Expression of *Escherichia coli otsA* in a *Saccharomyces cerevisiae tps1* mutant restores trehalose 6-phosphate levels and partly restores growth and fermentation with glucose and control of glucose influx into glycolysis

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The *TPS1* gene, encoding trehalose-6-phosphate synthase (TPS), exerts an essential control on the influx of glucose into glycolysis in the yeast *Saccharomyces cerevisiae*. The deletion of *TPS1* causes an inability to grow on glucose because of a hyper-accumulation of sugar phosphates and depletion of ATP and phosphate. We show that expression of the *Escherichia coli* homologue, *otsA*, in a yeast *tps1* mutant results in high TPS activity. Although the trehalose 6-phosphate (Tre6P) level during exponential growth on glucose was at least as high as in a wild-type yeast strain, growth on glucose was only partly restored and the lag phase was much longer. Measurement of the glycolytic metabolites immediately after the addition of glucose showed that in spite of a normal Tre6P accumulation there was still a partial hyperaccumulation of sugar phosphates. Strong elevation of the Tre6P level by the additional deletion of the *TPS2* gene, which encodes Tre6P phosphatase, was not able to cause a strong decrease in the sugar phosphate levels in comparison with the wild-type strain. In addition, in chemostat experiments the short-term response to a glucose pulse was delayed, but normal metabolism was regained over a longer period. These results show that Tre6P synthesis from a heterologous TPS enzyme can to some extent restore the control of glucose influx into glycolysis and growth on glucose in yeast. However, they also indicate that the yeast TPS enzyme, as opposed to the *E. coli otsA* gene product, is able to increase the efficiency of the Tre6P control on glucose influx into yeast glycolysis.

Key words: hexokinase, metabolic control, trehalose metabolism, yeast.

INTRODUCTION

In the yeast *Saccharomyces cerevisiae* trehalose is synthesized by trehalose-6-phosphate synthase (TPS), encoded by *TPS1*, and trehalose-6-phosphate phosphatase (TPP), encoded by *TPS2* [1,2]. The two enzymes reside in the trehalose synthase complex together with regulatory subunits encoded by *TSL1* and *TPS3* [3–5]. All subunits share a domain that is similar to Tps1. Inactivation of TPS causes an absence of trehalose accumulation but also a growth defect specifically on glucose and related sugars that are rapidly fermented [6,7]. When glucose is added to *tps1* cells pre-grown on non-fermentable or slowly fermentable carbon sources, a rapid hyperaccumulation of glucose 6-phosphate (Glu6P), fructose 6-phosphate (Fru6P) and especially fructose 1,6-bisphosphate (Fru1,6bisP) is observed concomitant with a rapid depletion of ATP and free phosphate. The flow through glycolysis gets stuck, apparently at the level of glyceraldehyde-3-phosphate dehydrogenase because all glycolytic intermediates upstream of this enzyme accumulate, whereas all intermediates downstream become depleted. In addition to an absence of growth on glucose, no ethanol is produced.

The rapid hyperaccumulation of sugar phosphates is consistent with overactive hexokinase activity *in vivo*. Deletion of *HXK2*, which encodes the most active hexokinase isoenzyme, restores growth of the *tps1* mutants on glucose, which supports the idea that the deregulation of glycolysis is due to overactive hexokinase activity [8]. In addition to the growth defect on glucose, the *tps1* mutants displayed a variety of other phenotypic defects, the most prominent being the complete absence of glucose-induced signalling effects [7,9]. However, the deletion of *HXK2* in a *tps1* mutant also suppressed the glucose-induced signalling defects [8]. This was consistent with hyperactive hexokinase activity as a primary consequence of the mutation, and the absence of glucose-induced signalling a secondary consequence of the rapid deregulation of glycolysis, in particular the rapid depletion of ATP.

It has been demonstrated that trehalose 6-phosphate (Tre6P) inhibits hexokinase activity *in vitro* and that the Tre6P level *in vivo* is in the same range (100–200 μM), at least under steady-state conditions, as the K_i for the inhibition of hexokinase by Tre6P [10]. Although this observation offers an attractive explanation for the apparent hyperactivity of hexokinase and the resulting deregulation of glycolysis in the *tps1* mutant, it also raises several questions. Inhibition of hexokinase by Tre6P *in vitro* is competitive with respect to glucose. If this were also true *in vivo*, any elevation of the free glucose level in the cells above a few hundred micromolar would tend to override the Tre6P control of hexokinase and cause the deregulation of glycolysis. In addition, Tre6P is formed as an intermediate of trehalose biosynthesis within the trehalose synthase complex. Controlled leakage of Tre6P from the complex would be required for the precise regulation of hexokinase activity. A 10-fold overexpression of hexokinase activity in a wild-type strain does not cause the deregulation of glycolysis and the growth defect with glucose as seen in the *tps1* mutant, which seems to indicate

Abbreviations used: Fru6P, fructose 6-phosphate; Fru1,6bisP, fructose 1,6-bisphosphate; Glu6P, glucose 6-phosphate; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase; Tre6P, trehalose 6-phosphate.

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mechanisms other than, or in addition to, allosteric control by Tre6P [11]. In contrast, the deletion of *TPS2* results in a very high Tre6P level and a decrease in the rate of sugar phosphate accumulation after the addition of glucose. This observation is consistent with an enhanced inhibition of hexokinase activity *in vivo* [12].

Two other mechanisms have been proposed for the regulation of hexokinase activity by Tps1. It has been suggested that the hyperaccumulation of Glu6P is prevented in wild-type cells by the deviation of Glu6P into trehalose biosynthesis [8]. This leads to a recovery of free phosphate, which is required as the substrate for glyceraldehyde-3-phosphate dehydrogenase and thus for the flux through glycolysis. This hypothesis is consistent with the observation that the stimulation of glycerol biosynthesis restores growth of the *tps1* mutant on glucose [13]. However, it has been contradicted by the demonstration that a decrease in phosphate recovery by the deletion of *TPS2* in a yeast strain with partial activity of Tps1 (*byp1*) improved growth on glucose [12]. A second alternative mechanism proposed that the *TPS1* gene product had a regulatory function for the inhibition of hexokinase activity in addition to its catalytic function in trehalose biosynthesis. Such a function would allow the uncoupling of the normal biosynthesis of trehalose from the restriction of hexokinase activity required for normal flux through glycolysis. Support for this idea has been obtained by the observation that the deletion of *TPS2* in a yeast mutant with a decreased amount of Tps1 restored the Tre6P level but not the normal control of glucose influx into glycolysis [12]. In addition, evidence has been obtained that Tps1 is present not only in the trehalose synthase complex but also as a free protein. The deletion of *TPS2* abolishes the trehalose synthase complex, resulting in only free Tps1 [5]. Recent studies on the reconstitution of yeast glycolysis in permeabilized cells of the *tps1* and *tps2* mutants have shown that, after the removal of Tre6P by the permeabilization procedure, the differences in flux and glycolytic intermediates from the wild-type strain remain partly present. This also supports an additional role of the Tps1 protein in the control of glycolysis [13a].

In *Escherichia coli*, trehalose is also synthesized by TPS and TPP but the two enzymes do not associate into a complex as they do in yeast. The two enzymes are encoded by the genes *otsA* and *otsB* respectively [14,15]. The *otsB* gene shows sequence similarity only to the presumed phosphatase domain encoded by the yeast *TPS2* gene, which might explain why *E. coli* TPS and TPP remain separate enzymes. Here we show that the replacement of *S. cerevisiae TPS1* with its homologue in *E. coli*, *otsA*, fully restores the Tre6P level in yeast cells but does not lead to the full restoration of growth on levels of glucose and glycolytic intermediates. This supports a specific role for Tps1 in the control of glucose influx into glycolysis.

MATERIALS AND METHODS

Yeast strains and plasmids

The *S. cerevisiae* strains used in this work are listed in Table 1. The pXKL1 vector contains promoter and terminator sequences of the 5′ and 3′ regions of the *PGK* gene. The 5′ and 3′ *PGK* regions are linked via a *Bgl*II site, used for the insertion of gene sequences containing an initiator codon. The *E. coli otsA* gene was obtained by amplification with PCR and cloned into this vector on a 1.4 kb fragment. For the chemostat experiments, the W303-1A and the *tps1*Δ strain with *otsA* were made completely prototrophic (ura$^+$ leu$^+$ his$^+$ ade$^+$ trp$^+$), to give strains PVD32 and PVD25 respectively. In the PVD25 strain the *otsA* gene was introduced into the genome so that it could not get lost during extended growth in the chemostat.

Table 1 *S. cerevisiae* strains used in this work

Yeast strains isogenic to W303-1A	Relevant genotype	Reference
W303-1A (wild type)	*Mata leu2-3,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC*	[27]
YSH 290	*tps1*Δ*::TRP1*	[8]
YSH 321	*byp1-3*	[9]
YSH 450	*tps2*Δ*::HIS3*	[9]
YSH 567	*tps1*Δ*::TRP1 tps2*Δ*::LEU2*	[12]
YSH 6.125-1A	*byp1-3 tps2*Δ*::LEU2*	[12]
PVD32 (wild type)	Prototrophic W303-1A	This study
PVD25	Prototrophic *tps1*Δ + *otsA*	This study

Growth conditions

Yeast cells were grown on minimal medium as described [16], supplemented with a carbon source as indicated. For growth curves and ethanol production in liquid medium, the cells were pregrown in 2% (w/v) galactose into stationary phase and then transferred to 2% (w/v) glucose or 2% (w/v) fructose. For metabolite determinations, cells were pregrown in minimal medium supplemented with 3% (w/v) glycerol and 0.1% galactose, harvested during late exponential phase and resuspended in fresh medium; glucose was then added to a final concentration of 100 mM. Trehalose concentration and TPS activities were measured after growth in 2% (w/v) galactose, 2% (w/v) glucose and 2% (w/v) fructose into either exponential, late exponential or stationary phase.

Biochemical determinations

Glycolytic metabolites were extracted and determined essentially as described in [17]. Tre6P was determined in the same extracts prepared for glycolytic metabolite determination. For Tre6P determination, glucose was first removed by anion-exchange chromatography; the Tre6P was then hydrolysed to glucose and Glu6P by treatment with phosphotrehalase (*Bacillus subtilis*). The glucose produced in this reaction was measured with glucose oxidase/peroxidase and *o*-dianisidine (C. Van Vaeck, S. Wera and J. M. Thevelein, unpublished work). Using the total amount of protein in the sample, as determined by the method described in [18], and with the assumption of a yeast cytosolic volume of 12 μl/mg of protein, cytosolic concentrations were calculated in mM. The ethanol concentration in the medium was determined with a commercial kit from Boehringer Mannheim. TPS activity was measured by the assay described in [19]. Trehalose was determined by the method described in [20]. Protein was quantified with the biuret method as described in [21].

Chemostat experiments

The cells were cultivated in chemostat cultures with a dilution rate of 0.10 h^{-1} using a defined medium as described by Albers et al. [21a] with ammonium sulphate as nitrogen source and a glucose concentration of 5 g/l. Steady-state conditions were established and maintained for more than 24 h before the addition of 200 mM glucose. At this point a glucose solution was poured into the chemostat (the pouring procedure lasted for approx. 1 min), resulting in a final concentration of 200 mM. In

addition, the feed medium was changed to a glucose concentration of 200 mM. One sample was taken before the addition of glucose and several samples after the addition of glucose over a period of either 20 h or 20 min.

RESULTS

E. coli otsA partly restores trehalose content and TPS activity

The *E. coli* homologue *otsA* of *S. cerevisiae TPS*1 was expressed in different *tps1* mutant strains by using either the *TPS1* promoter or the strong *PGK* promoter. The *tps1* mutant strains used were *tps1*Δ, *tps1*Δ *tps2*Δ and *byp1* (Table 1). The *byp1* strain contains a nonsense mutation in the *TPS1* gene that is to some extent read through by a nonsense tRNA suppressor, resulting in low Tps activity and low levels of Tre6P and trehalose [12,22]. When these strains were transformed with the *otsA* gene expressed from the *PGK* promoter, TPS activity could be restored by up to 15% in late-exponential-phase cells and 25% in stationary-phase cells. The trehalose content was even higher, up to 85% of that in the wild-type strain (Table 2). In the *tps1*Δ *tps2*Δ strain there is no *TPS2*-encoded Tre6P phosphatase activity; trehalose accumulation is therefore always very low. When the *otsA* gene was expressed from the *TPS1* promoter, TPS activity and trehalose content were somewhat lower (results not shown), indicating that the expression of *E. coli otsA* in a yeast *tps1* mutant results in significant TPS activity.

E. coli otsA partly restores growth on glucose

The *tps1*Δ, *tps1*Δ *tps2*Δ and *byp1* strains are all unable to grow on glucose in liquid medium (Figure 1A). The expression of *E. coli otsA* from the *PGK* promoter in these strains only partly restored growth on glucose. In the transformants the lag phase was also longer. The final cell density of the *tps1*Δ+*otsA* strain was the lowest. Ethanol production from glucose was virtually absent from the *tps1*Δ, *tps1*Δ *tps2*Δ and *byp1* strains (Figure 1B). The expression of *E. coli otsA* from the *PGK* promoter in these strains restored ethanol production somewhat less than it restored the growth on glucose. Similar results were obtained for growth and ethanol production on fructose-containing medium (results not shown). The introduction of *otsA* into a wild-type strain, in *byp1 tps2*Δ or in *tps1*Δ *tps2*Δ, in all cases made the strain sensitive to temperature for growth on glucose, fructose and galactose (results not shown). Since it is known that *tps2* mutant strains are temperature sensitive, because of the accumulation of Tre6P to toxic levels at high temperature [2], this supports the notion that the *otsA* gene product expressed in yeast is truly active *in vivo*.

E. coli otsA fully restores the Tre6P level

The Tre6P level in exponential-phase cells growing on glucose was measured. The Tre6P level in the *tps1*Δ and *byp1* strains expressing *otsA* was 2–3-fold higher than in the wild-type strain and 6–7-fold higher in the *tps1*Δ *tps2*Δ+*otsA* strain (Figure 1C). In addition, in a wild-type strain overexpressing *otsA* the Tre6P level was considerably elevated: approx. 4-fold that in the wild-type strain transformed with the empty vector. These results show that the *E. coli otsA* gene product was easily able to provide a normal and even a higher supply of Tre6P than the wild-type strain. Because the level of Tre6P is one or two orders of magnitude lower than the trehalose level and because the *otsA* gene product was able to sustain up to 85% of the normal accumulation of trehalose, this is not surprising. In addition, it is possible that the *otsA* gene product is unable to form a proper trehalose synthase complex with the yeast subunits Tps2, Tsl1 and Tps3. This could explain why the TPS activity is lower than would be expected from the level of trehalose accumulation. Free yeast Tps1 also has much lower TPS activity [5]. Tre6P formed within the trehalose synthase complex is probably hydrolysed more efficiently than Tre6P formed throughout the cytosol, which could explain why the *tps1*Δ+*otsA* strain accumulates an even higher level of Tre6P than the control strain.

E. coli otsA does not prevent the hyperaccumulation of sugar phosphates

Because the growth and fermentation activity of the *tps1*Δ strain on glucose-containing medium was only partly restored by *otsA*, we investigated the build-up of glycolytic metabolites after the

Table 2 Trehalose content and TPS activity in different *tps1* mutants transformed with *E. coli otsA*

TPS activity in a *tps1*Δ strain in exponential-phase cells was 0.000 and in stationary-phase cells was 0.001–0.002.

		Trehalose content (nmol/mg wet weight)			TPS activity (μkat/g of protein)		
Strain	Carbon source	Mid-exponential phase	Late exponential phase	Stationary phase	Mid-exponential phase	Late exponential phase	Stationary phase
Wild type + pXKL1	Galactose	4.3	38.3	55.7	0.09	0.48	0.56
	Fructose	3.6	45.5	61.2	0.11	0.50	0.52
	Glucose	3.1	44.7	56.3	0.11	0.42	0.58
*tps1*Δ + *otsA*	Galactose	17.4	31.0	36.4	0.001	0.02	0.06
	Fructose	12.4	32.0	52.1	0.004	0.06	0.16
	Glucose	10.4	32.1	46.0	0.003	0.05	0.14
*tps1*Δ*tps2*Δ + *otsA*	Galactose	1.8	2.0	3.1	0.001	0.01	0.08
	Fructose	2.1	2.4	2.6	0.004	0.07	0.17
	Glucose	3.3	2.8	3.5	0.003	0.05	0.14
byp1 + *otsA*	Galactose	16.1	33.3	32.2	0.002	0.02	0.08
	Fructose	11.0	37.1	49.8	0.004	0.07	0.16
	Glucose	8.9	33.1	42.1	0.004	0.06	0.15

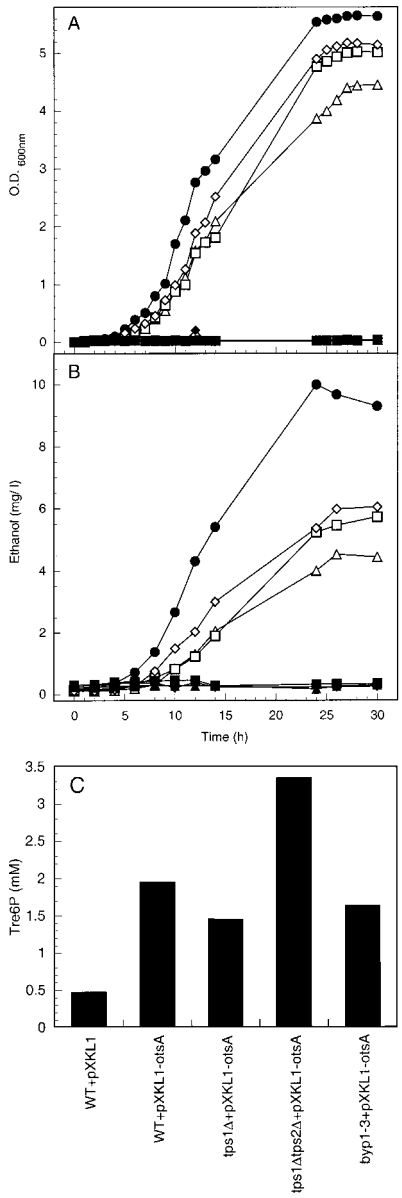

Figure 1 ***E. coli otsA*** **partly restores growth and ethanol production on glucose-containing medium in the** ***tps1*****Δ strain in spite of the presence of a high Tre6P level**

(**A**) Growth; (**B**) ethanol production; (**C**) Tre6P level. (The cells were grown on glucose into mid-exponential phase.) Strains: ●, wild type (W303-1A); ▲, *tps1*Δ; ■, *tps1*Δ *tps2*Δ; ◆, *byp1*; △, *tps1*Δ + p*otsA*; □, *tps1*Δ *tps2*Δ + p*otsA*; ◇, *byp1* + p*otsA*. O. D_{600nm}, attenuance at 600 nm.

addition of glucose. Figure 2 shows that after the addition of glucose to cells of the *tps1*Δ + *otsA* strain there is a normal accumulation of Tre6P. In spite of this there is a hyperaccumulation of Glu6P and Fru6P, as in cells of the *tps1*Δ mutant. In addition, the accumulation of Fru1,6bisP is higher in the *tps1*Δ + *otsA* strain than in the wild type, although not as markedly high as in the *tps1*Δ strain (Figure 2). This might be related to the partial restoration of growth and the formation of ethanol, because the decrease in ATP and in free phosphate levels are also more similar to those in the wild-type strain. These results show that the normal Tre6P level provided by the *otsA* gene is not able to control the build-up of the glycolytic intermediates as it is in the wild-type strain.

Very high Tre6P levels have only little effect on the accumulation of sugar phosphates

In the *tps2*Δ strain the basal Tre6P level is constitutively elevated at least 10-fold above the level in the wild-type strain and increases further after addition of glucose (Figure 2) [12]. In spite of this very high level, the increases in Glu6P, Fru6P and Fru1,6bisP were similar or only slightly smaller in comparison with those in the wild-type strain. The decrease in ATP and phosphate levels were similar to those in the wild-type strain. In previous experiments under somewhat different conditions, a stronger decrease in sugar phosphate accumulation had been observed in the *tps2*Δ strain [12]. The present results show that strongly elevated Tre6P levels do not necessarily result in a strong decrease in Glu6P, Fru6P and Fru1,6bisP levels compared with those in a wild-type strain.

The expression of *otsA* in the *tps1*Δ *tps2*Δ and *byp1-3 tps2*Δ strains resulted in much higher Tre6P levels than in a *TPS1* *tps2*Δ strain (Figure 3). The reason for this is not clear. A large difference in TPS activity does not seem to be involved. Although TPS activity in a *tps2*Δ strain, as measured *in vitro*, is only 20 % of that in a wild-type strain [5], this is still approximately the same as that measured for cells of the *tps1*Δ *tps2*Δ + *otsA* strain (Table 2). Therefore TPS activity, as measured *in vitro*, is comparable in the three strains. It is clear from Figure 3 that the high level of Tre6P in the *byp1-3* *tps2*Δ + *otsA* and *tps1*Δ *tps2*Δ + *otsA* strains compared with that in the *tps2*Δ strain has only little effect on the accumulation of Glu6P and Fru6P. Only the accumulation of Fru1,6bisP might be decreased somewhat in the two strains with *otsA* in comparison with the *tps2*Δ strain. In both strains the ATP level remains high, which is consistent with the restoration of growth and the production of ethanol in these strains.

The metabolites were also measured over a longer time period. At 5 h after the addition of glucose the metabolites in the strains that were able to grow on glucose were much closer, and in most cases very similar, to those in the wild-type cells. In the *tps1* strains unable to grow on glucose the sugar phosphates and in particular Fru1,6bisP accumulated to very high levels (results not shown).

Chemostat experiments with the *tps1*Δ + *otsA* strain

In a chemostat it is possible to grow micro-organisms for an extended period under substrate limitation, during which they usually develop a high capacity for substrate transport. The sudden addition of a high concentration of substrate can then lead to severe metabolic deregulation, causing 'substrate-accelerated death'. This has been observed with maltose in yeast [23]. In addition, it has been reported that in yeast cells the glucose transport capacity is enhanced substantially by growth under glucose limitation during a long duration in a chemostat. However, the cells still seem to be able to cope with a sudden load of glucose given afterwards [24,25]. We therefore investigated whether the *tps1*Δ + *otsA* strain would be able to cope with a sudden load of glucose given after an extended period of growth under glucose limitation in a chemostat. Although there was a significant delay in glucose consumption, CO_2 output, O_2 uptake and the production of ethanol and glycerol under these conditions in the *tps1*Δ + *otsA* strain in comparison with the

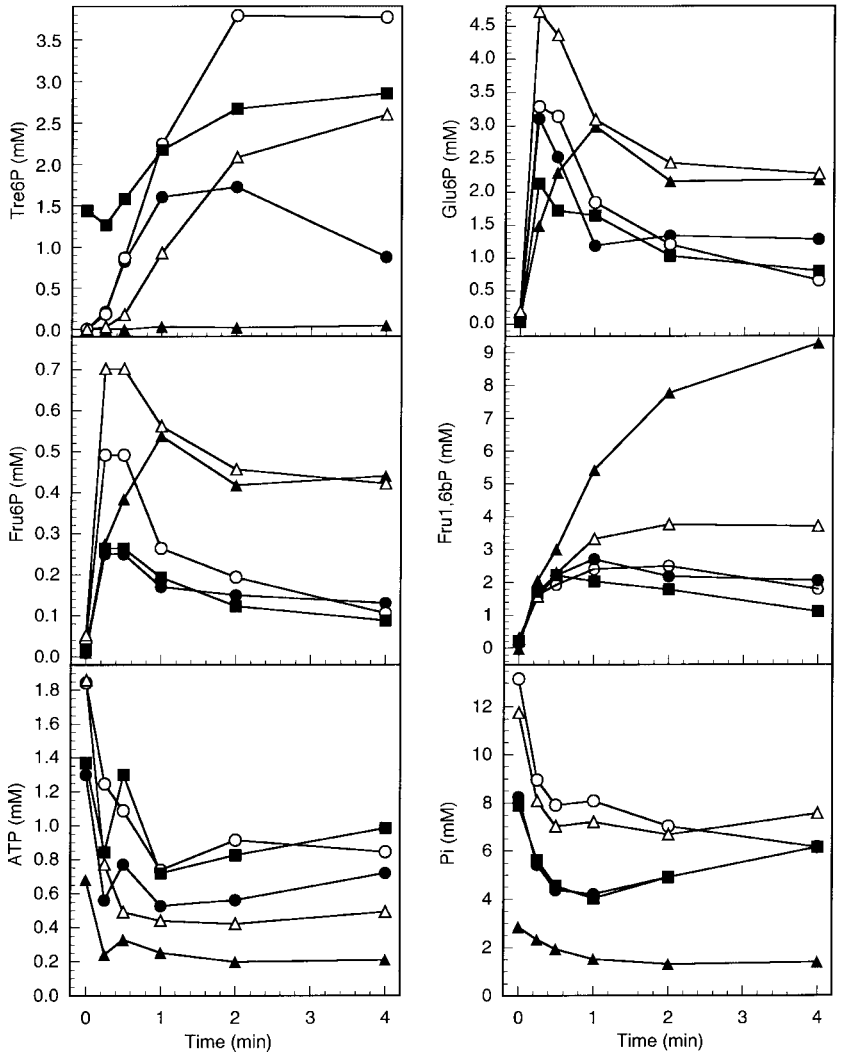

Figure 2 Levels of Tre6P, glycolytic intermediates, ATP and phosphate in the first 4 min after the addition of 100 mM glucose to derepressed cells

Strains: ●, wild type (W303-1A); ▲, *tps1*Δ; ■, *tps2*Δ; ○, wild type + p*otsA*; △, *tps1*Δ + p*otsA*.

wild-type strain, normal metabolism was regained over a longer period. The transformant was clearly not washed out (results not shown). The ATP level after the addition of glucose did not show a significant difference between the two strains. Apparently, the *tps1*Δ+*otsA* strain was relatively easily able to cope with a sudden glucose load under these conditions.

DISCUSSION

E. coli otsA restores the Tre6P level but not the proper control of glucose influx into glycolysis

Expression of the *E. coli* homologue, *otsA*, of *S. cerevisiae TPS*1 in the *tps1*Δ strain clearly causes a marked improvement in its capacity to grow on glucose. The simplest explanation for this is that the expression of *otsA* restores the Tre6P level, which down-regulates hexokinase activity and thereby prevents the hyper-accumulation of sugar phosphates and the concomitant depletion of ATP and P_i that occur in the *tps1*Δ mutant on glucose medium. A determination of TPS activity in cell extracts and trehalose content in cells of the *tps1*Δ+*otsA* transformant confirmed that *otsA* expression results in significant TPS activity. In stationary-phase cells *otsA* expression enhanced the trehalose level and the TPS activity more than in exponential-phase cells. Because the control of glucose influx into glycolysis was studied by measuring the accumulation of glucose metabolites in the first minutes after the addition of glucose, TPS activity in stationary-phase cells seems to be most relevant for this aspect.

Although the TPS activity level is not as high as in wild-type yeast cells (as also reflected in the lower trehalose content), it is apparently high enough to restore fully at least the normal Tre6P level in the cells. This was observed both for the basal level during growth on glucose and for the transient glucose-induced rise immediately after the addition of glucose. This can be explained in two ways. First, the Tre6P level is very low compared with the trehalose level, at least 1000-fold lower. Hence the capacity of the *otsA*-encoded TPS activity can be expected to be more than sufficient to generate a normal Tre6P level. The Tre6P level in the *tps1*Δ+*otsA* transformant was consistently higher than in the wild-type strain. Secondly, it is not known how the Tre6P level in *S. cerevisiae* is controlled. The *otsA* gene product

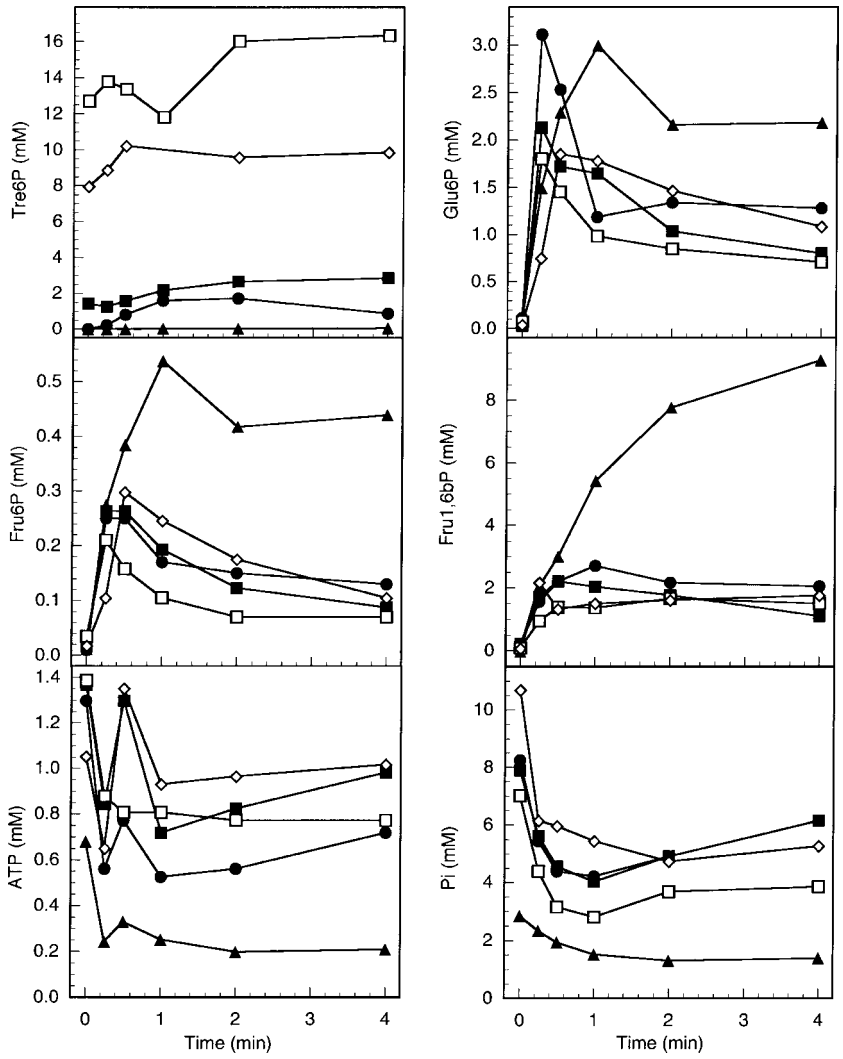

Figure 3 Levels of Tre6P, glycolytic intermediates, ATP and phosphate in the first 4 min after the addition of 100 mM glucose to derepressed cells

Strains: ●, wild type (W303-1A); ▲, *tps1*Δ; ■, *tps2*Δ; ◇, *byp1-3 tps2*Δ + p*otsA*; □, *tps1*Δ *tps2*Δ + p*otsA*.

might be less sensitive to product inhibition by Tre6P. In addition, understanding the regulation of the Tre6P level in yeast is complicated by the localization of Tps1, which is present within the trehalose synthase complex and also in a non-complex-associated form [5]. It is not clear which, or to what extent, the two forms contribute to the cellular Tre6P level. It seems reasonable to assume that the assembly of the TPS and TPP enzymes within one complex serves to improve the efficiency of trehalose synthesis by channelling the intermediate Tre6P within the complex. This raises the question of whether the Tre6P formed within the complex is released at all into the cytosol. In *E. coli*, *otsA*-encoded TPS and *otsB*-encoded TPP are separate enzymes [14]. It is unclear whether the *otsA* gene product in yeast is able to associate into the trehalose synthase complex together with the yeast subunits Tps2, Tps3 and Tsl1. If it cannot, or is less well able to incorporate into the complex, this might help to explain why the Tre6P level is higher in the *tps1*Δ + *otsA* transformant than in the control strain in spite of its lower trehalose content.

In a wild-type strain overexpressing *otsA* the Tre6P level was much higher than in the control wild-type strain. This could be due to TPP becoming rate-limiting for Tre6P hydrolysis. Alternatively, the *E. coli otsA*-encoded enzyme might not be incorporated properly into the trehalose synthase complex and therefore the Tre6P that it produces might not be accessible to the *TPS2*-encoded TPP activity. The higher accumulation of Tre6P might explain why this strain was temperature sensitive. Strains deleted for *TPS2* are temperature sensitive presumably because of Tre6P hyperaccumulation at higher temperatures [2].

Although the growth of the *tps1*Δ + *otsA* transformant was markedly improved compared with that of the *tps1*Δ strain, its growth rate remained clearly lower than that of the wild-type strain. Because the Tre6P level in the transformant was 2–3-fold that in the wild-type strain, this could have been due to a stronger inhibition of hexokinase and therefore a slower influx of glucose into glycolysis. This possibility seems rather unlikely for several reasons. First, it would mean that a slight increase in the Tre6P level would cause a considerable decrease in glycolytic flux. This is in contradiction with previous results obtained for the *tps2*Δ strain and for the wild-type strain just after the addition of glucose, in which the Tre6P level is much higher. Secondly, it is contradicted by other results in this paper (see below). Thirdly,

a determination of glycolytic metabolites in the first minutes after the addition of glucose revealed a hyperaccumulation of sugar phosphates rather than a decrease. It therefore seems that the inability of the *otsA* gene product to restore normal growth on glucose is due to its inability to take over the function of Tps1 fully.

E. coli otsA partly restores growth on glucose

In spite of the persistence of sugar phosphate hyperaccumulation in the *tps1*Δ+*otsA* transformant, the strain started to grow on glucose. This behaviour is similar to that of the *byp1 tps2*Δ strain, which also showed a partial restoration of growth on glucose in spite of an initial hyperaccumulation of sugar phosphates [12]. There are two possible explanations for this observation. The first is that the *otsA* gene product and/or Tre6P level are able to down-regulate glucose influx just enough to allow the growth of the cells after an adaptation period. Here it might be recalled that the *tps1*Δ mutants have been reported to grow on maltose after an initial adaptation period [26]. Alternatively, Tps1 and/or Tre6P might have a second function, for instance causing a stimulation of glycolysis at a point downstream of glyceraldehyde-3-phosphate dehydrogenase. We have checked whether Tre6P affected the activity of downstream enzymes of glycolysis *in vitro* but we could not detect a significant effect (W. Verheyden and J. M. Thevelein, unpublished work). It therefore remains unclear why the *tps1*+*otsA* strain starts to grow on glucose in spite of its initial hyperaccumulation of sugar phosphates.

Very high Tre6P levels have a marginal effect on the accumulation of sugar phosphates

The results obtained with the *tps2*Δ strains confirm the inability of the Tre6P level to down-regulate hexokinase activity solely, as shown by the hyperaccumulation of sugar phosphates *in vivo*. In the *tps2*Δ strain the initial Tre6P level was 10-fold that in the wild-type strain, without much effect on sugar phosphate accumulation *in vivo*. This result seems to contradict that reported previously, in which sugar phosphate accumulation and ethanol production were decreased in the *tps2*Δ strain [12]. However, the experimental set-up was also different. The present results show that a high Tre6P level does not necessarily lead to a decreased accumulation of sugar phosphates. Even the 100-fold higher initial Tre6P level in the *tps1*Δ *tps2*Δ+*otsA* and *byp1-3 tps2*Δ+*otsA* strains caused only a small decrease in sugar phosphate accumulation in glycolysis. These results support the notion that the Tps1 protein itself causes the inhibition of hexokinase activity and/or that it enhances the efficiency of the inhibition of hexokinase by Tre6P in a way that cannot, or at least can only much less efficiently, be mimicked by the *otsA* gene product. The Tps1 protein is present both in the trehalose synthase complex and in a non-complex-bound form; deletion of *TPS2* abolishes the formation of the trehalose synthase complex completely [5]. This could lead to a stronger inhibition of hexokinase by the free Tps1 protein.

Strain *tps1*Δ+*otsA* is able to cope with a sudden glucose load in a chemostat

Because of the hyperaccumulation of initial glycolytic intermediates in the *tps1*Δ+*otsA* transformant after the addition of glucose, we investigated whether the strain would show substrate-accelerated death after a glucose load to cells grown under glucose limitation in a chemostat. This did not occur. However, the *tps1*Δ+*otsA* transformant did show a reproducible delay in glucose consumption and in the production of ethanol and glycerol under this condition in comparison with the wild-type strain. In addition, the initial increases in CO_2 production and O_2 consumption after the addition of glucose were delayed. The partial growth problem observed in batch culture was apparently also present under the conditions in the chemostat and was not significantly aggravated. The ATP level after the addition of glucose to the cells growing in the chemostat was the same in the *tps1*Δ+*otsA* strain and the wild-type strain. These results indicate that the *tps1*Δ+*otsA* strain is able to cope quite efficiently with the sudden transition from glucose limitation to glucose affluence under these conditions. Hence in chemostat cultures the strain behaved in this respect in a similar way to batch cultures.

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