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The 70-kilodalton heat-shock proteins of the SSA subfamily negatively modulate heat-shock-induced accumulation of trehalose and promote recovery from heat stress in the yeast, *Saccharomyces cerevisiae*

Thomas HOTTIGER, Claudio De VIRGILIO, Walter BELL, Thomas BOLLER and Andres WIEMKEN
Botanisches Institut der Universität Basel, Switzerland

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In the yeast, *Saccharomyces cerevisiae*, the disaccharide trehalose is a stress-related metabolite that accumulates upon exposure of cells to heat shock or a variety of non-heat inducers of the stress response. Here, we describe the influence of mutations in individual heat-shock-protein genes on trehalose metabolism. A strain mutated in three proteins of the SSA subfamily of 70-kDa heat-shock proteins (hsp70) overproduced trehalose during heat shock at 37 °C or 40 °C and showed abnormally slow degradation of trehalose upon temperature decrease from 40 °C to 27 °C. The mutant cells were unimpaired in the induction of thermotolerance; however, the decay of thermotolerance during recovery at 27 °C was abnormally slow. Since both a high content of trehalose and induced thermotolerance are associated with the heat-stressed state of cells, the abnormally slow decline of trehalose levels and thermotolerance in the mutant cells indicated a defect in recovery from the heat-stressed state. A similar albeit minor defect, as judged from measurements of trehalose degradation during recovery, was detected in a *Δhsp104* mutant, but not in a strain deleted in the polyubiquitin gene, *UBI4*. In all our experiments, trehalose levels were closely correlated with thermotolerance, suggesting a thermoprotective function of trehalose. In contrast, heat-shock proteins, in particular hsp70, appear to be involved in recovery from the heat-stressed state rather than in the acquisition of thermotolerance. Cells partially depleted of hsp70 displayed an abnormally low activity of neutral trehalase when shifted to 27 °C after heat shock at 40 °C. Trehalase activity is known to be under positive control by cAMP-dependent protein kinases, suggesting that hsp70 directly or indirectly stimulate these protein-kinase activities. Alternatively, hsp70 may physically interact with neutral trehalase, thereby protecting the enzyme from thermal denaturation.

When exposed to a sudden rise in temperature (a heat shock), cells of virtually all organisms exhibit an adaptive response, the heat-shock response, that leads to induced thermotolerance, i.e. the ability to survive higher temperatures than unadapted cells. One of the key events in this adaptation process is a dramatic change in rates of synthesis of many individual polypeptides. Whilst the synthesis of most proteins is reduced, a small number of highly conserved proteins, the stress or heat-shock proteins (hsp), are strongly induced during heat shock (for reviews see Neidhardt et al., 1984; Lindquist, 1986; Lindquist and Craig, 1988; Schlesinger, 1990). The mechanism of hsp induction has been elucidated in considerable detail. In *Escherichia coli*, the synthesis of hsp is positively regulated by the product of the *htpR* gene (Neidhardt and VanBogelen, 1981, Neidhardt et al., 1983). *HtpR* encodes an alternative σ factor (σ^{32}) that enables RNA-polymerase to initiate transcription from heat shock promoters (Grossman et al., 1984). The concentration of σ^{32} within cells correlates well with the expression of hsp genes; in particular, it rapidly increases upon heat shock (Straus et al., 1987). Induction of hsp in eukaryotic cells requires that a special transcription factor, the heat-shock factor, be activated (Sorger et al., 1987). In yeast, heat-shock factor is encoded by a single essential gene (Sorger and Pelham, 1988). In mouse, two heat-shock-factor genes have recently been identified, and it was shown that their expression varies with tissue type (Sarge et al., 1991).

Whilst the induction of the heat-shock response is relatively well understood, little information is available concerning its termination and the process of recovery from the heat-stressed state. We are aware of only a few studies that have dealt with this question in more depth. In *E. coli*, it was found that repression of hsp genes and resumption of normal protein synthesis depends on the presence of an intact *dnaK* protein (Tilly et al., 1983). Similarly, restoration of the normal pattern of protein synthesis in heat-shocked cells of *Drosophila* has been suggested to depend on a critical level of 70-kDa hsp (DiDomenico et al., 1982). In mammalian cells, overexpression of hsp70 promotes recovery of nucleolar mor-

Correspondence to A. Wiemken, Botanisches Institut, Hebelstrasse 1, CH-4056 Basel, Switzerland
Fax: +41 61 261 53 18.
Abbreviation. hsp, heat-shock protein(s).

phology after heat shock (Pelham, 1984). More recently, genetic evidence has been presented that in *Saccharomyces cerevisiae* 70-kDa hsp repress their own synthesis, and it was suggested that this is part of the mechanism that terminates the heat-shock response (Stone and Craig, 1990).

The paucicity of data concerning recovery from induced thermotolerance and from the heat-stressed state may, in part, be due to difficulties in defining an appropriate biochemical marker for the heat-stressed state of cells. While, in principle, the rate of synthesis of hsp could be used for this purpose, it is technically demanding to accurately determine the production of individual polypeptides. An alternative marker of the heat-stressed state that is more amenable to quantitation would therefore be useful. In the yeast, *S. cerevisiae*, the non-reducing disaccharide trehalose potentially represents such a biochemical marker. Exponentially growing yeast cells accumulate large amounts of trehalose when subjected to heat shock; during recovery, the trehalose is rapidly degraded (Hottiger et al., 1987a, b). Trehalose accumulation is induced not only by heat shock but also by non-heat inducers of the stress response (Attfield, 1987; Hottiger et al., 1989; Hottiger, T., unpublished results). Furthermore, mutations that alter the activity of the RAS/adenylate-cyclase pathway affect trehalose metabolism and the heat-shock response in a parallel way (Hottiger et al., 1989; Shin et al., 1987). The trehalose content of cells and their thermotolerance have proven to be correlated under a wide variety of experimental conditions (Hottiger et al., 1987b, 1989; De Virgilio et al., 1990, 1991a). Thus, all evidence presently available suggests that trehalose accumulation is an element of the stress response. Here, the interrelationship between trehalose metabolism and heat-shock proteins was explored in two ways. Firstly, we studied the influence of mutations in individual hsp on the cells' ability to accumulate trehalose during heat shock and to degrade the disaccharide upon readjustment of the temperature. Secondly, the use of trehalose as a marker for the heat-stressed state was attempted in order to define the role of hsp during recovery from this state. Our experiments suggest that the 70-kDa heat-shock proteins of the SSA subfamily are major negative regulators of heat-induced trehalose accumulation and that their presence is crucial for recovery from the heat-stressed state. The data also indicate that hsp104 fulfils a function similar to that of hsp70 with respect to trehalose accumulation but is of comparatively minor importance. No evidence was found for a role of the polyubiquitin gene, *UBI4*, either in trehalose metabolism or during recovery from the heat-stressed state. Our results demonstrate the usefulness of trehalose as a biochemical marker of the heat-stressed state in yeast and suggest that trehalose accumulation contributes to induced thermotolerance.

MATERIALS AND METHODS

Strains, media and growth conditions

The characteristics of the yeast strains used in this study are given in Table 1. In most experiments, cells were cultivated on YPD medium (yeast extract 1%, peptone 2%, glucose 2%, mass/vol.). However, in experiments involving strain RH 1700 (Table 1), YPGal medium (yeast extract 1%, peptone 2%, galactose 2%, mass/vol.) was used. Cultures were grown in Erlenmeyer flasks at 27°C on a rotary shaker at 140 rpm. They typically contained $0.5-1\times10^7$ cells/ml when used for experiments. Stationary-phase cultures were obtained by growing cells for 3 d on medium with a reduced content of glucose (0.5% instead of 2%).

Table 1. Yeast strains

Strain	Genotype
SUB 62[a]	*MATαlys2-801 leu2-3, 112 ura3-52 his3Δ200 trp1-1(am)*
SUB 60[a]	*MATα lys2-801 leu2-3, 112 ura3-52 his3Δ200 trp1-1(am) ubi4-Δ2::LEU2*
RH 1700[b]	*MATα leu2 ura3 hys2 his4 ssa1-2::HIS3 ssa2-1::LEU2 ssa4-2::LYS2 bat 1-1 pGAL1:SSA1*
RH 144-3A[b]	*MATα his4 leu2 ura3 bar1-1*
W303[c]	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1*
Δhsp104[c]	[derived from W303 as reported by Sanchez and Lindquist (1990)]
C276[d]	*MATα/α*

[a] Obtained from A. Varshavsky.
[b] Obtained from H. Riezman.
[c] Obtained from S. Linquist.
[d] Obtained from J. R. Pringle.

Analytical procedures

Washed cells were extracted with ice-cold trichloroacetic acid (5%) as described (Hottiger et al., 1987a), and the trehalose content of the extracts was determined by the anthrone procedure (Lillie and Pringle, 1980). Since the anthrone assay is not specific for trehalose, the glucose content of every sample was determined with the glucose-oxidase-peroxidase kit (Boehringer) and deduced from the trehalose value. In general, the extracts contained no, or very little, glucose. In addition, in every experiment, some of the samples were subjected to HPTLC analysis as in Hottiger et al. (1987a). In no case were carbohydrates other than trehalose present in significant amounts in the extracts. Protein content was determined in samples treated with trichloroacetic acid (10%, mass/vol.) according to Peterson (1977), using bovine serum albumin as a standard. Neutral trehalase was assayed by the method of De Virgilio et al. (1991b).

Heat shock and thermotolerance

Heat shock was imposed by incubating cells at 40°C as in Hottiger et al. (1987a). For the determination of thermotolerance, cells were heated to 50.5°C (log-phase cells) or 56°C (stationary-phase cells) during an appropriate period of time (usually 8 min), rapidly cooled on ice, diluted with sterile water and plated on YPD agar (or, in experiments involving strain RH 1700, on YPGal agar). Details of the procedure have been described by Shin et al. (1987).

Transfer of heat-shocked cells to medium without glucose

Cultures were grown on medium with 2% glucose and subjected to heat shock (40°C) during exponential phase. At the desired time, the cells were rapidly collected by filtration, washed with warm (40°C) medium without glucose and resuspended in 1 vol washing medium. The entire procedure took approximately 1 min.

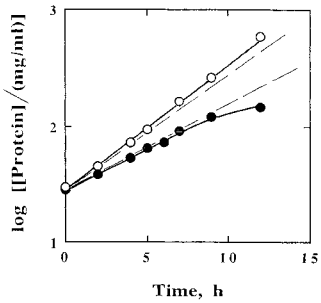

Fig. 1. Influence of glucose supply on growth of *S. cerevisiae* strains RH 144-3A (wild type, ○) and RH 1700 (*ssa1 ssa2 ssa4 pGal1:SSA1*, ●). YPGal medium was inoculated with cells which were grown to mid-exponential phase at 27°C. At 0 min, they were diluted with 1 vol fresh YPGal and supplied with glucose (4%) to repress expression of *SSA1* in the mutant. Growth was monitored by measuring cell protein. (- - -) Growth on YPGal as extrapolated from measurements of cell protein before glucose addition.

RESULTS

Effect of reduced levels of hsp70 on trehalose metabolism and thermotolerance

The SSA subfamily of 70-kDa hsp of *S. cerevisiae* comprises four genes, *SSA1–SSA4*. A mutant disrupted in three of these genes, (*SSA1, SSA2* and *SSA4*) is non-viable at all temperatures, indicating essential functions of SSA proteins (Deshaies et al., 1988). Conditional *ssa*⁻ strains can be constructed by introducing a plasmid with a wild-type *SSA1* gene under the control of the regulated *GAL1* promoter (see Deshaies et al., 1988) into an *ssa1ssa2ssa4* triple mutant. The plasmid-borne *GAL1:SSA1* gene allows for growth on media containing galactose as the only carbon source. Upon addition of glucose (4%), the *GAL1* promoter is switched off and the concentration of SSA1 protein gradually declines, reaching 40% of wild-type levels within 5 h (Deshaies et al., 1988; Zanolari, B. and Riezman, H., personal communication). The effect of inactivation of the plasmid-borne *SSA1* gene on growth of the conditional *ssa*⁻ mutant strain, RH 1700 (obtained from H. Riezman, see Table 1), is shown in Fig. 1. Growth of *ssa*⁻ cells was almost unaffected for up to 9 h following glucose addition; thereafter, a marked decline in growth rate was observed. Prolonged incubation of *ssa*⁻ cells in glucose medium resulted in cell death (unpublished results; see also Deshaies et al., 1988). In order to minimize the negative effects of SSA1 protein depletion on growth and viability, all further experiments were performed 5–9 h after glucose addition. During this time span, checks of plating efficiency gave no indication of decreased cell viability.

In the experiment shown in Fig. 2, wild-type and conditional *ssa*⁻ cells growing exponentially on YPGal medium were supplied with glucose (4%) for various periods of time then subjected to heat shock at 40°C for 50 min. Mutant cells cultivated on glucose for 5 h or even 7 h prior to temperature increase showed no defect in trehalose accumulation; in fact, they accumulated significantly more trehalose at 40°C than did wild-type cells (Fig. 2B, C). No difference in trehalose-accumulation capacity between wild type and mutant was seen when cells were subjected to heat shock immediately after glucose addition (Fig. 2A) or while growing exponentially on YPGal medium devoid of glucose. The relatively low concentrations of trehalose accumulated by galactose-adapted cells during exposure to 40°C (Fig. 2A) presumably reflect the fact that the biosynthetic precursors of trehalose, namely UDP-Glc and Glc6*P*, are more easily synthesized from glucose than from galactose.

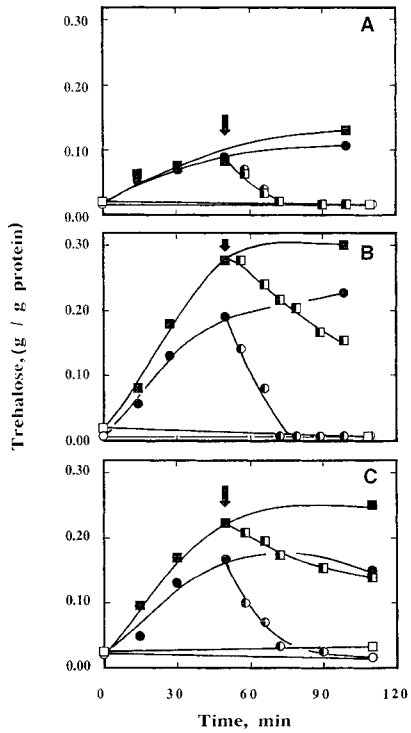

Fig. 2. Trehalose levels of *S. cerevisiae* strains RH 144-3A (wild type) and the conditional *ssa*⁻ mutant RH 1700 (*ssa1 ssa2 ssa4 pGal1:SSA1*) during heat shock and recovery. Cultures of parent strain RH 144-3A (circles) and mutant RH 1700 (squares) grown to early exponential phase on YPGal medium were supplemented with 4% glucose for different times in order to repress expression of *SSA1* in the mutant. At zero time, the cells underwent the following treatments. Cultivation at 27°C (control; ○, □); heat shock at 40°C (●,■); heat shock at 40°C for 50 min followed (arrow) by recovery at 27°C (◐, ◧). (A) Trehalose in cultures supplied with glucose (4%) at zero time. (B) Trehalose in cultures transferred to glucose (4%) 5 h before zero time. (C) Trehalose in cultures transferred to glucose (4%) 7 h before zero time.

After 50 min at 40°C, approximately 50% of the volume of the cultures subjected to heat shock was transferred to an Erlenmeyer flask at 27°C, and the kinetics of trehalose mobilization were monitored. In the conditional *ssa*⁻ strain, RH 1700, trehalose degradation after temperature decrease depended on how long the cells had been incubated with glucose prior to heat shock (Fig. 2). When heat shock was performed at the time of glucose addition (Fig. 2A), the half-life of the trehalose pool after temperature readjustment was 15 min, but it increased to 50 min and 80 min if the addition of glucose preceded heat shock by 5 h (Fig. 2B) or 7 h (Fig. 2C), respectively. In wild-type cells, the trehalose pool was mobilized with a half-life of approximately 15 min in all experiments shown in Fig. 2. Note that the different kinetics of trehalose degradation in wild type and mutant (Fig. 2B, C) are too large to be attributed simply to differences in growth rates during recovery (data not shown). Thus, sufficient levels of hsp70 are essential for normal mobilization of trehalose during recovery from the heat-stressed state.

A high content of trehalose appears to be a characteristic of cells in the heat-stressed state (see introduction). Therefore, the data in Fig. 2 suggested that cells devoid of normal levels of SSA proteins remain in the heat-stressed state for an abnor-

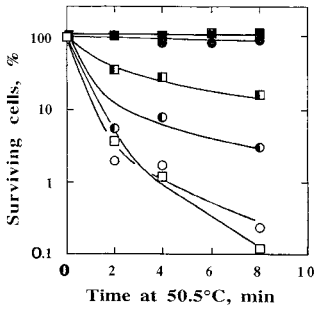

Fig. 3. Acquisition and loss of thermotolerance in wild-type RH 144-3A (circles) and mutant RH 1700 *(sal ssa2 ssa4 pGall:SSA1*, squares) as induced by heat shock and subsequent recovery. Cultures were grown to early exponential phase on YPGal medium and, after cultivation in the presence of glucose (4%) for 5 h, were subjected to the following treatments. Incubation at 27 °C for 50 min (control; ○,□); heat shock at 40 °C for 50 min (●,■); heat shock at 40 °C for 50 min, followed by recovery at 27 °C for 50 min (◐, ◧). At the end of these treatments, thermotolerance of the cells was assessed by incubating them at 50.5 °C for the indicated times and determining the fraction of surviving cells by plating.

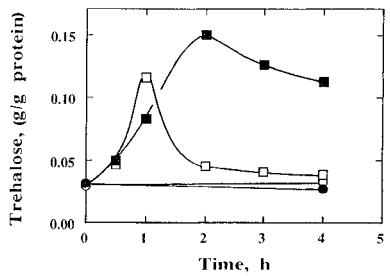

Fig. 4. Trehalose content of *S. cerevisiae* strains RH 144-3A (wild type; circles) and RH 1700 *(ssa1ssa2ssa4 pGall:SSA1;* squares) during a mild heat shock at 37 °C. Cells were grown to early exponential phase on YPGal medium and supplied with glucose (4%) 5 h before zero time. At zero time, the temperature was raised form 27 °C to 37 °C, and samples were taken for trehalose determination at the desired time points.

mally long period of time after transfer to the normal growth temperature. In order to substantiate this hypothesis, we studied induction and decay of thermotolerance, another attribute of cells in the heat-stressed state, in the conditional *ssa*$^-$ mutant, RH 1700. Cultures of wild-type RH 144-3A and mutant RH 1700 were grown in the presence of glucose for 5 h, as in Fig. 2B, in order to deplete the mutant cells of SSA1 protein. Thereafter, thermotolerance at 50.5 °C was assessed either directly, or after heat shock at 40 °C for 50 min, or after heat shock at 40 °C for 50 min followed by recovery at 27 °C for 50 min. As shown in Fig. 3, wild-type and mutant cells were equally sensitive to direct exposure to 50.5 °C (< 10% survivors after only 2 min). After a conditioning heat shock at 40 °C, both wild-type and mutant cells became thermotolerant (close to 100% survivors even after 10 min at 50.5 °C). However, as had been anticipated on the basis of the trehalose data shown in Fig. 2, mutant cultures subjected to heat shock at 40 °C and allowed to recover at 27 °C for 50 min were more thermotolerant than the corresponding wild-type cultures (Fig. 3). This demonstrates the usefulness of trehalose as a biochemical marker of the heat-stessed state of yeast cells and supports the concept that SSA proteins are essential for recovery from this state.

Recovery from the heat-stressed state takes place not only upon a shift from the heat-shock condition to the normal growth temperature but also during prolonged exposure to an elevated temperature that is still permissive for growth. Under such conditions (chronic mild heat shock) the heat-shock response is transient, i.e. the rate of synthesis of hsp declines after some time, indicating release of cells from the heat-stressed state. In *E. coli,* the *dnaK* protein, the only 70-kDa hsp of this organism, was found to mediate this process of recovery/adaptation (Tilly et al., 1983). We asked whether the SSA proteins fulfil a similar function in yeast. When YPGal cultures of the wild-type strain, RH 144-3A, were grown in the presence of glucose (4%) for 5 h then heated to 37 °C, the trehalose content rapidly increased during the first hour after the temperature increase then gradually decreased, almost reaching the low pre-heat-shock level after 4 h (Fig. 4). Similar results have been described previously (Hottiger et al., 1987a). When strain RH 1700 *(ssa1 ssa2 ssa4 pGal:SSA1)* was shifted to 37 °C after 5 h growth on glucose, significantly more trehalose was accumulated than in the wild-type strain and trehalose levels remained high even after 4 h cultivation at 37 °C (Fig. 4). This indicates that SSA proteins promote the restoration of a normal cell physiology during prolonged exposure to mild heat shock.

Trehalose metabolism and recovery from the heat-stressed state in yeast cells deleted in the polyubiquitin gene, *UBI4*, or in *HSP104*

In order to study whether a defect in recovery from the heat-stressed state was a general property of hsp mutants we looked at trehalose metabolism in a *ubi4* and in an *hsp104* strain. The phenotype of a yeast strain deleted in the polyubiquitin gene, *UBI4,* is superficially similar to that of an *ssa1 ssa2* double mutant (Finley et al., 1987; Craig and Jacobsen, 1984): *ubi4* cells are normal with respect to induced thermotolerance but are killed when exposed to a temperature close to the upper limit for growth for a prolonged time. It seemed of interest, therefore, whether *ubi4* cells might be similarly impaired in recovery from heat shock as conditional *ssa*$^-$ cells. *ubi4* cells growing exponentially on YPD medium at 27 °C were heated to 40 °C for 50 min then returned to 27 °C. The mutant cultures accumulated normal amounts of trehalose during heat shock and degraded the disaccharide at the same rate as the wild type during recovery (Fig. 5A). The mutant also behaved like wild type with respect to the induction and the decay of thermotolerance (Fig. 5B). We tested whether trehalose accumulation was durable in mutant cells incubated at 37 °C for a prolonged period of time. Again, no difference to the wild type was found (data not shown). However, we confirmed the observation by Finley et al. (1987) that the *ubi4* mutant is killed during prolonged exposure to 38.5 °C (data not shown). The data suggest that the *UBI4* gene is needed only for survival at the maximum temperatures of yeast growth, but neither for induced thermotolerance nor for recovery from the heat-stressed state.

The *HSP104* gene of *S. cerevisiae* has been demonstrated to be involved in the protection of heat-shocked cells against long-term exposure to temperatures around 50 °C (Sanchez and Lindquist, 1990). However, it has recently been shown that the need for hsp104 is greatly diminished when cells are offered a relatively severe conditioning heat shock before being exposed to 50 °C (De Virgilio et al., 1991a). The similarity between hsp104 and the clpB protein, the regulatory subunit of the ATP-dependent protease Ti of *E. coli* (Parsell

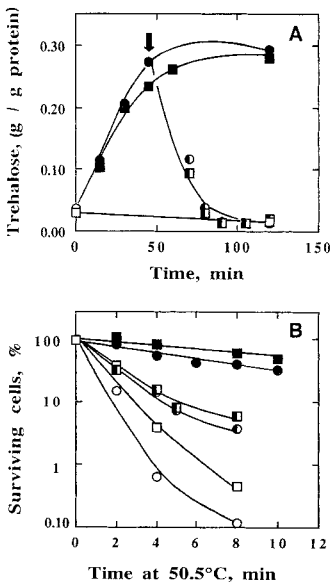

Fig. 5. Trehalose levels (A) and thermotolerance (B) of *S. cerevisiae* strains SUB 62 (wild type, circles) and SUB 60 (*Δubi4*, squares) during and after heat shock. (A) Cultures were grown to early exponential phase on YPD medium and, at zero time, subjected to the following treatments. Cultivation at 27°C (control; ○,□); heat shock at 40°C (●,■); heat shock at 40°C for 50 min followed by recovery at 27°C (◐, ◧). (B) Thermotolerance of log-phase cells after the following treatments. Incubation at 27°C for 50 min (○,□); heat shock at 40°C for 50 min (●,■); heat shock at 40°C for 50 min, followed (arrow) by recovery at 27°C for 50 min (◐, ◧). Thermotolerance was estimated from the percentage of cells surviving exposure to 50.5°C for the indicated times.

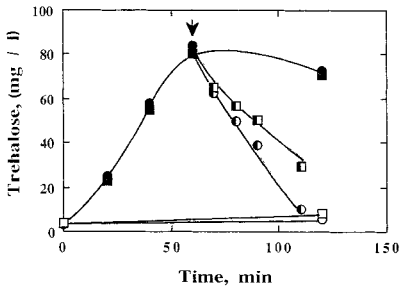

Fig. 6. Trehalose levels of *S. cerevisiae* strains W303 (wild type, circles) and *Δhsp104* (squares) during and after heat shock. Cultures were grown to early exponential phase on YPD medium and adjusted to the same cell densities by adding warmed YPD medium. At zero time, they were subjected to the following treatments. Cultivation at 27°C (control; ○,□); heat shock at 40°C (●,■); heat shock at 40°C for 50 min followed (arrow) by recovery at 27°C (◐, ◧). Trehalose levels are expressed as amount of trehalose/volume of culture in order to show that the small difference in trehalose-degradation rates during recovery is not due to a difference in growth rates.

et al., 1991), suggested to us that hsp104 might help degrade denatured proteins generated during heat shock, thereby promoting recovery. We therefore studied recovery from the heat-stressed state in an *Δhsp104* strain, using trehalose as a marker of this state. As shown in Fig. 6, a *hsp104* deletion strain and the corresponding wild type accumulated trehalose with comparable kinetics during heat shock at 40°C. However, trehalose degradation following temperature decrease to 27°C was reproducibly approximately 30% slower in the mutant

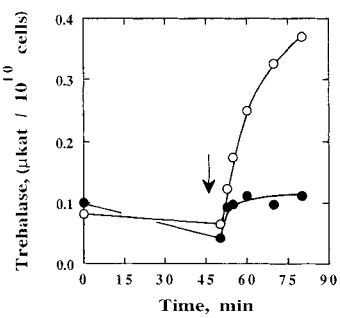

Fig. 7. Activity of neutral trehalase in strains RH 144-3A (wild type, ○) and RH 1700 (*ssa1 ssa2 ssa4 pGal1:SSA1*, ●) during heat shock at 40°C followed by recovery at 27°C (arrow). Trehalase was measured according to the method of De Virgilio et al. (1991b).

(Fig. 6). These results suggest that hsp104 is involved in recovery from the heat-stressed state, but that it plays a minor role compared to hsp70.

Depletion of hsp70 causes a defect in activation of neutral trehalase during recovery from heat shock

We next tried to answer the question why mutations in SSA genes interfere with trehalose mobilization during recovery from heat shock. The mobilization of trehalose in recovering wild-type yeast is brought about by a rapid activation of neutral trehalase, apparently due to phosphorylation of the enzyme by cAMP-dependent protein kinases (De Virgilio et al., 1991b and references cited therein). We asked whether partial depletion of hsp70 interfered with trehalase activation upon temperature decrease. Trehalase activities in cells growing at normal temperatures were comparably low in wild-type RH 144-3A and mutant RH 1700 and stayed at this low level during 50 min of heat shock at 40°C (Fig. 7). As expected, trehalase activity in wild-type cells rapidly increased approximately sixfold upon temperature decrease from 40°C to 27°C (Fig. 7). In contrast, strain RH 1700 partially depleted of SSA1 protein showed only a minor increase in trehalase activity during recovery from heat shock (Fig. 7). These data show that normal activation of neutral trehalase in recovering cells depends on the presence of functional hsp70. Whether this is due to a direct interaction of hsp70 with neutral trehalase or rather to an indirect effect of hsp70 depletion remains to be established.

Effect of glucose on recovery from the heat-stressed state

Growing yeast cells subjected to heat shock biochemically resemble stationary-phase cells. Both cell types contain high amounts of trehalose and are resistant against severe heat shock and against attack by cell-wall-degrading enzymes (Hottiger, T., unpublished results). When stationary phase cells were supplied with glucose or with fresh medium they rapidly degraded their trehalose reserves and, in parallel, lost thermotolerance (Fig. 8). These events are known to be preceded by a rapid, phosphorylation-induced activation of neutral trehalase (for reviews see Thevelein, 1984, 1988). Interestingly, the same set of events (trehalose mobilization, activation of neutral trehalase and loss of thermotolerance) is observed upon temperature decrease in cultures subjected to a heat shock (Figs 2, 3 and 7; Hottiger et al., 1987b; De Virgilio et al., 1991b). The biochemical similarities between glucose-depleted cultures re-fed with glucose and heat-shocked cul-

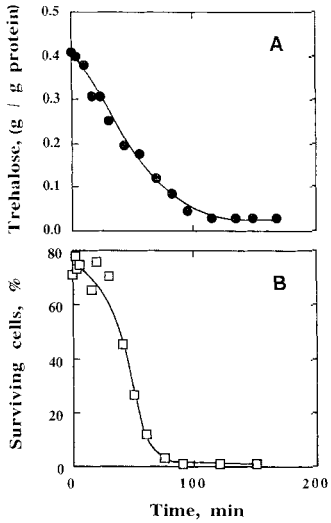

Fig. 8. Changes in trehalose content (A) and thermotolerance (B) of the prototrophic, diploid strain C 276, following transfer of stationary-phase cells to fresh medium. Stationary-phase cells were obtained as described in Materials and Methods. The cells were collected by centrifugation, resuspended in 1 vol fresh medium at zero time, and trehalose levels and thermotolerance (survival at 56 °C for 8 min) were determined at the indicated times.

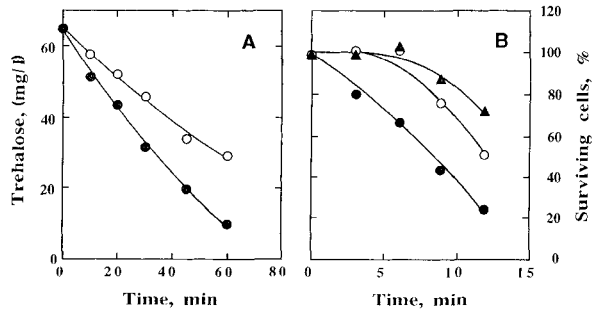

Fig. 9. Influence of glucose supply on recovery of *S. cerevisiae*, strain W303, from heat shock. Cells were grown to early exponential phase at 27 °C (YPD medium), heat shocked at 40 °C for 60 min, collected by filtration at 40 °C and transferred at zero time to YP medium at 27 °C containing 2% (●, ▲) or 0% (○) glucose. (A) Trehalose levels. (B) Thermotolerance after 0 min (▲) or 60 min (●,○) of recovery, assessed by incubating the cells at 50.5 °C for the indicated times and determining the fraction of surviving cells by plating.

tures returned to normal temperatures prompted us to investigate the role of glucose during recovery from heat shock. Cells of a wild-type strain, W303 (obtained from S. Lindquist), were grown to mid-exponential phase on YPD medium, subjected to heat shock at 40 °C and transferred to fresh medium with or without glucose at 27 °C. Glucose withdrawal significantly reduced the rate of trehalose mobilization after temperature decrease (Fig. 9A). Also, after 60 min at 27 °C the cells on the glucose-free medium were found to maintain a higher degree of thermotolerance than those on glucose (Fig. 9B). Since both trehalose accumulation and thermotolerance appear to be specifically associated with cells in the heat-stressed state, we conclude that the presence of glucose (or possibly another source of carbon and energy) is essential for normal recovery from this state.

DISCUSSION

Trehalose accumulation is known to be an element of the heat-shock response of baker's yeast (Attfield, 1987; Hottiger et al., 1987a, b, 1989; De Virgilio et al., 1991a). Here, we have studied the effect of mutations in individual hsp on trehalose metabolism in yeast cells subjected to heat shock. Our data indicate that the 70-kDa hsp of the SSA subfamily are major negative regulators of heat-induced trehalose accumulation. This is concluded from the fact that a conditional *ssa*$^-$ mutant grown under restrictive conditions overproduced trehalose when heated from 27 °C to either 37 °C or 40 °C and was impaired in trehalose degradation upon readjustment of the temperature (Figs 2 and 4). An albeit minor defect in trehalose degradation upon a shift from supraoptimal to optimal temperature was also observed in a *Δhsp104* strain (Fig. 6). Thus, both hsp70 and hsp104 appear to participate in the regulation of primary carbon metabolism. This suggests that hsp may fulfil an even more global function than has been previously envisaged.

While trehalose was originally thought to function as a storage carbohydrate of yeast (reviewed by Thevelein, 1984), recent data strongly indicate that it may rather be a stress-related metabolite (Van Laere, 1989; Wiemken, 1990). Therefore, we suspected that the defect in trehalose mobilization displayed by *ssa*$^-$ cells undergoing temperature decrease might be a symptom of a general defect in recovery from the heat-stressed state. To test this hypothesis, we studied the decay of thermotolerance in recovering *ssa*$^-$ cells and found it to be abnormally slow (Fig. 3). Since thermotolerance appears to be specifically associated with the heat-stressed state of the cells (see Lindquist, 1986), this finding strongly supports the view that *ssa*$^-$ cells are impaired in recovery from the heat-stressed state. This conclusion is also consistent with both the observation that cells partially depleted of SSA proteins showed durable, instead of transient, accumulation of trehalose when exposed to 37 °C (Fig. 4) and earlier findings that *ssa1ssa2* mutant cells are unable to repress the synthesis of some hsp even when growing at 25 °C (Craig and Jacobsen, 1984). Thus, we conclude that SSA proteins promote recovery from the heat-stressed state.

How might hsp70 (and the functionally related hsp104) mediate recovery and the shut-down of the stress response? The fact that normal recovery depends on the presence of an exogenous carbon source (Fig. 9) could mean that an adequate supply of ATP is of key importance. It has been demonstrated that members of the hsp70 family can bind to (partially) unfolded/denatured target proteins and catalyse their (re)folding in an ATP-dependent reaction (Gething and Sambrook, 1992). Thus, the need for both glucose and hsp70 may indicate that the hsp70-dependent and ATP-dependent repair of thermally damaged proteins is a central event in the process of recovery. This concept seems plausible in view of the fact that the presence of aberrant proteins has been proposed to be the actual trigger of the heat-shock response (Edington et al., 1989). This model also offers an explanation for the finding that cells partially depleted of hsp70 displayed abnormally low activities of neutral trehalase after a temperature decrease from 40 °C to 27 °C. It has been shown by protein blotting that the synthesis of trehalase continues during heat shock (De Virgilio et al., 1991b). Recent evidence suggests that 70-kDa hsp interact with newly synthesized proteins and that this normally transient interaction tends to be durable in heat shock (Beckmann et al., 1990). Thus, hsp70 might form a complex with newly synthesized neutral trehalase in cells sub-

jected to heat shock, thereby preventing trehalase from being misfolded. In this case, neutral trehalase would be one of the substrates of hsp70, of which only a few have been identified.

The above concept, albeit convincing at first sight, has two major weaknesses. It does not explain why cells undergoing temperature decrease in the absence of glucose did not use their trehalose reserves in order to meet a potential demand for ATP. Indeed, trehalose degradation was abnormally slow in glucose-deprived cells (Fig. 9). Also if the accumulation of thermally damaged proteins is the major burden imposed on cells by heat shock, it is surprising that we found no defect in recovery in a strain mutated in the polyubiquitin gene, *UBI4*. Given these inconsistencies, another hypothesis appears more attractive to us. We have shown, here (Fig. 7) and in an earlier publication (De Virgilio et al., 1991b), that neutral trehalase is rapidly activated during a temperature decrease from 40 °C to 27 °C. Since trehalase activation is mediated by cAMP-dependent protein kinases (Van Solingen and van der Plaat, 1975; Thevelein, 1984, 1988), this suggests that these protein kinases play an important role in recovery from the heat-stressed state. The fact that the activity of neutral trehalase remained abnormally low in recovering *ssa*$^-$ cells indicates that lack of hsp70 interferes with cAMP-dependent protein-kinase activity during recovery. Thus, hsp70 may be essential for maintaining the activity of components of the RAS/adenylate-cyclase pathway in cells subjected to heat shock. Interestingly, the effects of SSA-protein depletion (failure to activate neutral trehalase, slow mobilization of trehalose and delayed decay of thermotolerance) are mimicked by transfer to medium without glucose prior to recovery (Fig. 9; De Virgilio, C., unpublished results). Glucose is known to act as an activator of the RAS/adenylate-cyclase system in yeast (Thevelein, 1988; Mbonyi et al., 1988). Thus, the fact that lack of hsp70 or glucose results in a similar defect in recovery from the heat-stressed state may be due to the common ability of glucose and 70-kDa hsp to stimulate the RAS pathway. A similar role has previously been proposed for hsp90 (Piper, 1990). Therefore, the possibility emerges that hsp70, hsp90 and hsp104 all fulfil related functions.

In previous work, we have demonstrated that in yeast, trehalose levels are closely correlated with thermotolerance (Hottiger et al., 1987b, 1989; De Virgilio et al., 1990, 1991a). Here, we describe another striking example of this correlation. A *ssa1ssa2ssa4* triple mutant accumulated normal amounts of trehalose and, despite its defect in the synthesis of three major hsp, also developed normal (in fact, even unusually high; Hottiger, T., unpublished results) thermotolerance. Furthermore, the thermotolerance of *ssa*$^-$ cells decayed abnormally slowly, and this coincided with an unusually slow mobilization of trehalose (Figs 2 and 3). In wild-type yeast cells transferred to medium without a carbon source prior to temperature decrease, thermotolerance was maintained longer than in control cells supplied with glucose, and again this was paralleled by an abnormally slow mobilization of trehalose (Fig. 9). A correlation of trehalose levels and thermotolerance has previously been demonstrated even in an *hsp104* strain (De Virgilio et al., 1991a). We therefore suggest that trehalose accumulation has a thermoprotective function in yeast. Indeed, in *E. coli*, convincing genetic evidence has recently been provided that trehalose accumulation is required for thermotolerance of stationary-phase cells (Hengge-Aronis et al., 1991).

While our data suggest a role for trehalose in thermoprotection we could find no evidence for an involvement of hsp70 in induced thermotolerance. Indeed, the fact that thermotolerance decayed abnormally slowly in an *ssa1 ssa2 ssa4* triple mutant can be interpreted as evidence that hsp70 actually *counteracts* induced thermotolerance. This idea contradicts present concepts on the function of hsp (see e.g. Lindquist, 1986) but is compatible with recent genetic evidence that hsp synthesis is dispensable for induced thermotolerance of yeast (Smith and Yaffe, 1991). We suspect that the difficulties in assigning the role of hsp in induced thermotolerance may ultimately be connected with too narrow a view of the functions of the heat-shock response. It is presently thought that its principal function is to enhance thermotolerance, ensuring cell survival under extreme conditions of heat shock. However, based on the data presented here we tentatively suggest that induced thermotolerance also reflects a state of severe stress incompatible with growth and that one major function of the heat-shock response may be to counteract this state of stress and to allow cells to recover. We propose that hsp have no role in induced thermotolerance but are essential for the recovery of cells from the heat-stressed state and for the resumption of growth.

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