Autophosphorylation of Yeast Hexokinase PII

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(Received 3 March 1988; revised 15 May 1988)

Autophosphorylation of hexokinase PII was studied using an enzyme purified from *Saccharomyces cerevisiae*. Incubation of this enzyme preparation with [γ - 32 P]ATP and Mn^{2+} or Mg^{2+} gave a phosphoprotein of molecular mass 58 000 which corresponded to hexokinase PII. D-Xylose stimulated autophosphorylation of hexokinase PII. Dilution of hexokinase PII over a 10-fold concentration range did not change the specific activity of hexokinase PII autophosphorylation suggesting that it may occur by an intramolecular mechanism.

INTRODUCTION

Three glucose-phosphorylating enzymes, glucokinase and two hexokinase isoenzymes PI and PII have been described in yeast (Colowick, 1973; Barnard, 1975). In the last few years, special attention has been given to hexokinase PII, since it has both catalytic and regulatory properties (Frohlich *et al.*, 1984; Entian *et al.*, 1984; Fernández *et al.*, 1984). In particular, the carbon catabolic repression of invertase and maltase was relieved in yeast with xylose-inactivated hexokinase PII (Fernández *et al.*, 1984) and by a mutation in the hexokinase PII structural gene (Entian & Mecke, 1982). These results suggested a direct role of hexokinase PII in the regulation of carbon catabolite repression in yeast.

The detailed molecular mechanism of catabolite repression is unknown, but inactivation of yeast hexokinase PII by xylose is not paralleled by the loss of hexokinase PII protein (Fernández *et al.*, 1987). However, it has been suggested that xylose-induced phosphorylation of hexokinase PII *in vivo* may produce a change in enzyme conformation which could provide the triggering signal to activate an additional activity (Fernández *et al.*, 1987). We therefore examined autophosphorylation of hexokinase PII as a possible triggering signal to activate the carbon catabolite derepression process. Here we report the autophosphorylation of hexokinase PII and we characterize the mechanism and kinetics of this reaction.

METHODS

Materials. Yeast extract was obtained from Difco. Hydroxyapatite HA-Ultrogel was purchased from LKB. Chromatofocusing gel PBE94 was obtained from Pharmacia. Material for electrophoresis was from Bio-Rad Laboratories. [γ - 32 P]ATP (3000 Ci mmol $^{-1}$; 111 TBq mmol $^{-1}$) was from New England Nuclear. Sigma reagents and enzymes were used throughout.

Strain and growth conditions. *Saccharomyces cerevisiae* mutant strain D.308.3 (*MAT α hsk1 hsk2 glk1 ade1 trp1 his2 met14*), obtained from the Yeast Genetic Stock Center, (University of California, Berkeley, USA), was used as recipient in transformation experiments. Plasmid YRp/HXK 2-5 was kindly provided by K. D. Entian (University of Tübingen, FRG) (Entian *et al.*, 1985) and vector Yep1357 was used as a multicopy vector (Rodicio & Zimmermann, 1985). Hybrid plasmids Yep1357-HXK 2-5, complementing the *hsk2* mutation, were selected in a medium with glucose as carbon source as described by Fernández *et al.* (1987). Due to the multicopy effect of the vector Yep1357 an approximate 20-fold increase in the specific activity of hexokinase PII was observed in the Yep1357-HXK 2-5 transformant of D.308.3 compared to the wild-type strain. The Yep1357-HXK 2-5 transformant of D.308.3 was used as one of the sources of hexokinase PII.

The cells were grown in flasks with 300 ml medium containing 1% (w/v) yeast extract and 2% (w/v) glucose, supplemented with 30 mg adenine l⁻¹, on a rotatory shaker at 28 °C. Growth was followed by determination of the optical density at 600 nm. Yeast were harvested from cultures at an OD₆₀₀ of 0.9 when the sugar concentration reached 50 mM (repressed cells). The cells were washed twice with distilled water and resuspended in 10 mM-potassium phosphate, pH 7.0 for further treatment.

Sources of hexokinase PII. Two different sources of hexokinase PII have been used with identical results: (a) hexokinase PII from the Yep1357-HXK 2-5 transformant of D.308.3 purified using a three-step procedure (cell-free extract preparation, chromatofocusing and hydroxyapatite chromatography as described by Fernández *et al.*, 1986); (b) Sigma hexokinase PII (H-5875), which was further purified by chromatography on hydroxyapatite HA-Ultrogel (Moreno *et al.*, 1986).

The samples containing hexokinase PII (20 µg) from the last purification step were analysed by SDS-PAGE. We concluded that the hexokinase PII had been purified to homogeneity, from both sources, as the last enzyme preparation had only a single protein band (58 kDa) (Fernández *et al.*, 1987).

Autophosphorylation assay. Hexokinase PII, dialysed against 10 mM-potassium phosphate buffer, pH 7.0, was diluted 25-fold with 50 mM-HEPES/NaOH, pH 7.5, to give a final concentration of 172 µg protein ml⁻¹. Hexokinase PII (0.5 to 5 µg) was then incubated at 4 °C or 30 °C in 50 µl 50 mM-HEPES/NaOH, pH 7.5. Mn²⁺, Mg²⁺, D-xylose and [γ-³²P]ATP (1 × 10⁷ d.p.m.) were added at the concentrations indicated in the Figure legends. The reaction was started by addition of the enzyme protein and the incubation was continued for various time periods.

The reaction was terminated by adding 15 µl of 4-fold concentrated Laemmli sample buffer to obtain final concentrations of the following components: 2% (w/v) SDS; 0.5% (w/v) β-mercaptoethanol; 0.01% (w/v) bromphenol blue; 10% (v/v) glycerol; 2 mM-EDTA and 60 mM-Tris/HCl, pH 7.0 (Britton *et al.*, 1982). This mixture was heated immediately in a boiling water-bath for 3 min. The proteins were separated by SDS-PAGE using 3.5% (w/v) stacking and 12% (w/v) resolving polyacrylamide gels according to the method of Britton *et al.* (1982). Proteins were fixed and stained for 15 min at 55 °C with 45% (v/v) methanol, 10% (v/v) acetic acid containing 0.25% (w/v) Coomassie blue. The gels were destained at 55 °C for 5 h in 7.5% (v/v) acetic acid, 5% (v/v) methanol and dried *in vacuo* for 2 h at 45 °C.

Radioactivity was located in gel fragments by autoradiography and quantified by measuring the area of the peak obtained by scanning for absorbance at 560 nm with a Joyce Loebel scanner.

RESULTS

Effect of D-xylose, Mg²⁺ and Mn²⁺ on hexokinase PII autophosphorylation

An autoradiogram showing the *in vitro* autophosphorylation at 4 °C of hexokinase PII, purified to homogeneity from the Yep1357-HXK 2-5 transformant of D.308.3, is shown in Fig. 1. The phosphoprotein had a molecular mass of 58 kDa. An identical molecular mass has been described for hexokinase PII protein (Fernández *et al.*, 1987). In the absence of Mn²⁺ autophosphorylation occurred slowly and was not detected using Mg²⁺ under these experimental conditions. Xylose (100 mM) stimulated the incorporation of ³²P from [γ-³²P]ATP into hexokinase PII. Within 1 min, autophosphorylation of hexokinase PII, in the absence of xylose in the reaction mixture, reached 40% of the autophosphorylation value reached in the presence of the pentose.

Time-course of hexokinase PII autophosphorylation

Fig. 2 shows the time-course of *in vitro* autophosphorylation of hexokinase PII at 4 °C using 4 µM-ATP and Mn²⁺ (Fig. 2A) and at 30 °C using 4 mM-ATP and Mg²⁺ (Fig. 2B). D-Xylose stimulated the incorporation of ³²P from [γ-³²P]ATP into hexokinase PII under both sets of experimental conditions described. As shown in Fig. 2A, within 50 s autophosphorylation of hexokinase PII reached 50% of the maximum. Maximal phosphorylation was reached after about 2 min. Incubations for longer periods produced a decrease in ³²P incorporation into hexokinase PII. Fig. 2B shows the time-course of autophosphorylation of hexokinase PII at 30 °C. Within 5 min, autophosphorylation of hexokinase PII reached 50% of the maximum and a steady-state value was reached after about 20 min.

Effect of hexokinase PII concentration on autophosphorylation

Because the mechanism of hexokinase PII autophosphorylation could take place either by an intramolecular or by an intermolecular process, the influence of enzyme concentration on the

Fig. 1. Effect of D-xylose, Mg²⁺ and Mn²⁺ on the autophosphorylation of hexokinase PII. Purified hexokinase PII (5 µg), obtained from the Yep1357-HXK 2-5 transformant of D.308.3, was used to assay autophosphorylation. After 1 min incubation at 4 °C samples were taken for the measurement of ³²P incorporation into protein. The first reaction mixture contained 50 mM-HEPES/NaOH, pH 7.5, 4 µM-γ-³²P]ATP and 12 mM-MnCl₂. Additional components were 100 mM-D-xylose (lane b) or 50 mM-HEPES/NaOH, pH 7.5, in place of xylose (lane d). The second reaction mixture contained 12 mM-MgCl₂ in place of MnCl₂. Additional components were 100 mM-D-xylose (lane a) or 50 mM-HEPES/NaOH, pH 7.5, in place of xylose (lane c). The reactions were terminated by heating with SDS-gel electrophoresis sample buffer for 3 min. The reactions were separated by SDS-PAGE. Gels were dried and exposed to X-ray films for autoradiography. Identical results were obtained by using the two different sources of hexokinase PII indicated in Methods.

reaction rate was investigated. In an intramolecular reaction, the velocity at a constant ATP concentration would be linear (first-order) with respect to enzyme concentration, and parabolic (second-order) in an intermolecular reaction (Huang *et al.*, 1986). Furthermore, a plot of log velocity versus log enzyme concentration (a van't Hoff plot) would yield a slope equal to the enzyme stoichiometry in the reaction. Such a plot would have a slope of 1 for an intramolecular or a slope of 2 for an intermolecular reaction. The results obtained demonstrated that the specific activity of hexokinase PII autophosphorylation is independent of the protein concentration over a 10-fold dilution range (data not shown). Analysis of the relationship between reaction rate and protein concentration by means of a van't Hoff plot (Fig. 3) shows that the slope equals 1.0. These findings indicate that the autophosphorylation of hexokinase PII is intramolecular.

DISCUSSION

The results presented in this report demonstrate that hexokinase PII can undergo autophosphorylation. The rate of autophosphorylation and also the final extent of ³²P incorporation were stimulated by the presence of D-xylose in the assay mixture.

Protein autophosphorylation is usually measured at 4 °C using Mn²⁺ and a low ATP concentration. Hexokinase PII autophosphorylation under these assay conditions was saturated within 2 min and then declined (Fig. 2A). The reversal of autophosphorylation was only observed at very low ATP concentrations. The kinetics were similar to those observed for the

Fig. 2. Autophosphorylation of hexokinase PII under different assay conditions. A, Time-course of autophosphorylation under different assay conditions using 5 μ g hexokinase PII. At various times, samples were taken for the measurement of 32 P incorporation into protein. The basic reaction mixture contained 50 mM-HEPES/NaOH, pH 7.5, 4 μ M-[γ - 32 P]ATP and 12 mM-MnCl₂. Additional components were (1) 100 mM-D-xylose (●) or (2) 50 mM-HEPES/NaOH, pH 7.5, in place of xylose (▲). Below A is an autoradiogram of hexokinase PII samples separated by SDS-PAGE after incubation at 4 °C for 30 s (lane a), 2 min (lane b), 5 min (lane c) or 10 min (lane d), under condition 1. B, Time-course of autophosphorylation under different assay conditions using 5 μ g hexokinase PII. At various times, samples were taken for the measurement of 32 P incorporation into protein. The basic reaction mixture contained 50 mM-HEPES/NaOH, pH 7.5, 4 mM-[γ - 32 P]ATP and 12 mM-MgCl₂. Additional components were (1) 100 mM-D-xylose (○) or (2) 50 mM-HEPES/NaOH, pH 7.5, in place of xylose (△). Below B are autoradiograms of hexokinase PII samples separated by SDS-PAGE after incubation at 30 °C for 10 min (lane a), 20 min (lane b), 30 min (lane c) or 60 min (lane d) under condition 1 or condition 2. Identical results were obtained by using the two different sources of hexokinase PII indicated in Methods. The radioactivity located in gels by autoradiography was quantified by measuring the area of the peak obtained by scanning for absorbance at 560 nm and expressed as relative units.

reversal of autophosphorylation of type II cAMP-dependent protein kinase. This reversal is attributed to the increase of ADP concentration in the reaction mixture (Erichman *et al.*, 1983). Under the other assay conditions studied, protein phosphorylation slowly increased throughout a long incubation period reaching a steady state (Fig. 2B). These differential kinetics could be explained if hexokinase PII were a protein kinase presenting autophosphorylation (Blowers & Trewavas, 1987).

Fig. 3. Effect of enzyme concentration on autophosphorylation. Relationship between log hexokinase PII autophosphorylation velocity and log enzyme concentration (van't Hoff plot). The reaction rates were determined under the standard assay conditions of Fig. 2A containing 100 mM-D-xylose and 0.5 to 5 μg hexokinase PII (●) or were determined under the standard assay conditions of Fig. 2B containing 100 mM-D-xylose and 0.5 to 5 μg of hexokinase PII (○). The slope is 1.0.

The mechanism of hexokinase PII autophosphorylation appears to be intramolecular since phosphorylation was first-order with respect to enzyme concentration. This approach was used previously to determine the mechanism of autophosphorylation of the type II cAMP-dependent protein kinase (Erlichman *et al.*, 1983), the insulin receptor-kinase (Shia *et al.*, 1983), Ca^{2+} -calmodulin-dependent protein kinase (Lai *et al.*, 1986), and protein kinase C (Huang *et al.*, 1986). These studies have led to the conclusion that all of these kinases undergo intramolecular autophosphorylation.

The essential role of hexokinase PII for triggering glucose repression seems to be established (Frohlich *et al.*, 1984; Entian *et al.*, 1984; Fernández *et al.*, 1984), but the molecular mechanism is still unknown. Work in this laboratory has established that inactivation of yeast hexokinase PII by xylose is not correlated with the loss of hexokinase PII protein. However, derepression of maltase and invertase synthesis takes place when xylose is present in the culture medium (Fernández *et al.*, 1984). It was suggested that xylose-induced phosphorylation of hexokinase PII *in vivo* may produce a change in the enzyme conformation which could provide the triggering signal to activate an additional activity (Fernández *et al.*, 1987). The present results suggest that hexokinase PII has a xylose-induced protein kinase activity which autophosphorylates the enzyme and could catalyse the phosphorylation of an inactive derepressing signal to an active one.

We thank Professor S. Gascón for his interest in this work and Dr Ma. J. Mazón for the many interesting suggestions. This work was supported by grants from the CAICYT (340/84) and the Fondo de Investigaciones Sanitarias de la Seguridad Social.

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