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IDENTIFICATION AND PROPERTIES OF AN INDUCIBLE MANNOKINASE FROM *STREPTOMYCES VIOLACEORUBER*

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SUMMARY

1. Crude extracts from cells of several species of *Streptomyces* grown on different sources of carbon and energy, showed a constitutive ATP-dependent phosphorylating activity on glucose. Specifically inducible kinase activities for mannose and fructose were found in cells grown on each of these sugars.

2. The activity on glucose corresponded to a typical bacterial glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2). The activities on mannose and fructose corresponded to two enzymes of a novel pattern.

3. The activity on mannose was due to a mannokinase (ATP:hexose 6-phosphotransferase, EC 2.7.1.7), which has been purified about 100-fold from extracts of *Streptomyces violaceoruber*. The K_m of this enzyme for mannose was 0.05 mM. Glucose was also phosphorylated by this enzyme although with low affinity (K_m = 4 mM). Fructose was neither a substrate nor an inhibitor.

4. The inducible activity on fructose corresponded to a highly specific fructokinase (ATP:D-fructose 6-phosphotransferase, EC 2.7.1.4), which is described in the accompanying report [B. Sabater, J. Sebastián and C. Asensio, *Biochim. Biophys. Acta*, 284 (1972) 414].

INTRODUCTION

Growth of different species of *Streptomyces* on mannose or fructose usually shows a lag as compared with that on glucose[1]. To ascertain the biochemical bases of this behaviour we have examined the phosphorylation of these sugars by extracts from cells grown under a variety of nutritional conditions. This investigation led to the identification of three types of hexose kinase, namely, glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2), mannokinase (ATP:D-mannose 6-phosphotransferase, EC 2.7.1.7), and fructokinase (ATP:D-fructose 6-phosphotransferase, EC 2.7.1.4). The mannokinase is apparently a new type of phosphorylating enzyme in regard to its sugar specificity pattern. The fructokinase presents complex kinetics in

Abbreviation: PEP, phosphoenolpyruvate.

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relation to divalent metals and the concentrations of both the sugar and the nucleotide triphosphate. The present communication reports the identification and properties of the mannokinase of *Streptomyces violaceoruber*. The fructokinase is described in the accompanying report[2].

MATERIALS AND METHODS

Chemicals

The sugars used in this work (all of the D-configuration unless stated otherwise) were purchased from Pfanstiehl except tagatose which was from L. Light. Samples of altrose and talose were gifts of Dr N. K. Richtmyer, of the National Institutes of Health, Bethesda. The sugar-phosphates and other phosphoryl compounds were obtained from Sigma. NADH, $NADP^+$, and the auxiliary enzymes utilized in the kinetic measurements, were obtained from Boehringer and Soehne.

Microorganisms

Strains of *S. violaceoruber* (3086), *S. griseus* (3107 and 3154), and *S. albus* (3087), were provided by the Spanish Type Culture Collection (C.E.C.T.).

Growth and harvesting of the cells

Cells were usually grown in minimal medium "63"[3] except that 1 g of $MgSO_4 \cdot 7 H_2O$ and 0.1 mg of nicotinamide per l were added. The sources of carbon and energy were routinely added at 1%. The media were adjusted to pH 7 with KOH. A spore suspension (10 ml), prepared as described by Sistrom and Machlis[4], was normally used as inoculum for each 2-l flask containing 600 ml of the medium, and the flasks were maintained at 30 °C with shaking. Cells were collected by filtration at the end of the exponential phase, and washed with distilled water. About 13 g of wet mycelium per l were usually obtained.

Preparation of crude extracts

Crude homogenates were obtained by grinding the mycelium in a Virtis grinder for 5 min in the cold with 3 times its weight of an extractive medium containing 50 mM potassium phosphate (pH 7), 1 mM EDTA, and 1 mM dithioerythritol. The supernatant fluid ("crude extract") collected after centrifugation at 18 000 $\times$ *g* for 10 min, contained approximately 3 mg protein/ml.

Enzymic assays

For the estimation of hexose kinase activities two spectrophotometric methods were employed, one (Method A) coupling hexose 6-phosphate production to $NADP^+$ reduction, another (Method B) coupling ADP production to NADH oxidation. In Method A the assay mixture for glucokinase contained in a final volume of 1 ml (μmoles): imidazole–HCl (pH 7.5), 50; $NADP^+$, 0.4; hexose, 5; ATP, 5; $MgCl_2$, 10; 1500 units of glucose-6-phosphate dehydrogenase (EC 1.1.1.49); and amounts of enzyme preparation as appropriate. When fructokinase was assayed, 1500 units glucosephosphate isomerase (EC 5.3.1.9) were added. For mannokinase assay 1500 units of both glucosephosphate isomerase and mannosephosphate isomerase (EC 5.3.1.8) were added. In Method B the assay mixture contained in a final volume of 1

ml (μmoles): imidazole–HCl (pH 7.5) 50; KCl, 100; phosphoenolpyruvate (PEP), 2; NADH, 0.15; 1500 units of pyruvate kinase (EC 2.7.1.40); 1500 units of lactate dehydrogenase (EC 1.1.1.27); and amounts of enzyme preparation, sugar, ATP, and $MgCl_2$ as in Method A. For the estimation of other enzymic activities a final volume of 1 ml with 50 μmoles imidazole–HCl (pH 7.5) was also used. 6-Phosphogluconate dehydrogenase (EC 1.1.1.43), glucose-6-phosphate dehydrogenase, glucosephosphate isomerase, mannosephosphate isomerase, phosphoglucomutase (EC 2.7.5.1) and a presumptive phosphofructo(or manno)mutase, were assayed by following the reduction of 0.4 mM NADP with the appropriate substrate (0.5 mM) and a coupling system as in Method A. Adenylate kinase (EC 2.7.4.3) was assayed by coupling ATP formation to NADP reduction in a system containing (μmoles): ADP, 5; $MgCl_2$, 10; glucose, 5; NADP, 0.4; and 1500 units of each of glucose-6-phosphate dehydrogenase and yeast hexokinase (EC 2.7.1.1). The activity of ATPase (EC 3.6.1.3) was determined by measuring the ADP appearance from ATP, with (μmoles): KCl, 100; phosphoenolpyruvate (PEP), 2; NADH, 0.15; and 1500 units of each of pyruvate kinase and lactate dehydrogenase. Changes in NADH and NADPH concentrations were monitored at 340 nm in a Beckman DB or Cary Model 15 spectrophotometer with recording. The PEP–phosphotransferase system[5] was assayed by following both the sugar disappearance[6] and the appearance of sugar phosphates as in Method A.

All the assays were carried out at room temperature (approx. 25 °C). No significant differences were found when Methods A and B were employed in parallel. One unit of activity is defined as the amount of enzyme that catalyses the transformation of 1 nmole of substrate per min under the assay conditions. Specific activity is expressed as units/mg protein. The method of Lowry *et al.*[7] for estimation of proteins was routinely used.

RESULTS AND DISCUSSION

Growth of S. violaceoruber in hexoses

As a representative of the genus *Streptomyces*, *S. violaceoruber* exhibits different patterns when grown in a minimal medium with different sugars, namely, glucose, mannose and fructose. Although the generation time is about the same with the three hexoses (20 h), growth in mannose and fructose showed initial lags significantly different from those found with glucose. The length of these lags is largely dependent on the size of the inoculum. For a typical inoculum of $1 \cdot 10^8$ viable spores per l, initial lags of 40, 65 and 100 h were obtained for glucose, mannose and fructose, respectively. Maximal growth, however, was about the same for the three sugars and corresponded to a yield of 25–30% of the ratio dry cell weight/sugar used.

Phosphorylating activity of crude extracts from S. violaceoruber

Phosphorylating activities were determined in crude extracts obtained from several *Streptomyces* strains (see Materials) grown in either nutrient broth or in minimal medium with each of the following compounds: glycerol, glucose, mannose and fructose. A fairly constant ATP-dependent phosphorylating activity on glucose, of approximately 150 units/mg protein, was present in all the extracts. However, the capacity to phosphorylate mannose and fructose only markedly appeared when the cells were grown in each of these sugars, respectively. The results obtained with *S.*

violaceoruber are summarized in Table I. Activities on glucose and on the other sugars were grossly additive when present in the same extract. Filtration through Sephadex G-200 of crude extracts from *S. violaceoruber* grown on mannose or fructose allowed the separation of the inducible phosphorylating activities for each of these sugars from the constitutive one that phosphorylates glucose. No PEP-dependent phosphotransferase activity[5] was detected; Romano *et al.*[8] obtained similar results with other strictly aerobic bacteria.

TABLE I

PHOSPHORYLATING ACTIVITY OF CRUDE EXTRACTS FROM *S. violaceoruber*

The two spectrophotometric methods were used. For details see Methods.

Nutritional conditions		*Activity (units/mg protein) on*		
		Glucose	*Mannose*	*Fructose*
Minimal medium *plus*	glycerol	120	<1	10
	glucose	180	<1	5
	galactose	180	<1	5
	mannose	120	70	10
	fructose	150	10	100
Nutrient broth		120	10	<1

The activity on glucose was characterized in crude extracts of cells grown in this sugar. The Michaelis constant for the phosphorylation of glucose with ATP was 0.3 mM. Glucosamine was also a substrate (K_m = 7 mM; V, 75% of that for glucose). The K_m for ATP was 0.7 mM. Fructose, mannose, galactose, 2-deoxyglucose, lyxose and L-sorbose were negative, either as substrate or inhibitor. Xylose behaved as inhibitor (K_i = 20 mM). Glucokinase did not use GTP, CTP, ITP or UTP as phosphoryl donor (less than 5% as referred to the activity with ATP). The above results were obtained as for the mannokinase constants (see below). The activity on glucose seems to correspond to a typical bacterial glucokinase[9,10].

Purification of mannokinase from S. violaceoruber

The purification of mannokinase was carried out by starting with mycelium grown in minimal medium *plus* mannose. All the operations described below were performed in the cold (0–4 °C). Centrifugations were made at 18 000 × *g* for 10 min. Table II summarizes the results obtained starting from 35 g of fresh mycelium.

TABLE II

PURIFICATION OF *S. violaceoruber* MANNOKINASE

Crude extracts were prepared from *S. violaceoruber* grown in mannose. More details in the text.

Fraction	*Vol. (ml)*	*Total protein (mg)*	*Spec. act. (units/mg)*	*Recovery (%)*	*Purification (-fold)*
Crude extract	100	290	70		
Heat treatment (65 °C, 5 min)	100	250	80	100	1
$(NH_4)_2SO_4$ fractionation (30–50%)	13	10	1500	75	20
Alumina C_γ treatment	22	1.7	7500	65	110

Heat treatment. The activity on mannose of the crude extracts was lost after heating for 5 min at 65 °C. However, as shown in Fig. 1, this inactivation was specifically prevented by the presence of mannose. Under these conditions, treatment for 5 min at 65 °C fully inactivated the constitutive glucokinase (Fig. 1) and markedly improved the efficiency of the following step. This treatment was carried out on the total bulk (100 ml) of material, with gentle mixing.

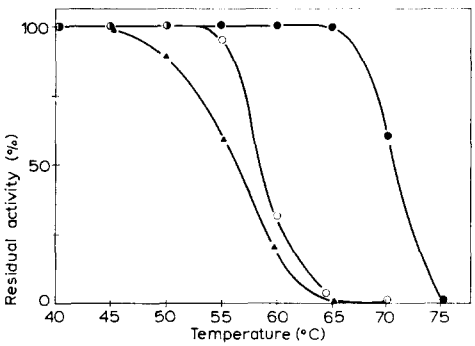

Fig. 1. Heat inactivation of mannokinase and glucokinase, and protection of the mannokinase by mannose. Aliquots of 1 ml of crude extract from *S. violaceoruber* grown on mannose were treated for 5 min at the indicated temperatures, with or without the addition of 10 mM mannose. After rapid cooling, phosphorylating activities on mannose and glucose were estimated. ○—○, Mannokinase activity after heat treatment without added mannose; ●—●, same in the presence of mannose; ▲—▲, glucokinase activity, with or without added mannose in the heat treatment. Glucokinase activity was estimated by difference between the phosphorylation of glucose with ATP and with ITP.

Ammonium sulphate fractionation. To collect the precipitate produced at between 30 and 50% of saturation, to the supernatant obtained after centrifugation of the heated preparation, solid $(NH_4)_2SO_4$ was added under careful mixing.

Alumina C_γ treatment. The above precipitate was resuspended in 12 ml of extractive medium (see Materials and Methods) and mixed with 16 ml of a 2.5% aqueous suspension of alumina C_γ (Sigma), giving a ratio of alumina/protein of 40. Most of the activity on mannose remained in the supernatant fluid collected after centrifugation of the suspension.

The above preparation represented a 110-fold purification of the activity on mannose (Table II) and was free of other enzymic activities that could interfere with the measurement of mannokinase (less than 1% as referred to the mannokinase reaction), namely, glucokinase, ATPase, hexosephosphate isomerases, phosphofructokinase, adenylate kinase and phosphogluconate dehydrogenase. The preparation contained 4% activity on fructose not inhibitable by mannose, which was 14% in the crude extract. This activity showed the same affinity for fructose in both the original extract and the purified fraction (K_m = 0.5 mM), and was presumably due to contaminating fructokinase[2].

Properties of the enzyme

Stability. The purified preparations are rather stable. They retain 75% of their activity after being kept for eight months at −15 °C or after 1 month between 0 and 2 °C.

Phosphoryl acceptor specificity. 31 different sugars or derivatives were assayed, either as substrates (Method B) or inhibitors (Method A). The results are shown in Table III. The kinetic data obtained with mannoheptulose and 2-deoxymannose are shown in Fig. 2. The activity on the compounds listed in Table III as substrates were not additive with that on mannose, when assayed at near saturation concentrations of both sugars. Inhibitions were all of a competitive type and the apparent K_i values of substrates were similar to their corresponding K_m values.

TABLE III

SUGAR SPECIFICITY OF *S. violaceoruber* MANNOKINASE

Besides the compounds listed above, the following were assayed and proved to be inert ($K_i >$ 0.1 M): *N*-acetylmannosamine, mannitol, galactose, fructose, fucose, L-fucose, mannose 1-phosphate, arabinose, altrose, L-sorbose, L-rhamnose, L-mannose, sedoheptulose, α-methyl glucopyranoside, β-methyl glucopyranoside, ribose, xylose, L-xylose, talose, tagatose, sorbitol, glucosamine, and *N*-acetylglucosamine. All the compounds were usually assayed as substrates at a concentration of 50 mM or as inhibitors at the same concentration with 0.2 mM mannose as substrate, by Method B. K_m values for substrates were determined by Method B, and apparent K_i values were determined by measuring the inhibition of mannose phosphorylation by Method A. For glucose phosphorylation, inhibition by mannose was assayed by Method A without added phosphohexose isomerases.

Compound	*V (relative values)*	*K_m (or K_i) (mM)*	*Phosphorylation coefficient*[11]
D-Mannose	100	0.05	100
D-Glucose	80	4	1
D-Mannoheptulose	10	0.4	1.25
2-Deoxy-D-mannose	25	15	0.1
D-Mannosamine	15	20	0.04
α-Methyl-D-mannosamine	<1	30	
D-Lyxose	<1	15	
N-Acetyl-D-mannosamine	<1	75	

Phosphoryl donor specificity. The phosphoryl donor specificity was studied with each of the five acceptor substrates listed in Table III at 5 mM concentration, with 10 mM $MgCl_2$, by using both crude extracts and purified preparations. Relative

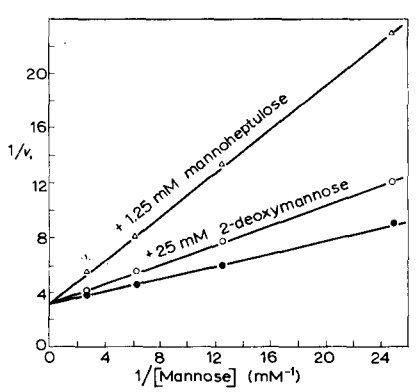

Fig. 2. Double-reciprocal plots for inhibition of mannokinase by mannoheptulose and 2-deoxymannose. Method A was used, except that mannose concentration varied as indicated, and appropriate amounts of the inhibitors were added.

velocities were approximately: ATP, 100; ITP, 90; GTP, 40; and CTP, 10. Activities with ITP, GTP and CTP were apparently not due to the presence of nucleosidediphosphate kinase (EC 2.7.4.6) since, as stated above, the glucokinase activity of the crude extracts did not use any nucleoside triphosphate but ATP. Under similar experimental conditions the following phosphoryl donors were inert (<1%) for the reaction: PEP, acetyl phosphate, phosphoramidate, glucose 1-phosphate, mannose 1-phosphate and fructose 1-phosphate. The K_m for MgATP (0.4 mM) and mannose (0.05 mM) did not change at different concentrations of the other substrate (Fig. 3).

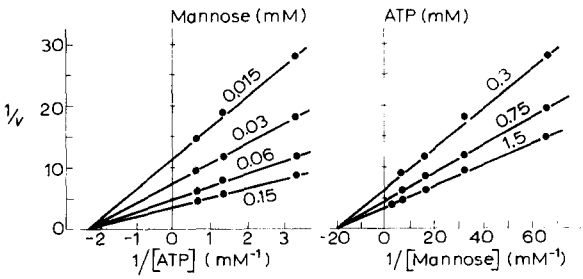

Fig. 3. Double-reciprocal plots for determining the K_m values of the mannokinase for mannose and MgATP. The method coupled to pyruvate kinase was used, except that MgATP and hexose concentrations were varied as indicated. The concentration of $MgCl_2$ was always twice that of ATP.

Optimal pH and metal specificity. The optimal pH for the reaction was about 7.5. The activity of the enzyme fell rapidly outside the range 7–9. The optimal ratio of Mg^{2+}/ATP for activity with 5 mM ATP, was 2. Co^{2+} and Mn^{2+} also served as metals with relative velocities of 40 and 25%, respectively, as compared with that of Mg^{2+}.

Reaction product identification. Phosphorylation by mannokinase occurs most likely at C-6, as indicated by the immediate coupling of the reaction to the mannose-6-phosphate isomerase dependent assay method and the lack in the purified preparation of a presumptive mannose-1-phosphate mutase activity.

Product inhibition. MgADP markedly inhibited the phosphorylation of mannose with a $K_i = 0.1$ mM. This experiment was carried out with Mg^{2+} at a concentration twice that of ATP and ADP. The inhibition proved to be strictly competitive. On the other hand, mannose 6-phosphate did not inhibit the reaction when assayed at concentrations up to 20 mM, with 0.15 mM mannose and 1 mM MgATP.

The inducible character of the mannokinase of *Streptomyces* strongly suggests that it is responsible for the phosphorylation of mannose in these organisms. In fact, the activity found in fully induced samples accounts for the rate of mannose utilization, according to the data obtained from the doubling time of the cells. This inducibility could also account for the lag observed in the growth of these organisms in mannose as compared with glucose, although the permeation step might also be involved. Current work in our laboratory is designed to clarify the participation of this step in the adaptation process.

The mannokinase of *S. violaceoruber* described in this report is the most specific one so far characterized. In fact, it is the first enzyme that could properly be named mannokinase. In this regard it stands in contrast to the mannose phosphorylating

enzyme previously found in other bacteria[12–14] which acts on fructose as effectively as on mannose.

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