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PURIFICATION AND SOME PROPERTIES OF HYDROXYPYRUVATE ISOMERASE OF *BACILLUS FASTIDIOSUS*

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Summary

Hydroxypyruvate isomerase of *Bacillus fastidiosus* is a novel enzyme (Braun, W. and Kaltwasser, H. (1979) Arch. Microbiol. 121, 129—134) which catalyzes the reversible conversion of tartronate semialdehyde into hydroxypyruvate.

The enzyme was purified to homogeneity. The native molecule had a molecular weight of 265 000—280 000 and was composed of six subunits with a molecular weight of 45 000.

The enzyme showed optimal activity at pH 6.6—7.4 and 57°C. Hydroxypyruvate isomerase is stable on heating for 10 min at 67°C.

The enzyme appeared to be specific for tartronate semialdehyde and hydroxypyruvate and no cofactors were involved in the reaction. The equilibrium constant

$$K = \frac{[\text{tartronate semialdehyde}]}{[\text{hydroxypyruvate}]}$$

was found to be 2.5 at pH 7.1, and 30°C.

Introduction

Bacillus fastidiosus is a microorganism which can use only urate, allantoin or allantoate for growth [1,2]. All strains of *B. fastidiosus* studied so far decompose urate along the same catabolic pathway yielding glyoxylate, ammonia and urea; the latter is further split into ammonia and carbon dioxide if the strain is urease-positive [2,3]. The conversion of glyoxylate was studied by Braun and Kaltwasser [4]. They observed that glyoxylate was first converted into

tartronate semialdehyde by glyoxylate carboligase (glyoxylate carboxy-ligase (dimerizing), EC 4.1.1.47), followed by an enzymatic isomerization of this compound to hydroxypyruvate which in turn was converted to pyruvate via serine.

$$CHOCHOHCOO^- \rightleftharpoons CH_2OHCOCOO^-$$

tartronate semialdehyde hydroxypyruvate

A conversion of tartronate semialdehyde into hydroxypyruvate was earlier observed with cell-free extracts of *Escherichia coli* [5] but was not further investigated.

In this communication we describe the purification and some properties of the enzyme which catalyzes the reversible isomerization of tartronate semialdehyde and hydroxypyruvate in *B. fastidiosus*.

Materials and Methods

Bacterial strains and growth conditions. Bacillus fastidiosus DSM 83 was obtained from Dr. Kaltwasser, Saarbrücken, F.R.G. The organism was grown on an allantoin medium as described before [2]. *Escherichia coli* B was grown on glycolate as described by Krakow et al. [5].

Preparation of cell-free extracts. Cells were harvested by centrifugation and washed twice with 0.13 M phosphate buffer (pH 7.1). The cells were suspended in 30 ml of this buffer and the cell suspension (absorbance at 600 nm was 17) was disrupted by sonication at 0°C for a total of 4.5 min in a MSE 150 W ultrasonic disintegrator at maximum output with intermittent cooling. The broken cell suspension was centrifuged at 100000 × *g* for 30 min at 4°C. The supernatant fraction (crude extract) was stored at −20°C.

Enzyme assays. Hydroxypyruvate isomerase activity was measured at 30°C in incubation mixtures which contained per ml, 130 μmol phosphate buffer (pH 7.1)/12 μmol hydroxypyruvate/0.1—10 μg protein dependent on the purity of the enzyme preparation. Initially incubation and sampling procedures were carried out under strictly anaerobic conditions (N_2 atmosphere) as used for methanogens [6], since it was shown that both hydroxypyruvate and tartronate semialdehyde can be oxidized nonenzymically [7]. However, the initial reaction velocity and the spectral properties of the 2,4-dinitrophenylhydrazone formed were identical both under aerobic and anaerobic conditions during the first 10 min incubation. Therefore, anaerobic conditions were applied only when longer incubation periods were needed. The specific activity is defined as μmol hydroxypyruvate converted (or μmol tartronate semialdehyde formed) per min per mg protein. Protein was determined by the method of Lowry et al. [8] using bovine serum albumin as standard.

Glyoxylate carboligase was assayed at 30°C under anaerobic conditions [5]. The disappearance of glyoxylate was followed [9] and the tartronate semialdehyde formed did not interfere in the assay method [10]. One unit of carboligase is defined as the amount which catalyzes the conversion of 2 μmol glyoxylate per min in 1 μmol tartronate semialdehyde.

Hydroxypyruvate reductase was isolated from parsley leaves and assayed as

described before [11,12]. The enzyme preparation did not show any activity with tartronate semialdehyde as substrate.

Purification of hydroxypyruvate isomerase. Crude extract (5 ml containing 26.2 mg protein) was heated for 6 min at 67°C in thin-walled preheated glass tubes and the precipitate was removed by centrifugation for 10 min at 28 000 × *g*. The supernatant fraction was applied to a column (39 × 1.6 cm) of DEAE-cellulose (Whatman DE-52) which had been equilibrated with 0.2 M NaCl in 13 mM phosphate buffer (pH 7.1). The protein was eluted at 4°C with a linear gradient of NaCl (0.2—0.5 M) in 13 mM phosphate buffer (pH 7.1). The isomerase was eluted between 0.24 and 0.26 M NaCl. The active fractions (126 ml) were pooled, dialyzed for 18 h at 4°C against 3 l 2 mM phosphate buffer (pH 7.1) and applied at 4°C to a column (8.5 × 1.5 cm) of hydroxyapatite (Serva) equilibrated with the same buffer. The isomerase was eluted with 2 mM phosphate buffer (pH 7.1), while contaminating proteins remained bound to the hydroxyapatite.

Preparation of tartronate semialdehyde. Tartronate semialdehyde was prepared enzymatically from glyoxylate with *E. coli* glyoxylate carboligase. About 85% of the glyoxylate was converted. The *E. coli* enzyme was used, since the specific activity of the *B. fastidiosus* enzyme was about 2 times lower in crude extracts (specific activity 0.09) and all activity was lost during purification. Glyoxylate carboligase from *E. coli* was purified 40-fold (specific activity 8) by the procedure of Gupta et al. [13]. This enzyme preparation was devoid of hydroxypyruvate isomerase activity.

Preparation of tartronate semialdehyde bis-2,4-dinitrophenylhydrazone. Tartronate semialdehyde was prepared enzymatically from hydroxypyruvate by the action of hydroxypyruvate isomerase in an incubation mixture containing per ml, 130 μmol phosphate buffer (pH 7.1)/31 μmol hydroxypyruvate/7 μg protein (crude extract). After 5 min incubation at 30°C, 10 ml 0.012% 2,4-dinitrophenylhydrazine in 2 N HCl was added. After 5 min standing at 30°C the orange-red bishydrazone crystals formed were separated by centrifugation for 2 min at 50 000 × *g*. The crystals were resuspended in 2 N HCl, washed three times with 2 N HCl and 2 times with glass-distilled water. After this washing procedure the wash fluid was colorless. The hydrazone preparation was neither contaminated with the hydrazone of hydroxypyruvate, since this compound did not precipitate under the conditions used, nor with the bishydrazone of glycolaldehyde formed as a result of nonenzymatic decarboxylation of tartronate semialdehyde [7]. The latter was evidenced from the following observations: (i) under the conditions used only about 4% of the tartronate semialdehyde was decarboxylated to glycolaldehyde; (ii) the conditions used for the preparation of 2,4-dinitrophenylhydrazone derivatives did not result in the formation of the bisderivative of glycolaldehyde (λ_{max} = 580 nm) but instead a compound with a λ_{max} of 445 nm was formed, which was unstable and did not separate from the solution even after several hours; (iii) crystals of the bisderivative of glycolaldehyde, prepared according to Dagley et al. [14] were formed after 1 h standing at 30°C. The absorption spectrum of the tartronate semialdehyde hydrazone in alkali showed a maximum at 565 nm, indicative of a bisderivative.

Determination of hydroxypyruvate and tartronate semialdehyde. Hydroxy-

pyruvate and tartronate semialdehyde were measured as their 2,4-dinitrophenylhydrazone derivatives [15]. The hydrazone of hydroxypyruvate (ϵ_{445} = $1.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) shows 42% of its maximal absorbance at 565 nm, while the bishydrazone of tartronate semialdehyde ($\epsilon_{565} = 3.5 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) shows 28% of its maximal absorbance at 445 nm. In mixtures containing both hydroxypyruvate and tartronate semialdehyde each compound can be determined by the use of the above-mentioned data. The validity of this method was proven by determination of hydroxypyruvate with the use of hydroxypyruvate reductase, a good agreement in the amount of hydroxypyruvate present was observed between the two methods.

Determination of glycolaldehyde. Glycolaldehyde was measured colorimetrically at 500 nm by a modification of the naphtoresorcinol method of Dickens and Williamson [12]: 2,7-dihydroxynaphthalene instead of 1,3-dihydroxynaphthalene was used and the assay was carried out at room temperature. The advantage of this modification was that both hydroxypyruvate and tartronate semialdehyde did not interfere, while the sensitivity remained about equal.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed according to the method of Davis [16]. Sodium dodecyl sulphate gel electrophoresis was performed according to Weber and Osborn [17]. Protein was stained with Coomassie blue.

Molecular weight determination. Columns of Sephadex G-200 (100 × 2.4 cm) and Sepharose B (39 × 1.6 cm) equilibrated and eluted with 0.13 M phosphate buffer (pH 7.1) were used to estimate the molecular weight of the native enzyme. Markers were urease (Sigma) (M_r 480 000); catalase (Boehringer) (M_r 240 000); alcohol dehydrogenase (Boehringer) (M_r 150 000); bovine serum albumin (Merck) (M_r 68 000) and cytochrome *c* (Boehringer) (M_r 12 500). The molecular weight of the subunits was determined by means of sodium dodecyl sulphate gel electrophoresis. Markers were bovine serum albumin (M_r subunits 68 000); glutamate dehydrogenase (Boehringer) (M_r subunits 53 000); aldolase (Boehringer) (M_r subunits 40 000) and ribonuclease A (Boehringer) (M_r subunits 13 700).

Chemicals. Hydroxypyruvate and phosphohydroxypyruvate were obtained from Sigma Chemical Co., St. Louis, MO. Other chemicals were purchased from Merck A.G., Darmstadt, F.R.G.

Results

Purification and properties of hydroxypyruvate isomerase

Hydroxypyruvate isomerase of *B. fastidiosus* was purified 117-fold by means of heat treatment, DEAE-cellulose and hydroxyapatite chromatography (Table I). A marked increase of activity was observed after heat treatment (see below).

The purified enzyme exhibited only one protein band on gel electrophoresis (Fig. 1).

The molecular weight was determined by means of Sephadex G-200 and Sepharose 6B gel filtration and found to be 265 000—280 000. Gel electrophoresis in the presence of sodium dodecyl sulphate showed the presence of identical subunits having a molecular weight of 45 000 (Fig. 1). The native

TABLE I

PURIFICATION OF HYDROXYPYRUVATE ISOMERASE FROM *B. FASTIDIOSUS*

Purification step	Activity (units)	Protein (mg)	Specific activity (units/mg protein)	Recovery (%)	Purification (-fold)
Crude extract	337.5	12.6	26.8	100	1
Heat treatment at 67°C	1340	6.2	216	397	8.1
DEAE-cellulose chromatography	970	0.85	1141	288	42.6
Hydroxyapatite chromatography	848	0.27	3125	251	117

enzyme therefore is composed of six identical subunits.

The purified enzyme could be stored at 4°C for 1 month without loss of activity. Most of the activity was lost be vacuum dialysis at 4°C or by freezing.

Enzymatic characterization of hydroxypyruvate isomerase

Table II shows that the enzyme catalyzes the stoichiometric conversion of

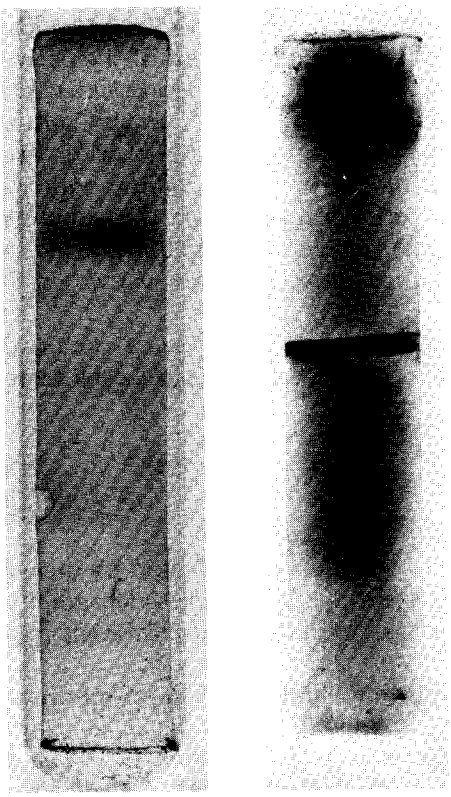

Fig. 1. Gel electrophoresis of purified hydroxypyruvate isomerase. Left: 7.5% polyacrylamide gel after electrophoresis in 0.1 M glycine adjusted to pH 8.4 with Tris. Right: 8% gel in the presence of 0.1% sodium dodecyl sulphate. Protein was 50 μg. Migration was from top to bottom.

TABLE II

ENZYMATIC CONVERSION OF HYDROXYPYRUVATE BY PURIFIED HYDROXYPYRUVATE ISOMERASE

The incubation mixture contained 390 μmol phosphate buffer (pH 7.1)/44 μmol hydroxypyruvate/3 μg purified hydroxypyruvate isomerase. Incubation was at 30°C under anaerobic conditions. At the times indicated, samples were taken and analyzed for the amount of hydroxypyruvate, tartronate semialdehyde and glycolaldehyde present. Hydroxypyruvate was measured both by 2,4-dinitrophenylhydrazone formation (1) and hydroxypyruvate reductase (2).

Incubation time (min)	Hydroxypyruvate converted (μmol)		Tartronate semialdehyde formed (μmol)	Glycolaldehyde formed (μmol)
	(1)	(2)		
0	0	0	0	0
0.5	4.4	4.6	4.3	0.3
2.5	8.4	8.7	7.9	0.4
5	13	12.9	12.2	0.8
10	18.9	19.2	17.2	1.1

hydroxypyruvate into tartronate semialdehyde. Part of the semialdehyde formed was decarboxylated nonenzymically to glycolaldehyde [7]. The equilibrium of the reaction was determined by using both hydroxpyruvate and tartronate semialdehyde as substrate; in the latter case glycolaldehyde and glyoxylate were present as a result of the preparation method of the semialdehyde, but these compounds did not interfere with the equilibrium position. The equilibrium constant

$$K = \frac{[\text{tartronate semialdehyde}]}{[\text{hydroxypyruvate}]}$$

was found to be 2.5 at pH 7.1 and 30°C.

The enzymatic activity is optimal at pH 6.6—7.4. Half optimal activities were observed at pH 5.4 and 8.5. No effect of buffer ions (citrate, phosphate, Tris, glycine, carbonate) on the reaction rate was observed. The optimal temperature for the enzymatic activity was found to be 57°C and the activation energy was 36.8 kJ. The purified enzyme was heat-stable for 10 min at temperatures up to 67°C. When crude enzyme preparations were treated in a similar way an increase of activity was observed which was optimal after 6 min; the activity was enhanced 3.9-fold as compared to the untreated enzyme preparation.

The apparent K_m value was 10 mM hydroxypyruvate. Since the tartronate semialdehyde preparations always contained some glyoxylate and decarboxylation of the semialdehyde during the reaction course could not be prevented, no attempt was made to determine the K_m value for tartronate semialdehyde. The enzymatic reaction was not inhibited by the glyoxylate present in the tartronate semialdehyde preparation, since glyoxylate (10 mM) did not inhibit the reaction rate in the standard assay.

The enzymatic activity was not stimulated by the addition of 0.5 mM pyridoxyl 5'-phosphate, 1 mM FAD and FMN or 20 mM NAD and ATP to the incubation mixture.

Metal ions were not required for enzymatic activity; activity was tested in a

N-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid buffer (pH 7.1). No effect was exerted by Co^{2+}, Ca^{2+}, Mg^{2+}, Mm^{2+}, Ni^{2+} or Zn^{2+} (all 10^{-4} M) while Cu^{2+} and Fe^{2+} (both 10^{-4} M) inhibited the activity 57 and 18%, respectively. EDTA (10^{-4} M) had no effect on the reaction rate. No effect was exerted by glutathione, thioglycolate, β-mercaptoethanol or dithiothreitol (all 1 mM) added to the incubation mixture. 0.7 mM iodoacetate, 2.5 mM NaCN and 20 mM hydroxylamine inhibited the reaction rate 100, 21 and 60%, respectively. 2.5 mM $NaBH_4$ and 10 mM glycolaldehyde had no effect on the reaction rate.

Phosphohydroxypyruvate, glyoxylate, glycolaldehyde, glycerate and dihydroxyacetone were not converted by the enzyme.

Discussion

The degradation pathway of uric acid, allantoin and allantoate to glyoxylate is well established in *B. fastidiosus* [2,3]. The conversion of glyoxylate was recently studied [4] and it was shown that the enzymes of the glycine-serine pathway, the oxalate pathway and the β-hydroxyaspartate pathway were not present in cell-free extracts. Tartronate semialdehyde is formed from glyoxylate by the action of glyoxylate carboligase, but this compound cannot further be utilized via the glycerate pathway since glycerate kinase is absent [4]. An enzymatic isomerization of the semialdehyde into hydroxypyruvate followed by further conversion to pyruvate was observed [4].

The enzyme responsible for this isomerization, hydroxypyruvate isomerase, was purified to homogeneity and was shown to be composed of six identical subunits with a molecular weight of 45 000. The conversion of tartronate semialdehyde into hydroxypyruvate is reversible and stoichiometric. The enzyme is specific for tartronate semialdehyde and hydroxypyruvate. No cofactors were required for full enzymatic activity.

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