Protective Role of Trehalose in Thermal Denaturation of Yeast Pyrophosphatase

Mauro Sola-Penna and José Roberto Meyer-Fernandes

Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, 21941-590, Rio de Janeiro, Brasil

Z. Naturforsch. **49c,** 327–330 (1994); received January 7, 1994

Thermal Denaturation, Trehalose, Yeast Pyrophosphatase, Water Activity, Carbohydrates

Trehalose, a disaccharide of glucose, is accumulated in yeast cytosol when this organism is submitted to a stress condition. Recently it was shown that the level of trehalose increase up to 15 times when yeast cells are submitted to heat shock (De Virgilio *et al.*, 1991). In this report we give evidence how trehalose may play an important role on the stress-survival of yeasts when submitted to a heat shock. We show that 1.5 M trehalose increases 13-fold the half-time for thermal inactivation ($t_{0.5}$) of yeast cytosolic pyrophosphatase at 50 °C. This thermal protection conferred by trehalose is dose-dependent, after 10 min at 50 °C, a condition which inactivated pyrophosphatase, the presence of 2 M trehalose preserves 95% of total activity. Other carbohydrates were tested but were not so effective as trehalose. The presence of trehalose at high concentrations in the reaction medium at 35 °C inhibits pyrophosphatase activity. This inhibition is less effective at 50 °C suggesting that under this condition the enzyme is temperature-protected and active.

Introduction

Trehalose, a non-reducing disaccharide of glucose, is widely distributed among living systems reaching high concentrations in cells cytossol when these organisms are under stress conditions (Crowe *et al.*, 1984). Such high concentrations can reach 35% of dry weight of the cells (Crowe *et al.*, 1984; Wiemken, 1990), that in hydrated cells reach the molar range. Trehalose is considered to have an important role in osmoregulation and in the ability of organisms such as baker's yeast to survive severe dehydration and low-temperature stress (Clegg and Filosa, 1961; Crowe *et al.*, 1984; Sussman and Lingappa, 1959). In some cells, trehalose accumulation is induced by heat shock. This phenomenon is reversible and trehalose is rapidly degraded upon return of the cells to physiological conditions. It has been proposed that in yeast, trehalose does not primarily function as a storage compound but as a highly efficient protecting agent to maintain the structural integrity of the cytoplasm under environmental stress conditions (De Virgilio *et al.*, 1991; Neves *et al.*, 1991; Wiemken, 1990).

We have shown that carbohydrates can modulate the catalytic cycle of ion-transporting ATPases. They decrease the K_m for Pi in the phosphorylation reaction catalyzed by $(Ca^{2+} + Mg^{2+})$ATPase from sarcoplasmic reticulum (Chini *et al.*, 1991) and increase the rate of ATP$\leftrightarrow$Pi exchange catalyzed by $(Ca^{2+} + Mg^{2+})$ATPase from renal plasma membranes (Vieyra *et al.*, 1989, 1991). Both reactions are stimulated by a decrease in water activity and in protein solvation (Chini *et al.*, 1991; de Meis, 1989). Recently it has been shown that a decrease in protein solvation promoted by sugars can regulate the equilibria among allosteric conformations of hemoglobin (Colombo *et al.*, 1992). These data indicate that the flexibility and dynamic nature of proteins are affected by protein-bound water molecules, in ways that can be modified by interaction with biological solutes. At high temperatures, carbohydrates can stabilize protein structure by driving the equilibrium between native and unfolding configurations in the direction of the native state.

The present report examines the role of trehalose in thermal denaturation and catalytic activity of yeast cytosolic pyrophosphatase and suggest why trehalose but not other carbohydrates are accumulated by yeast under stress conditions.

Reprint requests to José Roberto Meyer-Fernandes. Telefax (55-21) 270-8647.

0939–5075/94/0500–0327 $ 03.00

Materials and Methods

Yeast inorganic pyrophosphatase, trehalose and tris were purchased from Sigma Chemical Co. Other reagents were of the higher purity available.

Pyrophosphatase activity was determined by measuring the total Pi released in the end of reaction.

Data points show the means of four experiments, with standard errors of less than 10%. Fitting were done using the non-linear regression computer program Enzfitter (Elsevier, Biosoft). Goodness of fit was assessed by computing reduced chi squares for the fits, as described in (Motulsky and Ransnas, 1987).

Results

When yeast cytosolic pyrophosphatase was pre-incubated at 50 °C its activity measured in a standard assay medium of 35 °C, decreased as a function of pre-incubation time. The addition of trehalose to the pre-incubation medium protected the enzyme against inactivation, increasing its stability at high temperature (Fig. 1). In the absence of trehalose less than 20% of the original activity remained after pre-incubation for 25 min at 50 °C. The addition of 1.5 M trehalose to the pre-incubation medium preserved 80% of the original activity under the same conditions. The degree of protection depended on the trehalose concentration: after a 10 min pre-incubation at 50 °C in the presence of 0.4 M trehalose, half of the control activity without pre-incubation was retained; with 2 M trehalose, activity was 95% of the control value (Fig. 2).

The ability to stabilize pyrophosphatase at high temperature was specific to trehalose. Table I shows that other carbohydrates were not nearly so effective. Whereas 1.5 M trehalose increased the half-time ($t_{0.5}$) for temperature inactivation by 13-fold, the greatest effect detected with the other carbohydrates used was a 3.3-fold increase in $t_{0.5}$.

A second effect, also specific for trehalose, was also observed: the presence of trehalose in the

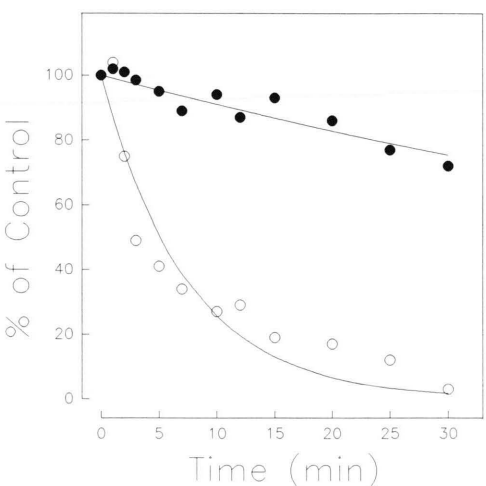

Fig. 1. Time course of thermal denaturation of pyrophosphatase at 50 °C. Yeast cytosolic pyrophosphatase (40 μg/ml) was pre-incubated for the times indicated in the abscissa in the absence (○) or in the presence (●) of 1.5 M trehalose. After pre-incubation, 50 vols. of reaction medium was added, at 35 °C. Final concentrations in the reaction medium were 100 mM Tris/HCl pH 7.5, 10 mM $MgCl_2$, 2 mM pyrophosphate, 0 or 30 mM trehalose and 0.8 μg/ml pyrophosphatase. The reaction was quenched after 1 min by addition of 2 vols of 20% (w/v) trichloroacetic acid. After dilution, the activity (100% on the ordinate) was 615 ± 50 nmol Pi · mg^{-1} · min^{-1} with or without 30 mM trehalose. The data were fitted by non-linear regression to the equation $v = Vo \cdot e^{-kt}$, where Vo is the initial rate of pyrophosphate hydrolysis without pre-incubation; k is the decay constant; t is the pre-incubation time.

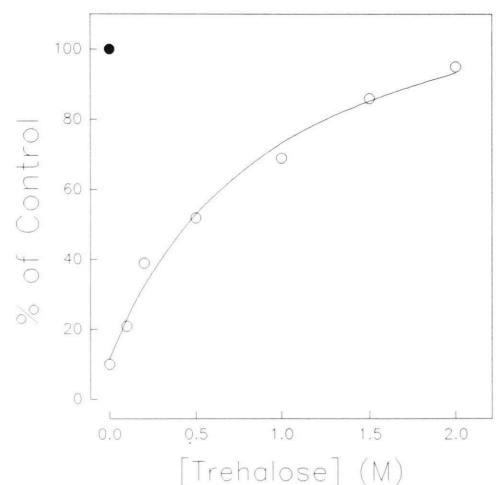

Fig. 2. Trehalose concentration dependence for protection against thermal denaturation. (○) The enzyme was pre-incubated for 10 min at 50 °C in the presence of the concentrations of trehalose indicated on the abscissa. Reaction was started as in Fig. 1, and the trehalose was diluted 50-fold by addition of reaction medium, at 35 °C. (●) Reaction measured without pre-incubation. Other conditions as in Fig. 1.

Table I. Thermal denaturation of pyrophosphatase in the absence and presence of different carbohydrates. The enzyme was pre-incubated for different times at 50 °C in the presence of the indicated concentrations of carbohydrates, then diluted in the reaction medium and tested for pyrophosphatase activity as described in Fig. 1. The fit and the equation used were the same as in Fig. 1. $t_{0.5}$ represents the half-time for loss of pyrophosphatase activity.

Carbohydrate present during pre-incubation	Concentration [M]	$t_{0.5}$ [min]
None	–	5.2 ± 0.5
Glucose	3	8.5 ± 0.9
Fructose	3	12.8 ± 1.1
Sucrose	1.5	16.9 ± 1.4
Trehalose	1.5	60.3 ± 0.8

reaction medium inhibited the enzymatic activity of pyrophosphatase (Fig. 3). At 35 °C, 2 M trehalose reduced the activity to 10% of the control value. Other carbohydrates were less inhibitory: with the same concentration of sucrose (or twice the concentration of glucose and fructose), 75% of the control activity remained (Fig. 3 A). When the reaction was carried out at 50 °C, trehalose inhibited less than at 35 °C (Fig. 3 B), whereas the other carbohydrates had the same effect as before.

Discussion

The ability of carbohydrates to stabilize proteins has been attributed to the preferential hydration of protein that occurs when it is dissolved in a carbohydrate solution (Arakawa and Timasheff, 1982, 1983; Lee and Timasheff, 1981). This phenomenon is due to the fact that polyols, in general, and unlike destabilizing agents such as guanidinium salts (Arakawa and Timasheff, 1984), solubilize in bulk water. An increase in the concentration of carbohydrate in bulk water engages more water molecules in solubilization and at high carbohydrate concentrations, protein and solutes begin to compete for the available water. This competition leads to a reduction in the protein solvation layer, which results in a decrease in protein apparent molal volume (Arakawa and Timasheff, 1982). As a result, the protein becomes more compact and less susceptible to the destabilizing forces of high temperature (Back *et al.*, 1979).

Preferential hydration studies have been done with several carbohydrates but not trehalose (Arakawa and Timasheff, 1982). The specificity of the effects of trehalose shown in this study suggest that this carbohydrate at high concentration can interact directly with the protein. It may be that this interaction stabilizes pyrophosphatase forming thermodynamically stable structures in such way that the resulting conformation has a low enzymatic activity (Klibanov, 1983). Inhibition of enzyme catalysis by trehalose was more effective at 35 °C than when activity was measured at 50 °C (Fig. 3), suggesting that at the higher temperature, increased protein mobility promoted recovery of the catalytic activity of this enzyme.

The results shown here are consistent with the idea that preferential hydration is not the only factor in stabilizing the native structure of proteins (Arakawa and Timasheff, 1985; Arakawa *et al.*, 1990; Lee and Lee, 1987). The magnitude of the stabilizing effect is shown here to depend on the

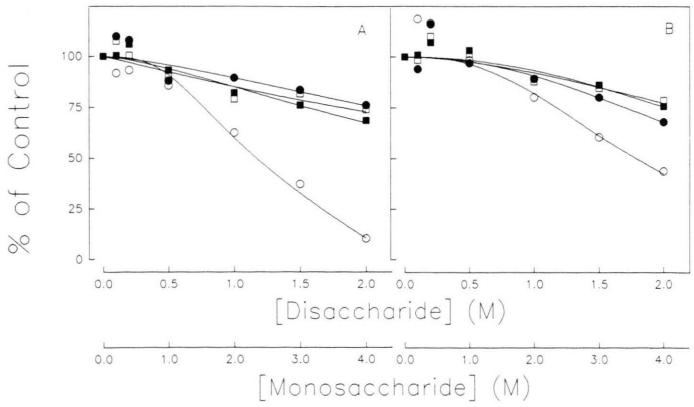

Fig. 3. Effects of different mono- and disaccharides on pyrophosphatase activity. (A) Reaction at 35 °C. The activity in the absence of sugars was 613 ± 48 nmol·mg^{-1}·min^{-1}. (B) Reaction at 50 °C. The activity in the absence of sugars was 942 ± 80 nmol·mg^{-1}·min^{-1}. The assay medium was the same as in Fig. 1. Reaction was started by addition of soluble yeast pyrophosphatase to a final concentration of 0.8 μg/ml, and concentrations of trehalose (○), sucrose (●), glucose (□) and fructose (■) are indicated on the abscissa. The reaction was quenched after 1 min, and pyrophosphatase activity was determined as in Fig. 1.

nature of the sugar, and may also depend on the nature of the protein. The ability to promote thermal stability and inhibit pyrophosphatase activity may be related to the different physical properties of the carbohydrates tested (Duda and Stevens, 1990).

The study of organisms that have adapted to conditions of dehydration, heat, or high osmotic activity provides a clue to how nature has approached the problem of maintaining a functional, viable organism under conditions that might normally be expected to denature or inactivate a large number of that organisms macromolecules. The specific effectiveness of trehalose in protecting against thermal denaturation of yeast pyrophosphatase may explain why these cells selectively accumulate trehalose and not other carbohydrates.

Acknowledgements

We thank Dr. Martha M. Sorenson for the discussions about this manuscript.

Arakawa T. and Timasheff S. N. (1982), Stabilization of protein structure by sugars. Biochemistry **21,** 6536–6544.

Arakawa T. and Timasheff S. N. (1983), Preferential interactions of proteins with solvent components in aqueous amino acid solutions. Arch. Biochem. Biophys. **224,** 169–177.

Arakawa T. and Timasheff S. N. (1984), Protein stabilization and destabilization by guanidinium salts. Biochemistry **23,** 5924–5929.

Arakawa T. and Timasheff S. N. (1985), Mechanism of poly(ethylene glycol) interaction with proteins. Biochemistry **24,** 6756–6762.

Arakawa T., Bhat R. and Timasheff S. N. (1990), Why preferential hydration does not always stabilize the native structure of globular proteins. Biochemistry **29,** 1924–1931.

Back J. F., Oakeenfull D. and Smith M. B. (1979), Increased thermal stability of proteins in the presence of sugars and polyols. Biochemistry **18,** 5191–5196.

Chini E. N., Meyer-Fernandes J. R. and Sola-Penna M. (1991), Monosaccharides and disaccharides decrease the K_m for phosphorylation of a membrane-bound enzyme ATPase. Z. Naturforsch. **46c,** 644–646.

Clegg J. S. and Filosa M. F. (1961), Trehalose in the cellular slime mould *Dictyostelium mucoroides*. Nature **192,** 1077–1078.

Colombo M. F., Rau D. C. and Parsegian V. A. (1992), Protein solvation in allosteric regulation: a water effect on hemoglobin. Science **256,** 655–659.

Crowe J. H., Crowe L. M. and Chapman D. (1984), Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science **223,** 701–703.

De Meis L. (1989), Role of water in the energy hydrolysis of phosphate compounds – energy transduction in biological membranes. Biochim. Biophys. Acta **973,** 333–349.

De Virgilio C., Piper P., Boller T. and Wiemken A. (1991), Aquisition of thermotolerance in *Saccharomyces cerevisiae* without heat shock protein hsp 104 and in the absence of protein synthesis. FEBS Lett. **288,** 86–90.

Duda C. A. and Stevens E. S. (1990), Trehalose conformation in aqueous solution from optical rotation. J. Am. Chem. Soc. **112,** 7863.

Klibanov A. M. (1983), Stabilization of enzymes against thermal inactivation. Adv. Appl. Microbiol. **29,** 1–28.

Lee J. C. and Lee L. L. Y. (1987), Thermal stabilization of proteins in the presence of poly(ethylene glycols). Biochemistry **26,** 7813–7819.

Lee J. C. and Timasheff S. N. (1981), The stabilization of proteins by sucrose. J. Biol. Chem. **256,** 7193–7201.

Motulsky H. J. and Ransnas L. A. (1987), Fitting curves to data using non-linear regression: a practical and non-mathematical review. FASEB J. **1,** 365–374.

Neves M. J., Jorge J. A., François J. M. and Terenzi H. F. (1991), Effects of heat shock on the level of trehalose and glycogen, and on the induction of thermotolerance in *Neurospora crassa*. FEBS Lett. **283,** 19–22.

Sussman A. S. and Lingappa B. T. (1959), Role of trehalose in Ascospores of *Neurospora tetrasperma*. Science **130,** 1343.

Vieyra A., Caruso-Neves C. and Meyer-Fernandes J. R. (1989), Regulation of the reversal cycle of the calcium pump from kidney proximal tubules. Methodological Surveys in Biochemistry and Analysis **19,** 31–41.

Vieyra A., Caruso-Neves C. and Meyer-Fernandes J. R. (1991), ATP↔^{32}Pi exchange catalyzed by plasma membrane Ca^{2+}-ATPase from kidney proximal tubules. J. Biol. Chem. **266,** 10324–10330.

Wiemken A. (1990), Trehalose in yeasts, stress protectant rather than reserve carbohydrate. Antonie van Leeuwenhock **58,** 209–217.