A Contribution of the Mitochondrial Adenosinetriphosphatase Inhibitor Protein to the Thermal Stability of the F_0F_1-ATPase Complex

Jorge Saad-Nehme, Andre L. Bezerra, Luz Alba M. Fornells, Jerson L. Silva and José R. Meyer-Fernandes

Departamento de Bioquímica Médica, Universidade Federal do Rio de Janeiro, 21941–590, Rio de Janeiro, RJ, Brazil

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Thermal Stability, Mitochondrial Adenosinetriphosphatase Inhibitor Protein, F_0F_1-ATPase Complex

A complete inactivation is observed after a 3 min pre-incubation at 70 °C with mitochondrial F_0F_1-ATPase complex depleted of the ATPase natural inhibitor protein (ammonium-Sephadex submitochondrial particles) and activated MgATP-submitochondrial particles (particles that after a 4 h-pre-incubation at 42 °C released the endogenous inhibitor protein). However, latent MgATP-submitochondrial particles (particles containing the inhibitor protein) pre-incubated under the same conditions are totally inactivated only after 15 min of pre-incubation. When ammonium-Sephadex particles are reconstituted with 20 μg/ml of purified ATPase inhibitor protein there is an increase of 15-fold in the half-time for thermal inactivation ($t_{0.5}$), showing that the inhibitor protein protects the mitochondrial F_0F_1-ATPase complex against thermal inactivation.

Introduction

The question of why proteins are stable is of fundamental importance in biochemistry. It is central to understanding the relationship between amino acid sequence and three-dimensional structure, and ultimately in the prediction of a protein's structure from its sequence (Klibanov, 1983; Tanner *et al.,* 1996). Thermal inactivation of enzymes is caused by temperature-induced conformational transitions in the protein molecule (Klibanov, 1983; Zaks and Klibanov, 1984; Ahern and Klibanov, 1985; Somero, 1995; Tanner *et al.,* 1996). At elevated temperatures, cooperative intramolecular motions occur until a temperature is reached where noncovalent forces that maintain the native structure of the protein can no longer prevail against the increase in entropy (Klibanov, 1983; Zaks and Klibanov, 1984; Ahern and Klibanov, 1985; Somero, 1995; Tanner *et al.,* 1996). As a result, the protein loses most of its ordered secondary and tertiary structures, so the protein is denatured. When a protein unfolds, hydrophobic regions that were located in the interior become exposed to the solvent, a situation that is thermodynamically unfavorable (Klibanov, 1983). The thermal stability of a protein can be changed intrinsically by alteration of amino acids or extrinsically by the additions of suitable stabilizing effectors, eg., osmolytes, coenzymes, membranes and peptides (Argos *et al.,* 1979; Timasheff, 1992; Sola-Penna and Meyer-Fernandes, 1994; Somero, 1995).

The ATP synthase complex (H^+-ATPase, F_1F_0-ATPase) of submitocondrial particles from bovine heart catalyzes the synthesis of ATP coupled to an electrochemical gradient of H^+ as well the reversed reaction, hydrolysis of ATP. The enzyme is composed of a 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; Hatefi, 1985; Senior, 1988; Boyer, 1993). The F_1 sector is extramembranous, water soluble in isolated form, and is composed of five types of subunits in a stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$ (Hek-

Abbreviations: AS, ammonium-Sephadex submitochondrial particles; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, (ethylene-bis[oxyethylenenitrilo]) tetraacetic acid; FCCP, carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone; IF_1, mitochondrial ATPase inhibitor protein; Mes, 2-(N-Morpholino)-ethanesulfonic acid Tris, tris(hydroxymethyl)aminomethane.

Reprint requests to Dr. José R. Meyer-Fernandes, Department of Biochemistry, Biological Sciences, West Building, University of Arizona, Tucson, Arizona 85721 U.S.A.
Fax: 520-6219288.

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man *et al.,* 1991) and exhibits catalytic activity of hydrolysis. Preparations of the ATP synthase complex 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 (Hekman *et al.,* 1991; Abrahams *et al.,* 1994; Walker, 1994; Belogrudov *et al.,* 1995).

The mitochondrial ATPase inhibitor protein (IF_1), first discovered and isolated from bovine heart mitochondria by Pullman and Monroy (1963), is a water-soluble, acid- and heat-stable, trypsin-sensitive protein with a low molecular weight (10 kDa) (Asami *et al.,* 1970; Galante *et al.,* 1981; Frangione *et al.,* 1981; Schwerzmann and Pedersen, 1986). Similar inhibitor proteins have been isolated from rat liver mitochondria (Cintron and Pedersen, 1979), chloroplasts (Nelson *et al.,* 1972), *Candida utilis* (Satre *et al.,* 1975), and *Escherichia coli* (Nieuwenhuis *et al.,* 1974). It has been suggested that the ATPase inhibitor protein might be a physiological regulator of ATPase activity, that plays an active role in the mechanism of energy conservation in mitochondria by preventing the hydrolysis of newly synthesized ATP (Amzel and Pedersen, 1983; Tuena de Gómes-Puyou *et al.,* 1983; Schwerzmann and Pedersen, 1986). IF_1 also protects against cold inactivation of soluble F_1 ATPase (Horstman and Racker, 1970), but it has not been established its role in increasing the thermal stability when it is bound to the F_1-ATPase complex.

In this work, we study the role of the ATPase inhibitor protein as a thermal stabilizer of the mitochondrial F_1-ATPase complex, using preparations of submitochondrial particles with or without inhibitor protein.

Material and Methods

Biological preparations

Mitochondria were isolated from fresh bovine heart according to the procedure of Low and Vallin (1963). "Heavy" mitochondria stored at −70 °C were used for the preparation of MgATP- and ammonium-Sephadex submitochondrial particles. MgATP-submitochondrial particles were prepared 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. Ammonium-Sephadex submitochondrial particles (particles depleted of the endogenous inhibitor protein) were prepared as described by Horstman and Racker (1970), by sonication in the presence of 2 mM EDTA and 250 mM sucrose, at pH 9.2. The ATPase inhibitor protein was removed by passing submitochondrial particles through a column (35×2.5 cm), containing Sephadex G-50 medium gel, equilibrated with pH 8.0 elution buffer containing 30 mM Tris-SO_4, 2 mM EDTA, 250 mM KCl and 75 mM sucrose, at 30 °C. MgATP- and ammonium-Sephadex submitochondrial particles were stored in liquid nitrogen until use. ATPase inhibitor protein was isolated and purified from bovine heart mitochondria as described by Horstman and Racker (1970), using alkaline extraction, fractionation with ammonium sulfate, precipitation with trichloroacetic acid, precipitation with ethanol and heat treatment.

Activation of MgATP submitochondrial particles

The ATPase activity of the MgATP-submitochondrial particles is low (0.5–1.2 μmol $Pi \cdot min^{-1} \cdot mg^{-1}$), but it can be increased by pre-incubation of these latent particles at temperatures ranging from 16 to 43 °C, at high ionic strength or at alkaline pH (Béltran *et al.,* 1986). After activation at 42 °C in pH 8.0 activation buffer containing 25 mM Tris-acetate, 250 mM sucrose and 30 mM potassium acetate, during 4 h, their hydrolytic activity was 5.0–7.0 μmol $Pi \cdot min^{-1} \cdot mg^{-1}$, at 30 °C.

Reconstitution of Ammonium-Sephadex particles with purified ATPase inhibitor protein

The productive binding of the ATPase inhibitor protein to the F_1-ATPase complex requires several minutes' incubation in a slightly acid medium, in the presence of MgATP (Schwerzmann and Pedersen, 1986). Ammonium-Sephadex submitochondrial particles (1 mg/ml) were reconstituted with different amounts of purified ATPase inhibitor protein (1, 5, 10 or 20 μg/ml) in the presence of 10 mM Mes-Tris buffer at pH 6.5. The particles were incubated with the inhibitor protein at 30 °C for 15 min in the presence of 3 mM MgATP and then placed in an ice-bath to stop the reconstitution.

ATPase activity assay

The ATPase activity of MgATP- and ammonium-Sephadex submitochondrial particles was

determined according to Vercesi *et al.* (1990), by measuring the release of Pi from [γ-^{32}P]ATP (specific activity about 10^4 Bq/nmol ATP). The reaction medium contained 65 mM Tris-maleate, 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 total volume of 0.5 ml. The ATPase activity was assayed at 30 °C. The reaction was started by the addition of 10 mM $MgCl_2$ after 5 min pre-incubation 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 rpm 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.

Protein concentrations

Concentrations of MgATP- and ammonium-Sephadex submitochondrial particles were determined using the Folin phenol reagent (Lowry *et al.*, 1951), and bovine serum albumin as standard. Concentration of the ATPase inhibitor protein was determined by the method of Bradford (1976), using Coomassie Blue.

Reagents

All reagents were purchased from Sigma Chemical Co., St. Louis, MO. [γ-^{32}P]ATP was prepared as described by Glynn and Chappel (1964).

Results

Reconstitution of AS-submitochondrial particles with purified ATPase inhibitor protein

Incubation of ammonium-Sephadex submitochondrial particles with different concentrations of inhibitor protein in the presence of MgATP led to a progressive inhibition of ATPase activity. Maximal inhibition (about 80%) was observed with 5–20 μg/ml of the inhibitor protein (Fig. 1A). This inhibition was reversible, since these particles reconstituted with the inhibitor protein were activated in the same way as latent MgATP-submitochondrial particles, when pre-incubated at 42 °C for 4 h (Fig. 1B). This activation process must be related to release of the inhibitor protein, since no activation could be seen with AS-particles (depleted of inhibitor protein) under the same conditions (○) (Fig. 1B).

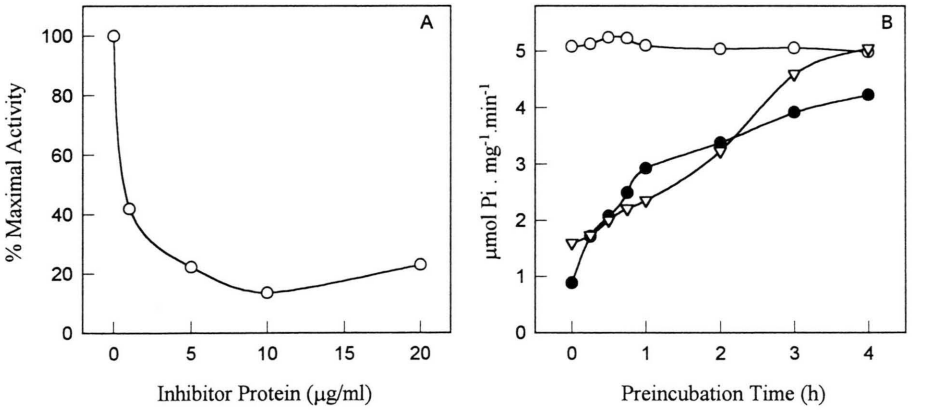

Fig. 1. Inhibition of AS-particles (A) and activation of MgATP-particles, AS-particles and inhibited AS-particles (B). (A) AS-particles (1 mg/ml) were reconstituted with inhibitor protein at the concentrations indicated on the abscissa, as described in Methods (100% maximal activity = 5.2 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$). (B) MgATP-particles (0.5 mg/ml) (●), AS-particles (0.5 mg/ml) (○) and AS-particles reconstituted with 20 μg/ml of inhibitor protein (▽) were preincubated at 42 °C for the times indicated on the abscissa in pH 8.0 activation buffer 25 mM Tris-acetate, 250 mM sucrose and 30 mM potassium acetate. After each interval an aliquot of 10 μl was withdrawn to assay ATPase activity, as described in Methods ($n=4$).

Time course of thermal inactivation of submitochondrial particles in the presence and absence of inhibitor protein

Activated MgATP- (▽) and AS-submitochondrial particles (○) were almost totally inactivated by a 2 min pre-incubation at 70 °C, while latent MgATP-particles (●) still preserved more than 80% of ATPase activity (Fig. 2A). The latent particles exhibited a slight activation (about 25%) in the first 40 sec of pre-incubation, possibly reflecting release of the inhibitor protein during the first few seconds at 70 °C. The different sensitivities of AS- and activated MgATP-particles to this temperature in comparison with latent MgATP-particles, suggest that the presence of the inhibitor protein in the latent particles might protect them against thermal inactivation (Fig. 2A).

In Fig. 2B, AS-particles reconstituted with increasing amounts of inhibitor protein showed a progressive increase in thermal stability. Particles reconstituted with 20 μg/ml inhibitor protein (▼) retained more than 70% of their activity after 5 min at 70 °C, while particles depleted of inhibitor protein (○) were completely inactivated under the same conditions.

Table I shows the half times ($t_{0.5}$) for thermal inactivation of particles containing or lacking inhibitor protein. Latent MgATP-particles showed a 4.7-fold increase in $t_{0.5}$ for temperature inactivation compared with AS-particles; particles reconstituted with 20 μg/ml of the inhibitor protein exhibited a 15-fold increase in $t_{0.5}$.

Table I. Thermal inactivation of submitochondrial particles containing or lacking inhibitor protein.

Particles	$t_{0.5}$ [min]
Latent MgATP	3.8
Activated MgATP	1.0
Ammonium-Sephadex (AS)	0.8
AS + 1 μg/ml IF_1	2.0
AS + 10 μg/ml IF_1	3.4
AS + 20 μg/ml IF_1	12.1

Submitochondrial particles (AS) containing or lacking inhibitor protein were pre-incubated for different times at 70 °C then diluted in the reaction medium and tested for ATPase activity as described in Fig. 2. The data were fitted by non-linear regression to the equation $v = V_o.e^{-kt}$, where V_o is the initial rate of ATP hydrolysis without pre-incubation; k is the decay constant; t is the pre-incubation time. $t_{0.5}$ represents the half-time for loss of ATPase activity.

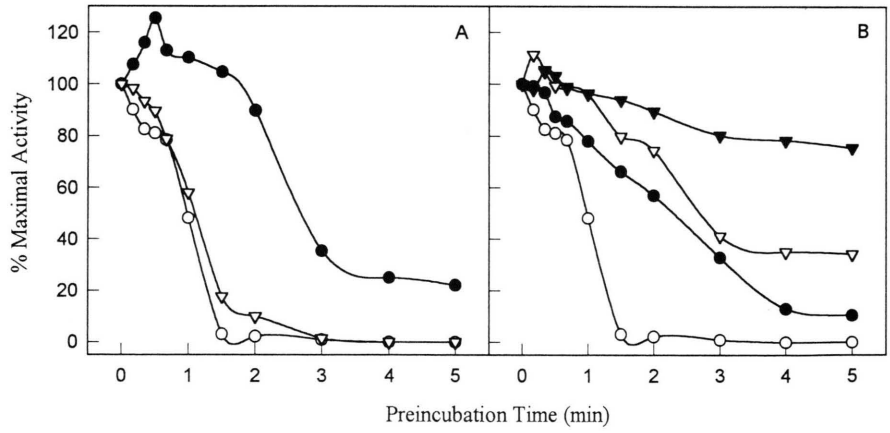

Fig. 2. Time course of thermoinactivation of submitochondrial particles containing or not inhibitor protein (A) and thermoinactivation of AS-particles and AS-particles reconstituted with inhibitor protein (B). (A) Latent (0.5 mg/ml) (●) and activated MgATP-particles (0.5 mg/ml) (▽) and AS-particles (0.5 mg/ml) (○) were preincubated at 70 °C for the times indicated on the abscissa in the activation buffer described in Fig. 1B. After each interval, an aliquot of 10 μl was withdrawn to assay ATPase activity. (100% of maximal activity corresponds to 0.9 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ for latent MgATP-particles, 4.6 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ for activated MgATP-particles and 5.2 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ for AS-particles). (B) AS-particles (0.5 mg/ml) (○) and AS-particles reconstituted with 1 μg/ml (●), 10 μg/ml (▽) or 20 μg/ml (▼) of inhibitor protein were preincubated at 70 °C for the times indicated on the abscissa, as described above. After each interval, an aliquot of 10 μl was withdrawn to assay ATPase activity. (100% of maximal activity corresponds to 6.4 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ for AS-particles and 2.9, 0.8 and 1.2 μmol $Pi \cdot mg^{-1} \cdot min^{-1}$ for AS-particles reconstituted with 1, 10 or 20 μg/ml of inhibitor protein).

Discussion

We have shown here that the ATPase inhibitor protein can confer thermal stability on the mitochondrial F_0F_1-ATPase complex: AS- and activated MgATP-particles were more sensitive to high temperature than particles containing the inhibitor protein. It was also observed that latent MgATP-particles showed a slight activation at the first 40 sec of pre-incubation at 70 °C. We believe that this activation must be due to release of the inhibitor protein, so that after its release the complex started to inactivate. Various investigators (Horstman and Racker, 1970; Schwerzmann and Pedersen, 1986) described that the inhibitor protein was able to inhibit AS-particles even after several minutes' incubation at high temperatures, showing that the inhibitor protein itself is a heat-stable protein. The inhibitor protein at the same time that is able to prevent the hydrolysis of newly synthesized ATP (Fig. 1A), is also able to increase the thermal stability of the enzyme (Fig. 2, Table I). We infer that it helps to preserve the structure of F_1-ATPase. Early works suggested that the IF_1 could be a natural regulator of ATPase activity in mitochondria (Asami *et al.*, 1970; Tuena de Gómes-Puyou *et al.*, 1983). From physiological point of view, the need for tight control of hydrolysis reinforces the role of IF_1 on unidirectional catalytic cycle. The recent published structure of F_1 (Abrahams *et al.*, 1994) suggests that the site of binding of inhibitor protein is in the β-subunit (sequence β 394-400-DELSEED), a site that would also bind amphipatic cationic inhibitors (Abrahams *et al.*, 1994). More importantly, this site would form a "catch" of interaction with the γ-subunit, that might be important for the rotation of the α3β3 relative to the γ-subunit that permit interconversion of states (Abrahams *et al.*, 1994; Walker, 1994).

IF_1 also protects against cold inactivation of soluble F_1-ATPase (Horstman and Racker, 1970). The structures of the cold and heat denatured states of some proteins, are thermodynamically and conformationally equivalent (Privalov, 1990; Huang and Oas, 1996). Low and high temperature favor the substitution of protein-protein interactions by protein-water interactions, and this is true for inter as well as intraprotein interactions (Privalov, 1990; Silva and Weber, 1993; Huang and Oas, 1996). Some reports (Back *et al.*, 1979; Klibanov, 1983; Timasheff, 1992; Sola-Penna and Meyer-Fernandes, 1994, 1996) have described the role of water in the maintenance of noncovalent interactions in the enzymes. It has been reported that soluble mitochondrial F_1-ATPase (Garza-Ramos *et al.*, 1989) and MgATP-submitochondrial particles (Garza-Ramos *et al.*, 1990), in a system containing toluene and phospholipids, catalyze the hydrolysis of ATP at a rate that depends on the concentration of water. It has been proposed that the enhancement of catalytic rates by water is due to a higher conformational mobility of the protein, the same factor that causes a decrease in the thermal stability of the enzyme (Garza-Ramos *et al.*, 1989, 1990). The thermal protection conferred by the mitochondrial ATPase inhibitor protein (Fig. 2, Table I) probably involves a different mechanism, since it binds to a specific region (the β-subunit) of the F_1-ATPase (Frangione *et al.*, 1981; Schwerzmann and Pedersen, 1986; Abrahams *et al.*, 1994; Walker, 1994). The fact that the enzyme loses its hydrolytic activity suggests that the inhibitor protein may induce interactions among the subunits that lead to a low mobility state of the enzyme, increasing its thermal stability. It was recently shown that the minimal inhibitory sequence of the inhibitor protein consists of residues 14–47 (van Raaij *et al.*, 1996). Whether this minimal inhibitory sequence is also involved in the increase of the thermal protection conferred by the mitochondrial ATPase inhibitor protein remains to be elucidated.

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