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Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases

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Trehalose-6-phosphate (P) competitively inhibited the hexokinases from *Saccharomyces cerevisiae*. The strongest inhibition was observed upon hexokinase II, with a K_i of 40 μM, while in the case of hexokinase I the K_i was 200 μM. Glucokinase was not inhibited by trehalose-6-P up to 5 mM. This inhibition appears to have physiological significance, since the intracellular levels of trehalose-6-P were about 0.2 mM. Hexokinases from other organisms were also inhibited, while glucokinases were unaffected. The hexokinase from the yeast, *Yarrowia lipolytica*, was particularly sensitive to the inhibition by trehalose-6-P: when assayed with 2 mM fructose an apparent K_i of 5 μM was calculated. Two *S. cerevisiae* mutants with abnormal levels of trehalose-6-P exhibited defects in glucose metabolism. It is concluded that trehalose-6-P plays an important role in the regulation of the first steps of yeast glycolysis, mainly through the inhibition of hexokinase II.

Trehalose-6-phosphate; Hexokinase; Glycolysis; Yeast

1. INTRODUCTION

Control of the glycolytic flux in *Saccharomyces cerevisiae* has been considered to occur mainly at the level of phosphofructokinase and pyruvate kinase. Phosphofructokinase is regulated through activation by fructose-2,6-bisphosphate, phosphate (P) and AMP, and inhibition by ATP, while pyruvate kinase activity is modulated by its activation by fructose-1,6-bisphosphate (for a review see [1]). However, since phosphofructokinase does not catalyze the first irreversible step in the utilization of glucose, its regulation is not sufficient to control the rate of glucose utilization, and thus some mechanism should exist to regulate the rate of glucose transport, phosphorylation, or both. Regulation of glucose transport by glucose-6-P was suggested by Sols [2], but experimental results obtained using mutants affected in phosphoglucose isomerase activity did not support this idea [3,4]. Besides, none of the yeast kinases that phosphorylate glucose are sensitive to glucose-6-P, in contrast with the mammalian hexokinase [5].

The need for a regulation of the first steps of yeast glycolysis is illustrated by the pattern of accumulation of metabolites in certain yeast mutants upon addition of glucose [6–8]. Yeast strains carrying the mutations *fdp1* [6] or *cif1* [7] do not grow on glucose, although the glycolytic enzymes are operative. These mutations turned out to be allelic [9], and strains bearing them become depleted of ATP upon addition of glucose and accumulate fructose-1,6-bisphosphate up to 20 mM [6,7,10], suggesting that the rate of the first glycolytic steps exceeds the capacity of the glycolytic pathway. The sequence of the *CIF1* gene [10] encodes the small subunit of the trehalose-6-P synthase/trehalose-6-P phosphatase complex [11,12]. A plausible explanation for the growth behaviour and the metabolic defects of *cif1* strains could be that either trehalose or trehalose-6-P play a role in the regulation of the yeast glycolytic flux. We show in this article that trehalose-6-P inhibits sugar phosphorylation. The strongest inhibition is observed upon hexokinase II, which is the most abundant isoenzyme of hexokinase during growth of *S. cerevisiae* on glucose [13].

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Dedicated affectionately to the memory of Nico Van Uden who loved yeasts.

2. MATERIALS AND METHODS

2.1. *Yeast strains and growth conditions*

The following strains were used: W303-1A, *MAT***a** *ade2-1 his3-11,15 ura3-1 leu2-1 trp1-1* [14]; WDC-3A, *MAT***a** *ade2-1 his3-11,15 ura3-1 leu2-1 trp1-1 cif1::HIS3* (constructed for this work); S2072D, *MAT*α *arg4 leu1 trp1 thr4 gal2* [15]; ts1265, *MAT*α *arg4 leu1 trp1 thr4 gal2 tre1* [16]; CJM 018, *MAT***a** *HXK1 hxk2 glk1*; CJM 019, *MAT*α *hxk1 HXK2 glk1*; CJM118, *MAT*α *hxk1 hxk2 GLK1*; *Yarrowia lipolytica* INAG 35667, *MAT***a** *lys11-23 ura3-302 leu2-270*; *Kluyveromyces lactis* Y1140 (NRL) and *Zygosaccharomyces bailii*. They were grown at 30°C in 2% peptone, 1% yeast extract and 2% glucose, except WDC-3A which was grown with 2% galactose, and ts1265 which was grown at 24°C.

2.2. *Enzymatic assays and metabolite determination*

Extracts were obtained by shaking in a vortex 100 mg of cells in 0.5 ml of ice-cold 20 mM imidazol, pH 7, with 1 g glass beads of 0.5 mm diameter, for 4 periods of 1 min, with 1 min intervals in ice after each

period. The liquid was centrifuged for 10 min at 700 × *g* and the supernatant was used for enzyme assays. Phosphorylating activity on glucose or fructose was assayed spectrophotometrically as in [13].

To measure intracellular glucose, 200 mg of yeast cells (wet weight) were collected by rapid filtration, washed twice with 15 ml ice-cold water, and extracted according to Gamo et al. [17]. Glucose was assayed spectrophotometrically with hexokinase [18].

Trehalose-6-P was measured in extracts obtained as above, heated at 100°C for 10 min in the presence of 0.1 M NaOH, and neutralized with HCl. The concentration of trehalose-6-P was assessed by the inhibition produced in a partially purified preparation of hexokinase from *Y. lipolytica*. Trehalose-6-P inhibits this enzyme with an apparent K_i of 5 μM when assayed with 2 mM fructose. The enzyme was purified free from glucokinase as described by Hirai et al. [19]. A standard curve with pure trehalose-6-P (Sigma, St. Louis, MO) was run in parallel. Trehalose-6-P added to the extracts was recovered with yields superior to 90%, and the reproducibility of the procedure was good with a coefficient of variation of less than 10%. (A detailed description of the method will be published elsewhere.)

Dictyostelium discoideum was grown and extracted as in [20]. Rat brain homogenate was obtained as in [21].

2.3. *Other methods*

Transport was measured at 20°C with uniformly labelled glucose as described by Bisson and Fraenkel [22].

Fermentation was measured in a conventional Warburg respirometer at 30°C, using 50 mM glucose.

3. RESULTS AND DISCUSSION

3.1. *Inhibition of glucose-phosphorylating enzymes from* S. cerevisiae *by trehalose-6-P*

Yeast strains expressing only one of the three enzymes able to phosphorylate glucose were used to test the possible effect of trehalose-6-P. Fig. 1 shows the results obtained: trehalose-6-P inhibited the phosphorylation of glucose and fructose by hexokinase II. This inhibition was competitive with either substrate, and a K_i of 40 μM was calculated. The inhibition of hexokinase I activity was much weaker than that observed for hexokinase II (K_i of 200 μM). Glucokinase remained unaffected even when assayed at concentrations of trehalose-6-P as high as 5 mM. Trehalose itself had no effect, either on the phosphorylation of the sugars or on the inhibition produced by trehalose-6-P. The inhibition caused by trehalose-6-phosphate was non- competitive with respect to ATP (data not shown).

3.2. *Effect of trehalose-6-P on glucose-phosphorylating activity from other organisms*

Extracts from different organisms were tested to examine whether the glucose-phosphorylating activity was also inhibited by trehalose-6-P. The results are presented in Table I. In all the extracts tested where hexokinase was present there was inhibition by trehalose-6-P. In those cases where glucokinase was the predominant or the only phosphorylating activity, like *Y. lipolytica* [19] or *D. discoideum* [23], the inhibition was weak or absent. Partially purified hexokinase from *Y. lipolytica* free of glucokinase was particularly sensitive to trehalose-6-P and exhibited an apparent K_i of 5 μM when assayed with 2 mM fructose. Based on this property it was possible to develop a sensitive method of assaying trehalose-6-P (see section 2). The glucokinase from *Y. lipolytica* was not inhibited by trehalose-6-P up to 1 mM.

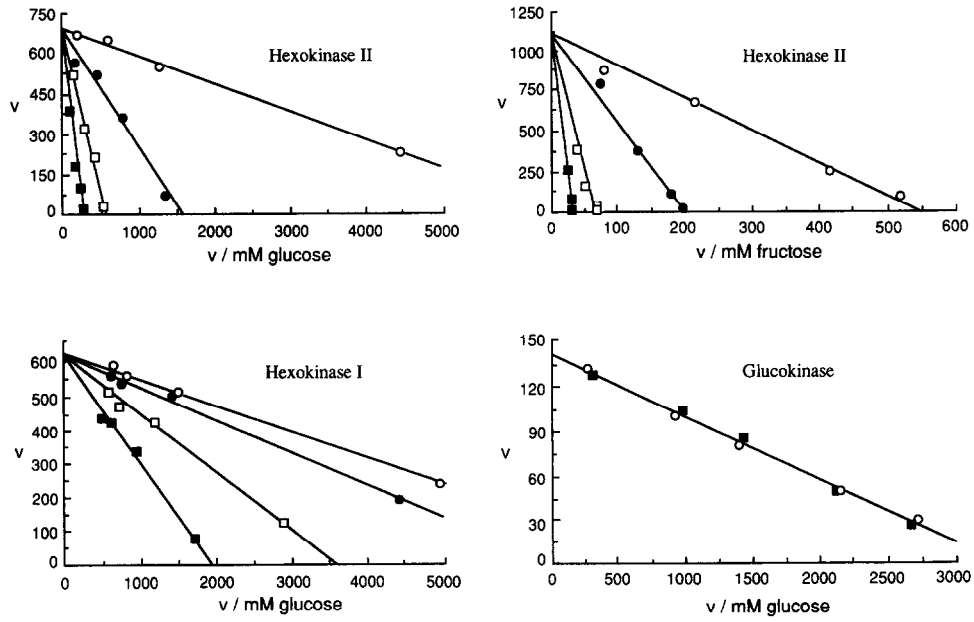

Fig. 1. Inhibition of hexokinases and glucokinase from *S. cerevisiae* by trehalose-6-P. Phosphorylating activities were assayed, as described in section 2, in extracts from strains CJM019, CJM018 or CJM118 that express only hexokinase II, hexokinase I or glucokinase, respectively. The concentrations of added trehalose-6-P were as follows: ○, none; ●, 0.1 mM; □, 0.4 mM; ■, 1 mM. *v* is expressed in nmol/min · mg protein.

3.3. *Possible physiological value of the inhibition*

Changes in the activity of an enzyme in response to a metabolite may have only a curiosity value. An important requisite for one effect to be of physiological significance is that the concentration needed to produce it in vitro, is reached in vivo under some physiological circumstances. We have measured the levels of trehalose-6-P under different metabolic conditions in different yeast strains with the results presented in Table II. In a wild-type strain, the concentration of trehalose-6-P during the exponential phase of growth on glucose is well within the range needed to obtain a significant effect. Under these conditions, hexokinase II, the enzyme most sensitive to the inhibition, is the most abundant hexokinase isoenzyme in the cell [13]. A remarkable result is the finding that although trehalose is accumulated by yeasts when they enter the stationary phase of growth [24], there was no increase in the concentration of trehalose-6-P when wild-type cells were harvested at this phase of growth (Table II).

The results presented predict that yeast cells affected in the synthesis or degradation of trehalose-6-P could have an altered glucose metabolism. To test this inference we chose two mutants affected in the metabolism of trehalose-6-P: one of them, *cif1*, lacks one of the subunits of trehalose-6-P synthase [11,12], while the other one, ts1265, accumulates high levels of trehalose-6-phosphate after heat shock ([16] and Table II).

In the *cif1* mutant, no trehalose-6-P was detected in cells harvested during either exponential or stationary phase (Table II). This mutant does not grow on glucose and is depleted of ATP, and accumulates hexose phosphates when given glucose [7,10]. This behaviour is in accordance with the prediction, and can be explained if the absence of trehalose-6-P caused an uncontrolled phosphorylation and therefore an imbalance between the energy consuming and energy producing glycolytic steps. Consistent with this idea is the fact that disruption of the *HXK2* gene encoding hexokinase II restores growth on glucose of *cif1* mutants [25], and that a dominant extragenic suppressor of the *cif1* mutation turned out to have a decreased activity of hexokinase II (Blázquez, M.A. and Gancedo, C., submitted for publication).

With the temperature-sensitive mutant that accumulates trehalose-6-P after a heat shock (Table II), we found that fermentation of glucose was very weak after 1 h at 42°C, while the parental strain fermented at a normal rate after the same treatment (Fig. 2). Cell viability of this mutant after heat shock, measured by flux cytometry, was about 80%; therefore cellular death can-

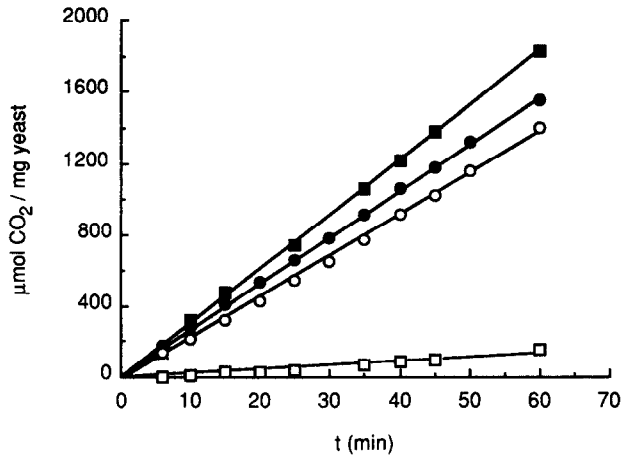

Fig. 2. Fermentation of glucose by *S. cerevisiae* strains S2072D (wild-type) and ts1265 after heat shock. Cultures were grown at 24°C until mid-exponential phase. At this moment they were divided into two aliquots: one was heated at 42°C for 1 h, while the other was kept at 24°C. After this time both samples were used to measure glucose fermentation as described in section 2. ●, S2072D 24°C; ○, S2072D after heat shock; ■, ts1265, 24°C; □, ts1265 after heat shock.

Table I

Effect of trehalose-6-P on hexose phosphorylating capacity in extracts from different organisms

Organism	% Inhibition by 1 mM trehalose-6-P	
	1 mM glucose	10 mM fructose
Saccharomyces cerevisiae	60	80
Kluyveromyces lactis	66	66
Zygosaccharomyces bailii	89	89
Yarrowia lipolytica	23[a]	100
Rat brain	40	35
Dictyostelium discoideum	<3	–[b]

[a] The concentration of glucose used was 0.4 mM.
[b] No activity was observed with fructose.

Assays were carried out in extracts as described in section 2, with 1 mM trehalose-6-P and the sugar concentrations indicated. The percentage of inhibition was calculated by reference to a control without trehalose-6-P.

Table II

Internal concentration of trehalose-6-P (mM) in different yeast strains during growth and after heat-shock treatment

Strain	Phase of growth		
	Exponential	Stationary	Heat shock
W303-1A[a]	0.18	0.15	0.20
W303-1A[b]	0.32	0.30	n.d.
WDC-3A	<0.04	<0.04	n.d.
ts1265	1	25	14

Growth conditions were as described in section 2. The wild-type strain (W303-1A) was grown on glucose[a] or galactose[b]; WDC-3A (*cif1::HIS3*) was grown on galactose and ts1265 on glucose. Exponential cultures were harvested at about 4 mg/ml and stationary phase ones at about 25 mg/ml. For heat shock, exponentially growing cells were maintained at 42°C for 1 h. Trehalose-6-P determinations were as described in section 2. n.d., not determined.

not account for the loss of fermentative capacity. In addition, the mutant subjected to heat shock subsequently accumulated up to 50 mM internal glucose, while the concentration in the parental strain remained lower than 5 mM. These results are consistent with a strong inhibition of hexokinases by trehalose-6-P in the mutant while transport continues unabated. In fact, transport of glucose after heat shock was similar in the parental strain and the mutant (1 mmol/g/h measured with 2 mM external glucose, and 6 mmol/g/h measured with 50 mM). We also checked that in a wild-type strain 5 mM externally added trehalose-6-P did not affect the rate of transport.

The results obtained with the two mutants used are consistent with a regulatory role of trehalose-6-P in yeast glycolysis. This compound could play in yeast a role as feed-back inhibitor similar to that played by glucose-6-P in higher organisms [5]. Although the results obtained show the importance of the inhibition of hexokinases, it remains to be established whether this inhibition is sufficient to completely control the early steps of yeast glycolysis, or whether additional regulatory mechanisms at the level of transport are necessary.

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