^{3}H]androstenedione formed from [^{3}H]testosterone had been found, in comparison to the level of [^{3}H]androstenedione formed in the absence of androstanedione. Simultaneously, no radioactive 5α-DHT or 5α-androstanedione could be traced. Therefore, 5α-androstanedione seems to inhibit 5α-reductase activity and to stimulate 17β-HSD *in vitro*. This result is opposite to the inhibitory action of 5α-androstanedione on 17β-HSD published previously [3].

Considerable activities of 5α-reductase could only be found in the cytosolic fractions from those

cells which had been cultivated in the presence of estradiol-17β. If the inducing steroid was omitted from the culture medium, only traces of testosterone were converted into 5α-androstane derivatives. So, estradiol-17β stimulated enzyme synthesis of both 17β-HSD as well as 5α-reductase in *Streptomyces hydrogenans*.

Both enzyme activities were strongly diminished in the cytosolic fractions *in vitro* when the cells were cultivated in the presence of 0.34 mM testosterone acetate + 0.09 mM estradiol-17β. The mechanism of action of testosterone acetate as inhibitor of both enzyme syntheses is still unkown. 17β-HSD is not inhibited by testosterone acetate *in vitro* [3].

Discussion

By the systematic analysis of testosterone metabolites formed *in vitro*, the presence of a 5α-reductase activity could be shown in certain fractions of the cell homogenate from *Streptomyces hydrogenans*. Previous studies on the formation of 5α-pregnanes from pregnenolone *in vitro* came to the same conclusion [7].

Considerable amounts of 5α-reductase activity can only be found *in vitro*, if the cells are cultivated in the presence of estradiol-17β. Therefore, 5α-reductase is the third steroidmetabolizing enzyme of *Streptomyces hydrogenans*, the synthesis of which is induced by steroids *in vivo*. Whereas, 5α-reductase activity of *Mycobacterium smegmatis* cannot be induced by testosterone or 4-chloro-17α-methyl-17β-hydroxy-1,4-androstadien-3-one [8].

Under the conditions employed, certain fractions of the cell cytosolic fraction convert 8% of testosterone into androstenedione plus DHEA, whereas 2% are identified as 5α-androstanedione plus 5α-DHT. *In vivo*, *Streptomyces hydrogenans* converts 80% of testosterone of the culture medium into androstenedione within 2 h [2]. 5α-Reductase from *Streptomyces hydrogenans* accepts only NADH as H-donor and is not active in the presence of NADPH. NADH is also shown as co-substrate for 5α-reductase in *Nocardia restrictus* [9]. However, for the 5α-reductase activity of *Mycobacterium smegmatis* FAD seems to act as co-substrate [10]. In the case of 5α-reductase of *Nocardia corallina*, the essential H-donor is NADPH [11].

Opposite to our assumption, 17β-HSD activity could not be inhibited after addition of 5α-androstanedione. Because this enzyme activity always contaminates the chromatographic fractions containing 5α-reductase and ketosteroid isomerase, we expected an increase of 5α-DHT resulting from the simultaneous inhibition of 17β-HSD by 5α-androstanedione [3]. However, [^{3}H]testosterone conversion to [^{3}H]androstenedione is significantly stimulated in the presence of 5α-androstanedione. But we cannot exclude the possibility that perhaps there is a bacterial NADH-oxidase in the same cytosolic fractions which results in unusual reactions sequences [12].

Previous investigations have shown a considerable conversion of pregnenolone to progesterone by *Streptomyces hydrogenans in vitro* [7, 13] which indicates a ketosteroid isomerase activity in the cell-free homogenate of this microorganism. Now, we are able to demonstrate a ketosteroid isomerase activity *in vitro* which accepts C_{19}-steroids as substrates. Under the conditions used, approximately 2% of [^{3}H]testosterone are converted to DHEA. Because DHEA can only be formed after reduction at the C-3 of 5-androstene-3,17-dione, an additional 3β-hydroxysteroid dehydrogenase must be present in the cytosolic fraction. Moreover, the production of DHEA confirms the reversibility of the 5-ene – 4-ene-isomerization. The same conclusion was drawn by Lamontagne *et al.* [14] who found a reformation of pregnenolone from progesterone by *Tetrahymena*. The formation of DHEA from androstenedione by adrenal microsomes was shown by Ward and Engel [15]. Whether *Streptomyces hydrogenans* contains different ketosteroid isomerases for C_{21}- and C_{19}-steroids has not yet been studied. In order to elucidate further enzymological data on 5α-reductase and ketosteroid isomerase, both enzymes need to be purified extensively.

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