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STEROID TRANSFORMING ENZYMES FROM MICROORGANISMS IV. PURIFICATION AND COFACTOR REQUIREMENT OF THE 4-ENE-3-OXOSTEROID-5α-REDUCTASE FROM *MYCOBACTERIUM SMEGMATIS*

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SUMMARY

4-Ene-3-Oxosteroid-5α-reductase of *Mycobacterium smegmatis* is bound with the subcellular structure of the microbial cell, although about 50% of activity can be released by sonication. By using some procedures of protein fractionation separation of nucleic acids, salt precipitation, gel filtration and ion exchange chromatography—the enzyme was purified about 60-fold with a recovery of 36%. 5α-Reductase activity was not stimulated by NADH or NADPH. The FAD-dependent character of the enzyme was deduced from its activation by dithionite, inhibition by acriflavin and restoration of activity after inactivation by acidic ammonium sulphate. The conception of a possible role of the flavin is discussed.

INTRODUCTION

In the previous papers[1, 2] we have considered the preparation of cell-free extracts and consequent $(NH_4)_2SO_4$ extract preparations from *Mycobacterium smegmatis* with 4-ene-3-oxosteroid-5α-reductase activity. Some properties of the enzyme have also been described. The reaction was shown to depend on sodium dithionite presence in the assay system. Due to inhibition effect of acryflavin the reduced flavin nucleotide appeared to participate in the 5α-reduction by the enzyme. To investigate the mechanism of the 5α-reductase action the attempt to purify the enzyme was undertaken.

MATERIALS AND METHODS

M. smegmatis 98 was obtained from the Type Culture Collection of the Central Institute of Microbiology and Experimental Therapy, Jena, GDR; [4-^{14}C]-4-androstene-3,17-dione (56 mCi/mM) from the Radiochemical Centre, Amersham, England; Sephadex G-100, Sephadex G-200 and DEAE-Sephadex A-50 from Pharmacia, Uppsala, Sweden; FAD and FMN from Serva, Heidelberg, Germany; $NADH_2$ and $NADPH_2$ from VEB Arzneimittelwerk, Dresden, GDR.

Growth of M. smegmatis. The medium for cultivation of the microorganism and the procedure for growing, harvesting and preparing of cell-free extract have already been described[2].

Assay of enzyme activity. The conditions for enzymic 5α-reduction and the measurement of 5α-reductase activity has also been described in detail previously[2]. One unit of the enzyme activity is the amount of the enzyme that catalyzes the formation of 1 μg of 4-androsten-3,17-dion/h under the condition specified. Specific activity is defined in terms of unit/mg protein.

Assay for protein. Protein was determined spectrophotometrically by U.V. absorbtion at 215 and 225 nm[3] or by the method of Hesse *et al.*[4].

Resolution of enzyme. Partial purified preparations of 5α-reductase were treated with acid ammonium sulfate according to the procedure of Warburg and Christian[5].

RESULTS

Differential centrifugation of sonicated cell suspensions

5α-Reductase activity was detected in the supernatants after centrifugation of cellular sonicated suspensions at 15,000 *g* for 20 min. As the supernatants were rather turbid we centrifuged them repeatedly at higher speeds (Table 1). Upon centrifugation at 105,000 *g* the supernatant fraction lost about 40% of original protein and activity. Further centrifugation (at 200,000 *g*) produced a high speed supernatant fraction which again lost part of the protein and a corresponding part of the enzyme activity. These findings indicate that protein and 5α-reductase were progressively sedimented by longer high speed centrifugation. Thus, we failed to purify 5α-reductase by this procedure as done by Germain *et al.*[6] in experiments with *Nocardia corallina*.

Table 1. Protein and 5α-reductase activity of supernatant fractions after differential centrifugation of sonicated cell suspensions

Fraction	Protein mg/ml	5α-reductase activity units/ml	S.A. of 5α-reductase
15,000 *g* supernatant	22.6	72.6	3.3
105,000 *g* supernatant	11.6	40.6	3.5
200,000 *g* supernatant	9.6	32.6	3.4

Table 2. Ammonium sulfate fractionation of streptomycin sulfate supernatant

Fraction	Protein mg	5α-reductase activity	
		Total	S.A.
Streptomycin sulfate supernatant	915	4,460	4.9
0–30% $(NH_4)_2SO_4$ precipitate	340	600	1.7
30–45% $(NH_4)_2SO_4$ precipitate	360	2,740	7.6
45–60% $(NH_4)_2SO_4$ precipitate	200	250	1.2
60–80% $(NH_4)_2SO_4$ precipitate	30	40	1.3

Purification of the enzyme

105,000 *g* Supernatant of sonicated cells was used as a source of 5α-reductase. All steps of the enzyme purification were performed at 4°C. Standard buffer used throughout the purification was 0.067 M Sörensen phosphate buffer, pH 7.0. The results of the purification are summarized in Table 3 with average specific activity and recovery estimates for each fraction step. Individual steps have been performed sometimes and have not deviated markedly from the average results presented in the table.

Precipitation of nucleic acids. A neutralized solution of streptomycin sulfate was added dropwise while stirring so that its final concentration was 1%. Stirring was continued for 15–20 min; the precipitate was collected by centrifugation at 10,000 *g* for 20 min and discarded.

Ammonium sulfate precipitation. Solid ammonium sulfate was added slowly and with continuous stirring for about 20–30 min after all the ammonium sulfate had dissolved. The addition was carried out at neutral pH adjusted with diluted ammonium hydroxide solution. The proteins precipitated between 0 and 30% salt saturation were centrifuged at 10,000 *g* for 15 min and discarded as the activity of the fraction was maximum 12% of the total activity (Table 2). The major part of the enzyme activity was revealed in the protein fraction precipitated between 30 and 45% of saturation with ammonium sulfate. The precipitate was dissolved in standard buffer and used as a material for the next step of the enzyme purification. This fraction contained about 60% of total 5α-reductase activity. The fraction of 45–60% salt saturation had only 5% of the total activity and the fraction of 60–80% saturation was practically inactive (Table 2).

Table 3. Purification of steroid-5α-reductase

Fraction	Total protein mg	5α-reductase activity			
		Specific	Total	Recovery	Purification
Initial extract (105,000 *g* supernatant)	1,004	4.9	4,914	100	—
Streptomycin sulfate supernatant	910	4.9	4,570	93	—
30–45% Ammonium sulfate precipitate	396	7.7	3,047	62	1.6
Sephadex G-100 (pooled fractions)	58	50.8	2,948	60	10.6
DEAE-Sephadex A-50 (pooled fractions)*	6.5	269.2	1,023	36	57.6

* Activity and recovery have been normalized assuming that all of the active Sephadex G-100 fraction was used.

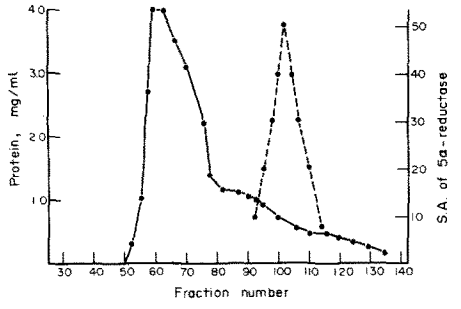

Fig. 1. Sephadex G-100 gel filtration of $(NH_4)_2SO_4$ extract precipitate (30–45% saturation). Details of the column and its operation are given in the text. The graph shows concentration of protein (●—●) and enzyme activity (●---●).

Sephadex G-100 gel filtration. The enzyme material after ammonium sulfate precipitation (30–40% saturation) was layered onto a column (4 × 100 cm) of Sephadex G-100 (Special examination showed that no preliminary material desalting was required). The column was equilibrated and eluated with the standard buffer. Flow rate at about 0.4 ml/min was maintained and 3.0 ml fractions were collected.

Figure 1 shows the pattern of eluated proteins and activity according to fractions in the course of gel filtration. More than 85% of ballast protein was separated at this step. Fractions, containing the bulk of 5α-reductase activity (about 95% of enzyme activity applied to the column) were pooled.

DEAE-Sephadex chromatography. The Sephadex G-100 eluate was applied to a column (1.5 × 35 cm) of DEAE-Sephadex A-50 previously equilibrated with standard buffer. After washing the column with approximately 70 ml of equilibrating buffer it was developed with 340 ml linear gradient of NaCL (from 0.4–0.55 M) in equilibrating buffer. Figure 2 depicts the chromatography on the ion exchanger Sephadex. About 90% of the proteins placed on the column did not reveal enzyme activity. The 5α-reductase was eluated at a NaCL concentration between 0.46 and 0.48 M NaCL. Thus, according to the data, showed

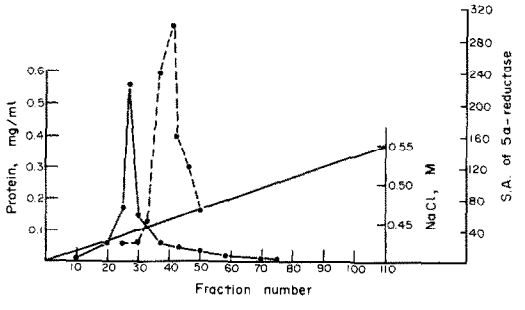

Fig. 2. Pattern of elution of proteins and 5α-reductase activity from DEAE-Sephadex A-50 column following chromatography. Experimental conditions as described in text. Concentration of protein (●——●), enzyme activity (●---●).

in Table 3, 5α-reductase was purified 58-fold with a recovery of 36%.

Cofactor requirement

The addition to the reaction mixture of such natural hydrogen donors as $NADH_2$ and $NADPH_2$ did not result in the increasing of enzymatic 5α-reduction of androstendione in the experiments with crude extracts and $(NH_4)_2SO_4$ precipitate extracts.

Special examination revealed the presence of active $NADH_2$-oxidase in these enzyme preparations. Incubation of 3 ml samples, containing crude extracts (7 mg protein), 50 μg $NADH_2$ and 0.067 M Sörensen phosphate buffer, pH 7.0, resulted in complete oxidation of $NADH_2$ in 1 min. In experiments with ammonium sulfate preparation (5 mg protein) under the same conditions 60% $NADH_2$ were oxidized in 1 h (measurements by the absorbtion at 340 nm). The amount of protein in the samples corresponded to that in the experiments on 5α-reduction of the steroid.

However, no 5α-reductase activity above control was observed with $NADH_2$ or $NADPH_2$ addition to the system under extremely anaerobic condition (nitrogen atmosphere) or with enzyme preparation after Sephadex G-200 column which had no pyridin-nucleotide oxidase activity.

In a previous communication[2] we have reported that the inhibition of 5α-reductase activity in the presence of dithionite was suggestive of the involvement of a flavin prosthetic group and this view was strengthened by the fact that acriflavin inhibition of the enzyme activity. The inhibition of many riboflavin-containing enzymes by acriflavin is well known.

Partially purified 5α-reductase from *M. smegmatis* was inhibited at a final concentration of 10^{-3} M acriflavin to the extent of 80%. The acriflavin inhibition was partially reversed by 10^{-5} M FAD, but not riboflavin phosphate (Table 4).

To prove the possible role of flavin as a prosthetic group of 5α-reductase from *M. smegmatis* the preparation of apoenzyme was carried out according to the procedure of Warburg and Christian[5]; when the flavin prosthetic group is extracted from an enzyme by precipitation of the apoenzyme with acidic ammonium sulfate the activity decreases or vanishes and it can be restored by the addition of low flavin nucleotide concentrations.

For acidic ammonium sulfate extraction of flavin 8 ml enzyme solution (the preparation after the Sephadex G-200 step), containing 3 mg protein/ml were mixed rapidly, but under careful stirring, with solid $(NH_4)_2SO_4$ to get 55% salt saturation. The suspension then was obtained by dropwise additioned 0.8 ml 1 N H_2SO_4 and $(NH_4)_2SO_4$ to 85% saturation. Stirring was continued for another 30 min and the suspension was centrifuged at 10,000 *g* for 10 min. The supernatant fluid, containing free flavin nucleotide, was removed completely by decanting and then wiping the inside of the tube with absorbant paper. The precipitate was dissolved in 8 ml 0.067 M

Table 4. Inhibition of the 5α-reductase by acriflavin and partial reversion by FAD

Acriflavin·HCl 3×10^{-3} M	FAD 6×10^{-5} M	FMN 10^{-3} M	5α-reductase activity %
–	+	–	100
–	–	+	108
+	–	–	20
+	+	–	34
+	–	+	22

For the 5α-reductase assay, standard conditions and the enzyme preparation after the Sephadex G-200 step were used. Before initiation of the reaction with substrate the samples were preincubated with acriflavin or/and flavin nucleotides for 15 min.

Sörensen phosphate buffer, pH 7.0, and a part of the precipitate, which could not be dissolved, was discarded.

In Table 5 the results of 5α-reductase activity of the initial material and the apoenzyme preparation and the effect of flavin nucleotides on native and resolved enzyme are summarized. In comparison with the initial enzyme preparation the resolved enzyme lost about 40% of its activity after such treatment. In the presence of dithionite FAD 2×10^{-5} M completely restored the activity of the resolved enzyme, although it had no influence on the native enzyme. FMN practically was without effect, even in concentration 50 times higher than those required for reactivation with FAD.

Finally, it was found that partially purified 5α-reductase preparations (after DEAE-Sephadex A-50 step) had lost about 90% of their activity during storage at −20°C for 6 weeks. The activity could be restored to 30% of the initial one by the addition of 15 μM FAD.

DISCUSSION

The present results, obtained by differential centrifugation at 15,000 *g* of crude extracts from *M. smegmatis*, gave evidence that 4-ene-3-oxosteroid-5α-reductase is bound with subcellular structures of the microbial cells, although about 50% activity can be released by sonication. Steroid-5α-reductase of *N. corallina* was found in cytosol[6].

By contrast, the corresponding enzymes of different mammalian tissues apparently fall into a class of proteins that are tightly integrated with the structure of nuclear and cytoplasmic membranes. It was one of the reasons for unsuccessful attempts to solubilize these 5α-reductases. At present we are aware of three recent publications on the solubilization of 5α-reductase from nuclei and microsomes of rat prostate and liver[7–9].

Applying certain techniques of protein fractionation—separation of nucleic acids, salt precipitation, gel filtration and ion exchanger chromatography—5α-reductase from *M. smegmatis* was purified about 60-fold with a recovery of 36%. So far purified microbial 5α-reductase preparations have not been obtained. We consistently observed that the enzyme activity became increasingly instable when the contaminating proteins are removed, particularly following DEAE-Sephadex chromatography. The 5α-reductase preparation had lost about 90% of its activity during storage for 6 weeks at −18°C, whereas crude extracts did not lose their activity during some months under the same conditions.

With regard to experiments on co-factor requirement we found that neither $NADH_2$ nor $NADPH_2$ increased the 5α-reductase activity of the enzyme preparations from *M. smegmatis* although they had no pyridine nucleotide oxidase activity.

On the other hand some findings suggested the possible role of flavin in the enzymatic 5α-reduction. The suggestion was consistent with the following facts: (a) In the presence of dithionite the 5α-reductase activity is highly increased[2]; (b) The purified enzyme is hardly inhibited by acriflavin and the inhibition can be reversed by FAD; (c) Precipitation of

Table 5. Effect of FAD and FMN on native and resolved 5α-reductase

Preparation	Additions	S.A. of 5α-reductase
Native enzyme	—	47
3.0 mg protein/ml)	FMN (10^{-3} M)	45
	FAD (10^{-4} M)	43
	—	28
Resolved enzyme	FMN (10^{-3} M)	31
(2.2 mg protein/ml)	FAD (10^{-4} M)	44
	FAD (2×10^{-5} M)	44

The effects of flavin nucleotides were examined in the regular assay system.

the enzyme with acidic ammonium sulfate resulted in a loss of enzyme activity. 5α-Reductase activity can be restored by the addition of FAD.

These results with *M. smegmatis* are in contrast with the data obtained with *N. corallina*: only $NADPH_2$, $NADH_2$ or their generating systems were found to be able to provide reducing equivalent for 5α-reductase from this microorganism[6]. Moreover mammalian 4-ene-3-oxosteroid-5α-reductases were believed by many to be $NADPH_2$-dependent enzymes[10–16].

However, 5α-reductase from rat liver microsomes appeared recently to be an enzyme system, consisting of two enzymes: (1) $NADPH_2$-co-enzyme Q_{10} oxydoreductase (flavin-dependent enzyme) and (2) steroid-5α-reductase, which transport hydrogen from the reduced co-enzyme Q_{10} to the steroid.

On account of these results our finding, which reflected the FAD-dependent character of 5α-reductase from *M. smegmatis*, are not extraordinary. At present we do not know whether the mechanism of action of 5α-reductase from *M. smegmatis* corresponds to the mechanism discovered by Staudinger and coworkers[9, 17]. It should be remembered that we did not observe the influence of reduced pyridine nucleotides on the enzymatic 5α-reduction. Apparently there are two possibilities: either the role of co-substrate in the microbial system (if it is a system) is carried out by other compound instead of pyridine nucleotides, or 5α-reductase of *M. smegmatis* contains FAD as its prosthetic group. In any case the conception is to be tested experimentally.

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