Effects of Naturally Occurring Polyols and Urea on Mitochondrial F_0F_1ATPase

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We show that urea inhibits the ATPase activity of MgATP submitochondrial particles (MgATP-SMP) with Ki = 0.7 M, probably as a result of direct interaction with the structure of F_0F_1-ATPase. Counteracting compounds (sorbitol, mannitol or inositol), despite slightly (10–20%) inhibiting the ATPase activity, also protect the F_0F_1-ATPase against denaturation by urea. However, this protection was only observed at low urea concentrations (less than 1.5 M), and in the presence of three polyols, the K_i for urea shift from 0.7 M to 1.2 M. Urea also increases the initial activation rate of latent MgATP-SMP in a dose-dependent-manner. However, when the particles (0.5 mg/ml) were preincubated in the presence of 1 M, 2 M or 3 M urea, a decrease in the activation level occurred after 1 h, 30 and 10 min, respectively. At high MgATP-SMP concentration (3 mg/ml) a decrease in activation was observed after 2 h, 1 h and 20 min, respectively. These data indicate that the effect of urea on the activation of MgATP-SMP depends on time, urea and protein concentrations. It was also observed that polyols suppress the activation of latent MgATP-SMP in a dose-dependent manner, and protect the particles against urea denaturation during activation. We suppose that a decrease in membrane mobility promoted by interactions of polyols with phospholipids around the F_0F_1-ATPase may also increase the compactation of protein structure, explaining the inhibition of natural inhibitor protein of ATPase (IF_1) release and the activation of the enzyme.

Introduction

The mammalian kidney and all tissues of cartilaginous fishes accumulate urea under specific conditions (Yancey *et al.*, 1982; Somero, 1995). The accumulation of high concentrations of urea alone (0.4–0.9 M) (Knepper *et al.*, 1991) would be lethal to these tissues. However, almost in parallel with the increase in urea, counteracting compounds such as polyols, especially sorbitol, mannitol and inositol (Bagnasco *et al.*, 1986), also accumulate in these tissues. It is of interest that these osmolytes are the most effective among a series of polyhydric alcohols that stabilize oligomeric enzymes and enhance the reassembly of denatured subunits in the presence of high urea concentrations (Vieyra *et al.*, 1991; Lopes *et al.*, 1999).

Abbreviations: EGTA, (ethylene-bis[oxyethylenenitrilo]) tetraacetic acid; FCCP, carbonylcyanide-*p*-(trifluromethoxy)phenylhydrazone; F_0F_1-ATPase, adenosinetriphosphatase; IF_1, natural inhibitor protein of ATPase; MgATP-SMP, submitochondrial particles containing inhibitor protein; Tris, tris(hydroxymethyl)aminomethane.

The F_0F_1-ATPase (ATP synthase complex, H^+-ATPase) of submitochondrial particles from bovine heart catalyzes the synthesis of ATP coupled to an electrochemical gradient of H^+ as well to reversed reaction, hydrolysis of ATP (Amzel and Pedersen, 1983; Boyer, 1993). The enzyme is composed of an H^+-conducting F_0 sector that permits the transport of protons generated by the respiratory chain to the soluble factor F_1 (Amzel and Pedersen, 1983; Boyer, 1993). The F_1 sector is extramembranous; water soluble in isolated form, and is composed of five types of subunits in a stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ (Boyer, 1993) and exhibits catalytic activity of hydrolysis. Preparations of the F_0F_1-ATPase also contain substoichiometric amounts of the ATPase inhibitor protein (IF_1). In intact mitochondria, there is 1 mol of IF_1/mol of F_1 (Boyer, 1993; Walker, 1994).

First discovered and isolated from bovine heart mitochondria by Pullman and Monroy (1963), IF_1 is a water-soluble, acid- and heat-stable, trypsin-sensitive protein of low molecular weight (10 kDa) (Frangione *et al.*, 1981; Galante *et al.*, 1981;

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Schwerzmann and Pedersen, 1986). Early data (Beltrán *et al.*, 1986) showed that the low level of ATP hydrolysis (0.4–0.6 μmol Pi . mg^{-1} . min^{-1}) found in MgATP-submitochondrial particles (latent particles) is related to the presence of IF_1. The ATPase activity of latent particles can be increased (6–8 μmol Pi . mg^{-1} . min^{-1}) by preincubation of these particles at temperatures ranging from 16 to 43 °C, at high ionic strength or at alkaline pH (Beltrán *et al.*, 1986).

As recently reviewed by Walker (1994), the down-regulation of F_0F_1-ATPase by IF_1 in mitochondria has a crucial role in preventing the futile expenditure of energy. It is proposed that IF_1 binds to a specific domain in the β-subunit, the same one that provides a major contact between the catalytic β-subunit and the central α-helical coiled-coil structure in the γ-subunit (Walker, 1994). Recently we also demonstrated that the inhibitor protein protects mitochondrial F_0F_1-ATPase against thermal denaturation (Saad-Nehme *et al.,* 1997).

This paper presents the results of experiments involving polyols and urea on modulation of ATP hydrolysis and the activation of the mitochondrial F_0F_1-ATPase.

Material and Methods

Biological preparations

"Heavy" mitochondria isolated from fresh bovine heart according to the procedure of Low and Vallin (1963) were used for the preparation of latent MgATP-SMP as described by Ernster *et al.* (1979), by sonication in the presence of 6 mM ATP, 6 mM $MgSO_4$ and 250 mM sucrose, at pH 7.4. MgATP-SMP were stored in liquid nitrogen until use.

Activation of MgATP-SMP

Latent MgATP-SMP were activated by preincubation at 42 °C (Beltrán *et al.*, 1986) in buffer containing 25 mM Tris-acetate and 30 mM potassium acetate, pH 8.0, during 4 h.

ATPase activity assay

The ATPase activity of MgATP-SMP was determined according to Bernardes *et al.* (1997), by measuring the release of Pi from [γ^{32}P]ATP (specific activity ≈ 10^4 Bq/nmol ATP). The ATPase activity was assayed at 30 °C in the reaction medium containing 65 mM Tris-maleate, pH 7.5, 5 mM ATP, 10 mM $MgCl_2$, 20 mM KCl, 0.2 mM EGTA, 1 μM FCCP and 10 μg/ml MgATP-SMP, in a total volume of 0.5 ml. After 5 min preincubation of the particles in the reaction medium, it was started by the addition of 10 mM $MgCl_2$ and stopped after 5 min by adding 1 ml of activated charcoal (25 g% charcoal in 0.1 N HCl). The tubes were centrifuged at 1500×*g* for 20 min at 4 °C and an aliquot of 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 determinations from two different experiments with two different enzyme preparations. Standard errors were less than 10% in all cases and are not shown for clarity.

Protein concentrations

Concentrations of MgATP-SMP were determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Reagents

ATP, FCCP, Tris, sorbitol, mannitol, inositol and urea were purchased from Sigma Chemical Co. (St. Louis, MO). ^{32}Pi was obtained from the Brazilian Institute of Energy and Nuclear Research (São Paulo, S. P.). [γ-^{32}P]ATP was prepared as described by Glynn and Chappell (1964). Other reagents were of the highest purity available. Distilled water deionized by the MilliQ system of resins (Millipore Corp., Bedford, MA) was used in the preparation of all solutions.

Results and Discussion

Combined effects of urea and polyols on ATPase activity catalyzed by MgATP-SMP

Figure 1A shows that urea (open circles) inhibited the ATPase activity catalyzed by latent MgATP-SMP in a dose-dependent manner (K_i ≈ 0.7 M). The addition of sorbitol (close circles) in the reaction medium protected the MgATP-SMP from the inhibition by urea. This protection was observed at low urea concentrations (less than 1.5 M) and, in the presence of sorbitol, the K_i for urea shifted from 0.7 M to 1.2 M. Similar results were

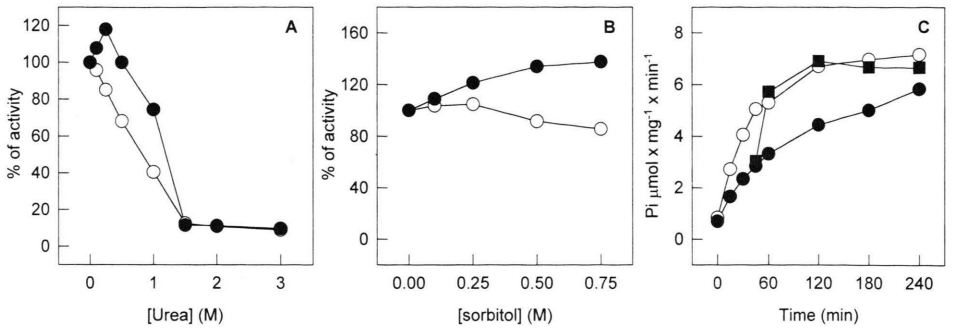

Fig. 1. Effects of sorbitol and urea on the ATPase activity and on the activation of the MgATP submitochondrial particles. (A) sorbitol protects against urea inhibition of latent MgATP-SMP ATPase activity. The reaction was performed in the medium described in Material and Methods in the presence of concentrations of urea varying as shown on the abscissa. The absolute enzyme activities measured in the absence of urea were 0.65 µmol Pi . mg^{-1} . min^{-1} for control experiments in the absence of sorbitol (open circle) and 0.54 µmol Pi . mg^{-1} . min^{-1} in the presence of 0.75 M sorbitol (closed circle). (B) Counteracting effects of urea on the inhibition promoted by sorbitol. The reaction was performed in the medium described in Material and Methods in the presence of concentrations of sorbitol varying as shown on the abscissa. The absolute enzyme activities were 0.65 µmol Pi . mg^{-1} . min^{-1} for control experiments in the absence of urea (open circle) and 0.44 µmol Pi . mg^{-1} . min^{-1} for the experiments in the presence of 0.5 M urea (closed circle). (C) The influence of sorbitol during time course of activation of the MgATP-SMP. MgATP-SMP (0.5 mg/ml) were activated as described in Material and Methods in the absence (○) or in the presence of (●) 1 M sorbitol. After 45 min preincubation at 42 °C one aliquot of each sample in the medium containing sorbitol was withdrawn and diluted 10-fold (■) into the activation buffer. At the times indicated on the abscissa the ATPase activity was measured. Standard errors were less than 10% in all cases and are not shown for clarity.

obtained when sorbitol was replaced by mannitol or inositol (data not shown).

It has been suggested that the use of stabilizing compounds alone might be as deleterious as the use of urea alone because high concentrations of polyols could make some proteins too rigid to function effectively (Yancey *et al.*, 1982). Figure 1B (open circles) shows that sorbitol, alone inhibited only slightly (10–20%) the ATPase activity catalyzed by latent MgATP-SMP. Note that, in the presence of a low urea concentration (0.5 M), sorbitol caused an increase in the MgATP-SMP ATPase activity (Fig. 1B, closed circles). Similar results were obtained when sorbitol was replaced by mannitol or inositol (data not shown).

Effects of polyols and urea on the activation of the MgATP-SMP

Figure 1C shows the time course of activation of latent MgATP-SMP at 42 °C (open circles). The addition of sorbitol (close circles) decreased this activation, but this effect was fully reversed when the preincubation medium containing sorbitol was diluted 10-fold with sorbitol-free mixture (close squares). Similar results were obtained when sorbitol was replaced by mannitol or inositol (data not shown). These polyols decreased the initial activation rate of latent MgATP-SMP in a dose-dependent manner (Fig. 2).

This activation process related to release of IF_1 (Saad-Nehme *et al.*, 1997) was dependent on mitochondrial protein concentration (Fig. 3). At a low protein concentration (0.15 mg/ml, open circles), the maximal activation was reached after 1 h of preincubation (Fig. 3A, open circles), and at a high protein concentration (3 mg/ml, open squares) the maximal activation was reached after 3 h. However, when latent MgATP-SMPs (0.15 mg/ml) were preincubated in the presence of 0.35 mg/ml (Fig. 3B, closed circles) or 2.85 mg/ml albumin (Fig. 3B, open squares), the maximal activation was reached in 1 h. These data indicated that osmolytes are able to promote compactation of the protein structure and then contribute to formation of enzyme aggregates and may modulate the release of the inhibitor protein.

Urea increased the initial activation rate of latent MgATP-SMP in a dose-dependent manner (Fig. 4). However, when the particles (0.5 mg/ml)

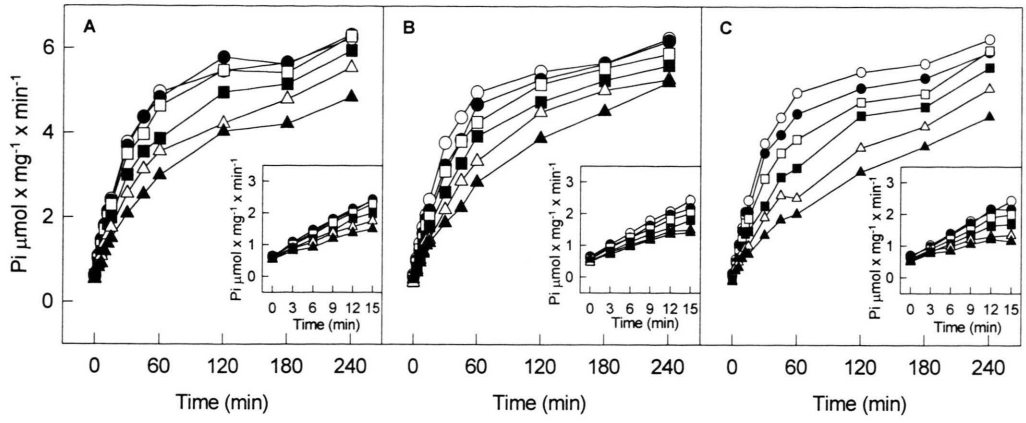

Fig. 2. Effect of polyols on the activation of the MgATP-SMP. MgATP-SMP (0.5 mg/ml) were activated as described in Material and Methods in the absence (○) or in the presence of (●) 0.1 M, (□) 0.25 M, (■) 0.5 M, (△) 0.75 M or (▲) 1 M sorbitol (panel A), mannitol (panel B) or inositol (panel C). *Insets:* initial rates of activation (expanded time scale). Standard errors were less than 10% in all cases and are not shown for clarity.

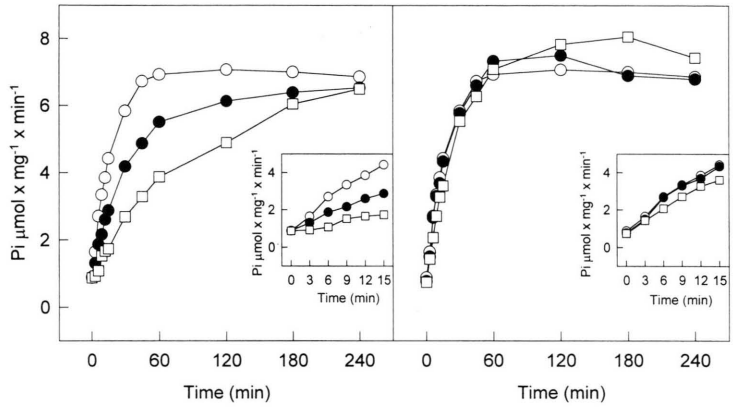

Fig. 3. Dependence on protein concentration on the activation of latent MgATP-SMP. MgATP-SMP were activated as described in Material and Methods. At the times indicated on the abscissa, the ATPase activity was measured. (A) major panel: (○) 0.15 mg/ml, (●) 0.5 mg/ml or (□) 3 mg/ml. *Inset:* initial rates of activation (expanded time scale).(B) Major panel: MgATP-SMP (0.15 mg/ml) were suspended in buffer described in Material and Methods and activated in the absence (○) or in the presence of (●) 0.35 mg/ml or (□) 2.85 mg/ml albumin. *Inset:* initial rates of activation (expanded time scale). Standard errors were less than 10% in all cases and are not shown for clarity.

were preincubated in the presence of 1 M, 2 M or 3 M urea (Fig. 4A), a decrease in the activation level was observed after 1 h, 30 min and 10 min, respectively. This inhibitory effect observed in the presence of 3 M urea was abolished when a mixture containing 3 M urea was diluted 30-fold with urea-free medium after incubation for 3 min, but not after incubation for 45 min (data not shown). This phenomenon may be related to denaturation of the enzyme.

Interestingly, when high MgATP-SMP concentrations (3 mg/ml) were preincubated in the presence of the same urea concentrations (Fig. 4B), the inhibition of activation was observed only after 2 h, 1 h and 20 min in the presence of 1 M, 2 M, and 3 M urea, respectively. These data show that urea denaturing effect on the activation of MgATP-SMP is time-, urea- and protein concentration-dependent. When latent MgATP-SMP (0.5 mg/ml) was preincubated in the presence of sorbitol and urea, the inhibition observed in the presence of urea at 1 M (Figs. 5A and 5C, closed circles) or at 3 M (Figs. 5B and 5D, closed circles) was minimized and the protection promoted by

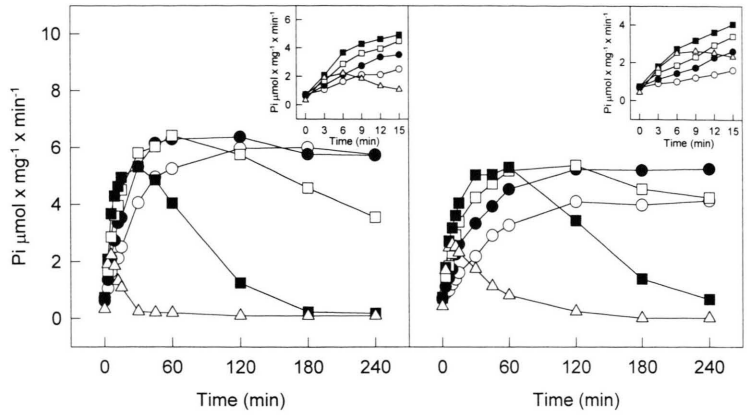

Fig. 4. Time course of activation of the MgATP-SMP. Dependence on urea concentration. MgATP-SMP (0.5 mg/ml, panel A or 3 mg/ml, panel B) were activated as described in Material and Methods in the absence (○) or in the presence of (●) 0.5 M, (□) 1 M, (■) 2 M or (△) 3 M of urea. *Inset:* rates of activation (expanded time scale). Standard errors were less than 10% in all cases and are not shown for clarity.

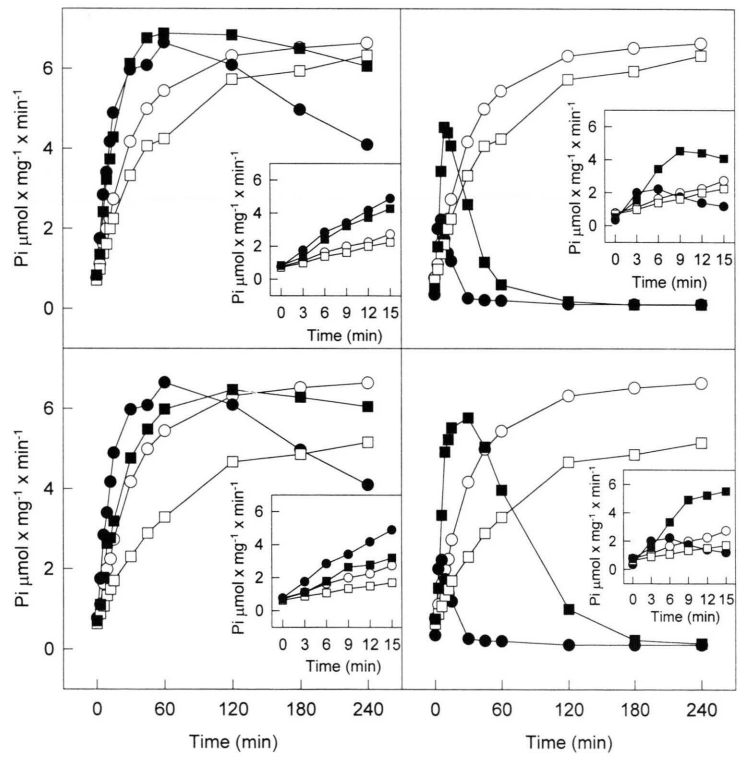

Fig. 5. Counteracting effects of sorbitol and urea in the time course of activation of the MgATP-SMP. Major panel: MgATP-SMP (0.5 mg/ml) were activated as described in Material and Methods in the absence (○) or in the presence of (●) 1 M (panels A and C) or 3 M (panels B and D) urea, (□) 0.5 M (panels A and B) or 1 M (panels C and D) sorbitol, (■) 0.5 M sorbitol plus 1 M (panel A) or 3 M urea (panel B) and 1 M sorbitol puls 1 M (panel C) or 3 M urea (panel D). *Insets:* initial times of activation of the MgATP-SMP in the same conditions described for the respective major panels. Standard errors were less than 10% in all cases and are not shown for clarity.

sorbitol was dose-dependent. The same protection observed in the presence of sorbitol was observed in the presence of mannitol or inositol (data not shown).

It has been demonstrated that some osmolytes protect against the effects of urea and guanidinium chloride on the function of different enzymes (Dreyfus and De Meis, 1989; Vieyra *et al.*, 1991; Sola-Penna and Meyer-Fernandes, 1996; Sola-Penna *et al.*, 1994, 1995, 1997; Felix *et al.,* 1999). We have previously shown that the osmolytes that protect against the effects of urea on membrane-bound (Ca^{2+} + Mg^{2+})ATPases from sarcoplasmic reticulum and renal plasma membrane also modulate independently the catalytic cycle of these Ca^{2+}-transporting ATPases (Chini *et al.*, 1991; Vieyra *et al.*, 1991; Sola-Penna *et al.*, 1994, 1995). It has been postulated that they modify the equilibrium between the different conformations of the enzyme (Chini *et al.*, 1991), and that they may exert their influence through a decrease in water activity and in protein solvation (Chini *et al.*, 1991; Sola-Penna and Meyer-Fernandes, 1994, 1998).

In this paper we show that urea inhibits the MgATP-SMP ATPase activity (Fig. 1A). This phenomenon is probably a result of a direct interaction of urea with the structure of F_0F_1-ATPase (Figs 4 and 5). The hydrogen bonds between this compound and proteins are stronger than those between water and proteins (Makhatadze and Privalov, 1992). In this way urea destabilizes intramolecular hydrogen bonding and causes unfolding of proteins (Makhatadze and Privalov, 1992). Despite its ability to perturb the structure and assembly of proteins, urea is accumulated by some organisms as a major osmolyte (Yancey *et al.*, 1982; Somero, 1995). Nature's strategy for counteracting urea effects is thought to be the co-accumulation of compounds such as polyols that counterbalance the effects of this perturbing osmolyte (Yancey *et al.*, 1982; Somero, 1995). Here we show that counteracting compounds (sorbitol, mannitol and inositol), despite slightly (10–20%) inhibiting the ATPase activity, also protect the F_0F_1-ATPase from deleterious urea effects (Figs. 1A, 1B and 5). The finding that polyols protect the F_0F_1-ATPase from the effects of urea in a manner similar to that observed with organic solvents (Dreyfus and De Meis, 1989) may indicate that these osmolytes, by promoting changes in water structure (Timasheff, 1993; Sola-Penna and Meyer-Fernandes, 1998) can contribute to protection of the cell from the deleterious effect of urea.

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