Mechanism of Inactivation of Hexokinase PII of *Saccharomyces cerevisiae* by D-Xylose

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The mechanism of inactivation of hexokinase PII of *Saccharomyces cerevisiae* by D-xylose was characterized. Inactivation was dependent on the presence of MgATP and was irreversible. Inactivation involved phosphorylation of the protein. Observation of the carbon catabolite repression of selected enzymes showed that invertase and maltase synthesis were not repressed when hexokinase PII was phosphorylated.

INTRODUCTION

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) is the first enzyme of the glycolytic pathway. The enzyme isolated from yeast exists as two non-interconvertible isoenzymes, PI and PII, coded for by two different structural genes (Colowick, 1973). In addition to its hexose phosphorylating activity, yeast hexokinase PII seems to predominate during growth on glucose (Fernández *et al.*, 1985) and its involvement in the regulation of the carbon catabolite repression of some enzymes has been demonstrated both with PI and PII transformants (Fröhlich *et al.*, 1984; Entian *et al.*, 1984) and by the D-xylose-induced decrease in hexokinase PII activity (Fernández *et al.*, 1984). According to Entian & Mecke (1982) hexokinase PII should be a bifunctional enzyme with both a catalytic and a regulatory domain.

D-Xylose is not metabolized by yeast but it non-competitively inhibits the hexokinase PII reaction and inactivates the enzyme (De la Fuente *et al.*, 1970; Fernández *et al.*, 1985). Yeast hexokinase PI is inactivated by D-xylose due to the phosphorylation of the enzyme protein (Menezes & Pudles, 1976). The physiological roles of the hexokinase isoenzymes are rather different. Hexokinase PI is synthesized constitutively and hexokinase PII has both catalytic and regulatory functions. The interaction between D-xylose and the PII enzyme produces a structural change which triggers derepression by an as yet unknown mechanism.

This paper deals with the characterization of an ATP-dependent, irreversible inactivation of hexokinase PII by D-xylose.

METHODS

Materials. Sigma reagents and enzymes were used throughout. Yeast extract was obtained from Difco. Materials for electrophoresis and Bio-Gel P-10 were obtained from Bio-Rad. [γ - 32 P]ATP was from New England Nuclear.

Organism and growth conditions. *Saccharomyces cerevisiae* G-517 (CECT 1317) was inoculated into flasks with 300 ml medium containing 1% (w/v) yeast extract and 2% (w/v) glucose and incubated in a rotatory shaker at 28 °C. Growth was measured as the OD₆₀₀. Yeast cells were harvested at an OD₆₀₀ of 2.4 and a glucose concentration of 0.5%. The cells were washed twice with distilled water and resuspended in 10 mM-potassium phosphate buffer, pH 7.0. Under these conditions hexokinase PII represents 64–70% of the total phosphotransferase activity of the cell (Fernández *et al.*, 1985).

Purification of hexokinase PII. Hexokinase PII was purified at 4 °C by the following procedure. *S. cerevisiae* cells were harvested as described above and homogenized for 20 min in a cell homogenizer (Vibrogen cell mill) with

Table 1. *Purification of hexokinase PII from S. cerevisiae*

Step	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)	Purification (-fold)	Yield (%)
Cell-free extract	120	558	228	0.4	1	100
Hydroxylapatite chromatography	30	82.5	163	2.0	5	71
Chromatofocusing	13.5	2.3	99	43	107	43
DEAE-Sephadex A-50 chromatography	15.5	0.45	59	132	330	26

glass beads (0.5 mm in diameter) in 25 ml 10 mM-potassium phosphate buffer, pH 7.0. The beads were removed and washed with 100 ml buffer. The washings and extract were pooled, and centrifuged at 39000 *g* for 20 min; the pellet was then discarded (this type of cell-free extract was used in the experiment shown in Table 2). The extract was applied to a hydroxylapatite HA-Ultrogel column (2.5 × 15 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. Hexokinase PII activity was eluted with a 500 ml linear gradient of 10–350 mM-potassium phosphate buffer, pH 7.0. The fraction volume was 5 ml. Fractions containing hexokinase PII activity were pooled and desalted over Bio-Gel P-10 with a buffer change (25 mM-histidine/HCl, pH 6.2). The effluent was applied to a PBE 94 column (1 × 36 cm), equilibrated with 25 mM-histidine/HCl buffer, pH 6.2, and fractionated with Polybuffer 74 (pH 4.0) (Pharmacia) in a pH gradient ranging from 6.2–4.0 (fraction size 2.5 ml). The fractions from the chromatofocusing step that had hexokinase PII activity were pooled and ampholytes removed by filtration over Bio-Gel P-10 with a buffer change (10 mM-potassium phosphate, pH 7.0). The effluent was applied to a DEAE-Sephadex A-50 column (1.5 × 25 cm) equilibrated with 10 mM-potassium phosphate buffer, pH 7.0. Hexokinase PII activity was eluted with a linear gradient of 0–1 M-NaCl in 500 ml 10 mM-potassium phosphate buffer, pH 7.0. The active fractions were pooled and stored at 4 °C. The result of this purification is summarized in Table 1.

Analytical methods. Hexokinase PII was assayed as in Fernández *et al.* (1985), glutamate dehydrogenase (NAD-dependent) as in Doherty (1970), NADH dehydrogenase as in Polakis *et al.* (1965), total malate dehydrogenase as in Ochoa (1955), isocitrate lyase as in Dixon & Kornberg (1959), maltase as in Zimmermann & Eaton (1974) and invertase as in Gascón & Lampen (1968). Protein concentration was determined by the method of Lowry with bovine serum albumin as standard.

Inactivation of hexokinase PII. The inactivation mixture contained the following in a final volume of 1 ml: 10 mM-potassium phosphate buffer, pH 7.0; 5 mM-magnesium chloride; 0, 25 or 100 mM-D-xylose; 0 or 4 mM-ATP; and 100 milliunits (mU) of hexokinase PII. The mixture was incubated at 30 °C. At appropriate time intervals, samples were taken and assayed for hexokinase PII as described above.

Phosphorylation assay. The incubation mixture contained the following in a final volume of 1 ml: 10 mM-potassium phosphate buffer, pH 7.0; 5 mM-magnesium chloride; 100 mM-D-xylose; and 1 U hexokinase PII. The assay was started by the addition of 5 µCi (185 kBq) [γ -³²P]ATP [10.6 Ci mmol⁻¹ (392.2 GBq mmol⁻¹)]. At appropriate times after incubation at 30 °C, 20 µl samples were taken for the assay of enzyme activity and 15 µl samples for the phosphorylation assay. The latter samples were rapidly mixed with 5 µl SDS buffer (8%, w/v, SDS; 250 mM-Tris/HCl, pH 6.8; 8 mM-EDTA; 35%, v/v, glycerol; 2.5%, v/v, 2-mercaptoethanol; 0.002% bromphenol blue) and immediately boiled for 7 min. The samples were subjected to SDS-PAGE on slab gels (Britton *et al.*, 1982). Gels were stained, dried, and exposed to X-Omat S X-ray film for 8 d. The incorporation of ³²P into hexokinase PII was measured by densitometry with a Chromoscan 3 (Joyce Loebel).

RESULTS

Inactivation of hexokinase PII induced by D-xylose and MgATP

Incubation of purified hexokinase PII with D-xylose (100 mM) in the presence of MgATP (4 mM) resulted in a loss of activity of hexokinase PII (Fig. 1). The inactivation was dependent on the presence of MgATP and proceeded until all enzyme activity was lost. Under these conditions the inactivation kinetics were pseudo-first-order with an apparent rate constant $K = 3.87 \text{ h}^{-1}$. The native enzyme treated under the same conditions but in the absence of MgATP lost 50% of the initial activity with an apparent inactivation rate constant $K = 0.69 \text{ h}^{-1}$.

The ATP-dependent inactivation of hexokinase PII produced by D-xylose was irreversible (Fig. 2). Filtration of D-xylose-inactivated hexokinase PII through a Bio-Gel P-10 column removed the pentose from the inactivated protein but did not restore enzyme activity. Dialysis

Fig. 1

Fig. 2

Fig. 1. ATP-dependent inactivation of hexokinase PII by D-xylose. Enzyme preparations were incubated at 30 °C in 20 mM-Tris/HCl buffer, pH 7.5, with 25 mM-xylose (Δ , $\blacktriangle$) or 100 mM-xylose ($\square$, $\blacksquare$). Controls without pentose were processed simultaneously ($\circ$, $\bullet$). $\blacktriangle$, $\blacksquare$, $\bullet$, Preincubation with 4.0 mM-MgATP; Δ , $\square$, $\circ$, preincubation without MgATP. The results are expressed as the percentage of activity remaining after the treatment.

Fig. 2. Irreversibility of the 'in vitro' inactivation of hexokinase PII by D-xylose. Purified hexokinase PII was incubated at 30 °C for 90 min with 100 mM-xylose and 4 mM-MgATP. The inactivated preparation was applied to a Bio-Gel P-10 column (1 $\times$ 40 cm) and protein was eluted with 10 mM-potassium phosphate buffer, pH 7.0, in 2 ml fractions. Each fraction was assayed for hexokinase PII activity ($\circ$) and reducing sugars (xylose) ($\blacktriangle$). $\bullet$, Hexokinase activity of untreated enzyme.

Fig. 3. Incorporation of ^{32}P into hexokinase PII induced by D-xylose. Purified hexokinase PII was incubated at 30 °C for 90 min with 100 mM-xylose and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The inactivated preparation was applied to a Bio-Gel P-10 column (1 $\times$ 40 cm) and protein was eluted as described in Fig. 2. Hexokinase PII activity was determined without ($\bullet$) or with xylose ($\circ$). $\blacktriangle$, ^{32}P radioactivity.

of D-xylose-inactivated hexokinase PII against 10 mM-potassium phosphate buffer, pH 7.0, also did not restore enzyme activity.

Incorporation of phosphate into hexokinase PII

When purified hexokinase PII was incubated in the presence of D-xylose and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, ^{32}P was incorporated into hexokinase PII (Fig. 3). No incorporation was observed in the absence of

Fig. 4. Inactivation and phosphorylation of yeast hexokinase PII 'in vitro'. Purified hexokinase PII was incubated with D-xylose and [γ - 32 P]ATP as described in Methods. At different times, samples were taken to measure enzyme activity and incorporation of radioactivity as described in Methods. (a) Autoradiogram showing 32 P incorporation into hexokinase PII. Lanes 1–6, complete mixture after 0, 5, 10, 30, 45 and 60 min incubation, respectively. The mixture without D-xylose (after 60 min incubation) gave the same result as lane 1. Arrow indicates the position of hexokinase PII in the gel. (b) Relationship between the loss of hexokinase PII activity (■) and the incorporation of 32 P (▲).

Table 2. Effect of xylose on the catabolic repression of some enzymes from *S. cerevisiae*

Enzyme	Specific activity (mU mg ⁻¹)			
	2 h culture		6 h culture	
	Glc	Glc + Xyl	Glc	Glc + Xyl
Invertase	107.0	321.0	260.0	560.0
Maltase	9.0	33.0	31.0	180.0
Glutamate dehydrogenase	0.0	0.0	16.0	18.0
NADH dehydrogenase	7.3	8.2	21.0	21.0
Malate dehydrogenase	1250	1730	4500	4100
Isocitrate lyase	0.0	0.0	780	770

D-xylose. As shown in Fig. 4 the incorporation of 32 P into the protein can be correlated with the loss of activity. From the radioactivity incorporated, the specific radioactivity of [γ - 32 P]ATP, and the molarity of the enzyme in the incubation mixture, it can be calculated that 1 mol of phosphate per mol of enzyme was incorporated after 90 min.

Effect of xylose on the catabolic repression of selected enzymes

The relationship between the xylose-induced decrease in hexokinase PII activity and the derepression of invertase synthesis in yeast has been described by Fernández *et al.* (1984). When xylose was added to cells growing in a yeast nitrogen base (or yeast extract) plus glucose medium, catabolic repression was suppressed, as is shown by the large increase in invertase levels even if the glucose concentration remained high. Table 2 summarizes the effect of xylose on the activity of enzymes selected as representative of different pathways: only invertase and maltase were freed from catabolite repression.

DISCUSSION

Hexokinase PII has been reported to be of major importance in carbon catabolite repression (Fröhlich *et al.*, 1984; Entian *et al.*, 1984; Fernández *et al.*, 1984). The hexokinase PII molecule provides a repression signal for certain enzymes when glucose is present in the culture medium. This requires some kind of change in enzyme conformation in response to the presence of carbon sources such as glucose or fructose.

Work on *hex-1r* mutants indicated that these are two different domains in the hexokinase PII molecule, one with catalytic properties, the other with regulatory properties (Entian & Fröhlich, 1984). The catalytic centre of hexokinase PII was unaffected in these mutants. The mutation affected the regulatory domain, which is responsible for the glucose repression of invertase, maltase, malate dehydrogenase and respiratory enzymes.

Our results show that the inactivation of yeast hexokinase PII by xylose and MgATP is related to the phosphorylation of the protein. Simultaneous with the phosphorylation, the synthesis of invertase and maltase was derepressed but the synthesis of malate dehydrogenase, NADH dehydrogenase, glutamate dehydrogenase and isocitrate lyase was repressed.

These experiments also showed that since malate dehydrogenase levels were unaffected, the carbon catabolite repression signal produced by the phosphorylated enzyme was different from that produced in *hex-1r* mutants. The xylose-induced phosphorylation of hexokinase PII may produce a conformational change in the enzyme which could provide the triggering signal for invertase and maltase derepression. Although the essential role of hexokinase PII in triggering glucose repression seems to be established, the molecular mechanism is still unknown. The identification of phosphorylated hexokinase PII isoenzyme under culture derepression conditions could give a direct physiological role to the observed covalent modification of the enzyme.

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