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Metabolic regulation of the trehalose content of vegetative yeast

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We have investigated the mechanism by which heat shock conditions lead to a reversible accumulation of trehalose in growing yeast When cells of *S cerevisiae* M1 growing exponentially at 30°C were shifted to 45°C for 20 min, or to 39°C for 40 min, the concentration of trehalose increased by about 25-fold, an effect reversed upon lowering the temperature to 30°C This was compared to the more than 50-fold rise in trehalose levels obtained upon transition from the exponential to the stationary growth phase Whereas the latter was paralleled by a 12-fold increase in the activity of trehalose-6-phosphate synthase, no significant change in the activities of trehalose-synthesizing and -degrading enzymes was measured under heat shock conditions Accordingly, cycloheximide did not prevent the heat-induced accumulation of trehalose However, the concentrations of the substrates for trehalose-6-phosphate synthase, i e glucose-6-phosphate and UDP-glucose, were found to rise during heat shock by about 5–10-fold Since the elevated levels of both sugars are still well below the K_m-values determined for trehalose-6-phosphate synthase in vitro, they are likely to contribute to the increase in trehalose under heat shock conditions A similar increase in the steady-state levels was obtained for other intermediates of the glycolytic pathway between glucose and triosephosphate, including ATP This suggests that temperature-dependent changes in the kinetic parameters of glycolytic enzymes vary in steady-state levels of intermediates of sugar metabolism, including an increase of those that are required for trehalose synthesis Trehalose, glucose-6-phosphate, UDP-glucose, and ATP, were all found to increase during the 40 min heat treatment at 39°C Since this also occurs in a mutant lacking the heat shock-induced protein HSP104 (*Δhsp104*), this protein cannot be involved in the accumulation of trehalose under these heat shock conditions However, mutant *Δhsp104*, in contrast to the parental wild-type, was sensitive towards a 20 min incubation at 50°C Since this mutant also accumulated normal levels of trehalose, we conclude that HSP104 function, and not the accumulation of trehalose protects *S cerevisiae* from the damage caused by a 50°C treatment

Trehalose assay with acid trehalase, Neutral trehalase, Trehalose 6-phosphate synthase, Heat shock, Regulation of trehalose concentration Metabolic regulation

1 INTRODUCTION

The relatively low concentration of trehalose in exponentially growing yeast cells at 30°C is rapidly increased by a temperature stress at 39–45°C for a few minutes [1–3] The 'temperature stress' also induces synthesis of a set of proteins referred to as heat-shock or stress proteins [4] The biological significance of the increase in trehalose concentration induced by temperature stress is seen by Hottiger et al [3] as the protection of the cells from death by 50°C heat treatment or by overnight desiccation. A change in the activity of the trehalose synthesizing enzyme, trehalose-6-phosphate synthase [5,6], or in the trehalose degrading enzymes, neutral trehalase [7] and acid trehalase [8], could not be observed [9] under temperature stress conditions In this paper we show that it is not a change in the activities of the trehalose metabolizing enzymes, but rather a drastic increase in the concentration of the substrates for trehalose-6-phosphate synthase (UDP-glucose and glucose-6-phosphate), observed during temperature stress of the exponentially growing cells, that causes the increase in trehalose concentration

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2. MATERIALS AND METHODS

2 1 *Reagents*

Auxiliary enzymes and biochemicals were purchased from Boehringer-Mannheim (Mannheim Germany), Merck (Darmstadt, Germany), and Sigma (Deisenhofen, Germany)

2 2 *Organism, growth conditions and heat treatment*

The diploid yeast *Saccharomyces cerevisiae* M1 (from Dr A W Linane Clayton, Australia) was used in most experiments of this study The mutant *Δhsp104* and the corresponding wild-type W303 were provided by S Lindquist (University of Chicago, IL) [10] Cells were grown on YEPD medium (1% Bacto yeast extract, 2% Bacto peptone and 2% glucose) at 30°C for 6 h (exponential growth) or 24 h (stationary phase) Heat treatment was performed by transfer of part of the growing cultures under aerobic shaking, for 20 or 40 min to 45°C or 39°C, respectively

2 3 *Assay of enzyme activity*

Extracts were prepared by shaking the centrifuged and washed cells with glass beads as described previously [11] For assay of ATPase at different pH values the homogenate obtained was separated from the beads by decantation and directly used For all other assays the clear supernatant from the centrifuged (20 min, 33 500×g) homogenates was used ATPase activity was assayed at pH 5 5, 6 9 and 9 0 as described by Uchida et al [12], alkaline phosphatase was assayed according to [13], acid phosphatase according to [14], neutral trehalase according to [7], acid trehalase according to [8], trehalose-6-phosphate synthase according to Van der Cammen et al [5], glucose-6-phosphate dehydrogenase according to [15]

2 4 *Assay of metabolites*

Cells were extracted with 35% $HClO_4$ and neutralized as described

previously [16] UDP-glucose was analyzed with UDP-glucose dehydrogenase and NAD as described by Keppler and Decker [17], ATP, glucose-6-phosphate, fructose-1,6-bisphosphatase, dihydroxyacetone phosphate, and pyruvate, as described by Hinze and Holzer [16], glycerate-3-phosphate according to [18] and inorganic phosphate according to Ames [19] The concentrations are given in mM, calculated with the assumption that 1 g of yeast cells packed by centrifugation for 10 min at 2000×g contains 0 7 ml dissolving phase, i e 'cell sap'

2 5 *Assay of trehalose with acid trehalase*

Yeast cells washed free from glucose with ice-cold water were suspended in water (1 g yeast wet weight plus 1 ml H_2O) and heated for 20 min at 95°C After centrifugation, 3 μl of the supernatant were incubated for 60 min at 37°C in a total volume of 7 5 μl with 2 μl 200 mM citrate/NaOH buffer (pH 4 5) and 2 5 μl trehalase solution containing 2 5 mU acid trehalase After addition of 742 5 μl of glucose-oxidase-Perid II reagent (37°C) (Boehringer-Mannheim, Mannheim, Germany) and further incubation at 37°C for 30 min at pH 7 0, the glucose assay was terminated by adding 250 μl 6 N HCl Absorption was measured at 436 nm and glucose formation calculated from a standard containing 20 nmol glucose instead of yeast extract Controls showed that, under these conditions, 10 nmol of trehalose is hydrolyzed to 20 nmol of glucose ± 5% The acid trehalose used in this assay does not hydrolyze sucrose, maltose, lactose, cellobiose, raffinose, or glucose-6-phosphate [8]

3. RESULTS AND DISCUSSION

As seen in Table I, the low concentration of trehalose under exponential growth conditions (0 6 mM) is increased 25-fold on transition from 6 h growth at 30°C to 20 min at 45°C. The relatively high concentration of trehalose under stationary growth conditions (39 mM) (see also [20]) is not significantly changed by a 20 min treatment at 45°C To understand the mechanism of the drastic increase of trehalose during short-term temperature stress of exponentially growing cells we have analyzed activities of the enzymes catalyzing degradation and synthesis of trehalose neutral trehalase [7], acid trehalase [8] and trehalose-6-phosphate synthase [5] As shown in Table I, the changes of the activities of the trehalose metabolizing enzymes observed after 20 min treatment at 45°C of the exponentially growing cells do not explain the drastic increase in the trehalose concentration: the trehalose-6-phosphate synthase activity is not significantly changed and the activity of the trehalose-degrading neutral trehalase increases instead of an expected decrease.

In the presence of cycloheximide in concentrations which completely inhibit protein synthesis the 20-fold increase of trehalose observed after a 20 min treatment at 45°C is the same as in the absence of cycloheximide (data not shown). This indicates, in agreement with the measurements of the activity of trehalose metabolizing enzymes (Table I), that protein synthesis does not participate in the increase in trehalose levels during heat treatment at 45°C. The 60-fold increase in trehalose concentration from 0.6 to 39 mM during the transition from exponential growth (6 h at 30°C) to stationary phase (24 h at 30°C), shown in Table I, can be explained as a consequence of the 12-fold increase of the activity

Table I

Concentration of trehalose and activity of trehalose metabolizing enzymes in growing or stationary cells of *Saccharomyces cerevisiae* M1 For growth conditions and assay of trehalose and enzymes see Methods

	Trehalose [mM]	Neutral trehalase [mU/mg]	Acid trehalase [mU/mg]	Trehalose-6-phosphate synthase [mU/mg]
Exponential (6 h, YEPD, 30°C)	0 6	7 5	n d *	20
Subsequently 20 min at 45°C)	15	13 5	n d *	18
Stationary (24 h YEPD, 30°C)	39	28	12	248
Subsequently 20 min at 45°C	36	31	11	222

* not detectable

of trehalose-6-phosphate synthase, in comparison to the 4-fold increase in neutral trehalase Acid trehalase increased over the 'not detectable level', however, the sum of the trehalose degrading activities is about 40 mU per mg extractable protein, whereas the trehalose synthesizing activity is in the range of 200–250 mU per mg protein.

An explanation for the 'short-term' regulation of trehalose concentration during 20 min heat treatment of the exponentially growing yeast cells has been found by analyzing the steady-state concentrations of the substrates of trehalose-6-phosphate synthase (UDP-glucose and glucose-6-phosphate). As shown in Table II, glucose-6-phosphate increases by about 10-fold and UDP-glucose by about 4-fold during temperature stress. In addition, the observed decrease from 20 to 11 mM in the concentration of inorganic phosphate, which is an inhibitor of trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase [5], may favour the rate of synthesis of trehalose Measurements with partially

Table II

Concentration of metabolites in exponentially growing or stationary cells of *Saccharomyces cerevisiae* M1 before and after temperature stress for 20 min at 45°C For growth conditions and assay of metabolites see Methods

Growth phase	Trehalose [mM]	Glc-6-P [mM]	UDP-Glc [mM]	Inorganic phosphate [mM]
Exponential (6 h, YEPD, 30°C)	0 6	0 04	0 04	20
Subsequently 20 min at 45°C)	15	0 45	0 15	11
Stationary (24 h YEPD, 30°C)	39	0 031	0 17	17
Subsequently 20 min at 45°C	36	0 063	0 20	14

Table III

Concentration of trehalose and intermediates of glycolysis in exponentially growing cells of *Saccharomyces cerevisiae* M1 (6 h at 30°C in YEPD medium) before and after temperature stress for 40 min at 39°C

Treatment at 39°C subsequent treatment at 30°C	0 min 0 min (mM)	40 min 0 min (mM)	40 min 40 min (mM)
Trehalose	0 6	27 2	1 7
Glc-6-P	0 02	0 07	0 01
UDP-Glc	0 02	0 1	0 03
Fru-(1,6)-P_2	0 04	0 18	0 04
Dihydroxy-acetone phosphate	0 03	0 18	0 07
ATP	0 11	0 92	0 27
Glycerate-3-P	0 33	0 07	0 23
Pyruvate	0 28	0 13	0 22

purified trehalose-6-phosphate synthase indicate the following concentrations for half-maximal velocity. 1 mM glucose-6-phosphate, 0 5–1 mM UDP-glucose, and 2 mM for inhibition by inorganic phosphate [5] The changes in the concentrations of glucose-6-phosphate and UDP-glucose depicted in Table II are below the concentrations for half-maximal activity of trehalose-6-phosphate synthase Therefore, they very probably contribute linearly (or, in the case of sigmoidal dependence of rate on substrate concentration, to a greater degree) to the increase of the rate of synthesis of trehalose during short-term temperature stress of exponentially growing cells. The measured concentrations of inorganic phosphate are between 11 and 20 mM (Table II) Trehalose-6-phosphate synthase/trehalose-6-phosphate phosphatase would then be inhibited under all conditions studied. These measurements in perchloric acid extracts of the yeast cells, however, may not report on the actual levels of 'true, free inorganic phosphate' because of the presence of 'acid labile phosphate compounds', which are recorded as inorganic phosphate [21]. Therefore, it could be that changes in the concentration of inorganic phosphate participate in the control of trehalose-6- phosphate synthase. It is evident that the low level of activity of trehalose-6-phosphate synthase is due to the relatively small steady-state concentrations of substrates in growing as well as in stationary cells. However, activity can be rapidly increased under temperature stress by increasing the concentrations of glucose-6- phosphate and UDP-glucose In stationary cells the steady-state concentrations of glucose-6-phosphate and UDP-glucose do not change significantly during the 20 min at 45°C treatment. This is in agreement with the observation that the high concentration of trehalose in stationary cells shows no further change during the 20 min temperature stress

As shown in the first two columns of Table III, not only glucose-6-phosphate and UDP-glucose increase during the 39°C temperature stress for 20 min, but also other intermediates of the glycolytic pathway. fructose-1,6-bisphosphate and dihydroxyacetone phosphate. In contrast, the intermediates after the step of glyceraldehyde-3-phosphate dehydrogenase show a decrease after heat treatment. glycerate-3-phosphate and pyruvate. Another drastic change consists of the about 8-fold increase in the ATP concentration These observations point to a block in the glycolytic pathway at the step of oxidation of glyceraldehyde-3-phosphate to glycerate-3-phosphate and/or a decrease in the ATP hydrolyzing activities as compared to ATP synthesis. Measurements at 25°C in broken cells of the ATP hydrolyzing activities effected by the three different types of ATPase operating at pH 5 5, pH 6 9 and pH 9 0 [12], or by the ATP hydrolyzing alkaline phosphatase (measured at pH 9 0 with *p*-nitrophenylphosphate as substrate), or acid phosphatase (measured at pH 3.8) showed after the 20 min temperature stress of the intact cells at 45°C a slight increase, rather than a decrease, of these five ATP-splitting activities (data not shown) Only a small increase of activities was observed after temperature stress when measuring glyceraldehyde-3-phosphate dehydrogenase and glycerate-3-phosphate kinase, the two enzymes catalyzing the oxidation of glyceraldehyde-3-phosphate to glycerate-3-phosphate (data not shown) Heat induced inactivation of the enzymes catalyzing the oxidation of triosephosphate is therefore not the explanation for the changes in steady-state concentrations shown in the first two columns of Table III.

The activities of the trehalose synthesizing and degrading enzymes (cf. Table I) and of the ATP-degrading enzymes as well as the glyceraldehyde phosphate dehydrogenating enzymes were not significantly changed after 20 min temperature stress at 45°C Furthermore, since the temperature stress induced increase of trehalose is insensitive to cycloheximide (data not shown), we conclude that it is not a change in the synthesis and/or the degradation of enzymes, but a temperature-dependent change in the kinetic constants that is responsible for the observed variation in the steady-state concentrations of metabolites The different temperature dependencies of the reaction rates of enzymes (as characterized by the different values of activation energy) and the differences in the temperature dependence of the various Michaelis constants (concentration for half-maximal activity) of the different enzymes for their substrates, coenzymes, and effectors, cause the establishment of different steady-state concentrations of metabolites when changing the temperature of the glucose metabolizing cells These effects of the temperature shift on the steady-state concentrations of metabolites are expected to be reversed when the temperature is reduced to 30°C. It is shown in the third column of Table III that this is indeed the case when the temperature stress is performed at 39°C A similar experiment with temperature stress at 45°C and then reversion to 30°C revealed

that the steady-state concentrations in question are only partially reversed (data not shown) Our explanation is that after a 45°C treatment not only the reversible effects on the rate of enzyme activity and on the Michaelis constants are taking place, but also irreversible effects, such as partial denaturation of certain enzymes, etc. It should be mentioned here that heat treatment at 39°C represents the standard conditions for production of the heat shock response in yeast cells [4].

In addition to the kinetic parameters stated above, reversible protein–protein interactions of heat shock proteins with enzymes involved in carbohydrate metabolism might participate in the drastic changes in steady-state concentrations of metabolites. It is well known that protein–protein interactions could result in activation or inactivation of enzymes as well as in changes of the affinity of enzymes for their substrates or coenzymes [22,23]. A mutant deficient in the heat shock protein HSP104 (*Δhsp104*) obtained from Y Sanchez and S L Lindquist [10] has been used to investigate this possibility. It is shown in Table IV that the 40 min temperature stress at 39°C causes a 20-fold accumulation of trehalose in the *Δhsp104* mutant, the parental wild-type W303, and also in the wild-type *S cerevisiae* M1 (cf Table III). Also, the heat treatment induced increase of the steady-state concentrations of ATP, glucose-6-phosphate and UDP-glucose, observed with the *Δhsp104* mutant are similar to those in W303 (Table IV) and *S cerevisiae* M1 (Table III) In contrast to the 'normal' behaviour of the *Δhsp104* mutant with respect to accumulation of trehalose during the 39°C temperature stress, the mutant has lost its resistance to heat treatment at 50°C (cf Table IV); as previously described [10] We conclude that it is not the accumulation of trehalose, which results from the increase of steady-state concentrations of UDP-glucose and glucose-6- phosphate, but the presence of the heat shock protein HSP104 that protects the cells from killing by a 50°C treatment The biological usefulness of this trehalose accumulation may consist of aiding the cell in surviving the temperature stress at 39°C or 45°C, during which time production of heat shock protein(s) takes place [4] This 'preconditioning' of the cells is necessary for acquirement of resistance to a 50°C treatment

Table IV

Concentration of trehalose, glucose 6-phosphate, UDP-glucose and ATP in exponentially growing wild-type W303 and mutant *Δhsp104* cells at 25°C before and after 40 min temperature stress ('preconditioning') at 39°C After preconditioning at 39°C cells were subjected to 20 min heat treatment at 50°C, plated on YEPD agar gel and the colonies formed from surviving cells counted

	Trehalose [mM]	Glc-6-P [mM]	UDP-Glc [mM]	ATP [mM]
Wild-type (W303) exponential (16 h YEPD 25°C)	< 1	0 07	0 016	0 14
Subsequently 40 min, 39°C	24	0 21	0 10	0 73
Subsequently 20 min, 50°C	survival in % of cells without 50°C treatment 39%			
Mutant strain (W303 *Δhsp104*) exponential (16 h YEPD 25°C)	< 1	0 10	0 006	0 11
Subsequently 40 min, 39°C	25	0 17	0 067	0 80
Subsequently 20 min, 50°C	survival in % of cells without 50°C treatment 1 1%			

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