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The role of trehalose synthesis for the acquisition of thermotolerance in yeast
II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro*

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In baker's yeast (*Saccharomyces cerevisiae*), accumulation of the non-reducing disaccharide, trehalose, is triggered by stimuli that activate the heat-shock response. Previously, trehalose levels have been shown to be closely correlated with thermotolerance, suggesting a protective function of this substance. Genetic evidence in support of this view is presented in an accompanying paper [De Virgilio, C., Hottiger, T., Dominguez, J., Boller, T. & Wiemken, A. (1993) *Eur. J. Biochem. 219*, 179–186]. In this study, we have examined the effect of trehalose on the thermal stability of proteins, a parameter thought to be a major determinant of thermotolerance. Physiological concentrations of trehalose (up to 0.5 M) were found to efficiently protect enzymes of yeast (glucose-6*P*-dehydrogenase, phosphoglucose-isomerase) as well as enzymes of non-yeast origin (bovine glutamic dehydrogenase, *Eco*RI) against heat inactivation *in vitro*. Trehalose also reduced the heat-induced formation of protein aggregates. The disaccharide proved to be a compatible solute, as even at very high concentrations (up to 1 M) it did not significantly interfere with the activity of test enzymes. Trehalose was at least as good or better a protein stabilizer than any of a number of other compatible solutes (including sugars, polyalcohols and amino acids), while the structurally related trehalose-6*P* was devoid of any protective effect. Thermoprotection of enzymes by trehalose was evident even in solutions containing high concentrations of yeast protein or substrate. The data indicate that trehalose accumulation may increase the thermotolerance of yeast by enhancing protein stability in intact cells.

Induced (acquired) thermotolerance of cells or organisms has been defined as a transient, non-inheritable increase in resistance to heat brought about by exposure to elevated temperatures or by treatment with a variety of non-heat stress factors (Lindquist, 1986). Thermotolerance induction represents an apparently universal response of biological systems to certain types of stress (Lindquist, 1986; Lindquist and Craig, 1988). The biochemical adaptations underlying the phenomenon are incompletely understood. However, it is well documented that the acquisition of thermotolerance is closely linked to the synthesis of a small set of proteins, the stress or heat shock proteins (hsp). Hsp have been strongly conserved throughout evolution, indicating biologically important functions. Indeed, they have recently been shown to participate in the folding and assembly of newly synthesized proteins as well as in their transport across biological membranes (Gething and Sambrook, 1992). The fact that, as a rule, thermotolerance is induced coordinately with hsp and that hsp levels correlate well with heat resistance has led to the hypothesis that hsp synthesis may be the ultimate cause for thermotolerance induction (Nover, 1984; Lindquist, 1986). Whilst there are good arguments to support this concept (Sanchez et al., 1992), there are also some notable contradictions. Cases have been reported where hsp were synthesized without a concomitant increase in thermotolerance (VanBogelen et al., 1987), whereas in other situations thermotolerance was induced in the absence of detectable hsp synthesis (Hall, 1983; Delaney, 1990; Barnes et al., 1990; Coote et al., 1991; Smith and Yaffe, 1991; De Virgilio et al., 1991). The most plausible explanation for these contradictory findings is that thermotolerance is due to more than one protective mechanism, alternative factors offering thermoprotection in the absence of hsp synthesis. Indeed, in the case of the yeast *Saccharomyces cerevisiae*, all of the following processes have been suggested to contribute to acquired thermotolerance: (a) induction of hsp104 (Sanchez and Lindquist, 1990; Sanchez et al., 1992); (b) maintenance of pH and ion gradients across the plasma membrane via the activity of the plasma membrane ATPase (Coote et al., 1991; Panaretou and Piper, 1990, 1992); (c) a decrease in the activity or changes in the distribution of water in the cytosol (Komatsu et al., 1992); (d) detoxification of active oxygen species (Wieser et al., 1991); and (e) the heat-induced accumulation of the non-reducing disaccharide trehalose (Hottiger et al., 1987b, 1989, 1992; De Virgilio et al., 1991). Clearly, to evaluate potential contributions of these mechanisms to induced thermotolerance, it is important to study their effects on and interactions with the cellular targets of heat-induced damage.

Although the primary target(s) of thermal killing are not yet known with certainty, there is general agreement that pro-

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Abbreviations. hsp, heat-shock protein(s); Glc6*P*DH, glucose-6-phosphate dehydrogenase; T_{150}, temperature at which an enzyme loses 50% of its activity under the specified conditions.

teins are among the cell components most prone to heat-induced damage, and it has been proposed that protein denaturation is the actual cause of thermal killing (Rosenberg et al., 1971). In hamster cells, changes in protein structure are induced at temperatures as low as 39°C (Lepock et al., 1988), and in bacteria thermal death coincides with heat-inactivation of ribosomes (Stephens and Jones, 1993). Also, it has been shown that the accumulation of abnormal proteins within cells induces a heat shock response even at optimal growth temperatures (Goff and Goldberg, 1985; Hiromi and Hotta, 1985; Ananthan et al., 1986). Collectively, the above findings are indirect but compelling evidence that protein stability is among the most important determinants of thermotolerance. Therefore, biochemical mechanisms contributing to induced thermotolerance are expected to increase protein stability.

In this study, we have examined the effect of a potential thermoprotectant of yeast, namely the disaccharide trehalose, on heat-induced protein denaturation. Trehalose accumulation in *S. cerevisiae* is strongly induced by heat shock and has been suggested to be an element of the heat-shock response (Attfield, 1987; Hottiger et al., 1989, 1992). Indeed, at least two subunits of the trehalose-6*P*-synthase complex are actively synthesized during heat shock and therefore, by definition, are hsp (Bell et al., 1992; De Virgilio et al., 1993a). Also, trehalose levels are modulated by the availability of hsp70 in much the same way as are the levels of hsp (Hottiger et al., 1992; Craig and Gross, 1991). A correlation between intracellular trehalose content and thermotolerance has been found in *S. cerevisiae* (Hottiger et al., 1987b, 1989, 1992; De Virgilio et al., 1991), *Schizosaccharomyces pombe* (De Virgilio et al., 1990), *Dictyostelium discoideum* (Killick and Wright, 1972) and *Neurospora crassa* (Neves et al., 1991). Furthermore, genetic evidence has recently been presented that trehalose contributes to the stationary-phase thermotolerance of *Escherichia coli* (Hengge-Aronis et al., 1991), and in an accompanying study, De Virgilio et al. (1993b) present similar data for *S. cerevisiae*. Taken together, these findings suggest that trehalose accumulation in microorganisms may serve (thermo)protective functions. In this study, we show that physiological concentrations of trehalose efficiently stabilize proteins against thermal denaturation *in vitro*, and that the disaccharide is superior to other micromolecules in this respect. We propose that trehalose contributes to the thermotolerance of microorganisms by acting as a stabilizer of proteins.

MATERIALS AND METHODS

Organism and cultivation

The prototrophic diploid strain C276 of *S. cerevisiae* was grown in minimal medium with 2% glucose as the carbon source (Hottiger et al., 1987a). The cultivation temperature was 27°C. Exponentially growing cultures (approximately 10^7 cells/ml) were used in all experiments.

Induction and assessment of thermotolerance

For thermotolerance induction, cultures were incubated at 40°C for 60 min in a waterbath. Thermotolerance was assessed by heating samples (1 ml) to the desired temperature for 8 min. The fraction of surviving cells was determined by plating cells on YPD agar (20 g glucose, 10 g yeast extract, 10 g peptone, 20 g agar in 1 l distilled water) as described in Hottiger et al. (1987b).

Determination of the trehalose concentration in thermotolerant yeast cells

A mid-log-phase culture of *S. cerevisiae* C276 was heat shocked at 40°C for 60 min, and a sample was withdrawn for trehalose analysis (performed in triplicate) as described by Hottiger et al. (1987a). The remainder of the culture was rapidly chilled by the addition of ice, the cells were collected by centrifugation (10 min at 3000×*g*), washed three times with ice water and resuspended in 0.25 vol. ice water supplemented with [U-^{14}C]mannitol (15000Bq/ml). Four samples (20 ml each) were filtered over previously weighed nitrocellulose filters (0.45 µm pore size) and were immediately (without washing) placed in a previously tared petridish and weighed. Thereafter, samples were dried at 95°C, weighed again and extracted with 0.8 M NaOH/10% SDS. The radioactivity of the extracts was determined by liquid-scintillation counting and used to determine the contribution of extracellular water to the fresh mass. Mannitol uptake by cells was found to be negligible. The intracellular molarity (*M*) of trehalose was calculated as follows: *M* = µmol trehalose/µl cell water in sample = µmol trehalose/(cell wet mass − cell dry mass − mass of extracellular water).

Preparation of yeast protein

Cells of an exponentially growing culture were collected by centrifugation at 3000 × *g* for 10 min and suspended in a small volume of 25 mM Tricine (K^+), pH 7.0. Homogenization was performed by shaking cells with glass beads as described in Hottiger et al. (1987a). The extract was desalted over Sephadex G-25 (equilibrated with 25 mM Tricine (K^+), pH 7.0) before use. The concentration of protein in the homogenate was determined by the method of Bradford (1976).

Influence of trehalose or other compounds on the thermal stability of enzymes *in vitro*

Yeast glucose-6-*P* dehydrogenase (Glc6*P*DH), phosphoglucose isomerase, as well as NAD-dependent glutamate dehydrogenase from bovine liver, were purchased from Merck. The commercial preparations [supplied as suspensions in $(NH_4)_2SO_4$] were desalted (Sephadex G-25) and diluted approximately 100-fold with 50 mM Tricine (K^+), pH 7.0. Aliquots (100 µl) of enzyme were mixed with an equal volume of test substance (e.g. trehalose) at the desired concentration and were heated to temperatures between 30°C and 70°C for 8 min. After chilling samples on ice, the remaining enzyme activity was measured and compared to that of an unheated control (= 100% activity). Enzyme assays were performed according to Bergmeyer (1974). For further details, see Hottiger (1988).

The thermal stability of the restriction endonuclease, *Eco*RI (Boehringer), was assessed by heating a solution of the enzyme in 1× restriction buffer H (Boehringer) to 60°C for different times. Thereafter, the samples were chilled on ice, mixed with 1 µl (approximately 0.5 µg) of plasmid YEp13 (three *Eco*RI sites) and incubated at 37°C for 60 min. The DNA fragments were separated by agarose electrophoresis according to standard procedures (Sambrook et al., 1989).

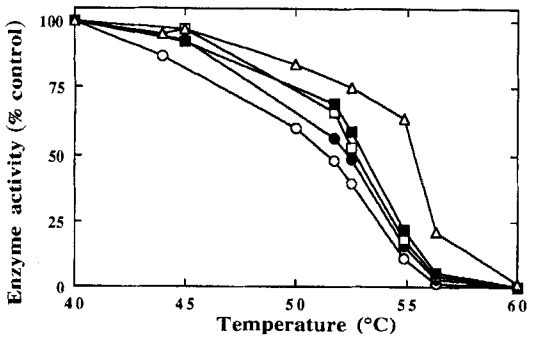

Fig. 1. Thermal stability of yeast Glc6*P*DH as influenced by trehalose addition. Purified Glc6*P*DH in 25 mM Tricine (K^+), pH 7.0, was incubated for 8 min at the indicated temperatures, either in the absence of trehalose (○), or in the presence of 25 mM (●), 50 mM (□), 100 mM (■) or 500 mM (△) trehalose. Enzyme activities after the above treatments are expressed relative to the activity of control samples of the same composition but maintained on ice for 8 min.

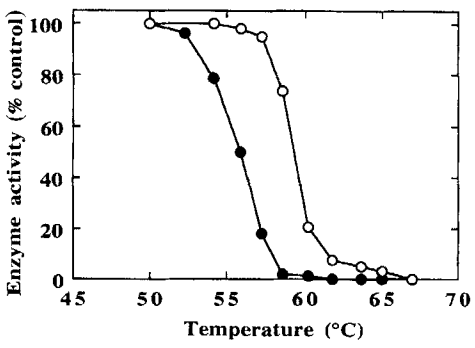

Fig. 2. Thermal stability of Glc6*P*DH as influenced by the addition of yeast protein and trehalose. Samples were heated to the indicated temperatures for 8 min, either in 25 mM Tricine (K^+), pH 7.0, supplemented with yeast protein at a concentration of 10 g/l (●), or in the same medium containing 0.5 M trehalose (○). Further details are described in the legend of Fig. 1.

RESULTS

To determine the concentration of trehalose in thermotolerant yeast cells, an exponentially growing culture of the prototrophic, diploid strain C276 of *S. cerevisiae* was heat shocked at 40°C for 60 min, subjected to trehalose analysis as described in the Materials and Methods section. The trehalose concentration in the cell water was found to be approximately 0.18 M. Trehalose is localised in the cytosol of yeast cells and is undetectable in vacuoles (Keller et al., 1982; Hottiger, T., unpublished results). Assuming that the vacuoles occupy 25% of the cell volume and contain negligible amounts of trehalose, one can calculate that the average concentration of trehalose in the cytosol of heat-shocked yeast cells is approximately 0.25 M. Since (a) up to 1 g trehalose/g protein can be found in yeast (Hottiger et al., 1987a) whereas only 0.36 g was accumulated in the experiment described above and (b) the disaccharide may not be evenly distributed in the cytosol we conclude that the maximal trehalose concentrations encountered *in vivo* are ≥ 0.5 M.

Using yeast Glc6*P*DH as a model protein, we considered whether physiological concentrations of trehalose (up to 0.5 M) would protect proteins/enzymes against heat denaturation *in vitro*. Samples containing purified yeast Glc6*P*DH in Tricine and trehalose at the desired concentration were incubated for 8 min at temperatures of 40°C–60°C. After chilling on ice, the Glc6*P*DH activity was measured and compared to that of control samples without trehalose. As shown in Fig. 1, trehalose concentrations well below those encountered in thermotolerant yeast cells clearly enhanced the thermal stability of Glc6*P*DH *in vitro*. At 0.5 M trehalose, i.e. approximately the maximal concentration found *in vivo*, the T_{150} value (temperature at which the enzyme loses 50% of its activity) increased by ≈ 4°C relative to the control without trehalose (Fig. 1). Thermotolerant yeast cells (containing high concentrations of trehalose) will survive exposure to temperatures ≈ 5°C above those required for killing sensitive cells (devoid of trehalose; Hottiger, 1988). Thus, the thermoprotection offered by trehalose is of an order of magnitude that may be biologically significant.

Whilst no protein besides the purified Glc6*P*DH was added to the samples in the experiment shown in Fig. 1, living cells are complex mixtures of highly concentrated proteins. In an *in vivo* situation, protein/protein interactions are

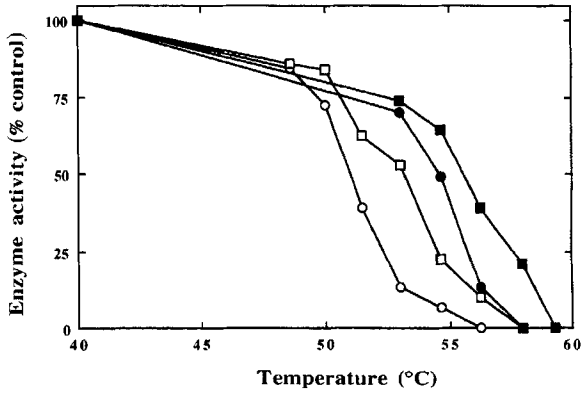

Fig. 3. Thermal stabiliy of yeast Glc6*P*DH as influenced by the addition of trehalose and/or substrate (Glc6*P*). Enzyme samples were heated to the indicated temperatures for 8 min in media of the following composition: 25 mM Tricine (K^+), pH 7.0, (○, control); 25 mM Tricine (K^+), pH 7.0, plus 2.5 mM Glc6*P* (□); 25 mM Tricine (K^+), pH 7.0, plus 0.5 M trehalose (●); 25 mM Tricine (K^+), pH 7.0, plus 2.5 M Glc6*P* and 0.5 M trehalose (■). Further details are described in the legend of Fig. 1.

expected to markedly influence the thermal stability of enzymes, and it seemed therefore important to determine whether addition of protein to the *in vitro* tests shown in Fig. 1 would annihilate the positive effect of trehalose on the thermal stability of Glc6*P*DH. Yeast protein was prepared as described in the Materials and Methods section and was added at a concentration of 10 g/l to samples otherwise identical to those used in the experiment in Fig. 1. As expected, the thermal stability of Glc6*P*DH was significantly increased by the addition of yeast protein (Figs 1, 2). However, trehalose offered still further protection, and the effect of trehalose addition (as judged by comparing T_{150} values) was comparable in the presence or absence of protein. Similarly, if substrate (Glc6*P* at a concentration of 2.5 mM) was added during heating, the stability of Glc6*P*DH was found to increase, but still better protection was obtained by adding trehalose together with Glc6*P* (Fig. 3).

We next considered whether trehalose would also stabilize enzymes other than Glc6*P*DH. Yeast phophoglucose isomerase (Fig. 4A), bovine glutamate dehydrogenase (Fig. 4B), and the restriction endonuclease *Eco*RI from *E.*

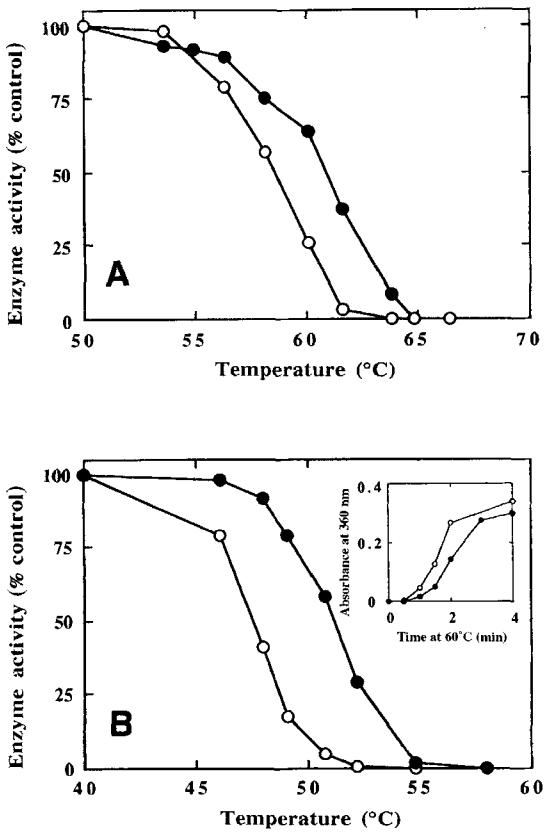

Fig. 4. Thermal stability of yeast phosphoglucose isomerase (A) and bovine glutamate dehydrogenase (B) in the absence (○) or presence (●) of 0.5 M trehalose. Details as in the legend of Fig. 1. Inset in B, aggregation of glutamate dehydrogenase (as evidenced by the time-dependent increase in absorbance at 360 nm) in the absence (○) or presence (●) of 0.5 M trehalose.

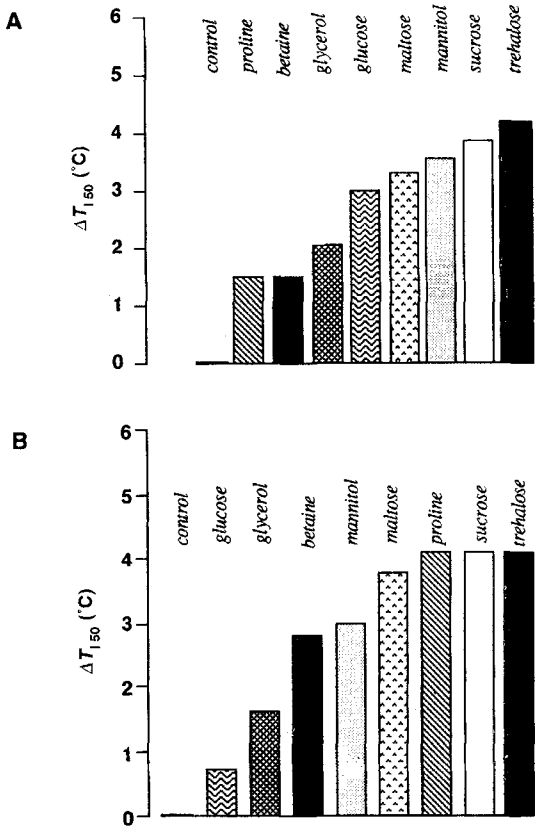

Fig. 5. Thermal stability of yeast Glc6*P*DH and bovine glutamate dehydrogenase as influenced by the presence of various compatible solutes. Enzymes in 25 mM Tricine (K^+), pH 7.0, plus 0.5 M effector were heated to temperatures between 30°C and 70°C. The temperature at which 50% of the enzyme was inactivated (T_{150}) was determined from plots as shown in Fig. 1. The graph shows ΔT_{150} values, i.e. T_{150} measured in buffer plus effector − T_{150} measured with buffer only.

coli (Fig. 6) were chosen as further examples. Trehalose at a concentration of 0.5 M clearly increased the thermal stability *in vitro* of all these enzymes. In the case of glutamate dehydrogenase, we checked whether trehalose would antagonize the heat-induced formation of protein aggregates. Indeed, the turbidity of glutamate dehydrogenase samples (0.25 mg protein/ml) heated to 60°C increased more slowly in the presence of trehalose than in its absence (Fig. 4B, inset), demonstrating that aggregate formation was reduced. In all our experiments, trehalose addition only minimally reduced enzymic activities *in vitro* (data not shown). This shows that the disaccharide fulfils the definition of a compatible solute (MacKay et al., 1984), i.e. even when accumulating to very high concentrations it does not inhibit metabolic reactions and therefore is relatively non-toxic. Interestingly, this is not the case for the structurally related trehalose-6*P* (see below).

We proceeded to compare the performance of trehalose as a thermoprotectant to that of other compatible solutes. Yeast Glc6*P*DH or bovine glutamate dehydrogenase were mixed with 25 mM Tricine (K^+), pH 7.0, and one of the following substances (0.5 M): the disaccharides, sucrose or maltose; the monosaccharide, glucose; the polyalcohols mannitol or glycerol; the amino acid, proline, or the amino acid-derivative, glycine betaine. As shown in Fig. 5, all compounds tested increased the thermal stability of both Glc6*P*DH and glutamate dehydrogenase. However, the extent to which the enzymes were stabilized varied greatly depending on the molecule tested. For example, trehalose, sucrose or prolin (0.5 M each) increased the T_{150} value of glutamate dehydrogenase by ≈ 4°C, while the increase in T_{150} brought about by glycerol was only ≈ 1.5°C. Two compounds (glucose and proline) efficiently stabilized one enzyme but not the other. We assume that this is due to their similarity with the substrates (Glc6*P* or glutamate) of Glc6*P*DH and glutamate dehydrogenase, respectively. As a rule, it was found that disaccharides were the most efficient thermoprotectants (Fig. 5). Significantly, trehalose was as good a stabilizer as any other compound tested. This suggests that trehalose accumulation is a highly appropriate means to protect enzymes against thermal denaturation *in vivo*.

Cells mutated in the trehalose-6*P* phosphatase (*tps2*) gene accumulate Tre6*P* instead of trehalose when heat shocked and, at the same time, become less thermotolerant than wild-type cells (De Virgilio et al., 1993b). It seemed of particular interest to investigate whether Tre6*P* could substitute for trehalose in stabilizing proteins against thermal inactivation *in vitro*. The restriction endonuclease *Eco*RI was chosen as a model protein for this experiment. *Eco*RI was incubated at 60°C in the presence of buffer only or with buffer supplemented with either 0.5 M trehalose or 0.5 M Tre6*P*. After incubating *Eco*RI for 2 min at 60°C in buffer alone, plasmid YEp13 was not now completely digested (Fig. 6A). When the buffer was supplemented with 0.5 M

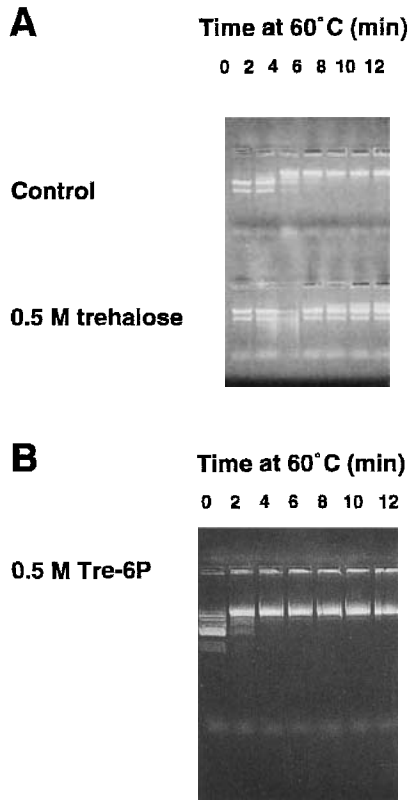

Fig. 6. Thermal stability of the restriction endonuclease *Eco*RI, in the presence of 1× restriction buffer (A, upper lanes), 1× restriction buffer containing 0.5 M trehalose (A, lower lanes), or 1× restriction buffer with 0.5 M trehalose-6 phosphate (B). Samples were heated to 60°C for the indicated times, supplemented with a fixed amount of plasimd YEp13 (three *Eco*RI sites) and incubated at 37°C for 30 min. The DNA fragments formed were separated by gel electrophoresis on a 0.8% agarose gel; two of the fragments run as a double band under the chosen conditions. Note that the pH of the trehalose and Tre*6P* samples was checked before the experiment and found to correspond to that of the restriction buffer.

trehalose, however, *Eco*RI was entirely stable for at least 6 min at 60°C and still displayed partial activity after 12 min (Fig. 6A). No comparable stabilization effect was observed when trehalose was replaced by Tre*6P* (Fig. 6B). In fact, Tre*6P* strongly reduced the activity of *Eco*RI even in the absence of a heat treatment (Fig. 6B), probably due to ionic effects. This result clearly demonstrates that in contrast to trehalose, Tre*6P* is unsuitable as a protein stabilizer.

DISCUSSION

In earlier studies, we have suggested that the cytosolic disaccharide, trehalose, is a stress-related metabolite of yeast that may contribute to induced thermotolerance (Hottiger et al., 1987a, b, 1989, 1992; De Virgilio et al., 1990, 1991). This hypothesis was initially based on the finding that trehalose levels and thermotolerance are correlated under a wide variety of experimental conditions. Meanwhile, it has been shown by genetic methods that trehalose contributes to the thermotolerance of both *E. coli* (Hengge-Aronis et al., 1991) and *S. cerevisiae* (De Virgilio et al., 1993b). This strongly indicates that trehalose indeed fulfils a thermoprotective function, an interpretation that is consistent with recent findings that at least two subunits of the trehalose-6*P*-synthase complex are hsp (Bell et al., 1992; De Virgilio et al., 1993a).

In this study, we have addressed the question of how trehalose might offer thermoprotection. For this purpose, the influence of trehalose on what is supposed to be a major determinant of thermotolerance, namely protein stability at high temperatures, was studied. Physiological concentrations (up to 0.5 M) of trehalose were effective in protecting a number of widely different proteins against thermal inactivation *in vitro* (Figs 1–5). On a molar basis, trehalose was at least as efficient a thermoprotectant as any of a number of other compounds considered to be compatible solutes (Fig. 5). In the case of yeast Glc6*P*DH, it was found that trehalose offered protection even when the enzyme was heated in the presence of yeast protein and/or substrate (Glc6*P*, Fig. 3). Trehalose concentrations in the molar range did not significantly inhibit the *in vitro* activities of our model enzymes (data not shown). Therefore, it is concluded that trehalose accumulation in heat-shocked yeast cells is an appropriate means to keep thermolabile proteins in an active state. Tre6*P*, despite its structural similarity to trehalose, proved unsuitable as a protein stabilizer. Not only was this substance devoid of any effect on the thermostabiliy of the restriction endonuclease *Eco*RI, but it even strongly inhibited the activity of this enzyme (Fig. 6).

Could trehalose act complementarily to other thermoprotective systems of yeast, for example hsp? It has recently been reported that hsp bind to enzymes undergoing thermal inactivation *in vitro*, and that they thereby prevent these proteins from aggregating (Höll-Neugebauer et al., 1991; Horwitz, 1992; Taguchi and Yoshida, 1993). Interestingly, albeit effective in counteracting protein aggregation, hsp do not appear to stabilize their substrate proteins against thermal inactivation (Höll-Neugebauer et al., 1991; Horwitz, 1992; Taguchi and Yoshida, 1993). Thus, inactivation and aggregate formation are clearly separate events. Whilst trehalose accumulation may help prevent the first step, i.e. protein inactivation (this study), hsp act at the second step, i.e. they minimize aggregate formation by binding to polypeptides once they have been denatured (Gragerov et al., 1992). In addition, hsp are thought to mediate the ATP-dependent refolding/repair of bound substrate proteins. Indeed, in *E. coli* a heat-labile λ repressor protein rapidly renatures after heat inactivation in a process that depends on the presence of hsp (Gaitanaris et al., 1990). Thus, the functions of trehalose and hsp appear to be complementary in that trehalose acts early and hsp act late in the chain of protein denaturation. It should be noted that trehalose counteracts the heat-induced aggregation of glutamate dehydrogenase (Fig. 4B, inset). In our view, this most likely reflects an effect of trehalose at the denaturation step and not a direct effect of trehalose on aggregate formation.

Apart from its presence in heat-shocked yeast cells, trehalose also accumulates in stationary-phase cultures and spores of yeast and many other microorganisms (Kane and Roth, 1974; Lillie and Pringle, 1980; Wiemken, 1990). This has led to the assumption that trehalose may function primarily as a reserve carbohydrate. Given the increasing amount of evidence that the disaccharide may also act as a stress protectant (Wiemken, 1990; Eleutherio et al., 1993; De Virgilio et al., 1993b), it is suggested that the accumulation of a high content of trehalose by spores and other non-proliferating microbial cells may serve a dual function. Trehalose can provide a source of energy and carbon under conditions of prolonged starvation and, in addition, increases the resistance of

cells during exposure to harsh environmental conditions. Thus, a relatively simple molecule may fulfil remarkably diverse functions.

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