Purification and Properties of Arogenate Dehydrogenase from *Actinoplanes missouriensis*

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Actinoplanes missouriensis utilizes arogenate as an intermediate in L-tyrosine biosynthesis, while no evidence of prephenate dehydrogenase was observed. Arogenate dehydrogenase has been partially purified by a five-step procedure. The enzyme requires NAD as cofactor. The K_m values for NAD and arogenate are 0.2 mM and 0.15 mM, respectively. The molecular weight of arogenate dehydrogenase is about 68,000, and SDS gel electrophoresis indicates a composition of two identical subunits. The enzyme is not feedback inhibited by L-tyrosine and unaffected by L-phenylalanine, prephenate, phenylpyruvate, *p*-hydroxyphenylpyruvate or L-tryptophan. Arogenate dehydrogenase is quite sensitive to *p*-hydroxymercuribenzoate with 50% inhibition at 12.5 μM of the SH-specific reagent. The presence of malate in usually applied arogenate preparations is demonstrated and the consequence of an impure substrate on arogenate dehydrogenase studies is discussed.

Introduction

Actinoplanes missouriensis produces actaplanin [1], a new complex of broad spectrum Gram-positive glycopeptide antibiotics mainly composed of aromatic amino acids. As reported in a former article [2], tyrosine was found to be synthesized *via* arogenate, but not *via* prephenate. The high activity for arogenate dehydrogenase in contrast to chorismate mutase and prephenate dehydratase in this organism encouraged us to pursue following aims: (1) purification of arogenate dehydrogenase, (2) determination of physical, kinetic and regulatory properties of the enzyme, (3) elucidation of a possible source of error in the arogenate dehydrogenase test.

Materials and Methods

Strain and culture conditions

Actinoplanes missouriensis strain (DSM 43046) was obtained from O. Salcher, Bayer-AG, Wuppertal, Germany (Federal Republic). The organism was cultivated aerobically on a rotary shaker at 30 °C in a minimal glycerol medium according to Waksman [3].

Reagents

Arogenate (β-(1-carboxy-4-hydroxy-2,5-cyclohexadiene-1-yl)-alanine) was prepared from the culture supernatants of a triple mutant of *Neurospora crassa* ATCC 36373 [4] and prephenate according to Dayan and Sprinson [5].

Enzyme assays

Arogenate dehydrogenase activity was measured spectrophotometrically at 340 nm by determination of NADH formed during the conversion of arogenate to tyrosine. Reaction mixtures containing 50 mM K-phosphate pH 7.5, 20% glycerol, 0.5 mM EDTA, 0.5 mM dithioerythritol and 0.6 mM NAD were incubated 3 min at 37 °C. Arogenate at a final concentration of 0.45 mM was added to start the arogenate dehydrogenase reaction. As a control, arogenate preparations were quantitatively converted to phenylalanine by addition of 1 N HCl, reneutralized with NaOH and used as substrate in an additional assay. To confirm arogenate dehydrogenase activity, we used the OPA-HPLC-method to detect tyrosine, the product of enzyme reaction. After addition of sulfosalicylic acid to a final concentration of 10%, the reaction vessel was immersed in an ice bath for 30 min. The protein, precipitated by this procedure, was sedimented by centrifugation. Compounds of the supernatant reaction mixture possessing a primary amino group were derivatized with

o-phthalaldehyde (OPA), separated with reverse-phase high performance liquid chromatography (HPLC) and detected fluorometrically. The OPA-derivative of the enzymatically produced tyrosine was identified by comparison with authentic tyrosine also derivatized with OPA and applied on the same column.

Analytical techniques

Protein concentrations were estimated by the method of Bradford [6].

Purification procedures

All procedures were performed at 4 °C and K-phosphate buffer pH 7.5 with 20% glycerol, 0.5 mM EDTA and 0.5 mM dithioerythritol was used unless otherwise stated. 70 g of wet cell paste of *Actinoplanes missouriensis* was suspended in 90 ml 20 mM buffer and disrupted by ultrasonification (Branson sonifier). The cell debris was removed by centrifugation at $20,000 \times g$ for 1 h.

DEAE-cellulose chromatography: Crude extract was applied on a 2.5×20 cm DE-52 (Whatman) ion exchange column previously equilibrated with 20 mM buffer. The column was washed thoroughly with 20 mM buffer followed with 100 mM buffer, the en-

zyme was eluted with 100 mM buffer containing 0.1 M NaCl. The active fractions were pooled (Fig. 1) and concentrated by an Amicon ultrafiltration cell with PM 10 membrane (10 ml).

Phenyl-Sepharose tandem-chromatography: The enzyme solution was placed on a column (2.5×5 cm) of Phenyl-Sepharose CL-4B equilibrated with 100 mM buffer and washed with 100 mM buffer. Arogenate dehydrogenase activity was found in the first 7 fractions (21 ml). To this enzyme solution was added ammonium sulfate to 30% saturation and applied on a second Phenyl-Sepharose CL-4B column (2.5×5 cm) equilibrated with 100 mM buffer plus 30% ammonium sulfate.

The column was washed with the same buffer and the enzyme was eluted with 200 ml of a linear gradient of buffer with ammonium sulfate 30−0% saturation (Fig. 2). The active fractions were concentrated with an Amicon ultrafiltration unit and washed twice with 20 mM buffer.

FPLC-Mono Q: The enzyme solution was applied on a Mono Q HR 5/5 anion exchange column equilibrated with 20 mM buffer. The column was equipped with a Pharmacia fast-protein liquid chromatography

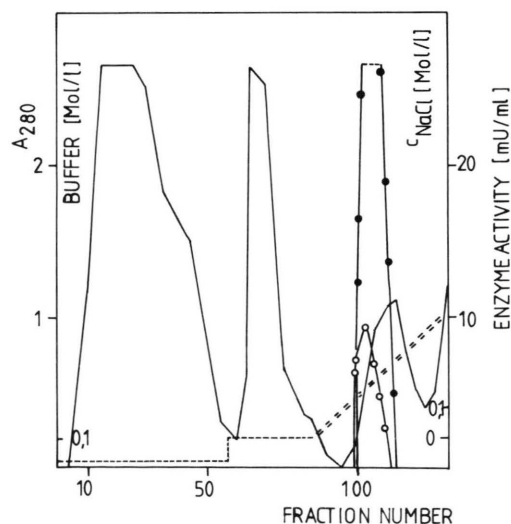Fig. 1. Chromatography of a crude extract of *Actinoplanes missouriensis* on DEAE-cellulose (DE-52 Whatman). Protein (——), buffer-gradient (----), NaCl-salt gradient (====), arogenate dehydrogenase (O—O—O), malate dehydrogenase (●—●—●).
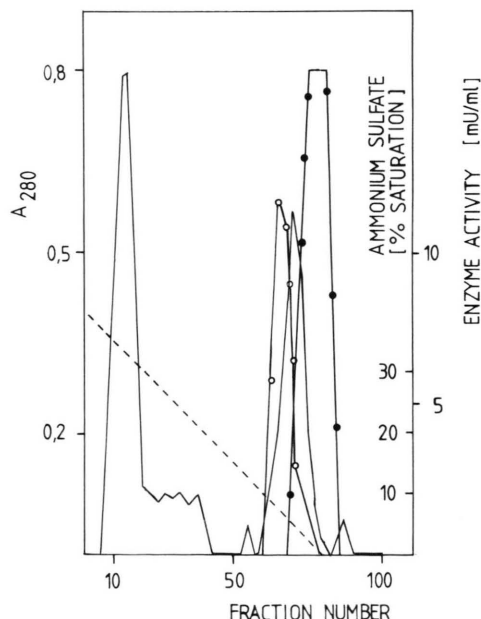

Fig. 2. Elution profile from Phenyl-Sepharose CL-4B chromatography. Protein (——), ammonium sulfate saturation (----), arogenate dehydrogenase (O—O—O), malate dehydrogenase (●—●—●).

system and developed at a flow rate of 1 ml/min with a gradient of NaCl (0–0.5 M) in 20 mM buffer. The enzyme was eluted with the buffer containing 0.2 M NaCl. The active fractions were combined and concentrated with an Amicon centricon 10 and washed twice with 50 mM buffer.

FPLC-Superose 12: The enzyme solution was applied on a Superose 12 HR 10/30 column equilibrated with 50 mM buffer. The column was equipped with a Pharmacia fast-protein liquid chromatography system and developed at a flow rate of 0.4 ml/min. The active fractions were combined, concentrated with an Amicon centricon 10 and stored at −20 °C.

Determination of molecular weight

Two chromatographic gels were applied:
a) a Superose 12 HR 10/30 column at a flow rate of 0.4 ml/min with FPLC at room temperature and an elution buffer consisting of 50 mM buffer and
b) a Sephadex G-100 (2.5 × 100 cm) column at 4 °C, at a flow rate of 6 ml/h and an elution buffer consisting of 100 mM buffer. The columns were calibrated with following proteins: γ-globuline (160,000 MG), bovine serum albumin (65,000 MG), ovalbumin (43,000 MG), α-chymotrypsinogen A (23,000 MG) and cytochrome C (13,000 MG).

Electrophoresis

Gel electrophoresis was performed according to system Nr. 6 of Maurer [7]. Protein was stained with Coomassie brillant blue G-250. Sodium dodecyl sulfate (SDS) electrophoresis was carried out as described in LKB application system 306.

Results

Purification of arogenate dehydrogenase, molecular weight and subunit structure

Purification of the enzyme resulted in a 354-fold enhancement of specific activity (Table I). Gel filtration of the purified enzyme on columns of Superose 12 (FPLC) and Sephadex G-100 yielded a single symmetrical peak of protein. The molecular weight was estimated to be 68,000.

When freshly purified enzyme preparations were electrophoresed on acrylamide and SDS-acrylamide gels, one major and a few minor bands of protein were detected. Activity staining of arogenate dehydrogenase was negative in a solution containing

Table I. Purification of arogenate dehydrogenase from *Actinoplanes missouriensis.*

	Total protein [mg]	Sp. act. [mU/mg]	Total activity [mU]	Yield [%]	Fold purification
Crude extract	860	–	–	–	
DEAE-Cellulose	80	13	1061	100	
Phenyl-Seph. 1	41	32	1370	129	2.5
Phenyl-Seph. 2	5.2	133	611	57	10
Mono Q	0.55	2057	520	49	158
Superose 12	0.1	4600	416	39	354

100 mM buffer pH 7.5 with 20% glycerol, 1 mM arogenate, 1 mM NAD, 0.3 mg/ml tetrazolium nitroblue and 0.05 mg/ml phenazine methosulfate. Probably, the enzyme was inactivated due to the absence of stabilizing glycerol during the electrophoresis run.

The molecular weight of the major band in SDS-gels was calculated to be 36.000, the minor bands were found to range between 32.000 and 42.000.

On the basis of data presented above, it was concluded that the protein is a dimer composed of two identical subunits.

Stability

Arogenate dehydrogenase was unstable, especially in the absence of glycerol: 90% of activity was lost when the preparation was allowed to stand for 7 days at 4 °C. Addition of 20% glycerol was found to stabilize the enzyme.

Effect of pH and temperature on enzyme activity

The enzyme showed a linear increase of activity from pH 6 to pH 8 in a 50 mM K-phosphate buffer supplemented with 20% glycerol. The maximum activity was found at pH 9.5 in a 50 mM glycine/NaOH buffer containing 20% glycerol (Fig. 3). All data of specific activities in this paper were estimated in 50 mM K-phosphate buffer pH 7.5 plus 20% glycerol. Glycine/NaOH buffer was avoided because glycine interfered in the OPA-HPLC-method.

Substrate specificity

Arogenate dehydrogenase is specific for NAD as cofactor and does not utilize NADP. The K_m values for NAD and arogenate are 0.2 mM and 0.15 mM, respectively.
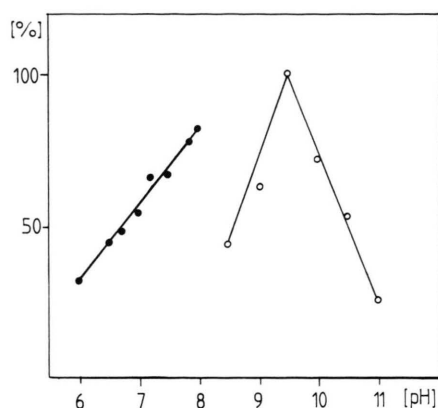
Fig. 3. Effect of pH on arogenate dehydrogenase of *Actinoplanes missouriensis*.
50 mM K-phosphate buffer and 20% glycerol (●—●—●),
50 mM glycine/NaOH buffer and 20% glycerol (○—○—○).

Prephenate, with a closely related structure to arogenate, was not used as substrate and had no inhibitory effect on arogenate dehydrogenase activity. L-Tyrosine, L-phenylalanine, L-tryptophan, phenylpyruvate and *p*-hydroxyphenylpyruvate (final concentration 1 mM) had no effect on arogenate dehydrogenase activity.

p-Hydroxymercuribenzoate was an effective inhibitor, 50% inhibition being achieved at 12.5 μM concentration.

Standard arogenate dehydrogenase assays with continuous spectrophotometric determination of NADH may be erroneous whenever arogenate preparations are contaminated with substrates of NAD-requiring dehydrogenases. Recently, we have found that *Neurospora crassa* accumulation media often contained as much malate as arogenate. Two anion exchange chromatography steps on Dowex-Cl additional to the isolation and purification method [4] were necessary to separate arogenate from malate. Malate was detected by enzymatic reaction with malate dehydrogenase of pig heart. Several control experiments were carried out with malate-free arogenate preparations, in order to rule out the possibility of a side reaction involving other impurities:

(i) Thin-layer chromatography and Ninhydrin test [4].

(ii) Acid conversion of arogenate to phenylalanine. Arogenate solutions that were treated with HCl, neutralized and included in reaction mixtures did not lead to a dehydrogenase activity, thus clearly

ruling out the presence of malate or any other possible substrate for a dehydrogenase. The dehydrogenase activities were lower than 0.5 mU/ml, calculated by the determination of formed NADH per minute (340 nm).

(iii) Quantitative analysis by HPLC indicated that the tyrosine produced by the enzymatic reaction was stoichiometrically equivalent to the NADH produced.

Discussion

Detection and purification of arogenate dehydrogenase is quite difficult, since arogenate preparations are often contaminated with impurities, in particular ketoacids of the aromatic amino acid biosynthesis. Prephenate [9], spiroarogenate [4], shikimic acid [9], prephenyllactate [10, 11], glutamate [4, 8] and recently malate were found to contaminate arogenate preparations. It is possible that malate is a product of malate dehydrogenase repression by glucose, since sucrose is added to the accumulation medium [12].

We found that malate and arogenate had similar chromatographic properties: they coeluted almost by anion exchange chromatography and gel filtration. Tests distinguishing between malate and arogenate and further purification steps were necessary for their separation.

All data on specific activities of arogenate dehydrogenases presented in the paper Hund *et al.* [2] express the specific activities of malate dehydrogenases, since at that time we had not detected contamination of arogenate with malate. For the same reason studies with arogenate [13–21] may be incorrect, a critical consideration being necessary.

We selected *Actinoplanes missouriensis*, one of the organisms presented in the paper of Hund *et al.* [2], to demonstrate arogenate dehydrogenase activity by detection of tyrosine, the product of reaction.

In this organism dialyzed crude extracts gave a positive arogenate dehydrogenase activity about 1 mU/mg by the OPA-HPLC method. This low activity could not be detected by measuring NADH formation at 340 nm due to high endogenous NADH-consumption. Malate dehydrogenase activity however was about 1.000 mU/mg and easily detectable. Arogenate and malate dehydrogenase of *Actinoplanes missouriensis* showed similar properties: the enzymes coeluted in anion exchange (Fig. 1) and gel chromatography, and the two enzymes could only be

separated by chromatography on Phenyl-Sepharose (Fig. 2). Purification of malate dehydrogenase of *Actinoplanes missouriensis* to homogeneity [22] was feasible because of the great quantity and high stability of this enzyme. Stability and amount of arogenate dehydrogenase, however, was considerably lower.

Detection of arogenate dehydrogenase and lack of prephenate dehydrogenase activity in extracts of *Actinoplanes missouriensis* indicate that the biosynthesis of tyrosine occurs *via* the arogenate route. The degree of specificity for arogenate and NAD is remarkable: prephenate, with a closely similar structure to arogenate, cannot be used as substrate and NAD cannot be replaced by NADP. The lack of regulatory properties (no inhibition of arogenate dehydrogenase by tyrosine) is consistent with the fact that the enzyme is not situated at a metabolic branch point (Fig. 4).
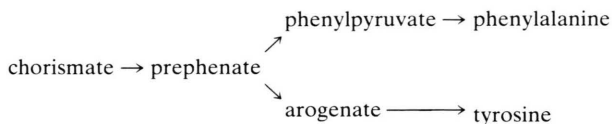
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                         phenylpyruvate → phenylalanine
                        ↗
chorismate → prephenate
                        ↘
                         arogenate ———————→ tyrosine
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Fig. 4. Phenylalanine and tyrosine pathway branchlets found in *Actinoplanes missouriensis* ([2], and this study).

Acknowledgements

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