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On the mechanism by which a heat shock induces trehalose accumulation in *Saccharomyces cerevisiae*

Maria-José NEVES* and Jean FRANÇOIS†
Laboratoire de Chimie Physiologique, International Institute of Cellular and Molecular Pathology, and Université Catholique de Louvain, Brussels, Belgium

When the temperature of exponential-phase cultures of *Saccharomyces cerevisiae* was abruptly raised from 28 to 40 °C, trehalose immediately accumulated, whereas the activities of trehalase and trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase complex increased after a lag period of about 10 min. Heat shock also induced a sudden rise in intracellular glucose, simultaneously with a decrease in the concentration of hexose phosphate and fructose 2,6-bisphosphate. The increase of trehalose-metabolizing enzymes, but not the accumulation of glucose and trehalose, was prevented by cycloheximide. Investigation of the kinetic properties of partially purified enzymes showed that both non-activated and cyclic AMP-dependent-protein-kinase-activated forms of trehalase are almost inactive in the absence of Ca^{2+} and that the concentration of free Ca^{2+} required for half-maximal activity increased with increasing temperature, being approx. 1 μM at 30 °C and 20 μM at 40 °C for the activated form of trehalase. In contrast, trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase were three times more active at 40 °C. It is proposed that the rapid accumulation of trehalose induced by heat shock may be in part explained by changes in the kinetic properties of trehalase and trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase.

INTRODUCTION

Trehalose (α-D-glucopyranosyl α-D-glucopyranoside) is a non-reducing disaccharide found in a wide variety of organisms including bacteria, fungi, nematodes and plants [1,2]. In the yeast *Saccharomyces cerevisiae*, trehalose accumulates during periods of reduced growth, for example during nitrogen, sulphur or phosphate starvation [3], as well as during the stationary phase of growth on glucose [3–5]. More recent studies have also established a strong correlation between the trehalose content of yeast cells and their resistance to temperature extremes, dehydration and freezing–thawing cycles (reviewed in [6]). From these data it has been proposed that the primary function of trehalose in yeast is not as an energy reserve, but as a protectant of cell membranes and proteins under conditions that deplete the activity of intracellular water [6,7].

Trehalose is synthesized from UDP-glucose (UDP-Glc) and glucose 6-phosphate (Glc6*P*) in a two-step reaction catalysed by the trehalose-6-phosphate synthase (Tre6*P* synthase)/trehalose-6-phosphate phosphatase (Tre6*P* phosphatase) complex [8,9]. It is hydrolysed into glucose by two distinct trehalases: an acid trehalase that is confined in the vacuoles [10], and a neutral trehalase located in the cytosol and which is activated by cyclic AMP-dependent phosphorylation [2]. In most cases, mobilization of trehalose is associated with the activation of neutral trehalase [2].

Investigating the metabolism of trehalose under heat-shock conditions, Hottiger *et al.* [11] observed a good correlation between fluctuations of trehalose pool and changes in Tre6*P* synthase activity after temperature shifts and concluded that Tre6*P* synthase rather than trehalase plays a key role in trehalose accumulation during a heat shock. More recently, the same group [12] had to correct their first conclusion, since they found that their sampling procedure actually leads to artificially high trehalase activities in crude extracts from heat-shocked cells. Using a permeabilization technique which apparently circumvented this problem, they reported rapid inactivation or re-activation of neutral trehalase depending on whether the cells were shifted from 27 to 40 °C or returned to 27 °C. Attfield [13] also suggested that storage of trehalose induced by heat shock required RNA synthesis *de novo*, since this accumulation was inhibited in the presence of Acridine Orange or ethidium bromide, two agents reported to inhibit RNA synthesis [14]. In deep contrast with these results, Winkler *et al.* [15] did not find any change in trehalose-metabolizing enzymes under heat-shock conditions and proposed that the heat-induced accumulation of trehalose may be a consequence of the drastic increase in the concentration of substrates (UDP-Glc and Glc-6*P*) for Tre6*P* synthase.

Here we show that the accumulation of trehalose required neither activation of Tre6*P* synthase and inactivation of trehalase, nor protein synthesis. This metabolic event may be primarily due to a temperature-dependent change in the kinetic properties of trehalase and Tre6*P* synthase/Tre6*P* phosphatase.

MATERIALS AND METHODS

Strain and growth conditions

The strain S288c of *Saccharomyces cerevisiae* was grown in a medium containing 2% bactopeptones, 1% yeast extract and 2% glucose (YEPD) at 28 °C. When cells reached an A_{600} of approx 1.0 unit (about 1.4×10^7 cells/ml), cultures were divided into portions of 100 ml and placed in a water bath maintained at

Abbreviations used: UDP-Glc, UDP-glucose; Glc*P*, glucose 6-phosphate; Tre6*P*, trehalose 6-phosphate; Fru6*P*, fructose 6-phosphate; Fru(1,6)P_2, fructose 1,6-bisphosphate.

* Present address: Laboratorium voor Cellulaire Biochemie, Katholieke Universiteit te Leuven, Kardinaal Mercierlaan, B-3030 Leuven-Heverlee, Belgium.

† Present address and address for correspondence: Institut National des Sciences Appliquées, Département de Génie Alimentaire, 31077 Toulouse Cédex, France.

60 °C such that they reached 40 °C in less than 3 min. The bath was then quickly maintained at 40 °C by the addition of ice.

Purification of trehalase and Tre6*P* synthase/Tre6*P* phosphatase complex

Trehalase was partially purified by the procedure of Londesborough & Varimo [16]. The enzyme was activated by incubation of 3–5 mg of partially purified protein in the presence of bovine heart type II cyclic AMP-dependent protein kinase (0.1 mg/ml; Sigma, St. Louis, MO, U.S.A.), 10 μM-cyclic AMP and 2 mM-ATPMg for 15 min at 30 °C. The mixture was then passed through 20 vol. of Sephadex G-25 equilibrated with 20 mM-Hepes, pH 7.1, containing 50 mM-KCl, 1 mM-dithiothreitol, 2 mM-EGTA and antipain (20 μg/ml). Tre6*P* synthase/Tre6*P* phosphatase protein complex was purified as described in [8], except that the last step was omitted. The most active fraction eluted from the Mono S column, which contained about 250 and 57 munits/mg of protein of Tre6*P* synthase and Tre6*P* phosphatase respectively, was used for kinetic studies.

Enzymic assays

The preparation of crude extracts and, unless otherwise stated, assay of trehalase [17], Tre6*P* synthase and Tre6*P* phosphatase [8] were performed as previously described. The glucose-phosphorylating activity was tested in an assay mixture containing 25 mM-Hepes, pH 7.1, 100 mM-KCl, 5 mM-$MgCl_2$, 2 mM-ATPMg and 2 mM-[U-^{14}C]glucose (500–1000 c.p.m./nmol; Amersham International) in a total volume of 100 μl. The reaction was started by the addition of 1–3 μl of extract and stopped at appropriate times (between 1 and 10 min) by spotting 20 μl portions on DE 81 papers, which were washed three times for 10 min in 10 % (v/v) ethanol (20 ml/paper) and then counted for radioactivity in the presence of 5 ml of scintillation cocktail (Opti phase HiSafe II; LKB, Loughborough, Leics., U.K.). The uptake of glucose by intact cells was measured in the presence of 20 mM-[U-^{14}C]glucose by the method of Bisson & Fraenkel [18]. One unit is the amount of enzyme that catalyses the conversion of 1 μmol of substrate in 1 min under the conditions of the assay.

Analytical procedures

Collection of cells and extraction of metabolites were done as described previously. Glucose [19], Glc6*P*, fructose 6-phosphate (Fru6*P*) [20], fructose 1,6-bisphosphate [Fru(1,6)P_2] [21], UDP-Glc [22] and ATP [23] were measured by the published procedures cited. Extraction for determination of Fru(2,6)P_2 [24] and trehalose [5,25] were performed in 0.5 ml of 0.25 M-Na_2CO_3 for 30 min at 80–90 °C. Concentrations of metabolites, trehalose and Fru(2,6)P_2 are expressed as mol/g wet weight. Protein synthesis was measured by the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material as follows. Early-exponential-phase cells from YEPD medium (A_{600} of approx. 1.0 unit) were harvested by low-speed centrifugation, washed twice with sterile water and resuspended in a new fresh YEPD medium. Cultures (1 ml) were radiolabelled by the addition of 10 μCi of [^{35}S]methionine (Amersham International). Radiolabelled protein was extracted with 0.125 M-NaOH for 15 min at 80 °C and subsequent precipitation with 10 % (w/v) trichloroacetic acid. The pellets were washed twice with ice-cold 5 % trichloroacetic acid, resuspended in 1 ml of 10 mM-NaOH and counted in the presence of 8 ml of scintillation cocktail (Aquasol-2; New England Nuclear–du Pont, Boston, MA, U.S.A.).

The concentration of free Ca^{2+} was calculated as described by Harrison & Bers [26], using EGTA as the chelator. Those authors showed that increasing the temperature from 1 °C to 36 °C led to a nearly linear increase in $K_{Ca\text{-}EGTA}$. Accordingly, at pH 7.00 and I0.1, the apparent Ca^{2+} association constant increases from 2.67 to 3.2 10^6 M^{-1} as the temperature increases from 28 to 40 °C. However, it can be calculated that this change in temperature has no effect on the calculated free Ca^{2+} concentration when this concentration varies over the range of 10^{-7}–to 10^{-3} M. The concentration of protein was determined by the method of Bradford [27], with bovine immunoglobulin G as a standard.

All results shown are representative of at least three independent experiments performed under similar conditions.

RESULTS

Changes in intracellular glucose, trehalose and metabolites after a temperature shift from 27 to 40 °C

During exponential growth on glucose at 28 °C, yeast cells contain only traces of glucose and trehalose, because the former is immediately phosphorylated as it enters the cell [28], whereas the latter is not synthesized because trehalase is found predominantly in its activated (phosphorylated) form and the activity of the complex Tre6*P* synthase/Tre6*P* phosphatase is

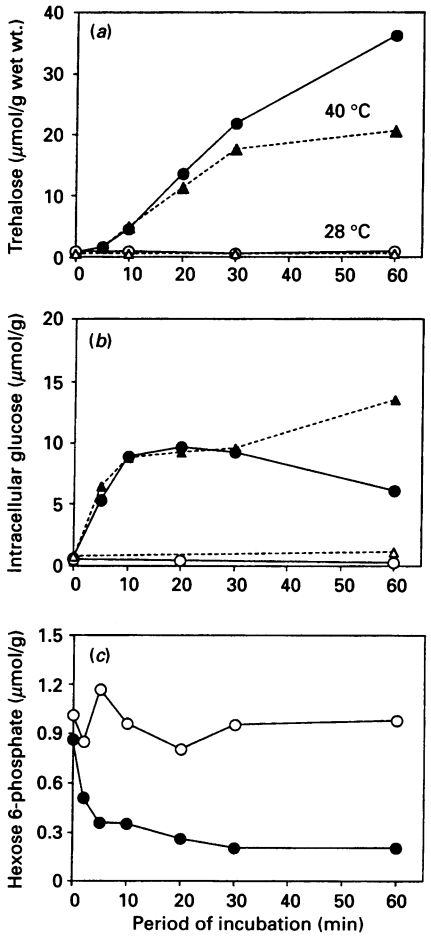

Fig. 1. Changes in the concentration of trehalose (*a*), glucose (*b*) and hexose 6-phosphate (*c*) induced by a temperature shift in exponential-phase cultures of *S. cerevisiae* in the absence or presence of cycloheximide

At zero time cultures were shifted from 28 (○, △) to 40 °C (●, ▲) in the absence (○, ●) or in the presence (△, ▲) of 50 μg of cycloheximide/ml. The drug was added 15 min before the temperature shift.

only 20% of that in stationary phase [5,29]. When the temperature of the cultures was abruptly raised to 40 °C, glucose and trehalose immediately accumulated at rates of approx. 1 μmol of glucose/min per g of wet cells (Figs. 1*a* and 1*b*), whereas the concentration of hexose 6-phosphate decreased by about 3-fold within 10 min after the shift (Fig. 1*c*). Addition of 50 μg of cycloheximide/ml to the cultures 15 min before the temperature shift did not significantly affect the intracellular accumulation of these sugars (Figs. 1*a* and 1*b*). Accumulation of glucose always preceded that of trehalose and reached a plateau of 10 μmol/g wet cells 10 min after the shift. In contrast, trehalose synthesis still continued after 60 min of incubation at 40 °C (Fig. 1*a*). When heat-shocked cells were returned to 27 °C, intracellular glucose and trehalose decreased to their initial levels at rates comparable with those of their accumulation (results not shown; but see also [11]).

Fig. 2 shows that the temperature stress caused a 3–5-fold decrease in the concentration of Fru(2,6)P_2 and Fru(1,6)P_2, as well as a progressive rise in the concentration of UDP-Glc (up to 5-fold after 20 min) and of ATP (2-fold after 20 min). Other metabolic changes caused by heat treatment were a 3–5-fold accumulation of cell-wall and membrane precursors ADP-glucose, GDP-mannose and UDP-*N*-acetylglucosamine (results not shown). It was also found that yeast cells lost their ATP pools upon incubation at 40 °C in the presence of 1 mM-Acridine Orange (results not shown).

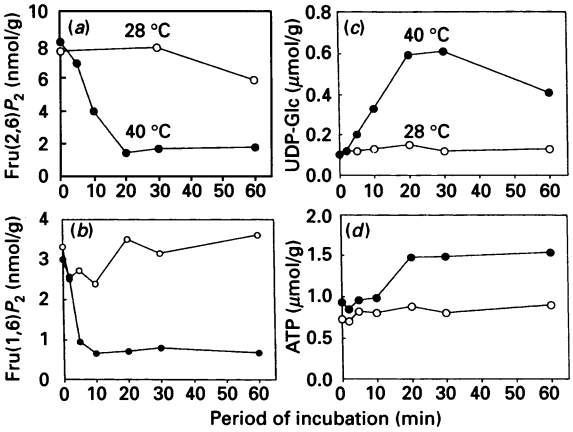

Fig. 2. Changes in the concentration of Fru(2,6)P_2, Fru(1,6)P_2, ATP and UDP-Glc after transfer of yeast cultures from 28 to 40 °C

The procedure and symbols were the same as those in Fig. 1.

Changes in the activity of the enzymes of trehalose metabolism after temperature shifts

As Fig. 3 shows, a sudden rise of temperature induced an increase of trehalase, Tre6*P* synthase and Tre6*P* phosphatase activities. However, this increase was preceded by a lag period of about 10 min and was prevented by addition of cycloheximide (50 μg/ml) 15 min before the temperature shift. Under this condition, cycloheximide inhibited by more than 95% the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material (Table 1). When yeast cultures were transferred back to 27 °C, the activity of Tre6*P* synthase and Tre6*P* phosphatase returned to their original value within 60 min, whereas that of trehalase showed a slight increase of about 30% (results not shown).

Because glucose rapidly accumulated in yeast incubated at 40 °C, we tested whether heat treatment affected the glucose-phosphorylating activity *in vivo*. For this purpose we prepared crude extracts from control cells and from cells that had been incubated at 40 °C for various periods of time and we measured the rate of formation of [^{14}C]hexose phosphate from [^{14}C]glucose and ATPMg, which reflected the activity of glucose phosphorylation. Under these assay conditions, an activity of 1.2 ± 0.3 μmol of hexose phosphate formed/min per mg of protein (mean for three separate experiments) was measured in the extracts from control and heat-shocked cells. These data suggest that the kinases (hexokinase I and II and glucokinase) responsible for glucose phosphorylation were not activated upon heat treatment.

Effect of temperature on the kinetic properties of trehalose metabolic enzymes

Since the increase in the activities of Tre6*P* synthase/Tre6*P* phosphatase complex was not a prerequisite for trehalose accumulation during heat shock, we investigated whether temperature by itself might dramatically affect the kinetic properties of the enzymes involved in trehalose metabolism.

Londesborough & Varimo [16] reported that the activity of neutral trehalase was dependent on the presence of Ca^{2+} or

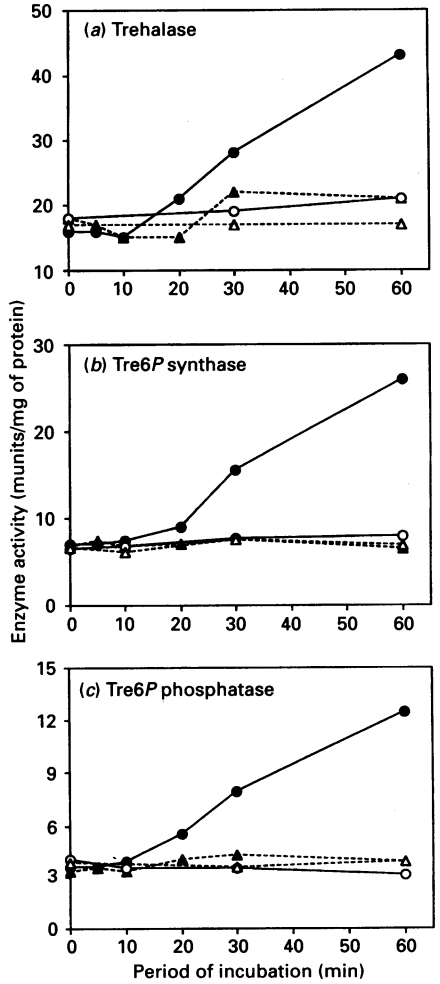

Fig. 3. Changes in the activities of trehalase (*a*), Tre6*P* synthase (*b*) and Tre6*P* phosphatase (*c*) during the incubation of yeast at 28 or 40 °C in the absence or presence of cycloheximide (50 μg/ml)

Cultures were shifted from 28 (○, △) to 40 °C (●, ▲) in the absence (○, ●) or in the presence (△, ▲) of 50 μg of cycloheximide/ml. Other procedures were as in Fig. 1.

Table 1. Effect of cycloheximide on the incorporation of [^{35}S]methionine in *S. cerevisiae* incubated at 28 or 40 °C

Exponential-phase cells were collected, washed with water and resuspended in new fresh YEPD medium at 2.0 units of A_{600} in the absence or presence of 50 μg of cycloheximide/ml. After 15 min of incubation at 28 °C, [^{35}S]methionine (10 μCi) was added and, simultaneously, part of the culture was adjusted to 40 °C.

Time (min)	10^{-3} × [^{35}S]Methionine incorporation into trichloroacetic acid-precipitable material (c.p.m./A_{600})			
	−Cycloheximide		+Cycloheximide	
	28 °C	40 °C	28 °C	40 °C
0	0	0	0	0
3	13	15	2.8	0
6	26	30	4.6	0.15
24	140	87	29	0.20

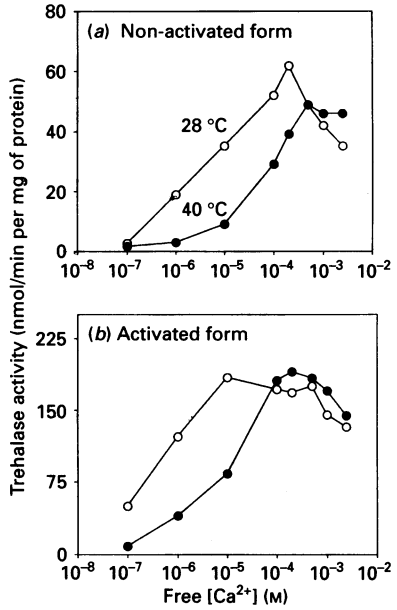

Fig. 4. Effect of Ca^{2+} on the activity of trehalase assayed at 28 °C or 40 °C

Partially purified trehalase (non-activated and activated by cyclic AMP-dependent protein kinase, ATP-Mg and cyclic AMP) was used. The reaction mixture contained 25 mM-Hepes/KOH, pH 7.1, 10 mM-trehalose, 1 mM-Ca-EGTA, 0.1 M-KCl and various concentrations of EGTA and $CaCl_2$ to give calculated free Ca^{2+} concentrations of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 2×10^{-4}, 5×10^{-4}, 10^{-3} and 5×10^{-3} M at 28 and 40 °C.

Mn^{2+}, whereas Mg^{2+} was ineffective. We therefore studied the effect of temperature on the sensitivity of the non-activated and the cyclic-AMP-dependent-protein-kinase-activated trehalase to Ca^{2+}. Free concentrations of Ca^{2+} were calculated using the $K_{Ca\text{-}EGTA}$ determined at 28 and 40 °C. However, as explained in the Materials and methods section, increasing the temperature from 28 to 40 °C does not influence the calculated free Ca^{2+} concentrations. As Fig. 4 shows, at 28 °C and in the presence of a calculated free Ca^{2+} concentration of approx. 10^{-7} M, the non-activated trehalase was completely inactive, whereas the activated

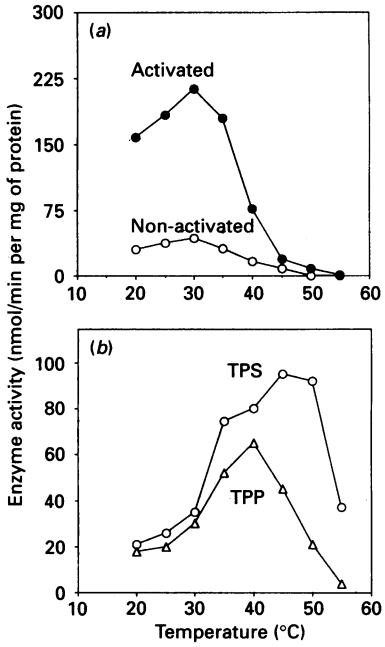

Fig. 5. Effect of temperature on the activities of trehalase (*a*), Tre6*P* synthase and Tre6*P* phosphatase (*b*)

The activities of the non-activated and activated trehalase were measured as described in Fig. 4 at a calculated free Ca^{2+} concentration of 10^{-5} M. The activity of Tre6*P* synthase was assayed in the presence of 1 mM-Glc6*P* and 0.5 mM-UDP-Glc. Abbreviations: TPS, Tre6*P* synthase; TPP, Tre6*P* phosphatase.

enzyme still hydrolysed trehalose at 25 % of its maximum activity (Fig. 4*a*). At this temperature, a half-maximum effect was obtained with 10 μM and 1 μM free Ca^{2+} for the non-activated and cyclic-AMP dependent-protein-kinase-activated trehalase respectively. Interestingly, the effect of increasing temperature was to reduce by more than 20-fold the affinity of both forms of trehalase to Ca^{2+} without affecting their maximal activity. For instance, at 40 °C and at 1 μM free calcium, the non-activated trehalase was nearly inactive and the activated form of the enzyme had only 25 % of its activity measured at 28 °C. A more complete temperature-dependent activity of the two forms of trehalase measured in the presence of 10 μM free calcium is reported in Fig. 5(*a*). It is important to stress that the low activity of trehalase measured at temperatures above 40 °C was not due to the denaturation of the protein, since normal activities were recovered by reincubation of the enzyme at 30 °C (results not shown). Other kinetic properties (K_m of 4 mM for trehalose, inhibition by neutral salts and by zinc ions) were not significantly modified at 40 °C, when the assays were performed at 10 μM free calcium (results not shown).

Fig. 5(*b*) also shows the effect of temperature on partially purified Tre6*P* synthase and Tre6*P* phosphatase activity. Maximal activity of Tre6*P* synthase was measured at temperatures ranging from 45 to 50 °C, whereas the activity of Tre6*P* phosphatase reached a maximum at 40 °C. Interestingly, these maxima for Tre6*P* synthase and Tre6*P* phosphatase activities coincided with maximal rate of trehalose synthesis ([11]; J. François, unpublished work). The K_m of Tre6*P* synthase for UDP-Glc (0.2 mM) and Glc6*P* (1.5 mM) and of Tre6*P* phosphatase for Tre6*P* (0.2 mM) were not significantly modified by raising the temperature from 30 to 40 °C (results not shown). We did not verify whether the loss of the activity of these two enzymes at temperature above 50 °C was reversible or not.

DISCUSSION

The rapid accumulation of trehalose in exponential-phase cells in response to a heat stress is now well documented (see [6] for a review). However, there is controversy as to whether this accumulation results from heat-induced changes in the activity of trehalose-metabolizing enzymes [11,12] or is a consequence of an increase in the concentration of the substrates for Tre6*P* synthase (UDP-Glc and Glc6*P*). Here we present several arguments against these two explanations and propose an alternative mechanism based on temperature-dependent modifications of the kinetic properties of trehalase and Tre6*P* synthase.

Although Tre6*P* synthase and trehalase activities increased during heat shock, as previously reported [11,12], this increase was preceded by a lag period of about 10 min and was inhibited by cycloheximide. In contrast, synthesis of trehalose took place immediately after heat shock and was insensitive to the protein-synthesis inhibitor. Taken together, our results exclude the induction of Tre6*P* synthase and/or a rapid inactivation of trehalase as a prerequisite for trehalose accumulation during heat shock. These results are at variance of those of De Vergilio *et al.* [30], who found that Tre6*P* synthase is still induced, albeit to a lesser extent, in a temperature-sensitive mutant which failed to initiate protein synthesis at 40 °C. To explain this result, those authors suggested that Tre6*P* synthase could be activated by a post-translational modification. However, their method for assaying Tre6*P* synthase is based on the measurement of the production of UDP. As discussed previously [8], this assay is not specific, since part of the UDP may come from the reaction catalysed by a glycogen synthase. The fact that agents known to inhibit RNA synthesis prevented trehalose accumulation is easily accounted for by our observation that these drugs actually caused a depletion of ATP, which prevents any further formation of trehalose precursors.

On the basis of metabolite determination and on the kinetic properties of Tre6*P* synthase, Winkler *et al.* [15] came to the conclusion that the rapid accumulation of trehalose upon heat shock can be explained in terms of increasing the concentration of glycolytic intermediates, including those required for trehalose synthesis. In our hands this explanation does not hold, since we found exactly the opposite results. This discrepancy concerns not only the effect of temperature on the concentration of metabolites, but also the absolute levels of these metabolites. The concentrations of Glc6*P* and ATP measured by Winkler *et al.* [15] in exponentially growing cells are respectively 50- and 10-fold lower than those commonly reported by others [17,28,29,31].

Because neither of the two explanations stated above could account for the heat-induced accumulation of trehalose, we wondered if temperature could not directly affect the activities of trehalase and Tre6*P* synthase/Tre6*P* phosphatase complex. Indeed, we found that the affinity of both forms of trehalase to Ca^{2+} ions was decreased by more than 20-fold at 40 °C. Conversely, Tre6*P* synthase and Tre6*P* phosphatase were three times more active at 40 °C. Assuming that the concentration of free Ca^{2+} inside the cytosol is between 0.1 μM and 1.0 μM [32] and that this concentration does not change with temperature, one can estimate that a rise of temperature from 28 to 40 °C should result in a relative gain of trehalose synthesis over its degradation of at least 15-fold. Thus our results support the notion that changes in the kinetic properties of trehalase and of Tre6*P* synthase/Tre6*P* phosphatase rather than changes in the synthesis and/or degradation of these enzymes could explain the rapid accumulation of trehalose induced by heat, and that, in particular, Ca^{2+} may play an essential role in this process.

As illustrated in Fig. 2, heat shock induced other severe perturbations of glucose metabolism, including a sudden rise in intracellular glucose concomitantly with a decrease in the concentration of hexose phosphate and Fru(2,6)P_2, which indicates a cross-over at the level of glucose phosphorylation and an inhibition of glycolysis. How these effects occur remains to be clarified, but so far we have been unable to find any differences in glucose uptake and glucose-phosphorylating activity between control and heat-shocked cells. Also, the finding of a rapid decrease in Fru(2,6)P_2 could not be explained only in terms of decreased concentrations of hexose 6-phosphate, but points to a stimulation by temperature of the enzymes involved in the degradation of this molecule [33–35], whereas the increase in the concentration of UDP-Glc upon heat shock may be due to the transient arrest of the cells in G1 phase of the cell cycle [11,36] and the subsequent inhibition of membrane and cell-wall biosynthesis.

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