Volume 213, number 1, 67–72 FEB 04479 March 1987

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

Received 4 November 1986

This study shows that the natural inhibitor protein of mitochondrial H^+-ATPase complex (IF_1) inhibits, in addition to the catalytic activity, the proton conductivity of the complex. The inhibition of ATPase activity by IF_1 is less effective in the purified F_1 than in submitochondrial particles where F_1 is bound to F_0. No inhibition of H^+ conductivity by F_0 is observed in F_1-depleted particles

H^+-ATPase; Inhibitor protein; Proton conductivity

1. INTRODUCTION

The natural ATPase inhibitor (IF_1), originally isolated by Pullman and Monroy [1] from beef-heart mitochondria, is a small, heat-stable protein (M_r 10500). 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 IF_1 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 F_1 [15]. The catalytic activity of membrane-bound F_1 is compulsorily coupled to transmembrane proton translocation through the membrane sector of F_0 [16]. Removal [17] or displacement of F_1 [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 IF_1 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 IF_1 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: F_0, membrane integral sector of the mitochondrial membrane; F_1, catalytic part of mitochondrial H^+-ATPase; IF_1, natural inhibitor protein of mitochondrial H^+-ATPase; ESMP, submitochondrial particles prepared in the presence of EDTA; Sephadex-ESMP, particles depleted of IF_1 by passing ESMP through a Sephadex column; USMP, particles depleted of F_1 by urea treatment of Sephadex-ESMP

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

Oligomycin and valinomycin were obtained from Sigma (St. Louis, MO); phospho*enol*pyruvate, 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).

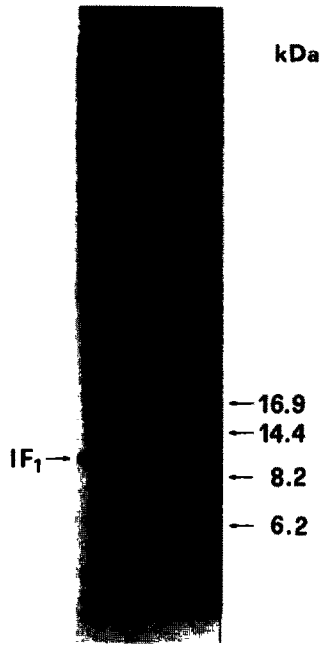

Fig.1. Gel electrophoresis of purified IF_1. IF_1 (10 μg), purified as described in section 2, was added to 50 μl aliquots of a solution containing 2.3% SDS, 5% 2-mercaptoethanol, 10 mM Tris-HCl, pH 6.8, and exposed to ultrasonic energy for 60 s. Electrophoresis was then performed on slabs of a linear 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).

2.3. *Measurements of ATPase activity and H^+ conductivity*

The ATPase activity was determined in the presence of added pyruvate kinase, phospho*enol*-pyruvate 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 μg valinomycin/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 μl/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 IF_1 on ATPase activity

	ATPase activity (μmol ATP hydrolyzed · min^{-1} · 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 μg/mg protein)	0.12	–	–

Submitochondrial particles, F_1 and IF_1 were prepared as described in section 2. Procedure: submitochondrial particles (5 mg/ml) were preincubated, when indicated, with IF_1, at 4 μg/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 phospho*enol*pyruvate, 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 $MgCl_2$ and 1.5 mg/ml F_1 (or 1.5 mg/ml F_1 + 50 μg/ml IF_1) 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 IF_1 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 IF_1, at 4 μg/mg particle protein, inhibited the ATPase activity of Sephadex-ESMP by 50%.

The ATPase activity of USMP was negligible. Addition of soluble F_1 to USMP resulted in oligomycin-sensitive ATPase activity which was inhibited by IF_1 to the same extent as that observed in Sephadex-ESMP. Separate controls showed that the amount of IF_1 causing 50% inhibition of ATPase activity in Sephadex-ESMP and in the reconstituted USMP plus F_1 inhibited the ATPase activity in ESMP by only 26%.

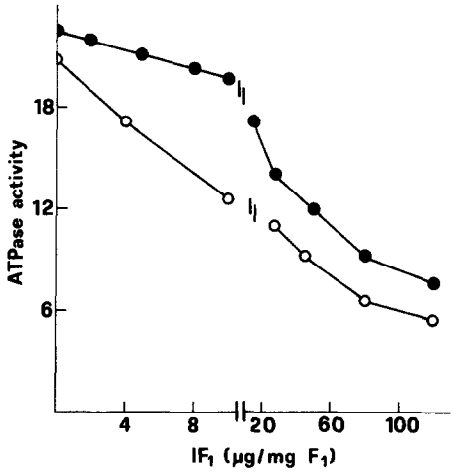

Fig.2. Titration curves for inhibition by IF_1 of the ATPase activity of purified F_1. F_1 and IF_1 were prepared as described in section 2. ATPase activity was determined as described in the legend to table 1 and the values shown are expressed as μmol ATP hydrolyzed · min^{-1} · mg^{-1} F_1 protein. For preincubation of F_1 with IF_1: F_1 (5 mg/ml) were preincubated with IF_1 (at the concentrations shown) for 15 min at room temperature in the absence (●—●) or presence (○—○) of 5 mM $MgCl_2$. Then 5 μg protein of purified F_1 were added to 1 ml reaction mixture for determination of the ATPase activity as described in the legend to table 1.

Titration of the inhibitory action of IF_1 on ATPase activity of soluble F_1 is shown in fig.2. The inhibitory titre of the present preparation of IF_1 was similar to that exhibited by other preparations on soluble F_1 [1,6], 50% inhibition being observed at around 50 μg IF_1 per mg protein soluble F_1 (corresponding to about 2 mol added IF_1 per mol F_1). The presence of Mg^{2+} during incubation of IF_1 with F_1 caused some promotion of the inhibitory effect of IF_1.

The results presented in table 2 and figs 3 and 4 reveal that IF_1 also regulates the proton conductivity of the H^+-ATPase complex in sonicated submitochondrial particles in the absence of ATP hydrolysis or synthesis. Removal of IF_1 (Sephadex-ESMP) resulted in a 2–3-fold enhancement of the rate of passive proton conduction measured as $1/t_{½}$, either from the anaerobic relaxation of the respiratory proton gradient or from the H^+ release

Table 2

Effect of IF_1 on passive proton conductivity in sonicated submitochondrial particles

Particles	$1/t_{1/2}$ (s^{-1})	
	Anaerobic release of respiratory $\Delta\mu H^+$	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 IF_1 were prepared as described in section 2. H^+ conductivity was measured potentiometrically as described in the text by following either anaerobic release of respiratory $\Delta\mu H^+$ or H^+ release induced by valinomycin addition in the presence of K^+. Where indicated particles (20 mg particle protein/ml) were preincubated with IF_1 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

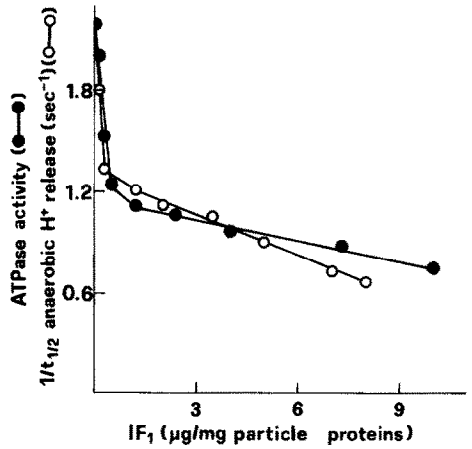

Fig.3. Titration curves for inhibition by IF_1 of the ATPase activity and passive proton conduction in Sephadex-ESMP. Sephadex-ESMP and IF_1 were prepared as described in section 2. ATPase activity was determined as described in the legend to table 1 and the values shown are expressed as μmol ATP hydrolyzed · $min^{-1} \cdot mg^{-1}$ 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 IF_1 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 IF_1/mg particle protein to Sephadex-ESMP depressed the rate of passive proton conductivity to the values observed in ESMP. Separate controls showed that IF_1 had, on the other hand, no effect on proton conduction in F_1-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 IF_1 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 IF_1/mg particle protein.

As observed by others [6] about 10-fold less IF_1 was required per mg protein of particles to cause 50% inhibition of the ATPase activity than with soluble F_1. This could reflect the fact that F_1 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

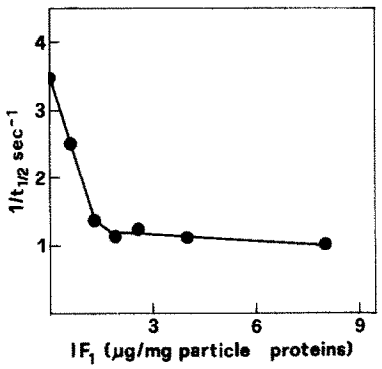

Fig.4. Titration curve for inhibition of anaerobic release of respiratory proton gradient by IF_1 in USMP after reconstitution of oligomycin-sensitive H^+-ATPase by addition of purified F_1. Preparations of USMP, F_1 and IF_1 were performed as described in section 2. For reconstitution USMP (15 mg/ml) were incubated with 0.1 mg F_1 per mg particle protein and, where indicated, with the amounts of IF_1 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.

ACKNOWLEDGEMENTS

The authors thank Dr F. Dabbeni-Sala for suggestions in the preparation of IF_1. The skilful technical assistance of A. Carlone is gratefully acknowledged. This work was supported by grant no.85.02288.44 of CNR, Rome, Italy, and a grant of the Joint Project of Community of Mediterranean Universities (CUM).

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