Purification and Characterization of a 3,17 β-Hydroxysteroid Dehydrogenase from *Streptomyces hydrogenans*

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Dedicated to Professor Adolf Wacker *on the Occasion of His 60th Birthday*

3,17 β-Hydroxysteroid Dehydrogenase, *Streptomyces hydrogenans*, 20 β-Activity

3,17 β-Hydroxysteroid dehydrogenase has been enriched and purified from cytosol of *Streptomyces hydrogenans*. After ammonium sulfate precipitation and filtration on Sephadex G-100 the enzyme was finally purified by preparative gel electrophoresis and DEAE-Sephadex A-50 chromatography. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate gave a single band of mobility corresponding to molecular weight of 70 200 ± 2 500. 3 β-, 17 β- as well as 20 β-hydroxy steroids were dehydrogenated by the enzyme in the presence of NAD^+. The dehydrogenation proceeded faster than the reduction of the corresponding ketosteroids in the presence of NADH. The enzyme does not accept $NADP^+$ or NADPH as co-substrates. The apparent K_m values were calculated to be 11 μM for 5 α-dihydrotestosterone, 20 μM for testosterone and 68 μM for epiandrosterone in the NAD^+-driven reaction, 1.8×10^{-4} M for NAD^+ and 1.9×10^{-4} M for NADH. The catalytic activity was influenced by the ratio of NAD^+/ATP. The inhibition by ATP appears to be of a competitive type with respect to NAD^+ (K_i 1.15×10^{-3} M).

After sucrose gradient centrifugation in a preparative ultracentrifuge the enzyme sediments with 4.1 ± 0.1 S as estimated in comparison to other proteins of known sedimentation coefficient. The isoelectric point was determined to be 3.9 with the LKB preparative isoelectric focusing column (pH 2 – 11) and 4.1 with the analytical flat bed polyacrylamide isofocusing (pH 3 – 5). The number of SH groups was determined to be 2 mol/mol enzyme. In the presence of 6 M urea the figure inceases to 3 mol SH/mol enzyme. In the presence of an excess of *p*-chloromercuribenzoate the enzyme activity decreases only partially.

Introduction

Streptomyces hydrogenans (ATCC 19 631) synthesizes 20 β-hydroxysteroid dehydrogenase as well as 3,17 β-hydroxysteroid dehydrogenase and 4-ene-3-oxosteroid-5 α-reductase in response to the addition of various steroids to the culture medium [1 – 4]. In the case of 3,17 β-hydroxysteroid dehydrogenase, the intracellular activity of the enzyme increases up to 10-fold in the presence of testosterone, 5 α-dihydrotestosterone, 17 β-estradiol or 17 α-methyltestosterone *in vivo* [5]. In order to investigate the biochemical properties of the enzyme in detail, further purification of the bacterial protein was attempted.

Materials and Methods

Chemicals

The steroids used were obtained from Steraloids, N. Y., USA. [7-^{3}H]Testosterone (25 Ci/mmol), [1,2-^{3}H]androst-4-en-3,17-dione (50 Ci/mmol) were obtained from New England Nuclear Corp., Dreieichenhain. [1,2-^{3}H]5 α-dihydrotestosterone (47 Ci/mmol) was obtained from Amersham & Buchler, Braunschweig. NAD^+, NADH, $NADP^+$ and NADPH were obtained from Boehringer, Mannheim. Sephadex G-100 and DEAE-Sephadex A-50 were from Pharmacia, Freiburg. Dimethyl suberimidate, PCMB, DTNB, Servalyt pH 2 – 11 and 3 – 5 and all chemicals for gel electrophoresis were products of Serva, Heidelberg. Silica gel plates without fluorescent indicator (layer thickness 250 μm), silica gel plates 60 F 254, Cellulose plates without fluorescent indicator (layer thickness 100 μm), solvents and staining chemicals were products of E. Merck AG, Darmstadt.

Cultivation of the cells

Streptomyces hydrogenans (ATCC 19 631) was cultivated in the presence of 4×10^{-3} M 17 β-estradiol for 3 h as described previously [1]. Protein content was determined by the method of Lowry [6]. Cells were harvested by vacuum filtration and wet cell material was homogenized sonically using a Branson

Abbreviations: SDS, sodium dodecylsulphate; PCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-Dithio-bis(2-nitrobenzoic acid).

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sonifier S 75 at a current of 4.5 A (power setting of 8) [7]. Cytosol was obtained by centrifugation of the cell homogenate at 105 000 × *g* for 120 min.

Enzyme purification and assay

Solid ammonium sulfate was added to 40 ml cytosol (4.6 mg protein/ml 10 mM Tris-buffer, pH 7.4) up to 40% saturation and the solution was stirred at 4 °C for 2 h. The precipitate was removed by centrifugation at 34 000 × *g* for 20 min. The sediment was dissolved in 2 ml 10 mM Tris-buffer, pH 7.4, and applied to a column of Sephadex G-100 (40 × 2.5 cm), previously equilibrated with the same buffer. Eluates from Sephadex G-100 were tested for enzyme activity with testosterone as substrate. The reaction mixture contained in a final volume of 3 ml: 0.45 ml 0.09 M glycine buffer, pH 9.0, 2 ml of a solution of 1 mM NAD^+ (or NADH) in glycine buffer, 50 µl of a solution of 18 mM testosterone in methanol (end conc. 300 µM testosterone) and 0.5 ml sample. Reaction mixtures were incubated at 30 °C. The initial rate of the enzymatic reaction was determined as ΔE/min at 366 nm.

Preparative gel electrophoresis

Pooled fractions containing the enzyme activity after Sephadex G-100 filtration were concentrated by ultrafiltration (Amicon XM-50) and separated by preparative gel electophoresis. 1000 µl containing 16.4 mg protein were applied to two slots of the 7% standard preparative polyacrylamide gel prepared according to the procedure of Maurer [8] in a DESAGA gel slab apparatus. Runs were performed in gel slabs using Tris-glycine buffer, pH 8.3, at 4 °C for 160 min. For the estimation of the enzyme activity, 2 mm thick gel slops were eluted with 10 mM Tris-buffer, pH 7.4, overnight at 4 °C.

Ion exchange chromatography

Eluates from the preparative gel electrophoresis containing the enzyme activity were separated on DEAE-Sephadex A-50 (4 × 1 cm). The ion exchange column was equilibrated with 10 mM Tris-buffer, pH 7.4, and 4.5 ml of the enzyme solution were applied to the column. After washing with the same buffer, chromatography was performed with 200 ml of a linear gradient of 0 – 1 M NaCl in 10 mM Tris-buffer, pH 7.4. Fractions of 2 ml were collected.

SDS gel electrophoresis

Electrophoresis in 10% gels containing 0.2% SDS was performed according to Maurer [8]. The gels were fixed and stained in 1% Coomassie Brillant Blue in 7% acetic acid for 1 h and destained electrophoretically. The electrophoresis buffer contained 2.4 g Tris, 11.4 g glycine, and 0.2% SDS, diluted to 1 l with dest. water.

Sucrose density gradient centrifugation

Ultracentrifugation was carried out with a preparative Spinco model 50 L 2 B. 0.5 ml samples containing 0.75 mg protein were applied to linear gradients of 10 – 35% sucrose in 10 mM Tris-buffer, pH 7.4, in 11 ml nitrocellulose tubes (Beckman). The gradients were centrifuged for 16 h at 41 000 rpm in a Spinco rotor SW 41 at 4 °C.

Isoelectric focusing

Fractions containing 3,17 β-hydroxysteroid dehydrogenase activity after gel filtration were pooled and separated on a LKB isoelectric focusing column (model 8101) with a 110 ml linear sucrose gradient (66% – 0%). A final potential of 400 V was applied for 50 h at 4 °C to the column containing 1% servalyt 2 – 11 [9]. Fractions of 1.1 ml were collected and measured for the enzyme activity, optical density, and pH, respectively.

Isoelectric focusing in polyacrylamide gels was performed at 2 °C in a DESAGA apparatus. The gel was polymerized from a solution, containing 38 ml 12.4% sucrose, 2 ml servalyt pH 3 – 5 and 10 ml of a solution of 29.1 g acrylamide, 0.95 g N,N′-methylen-bis-acrylamide and 20 mg ammonium persulfate in 100 ml water. The gel was transferred onto a cooling plate of the electrophoresis apparatus. The protein samples were adsorbed by small pieces of paper which were applied onto the gel surface. Marker proteins with definite I. P. were applied as well. After running, the proteins were fixed with 10% trichloroacetic acid over night, washed with water and stained with a solution of 0.2% Coomassie Brillant Blue R 250 in a mixture of acetic acid: methanol : water (1 : 9 : 10). The gel was destained with the same solvent mixture at 60 °C.

Steroid extraction

Identification of steroids formed in the presence of NADH and purified 3,17 β-hydroxysteroid dehydro-

genase from *Streptomyces hydrogenans* was performed after incubation in the presence of ^{3}H-labelled steroid substrates. The reaction mixture contained 0.5 ml enzyme, 2 ml 1 mM NADH in 0.09 M glycine buffer, pH 9.0, 0.5 ml = 0.1 mCi of ^{3}H-labelled steroid in the same buffer and 1 ml glycine buffer, pH 9.0. After incubation at 30 °C for 2 h the steroids were extracted with methanol : aceton (1 : 1) and dichloromethan : diethyl ether (3 : 1), successively, and separated by thin layer chromatography on silica gel plates (solvent system dichloromethane : diethyl ether, 85 : 15 v/v). For separation of the 3-hydroxy epimers of the steroids, chromatography was done on cellulose plates which were impregnated with 20% propanediol-1,2 in methanol (solvent system benzene : cyclohexane 50 : 50 v/v) [10].

Determination of SH groups

SH groups were determined by the method of Ellman [11] by incubation with DTNB.

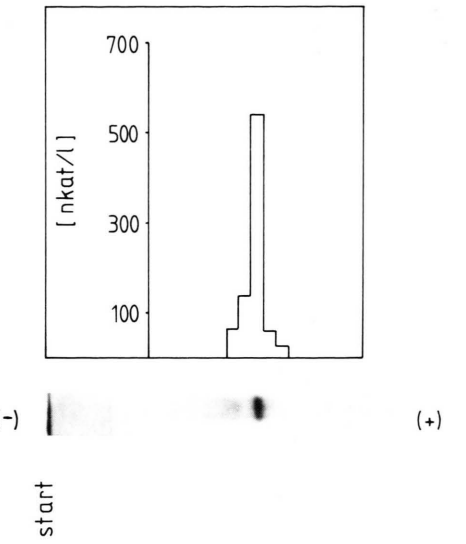

Fig. 2. Preparative gel electrophoresis of 3,17 β-hydroxysteroid dehydrogenase. Gel electrophoresis was performed after ammonium sulfate precipitation and gel filtration on Sephadex G-100 with 7% polyacrylamide gel for 135 min at 4 °C in a DESAGA gel slab apparatus. Estimation of the enzyme activity and staining of the gel as described in Materials and Methods.

Results

Purification of the enzyme

Ammonium sulfate was added to the high speed supernatant (cytosol) of the cell homogenate of *Streptomyces hydrogenans* which were cultivated in the presence of 4×10^{-3} M 17 β-estradiol for 3 h. After centrifugation of the precipitate obtained with 40% ammonium sulfate saturation, the sediment was dissolved in 2 ml 10 mM Tris-buffer, pH 7.4, and filtrated on Sephadex G-100 (Fig. 1). The eluates containing 3,17 β-hydroxysteroid dehydrogenase activity were concentrated to an end volume of 1 ml by ultrafiltration and subjected to preparative gel electrophoresis. After the run the gel was divided in 2 mm thick slopes which were eluted overnight at 4 °C with 3 ml 10 mM Tris-buffer, pH 7.4 (Fig. 2).

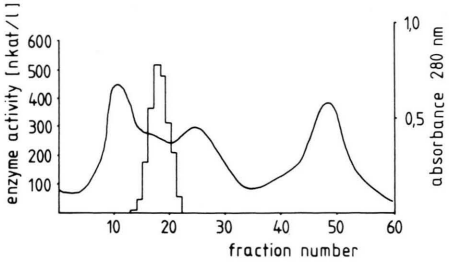

Fig. 1. Gel filtration of 2 ml of a protein fraction prepared from cytosol of *Streptomyces hydrogenans* by fractionated ammonium sulfate precipitation (0 – 40%). The precipitate was dissolved in 2 ml 10 mM Tris-buffer, pH 7.4, protein content: 18.2 mg/ml. Sephadex G-100 (40×2,5 cm). Elution with the same buffer, flow rate 15 ml/h, fraction volume 3 ml. Optical density was monitored continuously at 280 nm. Enzyme activity was determined in the eluate as described in Materials and Methods. Left ordinate: enzyme activity ——.

In order to achieve further purification, the eluates received after preparative gel electrophoresis were rechromatographed on DEAE-Sephadex A-50. The enzyme activity was eluted by 0.5 – 0.6 M NaCl. A summary of a representative purification is shown in Table I. With respect to the specific activity of the enzyme in the original cytosol, a 49-fold enrichment of the enzyme was achieved.

Molecular weight and electrophoretic properties of the enzyme

Using 7% analytical polyacrylamide gel electrophoresis, the enzyme migrated as a single band. The gel slice containing the enzyme was removed and heated for 5 min with 0.2 ml of a solution containing 1% SDS, 2.4 mg Tris, 3 mg dithiothreitol in 0.02 ml glycerol and 0.18 ml water. The mixture was subjected to 10% polyacrylamide gel containing 0.2% SDS

Table I. Purification of 3,17 β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans.*

Step	Total protein [mg]	Total activity [nkat]	Specific activity [mkat/kg]	Overall recovery [%]	Purification -fold
Cytosol	184.7	63	0.34	100	1
0 – 40% ammonium sulfate precipitate	36.5	31	0.8	49.2	2.3
Eluate from Sephadex G-100 filtration	22.7	32	1.4	50.8	4.1
Eluate from preparative gel electrophoresis	0.8	8	10.0	12.7	29
Eluate from DEAE-Sephadex A-50 chromatography	0.3	5	16.7	7.9	49

and run at 310 V for 60 min at 4 °C. The enzyme migrated again as a single band with the molecular weight of 70 200 ± 2 500 as judged by comparison with proteins of known molecular weight. Only very few weak minor bands could be detected after scanning of the stained gel (Fig. 3). The molecular weight of the crude enzyme was determined by gel filtration on Sephadex G-200 to be 130 000 [5]. Cross-linking of the native 3,17 β-hydroxysteroid dehydrogenase with dimethyl suberimidate followed by electrophoresis on 10% polyacrylamide gel in the presence of 0.2% SDS did not generate heavier protein bands.

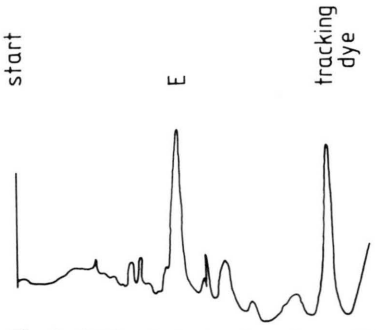

Fig. 3. SDS gel electrophoresis on 10% polyacrylamide gel containing 0.2% SDS. The gel slice containing the enzyme after 7% analytical gel electrophoresis was heated in the presence of 1% SDS at 95 °C for 5 min. After staining with Coomassie Brillant Blue R 250 and electrophoretic destaining the transmission of the stained gel slab was monitored by a continuously recording densitometer (Kipp & Zonen, model DD 2). E: 3,17 β-Hydroxysteroid dehydrogenase.

Sedimentation properties

The sedimentation coefficient of the enzyme was determined after sucrose gradient centrifugation. The linear dependence of migration distances on sedimentation coefficients was verified for different standard proteins with known sedimentation coefficient [12]. The sedimentation coefficient of the enzyme from *Streptomyces hydrogenans* was estimated to be 4.1 ± 0.1 (Fig. 4).

Determination of the isoelectric point

Analytical polyacrylamide gel isofocusing of the purified enzyme is shown in Fig. 5. In comparison with the marker proteins of definite I. P. the isoelectric point of the enzyme was estimated to be 4.1. There is a small difference between the I. P.s after analytical or preparative isofocusing, whereby I. P. was estimated to be 3.9.

Substrate specificity

3,17 β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* is capable to dehydrogenate the hydroxyl groups at positions 17 β, 20 β and 3 β of different steroids in the presence of NAD^+. However, the hydroxyl groups at 3 α, 17 α, 6 β, 20 α or 21 were not dehydrogenated. The acceptability of various steroids as substrates of 3,17 β-hydroxysteroid dehydrogenase is shown in Table II. The relative velocities of the dehydrogenation of the 17 β-hydroxy group of 5 α-dihydrotestosterone and 5 β-dihydrotes-

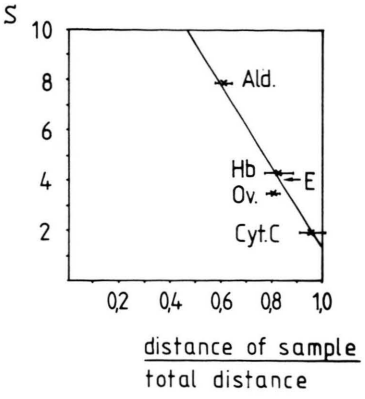

Fig. 4. Linear relationship between sedimentation coefficients of different marker proteins. 0.5 ml of marker proteins (1.5 mg protein/ml) were layered over separate 10 – 35% sucrose gradients in Tris-buffer, pH 7.4. Fractions of 0.42 ml were collected after centrifugation in a Spinco SW 41 rotor at 185 000 × *g* (41 000 rpm) for 16 h at 4 °C. Their absorbance was continuously monitored at 254 nm. Standard proteins used were: Aldolase (ald., 7.9 S), hemoglobin (Hb, 4.3 S), ovalbumin (Ov., 3.5 S), and cytochrome c (Cyt. c, 1.95 S). 3,17 β-Hydroxysteroid dehydrogenase (E, calculated 4.1 S).

tosterone were remarkably different. Testosterone and epiandrosterone (3 β-hydroxy-5 α-androstan-17-one) show nearly the same relative velocity for the dehydrogenation of the 17 β- and 3 β-hydroxy group, respectively. While 20 β-hydroxy-4-pregnen-3-one is transformed to progesterone by the enzyme, the corresponding epimer 20 α-hydroxy-4-pregnen-3-one remains unchanged.

The relative velocities for the hydrogenation of different ketogroups in the presence of NADH is shown in Table III. Testosterone was not converted to 3,17 β-dihydroxy-4-androsten. This was confirmed after incubation of [^{3}H]testosterone in the presence of NADH and the purified enzyme at 30 °C for 2 h. After extraction of the radioactive material and thin layer chromatography on silica gel no testosterone metabolites could be detected. Also progesterone remained unchanged and no reduction of the 20-ketogroup could be observed. 4-Androsten-3,17-dione and epi-androsterone (3 β-hydroxy-5 α-androstan-17-one) showed the lowest relative velocities. After incubation of [^{3}H]5 α-dihydrotestosterone or [^{3}H]4-androsten-3,17-dione in the presence of the purified enzyme and NADH for 2 h at 30 °C it could be

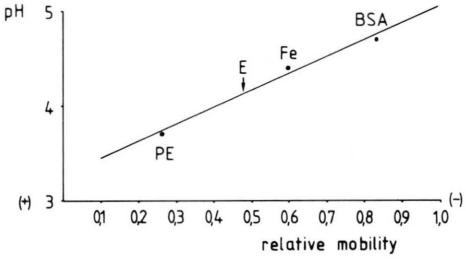

Fig. 5. Analytical polyacrylamide gel isofocusing of purified 3,17 β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*. 3,17 β-Hydroxysteroid dehydrogenase (E), Ferritin (Fe, I. P. 4.4), bovine serum albumin (BSA, I. P. 4.7), and pepsin (Pe, I. P. 3.7) were applied to polyacrylamide gel containing 4% Servalyt pH 3 – 5 and separated during 210 min as described in Materials and Methods.

Substrate	Relative velocity [%]
5 α-Dihydrotestosterone (17 β-hydroxy-5 α-androstan-3-one)	100
Testosterone (17 β-hydroxy-4-androsten-3-one)	63
epi-Androsterone (3 β-hydroxy-5 α-androstan-17-one)	68
20 β-Hydroxy-4-pregnen-3-one	44
17 β-Hydroxy-1,4-androstadien-3-one	38
5 β-Dihydrotestosterone (17 β-hydroxy-5 β-androstan-3-one)	36
Pregnenolone (3 β-Hydroxy-5-pregnen-20-one)	23
17 β-Estradiol	21
Dehydroepiandrosterone (3 β-hydroxy-5-androsten-17-one)	9
Testosterone acetate	–
17 α-Methyltestosterone	–
Androsterone (3 α-hydroxy-5 α-androstan-17-one)	–
6 β-Hydroxy-4-androsten-3-one	–
epi-Testosterone (17 α-hydroxy-4-androsten-3-one)	–
20 α-Hydroxy-4-pregnen-3-one	–
Ethanol	–
Deoxycorticosterone (21-hydroxy-4-pregnen-3,20-dione)	–

Table II. Relative velocities of the dehydrogenation of various steroids catalyzed by purified 3,17 β-hydrosteroid dehydrogenase from *Streptomyces hydrogenans*. End conc. of the steroids: 30 μM, ethanol: 0.2 M, NAD^+ : 0.66 mM. The initial reaction rate of 5 α-dihydrotestosterone was defined = 100% relative velocity. – no reaction.

Substrate	Relative velocity [%]
5 α-Dihydrotestosterone	100
5 α-Androstan-3,17-dione	45
Dehydroepiandrosterone (3 β-hydroxy-5-androsten-17-one)	55
Androsterone (3 α-hydroxy-5 α-androstan-17-one)	39
epi-Androsterone (3 β-hydroxy-5 α-androstan-17-one)	11
4-Androsten-3,17-dione	7
Testosterone	–
Progesterone	–

Table III. Relative velocities of the hydrogenation of various steroids catalyzed by purified 3,17 β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*. End conc. of the steroids: 30 μM, NADH: 0.66 mM. The initial reaction rate of 5 α-dihydrotestosterone was defined = 100% relative velocity. – no reaction

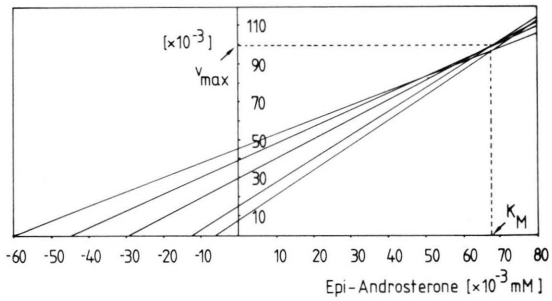

Fig. 6. Dehydrogenation of epi-androsterone (3 β-hydroxy-5 α-androstan-17-one) in the presence of 0.66 mM NAD^+ by purified 3,17 β-hydroxysteroid dehydrogenase (0.25 mg protein/ml) from *Streptomyces hydrogenans*. From the direct linear plot according to Eisenthal and Cornish-Bowden [13, 14] K_m can be calculated to 68 μM. Abscissa: substrate conc. [mM], ordinate: reaction rate [μmol · min^{-1}].

shown that 70 – 80% of 5 α-dihydrotestosterone were transferred to 3 β,17-dihydroxy-5 α-androstane. No formation of 3 α,17 β-dihydroxy-5 α-androstane was observed. About 50% of 4-androsten-3,17-dione was converted to testosterone. The ketogroup in position 3 was not accepted for hydrogenation if the steroid possessed 3-keto-Δ^4-configuration.

For the determination of the apparent K_m values the direct linear plot described by Eisenthal and Cornish-Bowden [13, 14] was used (Fig. 6 and 7). The following apparent K_m values were calculated: 5 α-dihydrotestosterone $0.11 \pm 0.02 \times 10^{-4}$ M, testosterone $0.20 \pm 0.08 \times 10^{-4}$ M, epi-androsterone $0.68 \pm 0.07 \times 10^{-4}$ M (each for the NAD^+-dependent reaction), 5 α-dihydrotestosterone $0.18 \pm 0.05 \times 10^{-4}$ M (for the NADH-dependent reaction), NAD^+ $1.84 \pm 0.41 \times 10^{-4}$ M, NADH $1.87 \pm 0.59 \times 10^{-4}$ M. These results are in good accordance with the K_m values estimated in the presence of the partial purified enzyme [5]. The enzyme does not accept NADPH or $NADP^+$ as co-substrate. The reaction rate for dehydrogenation is higher than for hydrogenation. In case of 5 α-dihydrotestosterone the turnover number is 383 and 217 mkat/mol enzyme for the NAD^+- or NADH-dependent reaction, respectively.

Inhibition by ATP

ATP is know to inhibit various dehydrogenases by competition with NAD^+ for the common binding site at the enzymes [15 – 17]. In fact, 3,17 β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* can also be inhibited by ATP. Using testosterone as substrate and increasing amounts of ATP the reaction was almost completely inhibited by an 100-fold excess of ATP over NAD^+. The K_i for the competitive inhibition was calculated by the Dixon plot [18] to be 1.15×10^{-3} M (Fig. 8).

Determination of SH groups and reaction with PCMB

The number of SH groups was calculated after reaction with DTNB to be 2 mol SH/mol enzyme protein (mol weight 70 200). In the presence of 6 M urea 3 mol SH/mol enzyme were detected. By the SH group blocking reagent PCMB the enzyme could not be completely inactivated (Table IV).

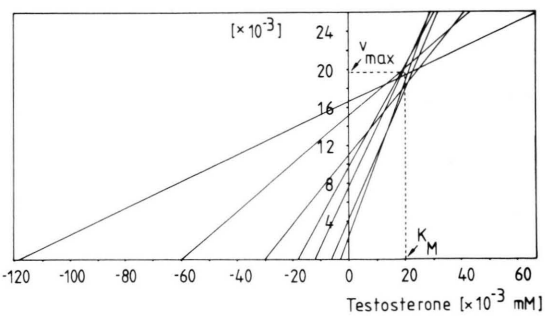

Fig. 7. Dhydrogenation of testosterone in the presence of 0.66 mM NAD^+ by purified 3,17 β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (0.28 mg protein/ml). From the direct linear plot according to Eisenthal and Cornish-Bowden K_m can be calculated to 20 μM. Abscissa: substrate conc. [mM], ordinate: reaction rate [μmol · min^{-1}].

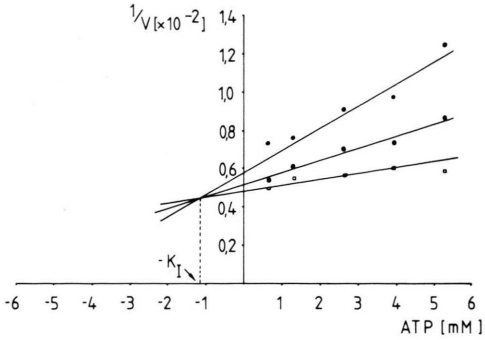

Fig. 8. Dixon-plot of 3,17 β-hydroxysteroid dehydrogenase-dependent dehydrogenation of testosterone inhibited by ATP. $K_i = 1.15$ mM. Abscissa: conc. of ATP [mM], ordinate: 1/reaction rate [min · μmol^{-1}]. Concentrations of NAD^+ were ● ● ● 0.085 mM, ■ ■ ■ 0.17 mM, □ □ □ 0.34 mM.

Table IV. Inhibition of 3,17 β-hydroxysteroid dehydrogenase-catalyzed dehydrogenation of 5 α-dihydrotestosterone in the presence of *p*-chloromercuribenzoate (PCMB)

PCMB [M]	Inhibition [%]
0.3×10^{-4}	0.5
1.5×10^{-4}	22
3×10^{-4}	22

Discussion

We have used a simple and rapid procedure for the purification of 3,17 β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* by means of which 10 mg-portions of cytosolic protein may be purified until an enzyme preparation of nearly homogeneity with respect to its electrophoretic behaviour. The overall recovery of 7.9% of the enzyme activity in relation to the activity in the cell cytosol is similar to the yield of 6% for 3,17 β-hydroxysteroid dehydrogenase activity obtained from *Pseudomonas testosteroni* [19]. The mass of the purified enzyme protein corresponds to 0.2% of the total protein in the cytosol of the cells of *Streptomyces hydrogenans.* There is no indication for multiple 3,17 β-hydroxysteroid dehydrogenases or families of isozymes as shown for *Pseudomonas testosteroni* [19].

Isoelectric focusing as well as affinity chromatography, using different steroid ligands and spacers, were tested as alternative methods for the fractionation of the cytosol proteins of *Streptomyces hydrogenans.* Both techniques yielded only low purification. Because of the rather high loss of proteinaceous material, the overall recovery was low. After isoelectric focusing the fraction containing the enzyme activity was still contaminated by further proteins. Moreover, the recovery of enzyme activity was low, perhaps due to the low isoelectric point of the enzyme. Supplements, which were recommended for avoiding or lessening of protein precipitation during isoelectric focusing [20] were not effective in our case. Various experimental protocols for purification of hydroxysteroid dehydrogenases from other sources also show rather low efficiencies [21, 22]. In our experiments specific activity of 3,17 β-hydroxysteroid dehydrogenase increased after affinity chromatography only 5-fold (results not shown).

The molecular weight of the enzyme protein estimated by SDS gel electrophoresis has been calculated to be 70 200. As cross-linking of the native enzyme did not generate heavier protein bands, the enzyme seems to be monomeric although gel filtration on Sephadex G-200 of the enzyme points to a protein twice as large. 3,17 β-Hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* was proved to be a tetrameric protein consisting of four similar subunits of molecular weight 23 500 [23].

If one compares the ability of different steroids to donate hydrogen for the formation of NADH, the specificity of the enzyme can be defined as 17 β-dehydrogenase (*e. g.* comparing testosterone/epi-testosterone), 3 β-dehydrogenase (*e. g.* epi-androsterone/androsterone) and 20 β-dehydrogenase (*e. g.* 20 β-hydroxy-4-pregne-3-one/20 α-hydroxy-4-pregnen-3-one). This property is opposite to the activity of 17 β-hydroxysteroid dehydrogenase from rabbit ovaries, which accepts 20 α-hydroxysteroids as hydrogen donors [24]. Although *Streptomyces hydrogenans* contains a very active 20 β-hydroxysteroid dehydrogenase, this enzyme activity is not responsible for the capability of the 3,17 β-hydroxysteroid dehydrogenase to dehydrogenate 20 β-hydroxy groups shown in this work. First of all, 20 β-hydroxysteroid dehydrogenase activity was separated completely by the purification procedure used. Furthermore, 20 β-hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* shows an additional activity to dehydrogenate 3 α-hydroxysteroids [25]. Such a 3 α-activity was absent in our 3,17 β-hydroxysteroid dehydrogenase preparation. 3,17 β-Hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* does not dehydrogenate the 21-hydroxy group of 11-deoxycorticos-

terone or 6 β-hydroxy group of 6 β-hydroxy-4-androsten-3-one and does not behave as alcohol dehydrogenase. The apparent K_m values estimated for the dehydrogenation of testosterone or 5 α-dihydrotestosterone are comparable to the figures calculated for dehydrogenation by animal dehydrogenases [26, 27] or are even lower [28]. In the presence of NADH the 3-keto group can be transformed to the 3 β-hydroxy group provided there is no double bond in ring A of the steroid substrate (*e. g.* 5 α-dihydrotestosterone or dehydroepiandrosterone). Therefore, testosterone will not be converted into 3 β,17 β-dihydroxy-4-androstene. 3,17 β-Hydroxysteroid dehydrogenase does not accept $NADP^+$ or NADPH as co-substrates.

In accordance to the inhibitory activity of ATP found for various NAD^+-dependent dehydrogenases [15 – 17] the dehydrogenation of testosterone by 3,17 β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* can be competitively inhibited by ATP as shown by the dixon plot [18]. This phenomenon points to the low specificity of the co-substrate binding site of the enzyme to discriminate between NAD^+ and ATP. 3,17 β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* contains at least 3 mol SH groups per mol enzyme protein, which seem to be of low importance for the activity of the enzyme because its activity was only partly inhibited after addition of 3×10^{-4} M PCMB. 17 β-Hydroxysteroid dehydrogenase of human placenta, however, can be inactivated completely in the presence of 10^{-5} M PCMB [27].

As *Streptomyces hydrogenans* can well grow in the absence of any steroid in the culture medium, the physiological importance of the numerous steroid-transforming enzymes and steroid-binding proteins within the cell is not clear [1, 4, 5, 9, 29, 30]. There may be the possibility that these microbial proteins take part in metabolic reactions *in vivo,* where steroids do not play any considerable role. A similar assumption has been made for several animal hydroxysteroid dehydrogenases [31 –33], but in case of the microbial 3,17 β-hydroxysteroid dehydrogenase a final suggestion is not yet possible.

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