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STEROID-1-DEHYDROGENASE OF *RHODOCOCCUS ERYTHROPOLIS*: PURIFICATION AND N-TERMINAL AMINO ACID SEQUENCE

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Summary—The inducible steroid-1-dehydrogenase from the bacterium *Rhodococcus erythropolis* IMET 7030 was purified to homogeneity using affinity chromatographic, electrophoretic, and ion exchange techniques. The spectrum of the pure enzyme is characterized by the associated FAD. The M_r of the enzyme is 56,000. The amino acid composition and the sequence of the 13 N-terminal amino acids are given.

INTRODUCTION

Microbial 1-dehydrogenation of 3-oxo steroids, leading to substances of therapeutic value, can be performed successfully by fermentation with members of the genera *Arthrobacter*, *Bacillus*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, or *Mycobacterium*.

Cell free extracts and partially purified enzyme fractions from these bacteria have been used to investigate substrate specificity [1–5], kinetics [6], and mechanism [7] of this enzymatic reaction. Recently, cloning of the 1-dehydrogenase of *Pseudomonas testosteroni* and expression in *E. coli* as well as in *Pseudomonas* species has been described [8]. However, the amino acid sequence has not been published. So far, the 1-dehydrogenases of two species have been enriched and purified: that from *Rhodococcus erythropolis* IMET 7030 [9–11], and that from *Nocardia corallina* [12]. Similarities of the 1-dehydrogenases as well as discrete differences have been found within these nocardioform bacteria using immunological methods [13]. Both enzymes, exactly designated 3-oxosteroid-(acceptor)-1-en-oxidoreductase, EC 1.3.99.4, are flavoproteins containing FAD [10, 12, 14, 15]. The enzymic reaction is reversible [16], and even a transhydrogenation between 1-ene- and 1-saturated 3-oxo steroids is possible [16, 17]. The enzyme protein of *R. erythropolis* was purified to homogeneity and the amino acid sequence of the N-terminus determined, which is the subject of this paper.

EXPERIMENTAL

Microorganisms

R. erythropolis IMET 7030 was obtained from the culture collection of the Division of Steroid Research of the CIMET Jena. The designation *Nocardia opaca*, used for this strain in earlier papers [9, 10, 14], had been corrected according to Goodfellow and Alderson [18].

Enzyme preparation

Cultivation, induction with 17α-methyltestosterone and ultrasonic disruption of the cells were done as described previously [10]. After treatment with streptomycine sulphate (1%) and precipitation by ammonium sulphate (30–55% saturation) the proteins were selectively adsorbed from 20 mM phosphate buffer pH 7.0 to columns of *N*-(4-androsten-3-on-17β-oxycarbonyl)-ε-aminocapronyladipinoyl-dihydrazido-Sepharose 4B [19]. The washed affinity gel was suspended in electrode buffer containing 10 mM DTT and loaded onto rod gels of PAA for electrodesorption [11] and separation (see disk electrophoresis). The enzyme zone was cut out, electroeluted into 50 mM ammonium bicarbonate using a dialysis bag,

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Abbreviations: DTT, dithiothreitol; PAA, polyacrylamide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate.

and protected by addition of 10% glycerol. Alternatively, the PAA gel pellet was subjected directly to SDS–PAGE.

Preparative SDS–PAGE

The enzyme containing pellets cut from disc electrophoresis gels were mashed, treated with SDS cocktail (SDS 4%, saccharose 20%, DTT 20 mM, Tris–HCl 0.1 M, pH 6.8), heated 3 min at 100°C and loaded onto SDS slab gels. After electrophoresis using 0.1 mM sodium thioglycolate in the electrode buffer as radical scavenger, the protein band was visualized by immersion in 0.5 M KCl or by Coomassie blue staining [20] (concentration lowered to 0.05%) and electroeluted into ammonium bicarbonate solution according to [20]. The protein was precipitated by 9 vol of methanol at −20°C and collected by centrifugation. Alternatively, electroblotting to ProBlott membranes [21] was performed using CAPS buffer [3-(cyclohexyl-amino)-1-propane-sulphonic acid, 10 mM, pH 11 with 10% methanol]. The protein zone was stained on the membrane with Ponceau S [22], and subjected directly to amino acid sequencing.

FPLC

Enzyme eluates containing 5% glycerol were chromatographed on a FPLC ion exchange column Mono Q (Pharmacia LKB) using a gradient of NaCl in 10 mM Tris–HCl pH 8.0. The main fractions were desalted by repeated dilution with 10 mM ammonium bicarbonate and ultrafiltration using Centriprep 30 units (Amicon).

Sequence analysis

Stepwise N-terminal degradation was performed either manually following the double coupling method of Chang *et al.* [23] with TLC detection of the released coloured amino acid derivatives, or automatically. The protein was freed from residual ammonium bicarbonate by precipitation with 9 vols of ethanol at −20°C or applied directly to Polybrene-coated glass-fibre filters and sequenced on a pulsed Liquid-phase sequencer A 477/A 120 (Applied Biosystems) with on-line HPLC analysis of the phenylthiohydantoine amino acids formed, following the manufacturer's instructions.

Amino acid analysis

The protein was hydrolysed in the gas phase with 6 M HCl/1% phenol, for 24 h at 110°C [24]. The amino acids released were derivatized with dabsylchloride and analysed by liquid chromatographic determination according to Knecht and Chang [25].

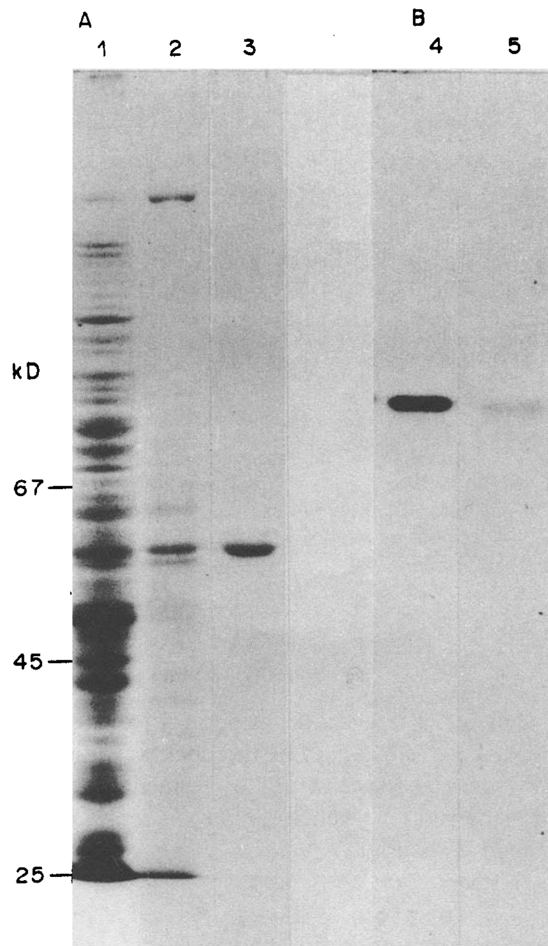

Fig. 1. Gel electrophoretic control of purification of steroid-1-dehydrogenase. (A) SDS–PAGE; gel 8%. Lane 1: after ammonium sulphate precipitation; 2: proteins adsorbed to affinity gel; 3: 1-dehydrogenase after disk electrophoresis. Staining: Coomassie blue. (B) Disk electrophoresis of the native enzyme; gel 15%. Lane 4: stained with Coomassie blue; 5: enzyme reaction.

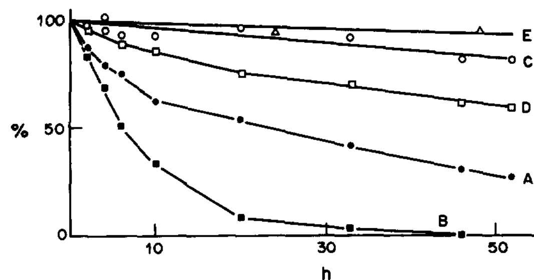

Fig. 2. Decrease of enzyme activity during storage in sodium phosphate buffer pH 8.0, at 0°C. Buffer concentrations: A, 100 mM; B, 10 mM; C, 100 mM + 20 μM FAD; D, 10 mM + 20 μM FAD and E, 50 mM ammonium bicarbonate containing 10% glycerol.

General methods

Disk electrophoresis was performed at 5–10°C using PAA gels of 10 or 15%, cross linking 2.7%, in 0.375 M Tris–HCl, pH 8.8, overlayered with stacking gels of 5% PAA in 0.08 M Tris–HCl, pH 8.8. Electrode buffer: Tris–glycine 0.025 M/0.192 M, pH 8.3. SDS–PAGE was performed using the Laemmli system [26].

Enzyme activity was measured at 25°C photometrically by dehydrogenation of 4-androstene-3,17-dione, 200 μM, in sodium phosphate, 100 μM, pH 9.0, containing 2% methanol, using the artificial electrone acceptor 2,6-dichlorophenolindophenol, 20 μM [2] ($E_{600\,nm}$ = 21,800 $cm^{-1}\,M^{-1}$).

Enzyme activity in PAA gels was detected by the dehydrogenation of androstenedione, 200 μM, with phenazine methosulphate, 200 μM, and nitrotetrazolium blue, 500 μM, at pH 7.4 [13].

Protein was determined by the Coomassie blue method [27] using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Properties of the steroid-1-dehydrogenase

Using an established purification protocol, including adsorption to an affinity gel, electrodesorption and disk gel electrophoresis [11, 13], the enzyme was obtained with overall yields of 20–30% in a purity of 80–90%, as shown by SDS–PAGE (Fig. 1, lanes 1–3). The enzyme exhibited a M_r of 56 kDa estimated by SDS–PAGE and gel filtration, and a pI of 4.5 (not shown). Only one zone with enzyme activity was found after disk electrophoresis (Fig. 1, lane 5). The sp.act. of 125 U/mg protein did not increase by further purification. The activity of the pure but diluted enzyme decreased by storage (Fig. 2, lane A), especially in solutions of low ionic strength (lane B). The enzyme could be protected by FAD (Fig. 2, lanes C and D) and by 10% glycerol (lane E), but only to a small degree by DTT. At −20°C in the presence of glycerol the activity was retained for more than 1 year.

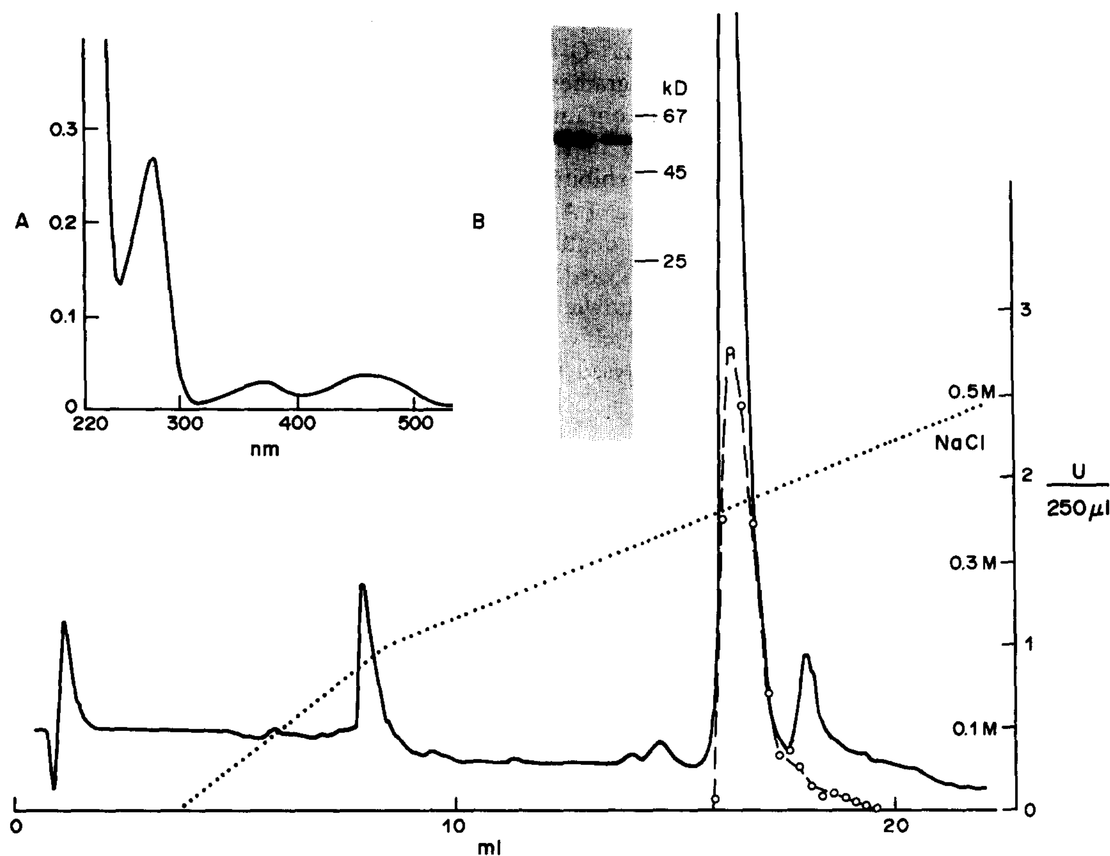

Fig. 3. Purification of steroid-1-dehydrogenase by FPLC on Mono Q. Absorbance (—), enzyme activity (-O-), and NaCl concentration of the eluate (· · ·). Insert A: spectrum of the main fraction; B: SDS–PAGE (gel 15%) of the main fraction.

Amino acid analysis and sequence

The enzyme preparation obtained as above was further purified by preparative SDS–PAGE. The protein zone at 56 kDa was accompanied sometimes by a minor one of an apparent M_r of 58 kDa which seemed to be formed during prolonged storage of the enzyme. Impurities of lower M_r had been removed. The protein was subjected to amino acid analysis. The composition, presented in Table 1, corresponds with the acidic pI and exhibits similarity to that of the 1-dehydrogenase of 60.4 kDa isolated from *Nocardia corallina* [12]. The SDS–PAGE purified protein was subjected to manual Edman degradation [23]. Only three steps were performed indicating the N-terminal amino acid sequence Met-Gln-Asp... Yields decreased drastically after the first degradation step, probably due to the partial cyclization of Gln in N-terminal position during acidic Edman conditions. Alternatively, the proteins were blotted to a ProBlott membrane and the 56 kDa band analysed in an automated sequencer. A more extended sequence could be detected, but there was also a drastic reduction in yields after the N-terminal methionine.

Using an ion exchange column Mono Q at pH 8, the enzyme was eluted by a NaCl gradient at 0.35 M and separated from some contaminating proteins (Fig. 3). The spectrum of the yellow main fraction (Fig. 3, insert A) exhibited the typical absorption maximum of a FAD containing enzyme [cf. 12]. Sequence identification of this pure material (see insert B) yielded the N-terminal amino acid sequence shown in Table 2, except the Cys in position 8, which was deduced from the DNA sequence analysed by Blei *et al.* [28]. This should open further possibilities for investigating this interesting steroid metabolizing enzyme.

Table 1. Composition of the steroid-1-dehydrogenases from *R. erythropolis* IMET 7030 and *N. corallina*

	Residues per mol enzyme	
Amino acid	*R. erythropolis**	*N. corallina* [12]
Asx	49	55
Glx	40	56
Ser	33	51
Thr	31	28
Gly	75	79
Ala	61	62
Val	24	37
Pro	33	35
Met	11	11
Arg	25	29
Ile	15	20
Leu	37	42
Phe	17	17
Lys	12	13
His	17	10
Tyr	17	18

*Cys and Trp were not determinated.

Table 2. N-terminal amino acids of the steroid-1-dehydrogenase from *R. erythropolis*

1	2	3	4	5	6	7	8	9	10	11	12	13
Met-	Gln-	Asp-	Trp-	Thr-	Ser-	Glu-	Cys-	Asp-	Val-	Leu-	Val-	Val-

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