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Multiple Effects of Trehalose on Protein Folding In Vitro and In Vivo

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Summary

The disaccharide trehalose is produced in large quantities by diverse organisms during a variety of stresses. Trehalose prevents proteins from denaturing at high temperatures in vitro, but its function in stress tolerance in vivo is controversial. We report that trehalose stabilizes proteins in yeast cells during heat shock. Surprisingly, trehalose also suppresses the aggregation of denatured proteins, maintaining them in a partially-folded state from which they can be reactivated by molecular chaperones. The continued presence of trehalose, however, interferes with refolding, suggesting why it is rapidly hydrolyzed following heat shock. These findings reconcile conflicting reports on the role of trehalose in stress tolerance, provide a novel tool for accessing protein folding intermediates, and define new parameters for modulating stress tolerance and protein aggregation.

Introduction

Protein aggregation occurs in many biological contexts, with dramatic and often devastating consequences. Its effects are manifest in phenomena ranging from the formation of inclusion bodies during heterologous expression of recombinant proteins, to the assembly of amyloid or prion protein fibrils characteristic of fatal neurodegenerative disorders (Wetzel, 1996). A common pathway, the noncovalent association of partially folded proteins, is now thought to underlie these diverse processes, offering the possibility that mechanisms for controlling them may be similarly broadly applicable.

Both aggregation and biological mechanisms to counteract it can be studied in vivo using a readily manipulable experimental system, that of heat shock. Exposure of cells to intense heat (or other protein denaturants) causes aggregation and ultimately death (Ang, et al., 1991; Parsell and Lindquist, 1994). But aggregation can be greatly reduced if cells first encounter moderate stress before being subjected to extreme conditions. Pretreatment at a mildly elevated temperature increases by 100- to 10,000-fold the ability of cells to survive a subsequent severe heat shock (Parsell and Lindquist, 1994). Such acquired tolerance is observed in all organisms, for a wide variety of stresses. It results from the induction of protective mechanisms collectively referred to as the heat-shock response (Ang et al., 1991; Piper, 1993).

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This response includes the production of specialized proteins and small organic compounds (Piper, 1993). The proteins, known as heat-shock proteins (HSPs) or molecular chaperones, prevent and repair protein aggregation damage, or promote the degradation of irretrievably damaged substrates (Ang et al., 1991; Parsell et al., 1993). Less well understood is the function of the large quantity of organic solutes synthesized in response to stress. Many such compounds are considered "compatible solutes," because their presence at high concentrations does not perturb enzyme structure or function (Somero and Yancey, 1997). A number of these organic molecules have been shown in vitro to stabilize native proteins at high temperatures (Hottiger et al., 1994). Their function in vivo, however, has been the subject of much dispute.

Saccharomyces cerevisiae provides an excellent model system in which to investigate how both facets of the heat-shock response contribute to survival. In addition to the genetic manipulations possible in this organism, its major protein and non-protein contributors to tolerance have been identified: the heat-shock protein HSP104, and the disaccharide trehalose (Sanchez and Lindquist, 1990; De Virgilio et al., 1994; Elliot et al., 1996).

HSP104 is present at very low levels during exponential growth but is strongly induced in stationary-phase cells, sporulating cells, and in cells exposed to mild stresses (Sanchez et al., 1992). Under all of these conditions, cells have high levels of stress tolerance, and this tolerance is severely compromised by deletion of *HSP104* (Sanchez and Lindquist, 1990; Sanchez et al., 1992). Unlike many other HSPs, HSP104 does not suppress the aggregation of thermally denatured proteins. Instead, it promotes the resolubilization and reactivation of proteins that have already begun to aggregate (J. Glover and S. L., unpublished data; Parsell et al., 1994; Vogel et al., 1995).

Like HSP104, trehalose is barely detectable in logarithmic-phase cells, but is very abundant in stationary-phase cells and spores (Kane and Roth, 1974; Lillie and Pringle, 1980). Trehalose was originally thought to serve as a reserve carbohydrate (Lillie and Pringle, 1980), but more recent work suggests an alternative role, as a stress protectant. Cells accumulate the disaccharide in response to mild heat pretreatment and other agents that induce stress tolerance (Attfield, 1987). In vitro, trehalose stabilizes proteins in their native state and preserves the integrity of membranes during stresses (Crowe et al., 1984; Colaco et al., 1994). Furthermore, mutation of the *tps1* gene, which encodes a subunit of trehalose synthase, renders cells unable to produce trehalose and sensitive to heat (De Virgilio et al., 1994).

Efforts to understand the role of trehalose, however, have been confounded by other, perplexing observations. The disaccharide is degraded very rapidly after stress, despite its stabilizing effects on proteins and its compatibility with enzyme activity (Attfield, 1987; Hottiger et al., 1987a; Hottiger et al., 1987b). More bewildering is the phenotype of mutants lacking NTH1, the protein responsible for trehalose degradation. Trehalose is

present at high levels in these cells, yet their ability to survive and recover from extreme heat is impaired (Nwaka et al., 1995a, 1995b). This and other work has led to doubt as to whether trehalose does protect cells from heat shock (reviewed by Nwaka and Holzer, 1997).

Consensus is similarly absent as to why *tps1* mutants show diminished thermotolerance. One suggestion is that it is not trehalose itself, but the TPS1 protein that is critical for stress resistance, by stimulating HSP expression (Hazell et al., 1995). A second model holds that trehalose protects cells at mildly elevated temperatures so that HSPs may be induced, which in turn provide protection from severe heat (Winkler et al., 1991; Nwaka et al., 1994). A third view, based on the ability of trehalose to stabilize proteins in vitro, posits a dichotomy of function in thermotolerance, with trehalose stabilizing proteins in their native conformation, and HSPs acting on those that have denatured (Hottiger et al., 1994).

We report that under moderate heat-shock conditions, mutation of *TPS1* does not affect HSP induction, yet these cells show greatly diminished tolerance to subsequent extreme heat. Our data indicates that trehalose acts directly during heat shock to stabilize proteins in the native state. Unexpectedly, we found that trehalose also reduces aggregation of proteins that have already denatured, a function previously thought to be carried out exclusively by HSPs. To our greater surprise, high concentrations of trehalose inhibit the reactivation of denatured proteins by molecular chaperones. This observation may explain why degradation of trehalose is necessary for full recovery from heat shock. Our work resolves many conflicts in the literature and provides a coherent explanation of the role of trehalose in stress tolerance. Moreover, these findings reveal novel properties of compatible solutes, with broad implications for the study of stress tolerance, protein folding, and aggregation.

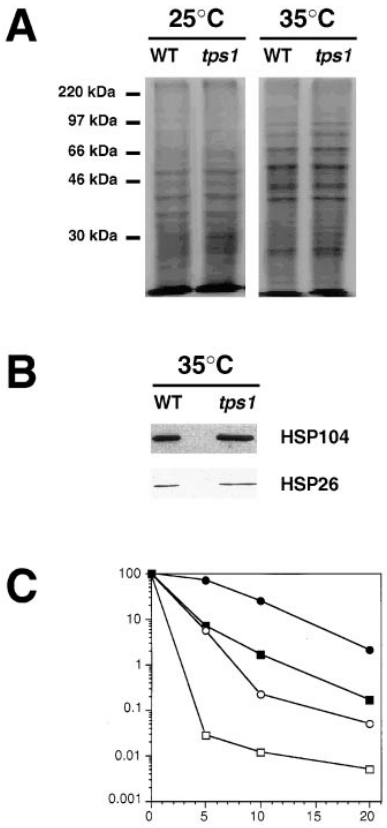

Figure 1. Mutation of *tps1* Does Not Affect Heat Shock Protein Expression or Function at Moderately Elevated Temperatures

(A) ^{35}S-labeled protein profiles of wild-type and *tps1* cells at 25°C and following a 60 min incubation at 35°C.

(B) Immunoblot of protein from wild-type and *tps1* cells after a 60 min incubation at 35°C, using antisera specific for HSP104 or HSP26.

(C) Survival of wild-type (closed circles), *tps1* (closed squares), *hsp104* (open circles), and *tps1 hsp104* (open squares) cells following severe heat shock at 50°C. Cells were pretreated for 60 min at 35°C prior to the heat shock. Following heat shock, cells were placed on ice, diluted in ice-cold YPGal liquid media, and plated to YPGal. Survival is shown relative to that of the same strain not exposed to 50°C.

Results

Mutation of *tps1* Does Not Affect Heat-Shock Protein Synthesis

The suggestion that the critical function of TPS1 in stress tolerance is to stimulate production of HSPs was based on the observation that *tps1* mutants produce significantly less *HSP* mRNA than do wild-type cells after 15 min at 39°C (Hazell et al., 1995). Using the same conditions with strains of the same genetic background, however, we observed that wild-type cells remained viable, but about half of the *tps1* cells did not survive. Thus, reduced *HSP* mRNA levels in the *tps1* mutants might be a secondary consequence of cell damage under those conditions.

We therefore compared HSP synthesis and accumulation in wild-type and *tps1* cells at normal temperature, and during a mild heat pretreatment (60 min at 35°C) selected to stimulate trehalose synthesis in wild-type cells, yet maintain full viability of the *tps1* mutants (data not shown). The profiles of ^{35}S-methionine and ^{35}S-cysteine incorporation into proteins were indistinguishable for the two strains, as were the accumulations of HSP104 and HSP26, determined by Western blotting (Figures 1A and 1B). (These two proteins provide the clearest measure of HSP induction, because they are barely detectable at normal temperatures.)

To determine whether the *tps1* mutation would affect acquired thermotolerance under conditions where HSP levels are equivalent, we pretreated the strains for 60 min at 35°C and subjected them to a severe heat shock at 50°C. Survival of cells lacking *tps1* was greatly impaired (Figure 1C). Finally, we examined whether TPS1 or trehalose might be required to capacitate HSPs for thermotolerance. If so, deletion of *HSP104* in a *tps1* mutant background should have little additional effect on thermotolerance. The dramatic loss of survival of the double mutant indicates that HSP104 makes a significant contribution to acquired thermotolerance in the *tps1* strain (Figure 1C). (Mutation of *HSP104* also impairs stationary-phase thermotolerance in *tps1* cells, although HSP accumulation was not examined in that case [Elliot et al., 1996].) The thermotolerance deficit of *tps1* cells,

rather than being an indirect consequence of impaired HSP expression or function, is most likely due to the absence of trehalose itself.

Stabilization of Proteins In Vivo

In vitro, trehalose has been shown to stabilize proteins in their native state at high temperatures. No such studies have been performed in vivo. The most critical heat-sensitive proteins in yeast, as in other organisms, are not known. However, because the heat-shock response is a general protective mechanism, this problem can be circumvented by using heterologous termperature-sensitive reporter proteins (Nguyen et al., 1989; Schroder et al., 1993; Parsell et al., 1994). This approach has the advantage of allowing analysis of damaged proteins under conditions where both the mutant and wild-type cells remain fully viable. We transformed wild-type and *tps1* strains with a plasmid directing constitutive expression of a temperature-sensitive bacterial luciferase fusion protein, whose activity can be determined by monitoring light emission in intact, living cells (Escher et al., 1989; Parsell et al., 1994). Cultures grown at 25°C were pretreated at 35°C for 60 min to induce the heat-shock response. Cycloheximide was then added to prevent any further luciferase synthesis, and cells were heat-shocked at 40°C for 60 min. (This heat shock does not affect the viability of either strain following the conditioning pretreatment.) Luciferase activity declined more rapidly and to a greater extent in the *tps1* strain than in wild-type cells (Figure 2A). This phenotype contrasts sharply with that of *hsp104* mutants, which lose bacterial luciferase activity at the same rate as wild-type cells, but differ in their ability to reactivate the protein during recovery from heat shock (Parsell et al., 1994).

To ascertain whether trehalose stabilization of proteins in vivo during heat shock is a general phenomenon, we also tested firefly luciferase. Like the bacterial protein, this enzyme is unstable at elevated temperatures, causes light to be emitted, and can be monitored in living cells. The two proteins are completely unrelated, however, in their amino acid sequences, structures, and reaction mechanisms. Thus, firefly luciferase provides an independent method for assessing the fate of heat-sensitive proteins. When firefly luciferase–expressing cells were treated as described above, activity decreased precipitously in the *tps1* mutant at high temperatures, while declining only slightly in wild-type cells (Figure 2B). Trehalose is therefore important for maintaining diverse proteins in their native state during heat shock in living cells.

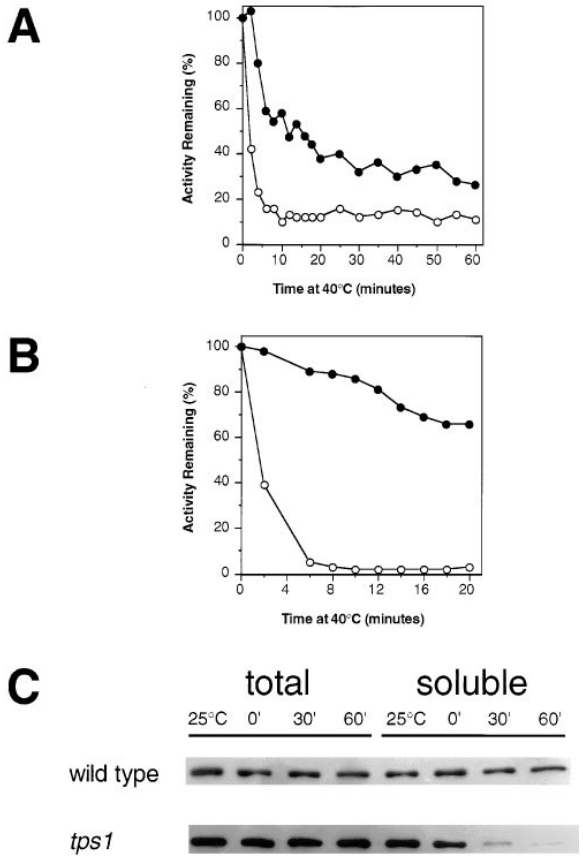

Figure 2. Trehalose Contributes to Thermotolerance by Stabilizing Native Proteins and Preventing Aggregation of Denatured Proteins

(A) Loss of bacterial luciferase activity in wild-type (closed circles) and *tps1* mutant (open circles) cells during heat shock. Cells were grown at 25°C to mid-log phase, given a 60 min preconditioning heat treatment at 35°C, then subjected to a mild heat shock at 40°C. Cycloheximide was added upon shift to 40°C to block further luciferase synthesis.

(B) Loss of firefly luciferase activity in wild-type (closed circles) and *tps1* mutant (open circles) cells at high temperature. Strains were treated as in (A).

(C) Bacterial luciferase aggregation in wild-type and *tps1* mutants. Cells were prepared as in (A), then lysed at various times during the 40°C heat shock. Total cellular protein was extracted, and aggregated proteins were sedimented by high-speed centrifugation. Total protein (total) and high-speed supernatants (soluble) were then electrophoretically separated and reacted with antiserum recognizing bacterial luciferase.

Suppression of Protein Aggregation In Vivo and In Vitro

Differential centrifugation of cell lysates revealed that the more rapid inactivation of bacterial luciferase in the *tps1* mutant was accompanied by greater aggregation of the enzyme. The heat-inactivated bacterial luciferase remained largely soluble in wild-type cells (Figure 2C). In contrast, the protein aggregated increasingly over time in the *tps1* mutant (Figure 2C). Disappearance of luciferase from the soluble fraction was not the result of degradation, since total levels of the protein were unchanged throughout the experiment (Figure 2C). The same phenomenon was also observed in cells expressing firefly luciferase (see below). Again, this result is at variance with the phenotype of *hsp104* mutants, in which aggregation is comparable to that in wild-type cells, but resolubilization is impaired (Parsell et al., 1994).

Because trehalose stabilizes proteins in their native state during heating, the increased aggregation in the mutant might solely be a consequence of having more denatured protein present to aggregate (Winzor et al., 1992; Hottiger et al., 1994). It is possible, however, that trehalose also directly inhibits aggregation of already-denatured proteins. We tested this possibility in vitro

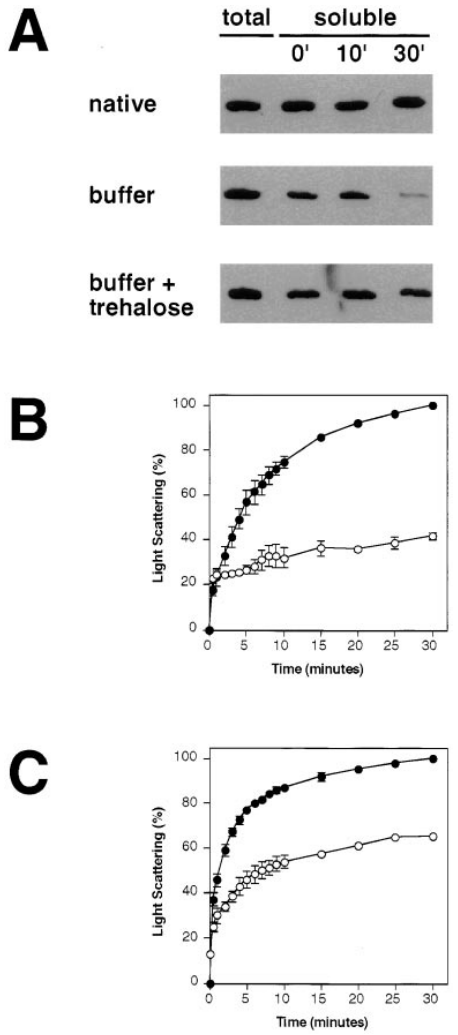

Figure 3. Trehalose Prevents Aggregation of Denatured Proteins In Vitro

(A) Purified firefly luciferase was denatured using guanidinium, then diluted into buffer alone or buffer containing 0.67 M trehalose. Native firefly luciferase was added to buffer alone. Sealed tubes were incubated at room temperature for the indicated times, after which the trehalose concentrations of all tubes were adjusted to 0.5 M and the tubes centrifuged to sediment aggregated protein. Supernatants before (total) and after (soluble) centrifugation were analyzed by Western blot, using antibody specific for firefly luciferase.

(B and C) Firefly luciferase (B) and rhodanese (C) were denatured in guanidinium, then diluted into quartz cuvettes containing either buffer C alone (closed circles) or buffer C with 0.5 M trehalose (open circles). Aggregation (light scattering) was measured as absorbance at 320 nm. Light scattering is expressed as a percentage of that observed for firefly luciferase 30 min after dilution into buffer C alone and is corrected for the effect of solute on the refractive index.

using purified firefly luciferase denatured with guanidinium. As previously described, denatured proteins aggregate when diluted into buffer alone (Buchner et al., 1991; Martin et al., 1991). In the presence of 0.5 M trehalose, a physiological concentration for yeast cells during heat shock (Hottiger et al., 1994), aggregation of firefly luciferase was substantially reduced. Aggregation was detected by two independent methods: determining the quantity of protein remaining soluble following high-speed centrifugation, and measuring the light scattering of such solutions (Figures 3A and 3B). In the centrifugation assay, concentrations of trehalose in all tubes were equalized immediately prior to sedimentation of aggregates. The decrease in soluble luciferase when denatured protein was added to buffer alone therefore indicates that trehalose inhibits aggregation, rather than promotes dissolution of existing aggregates. To test the generality of this effect, we used a second substrate, denatured rhodanese. The presence of trehalose also reduced aggregation of this protein, though to a lesser extent than that of firefly luciferase (Figure 3C).

Since different organisms produce different compatible solutes, we asked whether suppression of protein aggregation is a universal characteristic of these compounds. The intermediate effect of trehalose on rhodanese provided a means to determine whether other compounds are more or less effective than trehalose. Betaine, glucose, glycerol, maltose, mannitol, proline, sorbitol, and sucrose all stabilize enzymes in the native state in vitro at elevated temperatures (Colaco et al., 1994; Hottiger et al., 1994). Using rhodanese that had already been denatured, we observed that at 0.5 M, sucrose and maltose suppressed aggregation as well as did trehalose. Glucose and sorbitol had a smaller effect. Glycerol, proline, mannitol, and betaine had little or no effect (Figure 4). Thus, many but not all solutes can reduce aggregation of denatured proteins, with trehalose among the most effective.

Stabilization of Proteins in a Nonnative State

To determine whether trehalose had stabilized firefly luciferase in a nonnative state, or had simply promoted its refolding, we measured luciferase activity. As expected, trehalose did not inhibit the activity of native firefly luciferase (Figure 5A). Also not surprisingly, firefly luciferase that was diluted from denaturant to buffer lacking trehalose was inactive (Figure 5A). Denatured protein diluted into buffer containing trehalose, however, also lacked activity (Figure 5A). Thus, the unfolded protein diluted into trehalose-containing buffer is maintained in an inactive, nonnative state.

To gain further insight into that state, we utilized the fluorescent chromophore 1-anilino-naphthalene-8-sulphonate (ANS). Partially folded proteins displaying elements of secondary structure, but lacking tertiary structure, characteristically show high ANS fluorescence relative to their native or fully unfolded conformations (Martin et al., 1991). Trehalose-stabilized firefly luciferase also displayed increased ANS fluorescence compared to native or denatured protein (Figure 5B). (Since the fraction of luciferase that does aggregate under these conditions was removed by centrifugation prior to analysis, the increase in ANS fluorescence detected here is an underestimate of the total ANS fluorescence of an amount of trehalose-stabilized protein equivalent to that in the other tubes.) The presence of trehalose did not significantly alter the ANS fluorescence of the native protein (Figure 5B), or of the chromophore itself (data not shown).

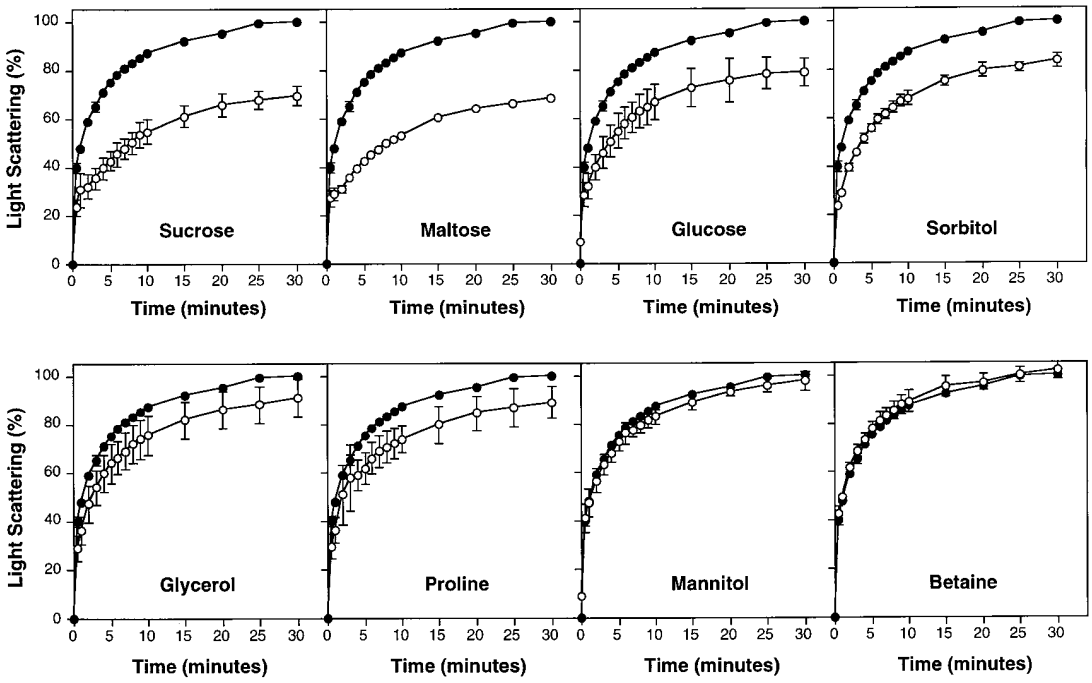

Figure 4. Cellular Solutes Vary in Their Ability to Prevent Aggregation of Denatured Rhodanese In Vitro

Guanidinium-denatured rhodanese was treated as in Figure 3C. Buffer C alone (closed circles); buffer C with 0.5 M of the indicated solute (open circles).

Trehalose-Stabilized Nonnative Proteins Are Efficiently Reactivated

We next assayed the reactivation potential of trehalose-stabilized firefly luciferase. For in vitro experiments, we utilized as a source of chaperones rabbit reticulocyte lysates, which lack the high protease activity characteristic of yeast lysates. In all cases, the test sample for reactivation was diluted 400-fold into the lysate, to reduce possible carry-over effects of the compounds in the test samples. As previously demonstrated, unfolded protein diluted directly from denaturant into the lysate is reactivated efficiently (Figure 6A and Nimmesgern and Hartl, 1993). Denatured firefly luciferase that had been first added to buffer lacking trehalose, and had therefore aggregated, was poorly reactivated (Figure 6A). However, denatured firefly luciferase that had been added to trehalose-containing buffer was refolded as efficiently as protein transferred directly from denaturant (Figure 6A). The same was true if denatured firefly luciferase was stabilized by another compatible solute, sucrose (data not shown).

To confirm that the increased reactivation was not a trivial effect of trehalose preventing adsorption of denatured protein to surfaces, the same experiments were performed, with or without trehalose, in buffer containing compounds to prevent adsorption (Buchberger et al., 1996; Warren et al., 1996). In all cases—with the detergent CHAPS, or a range of concentrations of the proteins BSA or IgG—reactivation was enhanced by the presence of trehalose (data not shown). When high temperature, rather than guanidinium, was used to denature firefly luciferase, we similarly observed efficient reactivation of protein heated in the presence of trehalose, but not of protein heated in buffer alone (data not shown). Thus, trehalose (and sucrose) stabilizes previously denatured proteins in a state that facilitates their reactivation by other cellular components.

Biological Effects of Trehalose Stabilization

We then examined the interplay of trehalose and HSP 104 functions in vivo, using cells expressing firefly luciferase. As in the experiments in Figure 2, cultures grown at 25°C were pretreated at 35°C for 60 min, received cycloheximide to block further luciferase synthesis, and then were subjected to a mild heat shock at 40°C. During the heat shock, firefly luciferase rapidly aggregated in the *tps1* mutant, as determined by differential centrifugation of cell lysates (Figure 6B). Over time, the protein was resolubilized (Figure 6B). Since HSP104 promotes resolubilization of aggregated proteins, we tested whether it is required for this process. Indeed, when both *hsp104* and *tps1* were mutated, firefly luciferase became and remained fully aggregated (Figure 6B). In contrast, little aggregation was observed in wild-type or *hsp104* cells (Figure 6B). These observations demonstrate the complementary functions of trehalose and HSP104 in thermotolerance: trehalose stabilizes native proteins during heat shock and reduces aggregation of denatured proteins, while HSP104 mediates resolubilization of those proteins that have aggregated.

Persistence of Trehalose Inhibits Protein Refolding

A major remaining question was why trehalose is degraded so rapidly after heat shock, while HSPs remain

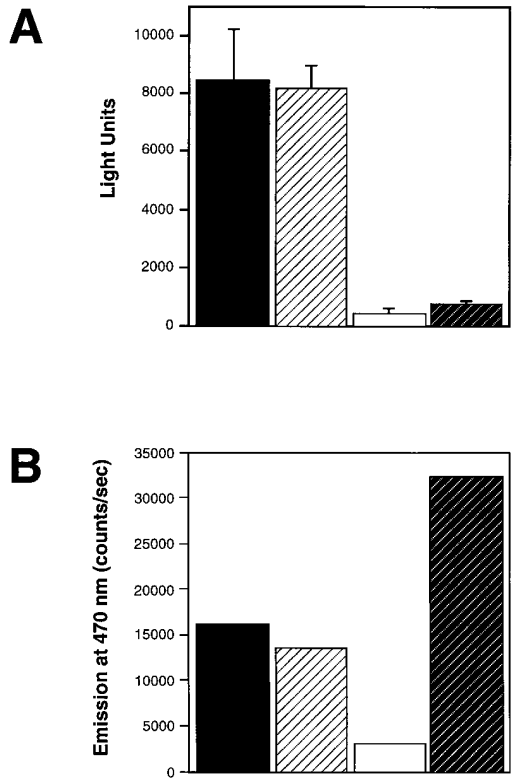

Figure 5. Trehalose Stabilizes Denatured Firefly Luciferase in an Inactive, Partially Folded Conformation

(A) Firefly luciferase activity. Native firefly luciferase was prepared in buffer C prior to dilution into buffer C alone (closed bar) or buffer C containing 0.5 M trehalose (open bar with hatching). Firefly luciferase was denatured by treatment with guanidinium and diluted into buffer C alone (open bar), or buffer C containing 0.5 M trehalose (shaded bar with hatching). After 30 min, aliquots were withdrawn and assayed.

(B) ANS fluorescence of native firefly luciferase in buffer C (closed bar); native in buffer C 0.5 M trehalose (open bar with hatching); unfolded in denaturant solution (open bar); and unfolded in buffer C 0.5 M trehalose (shaded bar with hatching). Firefly luciferase was prepared as in Figure 3B, but after 15 min, incubation at room temperature was centrifuged at high speed to sediment aggregates. ANS was then added and emission spectra (excitation, 390 nm; emission, 470 nm) were obtained by fluorimetery. Spectra were corrected for background fluorescence in the absence of firefly luciferase.

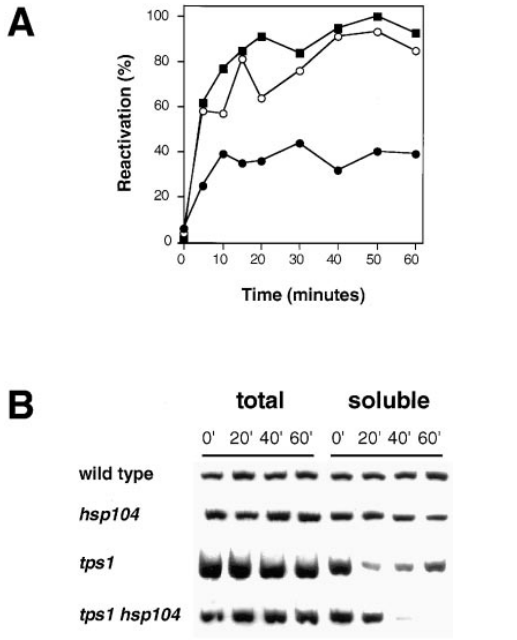

Figure 6. Reactivation of Trehalose-Stabilized Firefly Luciferase In Vitro and In Vivo

(A) Purified firefly luciferase was denatured in guanidinium and diluted into buffer C alone (closed circles), buffer C containing 0.5 M trehalose (open circles), or denaturant solution (closed squares). A sample was removed after 30 min and diluted into a refolding mixture containing rabbit reticulocyte lysate, MgATP, and an ATP-regenerating system. Reactivation is normalized to that of firefly luciferase reactivated directly from denaturant solution.

(B) Wild-type, *hsp104*, *tps1*, and *tps1 hsp104* strains expressing firefly luciferase were treated as in Figure 2A, then lysed at the indicated times during heat shock at 40°C. Protein was prepared as in Figure 2C and reacted with antibody detecting firefly luciferase.

for many hours. We considered that the ability of trehalose to stabilize denatured proteins in a nonnative conformation may interfere with spontaneous or chaperone-mediated refolding. The capacity of denatured firefly luciferase stabilized by trehalose to be refolded was therefore compared in the presence or absence of physiological concentrations of trehalose. When reticulocyte lysate was supplemented with 0.5 M trehalose, both the rate of reactivation and the ultimate yield of renatured protein was reduced (Figure 7A).

To test for a similar phenomenon in vivo, we compared reactivation of bacterial luciferase in wild-type cells and in *nth1* mutants. These mutants lack the major enzyme responsible for trehalose hydrolysis in *S. cerevisiae*, and are therefore unable to efficiently degrade trehalose following heat shock (Kopp et al., 1993). Heat shock at 40°C, following pretreatment at 35°C, caused comparable loss of bacterial luciferase activity in both strains (Figure 7B). (Viability was not affected in either case; data not shown.) During subsequent recovery at 25°C, reactivation was reduced in the *nth1* cells (Figure 7B). Thus, the continued presence of trehalose may inhibit protein reactivation in vivo as well as in vitro.

Discussion

Over the past decade, it has become evident that heat-shock proteins are key determinants governing the balance between aggregation and proper folding of cellular polypeptides damaged by stress. Our data indicate that an equally ancient and conserved mechanism, the production of organic solutes, also contributes critically to this balance. A considerable body of work had established that trehalose can protect native proteins from a wide variety of stresses in vitro (Colaco et al., 1994; Hottiger et al., 1994), but the role of trehalose in stress tolerance in vivo engendered much disagreement (Nwaka and Holzer, 1997). We have demonstrated for the first time that trehalose stabilizes proteins in their native state at high temperatures in living cells. We also observed that trehalose and certain other compatible solutes reduce the aggregation of denatured proteins

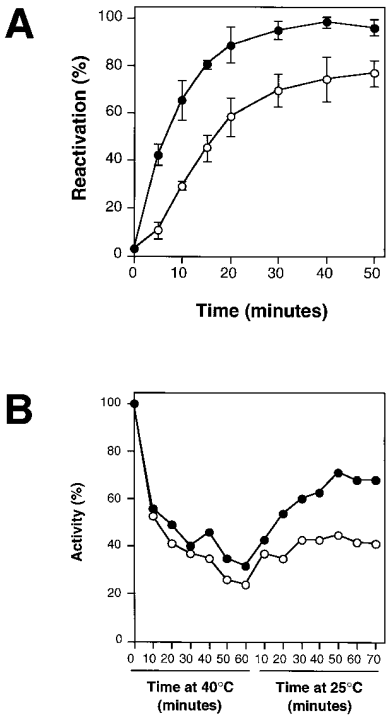

Figure 7. Persistence of Trehalose Inhibits Reactivation of Denatured Proteins In Vitro and In Vivo

(A) Purified firefly luciferase was denatured using guanidinium, then diluted out of denaturant into buffer C containing 0.5 M trehalose. Samples were added to refolding mixtures prepared as in Figure 6A, but either lacking (closed circles) or containing (open circles) 0.5 M trehalose. Reactivation is normalized to that of firefly luciferase in the refolding mixture without trehalose.

(B) Wild-type (closed circles) and *nth1* (open circles) strains expressing bacterial luciferase were incubated at 35°C for 60 min, treated with cycloheximide, heat shocked for 60 min at 40°C, then allowed to recover at 25°C. This experiment was repeated three times. The difference was consistent, although the absolute levels of reactivation varied.

that are unable to spontaneously refold. Trehalose-stabilized denatured proteins are maintained in a nonnative state, from which they can be efficiently reactivated by cellular factors. Finally, we found that reactivation of denatured proteins is inhibited in the presence of high concentrations of trehalose, which may explain the need for rapid degradation of the disaccharide during recovery from heat shock. These findings reconcile observations that previously appeared contradictory, and are of significance for stress tolerance, the evolution of different osmolyte systems, as well as for understanding protein folding and aggregation.

Our results provide a coherent picture of the interplay between HSPs and organic solutes in protecting cells from the vagaries of their environment. Stresses such as heat cause proteins to denature and adopt partially folded conformations that are prone to aggregation. Trehalose functions first against this process, by stabilizing proteins in their native state. Proteins that denature are bound by HSPs to prevent aggregation and promote refolding. Here, trehalose acts again, reducing aggregation of nonnative proteins when the protein repair machinery is overwhelmed. Substrates that elude both HSPs and trehalose, and begin to aggregate, can be resolubilized through a process involving HSP104. Thus, the relative importance of the HSP and nonHSP mechanisms of thermotolerance, which has been a long-standing subject of controversy (Hall, 1983; Watson et al., 1984; Widelitz et al., 1986; De Virgilio et al., 1990, 1991; Winkler et al., 1991; Nwaka et al., 1994; Nwaka and Holzer, 1997), is now clear. Our work, together with previous observations, establishes that both mechanisms are vital for maximal survival of severe heat shock. Cells lacking either HSP104 or trehalose retain a measure of thermotolerance, but cells lacking both are extremely vulnerable to heat (Figures 1C and 6B, and Elliot et al., 1996). Efforts to boost stress tolerance have thus far focused on manipulating levels of either HSPs or solutes (Welte et al., 1993; Holmstrum et al., 1996; Goddijn et al., 1997); a combination of the two is likely to prove far more efficacious.

Despite the ability of some small organic compounds to stabilize native proteins at high temperatures, we found that others have only a limited, if any, capacity to prevent aggregation of denatured protein. The variation in their properties and their accumulation in different organisms is reminiscent of the distinct molecular functions and patterns of expression of HSPs in different organisms (Parsell and Lindquist, 1994). Similarly, the regulation of trehalose levels and the inhibitory effects of its persistence parallel earlier findings with HSP70 in *Drosophila*. The major heat-shock protein in that organism, HSP70, is not produced at normal temperatures but becomes one of the most abundant cellular proteins during heat shock. Forcing expression of HSP70 in the absence of stress halts cell division, possibly because the protein interacts with factors critical for that process. Proliferation resumes only after HSP70 expression has ceased and existing protein is sequestered in granules (Feder et al., 1992). Thus, the evolution of diverse protein and nonprotein mechanisms of thermotolerance likely reflects not only differences in the stresses organisms encounter and in their most critical heat-sensitive structures, but also the need to balance beneficial and detrimental effects of different HSPs and solutes within the context of each organism's unique physiology.

Reduced aggregation in the presence of trehalose was previously noted during heat denaturation of a protein in vitro (Hottiger et al., 1994). That experiment, however, could not distinguish whether aggregation was decreased because trehalose was stabilizing the native protein, or an intermediate; the authors concluded the former, an interpretation in keeping with the existing literature. We have demonstrated that a compatible solute can stabilize denatured proteins in a nonnative state. Our finding is consistent with the report of diminished aggregation of partially folded intermediates of TEM-β-lactamase, a protein that spontaneously refolds, when allowed to renature in the presence of sucrose (Georgiou et al., 1994). In our study, a protein that requires chaperones for refolding can be maintained in a refoldable state for at least 30 min if trehalose is present. This observation suggests a number of important uses. Organic solutes may serve as general tools for the isolation and analysis of previously inaccessible protein folding

intermediates. Structural studies of such nonnative states can provide insight into normal protein folding pathways, as well as features predisposing proteins to misassembly and aggregation (Wetzel, 1996). Overproduction of trehalose in heterologous expression systems such as *E. coli*, which produces trehalose in response to stress, may prevent recombinant proteins from forming inclusion bodies. Finally, an increasing number of human diseases are now known to result from protein misfolding (Thomas et al., 1995; Wetzel, 1996). Compatible solutes have been proposed for therapeutic use, based on their ability in experimental systems to stabilize the proper conformation of labile proteins associated with cystic fibrosis and prion diseases (Brown et al., 1996; Tatzelt et al., 1996; Welch and Brown, 1996; DebBurman et al., 1997). Solutes should also be considered for the treatment of amyloid and similar disorders involving protein aggregation (Wetzel, 1996; Scherzinger et al., 1997). Design of such strategies, however, must take into account measures to overcome the potential interference of these solutes with normal forward-folding mechanisms. The emerging understanding of these compounds and their unusual properties opens broad avenues for further inquiry and promising applications.

Experimental Procedures

Yeast Strains, Plasmids, Media, and General Methods

All strains are of the W303 genetic background. The *tps1* mutant was the gift of S. Hohmann and J. Thevelein. The *hsp104* mutant was constructed by transforming cells with the Xba1/Xho1 fragment of plasmid 5503 pJTDP104. The *tps1 hsp104* strain was created by transforming *hsp104* mutants with plasmid P/TPS1/LEU2 (Vuorio et al., 1993), the gift of J. Londesborough. Strain *nth1::URA3* was created by transformation of W3031A with the *nth1* disruption construct pnth1::URA3 (Kopp et al., 1993), the gift of H. Holzer.

Cells producing the bacterial (*Vibrio harveyii*) luciferase fusion protein (Escher et al., 1989) were obtained by transformation with either 5687 pGPDluxAB(URA) or 5636 pGPDluxAB(HIS), 2 μ plasmids carrying the *URA3* and *HIS3* selectable markers, respectively. In both cases, luciferase expression is driven by the constitutive *GPD* promoter.

Cells expressing firefly (*Photinus pyralis*) luciferase were obtained by transformation with the centromeric plasmid 6092 pGAL-FFL[SEL], and selection for growth on media lacking uracil. Because firefly luciferase is normally targeted to peroxisomes, we utilized an otherwise normal allele in which the peroxisomal targeting signal is mutated, causing the enzyme to remain in the cytoplasm (Distel et al., 1992). Expression is driven by the *GAL1* promoter.

Transformations were carried out using a Gene Pulser electroporator (BioRad).

Media were prepared according to Rose et al., 1990. Galactose-containing medium was used in all cases except experiments involving *nth1* mutants, for which glucose was substituted. (*tps1* mutants will not grow in glucose [Bell et al., 1992; Van Aelst et al., 1993; Vuorio et al., 1993].)

Cellular proteins were extracted, separated, and examined by Western blotting as described in Nathan and Lindquist, 1995.

Metabolic Labeling and Thermotolerance

25 ml cultures of wild-type and *tps1* cells were grown at 25°C to mid-log phase in YPGal, were transferred to a 35°C shaking water bath, and received 550 μCi of Express Protein Labeling Mix (DuPont-NEN) after 35 min. Cells were harvested following 60 min at 35°C. Labeled proteins were visualized in dried gels using a PhosphorImager (Molecular Dynamics).

Thermotolerance was assayed as described in Sanchez et al., 1992.

Luciferase Activity and Aggregation In Vivo

Bacterial luciferase activity was assayed as described in Parsell et al. (1994). Light output of cells expressing firefly luciferase was determined following addition of 100 μl of 0.5 mM Dluciferin (Sigma) to 100 μl of culture (Schroder et al., 1993). Aggregation was assayed as in Parsell et al. (1994).

Centrifugation Assay to Determine Aggregation In Vitro

One volume of firefly luciferase (Sigma) in storage buffer (30 mM Tris [pH 7.2], 50 mM KCl, 10 mM DTT, 10% glycerol) was denatured by addition of 2 vol of buffer A (7.5 M guanidinium-HCl, 30 mM Tris [pH 7.2], 50 mM KCl, 10 mM DTT) and diluted 100-fold (0.15 μM final concentration; concentration of all protein stock solutions were determined by amino acid analysis at the University of Michigan Protein and Carbohydrate Structure Facility) into polycarbonate tubes (Beckman) containing buffer C (30 mM Tris [pH 7.2], 50 mM KCl, 10 mM DTT) or buffer C with 0.67 M trehalose. Tubes were sealed with Parafilm (American National Can) and incubated at room temperature for 0, 10, or 30 min, after which trehalose concentrations in all tubes were adjusted to 0.5 M. A volume was removed (total protein) and tubes were centrifuged for 5 min in a Beckman Optima TL ultracentrifuge at 386,000 g to sediment aggregated protein. The supernatant (soluble protein) was removed and analyzed by Western blotting.

Light Scattering Measurements

Firefly luciferase (final concentration 0.15 μM) or rhodanese (final concentration 0.17 μM) was prepared as for the centrifugation assay, then diluted into quartz cuvettes (NSG Precision Cells) containing either buffer C alone or buffer C with 0.5 M trehalose or other solute. Aggregation (turbidity) was measured as light scattering at 320 nm (Martin et al., 1991), using a Perkin-Elmer Lambda 6 spectrophotometer. Light scattering is expressed as a percentage of that observed for the particular protein 30 min after dilution into buffer C alone.

All light scattering measurements were corrected for the refractive index of the solution. That effect was determined by diluting the denatured substrate into buffer C alone and allowing the protein to aggregate for 30 min. Afterwards, either buffer C alone was added, or buffer C containing the solute at a concentration such that the final solute concentration was 0.5 M. The percent difference in final turbidity between these two conditions was taken as the effect of refractive index.

Firefly Luciferase Activity In Vitro

Native firefly luciferase was prepared in buffer C prior to dilution into buffer C alone or buffer C containing 0.5 M trehalose. Denatured firefly luciferase was prepared as described for the centrifugation assay and diluted into buffer C alone, or buffer C containing 0.5 M trehalose. After 30 min, 0.5 μl aliquots were withdrawn, added to 40 μl of luciferase assay buffer (Promega), and analyzed by luminometer (Nimmesgern and Hartl, 1993). Error bars indicate the standard deviation of at least three measurements.

ANS Fluorescence

Firefly luciferase was prepared as for light scattering, but after 15 min, incubation at room temperature was centrifuged at 386,000 g for 5 min. ANS was then added at 30 μM final concentration and emission spectra (excitation, 390 nm; emission, 470 nm) were obtained (Martin et al., 1991) using a fluorimeter (Photon Technologies International). Spectra were corrected for background fluorescence in the absence of firefly luciferase.

Firefly Luciferase Reactivation In Vitro

Firefly luciferase was denatured as for the centrifugation assay, then diluted in buffer C alone, buffer C containing 0.5 M trehalose, or denaturant solution (solution D, 2 vol of buffer A: 1 vol of buffer C). After 30 min, 0.5 μl of each was removed and diluted 400-fold into a refolding mixture containing untreated rabbit reticulocyte lysate (Green Hectares), 5 mM $Mg(OAc)_2$, 1 mM ATP, and an ATP regenerating system consisting of 10 mM phosphocreatine and 0.15 mg/ml phosphocreatine kinase (Sigma) final concentration (Nimmesgern

and Hartl, 1993). Final concentrations of trehalose and guanidinium were equalized in all reactions. At the indicated times, 0.5 μl was withdrawn and assayed. Reactivation is normalized to that of firefly luciferase from solution D.

For reactivation in the presence of trehalose, the final luciferase concentration was 0.07 μM and after denaturation protein was diluted only into buffer C containing 0.5 M trehalose. Samples were then added to refolding mixtures prepared as above, but either containing or lacking 0.5 M trehalose. Reactivation is normalized to that of firefly luciferase in the refolding mixture without trehalose.

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