

Regulatory role of the ATPase inhibitor protein on proton conduction by mitochondrial H⁺-ATPase complex

Ferruccio Guerrieri, Rosanna Scarfò, Franco Zanotti, Yan Wu Che* and Sergio Papa

Institute of Medical Biochemistry and Chemistry and Centre for the Study of Mitochondria and Energy Metabolism, CNR, University of Bari, Bari, Italy

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This study shows that the natural inhibitor protein of mitochondrial H⁺-ATPase complex (IF₁) inhibits, in addition to the catalytic activity, the proton conductivity of the complex. The inhibition of ATPase activity by IF₁ is less effective in the purified F₁ than in submitochondrial particles where F₁ is bound to F₀. No inhibition of H⁺ conductivity by F₀ is observed in F₁-depleted particles

H⁺-ATPase; Inhibitor protein; Proton conductivity

1. INTRODUCTION

The natural ATPase inhibitor (IF₁), originally isolated by Pullman and Monroy [1] from beef-heart mitochondria, is a small, heat-stable protein (*M_r* 10 500). A similar protein was subsequently isolated from chloroplasts [2], bacteria [3], yeast [4,5] and liver [6,7]. The amino acid composition and sequence of IF₁ from beef heart [8] and yeast

[9] have been determined, showing a high degree of homology [10]. This protein has been shown to affect the catalytic properties of the membrane-bound mitochondrial H⁺-ATPase complex (reviews [11–14]) by binding to the β-subunit of F₁ [15]. The catalytic activity of membrane-bound F₁ is compulsorily coupled to transmembrane proton translocation through the membrane sector of F₀ [16]. Removal [17] or displacement of F₁ [18–22] from its binding site(s) results in passive proton diffusion through the membrane sector in the absence of ATP hydrolysis or synthesis. In our laboratory a systematic study of the mechanism of proton conduction by the H⁺-ATPase complex has been carried out, following the anaerobic release of the transmembrane proton gradient generated by respiration in sonicated submitochondrial particles with various degrees of resolution of the complex [18–22]. From these studies it was shown that removal of IF₁ from submitochondrial particles results in enhancement of proton conductance of the H⁺-ATPase complex [18,19].

Here, a kinetic analysis is presented of the role of IF₁ in the regulation of proton conductivity of the ATPase complex in sonicated submitochondrial particles after resolution and reconstitution of the system.

Correspondence address: F. Guerrieri, Institute of Medical Biochemistry and Chemistry and Centre for the Study of Mitochondria and Energy Metabolism, CNR, University of Bari, Bari, Italy

* Permanent address: Institute of Biophysics, Academia Sinica, Beijing, China

Abbreviations: F₀, membrane integral sector of the mitochondrial membrane; F₁, catalytic part of mitochondrial H⁺-ATPase; IF₁, natural inhibitor protein of mitochondrial H⁺-ATPase; ESMP, submitochondrial particles prepared in the presence of EDTA; Sephadex-ESMP, particles depleted of IF₁ by passing ESMP through a Sephadex column; USMP, particles depleted of F₁ by urea treatment of Sephadex-ESMP

2. MATERIALS AND METHODS

Oligomycin and valinomycin were obtained from Sigma (St. Louis, MO); phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, β -NAD reduced form, adenosine 5-triphosphate and catalase from Boehringer (Mannheim). All other chemicals were of high-purity grade.

2.1. Preparation of submitochondrial particles

ESMP were prepared as described by Lee and Ernster [23] by exposure of bovine heart mitochondria [24] to ultrasonic energy in the presence of EDTA at pH 8.5. The ATPase protein inhibitor was removed by passing ESMP through a Sephadex column (Sephadex-ESMP) [25]. USMP were prepared by urea treatment of Sephadex particles as described in [18].

2.2. Purification of F_1 and IF_1

Purification of F_1 was performed by the chloroform extraction described by Beechey et al. [26]. This procedure is particularly convenient for being easy and rapid; however, it results in a lower activity of the ATPase as compared to other preparations [26]. IF_1 was prepared from Mg-ATP sonicated submitochondrial particles [24] by the method of Kanner et al. [27] generally used for isolation of IF_1 from purified ATPase complex [28–30]. Polyacrylamide gel electrophoresis of the purified protein performed on slab gels of a linear polyacrylamide gradient (12–20%) as in [22] showed only one band of apparent M_r 10500, characteristic of IF_1 preparations [1,6,10] (fig.1).

2.3. Measurements of ATPase activity and H^+ conductivity

The ATPase activity was determined in the presence of added pyruvate kinase, phosphoenolpyruvate and lactate dehydrogenase by following NADH oxidation spectrophotometrically as in [22].

For measurement of proton conduction the relaxation of the respiratory proton gradient was followed in sonicated particles (3 mg protein/ml) incubated in a reaction mixture containing 250 mM sucrose, 30 mM KCl, 0.5 μ g valinomy-

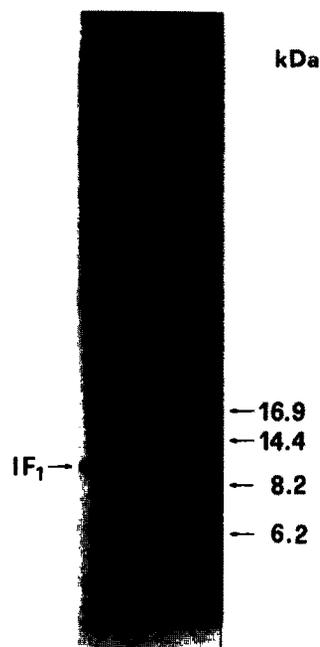


Fig.1. Gel electrophoresis of purified IF_1 . IF_1 (10 μ g), purified as described in section 2, was added to 50 μ l aliquots of a solution containing 2.3% SDS, 5% 2-mercaptoethanol, 10 mM Tris-HCl, pH 6.8, and exposed to ultrasonic energy for 60 s. Electrophoresis was then performed on slabs of a linear gradient polyacrylamide (12–20%) gel as in [22]. The apparent M_r was determined by comparison with the following standard mixture of proteins: LKB 1860-101 M_r markers containing: myoglobin (M_r 16949), myoglobin I and II (M_r 14404); myoglobin I (M_r 8159); myoglobin II (M_r 6214) and myoglobin III (M_r 2512).

cin/mg protein, 0.2 mg/ml purified catalase and 20 mM succinate as respiratory substrate (final volume 1.5 ml, pH 7.5). Incubation was carried out in a glass vessel, under a constant stream of N_2 , at 25°C. Respiration-driven proton translocation was activated by repetitive pulses of 1–3% H_2O_2 (5 μ l/ml). The pH changes monitored potentiometrically were converted into proton equivalents by double titration with standard HCl and KOH [31]. H^+ conductivity in the membrane was also measured by following potentiometrically the H^+ release induced by a diffusion potential (positive inside) imposed by valinomycin-mediated K^+ influx [32]. Protein concentration was determined by the method of Lowry et al. [33].

Table 1

Resolution and reconstitution of H⁺-ATPase complex in sonicated submitochondrial particles: effect of IF₁ on ATPase activity

	ATPase activity ($\mu\text{mol ATP hydrolyzed} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$)		
	Control	+ IF ₁	% inhibition
ESMP	1.25	0.92	26
Sephadex-ESMP	2.18	0.98	55
USMP	0.08	0.08	—
USMP + F ₁	1.00	0.50	50
USMP + F ₁ + oligomycin (2 $\mu\text{g}/\text{mg}$ protein)	0.12	—	—

Submitochondrial particles, F₁ and IF₁ were prepared as described in section 2. Procedure: submitochondrial particles (5 mg/ml) were preincubated, when indicated, with IF₁, at 4 $\mu\text{g}/\text{mg}$ particle protein, for 15 min at room temperature. Then 50 μg particle protein were added to 1 ml of a mixture containing 250 mM sucrose, 50 mM KCl, 20 mM Tris-HCl (pH 7.5), 0.025 mM NADH, 0.5 μg rotenone, 1 mM phosphoenolpyruvate, 2.5 U lactate dehydrogenase, 2 U pyruvate kinase and ATPase activity was determined by following NADH oxidation at 340 nm. For reconstitution of oligomycin-sensitive ATPase activity, USMP (15 mg protein/ml) were incubated with 5 mM MgCl₂ and 1.5 mg/ml F₁ (or 1.5 mg/ml F₁ + 50 $\mu\text{g}/\text{ml}$ IF₁) for 15 min, then 50 μg particle protein were added to the reaction mixture and the ATPase activity determined as described above.

3. RESULTS

Table 1 shows that removal of IF₁ from sonicated submitochondrial particles, effected by passing ESMP through a Sephadex column, resulted in an about 2-fold increase in ATPase activity. Addition of purified IF₁, at 4 $\mu\text{g}/\text{mg}$ particle protein, inhibited the ATPase activity of Sephadex-ESMP by 50%.

The ATPase activity of USMP was negligible. Addition of soluble F₁ to USMP resulted in oligomycin-sensitive ATPase activity which was inhibited by IF₁ to the same extent as that observed in Sephadex-ESMP. Separate controls showed that the amount of IF₁ causing 50% inhibition of ATPase activity in Sephadex-ESMP and in the

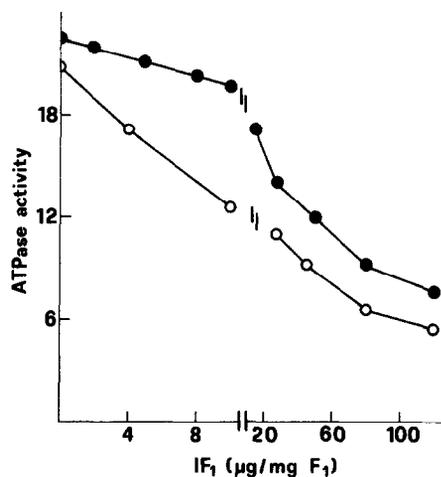


Fig. 2. Titration curves for inhibition by IF₁ of the ATPase activity of purified F₁. F₁ and IF₁ were prepared as described in section 2. ATPase activity was determined as described in the legend to table 1 and the values shown are expressed as $\mu\text{mol ATP hydrolyzed} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ F₁ protein. For preincubation of F₁ with IF₁: F₁ (5 mg/ml) were preincubated with IF₁ (at the concentrations shown) for 15 min at room temperature in the absence (●—●) or presence (○—○) of 5 mM MgCl₂. Then 5 μg protein of purified F₁ were added to 1 ml reaction mixture for determination of the ATPase activity as described in the legend to table 1.

reconstituted USMP plus F₁ inhibited the ATPase activity in ESMP by only 26%.

Titration of the inhibitory action of IF₁ on ATPase activity of soluble F₁ is shown in fig. 2. The inhibitory titre of the present preparation of IF₁ was similar to that exhibited by other preparations on soluble F₁ [1,6], 50% inhibition being observed at around 50 μg IF₁ per mg protein soluble F₁ (corresponding to about 2 mol added IF₁ per mol F₁). The presence of Mg²⁺ during incubation of IF₁ with F₁ caused some promotion of the inhibitory effect of IF₁.

The results presented in table 2 and figs 3 and 4 reveal that IF₁ also regulates the proton conductivity of the H⁺-ATPase complex in sonicated submitochondrial particles in the absence of ATP hydrolysis or synthesis. Removal of IF₁ (Sephadex-ESMP) resulted in a 2–3-fold enhancement of the rate of passive proton conduction measured as $1/t_{1/2}$, either from the anaerobic relaxation of the respiratory proton gradient or from the H⁺ release

Table 2

Effect of IF₁ on passive proton conductivity in sonicated submitochondrial particles

Particles	1/t _{1/2} (s ⁻¹)	
	Anaerobic release of respiratory ΔμH ⁺	H ⁺ release induced by valinomycin addition
ESMP	1.00	0.50
Sephadex-ESMP	2.20	1.43
Sephadex-ESMP + IF ₁ (4 μg/mg particle protein)	1.00	0.50

Particles and IF₁ were prepared as described in section 2. H⁺ conductivity was measured potentiometrically as described in the text by following either anaerobic release of respiratory ΔμH⁺ or H⁺ release induced by valinomycin addition in the presence of K⁺. Where indicated particles (20 mg particle protein/ml) were preincubated with IF₁ at the concentrations shown for 15 min at room temperature, 0.23 ml of the mixture then being added to 1.27 ml reaction mixture

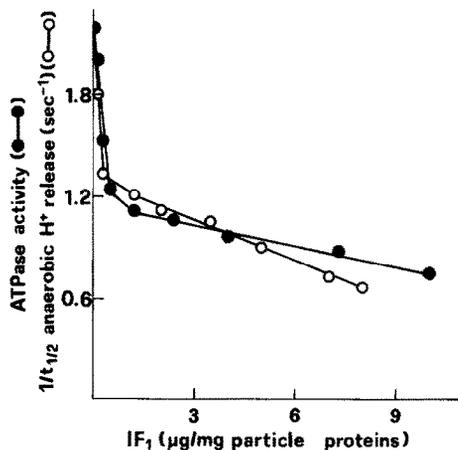


Fig.3. Titration curves for inhibition by IF₁ of the ATPase activity and passive proton conduction in Sephadex-ESMP. Sephadex-ESMP and IF₁ were prepared as described in section 2. ATPase activity was determined as described in the legend to table 1 and the values shown are expressed as μmol ATP hydrolyzed · min⁻¹ · mg⁻¹ particle protein. H⁺ conductivity was analyzed by following anaerobic release of the respiratory proton gradient as described in the legend to table 2. For preincubation of Sephadex-ESMP with IF₁ see legends to tables 1 and 2. (●—●) ATPase activity, (○—○) H⁺ conduction.

induced by a K⁺ diffusion potential set up by valinomycin (table 2). Addition of 4 μg IF₁/mg particle protein to Sephadex-ESMP depressed the rate of passive proton conductivity to the values observed in ESMP. Separate controls showed that IF₁ had, on the other hand, no effect on proton conduction in F₁-depleted particles (USMP). Oligomycin caused about 80% inhibition of passive proton conduction in Sephadex-ESMP (as well as in ESMP and USMP) (see also [17–22]).

Fig.3 shows the titration curves for the inhibitory action of IF₁ on ATPase activity and proton conductivity of Sephadex-ESMP. The two curves practically coincide and are both biphasic, showing 50% inhibition for the two processes at about 4 μg IF₁/mg particle protein.

As observed by others [6] about 10-fold less IF₁ was required per mg protein of particles to cause 50% inhibition of the ATPase activity than with soluble F₁. This could reflect the fact that F₁ represents only a fraction of the proteins of the inner mitochondrial membrane.

Fig.4 shows that the H⁺ conduction in USMP, after reconstitution of oligomycin-sensitive H⁺-ATPase by addition to the particles of purified

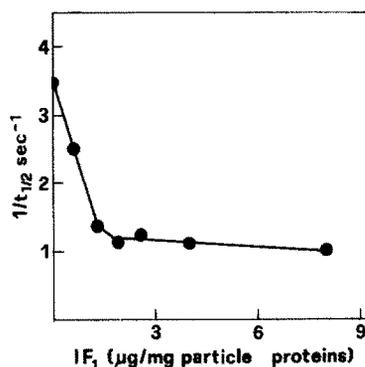


Fig.4. Titration curve for inhibition of anaerobic release of respiratory proton gradient by IF₁ in USMP after reconstitution of oligomycin-sensitive H⁺-ATPase by addition of purified F₁. Preparations of USMP, F₁ and IF₁ were performed as described in section 2. For reconstitution USMP (15 mg/ml) were incubated with 0.1 mg F₁ per mg particle protein and, where indicated, with the amounts of IF₁ reported on the abscissa. After 15 min incubation 0.2 ml of the particle suspension were added to 0.8 ml reaction mixture, reported in the text, and anaerobic release of respiratory proton gradient was measured potentiometrically.

F_1 (see table 1), was also inhibited by IF_1 , with a titration curve resembling that observed for Sephadex-ESMP. In this system the first phase of inhibition, observed at lower IF_1 concentrations, was, however, more extensive than in Sephadex-ESMP. 50% inhibition was observed at a concentration of IF_1 corresponding to 1.0 μg per mg protein USMP and 10 μg per mg protein added F_1 .

4. DISCUSSION

The present data show that IF_1 , besides controlling the catalytic activities of mitochondrial H^+ -ATPase, plays a regulatory role in the proton conductivity by the F_0F_1 complex in the absence of ATP hydrolysis or synthesis. Removal of IF_1 from submitochondrial particles results in stimulation of oligomycin-sensitive hydrolytic activity (see also [18,19]) and of passive proton conductivity of the ATPase complex, which is reversed in both cases by adding back IF_1 .

The inhibitory effect exerted by IF_1 on proton conductivity is not due to unspecific binding of the polypeptide to the membrane as shown by the specific requirement of F_1 for this effect. In fact the inhibitory action exerted by IF_1 on passive H^+ conduction, lost in sonicated submitochondrial particles deprived of F_1 (USMP), is recovered after reconstitution of the system by addition of soluble F_1 to USMP. Thus the inhibitory action exerted by IF_1 on proton conduction depends on the presence of F_1 .

IF_1 could favour binding of F_1 to F_0 with depression of proton conductance. This may derive from a conformational change induced in F_1 by the binding of IF_1 to the β -subunit [34,35]. It is, alternatively, possible that IF_1 mediates the binding of F_1 to the membrane sector, thus acting as a component of the gate of the H^+ -ATPase complex. It can, in fact, be recalled in this respect that Hashimoto et al. [36–38] have isolated from yeast mitochondria two protein factors which stabilize the formation of an F_0F_1 - IF_1 complex, but do not exert any effect on the binding of IF_1 to soluble F_1 .

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