Proteolysis of Hexokinase PII is Not the Triggering Signal of Carbon Catabolite Derepression in *Saccharomyces cerevisiae*

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The role of hexokinase PII in mediating carbon catabolite derepression in yeast has been examined. Hexokinase isoenzyme PII (EC 2.7.1.1) was partially degraded when protease inhibitors were omitted from the buffer used for preparation of cell-free extracts. The hexokinase PII inactivation induced by D-xylose was correlated with derepression of maltase (EC 3.2.1.20) in the wild-type strain *Saccharomyces cerevisiae* G-517 and in D.308.3, a strain that contains the cloned hexokinase PII gene on a multicopy plasmid. This inactivation was not correlated with the loss of hexokinase PII protein as assayed by immunoblotting. We conclude that during the derepression process there is no release of proteolytic peptides from hexokinase PII.

INTRODUCTION

In the yeast *Saccharomyces cerevisiae* low levels of hexokinase isoenzyme PII (EC 2.7.1.1) cause an increase in invertase (EC 3.2.1.26) and maltase (EC 3.2.1.20) synthesis (Fröhlich *et al.*, 1984; Entian *et al.*, 1984; Fernández *et al.*, 1984). It is possible to obtain a low level of hexokinase PII by a mutation in the hexokinase PII structural gene (*hex1* mutants) (Entian & Mecke, 1982) or by addition of D-xylose to the culture medium (Fernández *et al.*, 1984). When xylose is added to the culture medium, the growth rate, as well as the rate of glucose consumption, are similar to those of yeasts grown without xylose (Fernández *et al.*, 1985). These results indicate that hexokinase PII is not essential for glucose phosphorylation. However, carbon catabolite repression of invertase and maltase is regulated through hexokinase PII (Fröhlich *et al.*, 1984; Entian *et al.*, 1984; Fernández *et al.*, 1984).

The essential role of hexokinase PII for triggering glucose repression seems to be established, but the molecular mechanism is still unknown. There is evidence that the conformation of hexokinase changes during hexose phosphorylation (De la Fuente *et al.*, 1970; De la Fuente & Sols, 1970; Shill & Neet, 1975) and that hexokinase is a good substrate for proteases (Kenkase & Colowick, 1965; Pringle, 1970; Colowick, 1973; Barnard, 1975). Accordingly, two alternative hypotheses based on these known properties of hexokinase are proposed. These speculative hypotheses are also similar to those presented by Entian *et al.* (1985) (Fig. 1). (a) When glucose disappears from the culture medium, a proteolytic activity is synthesized and a peptide released from hexokinase PII acts as a derepressing signal. Several studies on the regulation of proteinases during growth have shown a derepression of proteinase activities upon glucose exhaustion in the cells (Hansen *et al.*, 1977). (b) A conformational change of hexokinase PII acts as a regulatory signal. The conformational change induced by a low level of glucose reveals a site for proteolytic cleavage. The peptide released could be the derepressing signal. Another

Abbreviation: PMSF, phenylmethylsulphonyl fluoride.

Fig. 1. Hypotheses that may explain the mechanism of carbon catabolite repression for invertase and maltase. (a) Mechanism based on the activation of endogenous proteases. (b) A conformational change of hexokinase PII acts as a regulatory signal, inducing proteolysis (1) or activating an additional unknown activity (2).

possibility is that the conformational change activates an additional activity of hexokinase PII, which could catalyse the modification of an inactive derepressing signal to an active one.

Here we report studies on the possible proteolytic regulation of a peptide derepressing signal released from hexokinase PII.

METHODS

Micro-organisms. *Saccharomyces cerevisiae* G517 (CECT 1317) was used as a wild-type strain. The mutant strains P2T22D (*MAT α ade1 hxx1 glk1*), lacking hexokinase PI and glucokinase, and D. 308.3 (*MAT α hxx1 hxx2 glk1 ade1 trp1 his2 met14*) were obtained from the Yeast Genetic Stock Center. D. 308.3 was used as recipient in yeast transformation experiments. *Escherichia coli* HB101 [*F⁻ hsdS20 (r_B⁻ m_B⁻) recA13 ara-14 proA2 lacY1 galK2 rpsL20(Sm^r) xyl-5 mtl-1 supE44 λ^{-}*] was used for amplification of recombinant plasmids.

Preparation of cell-free extracts. The yeasts were grown in flasks with 300 ml medium containing 1% (w/v) yeast extract and 2% (w/v) peptone, supplemented with either 2% (w/v) glucose or 2% (w/v) glucose plus 500 mM-D-xylose, on a rotatory shaker at 28 °C. Growth was followed by the determination of the optical density at 600 nm. Samples (20 ml) were taken at various times and the cells were harvested and washed twice with distilled water. The cells were resuspended in 500 μ l 10 mM-potassium phosphate buffer pH 7.0, containing 1 mM-EDTA and 4 mM-phenylmethylsulphonyl fluoride (PMSF), and were broken by shaking in a vortex mixer with 1 g glass beads. The cells were shaken five times for 1 min each, with 1 min intervals of cooling on ice. The liquid was carefully removed and centrifuged at 12000 *g* for 10 min. The supernatants were used for enzyme assays and for immunoblotting.

***E. coli* media.** LB-medium 1%, w/v, bacto-peptone, 0.5% yeast extract and 0.5%, w/v, NaCl was used. Transformants were selected on LB-medium supplemented with 40 μ g ampicillin ml⁻¹.

Transformation experiments. Plasmid YRp/HXK 2-5 (a plasmid that contains the bacterial ampicillin resistance gene and the yeast *TRP1* gene) was kindly provided by K. D. Entian (University of Tübingen, FRG) (Entian *et al.*, 1985) and vector Yep1357 (constructed from vectors YIp5, YEp13 and YRp7, containing both ampicillin and tetracycline resistance for *E. coli* and *URA3* and *TRP1* for yeast as well as a fragment of the 2 μ m circle with the replication origin) was used as a multicopy vector (Rodicio & Zimmermann, 1985). Transformation of yeast was carried out by the procedure of Beggs (1978). Hybrid plasmids Yep1357-HXK 2-5, complementing *hxx2* mutation, were selected on a medium with glucose as carbon source. *E. coli* cells were transformed by the calcium chloride procedure described by Cohen *et al.* (1972). Plasmids were isolated from *E. coli* in large scale by the method of Birnboim (1983).

Immunization of rabbits. Antiserum against hexokinase PII was raised in white rabbits by intramuscular injection of purified hexokinase PII, as described by Moreno *et al.* (1986). At 7 d intervals each rabbit received four injections containing 0.1 mg of purified enzyme, diluted with 0.5 ml distilled water containing 0.9% NaCl, mixed with 0.5 ml complete Freund's adjuvant. Control sera were obtained from the same rabbits prior to the first injection of antigen and stored frozen at -70°C . Titre of antisera was determined by Ouchterlony double diffusion analysis (Ouchterlony & Nilsson, 1978) of serially diluted samples against purified enzyme. Maximal titre was attained about 5 weeks after the first injection. At this time blood was collected from the carotid artery and allowed to clot. The serum was precipitated with 50% (w/v) ammonium sulphate after centrifugation at 12000 *g* for 15 min. The precipitate was suspended in 0.9% NaCl and stored at -70°C until use.

Double diffusion analysis demonstrated that hexokinase PII, hexokinase PI and glucokinase are not immunologically related. The hexokinase PI and glucokinase purified proteins did not react with the antiserum to hexokinase PII (data not shown).

Immunoblotting. Cell-free extracts containing 50 μg protein were electrophoresed in a SDS-polyacrylamide slab gel (Britton *et al.*, 1982). Proteins were electrophoretically transferred from the gel to a nitrocellulose sheet using a BioRad transblot cell unit. The nitrocellulose sheet was washed with TBS (20 mM-Tris/HCl pH 7.4, 150 mM-NaCl) and incubated with TBS containing 3% (w/v) bovine serum albumin (BSA) for 2 h at room temperature. The TBS-BSA was removed and the sheet was washed once in 5 ml TBS for 2 min. The nitrocellulose sheet was further incubated with antiserum containing TBS plus 3% BSA for 2 h, and washed five times, 5 min each, with wash buffer (TBS containing 0.6% NaCl and 0.3% NP-40). Then the nitrocellulose sheet was incubated at 25°C for 1 h with a second antirabbit antibody peroxidase conjugate. After incubation the nitrocellulose sheet was washed three times with wash buffer and twice with TBS, then incubated at 25°C with 3 mg 4-chloro-1-naphthol dissolved in 1 ml methanol, 5 ml TBS and 10 μl 30% (v/v) H_2O_2 . The reaction was stopped by washing the nitrocellulose sheet several times with water and the strip was dried on filter paper.

Analytical methods. Hexokinase and maltase were assayed as previously described (Fernández *et al.*, 1985; Zimmermann & Eaton, 1974). Protein concentration was determined by the method of Lowry.

RESULTS

Proteolysis of hexokinase PII

The presence of protease inhibitors in the buffer used for cell-free extract preparation protects hexokinase PII from proteolysis. When EDTA and PMSF were omitted from the buffer, the resulting protease activity resulted in a partial degradation of hexokinase PII, and the appearance of peptides with molecular masses ranging from 19 to 60 kDa (Fig. 2). Since the cleavages produced may be compatible with high retention of activity, the initial degradation can be easily overlooked unless the enzyme is detected by immunoblotting procedures. This degradation can be countered by the addition of inhibitors of serine and metal proteases (Figs 3 and 4). The proteolytic degradation of hexokinase PII is prevented by these inhibitors, although not all the serine or metal proteases of yeast are sensitive to them. These results show the high susceptibility of hexokinase PII to proteolysis and indicate that a peptide produced by limited proteolysis of the enzyme could be a good candidate for signalling catabolite repression in yeast.

Changes in hexokinase PII protein during carbon catabolite repression

The relationship between the xylose-induced decrease in hexokinase PII activity and the derepression of maltase synthesis in *S. cerevisiae* G-517 and a Yep1357-HXK 2-5 transformant of D.308.3 is shown in Tables 1 and 2 respectively. These results are in agreement with those obtained previously for derepression of invertase synthesis (Fernández *et al.*, 1984). In *S. cerevisiae* G-517 (Table 1), xylose induced a 67% loss of hexokinase PII activity within 8 h, reaching a 98% inactivation after 10 h; the enzyme activities detected at 10 h and 24 h culture were the same. In cultures in which hexokinase PII had been inactivated, maltase activity increased by approximately 27-fold, reaching this maximum after 10 h. The magnitude of hexokinase PII inactivation did not seem to be dependent on the enzyme level. A 20-fold increase in hexokinase PII specific activity was found in the Yep1357-HXK 2-5 transformant of D.308.3 (Table 2), but the inactivation induced by xylose followed identical kinetics to those observed in the *S. cerevisiae* wild-type, and maltase activity increased approximately fourfold within 10 h. The D.308.3 strain is unable to ferment maltose. However, it has basal levels of maltase activity which are also affected by xylose. The loss of hexokinase PII activity did not

Fig. 2. Immunoblot detection of hexokinase PII from P2T22D mutants. Yeast cells were grown for 12 h in YPG medium supplemented with 30 mg adenine l^{-1} and harvested and washed twice with distilled water by centrifugation. The cells were resuspended in 0.5 ml 10 mM-potassium phosphate buffer pH 7.0, and broken by shaking as described in Methods. The cell-free extract was electrophoresed and hexokinase PII was detected by immunoblotting. Lane 1, molecular mass calibration proteins: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa). Lane 2, 20 μ g purified hexokinase PII. Lane 3, crude extract from P2T22D mutant; the gel was stained with Coomassie blue after separation. Lane 4, immunoblotting detection of hexokinase PII contained in the crude extract of P2T22D.

correlate with the loss of hexokinase PII protein, as shown in Fig. 3 for the wild-type strain *S. cerevisiae* G-517, and in Fig. 4 for the transformed strain.

No proteolytic peptide intermediates of hexokinase PII were found in either yeast strain during catabolite derepression induced by D-xylose. Samples (50 μ g) of total protein were fractionated in duplicate on a 12% polyacrylamide gel. The proteins from one slot were transferred from the gel to a nitrocellulose sheet (see Methods). The hexokinase PII protein present on the nitrocellulose sheet was determined from the area of the peak obtained by scanning for absorbance at 560 nm with a Joyce Loebl scanner. As can be seen in Tables 1 and 2, there were no changes in the content of hexokinase PII protein in yeast harvested at different culture times from repressing or derepressing media.

Due to the multicopy effect of the vector Yep1357 an approximately 20-fold increase in the specific activity of hexokinase PII was observed in the transformants compared to the wild-type (Tables 1 and 2). This overproduction of the enzyme was substantiated by SDS-PAGE (Fig. 4). Crude extracts were prepared at different times from the transformed strain growing with or without xylose in the culture medium. After electrophoresis the gel was stained with Coomassie blue. A protein band of molecular mass 60 kDa appeared to have a significantly increased

Fig. 3. Separation of crude extracts from *S. cerevisiae* G-517 by SDS-PAGE and immunoblot detection of hexokinase PII. Cell extracts were prepared from yeasts growing in YPG (G) or YPG plus 500 mM-D-xylose (X). Samples taken at the times indicated were used. Total protein (50 µg) was applied per slot. Each sample was applied in duplicate: half of the gel was stained with Coomassie blue after electrophoresis (a); proteins from the other half of the gel were electrophoretically transferred to a nitrocellulose sheet for hexokinase PII detection by immunoblotting (b). St corresponds to the molecular mass calibration proteins indicated in Fig. 2. HK-PII, 20 µg purified hexokinase PII.

Table 1. Hexokinase PII inactivation and maltase derepression of *S. cerevisiae* G-517

Addition	Culture time (h)	Glc concn (mM)	Hexokinase				Maltase activity (mU mg ⁻¹)
			Total activity (mU mg ⁻¹)	HK-PII activity (mU mg ⁻¹)	HK-PII inactivation (%)	HK-PII protein (RU*)	
Glc	5	46	0.8	0.5		1.1	20
Xyl	5	45	0.6	0.3	40	1.1	35
Glc	8	2	1.0	0.6		1.4	22
Xyl	8	1	0.6	0.2	67	1.5	76
Glc	10	0	1.0	0.5		1.5	20
Xyl	10	0	0.6	0.01	98	1.5	480
Glc	24	0	1.0	0.5		1.5	18
Xyl	24	0	0.6	0.008	98	1.6	482

* Protein is expressed as relative units (RU) from the area of the peak obtained by scanning at 560 nm.

Fig. 4. Separation of crude extracts from a Yep1357-HXK 2-5 transformant of D.308.3 by SDS-PAGE (a) and immunoblot detection of hexokinase (b). Yeast cell extracts (50 µg total protein) prepared as described in Fig. 3 were electrophoresed and hexokinase PII was detected by immunoblotting as described in Methods. D.308.3, extract prepared from D.308.3 cells which lacked glucose-phosphorylating enzymes. HK-PII, 20 µg purified hexokinase PII.

Table 2. *Hexokinase PII inactivation and maltase derepression of Yep1357-HXK 2-5 transformant of D.308.3*

Yep1357-HXK 2-5 is a multicopy plasmid constructed by replacing the 1.45 kb *EcoRI* fragment of YRp-HXK 2-5 containing the *TRP1* gene by the 2.8 kb *EcoRI* fragment of Yep1357 containing the *TRP1* gene and the 2 µm circle with the replication origin.

Addition	Culture time (h)	Glc concn (mM)	Hexokinase PII			Maltase activity (mU mg ⁻¹)
			Activity (mU mg ⁻¹)	Inactivation (%)	Protein (RU*)	
Glc	5	87	9.1		2.0	14
Xyl	5	90	5.7	38	2.0	20
Glc	8	56	8.7		2.6	15
Xyl	8	61	3.3	62	2.6	32
Glc	10	36	9.7		2.4	13
Xyl	10	39	0.8	92	2.4	45
Glc	24	0	9.8		2.2	13
Xyl	24	0	0.7	93	2.3	46

* Protein is expressed as relative units from the area of the peak obtained by scanning at 560 nm.

intensity compared to the wild-type (Fig. 3). The intensity of the 60 kDa band, determined from the area of the peak obtained by densitometer scanning, showed that the amount of hexokinase PII protein was constant in both cultures, but hexokinase PII activity was inhibited in the xylose culture and carbon catabolite repression was relieved in these conditions.

DISCUSSION

Two main hypotheses are proposed to explain the molecular mechanism by which hexokinase PII regulates glucose repression in yeasts. These hypotheses are mainly based on a possible proteolytic regulation of a peptide derepressing signal released from hexokinase PII. Our results strongly suggest that during the derepression process there was no release of proteolytic peptides from hexokinase PII. Although generation of a peptide of 20 kDa or less would not be detected on the SDS-PAGE gels shown in Figs. 3 and 4, the residual hexokinase PII peptide with a molecular mass of 40 kDa or more would be detected on these gels. No peptide intermediates of hexokinase PII were found in either yeast strain during catabolite derepression induced by D-xylose.

Work on *hex1r* mutants indicated that there are two different domains in the hexokinase PII molecule, one with catalytic properties and the other with regulatory properties (Entian & Fröhlich, 1984). The catalytic site of hexokinase PII was unaffected in these mutants. The mutation affected the regulatory domain, which is responsible for glucose repression of invertase and maltase.

Our results show that inactivation of yeast hexokinase PII by xylose was not correlated with the loss of hexokinase PII protein. The glucose phosphorylating activity of hexokinase PII is not implicated in the carbon catabolite repression, as was demonstrated by work on *hex1r* mutants (Entian & Fröhlich, 1984). However, derepression of maltase and invertase synthesis takes place when xylose is present in the culture medium.

The inactivation of hexokinase PII by D-xylose and MgATP *in vitro* is related to the phosphorylation of the protein (Fernández *et al.*, 1986). The 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, as yet unknown, activity. This activity could catalyse the modification of an inactive derepressing signal to an active one, or some other unknown mechanism may occur.

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