Trehalose Protects Yeast Pyrophosphatase against Structural and Functional Damage Induced by Guanidinium Chloride

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Trehalose is accumulated at very high concentrations in yeasts when this organism is submitted to a stress condition. This report approaches the question on the protective effect of trehalose and its degradation product, glucose, against structural and functional damage promoted by guanidinium on yeast cytosolic pyrophosphatase. Here it is shown that both, 1 M trehalose or 2 M glucose, are able to attenuate at almost the same extent the conformational changes promoted by guanidinium chloride on the pyrophosphatase structure. On the other hand, while 1 M trehalose increases 3.8 times the K_i (from 0.15 to 0.57 M) for guanidinium chloride inhibition of pyrophosphatase activity, 2 M glucose did not even duplicate this parameter (from 0.15 to 0.25 M). These data support evidences for a functional reason for the accumulation by yeasts of trehalose, and not other compound, during stress conditions.

Introduction

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), a non-reducing disaccharide of glucose, is widely distributed among living organisms. It is accumulated at very high concentrations (35% of dry weight of the cells, reaching the molar range in hydrated cells) by several organisms, as yeasts, when submitted to stress conditions (Crowe *et al.*, 1984; Wiemken, 1990). 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- and high temperature stresses (Crowe *et al.,* 1984; Sussman and Lingappa, 1959; Clegg and Filosa, 1961; DeVirgilio *et al.,* 1991, 1994; Neves *et al.,* 1991; Hottiger *et al.,* 1994). It has been proposed that in yeasts, trehalose does not primarily function as a reserve but as a highly efficient protecting agent to maintain structural integrity of the cytoplasm under environmental stress conditions (Wiemken, 1990; DeVirgilio *et al.,* 1991, 1994; Neves *et al.,* 1991; Hottiger *et al.,* 1994). However, the studies concerning the role of the accumulation of trehalose by yeasts did not examine about the effects that this carbohydrate can exert on cell function (Sola-Penna and Meyer-Fernandes, 1994).

Recently, we showed that polyols, considered to be unable to affect enzyme function, modify the activity of a variety of enzymes (Vieyra *et al.,* 1989, 1991; Chini *et al.,* 1991; Sola-Penna and Meyer-Fernandes, 1994; Sola-Penna *et al.,* 1994, 1995a, 1995b). It has been shown that trehalose at high concentrations, but not other carbohydrates, protects yeast cytosolic enzymes against thermal inactivation (Hottiger *et al.,* 1994; Sola-Penna and Meyer-Fernandes, 1994), suggesting why yeasts accumulate trehalose but not other carbohydrates when submitted to a heat shock (Wiemken, 1990; DeVirgilio *et al.,* 1991, 1994; Neves *et al.,* 1991; Hottiger *et al.,* 1994; Sola-Penna and Meyer-Fernandes, 1994).

In this paper we show that trehalose and glucose can efficiently protect pyrophosphatase structure against conformational changes promoted by guanidinium chloride. Only trehalose protected from functional injury induced by this potent destabilizing compound.

Materials and Methods

Yeast inorganic pyrophosphatase (EC 3.6.1.1), was purchased from Sigma Chemical Co. (St. Louis,

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MO, USA), and exhibit high purity (99.5%). Trehalose, glucose, tetrasodium pyrophosphate, guanidinium chloride and Tris (tris[hydroxymethyl]aminomethane) were also purchased from Sigma Chemical Co (St. Louis, MO, USA). Other reagents were of the highest purity available.

Pyrophosphatase activity was determined by measuring the total Pi released at the end of incubation. Activity experiments were performed in a medium containing 100 mM Tris-HCl (pH 7.5); 10 mM $MgCl_2$, 2 mM tetrasodium pyrophosphate and 0.8 μg of purified enzyme per ml of reaction medium. The reactions were quenched after 1 min at 25 °C by addition of 2 vols. of 20% (w/v) trichloroacetic acid.

Steady-state fluorescence measurements were performed on an ISS/K2 spectrofluoremeter (ISS, Champaign, IL, USA). The pyrophosphatase concentration was fixed at 10 μg/ml in 100 mM Tris-HCl (pH 7.5). Appropriate reference spectra were subtracted from the data to correct for background interferences which were always less than 5% of the fluorescence signal. All experiments were performed at 25 °C, with magnetic stirring in the cuvette. The excitation wavelength was set at 280 nm and the emission was scanned at 300–400 nm. Center of mass (average emission wavelength) was calculated using software provided by ISS Inc. as follows:

$$\text{Center of mass (nm)} = \int \lambda I(\lambda) d\lambda / \int I(\lambda) d\lambda$$

where λ is the emission wavelength (nm) and $I(\lambda)$ is the fluorescence intensity at a given wavelength.

Fits were done by non-linear regression using the software Sigmaplot for Windows 1.0 (Jandel Scientific, USA). Curves and Ki were calculated using:

$$\% \text{ of control} = 100 \cdot \frac{K_i}{K_i + G}$$

where K_i is the inhibition constant and G is the guanidinium chloride molar concentration.

Results

The effect of guanidinium chloride on the intrinsic fluorescence emission of yeast cytosolic pyrophosphatase was investigated. The centers of mass (average emission wavelength) of the spectra were increased by addition of guanidinium chloride (Fig. 1. open circles). This result indicates that guanidinium chloride did change the average exposure of the tryptophan residues of the pyrophosphatase to the aqueous medium (Lakowicz, 1983), indicating that guanidinium chloride did promote unfolding of the protein structure.

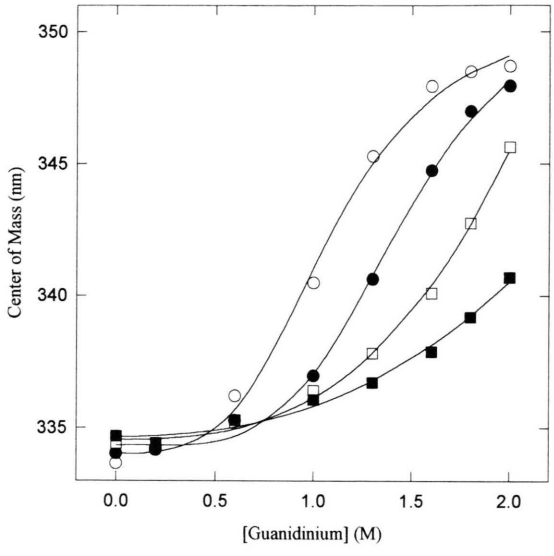

Fig. 1. Effect of guanidinium chloride on pyrophosphatase fluorescence and protective effect of trehalose. The center of mass of the emission spectra is shown as a function of guanidinium chloride concentration in the absence (○) and in the presence of 0.5 M (●), 1.0 M (□), and 1.5 M (■) trehalose. The experiments were performed in the presence of 100 mM Tris-HCl (pH 7.5) and 10 μg/ml of yeast cytosolic pyrophosphatase. Data points correspond to one representative experiment of a series of three.

This red-shift promoted by guanidinium chloride in the average emission wavelength of pyrophosphatase fluorescence was strongly attenuated by the presence of trehalose in the medium (Fig. 1). This protective effect of trehalose in the pyrophosphatase unfolding promoted by guanidinium chloride was dependent on the sugar concentrations. In figure 1 it can be seen that raising concentrations of trehalose decreased the red-shift promoted by guanidinium chloride.

A similar protective effect against unfolding of pyrophosphatase promoted by guanidinium chloride can be achieved with double concentration of glucose (Table I). This monosaccharide, that is the degradation product of trehalose and is utilized as primary energy source by yeasts, can partially prevent the red-shift promoted by guanidinium chlo-

Table I. Differences between protection conferred by sugars on structure and function of pyrophosphatase in the presence of guanidinium chloride. Fluorescence experiments were performed as indicated in the legend of Fig. 1. Colun c was calculated by the relative value of the difference between b and a to the values with no addition (d and g). No statistical differences were observed between d, e and f; nor between h and i, ($p > 0.05$). On the other hand, the differences between g and h, or g and i indicate a protection promoted by the sugars ($p < 0.001$). The same is observed for the Pyrophosphatase activity was measured as indicated under Material and Methods. Absolute values in the absence of guanidinium chloride were 615 ± 15 nmol·μg^{-1}·min^{-1} for control experiments, 480 ± 10 nmol·μg^{-1}·min^{-1} in the presence of 2 M glucose, and 390 ± 10 nmol·μg^{-1}·min^{-1} in the presence of 1 M trehalose. Data correspond to the mean of five experiments ± standard errors (n=5). No significant difference were observed between j and l ($p > 0.05$), but k is statistically different from both j and l ($p < 0.001$).

	Fluorescence measurements (Center of mass, nm)			Enzyme inhibition (%)
Addition	Control[a]	Guanidinium chloride (2 M)[b]	% of control red-shift[c]	Guanidinium chloride (2 M)
None	333.7±0.4[d]	348.7±0.2[g]	100	99±2[j]
Trehalose (1 M)	334.2±0.2[e]	345.1±0.3[h]	72.7	83±1[k]
Glucose (2 M)	334.4±0.5[f]	345.6±0.3[i]	74.7	98±2[l]

ride. It can be seen that 1 M trehalose or 2 M glucose conferred almost the same protecting effect against pyrophosphatase unfolding induced by 2 M guanidinium (Table I).

The effects of guanidinium chloride on the pyrophosphatase activity were investigated. Table I also shows the effects of 2 M guanidinium chloride on the pyrophosphatase in the absence and in the presence of 1 M trehalose or 2 M glucose. The pyrophosphatase activity was inhibited by guanidinium chloride revealing a K_i of 0.15 M (Table II). K_i increases in the presence of 1 M trehalose (K_i = 0.57 M) and in the presence of 2 M glucose (K_i = 0.25 M) showing that these polyols attenuate the guanidinium chloride effects on the pyrophosphatase activity (Table II). However, different from the protection against the unfolding of the pyrophosphatase, the protection on the activity is much more pronounced with 1 M trehalose, where K_i increased 3.8 times, than with 2 M glucose where K_i just increased 1.7 times (Table II).

Table II. Inhibition constants for guanidinium chloride effects in pyrophosphatase activity. The values represent the means of five isolated experiments. The absolute value for pyrophosphate hydrolysis was 615 ± 15 nmol·μg^{-1}·min^{-1}. Experiments and fits were done as described under Materials and Methods. All differences were statistically significant showing a $p < 0.01$ (between a and b) and $p < 0.001$ (between a and c; and, b and c).

Addition	K_i [M]
None	0.15±0.01[a]
Glucose (2 M)	0.25±0.01[b]
Trehalose (1 M)	0.57±0.02[c]

Discussion

The protective role of organic solutes against biological damage by destabilizing agents has been attributed to their ability to preserve the structural and functional integrity of proteins, probably promoting compactation of the protein structure (Yancey *et al.*, 1982; Somero, 1986; Mashino and Fridovich, 1987; Jorge-Garcia *et al.*, 1988; Vieyra *et al.*, 1989, 1991; Lin and Timasheff, 1994; Coelho-Sampaio *et al.*, 1994; Sola-Penna *et al.*, 1995b). In this work, we show that trehalose, that is accumulated at high concentrations by yeasts when submitted to stress conditions (Crowe *et al.*, 1984; Wiemken, 1990), and glucose, the trehalose degradation product and primary energy source used by yeasts, are able to attenuate the unfolding of yeast cytosolic pyrophosphatase promoted by guanidinium chloride, a destabilizing agent (Robinson and Jencks, 1965).

Guanidinium chloride promoted a red shift in the average fluorescence emission wavelength of pyrophosphatase (Fig. 1) and an inhibition of pyrophosphatase activity (Table I). The red-shift in tryptophan fluorescence is indicative of increased exposure to a polar environment (Lakowicz, 1983), and likely indicates partial unfolding of the pyrophosphatase polypeptide chain in the presence of increasing concentrations of gua-

nidinium chloride. The red-shift and the activity inhibition promoted by guanidinium chloride on pyrophosphatase did not take a parallel pattern (Sola-Penna, M., Lemos, A.P., Ferreira-Pereira, A. and Meyer-Fernandes, J.R., unpublished observation), indicating that the activity inhibition observed is not just a function of protein unfolding, and probably involves other effects on the protein molecule not detected by intrinsic fluorescence measurements. This can explain the observation that although both 1 M trehalose and 2 M glucose protected, at almost the same extent, the unfolding promoted by 2 M guanidinium chloride on pyrophosphatase, only trehalose effectively attenuated the activity inhibition induced by this destabilizing agent. In this case we can postulate that the intrinsic properties of trehalose conferred it the capacity to prevent that modifications on pyrophosphatase that are not detectable by intrinsic fluorescence measurements but are essential to preserve the functional fitness. This data allow us to postulate that the modifications here observed on the intrinsic fluorescence spectra and the modulation on enzyme activity are not strictly coupled phenomena.

The protective role of trehalose is not just observed against chemical stresses, we had previously shown that this polyol is also the most effective preserving pyrophosphatase against thermal inactivation (Sola-Penna and Meyer-Fernandes, 1994). In addition, been protected specifically by trehalose is not a privilege of pyrophosphatase, Hottiger and co-workers (1994) had shown that other enzymes like glucose 6-phosphate dehydrogenase, and triose phosphate isomerase are also more effectively protected against high temperatures by trehalose than by other carbohydrates.

The capacity of several compounds, including sugars and guanidinium salts, to interact with soluble proteins was pointed out in several papers (Lee and Timasheff, 1981; Arakawa and Timasheff, 1982, 1984; Timasheff and Arakawa, 1989; Arakawa *et al.,* 1990). These authors demonstrated that carbohydrates and guanidinium chloride interact in different ways with proteins. While carbohydrates solubilize preferentially in the bulk water, a phenomenon that stabilizes protein structure (Arakawa and Timasheff, 1982, 1984; Timasheff and Arakawa, 1989; Arakawa *et al.,* 1990), guanidinium chloride solubilize preferentially in the viccinal water destabilizing protein shape (Arakawa and Timasheff, 1984; Timasheff and Arakawa, 1989; Arakawa *et al.,* 1990). Recently, a thermodynamic approach pointed out a reason for the simultaneous accumulation of urea (that, like guanidinium chloride, preferentially solubilize in viccinal water) and methylamines (that, like sugars, preferentially solubilize in bulk water) by some organisms (Lin and Timasheff, 1994). These authors demonstrated that the effects of urea and methylamines are thermodynamically additive, and since they are antagonists, the presence of both together neutralize the whole effect (Lin and Timasheff, 1994). In addition, we demonstrated that polyols that are accumulated by renal tissue in the same conditions that it accumulates urea, are able to counteract some damages promoted by urea and guanidinium chloride on this tissue cells function indicating a functional reason for the simultaneous accumulation of these compounds (Sola-Penna *et al.,* 1995b). These data can readily explain the observation that both trehalose and glucose are able to protect structure feature of pyrophosphatase against guanidinium-induced unfolding, once that sugars and guanidinium chloride are counteracting agents.

On the other hand, the different effectiveness of trehalose and glucose on preventing functional injuries caused by guanidinium chloride may be due to some differences in physical properties between the sugars tested (Duda and Stevens, 1990). Finally, this different effectiveness did not completely surprised us since this sugar has been shown to have special properties, being more effective on a series of effects like reversible inhibition of the pyrophosphatase activity (Sola-Penna and Meyer-Fernandes, 1994); uncoupling the plasma membrane calcium pump (Sola-Penna *et al.,* 1994) and protecting yeasts enzymes against thermal inactivation (Hottiger *et al.,* 1994; Sola-Penna and Meyer-Fernandes, 1994).

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