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A method to study the rapid phosphorylation-related modulation of neutral trehalase activity by temperature shifts in yeast

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Heat shock enhanced the synthesis of neutral trehalase in growing cells of *Saccharomyces cerevisiae*, as detected by immunological methods The activity of the enzyme was measured in extracts obtained by two methods cells were either harvested by filtration and subsequent disruption with glass beads at 0–4°C or immediately frozen with liquid nitrogen in the presence of Triton X-100, followed by thawing at 30°C The first procedure yielded artificially high activities of neutral trehalase in heat-shocked cells due to rapid (<1min) activation during handling at 4°C before homogenization Activity of the enzyme in these homogenates decreased 75–90% upon a treatment with alkaline phosphatase, indicating that activation was due to phosphorylation The second procedure yielded low trehalase activities for heat-shock treated cells, much higher activities for cells shifted back for some seconds to 27°C, and very low activities again for cells shifted from 27 to 40°C for a second time Thus, permeabilization of cells following rapid freezing in Triton X-100 is a method of choice to study post-translational modulation of the neutral trehalase of *S cerevisiae* by phosphorylation and dephosphorylation

Trehalase, Heat shock, Protein phosphorylation, Trehalose, *Saccharomyces cerevisiae*

1. INTRODUCTION

The neutral trehalase in *Saccharomyces cerevisiae* can be post-translationally activated by cAMP-dependent phosphorylation [1–4]. Conclusive evidence for this comes from biochemical and genetic studies of glucose-derepressed cells (e.g. cells grown on acetate, stationary phase cells and ascospores) which generally contain large pools of trehalose and low activities of neutral trehalase, in these cells, addition of fermentable sugars induces a cAMP-signal which triggers a protein phosphorylation cascade [5–7] Neutral trehalase is phosphorylated and thereby rapidly activated, which in turn leads to the rapid mobilization of the accumulated trehalose [3–5,7].

It has been reported that a short severe heat shock (1–3 min at 50–54°C) also causes an apparent activation of neutral trehalase in yeast ascospores but that this may be an artifact arising during sampling which consists in rapidly cooling the cells on ice-cold water [8]

Cells of *S cerevisiae* growing exponentially on glucose have a very small trehalose pool and a low activity of neutral trehalase [5,7] However, they rapidly accumulate large amounts of trehalose during a heat shock (1–2 h at 40–45°C) [9,10]. The accumulation of trehalose correlates with an increase in thermotolerance [11], suggesting that trehalose is a protective substance in yeast [12,13]. Enzyme measurements in extracts prepared by disruption of cells with glass beads at 4°C have yielded the surprising result that not only trehalose-6-phosphate synthase but also neutral trehalase activity rapidly increases upon heat shock, suggesting a high turn-over of trehalose during the heat shock [9] Considering the potential problems of the sampling procedure [8] mentioned above, we decided to reinvestigate the regulation of neutral trehalase under heat shock conditions

Here we report that the previously used extraction method [9] leads to artificially high activities under heat shock conditions apparently due to rapid phosphorylation during sampling. We describe how this problem can be circumvented with a permeabilization technique [14] in which the cells are rapidly frozen in liquid nitrogen and subsequently thawed in the presence of Triton X-100 This procedure yields a much lower, ca threefold increase of neutral trehalase activity during heat shock which parallels the increase in the amount of enzyme, as detected by immunological methods Using the rapid permeabilization technique, we demonstrate rapid activation of neutral trehalase after a temperature shift from 40 to 27°C, in correlation with the observed rapid degradation of the accumulated trehalose, and an equally rapid inactivation of neutral trehalase when the cells are shifted back to 40°C and again begin to accumulate trehalose.

2 MATERIALS AND METHODS

2 1 *Yeast strains and culture conditions*

The following strains of *S cerevisiae* were used YS18 (MATα his3-11,15 leu2-3,112 ura3 can^R) and C13-ABYS86 (MATα his3-11,15 leu2 3,112 ura3 can^R pra1-1, prb1-1 prc1-1 cps1-3) derived

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from YS18 [15] Both strains were kindly provided by Prof D H Wolf (Biochemisches Institut, Universität Freiburg, Germany

Stock cultures were kept on YPDA (yeast extract 1% bactopeptone 2%, glucose 2%, agar 2%) and grown at 27°C on a rotary shaker (140 rpm) in liquid YPD-medium (as above but no agar) Well adapted log-phase cultures (at least 5 generations of exponential growth) at a density of $<6\times10^6$ cells/ml were used in all experiments

2 2 *Heat shock conditions*

Log-phase cultures (6×10^6 cells/ml) were concentrated 10 times by centrifugation (10 min at 2000×g) and subsequent resuspension in fresh prewarmed YPD-medium The concentrated cultures (10 ml in 100 ml culture flasks) were then subjected to heat shock by transfer into a shaking water bath at 40°C and relieved from heat shock by transfer to a water bath at 27°C

2 3 *Enzyme extractions and assays*

Cell suspensions (0 5 ml) were harvested by filtration (Whatman GF/C), washed twice with 5 ml of ice-cold water and resuspended in an Eppendorf tube containing 0 5 ml of ice-cold 0 2 M Tricine(Na^+) buffer, pH 7 0 (assay buffer) Cell disruption was performed by shaking with glass beads as in [16] The homogenates were desalted on a Sephadex G-25 column (bed volume 2 ml) and used immediately for the trehalase assay

Alternatively, trehalase was measured in permeabilized cells [14] To this end 0 5 ml of the culture were mixed with 0 5 ml of 0 1% Triton X-100 (in assay buffer) and immediately frozen in liquid nitrogen After thawing (1–4 min at 30°C), the cells were centrifuged (1 min at 12 000×g), washed twice with 1 ml of ice-cold assay buffer and immediately used for the trehalase assay

The assay of trehalase was performed in 50 mM Tricine(Na^+), pH 7 0, 0 1 M trehalose, 0 2 mM $MnCl_2$ and enzyme extract or permeabilized cells in a total volume of 400 μl After incubation for 10–30 min at 30°C the reaction was stopped in a boiling water bath (3 min) Glucose was determined in the supernatant using GOD-test kit from Boehringer, Mannheim Germany

2 4 *Gel electrophoresis and immunological detection of neutral trehalase*

Total cellular proteins (<50 μg) were separated by electrophoresis on 10% polyacrylamide-SDS gels as in [17] and transferred electrophoretically to a nitrocellulose membrane Nonspecific binding to the nitrocellulose blots was blocked with 5% milk powder dissolved in Tris-buffered saline (TBS 150 mM NaCl, 50 mM Tris-HCl, pH 7 4) for 2 h The blots were washed with TBS followed by incubation for 3 h in the same solution containing a 1 10000 dilution of a monoclonal antibody specific for neutral trehalase in *S cerevisiae* (kindly provided by Gist-Brocades, Delft, The Netherlands)

The protein blot was washed again in TBS and incubated for 1–3 h with goat anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad) Finally the blots were washed in TBS and developed by an enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Boehringer)

2 5 *Dephosphorylation of neutral trehalase*

Cell homogenates (ca 50–100 μg protein) were incubated with 5 units of alkaline phosphatase from *E coli* (Fluka Buchs, Switzerland) in Tris-HCl buffer at pH 7 0 and 30°C (total volume 35 μl)

2 6 *Determination of protein*

Protein was determined as before [9] using bovine serum albumin as a standard

3 RESULTS AND DISCUSSION

In the initial experiments, the ABYS mutant [15] of *S cerevisiae*, deficient in vacuolar proteinases A and B and carboxypeptidases Y and S, was used in order to minimize the risk of proteolytic breakdown of neutral trehalase [3] Trehalase activities were measured in cells that had grown to mid-log phase on glucose at 27°C and were then subjected to heat shock at 40°C (Fig 1) When cells were extracted by homogenization with glass beads after chilling on ice, trehalase activity in the extracts was low at the beginning of the heat shock and increased quite abruptly about 5-fold between 20 and 40 min of the heat treatment (Fig 1), in accordance with previous results [9]

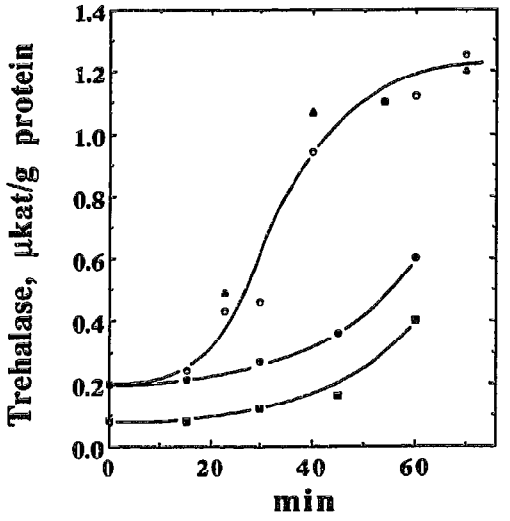

Fig 1 Activity of neutral trehalase in extracts from yeast cells (C13-ABYS86) subjected to heat shock (40°C) for different times Extracts were prepared by disrupting cells with glass beads at 4°C (○) or at 40°C (●) Extracts prepared at 4°C were subjected to the extraction procedure at 40°C (▲) or treated with alkaline phosphatase from *E coli* (■)

As it has been reported previously that trehalase activation can occur in heat-shocked yeast ascospores during the sampling procedure when they are cooled on ice [8], we harvested and subsequently disrupted heat shocked cells in an incubation chamber at 40°C In cells subjected to heat shock for 30–60 min, neutral trehalase, measured under these conditions, had an activity of only 30–50% compared to that obtained with the usual procedure of harvesting and rupturing the cells at

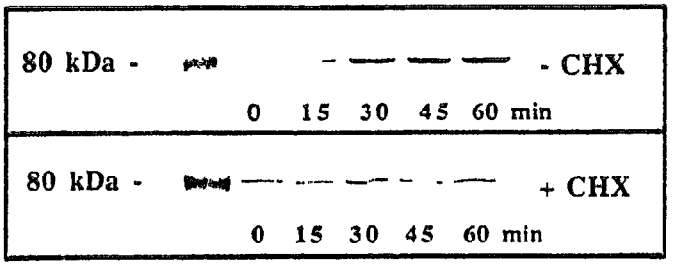

Fig 2 Immune blot of neutral trehalase in yeast cells (C13-ABYS86) subjected to heat shock at 40°C in the absence (−CHX) or in the presence (+CHX) of cycloheximide (50 μg/ml) for different times The position of the 80 kDa molecular weight standard (Prestained SDS-PAGE Standards, Bio-Rad) is indicated on the left

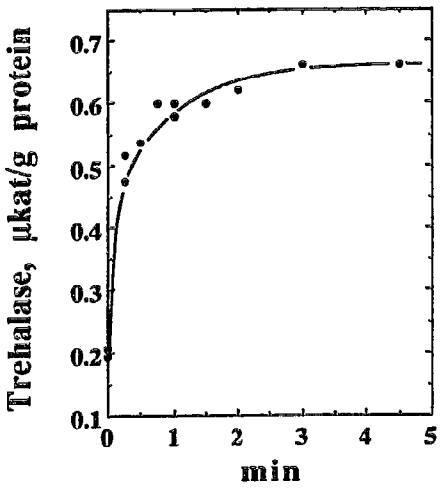

Fig 3 Activity of neutral trehalase in yeast cells (C13-ABYS86) heat shocked for 50 min at 40°C after a temperature shift to 4°C for the times indicated Activities were determined by the permeabilization method

4°C (Fig 1) In a control experiment we submitted homogenates obtained after cell disruption at 4°C to a further extraction procedure at 40°C These extracts had the same activity as the corresponding ones obtained by extraction at 4°C, demonstrating that the 40°C extraction procedure did not cause losses of neutral trehalase activity (Fig 1) On the basis of these data we suspected that the neutral trehalase might be activated during the sampling procedure upon shifting heat-shocked cells from 40 to 4°C prior to rupture Indeed, the activity of neutral trehalase in these homogenates decreased by 75–90 % upon incubation with alkaline phosphatase, indicating that the trehalase had been activated by phosphorylation (Fig 1) As discussed elsewhere [3], the activity remaining might be due to incomplete dephosphorylation or represent genuine activity of the dephosphorylated trehalase

As shown in Fig 1, both the trehalase activity in extracts prepared at 40°C and in phosphatase-treated extracts prepared at 0°C increased steadily during heat-shock, reaching a ca. 3-fold higher activity than control cells after 60 min Protein blots of these extracts stained with monoclonal antibodies against neutral trehalase revealed a similar increase in the amount of enzyme This increase was completely blocked by cycloheximide, suggesting that the enzyme was synthesized *de novo* upon heat shock (Fig 2)

In order to examine the activation of neutral trehalase in heat-shocked cells by the temperature shift to 0–4°C more closely, we employed a different harvesting and cell disruption technique, freezing the cells in liquid nitrogen in the presence of Triton X-100 and permeabilizing them during the subsequent thawing [14] Heat-shocked cells frozen immediately after addition of the Triton X-100 had a low activity of neutral trehalase (Fig 3) The activity increased at least 2-fold within 20 s of chilling to 4°C and reached 3–4-fold higher values 1–3 min later (Fig 3) In conjunction with the data shown in Fig 1, these results indicate that neutral trehalase is activated upon chilling in less than a minute by phosphorylation This is in accordance with data obtained in vitro demonstrating full activation of neutral trehalase by cAMP-dependent phosphorylation in less than a minute at 0°C [3] Furthermore, our study suggests that the permeabilization technique does not alter the activation state of trehalase, allowing accurate determination of its activity even during rapidly changing conditions

We used this permeabilization technique to examine the activity of neutral trehalase during alternating temperature shifts between 27°C and 40°C (Fig 4A) and to correlate them with trehalose accumulation and mobilization (Fig 4B) Upon a shift to 40°C, trehalase activity increased about 3-fold during the first hour and then remained constant for at least 2 h (Fig 4A). This trehalase activity is probably responsible for the previously demonstrated turnover of trehalose during incubation at 40°C [9] When the cells were shifted back to 27°C after 40 min at 40°C, trehalase activity quickly rose from 0 18 to 0 60 μkat/g protein during the first 3

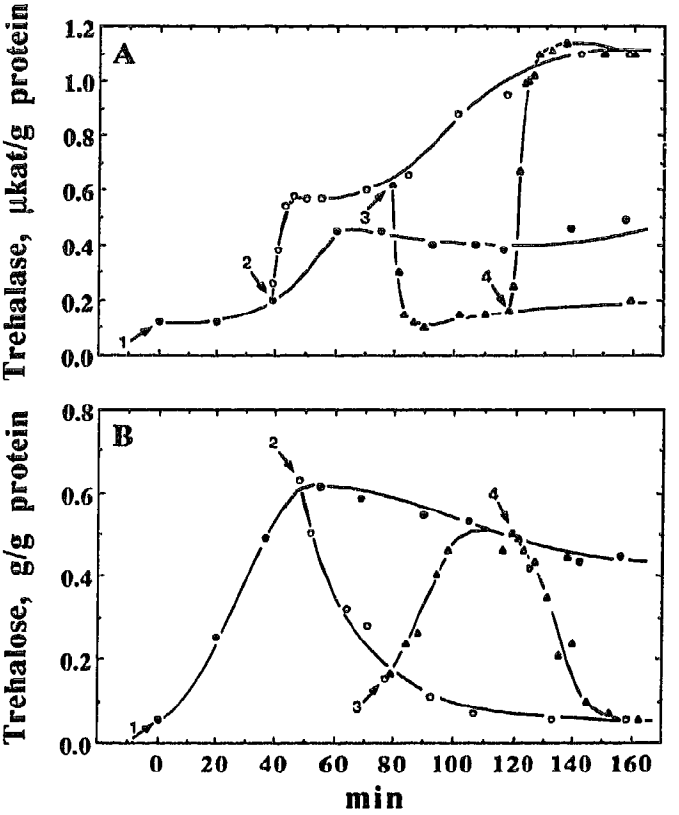

Fig 4 Activity of neutral trehalase (A) and trehalose content (B) in yeast cells (YS18) subjected to various temperature shifts At time zero (arrow 1), the temperature was shifted from 27 to 40°C (●) After 40 min (arrow 2), a portion of this culture was shifted back to 27°C ( ) After 80 min (arrow 3), a portion of the latter culture was shifted again to 40°C (▲) Finally, after 120 min, this culture was shifted back to 27°C ( )

min and then slowly increased until it reached 1 1 μkat/g protein after 2.5 h at 27°C The rapid activation of trehalase is probably a key factor for the rapid degradation of trehalose at 27°C (Fig 4B) Interestingly, a second heat shock 40 min after the shift back to 27°C resulted in an immediate inactivation (<3 min) of neutral trehalase, which then remained at a very low level for more than 1 h During this period, trehalose accumulated to the same high levels as in permanently heat-shocked cells (Fig. 4B) When these cells were cooled again to 27°C after 40 min of the second heat shock episode, neutral trehalase was activated in less than 3 min from 0.15 to 1 1 μkat/g protein (Fig. 4A) and trehalose disappeared rapidly (Fig 4B)

Thus far, regulation of neutral trehalase has been studied mainly with regard to the rapid activation by cAMP-dependent phosphorylation [4,7] The results presented show that cooling of heat-shocked cells is one of the conditions that cause such a rapid activation Much less is known about down-regulation of neutral trehalase activity [3,4,7]. In this regard, the rapid decrease of trehalase activity during the second heat shock is of particular interest it indicates that inactivation of trehalase, presumably by dephosphorylation, can proceed as rapidly as its induction

The diamatic fluctuations of the trehalose pool during temperature shift experiments were always inversely correlated with the activity of neutral trehalase (Figs 4A,B) Both trehalose and neutral trehalase are located in the cytosol in yeast [18–20] Thus, the neutral trehalase which can be so rapidly and finely tuned in its activity must play a key role in the regulation of the trehalose pool in yeast.

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