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Regulation of the yeast trehalose–synthase complex by cyclic AMP-dependent phosphorylation

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ABSTRACT

Background: Trehalose is an important protectant in several microorganisms. In *Saccharomyces cerevisiae*, it is synthesized by a large complex comprising the enzymes Tps1 and Tps2 and the subunits Tps3 and Tsl1, showing an intricate metabolic control.
Methods: To investigate how the trehalose biosynthesis pathway is regulated, we analyzed Tps1 and Tps2 activities as well as trehalose and trehalose-6-phosphate (T6P) contents by mass spectrometry.
Results: Tsl1 deficiency totally abolished the increase in Tps1 activity and accumulation of trehalose in response to a heat stress, whereas absence of Tps3 only reduced Tps1 activity and trehalose synthesis. In extracts of heat stressed cells, Tps1 was inhibited by T6P and by ATP. Mg^{2+} in the presence of cAMP. In contrast, cAMP-dependent phosphorylation did not inhibit Tps1 in *tps3* cells, which accumulated a higher proportion of T6P after stress. Tps2 activity was not induced in a *tps3* mutant.
Conclusion: Taken together these results suggest that Tsl1 is a decisive subunit for activity of the TPS complex since in its absence no trehalose synthesis occurred. On the other hand, Tps3 seems to be an activator of Tps2. To perform this task, Tps3 must be non-phosphorylated. To readily stop trehalose synthesis during stress recovery, Tps3 must be phosphorylated by cAMP-dependent protein kinase, decreasing Tps2 activity and, consequently, increasing the concentration of T6P which would inhibit Tps1.
General significance: A better understanding of TPS complex regulation is essential for understanding how yeast deals with stress situations and how it is able to recover when the stress is over.

1. Introduction

Trehalose (α-D-glucopyranosyl (1–1)-α-D-glucopyranoside) is a non-reducing disaccharide of glucose found in bacteria, fungi, plants and insects [1]. It was originally thought to serve as a reserve carbohydrate, but there are several works suggesting that it as an important stress protectant [2]. In the yeast *Saccharomyces cerevisiae*, trehalose can be accumulated up to 15% during adverse environmental conditions like nutrient starvation, heat, osmotic and ethanol shocks [3–5]. Trehalose seems to play a dual role. First, it can protect the plasma membrane. Second, it keeps proteins in their native state during a stress condition as well as it avoids protein aggregation. [6]. Furthermore, trehalose breakdown is critical and provides the energy necessary during stress recovery [7]. The reactivation of stabilized proteins by trehalose depends on its fast degradation after stress because the disaccharide can interfere in the ability of chaperones to reactivate damaged proteins [8]. The mechanisms by which trehalose protects biological molecules can be divided into three categories, namely water replacement, glass formation and chemical stability. These three mechanisms are not mutually exclusive and all may contribute to the stabilizing effects of trehalose [9].

The most usual pathway of trehalose synthesis involves two enzymes: trehalose-6-phosphate synthase (Tps1), which catalyzes the synthesis of trehalose-6-phosphate (T6P), and trehalose-phosphatase (Tps2), which dephosphorylates T6P to trehalose. The complex of trehalose synthesis (TPS) in yeast also includes two other proteins, Tsl1 and Tps3, which seem to have regulatory functions [10–12]. The TPS complex was initially thought to be formed by Tps1 (a 56-kDa subunit) [12], Tps2 (a 102-kDa subunit) [13] and Tsl1 (a 123-kDa subunit) [14]. Tps3, which is homologue of Tsl1, was discovered later. A two-hybrid approach revealed that Tsl1 and Tps3 do not interact with each other, but they interact with Tps1 and Tps2 which, in turn, interact with each other [15]. In order to analyze the properties of the TPS complex, isogenic mutants interrupted in *TPS1*, *TPS2*, *TPS3* and *TSL1* genes have been constructed [15,16]. The catalytic activity of Tps1 was severely reduced in a *tps2* mutant and in a *tps3 tsl1* double mutant. It means that Tps3 and Tsl1 could be responsible for stabilizing the TPS complex. According to previous works, Tps3 and Tsl1 are considered interchangeable regulatory subunits of the TPS complex with respect to theirs functions [5,15,16]. However, until now the

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precise role of both proteins has not been demonstrated. Therefore, in this work we aimed to clarify the regulatory mechanisms played by Tps3 and Tsl1 on trehalose synthesis during heat shock and stress recovery.

2. Materials and methods

2.1. Yeast strains and culture conditions

Wild-type strain BY4741 (*MATa*; *his3*; *leu2*; *met15*; *ura3*) and its isogenic mutants *tsl1* and *tps3*, harboring the genes *TSL1* or *TPS3*, respectively, interrupted by *KanMX4*, were acquired from Euroscarf, Frankfurt, Germany. Cells were grown at 28 °C/160 rpm until the middle of the first exponential phase in YPD2% (1% yeast extract, 2% peptone and 2% glucose) and then submitted to a heat treatment at 40 °C/160 rpm/1 h. In some cases, immediately after heat treatment, cells were cooled back to 28 °C, and incubated for 1 h.

2.2. Trehalose and trehalose-6-phosphate contents

Sugars were extracted by boiling ethanol from heat-shocked samples of WT, *tps3* and *tsl1*, and determined by mass spectrometry using the conditions described in [17]. Trehalose and trehalose-6-phosphate (Sigma-Aldrich), both at the concentration of 1 mM, were used as standards.

2.3. Determination of synthase (Tps1) and phosphatase (Tps2) activities

Total protein extracts were obtained by disruption of cells (50 mg, dry weight) with glass beads in 0.5 mL of MOPS buffer. Tps1 activity was evaluated from the formation of UDP as previously described [18]. To determine the effect of T6P and phosphorylation on Tps1 activity, part of the crude extracts obtained from heat stressed cells were treated either with T6P (Sigma) or with a phosphorylation cocktail during 10 min at 37 °C before analyzing Tps1 activity. The concentration of T6P in the assay was 125 μM. To test the effect of cAMP-dependent phosphorylation on Tps1 activity, cell extracts were incubated with 2 mM ATP, 20 mM $MgCl_2$, 50 μM cAMP, 50 mM NaF and 5 mM theophylline [19]. Tps2 activity was evaluated as previously described [13].

2.4. Real time PCR analyses — qRT-PCR

Analyses of expression were performed for TPS complex genes, namely *TPS1*, *TPS2*, *TPS3* and *TSL1*. *TAF10* (RNA pol II transcription factor activity/transcription initiation and chromatin modification) was used as an endogenous control [20]. Gene sequences were analyzed using File Builder® 3.1 v2.0 (Applied Biosystems, U.S.A.). TaqMan probes were synthesized by Life Technologies (Applied Biosystems, U.S.A.). QuickPrep mRNA Purification Kit (GE Healthcare Life Sciences) was used to extract mRNA from cells at 28 °C and after heat treatment at 40 °C. Ready to Go RT-PCR Beads (GE Healthcare Life Sciences) was used to cDNA synthesis. All real time PCR reactions were performed on a 7500 Fast System Real Time PCR cycler (Applied Biosystems, U.S.A.) according to the manufacturer's instructions. Gene expression fold changes between control group (28 °C) and heat-treated group (40 °C) were calculated by the delta Ct method.

3. Results and discussion

3.1. Effects of Tps3 and Tsl1 deficiencies

It is well known that cells growing exponentially on glucose at 28 °C contain no trehalose but rapidly accumulate large contents of this sugar when heat shocked at 37–42 °C due to Tps1 and Tps2 activation [7,8,15]. According to Fig. 1, after a heat treatment at 40 °C, both *tps3* and *tsl1* mutants showed reduced Tps1 activity. While Tps1 activity in the *tps3* mutant was significantly lower than in WT, synthase was not induced in the *tsl1* mutant. Cells deficient in Tps3 showed a reduced capacity to synthesize trehalose (Fig. 2). The different T6P levels observed between *tps3* and WT strain should be noted. Although the WT strain accumulated more T6P than the *tps3* mutant, T6P accumulation in this mutant was proportionally higher, which seems to be related to the absence of Tps2 activity observed in this strain (Fig. 2 — inset). As expected, in the WT strain, a variation from 28 °C to 40 °C induced a 2.5-fold increase in both Tps1 (Fig. 1) and Tps2 activities (Fig. 2 — inset), leading, therefore, to the accumulation of high levels of trehalose but low contents of T6P. In agreement with Tps1 activity, no significant levels of T6P neither of trehalose were observed in the *tsl1* mutant. Therefore, we may conclude that both Tps3 and Tsl1 subunits are required for the normal functioning of the TPS complex, under heat-shock.

Different groups have published conflicting data about the role of Tps3 and Tsl1 [11,15,16]. According to Reinders and collaborators [15], Tps1 activity is weakly affected in *tsl1* cells submitted to a heat stress. However, for the *tps3* mutant, their results were similar to ours: synthase activity was lower (40%) in the *tps3* mutant than in the WT strain [15]. An important point to be considered is the fact that they used galactose and we used glucose in the culture media. Since the level of catabolic repression determines the state of activation of the TPS complex [21,22] and, furthermore, taking into consideration that galactose is less effective as a repressor than glucose [23], this might explain the differences observed between the Tps1 activity of cells grown on glucose and that on galactose. Other groups did not observe any alteration in a *tps3* mutant with respect to Tps1 activity, however, an interruption in *TSL1* produced none or only a weak activation of Tps1 [11,16]. This last result is similar to the one presented in Fig. 1, although they used stationary phase cells instead of heat-shocked cells. Our results are also different from those obtained in previous works, since those do not point towards any significant changes either in trehalose or T6P levels when only *TPS3* or *TSL1* was disrupted. In their respective papers, the most dramatic effects were observed solely when both genes were interrupted [15,16].

Since the *tps3* mutant accumulated T6P and at the same time its Tps1 activity was lower when compared to the WT strain, we decided to evaluate whether T6P would be able to reduce synthase activity. According to Fig. 3, the presence of T6P caused a strong inhibitory effect on Tps1 activity, avoiding its activation in response to a heat stress. The activities of the extracts obtained from heat stressed cells and determined in the presence of T6P, were similar to those obtained from non-stressed cells (relative activity was almost 1.0), meaning that 125 μM T6P, which is near to physiological concentrations [24], totally abolished the increase

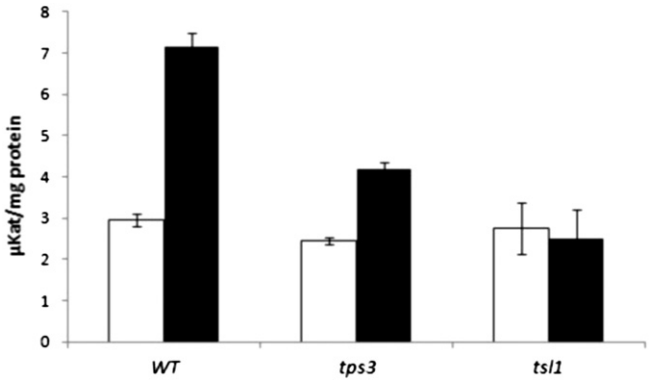

Fig. 1. *Trehalose-6-phosphate synthase activity during a temperature shift from 28 °C (white bars) to 40 °C (black bars).* Cell-free extracts were prepared for synthase activity determinations immediately before and after a heat treatment (40 °C/1 h). Data are shown as mean ± three independent experiments.

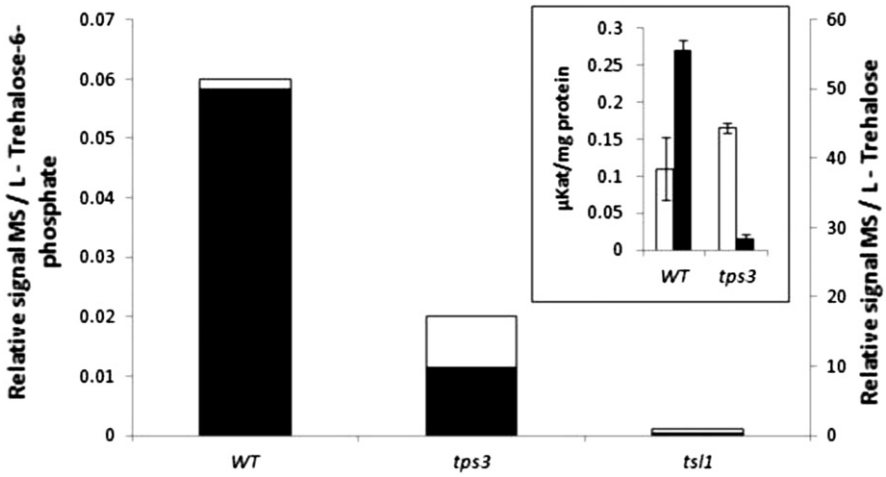

Fig. 2. *Effect of heat treatment on trehalose, trehalose-6-phosphate and Tps2 activity.* Trehalose (black bars) and trehalose-6-phosphate (white bars) were extracted from cells stressed at 40 °C/1 h as described in Methods and determined by mass spectrometry. *Inset*: Increase in Tps2 activity in response to the heat treatment in WT strain and in the *tps3* mutant. Phosphatase activity was determined in non-stressed (white bars) and stressed cells (black bars). The results represent the mean ± standard deviation of at least three independent experiments.

in Tps1 activity in response to a temperature shift. This is an interesting finding, since it is in accordance with our previous experiments (Figs. 1 and 2), in which an increase in the T6P level, caused by the absence of Tps2 activity in the *tps3* mutant, led to a lower Tps1 activity as observed in this mutant. Furthermore, corroborating the idea that T6P inhibits Tps1, it was previously demonstrated that an interruption in *TPS2* resulted in a significant reduction of synthase activity [12,15]. The inhibitory effect of T6P on Tps1 had never been observed before. Up to now, T6P accumulation was only associated with depletion of phosphate and inhibition of hexokinase, which helps to control the glucose influx into the cell [10,25]. Thus, our present result constitutes a new finding which can help in explaining the regulation of the TPS complex.

3.2. Effect of phosphorylation on Tps1

Accumulation of trehalose in response to a rise in temperature is a consequence of a significant increase in both Tps1 and Tps2 activities.

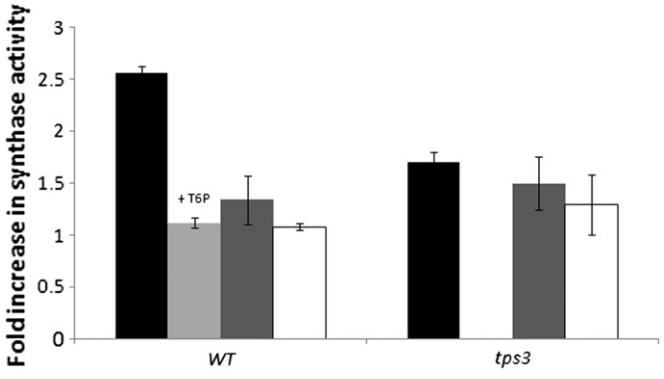

Fig. 3. *Effect of trehalose-6-phosphate (T6P) and cAMP dependent-phosphorylation on synthase activity of heat-stressed cells.* In the first three bars, the results were expressed as a relation between synthase activity of heat-stressed and non-stressed cells. The extracts of heat-stressed cells were divided into three parts: part was used to determine synthase activity without any other treatment (control — black bars); part was incubated with T6P (light gray bar — only for WT) and another part was treated with the phosphorylation cocktail (dark gray bars) before determination of activity. Extracts from heat-stressed cells shifted from 40 °C to 28 °C were also prepared (white bars). In this case, the results were expressed as a relation between synthase activity after stress recovery and activity of non-stressed cells. Data are shown as mean ± three independent experiments. The results obtained for *tps3* mutant showed not to be statistically different according to Student's *t*-test (control x phosphorylation treatment or stress recovery). The latter denotes homogeneity between experimental groups at $p < 0.05$.

To improve our understanding about the TPS complex regulation, we analyzed the effect of heat stress by alterations in the transcription of *TPS1*, *TPS2*, *TPS3* and *TSL1* applying the real time quantitative RT-PCR approach (qRT-PCR). Expression levels of *TPS1*, *TPS3* and *TPS2* were either not induced at all or showed a slight increase, while the expression of *TSL1* was greatly induced in response to the treatment at 40 °C (results not shown). Our results showed a similar expression profile to those previously obtained, using a similar stress condition (shift from 25 °C to 37 °C) but a different method (DNA microarray) [26]. In their work, the expression of *TPS1* increased 2-fold and *TPS3* expression remained unchanged, while *TPS2* and *TSL1* expressions increased almost 4- and 12-fold, respectively. Thus, trehalose levels increase during heat shock, at least in part, due to the induction of the expression of *TPS2* and, mainly of *TSL1*, which showed a strong influence on Tps1 activity (Fig. 1).

The activities of the enzymes partaking to the TPS complex increase with heat, but it is not certain whether the synthase activity is affected by phosphorylation. The efficacy in accumulating trehalose is a result of both, activation of the TPS complex and deactivation of trehalase, the hydrolyzing enzyme, which is regulated by cAMP-dependent phosphorylation [19]. Trehalase undergoes activation by phosphorylation and some results suggest that the synthesizing enzyme becomes less active when phosphorylated [18,19,27]. Therefore in our next step, using *in silico* analyses, we searched for phosphorylation sites in Tps3 and Tsl1 sequences given the fact that these subunits seem to be important for the modulation of the TPS complex activity.

Results obtained with the NetPhosYeast approach [28] showed that both Tps3 and Tsl1 have several putative phosphorylation sites on serine/threonine residues, while the enzymes Tps1 and Tps2 display only few ones (results not shown). According to PhosphoGRID, whose results are based on phosphoproteomic data [29], there are four and two phosphorylation sites in Tps3 and Tsl1, respectively, which are motifs for cAMP-dependent Protein Kinase (PKA), while Tps1 and Tps2 do not have PKA motifs. These observations strongly suggest that phosphorylation events occur on regulatory subunits of the TPS complex, instead of on catalytic subunits. Furthermore, they bring forward an important explanation for the metabolic control of the TPS complex when we bear in mind that the main trehalase, Nth1, becomes active when phosphorylated by PKA [19,30–33].

To validate the results obtained *in silico*, the effect of PKA dependent-phosphorylation on Tps1 activity was investigated using the strategy for evaluation of cryptic trehalase activity with some modifications [19]. According to Fig. 3, the high induction in Tps1 activity seen in the WT strain after a temperature shift from 28 to 40 °C was almost eliminated when extracts of heat stressed cells were incubated with ATP. Mg^{2+} in

the presence of cAMP, suggesting that synthase activity is inhibited when the TPS complex is phosphorylated by PKA. Interestingly, the reduction in synthase activity of heat stressed cells caused by phosphorylation was similar to the decrease observed after the stress recovery (temperature shift from 40 to 28 °C). In contrast, most of the synthase activity in *tps3* mutant extracts seemed to be already inhibited, since the treatment with the phosphorylation cocktail or the return of the cells to 28 °C practically did not change Tps1 activity of cells submitted to heat stress. These results indicate that Tps3 would be the target of cAMP dependent- phosphorylation, which seems to be important in inhibiting the TPS complex during stress recovery. Since no Tps1 activation in the *tsl1* mutant (Fig. 1) was observed, it was not possible to evaluate the phosphorylation effect on trehalose synthesis in this strain.

Previous works tried to explain the metabolic control of the TPS complex by establishing a relationship with phosphorylation events [27,34]. Panek's group presented a new insight for the control of the TPS complex by introducing the idea that trehalose synthesis could be regulated by phosphorylation [27]. A plausible suggestion, since trehalose degradation starts when Nth1 is in its phosphorylated form [19,31–33]. The authors concluded that trehalose synthesis is regulated by phosphorylation and this phosphorylation event occurs on Tps1 [27]. However our results suggest a different interpretation: the TPS complex could indeed be regulated by phosphorylation, but this event would seem to have the regulatory subunit Tps3 as target. On the other hand, Vandercammen and François (1989) [34] did not find any relationship between cAMP and Tps1 activity contrary to our results. We observed a Tps1 inactivation both when heat-stressed cells were shifted back to 28 °C (*in vivo* analyses) and when extracts of cells submitted to heat stress were incubated with the cAMP-dependent phosphorylation cocktail (*in vitro* analyses). In contrast, in all the conditions investigated (in intact cells as in the cell-free extract) trehalase activity was increased, confirming that PKA had been activated (data not shown). Therefore, our results suggest phosphorylation as a mechanism to rapidly decrease trehalose biosynthesis during stress recovery and Tps3 may act directly in this fast response.

3.3. Schematic representation of the TPS complex regulation

Based on results obtained so far, we propose a mechanism of the regulation of trehalose biosynthesis to explain the activity of the TPS complex found under three different conditions: non-stressed cells, under heat stress and during stress recovery. Non-stressed cells do not show significant Tps1 activity (Fig. 1) nor significant trehalose levels [18]. This could be due to the fact that the TPS complex is not completely formed in that condition (Fig. 4, upper panel). Our results and those previously obtained [26] suggest that Tsl1 is a decisive subunit of the TPS complex, since it is the only gene which has its expression strongly induced after heat shock. With the induction of *TSL1* expression, Tps1 activity would be activated and, consequently, trehalose would accumulate. Confirming this interpretation, under Tsl1 deficiency, Tps1 activity and trehalose levels did not increase in response to stress (Figs. 1 and 2). In this scenario, the TPS complex is completed, with its four subunits, and in its active state (Fig. 4, middle panel). After a stress condition, trehalose would not be required any longer. Moreover its presence would be very harmful because it hampers other repair mechanisms, like heat shock proteins [8]. We analyzed two situations in which stress conditions were suddenly interrupted: (i) by treating cell extracts with a phosphorylation cocktail or (ii) by submitting heat-shocked cells to a treatment at 28 °C/1 h. In this last case, glucose was still available, since cells were grown up to the middle of the first exponential phase. In both situations, we observed a significant decrease in Tps1 activity, as well as an increase in trehalase activity, which would explain the reduction of trehalose levels under stress recovery. Previous studies have shown an important relationship between trehalose breakdown and the RAS/PKA pathway [19,30–33]. Our results suggest an additional role for this signaling pathway besides activating Nth1: the inhibition

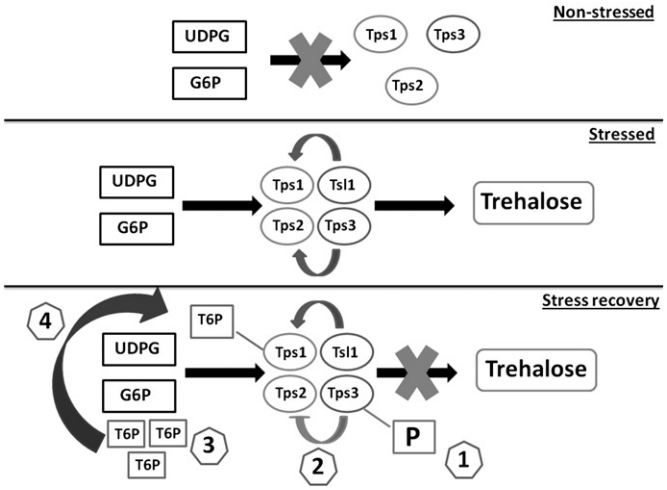

Fig. 4. *Schematic representation of the TPS complex regulation.* Under no stress conditions (upper panel) TPS complex is not completely formed and there is no trehalose synthesis from glucose 6-phosphate (G6P) and uridine diphosphate glucose (UDPG). Under stress conditions (middle panel), *TSL1* expression is induced, completing TPS complex. In this form, TPS complex is able to catalyze trehalose synthesis. Tps3 seems to influence Tps2 activation because without this subunit of TPS complex phosphatase activity is reduced. During a stress recovery situation (bottom panel), Tps3 is phosphorylated (1), inhibiting Tps2 activity (2). As a consequence, trehalose-6-phosphate (T6P) levels accumulate (3), leading to Tps1 inhibition (4) and, consequently, to the interruption of the trehalose synthesis.

of trehalose biosynthesis. According to our results, without Tps3, Tps2 is not induced after heat stress (Fig. 2). Besides, Tps1 activity of *tps3* cells neither decreased in response to a temperature shift from 40 °C to 28 °C nor it was reduced reduced when the extracts of heat-stressed cells were treated with a phosphorylation cocktail. Therefore, we raised the hypothesis of an important role played by the Tps3 subunit during stress recovery (Fig. 4, bottom panel). When the stress condition is over, Tps3 would be phosphorylated by PKA, leading to inhibition of Tps2 activity and, as a consequence, to T6P accumulation. Such an accumulation of T6P would further lead to Tps1 inhibition (Fig. 3), stopping trehalose synthesis. During recovery from a stress condition, the primary responsibility for trehalose hydrolysis would rest with the cytosolic trehalase Nth1. However, in such conditions the TPS complex should be inhibited to avoid a futile cycle. Consequently, trehalose would be degraded soon after a stress is over.

4. Conclusions

The trehalose pathway consists of only a few metabolites, which form a substrate cycle, yet is governed by a surprisingly complex control system that comprises several inhibiting or activating signaling mechanisms [29]. Several works, using different approaches, have attempted to explain this metabolic control [11,15,16,27,34]. The results presented in this paper reinforce the role of Tps3 and Tsl1 as regulatory subunits of the TPS complex. Furthermore, our conclusions endow these two proteins with more definite functions: Tps3 seems to play a regulatory role, being phosphorylated by PKA during stress recovery, while Tsl1 seems to play a structural role, since in its absence no Tps1 activity could be observed. Furthermore, after a heat shock, the gene expression profile corresponding to Tsl1 increased several times, suggesting it plays a decisive role in the formation of the TPS complex.

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