A Hexokinase Associated with Catabolite Repression in *Pachysolen tannophilus*

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A hexokinase (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1) associated with catabolite repression was isolated and purified from the yeast *Pachysolen tannophilus*. The enzyme phosphorylated D-fructose at a rate 1.5 times greater than that for D-glucose. The K_m values for D-glucose and D-fructose were 0.36 and 2.28 mM, respectively. Neither xylose reductase nor xylitol dehydrogenase were subject to catabolite repression in mutants defective in this enzyme.

INTRODUCTION

Two of the enzymes associated with xylose catabolism, aldose (xylose) reductase and xylitol dehydrogenase, have been purified from *Pachysolen tannophilus* and examined (Ditzelmüller *et al.*, 1984a, b, 1985; Verduyn *et al.*, 1985; Bolen *et al.*, 1986; Morimoto *et al.*, 1986, 1987). However, little is known about other enzymes in this yeast. We have recently demonstrated the existence of three hexose-phosphorylating enzymes in *P. tannophilus*; hexokinases A and B and a glucokinase specific for D-glucose (Wedlock *et al.*, 1989; accompanying paper). In this paper, we report the isolation and partial purification of a hexokinase (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1), designated A, from a mutant which is defective in the glucokinase. The absence of the glucokinase, which co-elutes with hexokinase A in the wild-type strain of *P. tannophilus*, aids the isolation of the hexokinase without contamination by the glucokinase. The hexokinase PII enzyme of *Saccharomyces cerevisiae* has been implicated in catabolite repression (Kopetzki & Entian, 1985; Hong & Botstein, 1986) as has a hexokinase isolated from *Schwanniomyces occidentalis* (McCann *et al.*, 1987). Xylose utilization by *P. tannophilus* is subject to hexose-sugar catabolite repression (Slininger *et al.*, 1987; Bicho *et al.*, 1988). The effect of the *P. tannophilus* hexokinase A on the activities of xylose reductase and xylitol dehydrogenase and on D-xylose utilization is described here.

METHODS

Yeast strains and growth conditions. The *Pachysolen tannophilus* strains used in enzyme analyses and sugar utilization experiments were P444-3 the wild-type strain, P510-5A defective in a glucokinase enzyme (*glu1*), P509-3C defective in hexokinase A (*hxx2*) and P509-1B defective in both hexokinase A and the glucokinase (*hxx2 glu1*). Strain P510-5A was also used in the purification of hexokinase A.

Cells were grown in 1 litre flasks containing 250 ml of YEP-glucose [1%, w/v, yeast extract (Difco), 2%, w/v, peptone (Difco) and 2%, w/v, D-glucose]. The culture was incubated for 42 h at 30 °C and 180 r.p.m. on a gyratory shaker. Cells grown for 48 h in YEP-glucose were used as the inoculum. For the measurement of xylose reductase and xylitol dehydrogenase activities, cells were grown for 16 h with either D-glucose, D-xylose, or D-glucose and D-xylose as carbon source.

Growth and sugar utilization were followed in YNB [0.67% yeast nitrogen base (Difco), 2%, w/v, sugar] liquid media. A 48 h YNB-xylose culture (100 ml) was washed with deionized water and resuspended in water to an optical density of 34–36 units at 600 nm (Cecil linear readout UV spectrophotometer, model CE272). The final medium (100 ml YNB-sugar in 250 ml Erlenmeyer flasks) was inoculated with 0.5 ml of the prepared inoculum and incubated at 30 °C and 180 r.p.m. Growth was followed by measurement of optical density at 600 nm and sugars were analysed by HPLC with an refractive index detector, using a Bio-Rad HPX-87H column at 65 °C and 5 mM-H₂SO₄ as the eluant.

Purification of hexokinase A. The culture was washed twice with deionized water and resuspended in 10 ml 100 mM-Tris/HCl, pH 7.5, containing the protease inhibitor PMSF (2 mM). Following two passages through a French pressure cell (82.8 MPa), the debris was removed by centrifugation at 17 500 *g* for 20 min. The supernatant was used as a cell-free extract. Streptomycin sulphate was added slowly to the extract to a final concentration of 0.7 g ml⁻¹; the extract was left for 20 min and then centrifuged at 17 500 *g* for 20 min. The supernatant was desalted by passage through a Sephadex G-25 column and applied to a DEAE-cellulose (Whatman DE-52) column (1.6 × 20 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.5. The column was washed with four column volumes of buffer and the enzyme eluted with a 0–0.2 M-NaCl gradient. Fractions (5 ml) were collected and the hexokinase activity measured. Active fractions (typically six) exhibiting a fructose to glucose phosphorylation ratio of 1.3/1.0 were pooled and dialysed overnight against 5 mM potassium phosphate buffer, pH 6.5, containing 5 mM-MgCl₂. The enzyme was further purified by affinity chromatography on a column (1.6 × 18 cm) of Cibacron FG-3A Blue Sepharose CL-6B. After washing with three column volumes of buffer, the enzyme was eluted with 10 mM-Tris/HCl, pH 8.5, containing 15 mM-ATP. All purification steps were done at 4 °C and all buffers contained 1 mM-EDTA and 2 mM-2-mercaptoethanol. The purified enzyme was used for the determination of *K_m* values. Polyacrylamide disc gel electrophoresis on the purified enzyme was done according to the method of Gabriel (1971).

Enzyme assays. Cell-free extracts were prepared as described for the purification of hexokinase A, except cells were resuspended in 100 mM-Tris/HCl, pH 7.5, containing 0.2 mM-PMSF, prior to breakage. Phosphorylation of D-glucose was measured by following the reduction of NADP⁺ at 340 nm (Joshi & Jagannathan, 1966). For the measurement of D-fructose phosphorylation, D-fructose (15 mM) was used as the substrate and phosphoglucose isomerase (0.6 units) was added to the reaction mixture. D-Xylose reductase (EC 1.1.1.21) (Suzuki & Onishi, 1973) and xylitol dehydrogenase (EC 1.1.1.9) (Touster & Montesi, 1962) were assayed at a substrate concentration of 100 mM. All assays were done at 30 °C. Specific activity was expressed as μmol cofactor consumed min⁻¹ (mg protein)⁻¹. Protein concentration was assayed by measurement of UV absorbance at 228.5 nm and 234.5 nm (Ehresmann, 1973) using bovine serum albumin as standard. All enzyme assays and protein concentration determinations were done in duplicate on two independently prepared cell-free extracts.

RESULTS AND DISCUSSION

The partial purification of hexokinase A from the glucokinase-less mutant of *P. tannophilus* is summarized in Table 1. The major glucose-phosphorylating activity in this strain was hexokinase A which constituted 90% of the overall activity; the remainder of the activity was due to the presence of hexokinase B. Fractionation of the two active hexokinases was achieved by chromatography on DEAE-cellulose (Wedlock *et al.*, 1989), and with subsequent use of Blue Sepharose affinity chromatography, a purification of about 55-fold was possible, with a yield of 10%. When the enzyme preparation was run on a polyacrylamide disc gel, one major band and three or more minor bands were detected after staining with Coomassie Blue (Fig. 1). Stability of the enzyme was enhanced by inclusion of ATP in the elution buffer during the chromatography step on Blue Sepharose. Following elution of the enzyme, addition of glycerol to a final concentration of 10% (v/v) also aided stability.

The *K_m* values for D-glucose and D-fructose were 0.36 mM and 2.28 mM, respectively as determined from a double reciprocal plot of initial reaction velocity, *V*₀, against substrate concentration, [S]. The maximum D-fructose/D-glucose phosphorylation (F/G) ratio was 1.5/1.0, as predicted from the *V*_{fructose}/*V*_{glucose} ratio. The F/G ratio at the 15 mM substrate concentration used in the enzyme assays was 1.3/1.0.

The partially purified enzyme from *P. tannophilus* appeared analogous to the hexokinase PII isoenzyme from *Saccharomyces cerevisiae*. The ratio of the rates of phosphorylation of D-fructose and D-glucose (Ramel *et al.*, 1971; Colowick, 1973; Entian & Mecke, 1982; Frohlich *et al.*, 1984; Fernández *et al.*, 1985) and the *K_m* values were similar to those reported for the PII isoenzyme in *Saccharomyces cerevisiae* (Entian *et al.*, 1985).

The activities of the xylose pathway enzymes, xylose reductase and xylitol dehydrogenase, were measured in cell-free extracts of *P. tannophilus* prepared from cells grown on D-xylose, D-glucose or on a mixture of D-xylose and D-glucose. Activities of these enzymes in the wild-type strain, P444-3D, grown on D-xylose/D-glucose, were much lower than those in D-xylose-grown cells (Table 2). Only very low xylose reductase and xylitol dehydrogenase activities were found in D-glucose-grown cells. Both these enzymes in *P. tannophilus* have been previously shown to be

Fig. 1. Polyacrylamide gel electrophoresis of hexokinase A. The enzyme preparation (45 μg protein) was electrophoresed at 32 mA for 1 h and stained with Coomassie Blue R-250 [20 mg ml⁻¹ in methanol/acetic/acid water (2:1:7, by vol.)].

Table 1. *Summary of partial purification of hexokinase A from strain P510-5A*

One unit (U) is 1 μmol NADP⁺ reduced min⁻¹ with D-glucose as the substrate.

Purification step	Total protein (mg)	Total activity (U)	Specific activity [U (mg protein) ⁻¹]	Yield (%)	Purification factor
Cell-free extract	245.0	216	0.9	100	1.0
Streptomycin sulphate	162.0	191	1.2	88	1.4
Anion-exchange chromatography	21.0	53	2.6	25	3.0
Affinity chromatography	0.4	21	48.4	10	55.0

induced by D-xylose (Maleszka *et al.*, 1983; Bolen & Detroy, 1985; Bicho *et al.*, 1988) and repressed by D-glucose (Maleszka *et al.*, 1983; Bicho *et al.*, 1988). By comparison, the activities found in cells of the mutant P509-1B, defective in both hexokinase A and a glucokinase and grown on both D-glucose and D-xylose, were only slightly lower than those found in D-xylose-grown cells. This was true also of P509-3C, which has an active glucokinase but is defective in hexokinase A. Strain P510-5A, defective in the glucokinase but retaining an active hexokinase A, showed greatly reduced activities when grown on the D-glucose/D-xylose mixture compared to D-xylose.

Fig. 2. Utilization of D-glucose and D-xylose by the wild-type strain P444-3D (a), and the mutant P509-1B (b), in YNB media. ●, D-Glucose (in mixed carbon source); □, D-xylose (in mixed carbon source); ▲, D-xylose (as sole carbon source).

Table 2. *Specific activities of xylose reductase and xylitol dehydrogenase in wild-type and mutant strains grown on different carbon sources*

Strain	Carbon source	Specific activity [μmols cofactor reduced or oxidized min ⁻¹ (mg protein) ⁻¹]	
		Xylose reductase	Xylitol dehydrogenase
P444-3D (wild-type)	Xylose	0.26	0.24
	Xylose + glucose	0.007	0.028
	Glucose	0.006	0.006
P510-5A (<i>glu1</i>)	Xylose	0.33	0.29
	Xylose + glucose	0.039	0.054
P509-3C (<i>hxx2</i>)	Xylose	0.34	0.28
	Xylose + glucose	0.21	0.20
P509-1B (<i>hxx2 glu1</i>)	Xylose	0.34	0.28
	Xylose + glucose	0.23	0.25

Characteristically, in media containing both D-glucose and D-xylose, the D-glucose was consumed preferentially by the wild-type strain (Fig. 2a). The utilization of D-xylose commenced when most of the D-glucose was depleted. This pattern has been reported previously (Detroy *et al.*, 1982; Slininger *et al.*, 1987; Bicho *et al.*, 1988). A different pattern of sugar utilization was observed with strain P509-1B, which was able to co-utilize D-glucose and D-xylose (Fig. 2b). This strain can utilize D-glucose slowly, due to the presence of the hexokinase B, but D-glucose does not appear to repress the utilization of D-xylose to the same extent as with the wild-type strain.

From these results, the presence of hexokinase A appears to be necessary for catabolite repression of both xylose reductase and xylitol dehydrogenase. Thus, strains P509-3C and P509-1B, which lack hexokinase A, showed only moderately lower xylose reductase and xylitol dehydrogenase activities when D-glucose was present in the medium. D-xylose utilization in strain P509-1B was no longer subject to repression by D-glucose. In *Saccharomyces cerevisiae*, there is evidence that hexokinase PII has a role in catabolite repression (Entian, 1980, 1981; Entian & Mecke, 1982; Entian *et al.*, 1984; Entian & Frohlich, 1984; Kopetzki & Entian, 1985; Hong & Botstein, 1986). Recently, a hexose-phosphorylating enzyme in the yeast *Schwanniomycetes occidentalis* has been associated with catabolite repression (McCann *et al.*, 1987). From the present studies we conclude that hexokinase A from *P. tannophilus*, in addition to sharing similar kinetic properties to the *Saccharomyces* hexokinase, also plays a role in catabolite repression. By cloning and sequencing the structural gene for hexokinase A, further understanding of the molecular basis for catabolite repression in *P. tannophilus* may be acquired.

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