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3-HEXULOSEPHOSPHATE SYNTHASE FROM *METHYLOMONAS AMINOFACIENS* 77a

PURIFICATION, PROPERTIES AND KINETICS

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Summary

3-Hexulosephosphate synthase (D-*arabino*-3-hexulose 6-phosphate formaldehyde lyase) was purified from an obligate methylotroph, *Methylomonas aminofaciens*, to homogeneity as judged by polyacrylamide gel electrophoresis and analytical ultracentrifugation. The molecular weight was determined to be 45 000—47 000 by sedimentation velocity and gel filtration. The enzyme appears to be composed of two identical subunits (M_r = 23 000). A bivalent cation is required for the activation and stabilization of the enzyme. The enzyme is specific for formaldehyde and D-ribulose 5-phosphate. The optimum pH is 8.0 (isoelectric point, pH 5.1) and the optimum temperature is 45°C.

Initial velocity studies are consistent with a sequential mechanism. The Michaelis constants are 0.29 mM for formaldehyde and 0.059 mM for D-ribulose 5-phosphate.

Introduction

The first step in the ribulose-monophosphate cycle of formaldehyde fixation in some methylotrophic bacteria is the condensation of formaldehyde and D-ribulose 5-phosphate to give D-*arabino*-3-hexulose 6-phosphate [1—3,6]. The reaction is catalyzed by 3-hexulosephosphate synthase (D-*arabino*-3-hexulose 6-phosphate formaldehyde lyase). The enzyme has been purified from a methane-utilizing bacterium, *Methylococcus capsulatus* [4], and the two methanol-utilizing bacteria, *Methylomonas* M15 [5] and *Methylomonas aminofaciens* [6].

We describe here the purification to homogeneity, the molecular characterization and the forward reaction (3-hexulosephosphate synthesizing reaction) kinetics of this enzyme from *Methylomonas aminofaciens* 77a.

Materials and Methods

Materials

Phosphoglucoisomerase, glucose-6-phosphate dehydrogenase, alcohol dehydrogenase, cytochrome *c*, chymotrypsinogen A, ovalbumin and bovine serum albumin were purchased from Boehringer Mannheim GmbH (Mannheim, West Germany). Phosphoriboisomerase, alkaline phosphatase (from calf intestinal mucose) and all sugar phosphates were products of Sigma Chemical Company (St. Louis, Mo. U.S.A.). DEAE-cellulose was purchased from Brown Co. Sephadex G-75, G-100 and DEAE-Sephadex A-50 were products of Pharmacia (Uppsala, Sweden) and collodion bags were obtained from Sartorius Membranfilter GmbH (Göttingen, West Germany). Phospho-3-hexuloisomerase was prepared from *Methylomonas aminofaciens* as described previously [6]. (In this enzyme preparation, no 3-hexulosephosphate synthase activity was detected.) D-*arabino*-3-hexulose 6-phosphate was prepared using the purified 3-hexulosephosphate synthase from *Methylomonas aminofaciens* [6]. Formaldehyde was prepared by heating 0.5 g of paraformaldehyde in 5 ml of water at 100°C in a sealed tube for 15 h.

Organism and cultivation

The obligate methylotroph, *Methylomonas aminofaciens* 77a, was maintained and grown under the same conditions as described previously [6].

Analyses

The formaldehyde solution was standardized with alcohol dehydrogenase according to Bernt and Gutman [7]. Formaldehyde in the reaction solution was determined by the method of Nash [8]. D-fructose 6-phosphate was assayed by the method of Land and Michai [9]. D-*arabino*-3-hexulose 6-phosphate was isomerized to D-fructose 6-phosphate with 1.0 unit of phospho-3-hexuloisomerase and the D-fructose 6-phosphate produced was assayed as described above. The protein concentration of the pure enzyme was determined by measuring the absorbance at 280 nm. $\epsilon^{1\%}_{1cm,280nm} = 1.75$ was calculated by comparing the absorbance with the protein determination according to the method of Lowry et al. [10], with bovine serum albumin as standard.

3-Hexulosephosphate synthase activity

The enzyme activity was assayed routinely by measuring the rate of D-ribulose 5-phosphate-dependent disappearance of formaldehyde according to the method of Ferenci et al. [4]. Method A. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 2 mM formaldehyde, 1 unit of phosphoriboisomerase, 4 mM D-ribose 5-phosphate (sodium salt) and an aliquot of the enzyme (0.05—0.2 unit) in a final volume of 0.5 ml. The spectrophotometric assay of the enzyme activity (Method B) was carried out as described previously [6]. When defined starting-concentrations of D-ribulose 5-phosphate were required in the assays, D-ribulose 5-phosphate was previously synthesized with phosphoriboisomerase [6]. 1 unit of the enzyme activity was defined as the amount of enzyme that catalyzed the removal of 1 μmol formaldehyde or the formation of 1 μmol NADPH per min and mg protein in the above assays.

Purification of the enzyme

All manipulations were carried out 0—6°C. All the buffers used were supplemented with 1 mM $MgCl_2$ and 0.01% 2-mercaptoethanol.

Step 1: cell-free extract. The washed cells, suspended in 50 mM Tris · HCl buffer (pH 8.2), were disrupted for 20 min with a KaijoDenki Ultrasonic oscillator (19 kHz). The supernatant, after centrifugation at 16 000 $\times g$ for 20 min and at 100 000 $\times g$ for 1 h, was precipitated with the addition of solid $(NH_4)_2SO_4$ (to 80% saturation). The resulting precipitate was dissolved in a small volume of 50 mM Tris · HCl buffer (pH 8.2) and dialyzed against the same buffer.

Step 2. DEAE-cellulose column chromatography. The dialysed solution was applied to a DEAE-cellulose column (5.6 × 57 cm) previously equilibrated with 50 mM Tris · HCl buffer (pH 8.2) and washed with 5 l of the same buffer. The enzyme was eluted with this buffer, containing 100 mM NaCl. The active fractions were combined and precipitated with $(NH_4)_2SO_4$ to 80% saturation.

Step 3: hydroxylapatite column chromatography. The precipitate was dissolved in 10 mM potassium phosphate (pH 7.4), buffer A, dialyzed against the same buffer, and layered on a hydroxyapatite column (2.2 × 22 cm) which had been equilibrated with the buffer A and the enzyme eluted with buffer A. The active fractions were pooled and precipitated with $(NH_4)_2SO_4$, as described above. The precipitate was dissolved in 1.5 ml 20 mM potassium phosphate buffer (pH 7.4), buffer B.

Step 4: 1st gel filtration. A column (2.3 × 110 cm) of Sephadex G-150 was equilibrated with buffer B. The enzyme solution was applied to the column and eluted with buffer B. The active fractions were pooled and concentrated by ultrafiltration with a collodion bag.

Step 5: 2nd gel filtration. The concentrated enzyme solution was placed on a column of Sephadex G-100 (2.1 × 110 cm) equilibrated with buffer B. The enzyme was, eluted with buffer B and the enzymically active fractions pooled.

Step 6: DEAE-Sephadex A-50 column chromatography. The enzyme solution from Step 5 was applied to a column of DEAE-Sephadex A-50 (1.6 × 12.5 cm) equilibrated with buffer B. After washing with buffer B, the protein was eluted with 125 ml linear gradient of 20—100 mM potassium phosphate buffer (pH 7.4). The active fractions were combined, concentrated by ultrafiltration, and stored at —18°C in small aliquots.

Acrylamide gel electrophoresis

Electrophoresis was carried out in 5% polyacrylamide gels in Tris/glycine buffer (pH 8.3) according to the method of Davis [11]. To estimate the molecular weight of the subunit(s), gel electrophoresis in 0.1% SDS was carried out as described Weber and Osborn [12]. The native enzyme and reference proteins were incubated in buffer B/1% SDS/25% glycerol/ 1.7% 2-mercaptoethanol for 2 h at 40°C. The following reference molecular weight markers were used: trypsin inhibitor from soy-bean, 215 000; bovine serum albumin, 68 000; RNA polymerase from *Escherichia coli*, β-subunit, 132 000; β′-subunit, 142 000.

Estimation of molecular weight by gel filtration

The molecular weight of the enzyme was also determined by gel filtration on Sephadex G-100 according to the method of Andrews [13]. The column (1 ×

54 cm) was equilibrated with buffer B/1 mM $MgCl_2$. The enzyme and the calibration proteins were eluted with buffer B. The following calibration proteins were used: cytochrome *c*, 12 500; chymotrypsinogen A, 25 000; ovalbumin, 45 000; bovine serum albumin, 67 000.

Isoelectric focusing electrophoresis

Isoelectric focusing was performed uisng an LKB 110 ml column, which was set up according to the method of Vesterberg and Svensson [14], 46 h, at 4°C, maximum load of about 1 W (2 mA, 500 V), using 0.77% Ampholite (pH 3—10).

Ultracentrifugal analysis

Sedimentation velocities were measured at 20°C in a Beckmann Spinco model E analytical ultracentrifuge at 59 780 rev./min. Diffusion measurements were also performed at 20°C with the same apparatus at 12 590 rev./min, employing synthetic boundary cells. The molecular weight of the enzyme was calculated from the sedimentation and diffusion coefficients, according to the equation of Svedberg and Pedersen [15]. All calculations used a value for the partial specific volume of 0.749 ml/g.

Kinetic data

The 3-hexulosephosphate synthase used for all kinetic experiments was the homogeneous preparation with a specific activity of 53 units/mg when assayed by Method A. The initial velocities were determined from the linear portion of the curves in Method B. The reaction velocities are expressed as μmol/min.

Results

Purification and stability of 3-hexulosephosphate synthase

Using the purification procedures described, the enzyme was purified 16-fold from the cell-free extract (Table I). The purified enzyme gave one single protein band that coincided with 3-hexulosephosphate synthase activity in analytical polyacrylamide gel electrophoresis (Fig. 1). In analytical ultracentrifuge, the enzyme protein migrated as a single, symmetrical boundary.

TABLE I

SUMMARY OF PURIFICATION OF 3-HEXULOSEPHOSPHATE SYNTHASE FROM *METHYLOMONAS AMINOFACIENS*

Fraction	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)
Cell-free extract	6031	20 010	3.3
DEAE-cellulose	1251	7 010	6.3
Hydroxyapatite	350	6 200	17.7
Sephadex G-150	78	3 744	48.0
Sephadex G-100	50	2 622	52.0
DEAE-Sephadex A-50	31	1 645	53.0

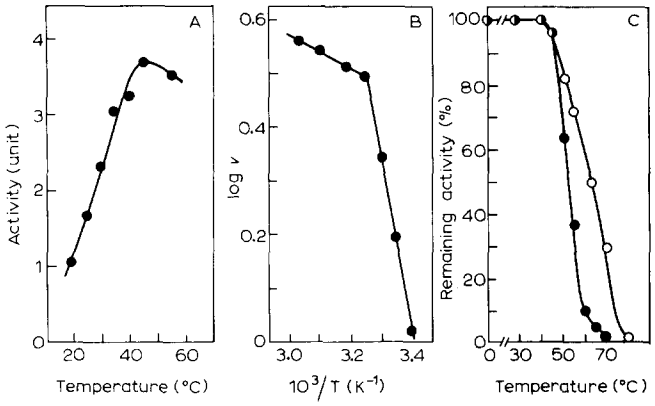


Fig. 1. Effect of temperature on (A) activity and (C) stability of 3-hexulosephosphate synthase; (B) Arrhenius plots of (A). (A) Enzyme activity was measured by Method A at various temperatures. (C) The enzyme (0.1 unit) in 20 mM potassium phosphate buffer (pH 7.4) was heated for 10 (—○—) or 30 min (—●—) at the temperature indicated. The remaining activity was measured by Method B.

Molecular weight and subunit structure

The molecular weight of the 3-hexulosephosphate synthase was determined by two techniques: The sedimentation coefficient in water at 20°C ($s^0_{20,w}$) was found to be 3.19 S, over a protein concentration range of 4—10 mg/ml, in buffer A, containing 1 mM $MgCl_2$/0.01% 2-mercaptoethanol. A diffusion coefficient (D) of $6.55 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ was determined for 7.3 mg/ml of protein in the same buffer. From these data the molecular weight was calculated to be 47 000.

The molecular weight of the native enzyme was also estimated by gel filtration on Sephadex G-100 to be 45 000.

The SDS gel profile of the enzyme showed a single band and a molecular weight of 23 000 for the subunit was estimated. The native enzyme probably consists of a molecule composed of two (possibly identical) subunits.

Isoelectric point

The pure 3-hexulosephosphate synthase was submitted to electrofocusing. Only one sharp peak of activity was found with a maximum at pH 5.1.

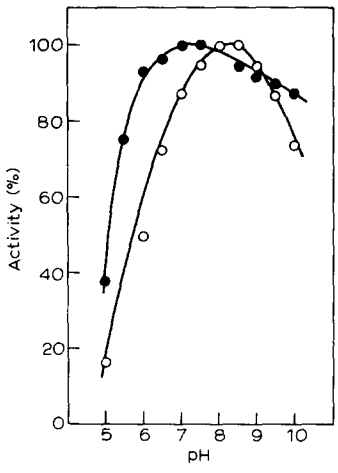
Effect of temperature and pH on the enzyme

As shown in Fig. 1A, the optimum temperature of the enzyme activity was found to be 45°C and the activity was relatively heat-stable. Almost full activity was retained after incubation at 40°C for 30 min, whereas at 80°C for 10 min the enzyme was completely inactivated (Fig. 1C). A changeover from a higher to a lower activation energy with increase in temperature was observed (Fig. 1B). The critical temperature was 35°C and the activation energies below and above 35°C were 12 420 and 1880 cal/mol, respectively.

The enzyme exhibited an optimum for activity at pH 8.0 (glycine/NaOH buffer), and was stable in neutral to slightly alkaline solutions (Fig. 2).

Metal ion requirement

The enzyme showed an essential requirement for Mg^{2+} or Mn^{2+} (5 mM). The



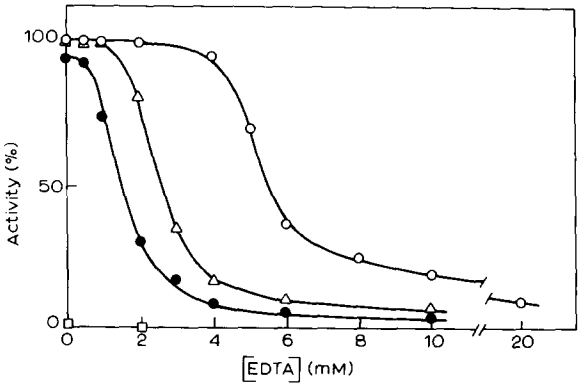


Fig. 2. Effect of pH on activity and stability of 3-hexulosephosphate synthase. The enzyme activity (—○—) was measured by Method A using 0.1 unit of enzyme. For the stability of the enzyme (—●—), the enzyme (0.1 unit) was incubated for 30 min at 45°C in 100 mM buffer. The solution was then adjusted to pH 7.4 with potassium phosphate buffer and the remaining activity was assayed by Method A. Acetate buffer, pH 5.0—6.0; potassium phosphate buffer, pH 6.5—7.5; glycine/NaOH buffer, pH 10.0.

Fig. 3. Reversibility of EDTA inhibition to 3-hexulosephosphate synthase. The enzyme was thoroughly dialyzed at 4°C for 14 h against 20 mM potassium phosphate buffer, 7.4, containing 0.01% 2-mercaptoethanol and then incubated with EDTA at the desired concentration at 30°C for 15 min. The restored activity was assayed by Method A except that $MgCl_2$ was supplemented as follows: —□—, 0 mM; —●—, 1 mM; —△—, 2 mM; —○—, 5 mM.

apparent K_m for $MgCl_2$ was found to be 0.17 mM. The enzyme activity was also promoted with 5 mM Co^{2+}, 85%; 1 mM Cd^{2+}, 71%; 5 mM Zn^{2+}, 45%; 1 mM Fe^{2+}, 35% and inhibited with 1 mM Hg^{2+}, 81%; 1 mM Pb^{2+}, 48%, 1 mM Cu^{2+}, 45%. The enzyme was completely inhibited in the presence of EDTA, but the activity was restored depending on the amount of $MgCl_2$ supplemented (Fig. 3).

Substrate specificity

It was found that 3-hexulosephosphate synthase was highly specific for D-ribulose 5-phosphate within the range of substrates tested. Studies with the pure enzyme by the formaldehyde-disappearance assay (Method A) showed no detectable activity with the following sugar phosphates; D-ribulose 1,5-bisphosphate, D-ribose 5-phosphate, D-erythrose 4-phosphate, D-fructose 6-phosphate, D-fructose 1,6-bisphosphate, D-glucose 6-phosphate, D-glucose 1-phosphate (2 mM each in the reaction system) and D-xylulose 5-phosphate (1 mM).

Inhibitions of the enzyme activity by aldehydes

Glycolaldehyde (49%), methylglyoxal (26%), glutaraldehyde (22%), glyceraldehyde (13%), and glyoxylate (12%) inhibited the enzyme activity by the indicated amounts under the standard assay conditions (Method B) except each aldehyde, 4 mM, was added and formaldehyde concentration was 0.8 mM. Glycolaldehyde was a competitive inhibitor with respect to both D-ribulose 5-phosphate and formaldehyde (Fig. 4). The inhibition constants (K_i) of the aldehyde

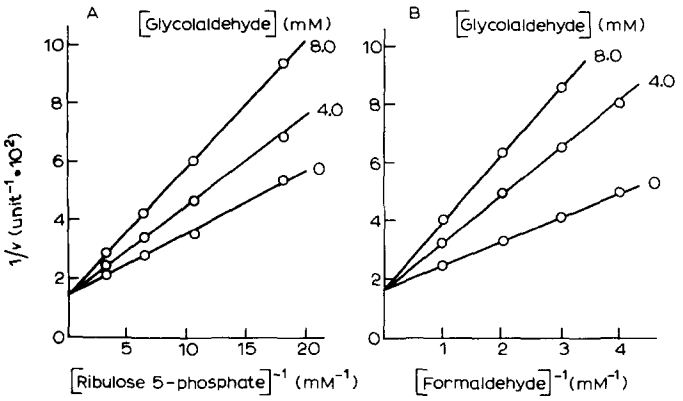


Fig. 4. Inhibition of 3-hexulosephosphate synthase by glycolaldehyde. Initial velocities were measured by Method B except that glycolaldehyde was added at the indicated concentrations. Glycolaldehyde inhibition with respect to (A) D-ribulose 5-phosphate in 3.1 mM formaldehyde is and with respect to (B) formaldehyde in 0.5 mM D-ribulose 5-phosphate.

obtained from a Dixon plot were 12 and 6.4 mM with D-ribulose 5-phosphate and formaldehyde, respectively.

Kinetics of the forward reaction of 3-hexulosephosphate synthase

Initial velocity data of the 3-hexulosephosphate synthase are shown in a double reciprocal plot in Fig. 5 with D-ribulose 5-phosphate as the variable and formaldehyde the fixed substrate. Intersecting lines in the double reciprocal plots indicate a sequential mechanism for the enzyme reaction. From the secondary plots of the data shown in Fig. 5 (s^{-1} vs. intercept, and s^{-1} vs. slope), the Michaelis constant for D-ribulose 5-phosphate and formaldehyde were calculated as 0.059 and 0.29 mM, respectively. The V of the enzyme is 59.3 μ mol/min per mg protein. The value corresponds to a molecular activity of 2730 molecules of D-*arabino*-3-hexulose 6-phosphate produced per min per molecule of enzyme, assuming a molecular weight of 46 000.

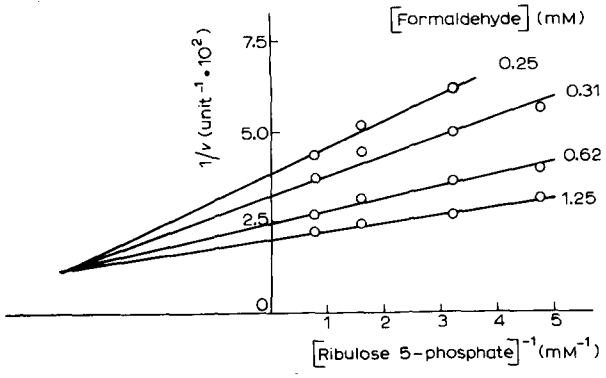


Fig. 5. Double reciprocal plots of initial velocities of 3-hexulosephosphate synthase with respect to D-ribulose 5-phosphate. Initial velocities were measured by Method B.

TABLE II

COMPARISON OF THE PROPERTIES OF 3-HEXULOSEPHOSPHATE SYNTHASES FROM VARIOUS METHYLOTROPHIC BACTERIA

Property	Unit	Value with enzyme from		
		Methylomonas aminofaciens	*Methylococcus capsulatus* (4)	*Methylomonas* M15 (5)
Distribution		soluble	particulate	soluble
Specific activity of final preparation	μmol/mg · min	53.0 (30°C, pH 7.4)	69.0 (37°C, pH 7.0)	66.5 (30°C, pH 7.5)
$s^0_{20,w}$	S	3.19	—	—
$D_{20,w}$	$cm^2 \cdot s^{-1}$	$6.53 \cdot 10^{-7}$	—	—
Molecular weight		45 000—47 000	310 000	43 000
Subunit structure type		homo (2 subunits)	homo (6 subunits)	homo (2 subunits)
Molecular weight		23 000	49 000	22 000
p*I*		5.1		
Heat stability *	°C	80 (10 min)	60 (5 min)	—
pH optimum		8	7	7.5—8.0
Apparent *K*m value for	mM			
$MgCl_2$		0.17	—	0.25
HCHO		0.29 (at $^{\infty}$ [Ru5*P*])	0.49 (at 0.59 mM [Ru5*P*])	1.1 **
Ru5*P* ***		0.059 (at $^{\infty}$ [HCHO])	0.083 (at 4 mM [HCHO])	1.6 **
Hu6*P* †		0.036	0.075	—

* Initial activity of the enzyme was total lost at this temperature for indicated period.

** No concentration of the other substrate was described.

*** D-ribulose 5-phosphate.

† D-*arabino*-3-hexulose 6-phosphate.

Discussion

Some enzymic and physicochemical properties of 3-hexulosephosphate synthase from *Methylomonas aminofaciens* are summarized in Table II with those from *Methylococcus capsulatus* [4] and *Methylomonas* M15 for comparison. The three enzymes hold common catalytic properties with respect to high specificities for formaldehyde and D-ribulose 5-phosphate, and bivalent cation requirement. The interesting differences in the properties of the three enzymes are the intracellular distributions of the enzyme, the molecular weights and subunit structures. The enzymes of obligate methanol-utilizers, *Methylomonas aminofaciens* and *Methylomonas* M15 [5] are soluble, in contrast the enzyme of a methane-utilizer, *Methylococcus capsulatus*, [1,4] which is particulate. The difference in localization of the enzymes in these species is in fitting with the lack of evidence for a particulate internal membrane system in methanol-utilizer, although such a system is observed in methane-utilizers [16].

The enzyme from *Methylococcus capsulatus* has been described as having a molecular weight of 310 000 and to be composed of 6 subunits [4]. On the other hand, the molecular weight of this enzyme from *Methylomonas aminofaciens* is 45 000—47 000 and is most probably composed of two indentical subunits. This molecular weight is very similar to that of *Methylomonas* M15 [5]. The difference in molecular weights of 3-hexulosephosphate synthase between methanol- and methane-utilizers can perhaps be interpreted as an indication of a large evolutionary gap between these groups of organisms. However, the apparent affinities for substrates of the enzyme from *Methylomonas aminofaciens* are closer to those from *Methylococcus capsulatus* than *Methylomonas* M15.

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