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PURIFICATION, PROPERTIES AND FORMATION OF ARGININE-α-KETOGLUTARATE TRANSAMINASE IN *ARTHROBACTER SIMPLEX*

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Summary

Arginine-α-ketoglutarate transaminase was purified 460-fold with 1.4% yield from *Arthrobacter simplex* grown on arginine as a carbon source. The preparation was more than 90% pure on polyacrylamide gel electrophoresis, and the molecular weight of the enzyme was calculated to be 110 000. The enzyme exhibited absorption maxima at 280, 330 and 370 nm. The 370 nm peak decreased with increase in the 330 nm peak on addition of arginine. K_m values for arginine, α-ketoglutarate, glutamate and α-keto-δ-guanidinovalerate were 2.9, 8.1, 25 and 0.30 mM, respectively. Those for pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were 0.25 and 0.57 μM. The enzyme reacted optimally at pH 8.0—8.5. The synthesis of arginine-α-ketoglutarate transaminase was inducible by arginine and α-keto-δ-guanidinovalerate.

Introduction

In the preceding paper [1], we reported a new catabolic pathway of arginine via α-keto-δ-guanidinovalerate and α-keto-δ-aminovalerate in *A. simplex*. Though the pathway resembles that in *Pseudomonas putida*, the data suggested that the conversion of arginine to α-keto-δ-guanidinovalerate proceeds via a transamination with α-ketoglutarate, whereas in *P. putida* arginine is deaminated by an arginine oxidase [2—4]. The degradation of α-keto-δ-guanidino-

Amino acids employed were L-isomers unless otherwise stated.

valerate also differed between these strains. Hitherto, transamination has been considered to play no role in arginine metabolism.

In this paper, we describe the purification, properties and formation of arginine-α-ketoglutarate transaminase in *A. simplex*.

Methods and Materials

Enzyme assay. The assay system for the forward (glutamate-forming) reaction consisted of 10 mM arginine, 10 mM α-ketoglutarate, 0.1 mM pyridoxal 5′-phosphate, 0.1 M Tris-HCl buffer (pH 8.3) and the enzyme preparation. For the reverse (arginine-forming) reaction, 10 mM glutamate and 10 mM α-keto-δ-guanidinovalerate were used as substrates. The reaction was initiated by addition of the enzyme. After incubation at 30°C, the reaction was terminated by immersing the reaction mixture in boiling water for 3 min, followed by centrifugation at 2270 × *g* for 10 min.

Formation of glutamate was assayed by ninhydrin colorimetry after paper chromatography [1]. α-Ketoglutarate was determined spectrophotometrically with glutamate dehydrogenase [5]. α-Keto-δ-guanidinovalerate in the reaction mixture exhibited no effect on the determination of α-ketoglutarate with glutamate dehydrogenase.

Protein was determined by the method of Lowry et al. [6].

Reagents. α-Keto-δ-guanidinovalerate was prepared by the method described previously [7]. Glutamate dehydrogenase was supplied by Sigma Chemical Co. Ltd. Other reagents were commercial products.

Enzyme purification. *A. simplex* IFO 12069 was grown at 28°C for 22 h in a 30 l jar fermentor containing 25 l of culture medium. Aeration and agitation rates were 25 l/min and 120 rev./min, respectively. The medium consisted of 1% arginine, 0.2% $(NH_4)_2SO_4$, 0.2% $(NH_4)_2HPO_4$, 0.05% $MgSO_4$ and 0.05% yeast extract in tap water (pH 7.0). The cells obtained from 100 l of broth were suspended in 0.01 M potassium phosphate buffer (pH 7.5) containing 0.1 mM α-ketoglutarate and 0.01% 2-mercaptoethanol. All buffers used for purification contained these reagents unless otherwise stated. All operations were carried out at 0—10°C.

(1) The cell suspension was disrupted in a Dyno-Mill, then centrifuged for 20 min at 10 000 × *g*. (2) 10 g of solid ammonium phosphate (dibasic) per 100 ml of supernatant (52.3 g protein) were added gradually, and the pH of the solution was maintained at 7.5 with dilute phosphoric acid. The solution was stirred for 1 h, then centrifuged (10 000 × *g*, 20 min). A further 20 g ammonium phosphate was added per 100 ml of the supernatant and the mixture was centrifuged. The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.5) and dialyzed against the buffer. (3) The dialysate was charged on a column of DEAE-cellulose (6.2 × 30 cm) equilibrated with the same buffer. After washing, the column was chromatographed with buffer and NaCl. Arginine-α-ketoglutarate transaminase was eluted with the buffer containing 0.24 M NaCl. (4) The active fractions were combined and concentrated with ammonium phosphate (40 g/100 ml). (5) The precipitate was dissolved in and dialyzed against the buffer and the dialysate was chromatographed on DEAE-Sephadex A-50 (column size: 2.6 × 36 cm) by gradient elution with NaCl in the

buffer, at a flow rate of 20 ml/h. The enzyme activity was eluted at 0.2—0.25 M NaCl. (6) The active fractions were concentrated with ammonium phosphate and dissolved in a small volume of the buffer. The solution was filtered through a column of Sephadex G-150 (2.4 × 103 cm). (7) The enzyme fractions obtained by gel filtration were combined and dialyzed against 0.001 M potassium phosphate buffer (pH 7.5), then subjected to a final chromatography on hydroxyapatite (0.9 × 5 cm). The enzyme was eluted with 0.005 M buffer. The eluate was concentrated to a small volume in a mechanical membrane concentrator.

Enzyme formation. Various carbon sources were substituted for arginine in the medium and their effect on the enzyme activity was investigated. The cultivation was carried out at 28°C for 20—22 h with 100 ml of culture medium in a 500-ml flask on a shaker. The cells were harvested, washed twice with cold saline and resuspended in 0.01 M potassium phosphate buffer (pH 7.5). Cell-free extracts obtained by the method described previously [1] were used as enzyme preparations.

To examine enzyme induction, cells were grown on glycerol as a carbon source for 18 h, collected, washed twice with cold saline, and resuspended at a concentration of 0.35 mg dry cells/ml in the induction medium (0.1 M carbon source, 10 mM potassium phosphate buffer (pH 7.5), 50 μM each of $FeSO_4$, $MgSO_4$ and 1 μM each of adenine, guanine, uracil and cytosine). Mixtures (10 ml in the test tubes, 25 mm diameter) were incubated at 28°C for 6.5 h with shaking. Under these conditions growth did not occur and any increase in enzyme activity was due to the de novo synthesis of the enzyme protein. After incubation, the cells were resuspended in 0.01 M potassium phosphate buffer (pH 7.5), then lysed with toluene at 37°C for 30 min and used as enzyme preparations.

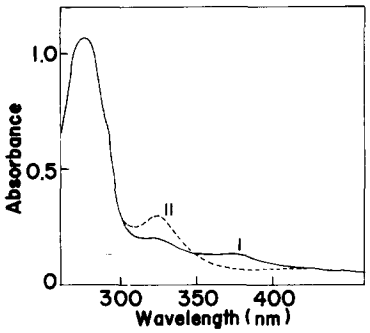
Results and Discussion

Enzyme purification. The enzyme was purified approx. 460-fold from the cell-free extracts with an overall yield of 1.4%. The final preparation appeared to be more than 90% pure on polyacrylamide gel electrophoresis. The molecular weight of arginine-α-ketoglutarate transaminase was calculated to be about 110 000 by gel filtration on Sephadex G-150.

Absorption spectrum. The enzyme solution dialyzed against 0.01 M potassium phosphate buffer (pH 7.5) without α-ketoglutarate and pyridoxal 5′-phosphate showed absorption maxima at 280, 330 and 370 nm (Fig. 1). No spectral change was observed when pH was varied from 5.5 to 9.0. The addition of arginine (4.2 mM) decreased the absorbance at 370 nm and increased it at 330 nm. According to knowledge about vitamin B_6 enzymes in general, the 370 nm peak is derived from the coenzyme bound to the ϵ-amino group of lysine residue of the enzyme protein and the absorption shift on addition of arginine reflects the conversion of pyridoxal 5′-phosphate to pyridoxamine 5′-phosphate [8].

Enzymatic properties. Arginine-α-ketoglutarate transaminase was most active in the pH range 8.0—8.5 with potassium phosphate and Tris-HCl buffers (Fig. 2).

When it was heated in 0.01 M potassium phosphate buffer (pH 6.5) at 50°C



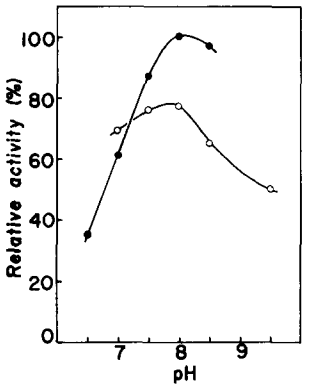


Fig. 1. Absorption spectrum of arginine-α-ketoglutarate transaminase. Curve I, the enzyme solution (approx. 0.7 mg/ml protein) dialyzed against 0.01 M potassium phosphate buffer (pH 7.5). Curve II, the enzyme solution incubated with 4.2 mM arginine for 5 min at room temperature. The spectra are taken against the buffer used for dialysis.

Fig. 2. Effect of pH on the enzyme activity. Solutions containing 4 μg/ml of enzyme were incubated for 10 min. ●———●, potassium phosphate buffer; ○———○, Tris-HCl buffer.

for 10 min, 30% of the native activity was lost. Addition of 0.1 mM α-ketoglutarate prevented the inactivation but arginine did not.

Table I shows the substrate specificity of the enzyme. Slight activities (less than 5% of that observed toward arginine) were observed toward ornithine, glutamine, leucine, methionine, phenylalanine and tryptophan. Glycine, valine, isoleucine, asparagine, serine, threonine, tyrosine, lysine, histidine, cysteine, proline, homocitrulline, D-asparagine, glyoxylate, hydroxypyruvate, α-ketobutyrate and α-ketoisocaproate were not effective as amino donors and acceptors. Of three vitamers (pyridoxal, pyridoxamine, pyridoxine) and their phosphorylated forms, only pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate were effective as coenzymes. K_m values are summarized in Table II. V for the forward and reverse reactions under the conditions used were 1.25 and 0.80 mM/15 min, respectively. K_{eq} was calculated to be 1.3 by the method of Velick and Vavra [9].

The enzyme was inhibited by some carbonyl- and SH-reagents. Phenylhydrazine, *p*-chloromercuribenzoate and $HgCl_2$ (each at 1 mM) decreased the activity to 15, 4 and 22% of the full activity.

TABLE I

SUBSTRATE SPECIFICITY OF ARGININE-α-KETOGLUTARATE TRANSAMINASE

A. α-Ketoglutarate is used as amino acceptor. B. Arginine is used as amino donor.

Substrate	Relative activity (%)
A. Arginine	100
Nitroarginine	22.5
Citrulline	15.8
Alanine	9.5
B. α-Ketoglutarate	100
Pyruvate (100 mM)	12

TABLE II

K_m VALUES FOR SUBSTRATES AND COENZYMES

K_m values were measured under the standard assay conditions. A. A solution of 3 μg/ml of enzyme was incubated for 15 min. K_m values were determined according to Velick and Vavra [9]. B. The enzyme was pretreated with 1 mM phenylhydrazine at 30°C for 20 min, then dialyzed against 0.01 M potassium phosphate buffer (pH 7.5) containing 0.01% 2-mercaptoethanol. The preparation had about 50% of the full activity without added pyridoxal 5'-phosphate. 80% of the activity lost was restored when pyridoxal 5'-phosphate was added.

Substrate or coenzyme	K_m
A. Arginine	2.9 mM
α-Ketoglutarate	8.1 mM
Glutamate	25 mM
α-keto-δ-guanidinovalerate	0.3 mM
B. Pyridoxal 5'-phosphate	0.25 μM
Pyridoxamine 5'-phosphate	0.57 μM

TABLE III

EFFECT OF CARBON SOURCES IN THE CULTURE MEDIA ON THE TRANSAMINASE ACTIVITY

Cells grown on peptone-meat extract-NaCl (1, 1, 0.5%) medium for 18 h were used as inoculum. Enzyme activities are shown as μmol of glutamate formed/mg protein of cell-free extract per h.

Carbon source in culture medium	Enzyme activity
A. Arginine (1%)	9.72
Arginine (1%) + glucose (1%)	4.88
Glucose (1%)	0.68
B. Arginine (1%)	9.22
Arginine (1%) + glycerol (1%)	4.46
Glycerol (1%)	2.28

Enzyme formation. Table III shows the enzyme activity in cells grown on various carbon sources. The activity was 5—10-times higher in arginine-grown cells than in glucose- or glycerol-grown cells. Addition of glucose or glycerol to arginine medium reduced the enzyme activity. The results in Table IV indicate that arginine and α-keto-δ-guanidinovalerate were the most effective inducers of the enzyme. γ-Guanidinobutyrate also showed a positive effect on the enzyme formation, which might be due to its structural similarity to the true inducers. The negative effect of glucose was reconfirmed in this experiment

TABLE IV

INDUCIBLE FORMATION OF THE TRANSAMINASE

Enzyme activities are shown as μmol of glutamate formed/mg cells per h.

Carbon source in induction medium	Enzyme activity
Arginine	8.93
α-Keto-δ-guanidinovalerate	7.35
γ-Guanidinobutyrate	6.05
Agmatine	0.91
Glucose	0.72

(data not shown). These facts suggest that the transaminase participates in some way in arginine metabolism of *A. simplex*.

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