Carbohydrates Protect Mitochondrial F_0F_1-ATPase Complex against Thermal Inactivation

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Organisms and cellular systems are required to adapt to stress conditions like high temperature, often responding by accumulating organic solutes, such as sugars. This accumulation is associated with the effectiveness of these osmolytes in minimizing protein denaturation and membrane damage under stress conditions. In this work, we have studied the effect of sugars on the protection against thermal inactivation of mitochondrial F_0F_1-ATPase complex, in preparations of submitochondrial particles containing or depleted of inhibitor protein. We observed that after 15 min of pre-incubation at 70 °C of latent MgATP-submitochondrial particles (with inhibitor protein) in the presence of 1.5 M of sucrose or trehalose, or 3.0 M of glucose or fructose, about 80% of enzyme activity remained active. In the same conditions, but in the absence of sugars, the activity of the particles was completely abolished. Submitochondrial particles depleted of the inhibitor protein (AS-particles) were almost completely inactivated after 3 min of pre-incubation at 70 °C in the absence of sugars and more than 60% of the enzyme activity remained active when these particles were pre-incubated in the presence of sugars. In such condition, the enzyme acquires a more compact and heat-stable conformation. Sugars, as well as the inhibitor protein, inhibit reversibly F_0F_1-ATPase complex activity and protect this enzyme against inactivation by high temperature. Interestingly, the protection, promoted by sugars, of particles containing inhibitor protein is higher than of particles depleted of inhibitor protein, suggesting a synergism between sugar and inhibitor protein.

Introduction

Organisms have evolved in a wide variety of aqueous environments with large differences in osmolarity. Today, a number of organisms can be found living in a hypertonic or hypotonic environment. Organisms living in environments of extreme osmolarity have evolved the ability to accumulate cytosolic compounds, called osmolytes, which preserve them against the osmotic stress, such as freezing or dessication, to which they are subjected (Somero, 1995; Crowe *et al.*, 1998).

The osmolytes can be divided into two groups based on the effect they exert on the structure and/or function of proteins: the stabilizers, such as polyols or sugars, and the non-stabilizers, such as urea or guanidine hydrochloride (Vieyra *et al.*, 1991; Sola-Penna *et al.*, 1994; 1995; 1997). Timasheff (1993) suggested that sugars, in general, do not interact directly with proteins, preferentially solubilizing in bulk water in a phenomenon called preferential hydration of protein. Preferential hydration of protein postulates that in a triphasic system consisting of water, protein and a cosolvent that can be a sugar a stabilizer (cosolvent) is excluded from viccinal water that composes the solvation layer of protein. As a result, the protein becomes preferentially hydrated, but the radius of the solvation layer and the apparent volume of protein decreases, in a phenomenon that leads to a more stable protein conformation (Timasheff, 1993; Sola-Penna and Meyer-Fernandes, 1994; 1996; 1998; Felix *et al.*,

Abbreviations: F_0F_1-ATPase, adenosinetriphosphatase; IF_1, inhibitor protein; FCCP, carbonylcyanide-*p*-(trifluoromethoxy)phenylhydrazone; MgATP-SMP, submitochondrial particles containing endogenous inhibitor protein; AS-particles, ammonium-Sephadex submitochondrial particles depleted of inhibitor protein; Tris, tris (hydroxymethyl)aminomethane.

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1999; Lopes *et al.*, 1999). On the other hand, the non-stabilizer compounds would make a preferential interaction with the protein and it could mobilize a certain amount of water molecules of the bulk water, increasing the solvation layer. This phenomenon contributes to decrease the forces that stabilize the tertiary and quaternary structures of the protein, making it more sensitive to changes in the medium (Timasheff, 1993; Sola-Penna *et al.*, 1997; Lopes *et al.*, 1999).

Several conditions, such as high temperature, are able to inactivate proteins (Klibanov, 1983; Somero, 1995). Thermal inactivation of enzymes may be caused by temperature-induced conformational changes in the protein molecule. At elevated temperatures, cooperative intramolecular motions may occur until a temperature is reached where non-covalent forces that maintain the native structure of the protein can no longer prevail against the increase in entropy (Klibanov, 1983; Somero, 1995; Felix *et al.*, 1999). As a result, the protein loses most of its ordered secondary and tertiary structures and is denatured. The thermal stability of proteins can be changed by the addition of suitable stabilizing effectors, eg., osmolytes, coenzymes, membranes and peptides (Timasheff, 1993; Sola-Penna and Meyer-Fernandes, 1994; Somero, 1995; Saad-Nehme *et al.*, 1997). In this report we show the protection of mitochondrial F_0F_1-ATPase complex promoted by sugars as well as the effect these compounds exert on the hydrolytic activity and on the activation process of this enzyme.

Material and Methods

Protein preparations

Heavy beef-heart mitochondria and latent submitochondrial particles (MgATP-SMP), containing endogenous inhibitor protein of ATPase (IF_1), were prepared as described by Low and Vallin (1963). Fully active ammonium-Sephadex submitochondrial particles (AS-particles), depleted of the endogenous inhibitor protein, were prepared as described by Horstman and Racker (1970).

Activation of latent MgATP submitochondrial particles

Latent MgATP-SMP were pre-incubated at 42 °C in 25 mM Tris (tris (hydroxymethyl)aminomethane)-acetate (pH 8.0), 250 mM sucrose and 30 mM potassium acetate, for the different intervals of time. The enzyme activity was then assayed at 30 °C.

Thermal inactivation assay

MgATP- and ammonium-Sephadex submitochondrial particles were pre-incubated at 70 °C in 25 mM Tris-acetate (pH 8.0), 250 mM sucrose and 30 mM potassium acetate, for different intervals of time. The enzyme activity was then assayed at 30 °C.

ATPase activity assay

The ATPase activity of MgATP- and ammonium-Sephadex submitochondrial particles was determined according to Bernardes *et al.* (1997), by measuring the release of Pi from [γ-^{32}P]ATP (specific activity about 10^4 Bq/nmol ATP). Standard conditions for measuring ATPase activity were: 65 mM Tris-maleate buffer (pH 7.5), 5 mM ATP, 10 mM $MgCl_2$, 20 mM KCl, 0.2 mM EGTA, 1 μM FCCP, 10 μg/ml MgATP-submitochondrial particles or 10 μg/ml AS-particles, in a final volume of 0.5 ml at 30 °C. The reaction was started by the addition of 10 mM $MgCl_2$ after 5 min preincubation of the particles in the reaction medium and it was stopped after 5 min of hydrolysis, by adding 1 ml of activated charcoal (25 g% charcoal in 0.1 N HCl). The tubes were centrifuged at 1500 x *g* for 20 min at 4 °C and then 0.4 ml of the clear supernatant was dried on filter paper and counted in a scintillation counter. Data points show the means of triplicate determination from three different experiments with three different enzyme preparations. Standard errors were less than 10% in all cases and are not shown for clarity.

Protein concentrations

Concentrations of MgATP- and ammonium-Sephadex submitochondrial particles were determined using the Folin phenol reagent (Lowry *et al.*, 1951), using bovine serum albumin as standard.

Reagents

All reagents were purchased from Sigma Chemical Co., St. Louis, MO. ^{32}Pi was obtained from the Brazilian Institute of Energy and Nuclear Re-

search (São Paulo, S. P.). [γ-^{32}P]ATP was prepared as described by Glynn and Chappell (1964). Distilled water deionized by the MilliQ system of resins (Millipore Corp., Bedford, M. A.) was used in the preparation of all solutions.

Table I. Influence of carbohydrates on the ATPase activity of MgATP-SMP. ATPase activity of latent MgATP-SMP (0.5 mg/ml) was assayed as described under Material and Methods at 30 °C in the presence of the indicated concentrations of carbohydrates. The absolute value for 100% ATPase activity was 0.8 μmol Pi . mg^{-1} . min^{-1}. Values shown are the mean of triplicate determinations from three different experiments with three different enzyme preparations. Standard errors were in all cases less than 10% of absolute values.

Carbohydrate	Concentration [M]	% Activity
None	–	100
Sucrose	0.5	92.3
Sucrose	1.5	0
Trehalose	0.5	93.9
Trehalose	1.5	28.3
Glucose	0.5	94.8
Glucose	1.5	35.1
Glucose	3.0	0
Fructose	0.5	82.3
Fructose	1.5	67.9
Fructose	3.0	12.4

Results and Discussion

It can be seen that the ATPase activity of MgATP-SMP is inhibited, reversibly, with the incubation of the particles in the presence of increasing concentrations of sucrose, trehalose, glucose or fructose (Table I). However, the concentration of the monosaccharides that inhibit 50% of the maximal hydrolytic activity of the particles ($I_{0.5}$) is higher than that promoted by the disaccharides (sucrose, $I_{0.5}$ = 0.9 M; trehalose, $I_{0.5}$ = 1.0 M; glucose, $I_{0.5}$ = 1.4 M and fructose, $I_{0.5}$ = 2.1 M).

The ATPase activity of latent MgATP-SMP is low and by the incubation of the particles at temperatures over 16 °C this activity increases, probably due to the release of the inhibitor protein (IF_1) from its site (Béltran *et al.*, 1986). In Table II, we observe that when latent MgATP-SMP are pre-incubated at 42 °C in the presence of high concentration of sucrose, trehalose, glucose or fructose, the activation of the particles is suppressed. This suppression of the activation is reverted when the concentration of sucrose (Fig. 1A) or trehalose (Fig. 1B) is diluted 5-fold and the enzyme returns to undergo activation. However, in the presence of glucose (Fig. 1C) or fructose (Fig. 1D), even af-

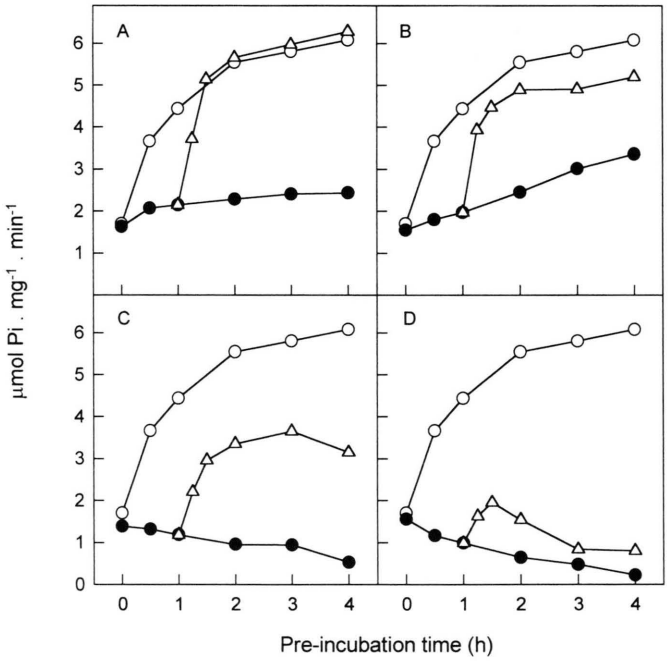

Fig. 1. The influence of the dilution of carbohydrates during pre-incubation of latent MgATP-SMP at 42 °C. Latent MgATP-SMP were activated as described under Material and Methods at 42 °C. Latent MgATP-SMP (0.5 mg/ml) were pre-incubated for the times indicated in the abscissa in the absence (○), or in the presence (●) of: (A) 1.5 M sucrose; (B) 1.5 M trehalose; (C) 3.0 M glucose; or (D) 3.0 M fructose. After 1-hour pre-incubation one aliquot of each sample in the medium containing sugar was withdrawn and diluted 5-fold into the activation buffer (△) (see Methods), except fructose (D) which was 10-fold diluted; then, the samples returned to pre-incubation at 42 °C for the times indicated in the abscissa. After pre-incubation, the samples that were firstly diluted in the activation buffer were diluted 10-fold into the assay medium (except in the presence of fructose which was diluted 5-fold) and the samples that were not diluted in the activation buffer were 50-fold diluted in the assay medium. The activity was measured at 30 °C (see Methods). Values shown are the mean of triplicate determinations from three different experiments with three different enzyme preparations. Standard errors were less than 10% in all cases and are not shown for clarity.

Table II. Influence of carbohydrates on the activation of latent MgATP-SMP. MgATP-SMP (0.5 mg/ml) were activated as described under Material and Methods at 42 °C. After pre-incubation, the samples were diluted 50-fold into the assay medium and the activity measured at 30 °C (see Methods). Values shown are the mean of triplicate determinations from three different experiments with three different enzyme preparations. Standard errors were less than 10% in all cases.

Pre-incubation time [h]	Carbohydrate	Concentration [M]	ATPase activity [μmol Pi . mg^{-1} . min^{-1}]
0	none	–	1.69
1	none	–	4.44
4	none	–	6.08
0	sucrose	1.5	1.67
1	sucrose	1.5	2.30
4	sucrose	1.5	2.41
0	trehalose	1.5	1.66
1	trehalose	1.5	1.84
4	trehalose	1.5	3.16
0	glucose	3.0	1.78
1	glucose	3.0	2.26
4	glucose	3.0	1.56
0	fructose	3.0	1.49
1	fructose	3.0	1.23
4	fructose	3.0	0.81

Table III. The influence of carbohydrates on the time course of thermal inactivation of AS-particles at 70 °C. AS-particles (0.5 mg/ml) were pre-incubated as described under Material and Methods at 70 °C for different times in the presence of the indicated concentrations of carbohydrates. After pre-incubation, samples were diluted 50-fold into the assay medium and the activity measured at 30 °C (see Methods). 100% activity corresponds to 7.0 μmol Pi . mg^{-1} . min^{-1}. Values shown are the mean of triplicate determinations from three different experiments with three different enzyme preparations. Standard errors were less than 10% in all cases.

Pre-incubation time [min]	Carbohydrate	Concentration [M]	% Activity
0	none	–	100
3	none	–	0.8
15	none	–	0
0	sucrose	1.5	100
3	sucrose	1.5	63.8
15	sucrose	1.5	29.8
0	trehalose	1.5	100
3	trehalose	1.5	65.8
15	trehalose	1.5	12.5
0	glucose	3.0	100
3	glucose	3.0	63.1
15	glucose	3.0	24.7
0	fructose	3.0	100
3	fructose	3.0	60.5
15	fructose	3.0	6.8

ter a 5-fold or a 10-fold dilution, respectively, the enzyme did not revert to activation. Since activation of the latent particles is directly involved with the release of the natural inhibitor protein (Béltran *et al.*, 1986), we believe that sugars could interact with F_0F_1-ATPase complex and so impeach the release of the inhibitor protein.

In order to exclude the possibility that the inhibitor protein itself could be protecting the enzyme against high temperature, a preparation of submitochondrial particles depleted of inhibitor protein (AS-particles) was used. In Table III we observe that AS-particles, in the absence of sugars, were almost totally inactivated after a 3-min pre-incuba-

tion at 70 °C. However, when these particles were pre-incubated in the presence of sucrose, trehalose, glucose or fructose, it retained more than 60% of ATPase activity. In Figure 2 we observe that submitochondrial particles containing inhibitor protein, in the absence of sugars (open circles), were totally inactivated only after a 15-min pre-incubation at 70 °C, like our previous data (Saad-Nehme *et al.*, 1997) about the contribution of the IF_1 to the thermal stability of the F_0F_1-ATPase complex. Now, these particles, after a 15-min pre-incubation at 70 °C in the presence of sucrose (panel A), trehalose (panel B), glucose (panel C) or fructose (panel D) (closed circles), remained about 80% of ATPase activity. In addition, a pronounced activation of the particles (about 50%) was observed in the first minutes of pre-incubation, possibly reflecting the release of the inhibitor protein, and, after its release, the particles become more sensitive to the temperature and start to inactivate (Saad-Nehme *et al.*, 1997). We have previously shown that IF_1 protects F_0F_1-ATPase against thermal (Saad-Nehme *et al.*, 1997) and pressure (Fornells *et al.*, 1998) inactivation. The higher protection promoted by sugars to the latent particles might be due to the ability of sugars to keep the inhibitor protein bound to the enzyme for a longer time, and both, synergistically, protect mitochondrial F_0F_1-ATPase against thermal inactivation.

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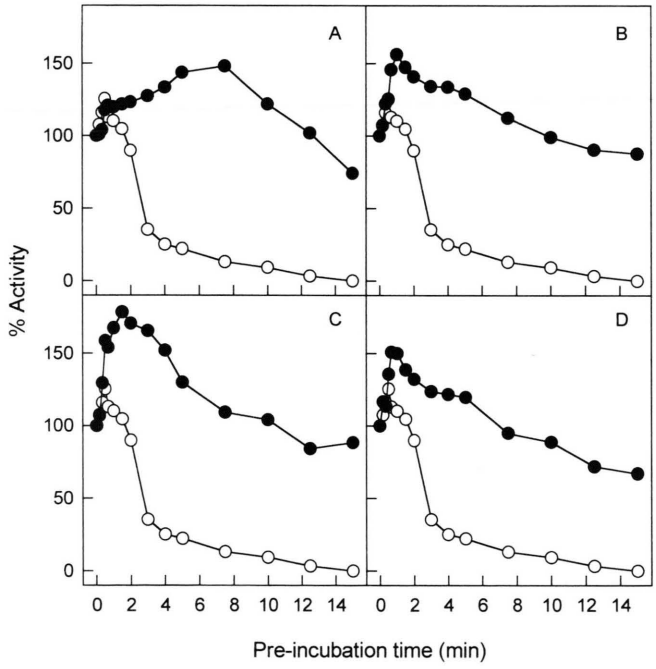

Fig. 2. The influence of carbohydrates on the time course of thermal inactivation of latent MgATP-SMP at 70 °C. MgATP-SMP were pre-incubated as described under Material and Methods at 70 °C. MgATP-SMP (0.5 mg/ml) were pre-incubated for the times indicated in the abscissa in the absence (○), or in the presence (●) of: (A) 1.5 M sucrose; (B) 1.5 M trehalose; (C) 3.0 M glucose; or (D) 3.0 M fructose. After pre-incubation, the samples were diluted 50-fold into the assay medium and the activity measured at 30 °C (see Methods). 100% activity corresponds to 0.8 μmol Pi . mg^{-1} . min^{-1}. Values shown are the mean of triplicate determinations from three different experiments with three different enzyme preparations. Standard errors were less than 10% in all cases and are not shown for clarity.

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