Invertase Secretion by *Phycomyces blakesleeanus*: Regulation by Carbon Catabolite Repression and by the pH of the Growth Medium

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(Received 10 February 1989; revised 13 June 1989; accepted 9 August 1989)

Carbon catabolite repression was studied in secreted (invertase) and mitochondrial (isocitrate dehydrogenase) enzymes of *Phycomyces blakesleeanus*. Both enzymes were subject to repression by glucose when the fungus was grown at pH 4.5. Repression of invertase but not of isocitrate dehydrogenase was overcome when xylose was added to the growth medium together with glucose. Derepression of invertase activity was obtained when the fungus was grown in a medium with glucose and at pH 7.3. This pH-dependent derepression was not due to enzyme redistribution or stimulation of activity. In contrast, isocitrate dehydrogenase activity was not derepressed at pH 7.3, but was significantly reduced either in the presence or absence of glucose.

INTRODUCTION

The Zygomycete fungus *Phycomyces blakesleeanus* can utilize a variety of compounds as carbon source, ranging from simple substrates such as acetate, lactate, or pyruvate to more complex ones such as sucrose (Otto, 1980). Growth on sucrose implies the production and secretion of invertase (sucrase: β -D-fructofuranoside fructohydrolase; EC 3.2.1.26) for splitting the disaccharide into glucose and fructose.

In *Saccharomyces cerevisiae* (Gascon & Lampen, 1968), *Schizosaccharomyces pombe* (Moreno *et al.*, 1985) and *Neurospora crassa* (Lee & Free, 1984) the synthesis of invertase is regulated by the presence of glucose in the culture medium. In yeasts, the repression of invertase and other enzymic activities by glucose, termed catabolite repression, is well-documented and constitutes a major regulatory system that adapts the metabolic activity of the cell to the most readily available carbon source. Glucose also represses several other enzymes, particularly those involved in gluconeogenesis (Gancedo *et al.*, 1965; Gancedo & Schwerzmann, 1976), and in the tricarboxylic acid (Polakis & Bartley, 1965) and the glyoxylate cycles (Barnett & Kornberg, 1960). Glucose inactivation, on the other hand, refers to the proteolytic inactivation of specific enzymes that follows the addition of glucose to the growth medium (Holzer, 1976).

Although *Phycomyces* is able to utilize sucrose as a carbon source, the presence of invertase has not been reported. We investigated the synthesis of invertase by *Phycomyces* and the response of the enzyme to catabolite repression, the pH of the growth medium and the presence of xylose. The regulation by glucose of a mitochondrial enzyme, isocitrate dehydrogenase (EC 1.1.1.41) was also studied.

METHODS

Strain. The wild-type strain NRRL 1555(–) of *P. blakesleeanus* was used throughout. Spores were obtained and cultures were propagated as described previously (Martínez-Cadena & Ruiz-Herrera, 1987).

Media and growth conditions. YP medium, containing 0.3% yeast extract and 1% (w/v) peptone was used as the basic medium, supplemented with 2% (w/v) glucose (Bartnicki-García & Nickerson, 1962) where indicated. The pH of the medium was adjusted to 7.3 with 1 M-NaOH or to 4.5 with 1 M-H₂SO₄, before autoclaving. Heat-

activated (48 °C, 15 min) spores were inoculated into medium in Erlenmeyer flasks at a final density of 5×10^5 ml⁻¹. Flasks were shaken aerobically at 24 °C for 12 h.

Invertase was repressed by growing cells in YP medium (pH 4.5) supplemented with 2% glucose (YPG). For derepression, glucose-grown cells were centrifuged, washed once and resuspended in YP medium (pH 4.5). The cell suspension was incubated at 24 °C and invertase activity was assayed at intervals as described below.

Invertase activity assay. Cells were centrifuged, washed twice with water by centrifugation and resuspended in 50 mM-sodium acetate buffer, pH 4.9. Invertase was assayed in whole cells as described by Goldstein & Lampen (1975). One unit (U) of invertase activity is defined as the amount of the enzyme that produces 1 µmol glucose min⁻¹ at 30 °C.

Isocitrate dehydrogenase assay. NAD⁺-dependent activity was measured in a mitochondria-enriched fraction as described by Schwitzguebel *et al.* (1981). One unit (U) of activity is defined as the amount of enzyme that reduces 10 nmol NAD⁺ min⁻¹.

Protein determination. This was done by the method of Lowry.

Reproducibility of results. All experiments were repeated at least three times with consistent results. Values given are means of three experiments done in duplicate.

RESULTS AND DISCUSSION

Invertase secretion and isocitrate dehydrogenase activity under repressed and derepressed conditions

When *Phycomyces* was grown in YPG medium at pH 4.5 for 12 h, and then transferred to YP medium at the same pH, invertase activity could be detected 30 min after the transfer, and sharply increased during the next 120 min [to 70 mU (mg protein)⁻¹]. The enzyme activity detected in cells maintained in YPG medium after the same time of incubation was 20-fold lower (3.5 mU mg⁻¹). These results clearly indicate the presence of invertase in the fungus and its regulation through catabolite repression. Similarly, Lee & Free (1984) also found that invertase activity could be detected 20 min after transfer of *N. crassa* to derepressing conditions. Under the same experimental conditions used for the analysis of invertase, we also observed an increase in isocitrate dehydrogenase activity following transfer of glucose-grown cells to a glucose-free medium. This increase was, however, much lower than that of invertase activity (16 mU mg⁻¹ in YPG medium compared with 42 mU mg⁻¹ in YP medium). These results indicate that the mitochondrial enzyme is also subject to catabolite repression as has been reported previously for the enzyme of *Sacch. cerevisiae* (Polakis & Bartley, 1965).

Effect of xylose on catabolite repression of invertase and isocitrate dehydrogenase

Hexokinase PII plays a crucial role in carbon catabolite repression in yeasts (Entian & Frohlich, 1984; Entian *et al.*, 1984; Fernandez *et al.*, 1984) by providing a signal to repress the synthesis of various enzymes in the presence of glucose. In *Sacch. carlsbergensis* (Fernandez *et al.*, 1984) and *Sacch. cerevisiae* (Fernandez *et al.*, 1986), xylose induces the derepression of invertase synthesis in the presence of glucose by inactivating hexokinase PII. Therefore, it was of interest to test whether xylose affected catabolite repression of invertase in *Phycomyces*. Table 1 shows that when xylose was added to a glucose-containing medium, invertase activity increased about 6-fold. In the absence of glucose, xylose led to a further increase in invertase activity. This could be due to the presence of some residual hexokinase PII activity in YP medium at pH 4.5, whose inactivation by xylose would increase the synthesis of invertase. The presence of hexokinase PII activity has been also described in *Sacch. carlsbergensis* grown with ethanol as carbon source (Fernandez *et al.*, 1984).

No difference in isocitrate dehydrogenase activity was observed in extracts from cells grown in YPG medium at pH 4.5 either in the presence or in the absence of xylose (Table 1). These results suggest the possible involvement in *Phycomyces* of an hexokinase-PII-like enzyme in glucose repression of invertase, but not in repression of isocitrate dehydrogenase.

Effect of pH on glucose repression

Most fungi can grow in media with a pH as acidic as 3.0 or as basic as 8.0. Since invertase and isocitrate dehydrogenase were regulated by carbon catabolite repression at acid pH, we investigated whether this also occurred at a basic pH.

Fig. 1. Time-course of secretion of invertase activity in different media. Spores of *Phycomyces* were inoculated in YPG medium pH 4.5 (●), YP medium pH 4.5 (○) and YPG medium pH 7.3 (△). Samples of the cultures were withdrawn at various times and assayed for invertase activity (—) and protein (---).

Table 1. *Effect of xylose on catabolite repression of invertase and isocitrate dehydrogenase*

Cells were grown for 9 h at 24 °C, collected and assayed for invertase activity. Isocitrate dehydrogenase activity was assayed in cells grown for 12 h as described in Methods. ND, Not determined.

Medium	Activity [mU (mg protein) ⁻¹]	
	Isocitrate dehydrogenase	Invertase
YPG	20.0	5
YPG + 4.5% (w/v) xylose	16.0	31.5
YP	ND	62.4
YP + 4.5% (w/v) xylose	ND	143.3

Table 2. *Distribution of invertase activity at different pH values*

Cells were grown for 12 h at 24 °C, collected and assayed for invertase activity. The activity of the cell-free medium was measured also. ND, Not determined.

Medium	Invertase activity	
	Whole cells [mU (mg protein) ⁻¹]	Cell-free medium (mU ml ⁻¹)
YPG, pH 4.5	0	ND
YPGlycerol, pH 4.5	99	16
YPG, pH 7.3	433	120
YPGlycerol, pH 7.3	40	15

When *Phycomyces* was grown first under repressing conditions (YPG medium) at pH 7.3, and then transferred to derepressing conditions (YP medium) at the same pH, there was a 2-fold increase in invertase activity in the cells (230 mU mg⁻¹ in YPG compared with 460 in YP medium). This increase is low compared with the 20-fold increase observed when the transfer experiment was done at pH 4.5. Invertase activity in the medium at pH 7.3 prior to transfer was 230 mU mg⁻¹ but was nearly undetectable at pH 4.5. The amount of residual glucose in the medium after growth in YPG medium at pH 4.5 and at pH 7.3 was measured to investigate whether derepression at pH 7.3 was a consequence of decreased glucose uptake. At either pH, the amount of glucose in the cell-free media was the same (data not shown). At pH 7.3, isocitrate dehydrogenase was not derepressed; instead, enzyme activity fell by about 50%.

Fig. 2

Fig. 3

Fig. 2. Derepression of invertase from cells grown in YPG medium pH 4.5 and transferred to different media. Cells were grown for 12 h in YPG medium pH 4.5 (●) and transferred to YP medium pH 4.5 (○) and YPG medium pH 7.3 (△). Samples of the different cultures were withdrawn at various times and assayed for invertase activity.

Fig. 3. Optimum pH for invertase activity from cells grown in YP medium pH 4.5 (○) and YPG medium pH 7.3 (△). Cells grown for 11 h in each medium were washed with water and invertase activity was assayed as follows: 50 mM-sodium acetate buffer pH 4.5 and pH 5; 50 mM-potassium phosphate buffer pH 6.65; 50 mM-Tris/HCl pH 7.5.

Table 3. *Effect of the pH of the growth medium on isocitrate dehydrogenase activity*

Spores of *Phycomyces* were inoculated into YPG medium at pH 4.5. After 12 h incubation at 24 °C, the culture was divided into two batches: one was maintained in the same medium and the other was washed and transferred to YPG medium at pH 7.3. After 2 h, cells were collected and isocitrate dehydrogenase activity was assayed in the mitochondria-enriched fraction as described in Methods.

Medium	Protein (mg)	Specific activity [U (mg protein) ⁻¹]	Total activity (U)
YPG, pH 4.5	1.8	22.2	39.1
YPG, pH 7.3	2.1	11.3	23.4

Time-course of invertase secretion and isocitrate dehydrogenase activity at different pH values

Spores of *P. blakesleeanus* were inoculated either in YPG medium at pH 4.5 or 7.3, or in YP medium at pH 4.5 (no growth being observed at pH 7.3 in the absence of glucose). The flasks were incubated at 24 °C and invertase activity was assayed at intervals. Fig. 1 shows that invertase activity in YP medium at pH 4.5 was very low and did not increase after 9 h of incubation whereas a sharp increase in enzyme activity was observed after 7 h in cells incubated in YPG medium at pH 7.3. As expected, no invertase activity was detected in cells grown in YPG medium at pH 4.5. Growth was similar in YP at pH 4.5 and YPG at pH 7.3 (Fig. 1). In addition to the effect of pH, one plausible explanation for the marked difference in invertase activity on the various media could be the different distribution of the enzyme. To check this, activity in whole cells and in cell-free growth medium was measured. Table 2 shows that on YPG medium at pH 7.3 much more invertase activity was detected both in whole cells and in cell-free medium than in YPGlycerol medium at pH 4.5. Growth was poor in YPGlycerol medium at pH 7.3, only 10–20% of cells germinating; the low invertase activity detected under these conditions might correspond to that of germinated cells.

When *Phycomyces* was grown first on YPG medium at pH 4.5 (repressing conditions) and

then transferred to YP medium at pH 4.5 or YPG at pH 7.3, invertase activity increased in parallel in both media up to 120 min post-transfer (Fig. 2). Thereafter, invertase activity in YP at pH 4.5 reached a plateau, whereas activity in YPG at pH 7.3 continued to increase. As expected, control cells left in YPG at pH 4.5 did not show significant levels of enzyme activity. Thus, it appears that the increase in invertase activity is a function of the pH of the derepression medium.

Another explanation for the noticeable difference in invertase activity could be due to the pH at which the enzyme is assayed. To discount this, we grew *Phycomyces* in YPG medium at pH 7.3 or in YP medium at pH 4.5 for 11 h. Separate batches of cells were washed twice with water and assayed for invertase at different pH values. The optimum pH for activity was pH 5.0 and at pH 7.0 and above there was no activity at all (Fig. 3).

In *Aspergillus nidulans*, alkaline phosphatase is produced at pH 8.0 but not at pH 5.0, whereas for acid phosphatase and phosphodiesterase the opposite occurs (Caddick & Arst, 1986). These enzymes are synthesized at the pH of the growth medium which is the optimum for activity. In contrast, we found that in *Phycomyces* invertase is overproduced at a pH of the growth medium at which it does not have any activity. The reason for this is not clear. Caddick *et al.* (1986) isolated *Aspergillus* mutants in which gene expression is altered by the extracellular pH. These mutations affect the regulation of structural genes specifying extracellular enzymes and permeases. The alkaline pH of the growth medium might exert a similar effect on invertase synthesis in *Phycomyces*.

When isocitrate dehydrogenase activity was measured following transfer of cells from YPG medium at pH 4.5 to YPG at pH 7.3, it decreased by approximately 50% (Table 3), possibly due to enzyme inactivation at the slightly basic pH.

In yeasts, glucose represses the enzymes of the tricarboxylic acid cycle (Polakis & Bartley, 1965). This phenomenon involves the repression of the synthesis of proteins coded by nuclear and mitochondrial genes (Schatz & Mason, 1974). The isolation of mutants resistant to carbon catabolite repression has been described. The *GLR1* mutants have a constitutively glucose-derepressed phenotype both of mitochondrial and of cytoplasmic enzymes (Michels & Romanowski, 1980). In *CCR* mutants the synthesis of some mitochondrial proteins appears to be constitutively derepressed, whereas repression of the synthesis of cytoplasmic enzymes still occurs (Boker-Schmitt *et al.*, 1982). We found that the pH of the growth medium gave rise to another type of response. Thus, a pH change derepressed a secreted (invertase) but not a mitochondrial (isocitrate dehydrogenase) enzyme.

Our results suggest two types of regulation of invertase biosynthesis: one by carbon catabolite repression and the other by a pH-dependent mechanism. The possibility that the pH of the growth medium regulates the overall glucose repression system is ruled out, since isocitrate dehydrogenase does not respond similarly. Work is now in progress to elucidate the mechanisms of regulation of the two enzymes.

Part of this work was supported by the DIGICySA of the Subsecretaria de Educacion Superior e Investigacion Cientifica, SEP; and the Consejo Nacional de Ciencia y Tecnologia, Mexico. Thanks are given to Drs Laura Ongay-Larios, J. Felix Gutierrez-Corona and E. Lopez-Romero for critical reading of the manuscript. We thank Carmelita Silva for typing the manuscript.

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