Inhibition and Inactivation of Glucose-phosphorylating Enzymes from Saccharomyces cerevisiae by D-Xylose

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Three glucose-phosphorylating enzymes were separated from cell-free extracts of Saccharomyces cerevisiae by hydroxylapatite chromatography. Variations in the amounts of these enzymes in cells growing on glucose and on ethanol showed that hexokinase PI was a constitutive enzyme, whereas synthesis of hexokinase PII and glucokinase were regulated by the carbon source used. Glucokinase proved to be a glucomannokinase with $K_m$ values of 0.04 mM for both glucose and mannose. D-Xylose produced an irreversible inactivation of the three glucose-phosphorylating enzymes depending on the presence or absence of ATP. Hexokinase PI inactivation required ATP, while hexokinase PII was inactivated by D-xylose without ATP in the reaction mixture. Glucokinase was protected by ATP from this inactivation. D-Xylose acted as a competitive inhibitor of hexokinase PI and glucokinase and as a non-competitive inhibitor of hexokinase PII.

INTRODUCTION

Yeasts produce three glucose-phosphorylating enzymes. Hexokinase PI and hexokinase PII have ratios of phosphorylation of fructose to glucose of approximately 2.5 and 1.5, respectively (Barnard, 1975; Gancedo et al., 1977). The third enzyme, glucokinase, does not use fructose as a substrate (Maitra, 1970). The physiological roles of the three enzymes remain unclear. Any one of them enables growth on glucose, and either of the two hexokinases enables growth on fructose (Gancedo et al., 1977; Lobo & Maitra, 1977) but very little is known about the regulation of their levels in yeasts. Hirai et al. (1977) reported the constitutive nature of hexokinase and glucokinase synthesis in Candida lipolytica. They also showed that in Candida tropicalis hexokinase was specifically induced by sugars, while glucokinase was a constitutive enzyme. On the other hand, no changes were observed for the hexokinase isoenzymes of Saccharomyces cerevisiae grown on non-fermentable carbon sources or glucose (Entian et al., 1984).

Hexokinase PII seems to predominate in growth on glucose (Gancedo et al., 1977) and its involvement in carbon catabolite repression has been proposed (Entian & Mecke, 1981). A decrease in hexokinase PII activity induced by D-xylose leads to the relief of carbon catabolite repression of invertase (Fernández et al., 1984). On the other hand, a highly specific inactivation of yeast and animal hexokinase induced by D-xylose has been reported (De la Fuente et al., 1970; Lazo & Sols, 1979) which is said to involve a potentially reversible paracatalytic phosphorylation of the enzyme at a serine residue (Menezes & Pudles, 1976). Xylose is a non-phosphorylatable glucose analogue that competitively inhibits the hexokinase reaction (De la Fuente et al., 1970).

This paper deals with the separation of the different glucose-phosphorylating enzymes from Saccharomyces cerevisiae. We have verified that the synthesis of the hexokinase PI is constitutive and that the levels of hexokinase PII and glucokinase are regulated by the carbon source. Moreover, the hexokinase isoenzymes and glucokinase are susceptible to specific inactivation induced by D-xylose in different ways.
METHODS

**Materials.** Sigma reagents and enzymes were used throughout. Yeast Nitrogen Base was obtained from Difco. HA-Ultrogel hydroxylapatite was purchased from LKB.

**Yeast strain, growth conditions and preparation of cell-free extracts.** Saccharomyces cerevisiae G-517 (CECT 1317) was inoculated into flasks with 300 ml medium containing 0.7% Yeast Nitrogen Base and 1% (w/v) glucose or 1% (v/v) ethanol and incubated in a rotatory shaker at 28 °C. Growth was followed by changes in optical density at 600 nm. Yeasts were harvested from glucose cultures, when the OD₆₀₀ was 1.2 and the sugar concentration reached 0.65%, (early exponential phase) or when glucose had been exhausted and the OD₆₀₀ was 2.5 (stationary phase). Cells were collected from ethanol cultures when the OD₆₀₀ was 0.8 and the carbon source concentration reached 0.50%, (early exponential phase). In all cases, the cells were washed twice with distilled water and resuspended in 10 ml 10 mM-potassium phosphate buffer, pH 7.0.

For cell disruption, equal volumes of yeast cell suspension and glass beads 0.45 to 0.50 mm in diameter were shaken at 4 °C for 10 min in a Vibrogen Cell Mill homogenizer. This treatment disrupted all the cells, as judged by light microscopy. The glass beads were removed by washing the broken cell suspension through a coarse sintered glass filter. Cell debris was removed by centrifugation at 10000 g for 20 min. The supernatant fluid was used as cell-free extract.

**Glucose and ethanol determination.** Glucose was determined by a glucose oxidase-peroxidase coupled assay. Ethanol was determined by following the reduction of NAD⁺ by ethanol in the presence of alcohol dehydrogenase (Fernández et al., 1984).

**Chromatographic separation of glucose-phosphorylating enzymes.** Samples from cell-free extracts obtained after different culture times, or grown in different carbon sources, were chromatographed on hydroxylapatite HA-Ultrogel columns (2.5 cm × 10 cm), equilibrated and eluted at 4 °C with 10 mM-potassium phosphate buffer, pH 7.0. The enzymes were separated by a 400 ml linear potassium phosphate gradient, 10 to 300 mM, at pH 7.0. Fractions (5 ml) were collected in an Ultrorac fractionator. The gradient shape was checked with a conductivity meter. The separated hexokinase isoenzymes and glucokinase were used for the inactivation and inhibition experiments.

**Enzyme assays.** Glucose-phosphorylating activity was followed by measurement of glucose 6-phosphate formation, coupling the reaction with NAD⁺ and an excess of glucose-6-phosphate dehydrogenase. NADPH formation was followed at 340 nm in a Perkin-Elmer spectrophotometer maintained at 25 °C in a medium containing (final concentrations): 4 mM-glucose; 20 mM-Tris/HCl buffer, pH 7.5; 7 mM-MgCl₂; 10 mM-ATP; 0.65 mM-NAD⁺; 2 units glucose-6-phosphate dehydrogenase and 10–50 μl of suitably diluted enzyme preparation. Assay systems lacking ATP were used as blanks. When fructose was used as a substrate, glucose 6-phosphate was measured by the addition of an excess of phosphoglucone isomerase. When mannose was used as a substrate, glucose 6-phosphate was measured by the addition of an excess of phosphoglucone isomerase and phosphomannose isomerase. The formation of 1 μmol glucose 6-phosphate min⁻¹ was taken as 1 unit.

**RESULTS AND DISCUSSION**

Three glucose-phosphorylating enzymes, with different specificities for glucose and fructose, were separated from the cell-free extracts of *Saccharomyces cerevisiae* by hydroxylapatite chromatography (Fig. 1). Two of them, which phosphorylated fructose 2·5 and 1·5 times faster than glucose, were identified as hexokinases PI and PII, respectively, and the third, with very low or no fructose-phosphorylating activity, was identified as glucokinase. *Kₘ* values with glucose were 0·12 mm for both hexokinases, and 0·04 mm for glucokinase. Using fructose as a substrate, hexokinases PI and PII had *Kₘ* values of 0·33 mm.

Chromatographic profiles similar to those in Fig. 1 were obtained from stationary phase cell extracts; however, the percentages of hexokinase PII and glucokinase changed with the physiological state of the yeasts (Table 1). In the presence of glucose, the amount of hexokinase PII represented 64% of the total phosphotransferase activity whereas glucose consumption led to a decrease of hexokinase PII activity to 30% of the total activity. The difference in activity was not due to difference in recoveries, which were 90% in the three columns. The differences in activity could have been due to differences in contaminating substances that affect the activity since the eluted enzymes were not pure. On the other hand, the recoveries were consistent with the total activity adsorbed to the hydroxylapatite columns.

From these results we concluded that hexokinase PI was synthesized constitutively but synthesis of hexokinase PII and glucokinase were regulated by culture conditions. The specific activity of glucokinase increased when the predominant metabolic conditions were gluconeo-
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Fig. 1. Separation of glucose-phosphorylating enzymes by hydroxylapatite chromatography. The cell-free extract was obtained from a 6 h culture. The glucose concentration in the supernatant was 37 mM at the time of harvesting. Samples containing 11 units of glucose-phosphorylating activity were chromatographed on a 2.5 cm x 10 cm column of HA-Ultrogel (LKB) and 90% activity was recovered from the column. After washing with 10 mM-potassium phosphate, the sample was eluted with a 400 mM linear gradient of potassium phosphate (-----), 10 to 300 mM, pH 7.0. Fractions (5 ml) were assayed spectrophotometrically as described in Methods with glucose (●) or with fructose (○) as substrate. The ratio of fructose phosphorylation to glucose phosphorylation at the peaks was 1.5 (hexokinase PII) and 2.5 (hexokinase PI). The potassium phosphate concentrations at the peaks were 48 mM (hexokinase PII), 100 mM (hexokinase PI) and 150 mM (glucokinase). This type of enzyme preparation was used throughout the work.

Table 1. Activity of glucose-phosphorylating enzymes of yeasts grown under different conditions

The yeast cells were grown and samples taken as described in Methods. The cells were broken and the cell-free extracts (11 units for glucose early exponential culture; 28 units for glucose stationary culture and 10 units for ethanol culture) were applied to a hydroxylapatite HA-Ultrogel column. The percentage of each enzyme was calculated on the basis of total glucose-phosphorylating activity applied to the column.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Stage of growth</th>
<th>Hexokinase PI ((%))</th>
<th>Hexokinase PII ((%))</th>
<th>Glucokinase ((%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Early exponential</td>
<td>9</td>
<td>64</td>
<td>26</td>
</tr>
<tr>
<td>Glucose</td>
<td>Stationary</td>
<td>9</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Early exponential</td>
<td>9</td>
<td>31</td>
<td>60</td>
</tr>
</tbody>
</table>

genic, while that of hexokinase PII decreased under these conditions. The total phosphorylating capacity of the yeasts was constant (data not shown) despite changes in the relative amount of each phosphotransferase.

Glucokinase predominated in growth on ethanol and it was identified as a glucomannokinase. Glucokinase was capable of phosphorylating mannose with a \(K_m\) of 0.04 mM which was shown to be a competitive inhibitor of glucose with a \(K_i\) of 0.06 mM. Inclusion of other sugars in the spectrophotometric assay in a proportion of 20:1 to the glucose molarity showed that glucokinase did not phosphorylate fructose, galactose, sorbose, rhamnose, arabinose, mannitol, sorbitol or ribose.

The 'in vitro' effect of D-xylose on the three partially purified kinases was also studied. D-Xylose inactivated the three kinases, the extent of this effect depending on the presence or absence of ATP. The enzyme preparations from hydroxylapatite chromatography were incubated with two concentrations of D-xylose with and without ATP. Samples were taken at different times and assayed for glucose phosphorylation as indicated in Methods. The results
Fig. 2. Time courses of the inactivation of glucose-phosphorylating enzymes by D-xylose. Enzyme preparations, obtained as described for Fig. 1, were incubated at 30 °C in 20 mM-Tris/HCl buffer, pH 7.5, with 25 mM-D-xylose (Δ, ▲) or 100 mM-D-xylose (□, ■). Controls without pentose were processed simultaneously (○, •). Filled symbols indicate preincubation with 4.0 mM-MgATP; open symbols indicate preincubation without MgATP. Samples were taken and glucose-phosphorylating activity was assayed as described in Methods. The results are expressed as the percentage of activity remaining after the treatment.

Fig. 3. Inhibition of glucose-phosphorylating enzymes by D-xylose. Enzyme preparations were obtained as described for Fig. 1. D-Glucose-phosphorylating activity was determined without D-xylose (○), with 40 mM-D-xylose (●), with 80 mM-D-xylose (▲) obtained with hexokinase P1 (Fig. 2a) are in agreement with those previously described for the animal enzyme (Lazo & Sols, 1979). The inactivation of the enzyme depended on the presence of ATP, suggesting that the mechanism of inactivation could be a phosphorylation of the protein (Menezes & Pudles, 1976). On the other hand, hexokinase P11 inactivation by D-xylose was independent of ATP (Fig. 2b) and glucokinase was inactivated by D-xylose but ATP protected the enzyme from inactivation (Fig. 2c).

In addition to the inactivation observed, D-xylose acted as an inhibitor of the three kinases (Fig. 3). D-Xylose was shown to be a competitive inhibitor of hexokinase P1 and glucokinase, with $K_i$ values of 25 mM and 0.85 mM, respectively, and a non-competitive inhibitor of hexokinase P11 with a $K_i$ of 80 mM.

These results explain the apparent inactivation observed (Fig. 2c) for glucokinase in the absence of ATP as an inhibition of the enzyme produced by D-xylose. Hexokinase P1 showed an inactivation pattern similar to the one described by Lazo & Sols (1979) for the animal hexokinase isoenzymes. Also, in this case it is now known why the inactivation does not approach completion. The inactivation of hexokinase P1 by D-xylose was 50% under all the metabolic conditions assayed. Neither inactivation rate nor inactivation percentage were altered by inclusion of 20 mM-MgATP during the preincubation period. Reactivation of the glucose-phosphorylating enzymes in xylose-inactivated cells by transfer into a xylose-free, glucose-containing medium was prevented by cycloheximide (Fig. 4). The reactivation was therefore dependent on 'de novo' protein synthesis. Dialysis of xylose-inactivated enzyme preparations did not reactivate the phosphotransferase activity.
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Fig. 4. Effect of cycloheximide on the reactivation of glucose-phosphorylating enzymes. Activity of glucose-phosphorylating enzymes after transfer from a medium with 1% glucose and 300 mM-D-xylose (○) to a medium with 1% glucose and 100 μg cycloheximide ml⁻¹ (△) or a medium with 1% glucose (△): ●, control culture. The arrow indicates the time at which cell transfers were done.

Our results suggest that the interaction of D-xylose with hexokinase PII took place at a site other than the catalytic one (non-competitive inhibitor), which is in agreement with the results obtained with mutants and provides evidence for hexokinase PII acting as a bifunctional enzyme (Entian & Fröhlich, 1984) with catalytic and regulatory properties. D-Xylose reverses catabolite repression of invertase and inactivates hexokinase PII (Fernández et al., 1984) but it is not clear whether this is due to a loss of catalytic activity or to changes in the protein conformation. Further studies are needed to define the molecular mechanism of catabolite repression in yeast.

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REFERENCES


