Purification of arogenate dehydrogenase from *Phenylobacterium immobile*

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Phenylobacterium immobile, a bacterium which is able to degrade the herbicide chloridazon, utilizes for L-tyrosine synthesis arogenate as an obligatory intermediate which is converted in the final biosynthetic step by a dehydrogenase to tyrosine. This enzyme, the arogenate dehydrogenase, has been purified for the first time in a 5-step procedure to homogeneity as confirmed by electrophoresis. The M_r of the enzyme that consists of two identical subunits amounts to 69000 as established by gel electrophoresis after cross-linking the enzyme with dimethylsuberimidate. The K_m values were 0.09 mM for arogenate and 0.02mM for NAD^+. The enzyme has a high specificity with respect to its substrate arogenate.

L-Tyrosine biosynthesis *Arogenate* *Arogenate dehydrogenase* Phenylobacterium immobile

1. INTRODUCTION

Biosynthesis of the amino acid L-tyrosine is known to proceed not only via the 4-hydroxyphenylpyruvate branchlet, which has been studied in many eubacteria [1–3]. In some organisms, however, prephenate is transaminated directly to arogenate, an immediate precursor of L-tyrosine initially described in species of cyanobacteria [4]. A dehydrogenase finally converts this 'pre-tyrosine' to L-tyrosine. The enzyme patterns of aromatic amino acid biosynthesis in various examined organisms, especially in pseudomonads, have been used as indicators of microbial relatedness [5]. For this reason it is important to analyse the appropriate enzymes in detail. The arogenate dehydrogenase of rather few microorganisms is characterized only in a partially purified state [6–8], but purification to homogeneity has not yet been accomplished.

This paper concerns the purification of the arogenate dehydrogenase from *Phenylobacterium immobile*, a gram-negative species within a new genus; the bacterium is able to degrade the herbicide chloridazon [9]. The arogenate pathway to tyrosine is, as shown previously, obligatory in this bacterium [10].

Dedicated to Professor Karl Decker on the occasion of his 60th birthday

2. MATERIALS AND METHODS

2.1. *Reagents*

Arogenate (β-(1-carboxy-4-hydroxy-2,5-cyclohexadien-1-yl)alanine) was prepared from the culture supernatants of a triple mutant of *Neurospora crassa* ATCC 36373 [11]. The purification procedure was a modified method of Zamir et al. [12]. Arogenate concentrations were estimated fluorometrically after quantitative conversion to L-phenylalanine at acidic pH [13]. Prephenate was purified from the accumulation medium of the tyr 19 mutant of *Salmonella typhimurium* [14] in a modified procedure. Chorismic acid was prepared according to Gibson [15].

2.2. *Enzyme assays*

Arogenate dehydrogenase activity was assayed spectrophotometrically by following the NADH formation at 340 nm. The reaction mixture con-

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tained 2 mM NAD^+, 0.2–0.3 mM arogenate and 50 mM glycine/NaOH buffer, pH 9.5, to a final volume of 0.5 ml. The enzyme was incubated in test buffer at 37°C for 1 min and the test was started with arogenate after addition of NAD^+. The enzymatic activity exhibited proportionality with respect to the enzyme concentrations under all conditions of assay used, and is expressed as μmol product (NADH) formed/min per mg protein = U/mg. Chorismate mutase and prephenate dehydratase assays are described in [7]. Protein concentrations were either estimated by the method of Lowry et al. [16], or according to Kalckar as described by Layne [17].

2.3. *Bacterial strain, cell-free extracts*

P. immobile DSM 1986 strain E was cultured in a 200-l fermenter according to Buck et al. [18]. Antipyrine (1-phenyl-2,3-dimethylpyrazolinone) was employed as carbon source. To prepare a crude extract, 45 g bacteria (wet wt) were suspended in 100 ml of 10 mM K-phosphate buffer, pH 7.0 (buffer A), containing 1 mM $MgCl_2$ and DNase. The suspension was disrupted in a French pressure cell press at 16000 lb/inch^2, and cell debris was removed by centrifugation at 23000 × *g* and 4°C for 60–80 min. All purification steps were performed at 4°C.

2.4. *Purification of arogenate dehydrogenase*

2.4.1. DEAE-cellulose chromatography

The crude extract was diluted (1 : 1) in buffer A and loaded onto a column containing Whatman DE 52-cellulose (2.5 × 24 cm) previously equilibrated in the same buffer. The column was then washed until $A_{280} \leq 0.05$, and arogenate dehydrogenase was eluted at 0.1 M K-phosphate, pH 7.0, employing a stepwise gradient procedure. Fractions containing arogenate dehydrogenase activity were pooled and adjusted to a protein concentration of 2 mg/ml.

2.4.2. $(NH_4)_2SO_4$ fractionation

The resulting protein solution was fractionated by addition of solid $(NH_4)_2SO_4$. Arogenate dehydrogenase was discovered in the 35–80% precipitate, and was collected by centrifugation. The sediment was redissolved in 0.1 M K-phosphate buffer, pH 8.0 (buffer B), and finally dialysed for 17 h against the same buffer.

2.4.3. Gel permeation chromatography on Ultrogel AcA 44

After concentration with an Amicon ultrafiltration cell using a PM 10 membrane the solution was passed through a column of Ultrogel AcA 44 (5 × 88 cm), equilibrated in buffer B. Fractions containing arogenate dehydrogenase were combined and again concentrated as above.

2.4.4. Isoelectric focusing

A preparative isoelectric focusing run was performed according to the LKB application system 198 with a Sephadex G-200 SF layer in pH 4–6 carrier ampholytes obtained from Pharmacia. Gel strips were taken from the tablet with the aid of a fractionating grid and protein was eluted with buffer B. The fraction containing the highest dehydrogenase activity was concentrated.

2.4.5. Gel permeation chromatography on Sephacryl S-200

In a last step a second gel filtration was performed on a column of Sephacryl S-200 in buffer B. The enzyme fractions eluted from the column were pooled and dialysed for 20 h against 10 mM Na-phosphate buffer, pH 7.2, concentrated thereafter by vacuum dialysis and stored at −20°C.

2.5. *Gel electrophoresis*

Gel electrophoresis was carried out as in [19] in a 10% polyacrylamide gel at 5°C. Gels were either stained for protein using Serva blue R or for arogenate dehydrogenase activity [20]. Electrophoresis in the presence of SDS was performed as in [21] with 5 and 10% polyacrylamide gels. Standard proteins for M_r determination of subunits were: phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14400).

3. RESULTS AND DISCUSSION

Details on the purification of arogenate dehydrogenase are given in table 1. In the first purification step, prephenate dehydratase, an enzyme for the biosynthetic route to L-phenylalanine, is separated completely from arogenate dehydrogenase (not shown). Chorismate mutase is

Table 1

Purification of arogenate dehydrogenase from *Phenylobacterium immobile*

Step	Volume (ml)	Protein (mg/ml)	Activity (units/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purification (-fold)
Crude extract	85	15.3	18.09	1538	100	1.18	1
DE52 cellulose	57	5.0	23.8	1357	88	4.76	4
$(NH_4)_2SO_4$ precipitation	57	2.5	22.65	1291	84	9.1	8
Ultrogel AcA 44	1.8	16.1	346.0	692	45	21.5	18
IEF	2.2	–	106.7	235	15	–	–
Sephacryl S-200	6.1	0.17	16.24	99	6	95.5	81

detectable in the same fractions as the dehydrogenase until it is passed through the AcA 44 gel column (fig.1): the arogenate dehydrogenase from *P. immobile* is not complexed with any other terminal enzyme of the biosynthetic pathway to L-tyrosine or L-phenylalanine. Such complexes are described in various organisms, for example, the isoenzymes of chorismate mutase/prephenate dehydrogenase and chorismate mutase/prephenate dehydratase in *E. coli* [22]. In fact, the molecular size of arogenate dehydrogenase does not attribute a complex structure as for arogenate/prephenate dehydrogenase in *Pseudomonas aeruginosa* [7].

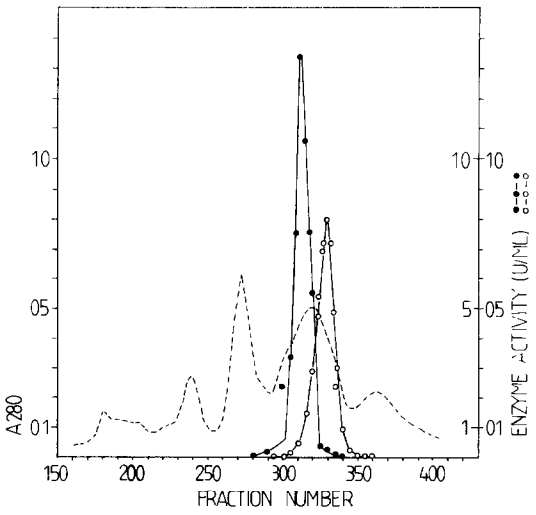

Fig.1. Elution profile from Ultrogel AcA 44 gel filtration protein (– – –), arogenate dehydrogenase (●—●), chorismate mutase (○—○). Both enzymatic activities are expressed in μmol/min. Fractions of 3.5 ml were collected.

Preparative isoelectric focusing is an important purification step in the presented procedure although it is related to a relatively high loss in enzymatic activity. Homogeneous enzyme, as judged by polyacrylamide gel electrophoresis under native conditions (fig.2) and in the presence of SDS (fig.3) was obtained only in 6% overall

Fig.2. Purification of arogenate dehydrogenase from *Phenylobacterium immobile*. Lanes: (A) crude extract, (B) DE 52 cellulose column, (C) $(NH_4)_2SO_4$ precipitation, (D) enzyme eluted from Ultrogel AcA 44 column, (E) enzyme eluted from Sephacryl S-200 after IEF.

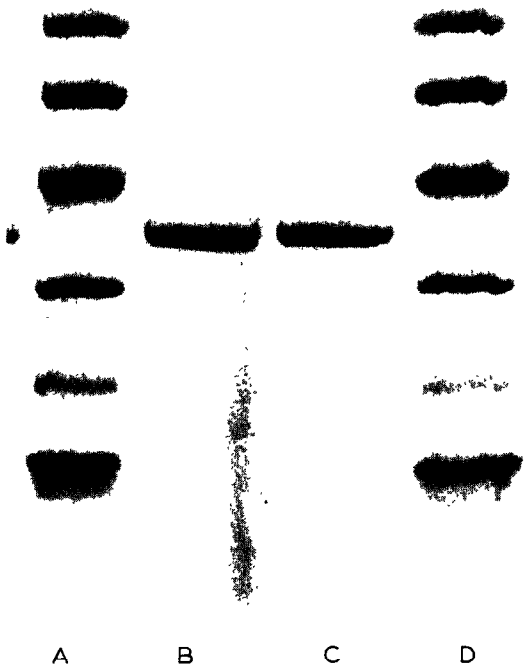

Fig.3. SDS-polyacrylamide gel run of a homogeneous arogenate dehydrogenase preparation.

Table 2

Amino acid composition of a subunit of arogenate dehydrogenase from *Phenylobacterium immobile*

Amino acid	Residues per mol subunit
Aspartic acid and asparagine	45.7
Threonine	15.6
Serine	20.8
Glutamic acid and glutamine	41.9
Proline	17.9
Glycine	34.7
Alanine	47.7
Valine	19.5
Methionine	1.9
Isoleucine	16.7
Leucine	29.1
Tyrosine	6.0
Phenylalanine	11.7
Histidine	4.2
Lysine	22.4
Arginine	16.2
Tryptophan	5.4

Tryptophan and cysteine are not detectable by the determination method used. Tryptophan was estimated by differential UV spectroscopy at 280 and 288 nm

yield; the overall purification factor was approx. 80. A polyacrylamide gel showing the protein components at each purification state is presented in fig.2. The second gel permeation chromatography on Sephacryl S-200 was used to remove the carrier ampholyte from the protein fraction and to estimate the native M_r of the arogenate dehydrogenase. The column was calibrated with the following proteins as M_r standards: bovine serum albumin (M_r 67000), ovalbumin (M_r 45000), chymotrypsinogen (M_r 25000) and cytochrome *c* (M_r 13500). The void volume was determined with ferritin. The M_r of arogenate dehydrogenase was estimated at 61700 by this procedure. The enzyme consists of two identical subunits with M_r 37700 corresponding to the protein standards cited in section 2. This dimeric structure is confirmed by another method for determination of the native M_r values of proteins: the cross-linking with dimethylsuberimidate (DMSI) [23]. On a 5% SDS-polyacrylamide gel only two protein bands are detected with M_r values corresponding to monomer and dimer: 38400 ± 700 and 68500 ± 1100, respectively, determined by gel electrophoresis. Comparable data from other arogenate dehydrogenases are for example the M_r of the enzyme from *Hansenula henricii*, 50000 [8]. In coryneform bacteria different M_r values were obtained for the enzyme: 68000 in *Brevibacterium ammoniagenes* and 158000 in *B. flavum* and *Corynebacterium flavum* [6].

The pH optimum found for arogenate dehydrogenase was 9.5 in 50 mM glycine/NaOH buffer. The value for the isoelectric point of p*I* 4.8 resulted from an analytical electrofocusing run. With respect to kinetic data and substrate specificity there are no remarkable differences between the enzymes described previously [6–8] and arogenate dehydrogenase studied here. The K_m values obtained were 0.091 mM for arogenate and 0.02 mM for NAD^+. Arogenate dehydrogenase is a quite specific enzyme. Even prephenate, with a closely similar structure to arogenate, cannot be used as substrate. Apparently, the amino function at the α-C atom of arogenate instead of the keto function of prephenate is involved in the reaction at the active site of the enzyme, but prephenate is bound to

the enzyme as shown by its inhibitory effect to the arogenate reaction.

The result of an amino acid analysis is presented in table 2.

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