Opposite roles of trehalase activity in heat-shock recovery and heat-shock survival in *Saccharomyces cerevisiae*

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A variety of results has been obtained consistent with activation of neutral trehalase in *Saccharomyces cerevisiae* through direct phosphorylation by cAMP-dependent protein kinase (PKA). A series of neutral trehalase mutant alleles, in which all evolutionarily conserved putative phosphorylation sites were changed into alanine, was tested for activation *in vitro* (by PKA) and *in vivo* (by glucose addition). None of the mutations alone affected the activation ratio, whereas all mutations combined resulted in an inactive enzyme. All mutant alleles were expressed to similar levels, as shown by Western blotting. Several of the point mutations significantly lowered the specific activity. Using this

series of mutants with different activity levels we show an inverse relationship between trehalase activity and heat-shock survival during glucose-induced trehalase mobilization. This is consistent with a stress-protective function of trehalose. On the other hand, reduction of trehalase activity below a certain threshold level impaired recovery from a sublethal heat shock. This suggests that trehalase breakdown is required for efficient recovery from heat shock, and that the presence of trehalase protein alone is not sufficient for efficient heat-stress recovery.

Key words: protein phosphorylation, trehalose, yeast.

INTRODUCTION

Trehalose (α -D-glucopyranosyl α -D-glucopyranoside) is well known to act as a stress protectant in yeast and other organisms and displays potent protective effects against denaturation of proteins and other cellular components *in vitro* [1,2]. In numerous studies a positive correlation has been observed between the trehalose level and stress resistance. Sublethal heat treatment induces trehalose accumulation in yeast simultaneously with heat resistance [3].

Remarkably, the *NTH1*-encoded neutral trehalase, which is responsible for trehalose breakdown, is also induced during sublethal heat treatment [4,5]. Since the *nth1* Δ mutant is impaired in recovery from heat shock, it has been proposed that mobilization of trehalose is important for recovery after heat stress.

This possibility has been supported by recent evidence that high trehalose levels inhibit efficient renaturation of partially unfolded proteins by molecular chaperones like Hsp104 [6]. Although the impaired recovery from heat stress of an *nth1* Δ mutant is consistent with a requirement for rapid mobilization of trehalose after heat shock, there is no direct evidence that it is the enzymic activity of trehalase, and therefore trehalose mobilization, that is important for heat-stress recovery. In this paper we provide such direct evidence by demonstrating that in a series of trehalase mutants with different specific activity levels there is a positive correlation of trehalase activity with heat-stress recovery, in spite of a negative correlation with heat-shock survival.

Three trehalase genes have been described in *Saccharomyces cerevisiae* (reviewed in [7]). Degradation of trehalose, which is present in the yeast cell as a storage carbohydrate and as a stress protectant, is mediated by neutral trehalase, encoded by the *NTH1* gene [8]. The function and the expression of the *NTH2*

gene, which is highly homologous to the *NTH1* gene, remains poorly understood. Neither deletion nor overexpression of *NTH2* influences trehalose levels or trehalase activity [4]. Trehalase activity at neutral pH is hence mediated by the *NTH1* gene product. *S. cerevisiae* also contains an acid trehalase encoded by the *ATH1* gene [9,10], which displays no sequence homology to neutral trehalase. Ath1 is located in the vacuole and appears important for growth on media containing trehalose as a carbon source [11]. Consistent with this function, acid trehalase has a pH optimum of ≈ 4.5 [12].

Neutral trehalase activity is low in derepressed cells. Upon addition of glucose, trehalase activity increases rapidly within a few minutes [13]. Several lines of evidence point to a role for the cAMP-dependent protein kinase (PKA) in this process: (i) active trehalase can be inactivated by incubation with alkaline phosphatase [7,14]; (ii) inactive trehalase can be re-activated by incubation with the catalytic subunit of PKA and this coincides with the incorporation of about 1 mol/mol phosphate into Nth1 [14]; (iii) inhibitors or mutations in the cAMP-PKA pathway prevent the activation of trehalase by glucose [15,16]; and (iv) mutations that increase PKA activity lead to increased trehalase activity [17,18].

At present it remains unclear which residue in Nth1 is responsible for the activation. PKA phosphorylates serine or threonine residues that are located in an R/KR/KXS/T consensus site, where X is any amino acid [19,20]. Besides these 'perfect' sites, PKA sites often contain only one basic residue at position -2 or -3 relative to the phosphorylated residue. Recently, we developed an EXCEL-based method that allows the prediction of potential Ser/Thr phosphorylation sites in proteins [21]. We used this method in combination with a sequence comparison of Nth1 with trehalases from *Kluyveromyces fragilis* [22].

Abbreviations used: PKA, cAMP-dependent protein kinase; Nth1, neutral trehalase; STRE, stress-responsive element.

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Figure 1 Sequence alignment of neutral trehalases from *S. cerevisiae* [4,8], *K. lactis* [22] and *C. albicans* [23]

The eight conserved putative phosphorylation sites and Thr260 are indicated in black. Regions that are identical between two or three proteins are shaded grey.

myces lactis and *Candida albicans*. Trehalases from the latter organisms are also known to be regulated by PKA-mediated phosphorylation [22,23]. Eight potential PKA phosphorylation sites appeared to be conserved in trehalases from all three organisms. In this study we describe the mutation of these eight residues as well as of the only additional 'perfect' PKA consensus site in *Saccharomyces* trehalase and the effect of these mutations on the basal activity and the activation ratio both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Yeast strains and media

All experiments were carried out using the M5 strain in which the *NTH1* gene was replaced by the *LEU2* gene ([24] α *leu2-3,112 ura3-52 trp1-92 nth1::LEU2*), and which were transformed subsequently with a multicopy expression vector encoding either the wild-type or the mutated forms of trehalase and harbouring a *TRP1* selectable marker gene. Transformed yeasts were cultured in synthetic medium without tryptophan and containing either 2% glucose or 2% glycerol as carbon source.

Site-directed mutagenesis

Site-directed mutagenesis of trehalase was carried out using the Quikchange mutagenesis kit (Stratagene) with the following primers (mutated residues are underlined): Ser20/21Ala, GGCCGTCAAAGAAGATTAGCAGCACTAAGTGAATTC; Thr58Ala, CCGTACGAGGGCTATGAGTGTTCGATAA-TG; Ser60Ala, CCGTACGAGGACTATGGCTGTTTCGATAATG; Ser83Ala, CAGACTAGACGTGGTGCTGAGGATGACACC; Thr135Ala, GTTTTGAAAGTCGGTCCGCAAACTCCTATG; Thr149Ala, CATATTAATATTAGGGGTGCGTATATGTTATCC; Thr260Ala, CCAAAAAAATA-GCGGCAGAAATACGTCAAATC; Ser475Ala, CCGTGCGCTTAGAGAGCTGGACACGACACTAC. A restriction fragment (the *NcoI*/*SpII* fragment containing Ser20Ala and Ser21Ala mutations, the *SpII*/*HindIII* fragment containing the Thr58Ala, Ser60Ala, Ser83Ala, Thr135Ala or Thr149Ala mutations, or the *SpII*/*SalI* fragment containing the Thr260Ala or Ser475Ala mutations) was, after sequencing by the dideoxy method, exchanged for the corresponding restriction fragment in the *Nth1*-expression vector. This vector is composed of the *Nth1* coding region cloned in the *NcoI*/*SalI* sites of pYX232 (R&D systems).

Activation of trehalase *in vitro*

Yeast cells were grown in glycerol-containing medium to exponential phase, harvested by centrifugation, washed once in ice-cold water and resuspended in 100 mM Mes buffer (pH 7) containing 50 μ M CaCl₂. Extracts were prepared by shaking with glass beads for 90 s followed by a centrifugation step (10 min at 20000 g).

Protein concentration was determined using the Bio-Rad assay kit with thyroglobulin as standard. The extracts were diluted to 1 mg/ml. Extract (2.5 μ l) was incubated for 30 min at 30 °C in a final volume of 30 μ l, either as such or in the presence of 0.1 mM ATP, 2 mM magnesium acetate and 1 unit of the catalytic subunit of PKA (Sigma). After the incubation, trehalase activity was measured as described previously [25].

Activation of trehalase *in vivo*

For activation studies *in vivo*, yeast cells overexpressing wild-type and mutant trehalases were grown to exponential phase in

selective medium containing 2% glycerol. Cells were harvested by centrifugation, washed in water and resuspended to $D_{600} = 6$ in 100 mM Mes buffer, pH 7, containing 50 μ M CaCl₂. Aliquots (750 μ l) were taken before and after the addition of 2% glucose (final concentration). Cells were harvested, washed in ice-cold water, resuspended in 500 μ l of buffer and extracted by shaking with glass beads. Trehalase activity was determined in the extracts as described previously [25].

Heat-shock-sensitivity and heat-shock-recovery assays

For heat-shock-recovery assays yeasts were grown to stationary phase on selective agar medium containing 2% glucose as carbon source. Replicas were taken and either incubated for 5 h at 50 °C and subsequently at 30 °C for 16 h (heat-shock recovery) or immediately at 30 °C for 21 h (controls).

For heat-shock-sensitivity assays cells were grown to stationary phase in liquid medium containing 2% glucose as carbon source. Each culture (50 μ l) was diluted in 1 ml of liquid medium containing 2% glucose, incubated for 1 h at 30 °C, re-diluted 20-fold in the same glucose-containing medium and incubated for another hour at 30 °C. Before heat shock (20 min at 51.5 °C), cells were diluted another 10-fold in glucose-containing medium to a final volume of 1 ml. Controls were diluted another 20-fold and not heat-shocked. Aliquots of 50 and 500 μ l of the control or heat-shocked cells were plated on glucose-containing medium and grown for 48 h. Colonies were counted and for each strain the number of colonies on the heat-shocked plate was expressed relative to the number of colonies on the control plate (100%).

RESULTS AND DISCUSSION

PKA recognition sites in *Nth1*

A close inspection of the amino acid sequence of *Saccharomyces* *Nth1*, using the algorithm described previously [21] revealed the presence of 17 potential PKA-recognition sites (defined as one or two basic residues at positions -2 or -3 relative to a Ser/Thr residue). Of these 17 potential sites only three sites are composed of two basic residues upstream of the potential phosphorylation site (Ser20, Ser83 and Thr260) and two of these correspond to an RRXS site (Ser20 and Ser83). As shown in Figure 1, eight of the 17 potential PKA sites (Ser20, Ser21, Thr58, Ser60, Ser83, Thr135, Thr149 and Ser475) are conserved in trehalases from *K.*

Table 1 Mutations in evolutionarily conserved PKA-recognition sites do not abolish activation of trehalase by PKA (*in vitro*) or by glucose (*in vivo*)

Shown are the ratios of specific trehalase activity after versus that before the activation of trehalase by the incubation of extracts with PKA, ATP and Mg²⁺ (*in vitro*) or by the addition of 2% glucose to glycerol-grown yeast cells (*in vivo*), as detailed in the Materials and methods section.

Nth1 mutant	Fold activation	
	<i>In vitro</i>	<i>In vivo</i>
Wild type	3.0	10.8
Ser20/21Ala	2.6	7.5
Thr58Ala	2.9	8.4
Ser60Ala	4.6	15.4
Ser83Ala	10.1	16.5
Thr135Ala	12.3	4.9
Thr149Ala	2.8	13.6
Thr260Ala	5.8	7.5
Ser475Ala	4.3	7.9

Figure 2 Mutation of several putative phosphorylation sites reduces specific trehalase activity

(Top panel) Extracts from glycerol-grown cells expressing the indicated trehalase mutant or wild-type allele were assayed for trehalase activity before (open bars) or after (black bars) incubation with PKA and MgATP as described in the text. A mean ($\pm$ S.E.M.) of three experiments is shown. (Bottom panel) Each extract (10 μ g) was loaded on SDS/PAGE, transferred on to nitrocellulose and developed with a monoclonal anti-trehalase antibody. F.M., fully mutated Nth1 (all eight conserved sites plus Thr260 mutated).

lactis and *C. albicans* and trehalases from these species are known to be regulated by reversible phosphorylation [22,23]. Hence, one or several of these eight sites are likely to be involved in the control of trehalase by reversible phosphorylation. We therefore decided to mutate the Ser or Thr residues in these eight sites one by one into alanine.

Activation of Nth1 mutants *in vitro*

Incubation of extracts from glycerol-grown cells overexpressing wild-type trehalase with the catalytic subunit of PKA and MgATP resulted in an increase in trehalase activity of about 3-fold (Table 1). Extracts from cells expressing trehalase in which one potential phosphorylation site was changed into alanine (Ser20/21Ala, Thr58Ala, Ser60Ala, Ser83Ala, Thr135Ala, Thr149Ala or Ser475Ala) showed a similar or even higher fold increase in trehalase activity upon incubation with the catalytic subunit of PKA and MgATP, indicating that none of the mutations alone abolished the activation potential of Nth1 (Table 1).

As shown in Figure 2, top panel, several of these mutations, however, lowered the basal activity of trehalase drastically. To exclude the possibility that these effects were due to a lowered stability and/or expression level of the mutant proteins, we performed a Western-blot analysis (Figure 2, bottom panel) with an anti-trehalase antibody. Since trehalase protein levels of all mutants were similar to the level of wild-type trehalase, our results might indicate that some of the mutated residues are important for the catalytic mechanism of the enzyme, or that their phosphorylation is important for the basal activity of trehalase.

Activation of Nth1 mutants *in vivo*

We also checked whether the mutations affected the activation potential of trehalase *in vivo*. Yeast cells were grown in glycerol-

Figure 3 Trehalase activity is negatively correlated with heat-shock survival

Correlation between the specific (basal) trehalase activity and the percentage of cells surviving a heat shock at 51.5 °C for 20 min. The following Nth1 mutants are plotted: S20/21A, T58A, S60A, S83A, T135A, T149A and S475A, together with the fully mutated construct (F.M.), wild-type Nth1 and the *nth1* deletion mutant.

A. Controls: no heat shock

B. Heat-shock recovery

Figure 4 Trehalase activity is required for efficient recovery from heat shock

Yeast cells overexpressing the indicated mutant allele of trehalase or containing the empty expression vector were tested for their behaviour in a heat-shock-recovery assay. Controls were not heat shocked. F.M., fully mutated Nth1 allele (all conserved sites plus Thr260 mutated).

containing medium and, at several time points before and after the addition of glucose to a final concentration of 2 %, samples were taken and specific trehalase activity was determined. The results (Table 1) largely confirmed the *in vitro* data. None of the mutations alone abolished the activation by glucose addition, but for several the basal activity of trehalase was again significantly lower in the mutants. The extent of activation was enhanced both *in vitro* and *in vivo* for some mutant alleles except for the Thr135Ala mutant allele, which behaved in the opposite way. The reason for this is not entirely clear, but the effect of the mutations on basal trehalase activity might offer an explanation.

As mentioned earlier, Nth1 contains three potential PKA-recognition sites composed of two basic residues at positions -2 and -3 relative to the phosphorylatable residue. Two of these (Ser20 and Ser83) are conserved in *K. lactis* and *C. albicans* and were already mutated. We also mutated the third one (Thr260) which is located in close proximity to the putative catalytic domain of Nth1. Nth1 carrying the Thr260Ala mutation, however, was activated *in vitro* (by incubation with PKA and MgATP) and *in vivo* (by addition of glucose to glycerol-grown cells) to the same extent as wild-type Nth1. Moreover, this mutation did not affect the basal Nth1 activity (results not shown).

Our results suggested that activation of trehalase is mediated by the phosphorylation of more than one site. We therefore constructed a mutant where all eight conserved sites and Thr260 were changed into Ala. The resulting mutant Nth1 allele was also expressed to the same level as the wild-type protein (Figure 2, bottom panel), but was totally inactive (Figure 2, top panel). Neither incubation with PKA and MgATP *in vitro* nor addition of 2 % glucose *in vivo* resulted in significant trehalase activity. Similar results were obtained when mutations of Ser20, Ser21 and Ser83 were combined (results not shown). These results indicate either that these three residues are essential for the catalytic mechanism of trehalase or that a certain extent of phosphorylation of these residues is required for basal activity of trehalase. At present we are unable to distinguish between these possibilities. Nevertheless, if Ser20, Ser21 and Ser83 represent phosphorylation sites, one might expect that their combined mutation into Asp would mimic a fully phosphorylated (and hence active) trehalase. However, the triple-Asp mutant (Ser20Asp, Ser21Asp, Ser83Asp) was totally inactive (results not shown), offering support for the hypothesis that these residues are essential for catalytic activity. Alternatively, the introduction of Asp residues might interfere with the conformation of the enzyme, or Asp residues might, in this particular case, not be able to mimic phosphoserines and hence result in the same effect as mutations into Ala. It is noteworthy that mutation of another conserved (but not phosphorylatable) residue, Gly632Ser, resulted in a 90 % decrease in trehalase activity (results not shown).

Trehalase activity correlates with heat-shock sensitivity and heat-shock recovery

Experimental evidence points to a role of trehalase in recovery from heat shock, and a *nth1Δ* strain displays a poor heat-shock-recovery phenotype [4,5]. Both mRNA levels and enzymic activity of Nth1 are increased upon mild heat stress. This might be mediated by one or more of the three STREs (stress-responsive elements) in the promoter region of the *NTH1* gene. According to the current model, during heat shock trehalose protects cellular proteins against denaturation and subsequent aggregation, but inhibits the solubilization of protein aggregates and the refolding of the partially denatured proteins during recovery from heat shock [6]. However, it has been unclear [7] whether

Nth1 is important because of its role in trehalose hydrolysis or whether Nth1 displays an additional (e.g. chaperone) function. Chemical stressors such as CuSO_4 induce *NTH1* [7,26], but do not lead to trehalose accumulation, which might suggest an additional function for Nth1. In this study we have extended our earlier observations [24] that strains lacking trehalase survive a heat-shock treatment better. Using the mutant trehalase alleles with different specific activities, a clear inverse correlation (correlation coefficient = -0.93) appears to exist between specific (basal) trehalase activity and heat-shock survival (Figure 3). A similar inverse correlation (correlation coefficient = -0.95) is obtained when the glucose-induced specific trehalase activity is correlated with heat-shock survival (results not shown). This supports the protective effect of high trehalose levels for survival of heat stress. However, a heat treatment at a somewhat lower temperature that allows most cells to survive reveals a clear difference in the time needed to recover from the shock for the mutants with reduced trehalase activity. The type of assay used has been described previously as a heat-shock-recovery assay [4]. In this recovery assay cells are in stationary phase before the heat shock and are hence loaded with trehalose, rendering them highly stress-resistant. In the sensitivity assay, cells are also allowed to grow to stationary phase, but 2 h prior to the heat shock they are diluted in fresh glucose-containing medium, and this treatment will initiate trehalose breakdown, at least in strains that contain active Nth1. As shown in Figure 4, *nth1*-deletion cells perform worse than the cells expressing wild-type trehalase in the recovery assay, confirming the poor heat-shock-recovery phenotype described previously for this mutant [4,5].

Since we obtained a series of trehalase mutants which are equally expressed, but display strikingly different specific activities, we were able to check whether the requirement for trehalase during heat-shock recovery was correlated to trehalase protein content or to trehalase activity. Moreover, expression of these plasmid-encoded mutants is not driven by the endogenous promoter, but is constitutive and high, resulting in constant basal trehalase activities that are not transcriptionally upregulated during heat treatment. Yeast cells expressing trehalase mutant alleles with a specific activity that is at least four times lower than the wild type (*nth1* Δ , Ser83Ala, Thr135Ala and the fully mutated Nth1 allele) fail to recover efficiently. It appeared that the specific activity of trehalase displays a threshold level below which cells do not recover efficiently from heat shock (compare Figure 2, top

panel, with Figure 4). Taken together, our results show that the trehalase activity of Nth1 is required for yeast cells to recover efficiently from heat shock.

S.W. is a postdoctoral fellow of the Fund for Scientific Research (FWO), Flanders, Belgium. I.G. is a recipient of an IWT predoctoral fellowship. We thank J. Morren for help with the preparation of Figure 1 and M. Versele for critical reading of the manuscript. This work was supported by grants from the Fund for Scientific Research, Flanders, and the Research Fund of the Katholieke Universiteit Leuven (Concerted Research Actions) to J.M.T.

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