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IDENTIFICATION AND PROPERTIES OF AN INDUCIBLE AND HIGHLY SPECIFIC FRUCTOKINASE FROM *STREPTOMYCES VIOLACEORUBER*

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SUMMARY

1. An inducible fructokinase (ATP:fructose 6-phosphotransferase, EC 2.7.1.4) found in *Streptomyces* is described. The enzyme has been purified 400-fold from extracts of *Streptomyces violaceoruber*.

2. This fructokinase phosphorylated fructose as the apparently unique sugar substrate, with a K_m of 0.5 mM under conditions of MgATP saturation. The K_m for MgATP was 0.2 mM. MgADP inhibited the enzyme competitively with MgATP (K_i, 0.2 mM). The other product, fructose 6-phosphate, did not inhibit the enzyme appreciably.

3. The enzyme showed complex kinetic properties: apparent activation by free Mg^{2+}, a sigmoid saturation curve for MgATP, and a double loop saturation curve for fructose at low MgATP concentrations.

INTRODUCTION

As reported in the preceding paper[1], the growth of *Streptomyces* species on fructose exhibits a lag as compared with that on glucose. During the study of the biochemical bases of this behaviour, a highly specific fructokinase (ATP:D-fructose 6-phosphatase, EC 2.7.1.4) was identified in extracts of *Streptomyces violaceoruber*. In the present report the purification, kinetics and other properties of this enzyme are described.

MATERIALS AND METHODS

Strains of *S. violaceoruber* (3086), *S. griseus* (3107 and 3154), *S. albus* (3078), and *S. fradiae* (3197) were provided by the Spanish Type Culture Collection (C.E.C.T.).

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Culture conditions, preparation of extracts, and estimation of enzymic activities were as previously described[1].

Proteins were estimated by the method of Lowry *et al.*[2], and by measuring the absorbance at 280 and 260 nm according to Kalckar[3]. One unit of enzyme is defined as the amount that transforms 1 nmole of substrate per min under the assay conditions; specific activity is expressed as units/mg of protein.

RESULTS

Growth of Streptomyces on fructose

Growth of *Streptomyces* on a minimal medium *plus* fructose usually shows a lag as compared with that on glucose[1]. By using spores of the different species the initiation of growth on fructose took from 40–100 h. The duration of the lag depended on both the strain and the size of the inoculum. When fresh mycelia were used no lag was observed. With *S. violaceoruber* the generation time for fructose and glucose was about 20 h, regardless of the initial lag for growing. All but one of the *Streptomyces* species used grew well on fructose. The exception was *S. fradiae*, which did not grow on this sugar even after 3 weeks.

Inducible fructokinase from Streptomyces

As reported in the preceding paper[1], fructokinase activity in *Streptomyces* species occurs substantially (100–150 units per mg protein) only when the cells are grown on minimal medium with fructose. In the special case of *S. fradiae* no activity (<1 unit/mg protein) was detected when the organism was grown on different sources of carbon and energy, with or without fructose added to the medium.

To characterize the inducible phosphorylating activity on fructose, a further investigation was carried out by using *S. violaceoruber* as the source material.

Purification of fructokinase from S. violaceoruber

Extracts of *S. violaceoruber* grown on fructose were made from cells collected at the end of the exponential growth. Crude extracts were prepared as previously described[1], except that the extractive solution contained 2 mM instead of 1 mM dithioerythritol.

The results of the purification, starting from 150 g of fresh mycelium, are summarized in Table I. All operations were carried out at 0–4 °C.

Ammonium sulphate fractionation

Most of the phosphorylating activity on fructose of the crude extracts precipitates between 35 and 45% of $(NH_4)_2SO_4$ saturation at pH 7.5. This fraction was dissolved in a small volume of the extractive medium.

Sephadex G-200 filtration

The above preparation was filtered through a column (45 cm $\times$ 2.4 cm) of Sephadex G-200 previously equilibrated with the extractive medium. Fractions of 3 ml were collected. Phosphorylating activity appeared as a single peak. Fractions containing the phosphorylating activity were pooled and concentrated 6-fold by precipitation with $(NH_4)_2SO_4$ at 60% saturation (pH 7.5). The precipitate was dissolved in a

TABLE I

PURIFICATION OF *S. violaceoruber* FRUCTOKINASE

Details in the text.

Fraction	*Vol. (ml)*	*Total protein (mg)*	*Spec. act. (units/mg protein)*	*Recovery (%)*	*Purification (-fold)*
Crude extract	460	1380	100	100	1
$(NH_4)_2SO_4$ fractionation (35–45%)	9	110	1 000	75	10
Sephadex G-200 filtration (concentrated)	5	26	2 800	55	28
First DEAE-Sephadex fractionation (concentrated)	2.8	3.1	14 000	30	140
Second DEAE-Sephadex fractionation (concentrated)	1.6	0.7	40 000	20	400

medium (Medium A) that contained 25 mM Tris–HCl, 1 mM EDTA, and 2 mM dithioerythritol (pH 7.5).

First DEAE-Sephadex fractionation

The above preparation was chromatographed on a column (30 cm × 1.8 cm) of DEAE-Sephadex equilibrated with Medium A, and eluted with a 300-ml linear gradient of KCl (0 to 2 M) dissolved in the same medium. The eluate was collected in fractions of 2 ml. A single peak of fructokinase activity was obtained at about 0.8 M KCl. Fractions containing the activity were pooled and concentrated 7-fold as indicated above, except that redissolution of the $(NH_4)_2SO_4$ precipitate in Medium A was supplemented with 10 mM $MgCl_2$.

Second DEAE-Sephadex fractionation

This step was carried out as the previous one, except that the column was equilibrated with Medium A *plus* 10 mM $MgCl_2$. The KCl for elution was also dissolved in Medium A *plus* 10 mM $MgCl_2$. Fructokinase was eluted at about 0.5 M KCl. Active fractions were pooled and concentrated as before. Finally, the preparation was dialyzed twice, for 2 h each time, against 500 ml of the extractive medium (with $MgCl_2$).

The purified preparation was free of (less than 1% as referred to the fructokinase reaction): glucokinase (EC 2.7.1.2), ATPase (EC 3.6.1.3), glucosephosphate isomerase (EC 5.3.1.9), phosphofructokinase (EC 2.7.1.11), phosphofructomutase, adenylate kinase (EC 2.7.4.3) and phosphogluconate dehydrogenase (EC 1.1.1.43).

Stability

The stability of the enzyme was markedly improved by dithioerythritol and Mg^{2+}. In the presence of these compounds purified preparations retained 75% of their activity when stored for 6 months at −20 °C, or after two weeks at about 2 °C and pH 7, but only 50% after 1 h at pH 6.

Identification of reaction product

Most probably the phosphorylation of fructose occurs at position C-6, as sug-

gested by the instantaneous coupling of the enzymic reaction with phosphoglucose isomerase. The assay for a presumptive phosphofructomutase in the fructokinase preparation was negative.

Phosphoryl acceptor specificity

The purified preparation did not phosphorylate (less than 1% as referred to the rate of fructose phosphorylation) the following compounds assayed at 50 mM: D-fructose 1-phosphate; D-glucose; D-mannose; D-galactose; 2-deoxy-D-glucose; sedoheptulose; D-tagatose; D-glucosamine; D-arabinose; L-sorbose; D-lyxose; D-fucose; D-mannoheptulose; D-xylose; D-talose; D-altrose; mannitol; D-mannosamine; *N*-acetyl-D-glucosamine; *N*-acetyl-D-mannosamine; D-fructosamine and sucrose. In each test, 20 units of fructokinase were used in the assay system. No significant inhibition was observed with any of these compounds when tested as inhibitors of the fructokinase reaction at a concentration of 50 mM with 1 mM fructose. Hence it may be concluded that this inducible enzyme from *S. violaceoruber* is a highly specific fructokinase. The K_m for fructose with 5 mM ATP and 10 mM $MgCl_2$ was 0.5 mM, at pH 7.5 and 25 °C.

Phosphoryl donor specificity

In the presence of 10 mM $MgCl_2$, 5 mM of the phosphoryl donor and 5 mM fructose, the following relative velocities were obtained using a purified preparation: ATP, 100; dATP, 80; ITP, 60; GTP, 50; CTP, 30; UTP, 20. Similar results were obtained by using crude extracts, in which the study of glucokinase phosphoryl donor specificity showed[1] a strict requirement for ATP, which rules out the possibility that the activity of fructokinase with other nucleoside triphosphates could be due to the presence of nucleosidediphosphate kinase activity. The following phosphoderivatives were inert (<1% as referred to the rate with ATP) for the fructokinase reaction: phosphoenolpyruvate, acetyl phosphate, carbamyl phosphate, glucose 1-phosphate, fructose 1-phosphate and mannose 1-phosphate. In the presence of 10 mM Mg^{2+} in excess over the concentration of ATP, the K_m for MgATP was 0.2 mM.

Metal specificity

In a system with 5 mM fructose, 5 mM ATP and 10 mM of metal, Co^{2+} showed 60% of the activity found with Mg^{2+}. No activity (<1%) was detected with Mn^{2+}, Ni^{2+}, Ca^{2+}, Cd^{2+} or Cu^{2+}.

Optimal pH

Fig. 1 shows the profile of fructokinase activity at different pH values. The fall of activity at low pH was not due to inactivation of the enzyme or/and to a limited operation of the coupling system for the assays. Fructokinase shows a lengthy plateau of optimal pH in the alkaline zone.

Product inhibition

MgADP inhibited fructokinase competitively with MgATP; K_i was 0.2 mM as measured with 5 mM fructose, 0.5 mM MgADP and 10 mM Mg^{2+} in excess of ATP and ADP concentrations. 20 mM fructose 6-phosphate did not appreciably inhibit fructokinase activity with 1 mM fructose, 0.5 mM ATP and 10 mM Mg^{2+}.

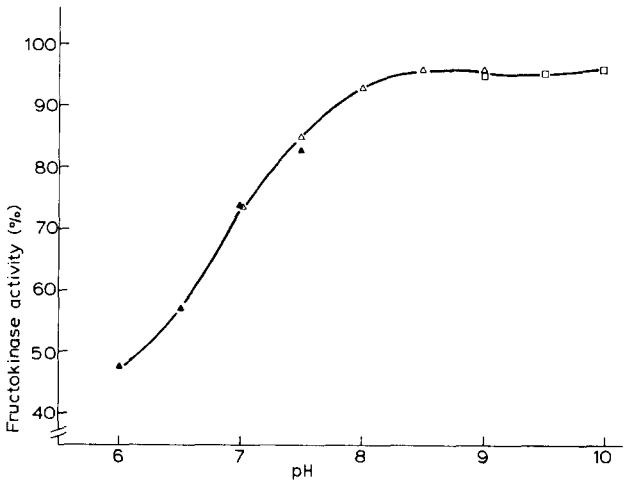


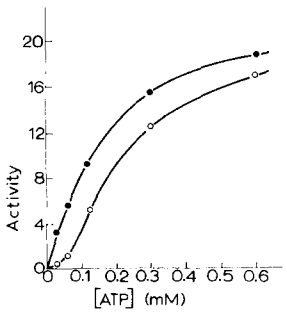
Fig. 1. pH profile of fructokinase activity. Methods A and B were used. Fructose was 10 mM; ATP, 5 mM and $MgCl_2$ 10 mM. Buffers were at 50 mM. ▲, imidazole buffer; △, Tris buffer; □, glycine buffer.

–SH groups

A preparation of fructokinase containing 20 units/ml was quickly inactivated at 30 °C by 1 μm *p*-chloromercuribenzoate. Partially purified preparations of fructokinase lost all their activity after standing at 0–4 °C during two weeks. The inactivated preparations recovered almost all their original activity after 3 days by the addition of 5 mM of reduced dithioerythritol at 0–2 °C.

Kinetic properties

Fig. 2 shows the saturation curve of fructokinase with MgATP at a ratio of $Mg^{2+}/ATP = 2$, and its activation by the further addition of Mg^{2+}. Yeast hexokinase



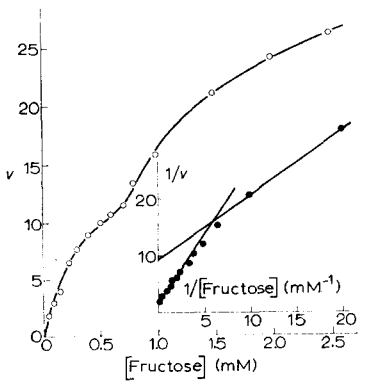


Fig. 2. Fructokinase activity at different ATP and $MgCl_2$ concentrations. Methods A and B were used. Fructose was 5 mM. ○, with a concentration of $MgCl_2$ twice that of ATP; ●, the same *plus* 5 mM $MgCl_2$.

Fig. 3. Fructokinase activity at different fructose concentrations with low ATP concentration. Method A was used. ATP was 0.2 mM and $MgCl_2$ 0.4 mM.

(EC 2.7.1.1) had the same K_m for MgATP, but showed a normal hyperbolic saturation curve in the same range of concentrations with or without an excess of Mg^{2+}. This was also true after yeast hexokinase was mixed with a fructokinase preparation (activity measured with glucose as substrate).

The kinetics of fructokinase in respect to fructose concentration also show an unusual pattern. As Fig. 3 shows, the saturation curve sensibly deviates from the hyperbolic type and a double-loop saturation curve is obtained at low ATP and Mg^{2+} concentrations. However, at high ATP and Mg^{2+} concentrations (over 2 and 4 mM, respectively) saturation curves of fructokinase with fructose show a hyperbolic pattern, from which a K_m for fructose of 0.5 mM is obtained.

DISCUSSION AND CONCLUSIONS

Very few systems are known for the phosphorylation of fructose with ATP in microorganisms. An ATP-dependent kinase able to phosphorylate mannose and fructose with the same efficiency has been characterized in *Leuconostoc mesenteroides*[4] and *Escherichia coli*[5]. An apparently specific ATP-dependent fructokinase that phosphorylates fructose at position C-6 has been described by Kelker *et al.*[6] in *Aerobacter aerogenes*. This enzyme is induced by sucrose. According to these authors, this enzyme is responsible for the phosphorylation of fructose in cells growing on sucrose but not on fructose. When *A. aerogenes* grows on fructose, this sugar is phosphorylated in position C-1 by the phosphoenolpyruvate–phosphotransferase system[7].

The fructokinase from *Streptomyces* seems to be responsible for the utilization of fructose by these organisms, as indicated by its inducible character, the amount of enzyme activity found in wholly induced cells and also by the behaviour of *S. fradiae*, which does not grow on fructose and apparently lacks the enzyme. Moreover, the phosphoenolpyruvate–phosphotransferase system[7] was not detected in these organisms[1]. The absence of this system has also been reported by Romano *et al.*[8] from other strictly aerobic bacteria. Sucrose does not support the growth of *S. violaceoruber* nor induce fructokinase activity when added to cells growing on glucose.

A type of highly specific fructokinase in peas[9] that phosphorylates at the position C-6 has been described. It was not studied in such detail as to allow a useful comparison with that of *Streptomyces*. To our knowledge, no one kinase that specifically phosphorylates fructose at position C-6 has thus far been thoroughly characterized. The enzyme from *S. violaceoruber* shows exceptional kinetic properties in respect to the three compounds, ATP, Mg^{2+}, and fructose. These unusual kinetics are presently under investigation in our laboratory. Besides its strict specificity for fructose (none of 22 analogues of fructose assayed being either substrate or inhibitor) it shows an unusual plateau for optimal activity in the alkaline range, similar to that previously reported for the *N*-acetylglucosamine kinase[10] of *E. coli*.

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