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ORIGINAL PAPER

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Regulation of maltose transport in *Saccharomyces cerevisiae*

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Abstract Solute transport in *Saccharomyces cerevisiae* can be regulated through mechanisms such as *trans*-inhibition and/or catabolite inactivation by nitrogen or carbon sources. Studies in hybrid membranes of *S. cerevisiae* suggested that the maltose transport system Mal61p is fully reversible and capable of catalyzing both influx and efflux transport. This conclusion has now been confirmed by studies in a *S. cerevisiae* strain lacking the maltase enzyme. Whole cells of this strain, wherein the orientation of the maltose transporter is fully preserved, catalyze fully reversible maltose transport. Catabolite inactivation of the maltose transporter Mal61p was studied in the presence and absence of maltose metabolism and by the use of different glucose analogues. Catabolite inactivation of Mal61p could be triggered by maltose, provided the sugar was metabolized, and the rate of inactivation correlated with the rate of maltose influx. We also show that 2-deoxyglucose, unlike 6-deoxyglucose, can trigger catabolite inactivation of the maltose transporter. This suggests a role for early glycolytic intermediates in catabolite inactivation of the Mal61 protein. However, there was no correlation between intracellular glucose-6-phosphate or ATP levels and the rate of catabolite inactivation of Mal61p. On the basis of their identification in cell extracts, we speculate that (dideoxy)-trehalose and/or (deoxy)-trehalose-6-phosphate trigger catabolite inactivation of the maltose transporter.

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Introduction

The utilization of maltose by *Saccharomyces cerevisiae* requires two gene products, a maltose transporter encoded by the *MALX1* gene (X stands for one of five unlinked MAL loci) and a maltase enzyme encoded by the *MALX2* gene (Needleman 1991). The maltose transporters facilitate the uptake of maltose in symport with a proton (Van Leeuwen et al. 1992). Studies on electrochemical proton-gradient-driven solute transporters in *S. cerevisiae* indicate that these systems are virtually unidirectional (Eddy 1982; Horak 1986). The apparent unidirectionality of some of these transporters can be explained by the accumulation of solutes, e.g. basic amino acids, in the vacuole, but for other transport systems the opposing directions of translocation are thought to be kinetically different. A plausible explanation for the unidirectionality of these transport systems is that accumulated solute inhibits further uptake. This so called *trans*-inhibition involves a decrease in transport activity as the intracellular substrate concentration increases and has been well-described for bacterial transport systems involved in osmoregulation (Poolman and Glaasker 1998). Studies in hybrid membranes with overexpressed Mal61p indicate that maltose transport is reversible, because influx as well as efflux transport can be observed (Van der Rest et al. 1995). Since the orientation of the maltose transporter in the hybrid membranes is most likely random, these experiments do not rule out the possibility that the maltose transport system is *trans*-inhibited or, for some other reason, kinetically different for uptake and efflux transport, as both modes of transport may be facilitated by oppositely oriented Mal61p molecules. We now report on the reversibility of maltose transport in a *S. cerevisiae* strain that lacks the maltase enzyme.

Another form of regulation of solute transport involves the inactivation of the transporter by chemical modifica-

tion and/or breakdown. For instance, the addition of glucose to a yeast culture leads to rapid inactivation of many transporters and enzymes, such as the maltose and galactose transporters, fructose-1,6-bisphosphatase and malate dehydrogenase. This process is termed "catabolite inactivation" (Holzer 1976). Catabolite inactivation of the maltose transporter Mal61p involves a rapid inactivation of the protein, most likely mediated by phosphorylation and subsequent degradation of the protein in the vacuole (Brondijk et al. 1998; Medintz et al. 1996; Riballo et al. 1995). At least two glucose-signaling pathways have been attributed to the catabolite inactivation of Mal61p (Jiang et al. 1997). The first signaling pathway is independent of glucose transport per se and requires the presence of the proposed low-affinity glucose sensor Rgt2p (Jiang et al. 1997; Özcan et al. 1996, 1998;). The second signaling pathway requires glucose transport via the high-affinity glucose transporters (Jiang et al. 1997). It has not been determined whether the transport of glucose by the hexose transporters is sufficient to trigger inactivation of the Mal61p via pathway 2 or whether it requires further metabolism of the sugar (Jiang et al. 1997). Thus, although the enzymes involved in endocytosis and the ultimate breakdown of maltose permease are well-characterized to date, the actual signal that triggers catabolite inactivation of the maltose transporter is still unknown.

Not only hexoses that are transported via the Hxt transporters, but also galactose and even maltose itself may elicit catabolite inactivation of the maltose transporter (Penalver et al. 1998; Robinson et al. 1996). It has been claimed that maltose alone cannot trigger catabolite inactivation of the maltose transporter, but that it enhances the catabolite inactivation inflicted by galactose (Robinson et al. 1996).

To address whether or not maltose is able to trigger catabolite inactivation of its own transporter, Mal61p, the rate of inactivation of maltose uptake was determined in cells with different levels of maltose permease. Furthermore, to establish whether or not the transport step is sufficient to trigger catabolite inactivation, we compared maltose-triggered inactivation of Mal61p in the presence and absence of maltose metabolism. Using glucose analogues and measurement of the intracellular concentration of sugar derivatives, we then determined the minimal number of metabolic steps required for catabolite inactivation. We suggest that the signal that triggers catabolite inactivation of the maltose transporter may be trehalose-6-phosphate or trehalose, possibly mediated via the Tps1 protein.

Materials and methods

Strains and growth conditions

Saccharomyces cerevisiae 6001BΔ11 (*Mat*a, *SUC1, mal11Δ*::*URA3, MAL12, MAL13, ura3–52, leu2–3,112*) (Charron et al. 1986) was a gift from Dr. C.A. Michels (Queens College, New York). *S. cerevisiae* 6001BΔ11Δ12 (*mal12Δ*) was constructed by disruption of the *MAL12* gene as described previously (Van der Rest et al. 1995). *MAL61* was expressed in both strains under control of the *ADC1* promoter, using plasmid pYEpY87 (Brondijk et al. 1998). Strains were grown in batch culture on YNB medium (Difco) plus 20 μg adenine ml^{-1}, supplemented with 0.5% (w/v) glucose, 0.5% (w/v) maltose or 0.5% (w/v) galactose. For growth in chemostat culture, the cells were grown in minimal medium containing per liter: 0.2 g $MgSO_4$, 1.0 g KH_2PO_4, 2.5 g $(NH_4)_2SO_4$ (pH 6.0), 1 ml of Vishniac solution and 1 ml of vitamin solution, supplemented with 0.5% (w/v) maltose and 20 μg adenine ml^{-1}. Chemostat cultures were operated at a dilution rate of 0.1 h^{-1} at 30 °C and pH 5.0; the pH was kept constant by titration with 0.5 M KOH.

Uptake and efflux studies in cells pre-loaded with maltose

Saccharomyces cerevisiae 6001BΔ11Δ12 pYEpY87 was grown to the late-exponential phase of growth in the presence of 0.5% galactose. Cells were harvested and resuspended in citrate buffer (100 mM citrate/K_2HPO_4, pH 5.0) containing the appropriate amount of maltose. To determine the internal maltose concentration, tracer amounts of [^{14}C]maltose (2,000–4,000 cpm×μl^{-1})were added and the accumulation of maltose was determined after incubation for 30 min at 30 °C. Samples of 15 μl were withdrawn, diluted into 2 ml 0.1 M LiCl and filtered immediately over 0.45 μm-pore-size cellulose-nitrate filters (Schleicher and Schuell). The filters were washed with 2 ml 0.1 M LiCl and transferred to 2 ml scintillation fluid. The total amount of label was determined by adding 15 μl of sample directly to the scintillation fluid.

The maltose uptake rate in the maltose-preloaded cells was measured after washing the cells three times with 1 ml of ice-cold citrate buffer to remove external maltose and resuspension of the cells to 1/6 of the original volume. Control experiments, using [^{14}C]maltose, showed that less than 1% of the accumulated maltose was released under these conditions. A 20-μl aliquot of this suspension was diluted six-fold in citrate buffer plus 50 μM [^{14}C]maltose at 30 °C. At given time intervals, 15-μl samples were withdrawn, diluted into 2 ml 0.1 M LiCl and treated as described above.

Efflux was determined after washing the cells three times with 1 ml ice-cold citrate buffer, resuspension in 1/10 the original volume of citrate buffer, followed by a 10-fold dilution in citrate buffer plus 10 μM carbonylcyanide *m*-chlorophenylhydrazone (CCCP) at 30 °C. At given time points, 50-μl samples were withdrawn and treated as described above.

Inactivation studies

Cells were harvested, washed and resuspended in buffer containing per liter: 0.2 g $MgSO_4$, 3.0 g NaH_2PO_4, 0.7 g K_2HPO_4 and 1.25 g K_2SO_4, pH 6.0, plus the appropriate amount of carbohydrate and 12.5 μg cycloheximide ml^{-1} to inhibit protein synthesis. The culture was incubated at 30 °C and at different intervals 1-ml samples were withdrawn. The cells were washed twice in 100 mM citrate/K_2HPO_4, pH 5.0) and concentrated 20-fold. Transport of maltose in whole cells was measured as described above, using a final [^{14}C]maltose concentration of 50 μM.

Identification of intracellular metabolites

Cells were harvested and washed with citrate buffer (100 mM citrate/K_2HPO_4, pH 5.0) and diluted to a final OD_{660} of approximately 40 in 500 μl citrate buffer containing 2 mM ^{14}C-2-deoxyglucose (2DOG) and incubated at 30 °C. At the appropriate times, 50-μl samples were withdrawn and quenched with 2 ml 60% MeOH at –40 °C (as described previously; Gonzalez et al. 1997). Cells were washed once with cold 60% MeOH and, subsequently, the metabolites were extracted by resuspending the cell pellets in 500 μl 75% EtOH, 250 mM K-HEPES, pH 7.5, kept at 80 °C. The mixture was incubated for 3 min at 80 °C with occasional vortexing. Cell extracts were concentrated by airflow evaporation and stored at –20 °C until further use. For use as a standard

in the TLC experiments, [^{14}C]2-DOG was diluted with unlabeled extract to about the same specific activity as that of the extract prepared from cells that had been incubated with [^{14}C] 2-DOG.

Five-µl samples of the labeled cell extract (~40,000 cpm) were diluted with 15 µl triethanolamine buffer containing 3 mM NADH and 10 mM ATP and incubated for 1 h at 30 °C in the presence of either glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), glucose-6-phosphate dehydrogenase plus hexokinase, glucose oxidase (E.C. 1.1.3.4.), or without additions. The extracts were then loaded on a Silicagel 60 TLC plate (Merck) and subjected to one-dimensional TLC using NH_4OH:1-propanol:H_2O (40.5:81:13.5) as the mobile phase, or two-dimensional TLC using NH_4OH:1-propanol:H_2O (40.5:81:13.5) in the first dimension and 1-propanol:1-butanol:H_2O (4:12:4) in the second dimension. After drying, the plates were autoradiographed at –80 °C using intensifier screens.

Quantification of intracellular metabolite concentrations

One-ml culture samples were withdrawn during the inactivation experiments, immediately added to 200 µl ice-cold 36% PCA and kept on ice for 30 min. Next, 170 µl of 5 M K_2CO_3 was added to bring the extracts to pH 7.5. The extracts were centrifuged to remove the potassium-perchlorate and cell debris, and 200-µl aliquots were stored at –20 °C until further use.

Glucose-6-phosphate was measured by following the conversion by glucose-6-phosphate-dehydrogenase to gluconate-6-P, which yields 1 mol of NADPH per mol of glucose-6-phosphate. The cell extracts were diluted into 200 mM triethanolamine, 5 mM $MgCl_2$, pH 7.6. The reaction was carried out in the presence of 100 µM $NADP^+$ and 0.07 U of glucose-6-phosphate dehydrogenase (EC 1.1.1.49, Boehringer Mannheim). The NADPH concentration was measured in a spectrofluorimeter (Aminco Bowman series 2) using excitation and emission wavelengths of 340 and 460 nm, respectively. The split widths were 4 and 8 nm, respectively.

ATP was determined using the luminometric luciferase assay. A 4-ml extract of 50 mg dried lanterns was added to 25 ml of 50 mM Tris-SO_4, 2 mM EDTA, pH 7.5 (buffer A); 1 mg of luciferin was dissolved into this solution and the resulting luciferin/luciferase mixture was stored in the dark. The cell extracts were diluted into buffer A. Fifty µl of the diluted extract was mixed with 50 µl buffer B (buffer A plus 20 mM $MgSO_4$ and 2 mg/ml BSA). The reaction was started by adding 100 µl luciferin/luciferase mixture. Emission of light was measured during 30 s in a luminometer (A5–2021A Lumi-Tec, St. John Associates).

Results

Inhibition of maltose uptake by intracellular maltose

Studies in hybrid membranes with overexpressed Mal61p indicate that the maltose transporter is fully reversible and capable of catalyzing both influx and efflux transport (Van der Rest et al. 1995). However, the orientation of the maltose transporter in the hybrid membranes is most likely random and therefore the possibility that Mal61p is *trans*-inhibited or kinetically different for influx and efflux transport cannot be ruled out based on these studies. The construction of a *S. cerevisiae* strain that had a disruption in the maltase gene (strain 6001BΔ11Δ12) enabled us to study the uptake of maltose in whole cells, that is with Mal61p in one orientation and without the interference of maltose metabolism. If the Mal61p is *trans*-inhibited, then the maltose uptake capacity of cells will decrease with increasing internal maltose concentrations. For the pre-loading with maltose, cells of *S. cerevisiae* 6001BΔ11Δ12 pYEpY87 were used that had been grown in batch culture on galactose. Maltose uptake in these cells reached a plateau after about 30 min at 30 °C (Fig. 1A), and these conditions were used for pre-loading the cells at different external concentrations of maltose. The internal maltose concentrations were determined (Fig. 1B) and the initial rates of maltose uptake into the maltose-loaded cells were determined as a function of the intracellular maltose concentration. Figure 1C shows that the initial rate of maltose uptake did not decrease significantly when the internal maltose concentration was increased from 0 to 35 nmol (mg protein)$^{-1}$, indicating that Mal61p is not *trans*-inhibited under these conditions.

To determine whether or not transport of maltose is fully reversible, efflux of maltose down the concentration gradient was determined. In the presence of a proton-motive force the intracellular maltose is retained by the cells (not shown). However, when the proton motive force was dissipated by the addition of 10 µM CCCP, efflux of maltose from the cells was observed. The rate of maltose efflux from the pre-loaded cells as a function of the internal maltose concentration is shown in Fig. 1D. Maltose efflux obeyed Michealis-Menten kinetics with a maximal rate of efflux ($V_{max\ efflux}$) of 0.6 nmol min^{-1} (mg protein)$^{-1}$. The maximal uptake rate ($V_{max\ uptake}$) at the external surface of the membrane was 4.2 nmol min^{-1} (mg protein)$^{-1}$. These studies clearly show that maltose transport in *S. cerevisiae* occurs in both directions under the conditions tested.

Catabolite inactivation triggered by maltose

Saccharomyces cerevisiae 6001BΔ11Δ12 cells pre-loaded with maltose, using external maltose concentrations up to 50 mM and equilibrated for 30 min, displayed maltose uptake rates essentially independent of the intracellular maltose concentration. A decrease in maltose transport rates would be expected when the presence of external or internal maltose by itself would be sufficient to trigger catabolite inactivation of Mal61p. This shows that external and/or internal maltose is insufficient for the triggering of catabolite inactivation of Mal61p. It was therefore decided to compare catabolite inactivation of Mal61p in the presence and absence of maltose metabolism, using isogenic strains without (6001BΔ11Δ12 pYEpY87) and with maltase enzyme (6001BΔ11 pYEpY87) and grown in batch culture on 0.5% galactose (Fig. 2). In the maltase-negative strain inactivation of the transporter was not observed over a period of 2 h in the presence of 100 mM maltose, whereas in the maltase-expressing strain inactivation of Mal61p with a $t_{1/2}$ of about 40 min was observed. The catabolite inactivation of Mal61p in the presence 50 mM glucose was similar in both strains with a $t_{1/2}$ of ±15 min for maltose uptake activity.

In the case of glucose activation of the cAMP pathway via the Gpr1-Gpa2 G-protein coupled receptor system, it has been observed that, in addition to binding of glucose to the Gpr1 receptor, glucose phosphorylation is required

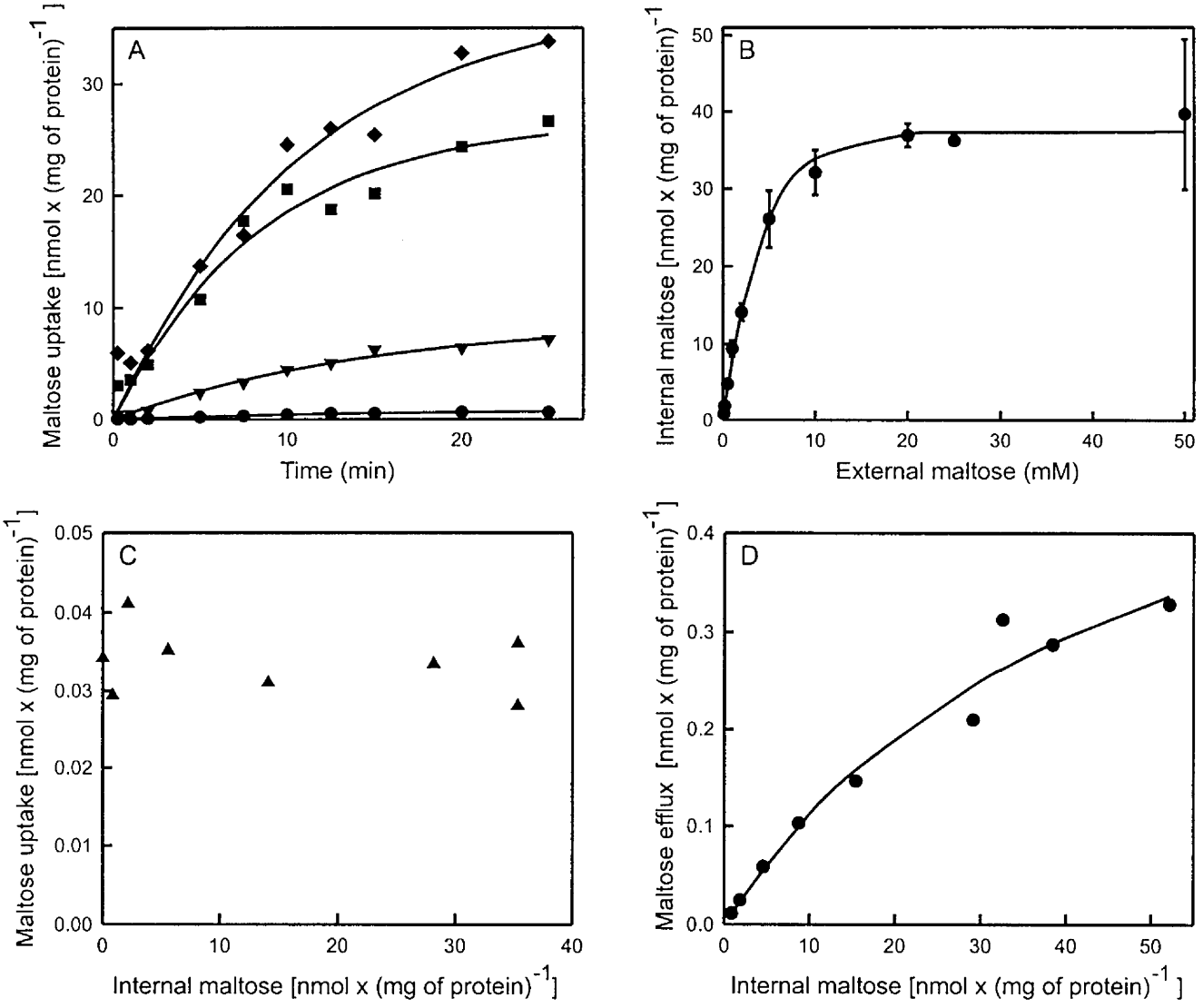

Fig. 1A–D Uptake and efflux of maltose in pre-loaded cells lacking the maltase *Saccharomyces cerevisiae* 6001BΔ11Δ12 pYEpY87 was grown in batch culture on 0.5% galactose, harvested and resuspended in 100 mM citrate/PO_4, pH5.0 with the indicated amounts of maltose. **A** Maltose uptake at.50 μM (●), 0.5 mM (▼), 5 mM(■) and 10 mM (◆) external maltose. **B** Internal maltose concentration after 30 min of incubation at 30°C. Tracer amounts of [^{14}C]maltose were used to monitor the internal maltose concentration. **C** Initial rates of maltose uptake as a function of the internal concentration of maltose. The external maltose concentration was 50 μM. **D** Rates of maltose efflux in the presence of 10 μM CCCP as a function of the internal maltose concentration

for activation of the cAMP pathway (Kraakman et al. 1999). Activation of the cAMP response via Gpr1 in a multiple hexose carrier deletion strain, which is a mutant essentially devoid of glucose uptake, can be elicited provided a low level (<1 mM) of maltose is present to fulfill the requirement for glucose phosphorylation. To exclude the possibility that this requirement also plays a role in catabolite inactivation triggered by maltose, we determined the inactivation of Mal61p in strain 6001BΔ11Δ12 pYEpY87 in the presence of 50 mM maltose plus 1 mM glucose. Also in this case no inactivation of the maltose transporter was observed ($t_{1/2}$ >6 h).

It has been observed previously that the expression of Mal61p from pYEp87 is much higher in cells from maltose-limited chemostat cultures than in batch grown cells (Brondijk et al. 1998; Van der Rest et al. 1995). The dependency of catabolite inactivation on sugar transport rates was therefore tested by measuring the inactivation of Mal61p at different concentrations of glucose and maltose, using cells from both maltose-limited chemostat and batch cultures grown in the presence of 0.5% maltose. The maximal rates (V_{max}) of maltose uptake in cells from chemostat and batch cultures were 4.2±0.9 and 46.1± 3.9 nmol min^{-1} (mg protein)$^{-1}$, respectively. The apparent affinity constants for maltose uptake were similar, that is 2.1±1.1 mM for batch grown cells and 4.3±0.7 mM for cells from the chemostat cultures.

Catabolite inactivation of Mal61p by maltose in cells from maltose-limited chemostat cultures was almost as rapid as the inactivation triggered by glucose; the $t_{1/2}$ values were 26 min for maltose and 29 min for glucose at a

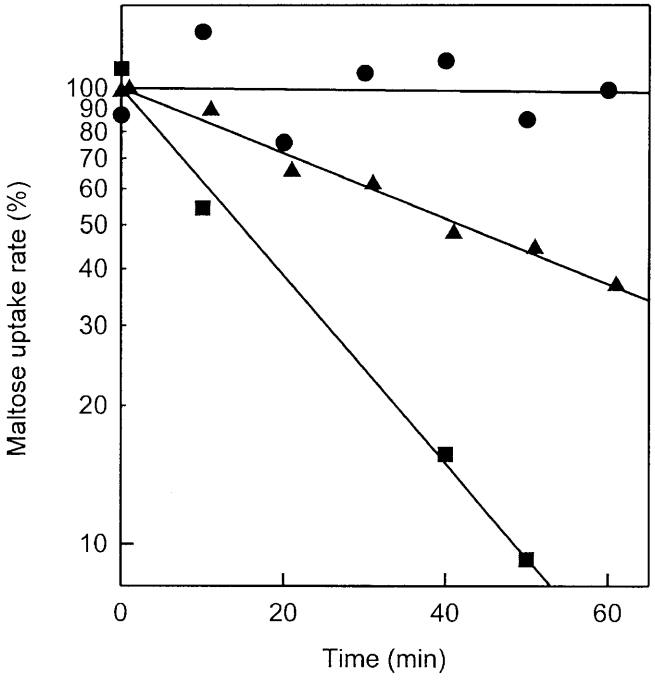

Fig. 2 Catabolite inactivation of Mal61p triggered by maltose *S. cerevisiae* 6001BΔ11 pYEpY87 (maltase$^+$) was grown in batch culture on 0.5% maltose and *S. cerevisiae* 6001BΔ11Δ12 pYEpY87 (maltase$^-$) was grown in batch culture on 0.5% galactose. At time zero, the cells were harvested and resuspended in nitrogen-deficient medium with the appropriate sugar plus 12.5 μg cycloheximide ml^{-1}. Inactivation of Mal61p in *S. cerevisiae* 6001BΔ11Δ12 pYEpY87 with 50 mM maltose (●) and 50 mM glucose (■) was determined and compared to the inactivation of Mal61p in *S. cerevisiae* 6001BΔ11 pYEpY87 with 50 mM maltose (▲). The maltose uptake rate at *t*=0 was set to 100% and corresponded to ±50 pmol×min^{-1}×mg of protein^{-1} for *S. cerevisiae* 6001BΔ11Δ12 pYEpY87 and 150 pmol×min^{-1}×mg of protein^{-1} for *S. cerevisiae* 6001BΔ11 pYEpY87

Table 1 Catabolite inactivation of Mal61p in cells cultivated under different conditions. *Saccharomyces cerevisiae* 6001BΔ11 pYEpY87 was grown in batch culture on 0.5% maltose or in a maltose-limited chemostat. At time zero, cells were harvested and resuspended in a nitrogen-deficient medium containing the appropriate sugar plus 12.5 μg/ml cycloheximide to prevent protein synthesis. Transport of [^{14}C]maltose was measured as described under Materials and methods. The $t_{1/2}$ values of inactivation were derived from plots of log activity vs time (see Fig. 2)

Substrate	$t_{1/2}$ batch culture	$t_{1/2}$ chemostat culture
100 mm Glucose	14 min	22 min
50 mm Glucose	16 min	–
25 mm Glucose	21 min	29 min
10 mm Glucose	>6 hours	38 min
100 mm Maltose	37 min	15 min
50 mm Maltose	39 min	33 min
25 mm Maltose	79 min	26 min
10 mm Maltose	113 min	44 min
5 mm Maltose	–	55 min
0.5 mm Maltose	–	>6 h

sugar concentration of 25 mM (Table 1). Catabolite inactivation of Mal61p was slower at lower maltose concentrations. In cells taken from a batch culture grown in the presence of 0.5% maltose, catabolite inactivation of Mal61p by glucose was slightly faster than that of cells from the chemostat, whereas the catabolite inactivation triggered by maltose was significantly slower at similar maltose concentrations. For instance, at 25 mM maltose, the $t_{1/2}$ of Mal61p was 26 min in cells from the maltose-limited chemostat and 79 min in cells from the batch culture. From these experiments, we conclude that the different rates of catabolite inactivation by maltose of Mal61p in chemostat and batch-cultured cells are due to different maltose uptake rates, and consequently different rates of maltose metabolism.

Glucose analogues

The next step was to determine whether the first metabolite of maltose metabolism, intracellular glucose, was sufficient to trigger catabolite inactivation of the maltose transporter. For this, the inactivation of Mal61p in the presence of some glucose analogues was determined (Fig. 3). In batch-grown cells, 50 mM glucose caused inactivation of the maltose transporter with a $t_{1/2}$ of 16 min. As has been shown previously (Brondijk et al. 1998), 2-deoxyglucose, which cannot be metabolized via the gly-

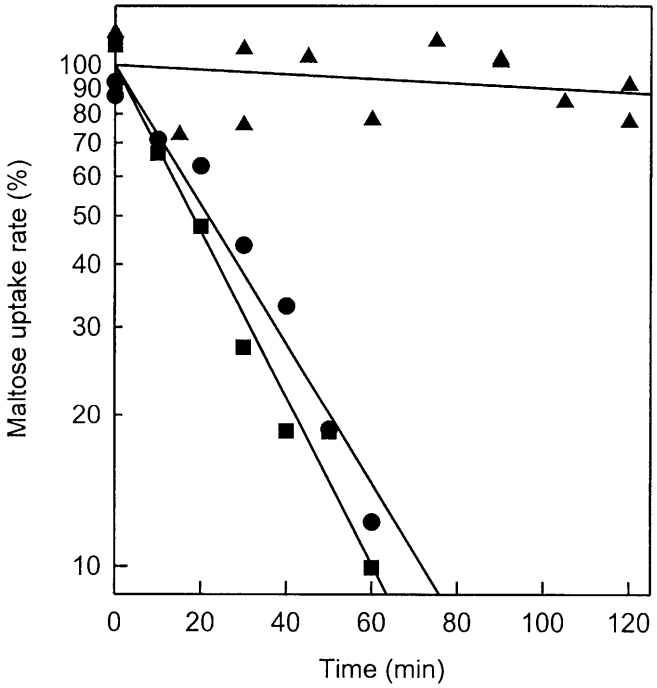

Fig. 3 Catabolite inactivation of Mal61p using different glucose analogues *S. cerevisiae* 6001BΔ11 pYEpY87 was grown in batch culture on 0.5% maltose and catabolite inactivation of Mal61p was measured as described in the legend to Fig. 2. Catabolite inactivation rates were determined in the presence of 50 mM glucose (●), 50 mM of 6-deoxyglucose (▲) 50 mM of 2-deoxyglucose (■)

Table 2 Intracellular metabolites under different conditions of catabolite inactivation. *S. cerevisiae* 6001BΔ11 pYEpY87 was grown in batch culture on 0.5% maltose. At time zero, cells were transferred to minimal medium containing the indicated amount of sugar and 12.5 μg/ml cycloheximide to inhibit protein synthesis. At different time points ,1-ml samples were withdrawn and treated as described in Materials and methods. ATP values are the means of three different measurements. Sugars were added at the indicated concentrations at time zero

	Time (min)	50 mM Glucose	10 mM Glucose	50 mM Maltose	100 mM Maltose
Glucose-6-phosphate (nmol/mg of protein)	–1	2.5	2.6	3.1	2.6
	0.25	19	12.7	4.3	3.0
	10	7.8	6.9	3.8	3.6
	30	5.3	5.3	3.1	3.6
	60	3.6	3.5	3.4	3.1
ATP (μmol/mg of protein)	–1	5.2±2.2	3.7±1.7	9.7±2.9	11.3±4.2
	0.25	6.4±1.7	10.6±2.6	7.5±2.3	7.9±2.1
	10	6.4±1.8	9.6±2.5	7.0±2.2	7.3±2.5
	30	5.3±1.8	7.7±1.9	7.4±1.7	7.1±3.0
	60	8.0±2.6	8.5±2.0	5.9±2.0	5.7±2.3

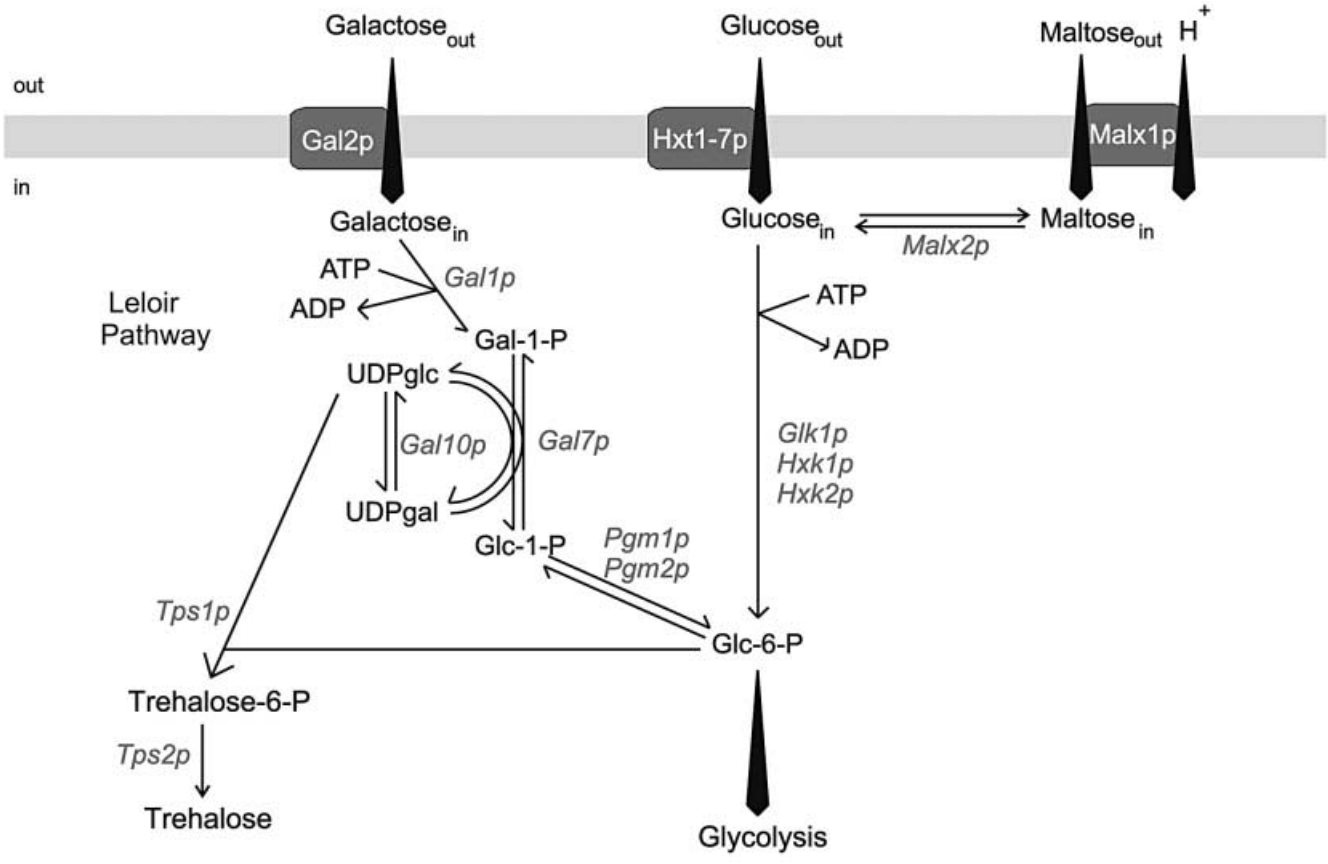

Fig. 4 The initial steps of sugar metabolism in *S. cerevisiae*. The initial steps of metabolism of maltose, glucose and galactose are shown. *Gal1p* Galactokinase, *Gal2p* galactose permease, *Gal7p* UDP-glucose-hexose-1-phosphate uridyltransferase, *Gal10p* UDP-glucose-epimerase, *Glk1p* glucokinase, *Hxk1p* hexokinase I, *Hxk2p* hexokinase II, *Hxt1–7p*, glucose transporters, *Malx1p* maltose , *Malx2p* maltase, *Pgm1p* phosphoglucomutase, *Pgm2p* phosphoglucomutase (major isoform), *Tps1p* trehalose-6-phosphate synthase, *Tps2p* trehalose-6-phosphate phosphatase

colytic pathway beyond 2-deoxyglucose -6-phosphate, caused inactivation of Mal61p with a $t_{1/2}$ of 18 min. In contrast, 6-deoxyglucose, which is taken up by the hexose transporters but not metabolized further, was unable to trigger catabolite inactivation of Mal61p. The slow decrease in maltose uptake capacity that was observed in the presence of 6-deoxyglucose is similar to that in the absence of sugar and probably reflects the normal turnover rate of the protein (data not shown). These studies show that glucose or maltose uptake is not sufficient to trigger

catabolite inactivation of Mal61p, but that glucose phosphorylation is required. Metabolism in the glycolytic pathway beyond glucose-6-phosphate is not required, since 2-deoxyglucose was capable of eliciting catabolite inactivation of Mal61p.

Pools of metabolites during catabolite inactivation

The intracellular pools of glucose-6-phosphate and ATP were determined under conditions that trigger the catabolite inactivation of Mal61p (Table 2). *S. cerevisiae* 6001BΔ11pYepY87 was grown in batch on 0.5% maltose to mid-exponential phase, whereafter cells were shifted to inactivation medium. When cells were incubated in the presence of 10 or 50 mM glucose, a transient increase in glucose-6-P levels was observed immediately following the addition of glucose. The rise in glucose-6-P levels was similar irrespective of whether 50 mM or 10 mM glucose was used, whereas catabolite inactivation of the maltose transporter was significantly higher in the presence of 50 mM glucose (Table 1). Hardly any change in glucose-6-P levels was observed in the presence of 100 mM or 50 mM maltose, whereas these conditions led to a significant inactivation of the maltose transporter (Table 1 and 2). It therefore seems unlikely that the level of glucose-6-P is the primary trigger for catabolite inactivation.

ATP is consumed in the initial steps of glycolysis, whereas it is formed further down in the glycolysis pathway, that is, in the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate and phospho*enol*pyruvate to pyruvate. An "overflow" of metabolism due to a sudden uncontrolled influx of sugar may therefore cause a drop in intracellular ATP levels, which could then be the trigger for the onset of catabolite inactivation of the sugar transporters. However, under conditions that trigger the catabolite inactivation of the maltose carrier, ATP levels remained essentially constant (Table 2).

By eliminating glycolytic intermediates beyond glucose-6-P as signal molecules in the process of catabolite inactivation, it seems plausible that metabolites of the Leloir and/or trehalose synthase pathway (Fig. 4) fulfill this role, as these are the only ones that can be formed from 2-deoxyglucose. On the other hand, the Leloir pathway and trehalose synthase complex are repressed during growth on glucose. Therefore, we determined whether any metabolites other than 2-deoxyglucose-6-phosphate were formed during 2-deoxyglucose-induced catabolite inactivation. Thin-layer chromatography was used to separate metabolites derived from ^{14}C-2-deoxyglucose (Fig. 5). Three different products were formed from 2-deoxyglucose (bands A, B and C in Fig. 5). We identified the different products by following their conversion upon incubation with one or more relevant enzymes (Fig. 5A).

As expected, the main metabolite formed was 2-deoxyglucose-6-phosphate. The 2-deoxyglucose-6P was identified by incubating the cell extract in the presence of glucose-6-phosphate dehydrogenase. This enzyme catalyzes the conversion of glucose-6P into glucono-δ-lac-

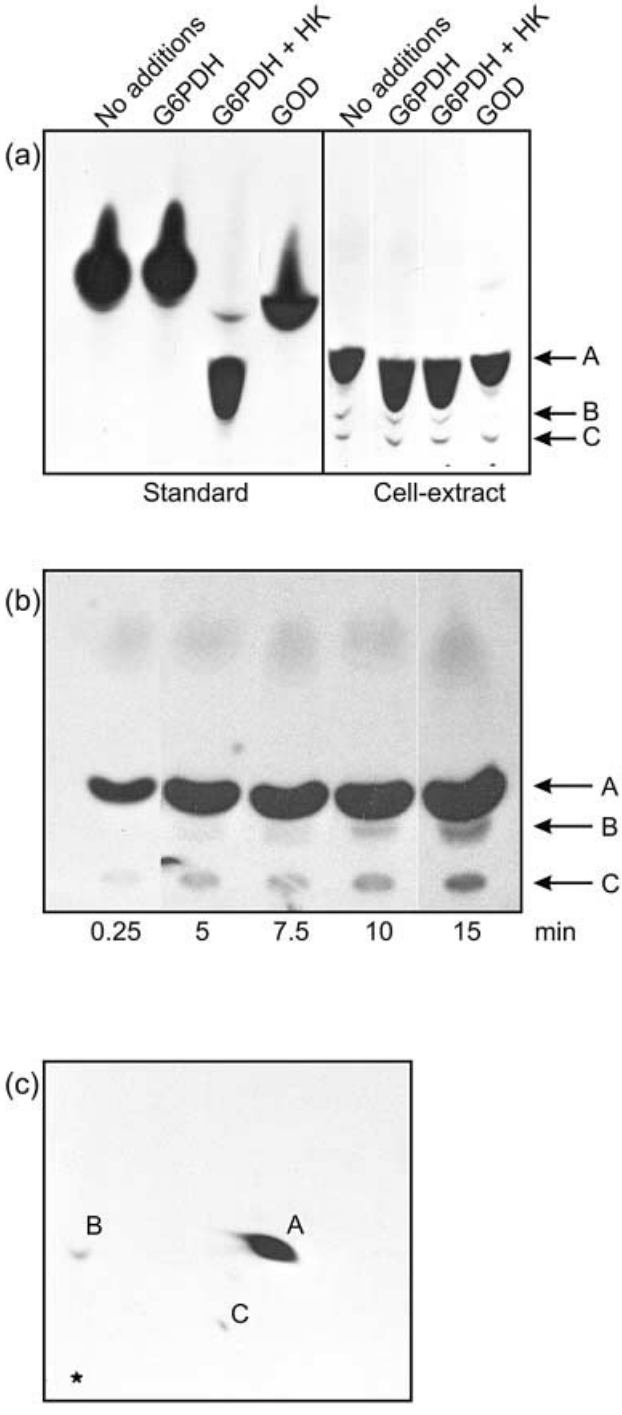

Fig. 5A–C Thin-layer chromatography of cells extracts of *S. cerevisiae* incubated with 2-deoxyglucose. **A** Five-µl samples of ^{14}C-2-deoxyglucose standard (*left*) and labeled cell extracts (*right*) were incubated without further additions, or with glucose-6-phosphate dehydrogenase (*G6PDH*), glucose-6-phosphate dehydrogenase plus hexokinase (*G6PDH+HK*) or glucose oxidase (*GOD*) for 1 h. **B** Cell extract isolated at different time points after the addition of [^{14}C]2-DOG to the cells. **C** A 5-µl sample of the labeled cell extract subjected to two dimensional TLC. The point of loading is indicated with ★. The spots are assigned as follows: *A* Deoxyglucose-6-phosphate, *B* dideoxy-trehalose, *C* dideoxytrehalose-6-phosphate. TLC plates were run as described in Materials and methods

tone-6-phosphate, but it also accepts 2-deoxyglucose-6P as substrate. After incubation with glucose-6P dehydrogenase the major band in the extract migrated at a position that was identical to that of the 2-deoxyglucose standard incubated with hexokinase plus glucose-6P dehydrogenase.

In addition to 2-deoxyglucose-6P, significant amounts of two other, slower-migrating components were observed. These components represented less than 1% of the label incorporated by the cells. On the basis of known metabolic pathways in yeast (Fig. 4), it is likely that the slower-moving components are either UDP-2-deoxyglucose, dideoxytrehalose-6-phosphate or dideoxytrehalose. The formation of deoxytrehalose from 2-deoxyglucose in glucose-grown cells has already been reported (Tran-Dinh et al. 1995; Wietzerbin et al. 1993). Dideoxytrehalose is a substrate of glucose-oxidase and since band B in the extract disappeared after incubation with glucose-oxidase (Fig. 5A), it is concluded that this product corresponds to dideoxytrehalose. The slowest-migrating component (band C) could either be UDP-2DOG or dideoxytrehalose-6P (Fig. 4). Since UDP-2-deoxyglucose is acid labile whereas band C appeared to be acid-stable (not shown), we tentatively assign this component to dideoxytrehalose-6P. This component was already present after 15 s of incubation of the cells with 2-deoxyglucose, whereas the dideoxytrehalose appeared after 5 min of incubation (Fig. 5B).

To determine whether or not bands A, B and C of Fig. 5 were composed of multiple components, the cell extracts were also analyzed on a 2-dimensional TLC (Fig. 5C). This appeared not to be the case. With very long exposure times we also observed trace amounts (<0.1%) of non-metabolized 2-deoxyglucose in the cell extract both in the 1-dimensional and 2-dimensional TLC experiments.

Discussion

It has previously been shown that maltose transport in hybrid membranes is fully reversible (Van der Rest et al. 1995). However, the orientation of the maltose transporter in the hybrid membranes is probably random, which could also explain this observation. Using a *S. cerevisiae* strain that cannot metabolize maltose due to a disruption of the *MAL12* gene, we were able to show that also in whole cells of *S.cerevisiae*, where the orientation of the protein is fully preserved, maltose transport is reversible, albeit with somewhat different kinetic parameters for the uptake and efflux reactions. This difference in kinetics is not surprising, as the internal pH is much lower in the efflux than in the uptake experiments. In contrast to the situation with amino acid transporters (Eddy 1982; Horak 1986), Mal61p does not appear to be inhibited by the presence of intracellular substrate and facilitates both entrance and exit transport.

We also show that maltose does not trigger inactivation of Mal61p in a strain that is unable to metabolize the sugar (6001BΔ11Δ12 pYEpY87), whereas it does so when maltose is metabolized. It thus seems likely that a metabolite derived from maltose, and common to one derived from glucose, triggers the catabolite inactivation of the maltose transporter. 2-Deoxyglucose is phosphorylated but cannot be metabolized further in the glycolytic pathway. As Mal61p is inactivated in the presence of 2-deoxyglucose, we conclude that the metabolism of glucose beyond glucose-6P is not required to trigger catabolite inactivation. Since 6-deoxyglucose was unable to trigger catabolite inactivation of Mal61p, it is unlikely that it is the accumulation of intracellular glucose that forms the trigger for catabolite inactivation.

Several steps in the degradation of plasma membrane proteins require metabolic energy, such as the transport to and fusion of endocytic vesicles with the vacuole. Since 2-deoxyglucose cannot provide the cells with metabolic energy, the required ATP must be originating from the breakdown of storage polymers in the cell. This is quite feasible as the experiments are performed over a relatively short periods of time (1 h) in resting cells of *S.cerevisiae*.

2-Deoxyglucose not only triggers catabolite inactivation of the maltose transporter, but also elicits catabolite repression. 2-Deoxyglucose is highly toxic to *S. cerevisiae* and cells are unable to grow on reduced carbon sources, like ethanol or lactate, in the presence of 2-deoxyglucose. Because of these properties, 2-deoxyglucose is often used to select for mutants that are resistant to catabolite repression. Randez-Gil et al. (1995) isolated a 2-deoxyglucose resistant mutant that showed no catabolite repression even at 0.5% 2-deoxyglucose(the concentration normally used for the selection is 0.03%), whereas it was still sensitive to glucose-induced catabolite repression (Randez-gil et al. 1995). The 2-deoxyglucose -resistant phenotype was caused by the overexpression of a specific 2-deoxyglucose-6P. The 2-deoxyglucose could enter the 2-deoxyglucose-resistant mutant and was phosphorylated with similar kinetics as was glucose, but, due to the overexpression of the 2-deoxyglucose-6P-phosphatase, the 2-deoxyglucose-6P was rapidly dephosphorylated and the free sugar diffused out of the cell (Randez-gil et al. 1995). Based on these results, the authors suggested that the accumulation of hexose phosphates was important for generating the signal for catabolite repression/inactivation.

Recently, Jiang et al. (2000) showed the importance of hexokinases, especially *HXK2*, for signaling the degradation of Mal61p via pathway 2. Based on these results and the fact that also maltose and galactose can trigger pathway 2 signaling, the authors concluded that the pathway 2 signal is generated by a high rate of sugar metabolism. This high rate of sugar metabolism is most likely reflected in the concentration of a certain intracellular metabolite, which could be monitored by a sensor protein that triggers signaling via pathway 2.

Our observations also confirm that high metabolic fluxes in the first steps of sugar metabolism are required to induce catabolite inactivation. We set out to identify the metabolite that activates the catabolite inactivation pathway. Glucose-6P would be a prime candidate as the trig-

ger for catabolite inactivation of Mal61p. However, we did not observe a correlation between glucose-6P levels and catabolite inactivation rates of the maltose transporter Mal61p. ATP is consumed during the initial steps of glycolysis and it is only produced in the later steps. Therefore, the rapid uptake of glucose or maltose could lead to a transient depletion of the ATP pool, but this was not observed under our experimental conditions. Previously, Penalver et al. (1998) also reported a lack of correlation between ATP levels and the rate of inactivation of the maltose transporter.

Although 2-deoxyglucose-6P cannot be metabolized further in glycolysis, it can be converted to intermediates of the Leloir pathway and into dideoxytrehalose-6P and dideoxytrehalose. Accumulation of any of these compounds may be the signal that causes the catabolite inactivation of Mal61p and most likely also the other high-affinity sugar transporters. Also galactose, which has been reported to trigger the catabolite inactivation of Mal61p, is metabolized via the Leloir pathway and could therefore produce the same signal. We have shown that cells incubated in the presence of 2-deoxyglucose accumulate not only 2-deoxyglucose-6P, but also two other components which correspond most likely to dideoxytrehalose-6P and dideoxytrehalose. These components are already present in significant amounts within 1–2 min after the addition of 2-deoxyglucose to the cells and could therefore very well be the prime trigger for the onset of catabolite inactivation.

There has been some speculation about the role of trehalose-6-phosphate synthase (Tps1p) in mediating the signal that triggers catabolite inactivation (Hohmann et al. 1996; Thevelein and Hohmann 1995). An unexplained phenotype of *tps1* mutants is that they are unable to grow on readily fermentable carbon sources such as glucose. This growth defect is caused by an uncontrolled influx of these sugars in *tps1* mutants. Three hypotheses have been proposed to explain the effect of the *tps1*mutation: (1) It has been suggested that trehalose production may act as a buffer system for glycolysis. When too much glucose-6P is formed, which could lead to a drop in ATP and free inorganic phosphate levels, conversion of excess glucose-6P into trehalose would recycle free inorganic phosphate for later use during glycolysis. (2) It has been observed that the sugar kinases are inhibited by trehalose-6P. This provides a feed-back mechanism on glucose influx. When glucose fluxes increase, trehalose-6P is formed, which inhibits the kinase activity and thus prevents uncontrolled influx of sugars into glycolysis. (3) It has been postulated that Tps1 has, in addition to its role in trehalose synthesis, a separate function in regulation of transport and/or phosphorylation of sugars. A significant portion of Tps1p has been found to be present in a form not bound by the trehalose synthase complex (Bell et al. 1998). This monomeric form of Tps1p has been suggested to act as a regulatory protein. Our results provide the first evidence for the accumulation of trehalose-6P and trehalose under conditions that trigger catabolite inactivation of the maltose transporter. Since we found no apparent correlation between catabolite inactivation rates and the levels of ATP or other metabolites, we favor the suggestion that Tps1p and/or trehalose-6P provide the signal that leads to the active degradation of the maltose and possibly other sugar transporters.

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References

Bell W, Sun W, Hohmann S, Wera S, Reinders A, De VC, Wiemken A, Thevelein JM (1998) Composition and functional analysis of the *Saccharomyces cerevisiae* trehalose synthase complex. J Biol Chem 273:33311–33319

Brondijk THC, Van der Rest ME, Pluim D, Stingl K, Poolman B, Konings WN (1998) Catabolite inactivation of wildtype and mutant maltose transport proteins in *Saccharomyces cerevisiae*. J Biol Chem 273:15352–15357

Charron MJR, Dubin A, Michels CA (1986) Structural and functional analysis of the MAL1 locus of *Saccharomyces cerevisiae*. Mol Cell Biol 6:3891–3899

Eddy AA (1982) Mechanisms of solute transport in selected microorganisms. Adv Microb Physiol 23:1–78

Gonzalez B, François JM, Renaud M (1997) Yeast functional analysis reports: A rapid and reliable method for metabolite extraction in yeast using boiling buffered ethanol. Yeast 13:1347–1355

Hohmann S, Bell W, Neves MJ, Valckx D, Thevelein JM (1996) Evidence for trehalose-6-phosphate-dependent and-independent mechanisms in the control of sugar influx into yeast glycolysis. Mol Microbiol 20:981–991

Holzer H (1976) Catabolite inactivation in yeast. Trends Biochem Sci 1:178–181

Horak J (1986) Amino acid transport in eukaryotic microorganisms. Biochim Biophys Acta 864:223–256

Jiang H, Medintz I, Michels CA (1997) Two glucose sensing/signaling pathways stimulate glucose-induced inactivation of maltose permease in *Saccharomyces*. Mol Biol Cell 8:1293–1304

Kraakman L, Lemaire K, Ma P, Teunissen-Aloys W-RH, Donaton-Monica CV, Van DP, Winderickx J, de-Winde JH, Thevelein JM (1999) A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol Microbiol 32:1002–1012

Medintz I, Jiang H, Han EK, Cui W, Michels CA (1996) Characterization of the glucose-induced inactivation of maltose permease in *Saccharomyces cerevisiae*. J Bacteriol 178:2245–2254

Needleman R (1991) Control of maltase synthesis in yeast. Mol Microbiol 5:2079–2084

Özcan S, Dover J, Rosenwald AG, Woelfl S, Johnston M (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc Natl Acad Sci USA 93:12428–12432

Özcan S, Dover J, Johnston M (1998) Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO J 17:2566–2573

Penalver E, Lucero P, Moreno E, Lagunas R (1998) Catabolite inactivation of the maltose transporter in nitrogen- starved yeast could be due to the stimulation of general protein turnover. FEMS Microbiol Lett. 166:317–324

Poolman B, Glaasker E (1998) Regulation of compatible solute accumulation in bacteria. Mol Microbiol 29:397–407

Randez-gil F, Prieto JA, Sanz P (1995) The expression of a specific 2-deoxyglucose-6P phosphatase prevents catabolite repression mediated by 2-deoxyglucose in yeast. Curr Genet 28: 101–107

Riballo E, Herweijer M, Wolf DH, Lagunas R (1995) Catabolite inactivation of the yeast maltose transporter occurs in the vacuole after internalization by endocytosis. J Bacteriol 177:5622–5627

Robinson KS, Lai K, Cannon TA, McGraw P (1996) Inositol transport in *Saccharomyces cerevisiae* is regulated by transcriptional and degradative endocytic mechanisms during the growth cycle that are distinct from inositol-induced regulation. Mol Biol Cell 7:81–89

Thevelein JM, Hohmann S (1995) Trehalose synthase: guard to the gate of glycolysis in yeast? Trends Biochem Sci 20:3–10

Tran-Dinh S, Wietzerbin J, Courtois A, Herve M (1995) A novel approach for investigating reaction mechanisms in cells. Mechanism of deoxy-trehalose synthesis in *Saccharomyces cerevisiae* studied by 1H-NMR spectroscopy. Eur J Biochem. 228: 727–731

Van der Rest ME, De Vries Y, Poolman B, Konings WN (1995) Overexpression of Mal61p in *Saccharomyces cerevisiae* and characterization of maltose transport in artificial membranes. J Bacteriol 177:5440–5446.

Van Leeuwen CCM, Weusthuis RA, Postma E, Van den Broek PJA (1992) Maltose/proton co-transport in *Saccharomyces cerevisiae*. Biochem J 284:441–445

Wietzerbin J, Herve M, Un S, Neumann JM, Namane A, Tran-Dinh S (1993) Influence of glucose on the deoxyglucose metabolism in *S. cerevisiae*: detection and identification of deoxyglucose and trehalose derivatives by 1H- and 13C-NMR spectroscopy. Biochimie 75:825–830