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Relevance of Divalent Cations to ATP-Driven Proton Pumping in Beef Heart Mitochondrial F_0F_1-ATPase[1]

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The ATP hydrolysis rate and the ATP hydrolysis-linked proton translocation by the F_0F_1-ATPase of beef heart submitochondrial particles were examined in the presence of several divalent metal cations. All Me–ATP complexes tested sustained ATP hydrolysis, although to a different extent. However, only Mg- and Mn-ATP-dependent hydrolysis could sustain a high level of proton pumping activity, as determined by acridine fluorescence quenching. Moreover, the K_m of the Me-ATP hydrolysis-induced proton pumping activity was very similar to the K_m value of Me-ATP hydrolysis. Both oligomycin and DCCD caused the full recovery of the fluorescence, providing clear evidence for the association of Mg-ATP hydrolysis with proton translocation through the F_0F_1-ATPase complex. In contrast, with other Me-ATP complexes, including Ca-ATP as substrate, the proton pumping activity was undetectable, implicating an uncoupling nature for these substrates. Attempts to demonstrate the involvement of the ϵ subunit of the enzyme in the coupling mechanism failed, suggesting that the participation of at least the N-terminal segment of the subunit in the coupling mechanism of the mitochondrial enzyme is unlikely.

KEY WORDS: F_1-ATPase; F_0F_1-ATPase; H^+-ATPase; ϵ subunit; energy coupling; tryptophan; phosphorescence.

INTRODUCTION

The F_0F_1-ATP synthases of energy-transducing membranes are reversible enzymes that couple transmembrane proton electrochemical gradients to ATP synthesis. The F_0 sector is an integral membrane protein complex capable of proton transport. F_1, the catalytic sector of the enzyme, is a peripheral protein, attached to F_0 by a stalk (Boyer, 1997; Weber and Senior, 1997; Pedersen and Amzel, 1993) and possibly by a very narrow polypeptide(s) segment (Ogilvie *et al.*, 1997; Engelbrecht and Junge, 1997). F_1 can be removed from the membrane, retaining the capability to hydrolyze ATP. It contains five different subunits α through ϵ, in order of decreasing molecular weight, with the stoichiometry 3:3:1:1:1 (Walker *et al.*, 1985). However, enzymes of the F-type are not identical, mainly because of differences in two of the subunits; δ and ϵ subunits of bacteria and chloroplasts are homologous to oligomycin sensitivity-conferring protein and δ of beef heart, respectively. Therefore, the ϵ subunit of mitochondria lacks a counterpart in bacteria and chloroplasts (Walker *et al.*, 1985). It is widely believed that the coupling of the reversible ATP synthesis and the flux of protons across F_0 is mediated by conformational changes transmitted from the membrane sector to the catalytic sector of the F_0F_1 complex, implying that polypeptide(s) of the stalk are involved (Pedersen

[1] Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; F_1, catalytic sector of the mitochondrial H^+-ATPase complex; F_0, membrane sector of the H^+-ATPase complex; SMP, coupled submitochondrial particles prepared by sonication of mitochondria; ΔpH, transmembrane proton concentration difference.

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and Amzel, 1993; Futai and Omote, 1996; Boyer, 1997; Fillingame, 1997 and references therein). Recent experimental evidence (Duncan *et al.*, 1995; Noji *et al.*, 1997; Junge *et al.*, 1997) support a mechanical coupling between the F_0 and the F_1 sectors based on the rotation of a central rotor, including the γ subunit, with respect to the $\alpha_3\beta_3$ hexamer. This model necessarily requires a structural link (stator) connecting the hexamer with the nonrotating part of F_0. Energy transduction in this model would occur through a proton flow-powered rotation within the F_0 sector during ATP synthesis. Analogously, a torque should be generated in F_1 coupled to the Me-ATP hydrolysis mechanism. The structural rearrangements concerned with this machinery might include the ϵ subunits of F_1 from both bacteria and mitochondria.

It has also been reported that the divalent cations bound to the nucleotide, which is the actual substrate of the enzyme, play a role in the coupling process. Thus, coupled photophosphorylation in plant thylakoid membranes required the presence of Mg^{2+} ions that could eventually be replaced by Co^{2+}, but not by Ca^{2+} (Whatley *et al.*, 1959). On the other hand, both bacterial photosynthetic membranes and the chloroplast-bound Ca-ATPase, unlike its Mg-ATPase, are not coupled to proton translocation (Pick and Weiss, 1988; Casadio and Melandri, 1996). Other results indicate that the divalent cations play an important role in the regulation of proton-coupled ATP synthesis and hydrolysis by the F_0F_1 complex in *Rhodospirillum rubrum* (Gromet-Elhanan and Weiss, 1989). Finally, studies carried out some years ago (Perlin *et al.*, 1984), aimed at assessing specificity of the enzyme for nucleotides in *Escherichia coli* membranes, suggest that the proton transport driven by Ca-ATP has nearly the same effectiveness as Mg-ATP. Although the coupling mechanism within the various H^+-ATPase complexes is believed to be the same, the involvement of Ca-ATP in coupling appears controversial. Besides, to our knowledge, the H^+-pumping activity of the F_0F_1-ATPase of either mitochondria or submitochondrial particles with Ca-ATP as substrate has never been investigated.

In the present study, the ATP hydrolysis and the proton-translocating activities of the F_0F_1-ATPase of submitochondrial particles were measured with different Me-nucleotides to obtain information about the coupling of catalysis in F_1 and the H^+ transport through F_0. The results reveal that several Me-nucleotides can effectively be hydrolyzed by the F_0F_1 complex with the onset of a transmembrane ΔpH, whereas some others, including Ca-ATP, cannot. The results also reveal interesting characteristics of the mitochondrial synthase and concur with the concept of a required specific coordination chemistry of the nucleotide-complexed metal in order to couple the chemical catalysis to the proton transport by the F_0F_1 complex.

Moreover, since recent data on the *E. coli* enzyme have evidenced the involvement of the smaller subunits of F_1 in the mechanism of coupling (Tang and Capaldi, 1996) and suggestion on the possible involvement of the ϵ subunit of the mitochondrial enzyme has also been reported (Guèlin *et al.*, 1993; Gabellieri *et al.*, 1997), this item was also investigated. The present work demonstrates that the conformation of the N-terminal segment of the ϵ subunit is similarly affected by each Me-ATP binding to F_1, therefore rising doubts about the participation of this domain of the subunit in the coupling mechanism of the beef heart enzyme.

MATERIALS AND METHODS

Materials

ATP, DCCD, FCCP, valinomycin, phosphoenolpyruvate, Tris, Hepes, reduced nicotinamide-adenine dinucleotide, pyruvate kinase, and lactate dehydrogenase in glycerol-containing buffer were purchased from Sigma Chemical Co. (St. Louis, MO). Diethylaminoethyl-Sephadex G-50 and blue Sepharose CL-6B were obtained from Pharmacia Biotech Inc. (Uppsala, Sweden). ACMA was purchased from Molecular Probes (Eugene, OR).

Enzyme Preparation

Coupled beef heart submitochondrial particles were obtained essentially according to Lee and Ernster (1968). In this preparation, a suspension of bovine heart mitochondria in 0.25 *M* sucrose and 2 m*M* EDTA, pH 8.5, was sonicated for 2 min at medium output on a MSE PB-254 sonic oscillator under N_2 stream. The temperature was kept between 4–10°C.

F_1 was extracted from sonicated submitochondrial particles and purified by DEAE-Sephadex G-50 ion-exchange chromatography according to Penefsky (1975). Further purification was achieved by affinity chromatography on a blue Sepharose CL-6B column as described by Baracca *et al.* (1995). The enzyme solution was stored at 4°C in a suspension containing

50% ammonium sulfate in the presence of 4 m*M* ATP. Under these conditions the enzyme activity remained stable for several weeks. The F_1 preparation used in phosphorescence measurements was obtained daily from the above suspension by centrifugation. The sedimented enzyme was dissolved in buffer containing 0.15 *M* sucrose, 1 m*M* KH_2PO_4, 10 m*M* Hepes, pH 8, and glycerol, 10% by mass. In all cases the final concentration of F_1 was about 3 μ*M*, assuming a molecular mass of 370 KDa for the enzyme.

ATP Hydrolysis Activity

The rate of ATP hydrolysis of submitochondrial particles suspensions was determined by detecting the inorganic phosphate released. The reaction mixture (0.25 ml final volume) contained 50 m*M* Tris, pH 8 and 0.2 mg of submitochondrial particles. The reaction was started by adding increasing amounts of Me-ATP and stopped 5 min later with 10% trichloroacetic acid. The temperature was kept at 27 ± 1°C. The divalent metal ions were added at equimolar concentrations to that of ATP. The final concentration of the substrates, Me-ATP, was estimated on the basis of the stability constant for the complexes reported in Table I and considering the multiple equilibria of binding between the cations and the nucleotides (Fregni and Casadio, 1993). The concentration of inorganic phosphate was detected after complexation with molybdate reagent (Taussky and Schorr, 1951) and measured using a calibration curve obtained under the same experimental conditions.

The ATPase activity of purified F_1 was determined using an ATP-regenerating system by following the decrease of NADH absorption at 340 nm in a Jasco 7850 spectrophotometer. The assay was carried out at saturating substrate conditions (steady-state) as described by Solaini *et al.* (1993). The reaction was started by adding 1–5 μg protein. The specific activity of the enzyme was about 80–100 units/mg protein at 20°C.

Table I. Kinetic Constants for ATP Hydrolysis of Submitochondrial Particles in the Presence of Several Divalent Metals[a]

Substrate	log $K_{Me\text{-}ATP}$ (mM^{-1})	V_{max} (μmol/min/mg)	K_m (m*M*)	V_{max}/K_m ($min^{-1}\cdot mg^{-1}$)
Mg-ATP	4.29	0.200	0.16	1.25
Mn-ATP	5.01	0.120	0.22	0.53
Ca-ATP	3.91	0.065	0.18	0.36
Co-ATP	4.97	0.051	0.085	0.60
Zn-ATP	5.16	0.042	0.073	0.57
Ni-ATP	4.86	0.045	0.030	1.50

[a] The stability constants of the Me-ATP complexes are reported as $\log K_{Me\text{-}ATP}$.

Proton-Pumping Activity

The proton-pumping activity coupled to the Me-ATP hydrolysis of submitochondrial particles was determined from the quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) fluorescence measured using a Jasco P450 fluorometer with excitation and emission at 412 and 510 nm, respectively (Casadio, 1991). The assay medium contained in 2-ml final volume: 0.25*M* sucrose, 50 m*M* Tris, 50 m*M* KCl, pH 8, 1.8 μg valinomycin, 0.5 μ*M* ACMA, and 0.4 mg of submitochondrial particles. The reaction, performed under continuous mixing, was started by the addition of both ATP and the divalent metal ion (as metal chloride) at equimolar concentration.

A calibration curve relating the fluorescence of the probe to the pH gradient formed across the membrane was obtained imposing known, artificially induced, ΔpHs to a submitochondrial particle suspension equilibrated at pH 5.5 (Fregni and Casadio, 1993). ΔpH dependence quenching (Q) of ACMA fluorescence was fitted using the following equation:

$$\Delta pH = AQ/(B - Q) \exp [Q/(B - Q) + CQ] \quad (1)$$

where A, B, and C are the empirical parameters determined by the calibration curve (Casadio, 1991). By using the same experimental conditions and the same amount of protein, the values obtained for the three parameters were: $A = 4.57$, $B = 126$, and $C = -0.035$.

Phosphorescence Decay Measurements

Phosphorescence decay in fluid solution at room temperature was monitored with a home-made apparatus suitable for lifetime measurements in the μs–ms range (Strambini, 1983). Pulsed excitation (λ_{ex} = 292 nm) was generated by a frequency-doubled flash pumped dye laser (UV500 M Candela) with a pulse duration of 1 μs and an energy/pulse typically of 1 to 10 mJ. The sample, placed in a vacuum-proof quartz cuvette that allows excitation of the solution from above, was extensively deoxygenated prior to analysis (Strambini and Gonnelli, 1995). The decay signal was

digitized by a computerscope system (ISC-16, RC Electronics) capable of averaging multiple sweeps. Subsequent analysis of decay curves in terms of the sum of exponential components was carried out by a nonlinear least-squares fitting algorithm implemented by the program Global Analysis (Global Unlimited, LFD University of Illinois, Urbana).

At the end of the phosphorescence measurements, the hydrolytic activity of all the samples of the enzyme examined was nearly unchanged, to indicate a conserved structure during the measurements.

Other Methods

Protein concentration of submitochondrial particle preparations was determined by the method of Bradford (1976), whereas the protein concentration of enzyme solutions was determined by the method of Lowry *et al.* (1951).

The concentration of the endogenous Mg^{2+} ions in the SMP was determined spectroscopically after complexation of the Mg^{2+} ions with Titan yellow (Orange and Rheim, 1951).

RESULTS

It is well known that in the presence of the physiological cofactor, Mg^{2+}, the hydrolytic activity of the F_0F_1-ATPase complex is coupled to the proton-pumping activity generating a transmembrane proton gradient. Moreover, it has been reported that other divalent cations are able to substitute Mg^{2+} in the hydrolytic activity of the enzyme, but yet it has not been established whether the hydrolysis of these Me-ATP complexes is coupled to the translocation of proton across the inner mitochondrial membrane.

In the present work, we used beef heart-coupled submitochondrial particles prepared in the presence of EDTA in order to obtain preparations with very low concentration of free Mg^{2+} that could interfere with the subsequent kinetic analysis employing different divalent cations. In fact, the spectroscopic determination of the endogenous Mg^{2+} ions indicated that the concentration of Mg^{2+} was below 2 μM, which is the lowest concentration that we could detect. Moreover, in the absence of exogenous Mg^{2+}, the submitochondrial particles showed no detectable hydrolytic activity upon addition of 0.01 to 1 mM ATP.

Me-Dependent ATPase Activity

The metal dependence of the ATP hydrolytic activity of coupled submitochondrial particles was investigated examining the kinetic constants in the presence of several Me-ATP complexes. In each set of experiments, the Me-ATP concentration was determined using the stability constants reported in Table I. All the divalent cations examined could induce a detectable hydrolytic activity of ATP, indicating that metal ions other than Mg^{2+} can support ATP hydrolysis. Figure 1 shows the concentration dependence of hydrolysis rate with either Mg-ATP, Ca–ATP, or Mn–ATP. All these substrates have similar affinity for the enzyme, expressed by K_m values in the range 0.16–0.22 mM (Table I). If ATP was added to the enzyme in the presence of Co^{2+}, Zn^{2+} or Ni^{2+}, the K_m was lower, falling in the range 0.030–0.085 (Table I), to indicate an enhanced affinity of these transition metal–ATP complexes for the enzyme. In this respect, membrane-bound F_1 behaves as it does when isolated in the soluble form (Dorgan *et al.*, 1984). The maximal activity of the enzyme in the presence of various ions was significantly lower with respect to that measured with the physiological substrate, Mg-ATP. Except for Mn-ATP, which V_m or K_{cat} values have been reported to be slightly higher than those of Mg-ATP, the values of

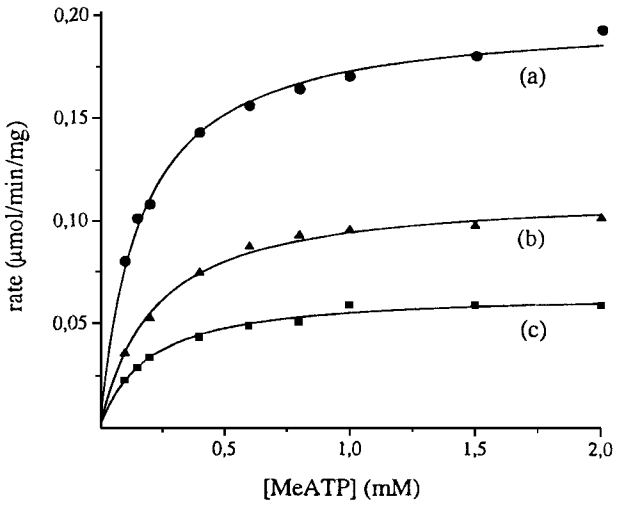

Fig. 1. Kinetic analysis of the ATP hydrolysis rate of submitochondrial particles in the presence of different divalent metals. Assay conditions are described in the section Materials and Methods. (a), Mg-ATP; (b), Mn-ATP; (c), Ca-ATP. Substrate concentrations were calculated on the basis of the stability constants of the metal–ATP complexes reported in Table I. The kinetic constants were determined by nonlinear regression analysis.

V_m for the other Me-ATP tested are lower than those of Mg-ATP, in agreement with reported values for the isolated F_1 (Dorgan *et al.*, 1984; Williams *et al.*, 1987; Baracca *et al.*, 1993).

Proton-Pumping Activity

Acridine fluorescent probes are widely used to detect transmembrane proton gradients in natural and model membrane systems. The quenching of ACMA fluorescence associated with the multiple equilibria distribution of the amine between two aqueous bulk phases and the membrane (Casadio, 1991) at different pHs can be used to qualitatively monitor the extent and the rate of the transmembrane ΔpH formation. This method was applied in order to determine whether the ATP hydrolysis observed in the presence of the metal ions examined was coupled to the generation of a proton gradient.

Adding Mg-ATP to a suspension of submitochondrial particles, a decrease in the fluorescence of the probe was observed until a steady state was reached (Fig. 2). Subsequent addition of either inhibitors of the F_0F_1-ATPase, oligomycin, or DCCD (not shown), or the ionophore, nigericin (not shown), induced the complete recovery of the fluorescence, indicating that Mg-ATP hydrolysis is coupled to the transmembrane proton flux. This conclusion is supported by the kinetic analysis of the proton-pumping activity in the presence of Mg-ATP: traces of fluorescence quenching similar to that reported in Fig. 2 were obtained at different concentrations of Mg-ATP. From these curves, on the basis of the calibration of ACMA response to ΔpH (see methods), it was possible to trace the time course of the ΔpH formation for each concentration of the substrate (Fig. 3). The rate of ΔpH generation appeared constant for the first sec allowing the interpolation of the initial rate of ΔpH formation. The subsequent kinetic analysis, shown in Fig. 4, resulted in a K_m of the proton-pumping activity of 0.2 m*M*. Therefore, it is very similar to the K_m value determined for the hydrolytic activity. This similarity is consistent with the coupling of the Mg-ATP hydrolysis with the proton translocation through the F_0F_1-ATPase complex.

Traces of fluorescence quenching similar to those of Mg-ATP were obtained in the presence of Mn-ATP (Fig. 2). The extent of decrease of the fluorescence intensity and, hence, the magnitude of the ΔpH generated is similar to that of Mg-ATP, as one could expect on the basis of the kinetics of the hydrolytic activity.

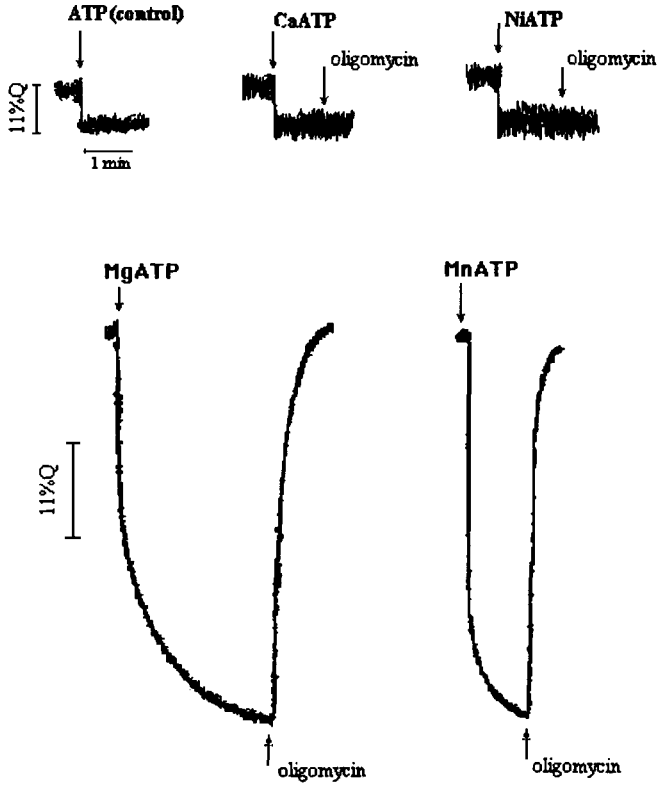

Fig. 2. Quenching of ACMA fluorescence induced in submitochondrial particles upon addition of different metal–ATP complexes. The assay medium contained 0.5 μ*M* ACMA and 0.4 mg submitochondrial particles and the reaction was started by addition of the substrates. (a), Control; (b), in the presence of 1 m*M* Ca-ATP; (c), 1 m*M* Zn-ATP; (d), 1 m*M* Mg-ATP; (e), 1 m*M* Mn-ATP. Oligomycin was added at a final concentration of 1 μg/mg protein.

Moreover, experiments performed in the presence of Zn-ATP and Co-ATP resulted in ACMA fluorescence quenching (not shown), suggesting that hydrolysis of these substrates can support a significant proton transport, which, however, has a magnitude considerably lower than that of Mg-ATP. Addition of oligomycin produced the complete recovery of ACMA fluorescence, whatever substrate was present.

When Ca-ATP and Ni-ATP were added to a suspension of submitochondrial particles, the fluorescence intensity of the probe resulted almost unchanged (Fig. 2), even when the incubation period was extended up to 20 min (data not shown). The small entity of quenching observed when Ca- and Ni-ATP were added was probably due to the interaction between ACMA and ATP, as it was present also in the control sample in which ATP was added in buffer. Moreover, the addition of either oligomycin or uncouplers had no effect on the quenching, clearly indicating that the quenching was not associ-

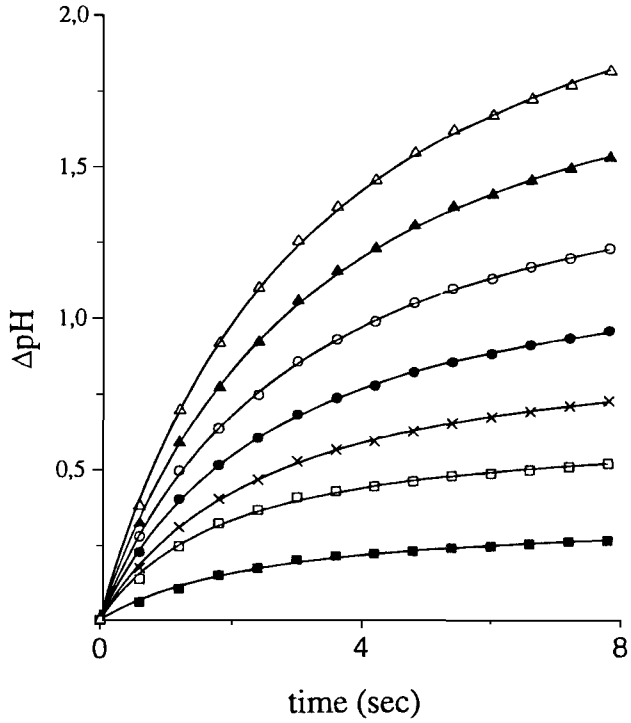

Fig. 3. Time course of pH gradient formation coupled to Mg-ATP hydrolysis. ΔpH is evaluated from traces of fluorescence quenching obtained at increasing substrate concentrations according to proper calibration. Different symbols indicate different substrate concentrations: filled square: 0.1 m*M* Mg-ATP; open square: 0.15 m*M* Mg-ATP; cross: 0.2 m*M* Mg-ATP; filled circle: 0.3 m*M* MgATP; open circle: 0.5 m*M* Mg-ATP; filled triangle: 0.8 m*M* Mg-ATP; open triangle: 1 m*M* Mg-ATP.

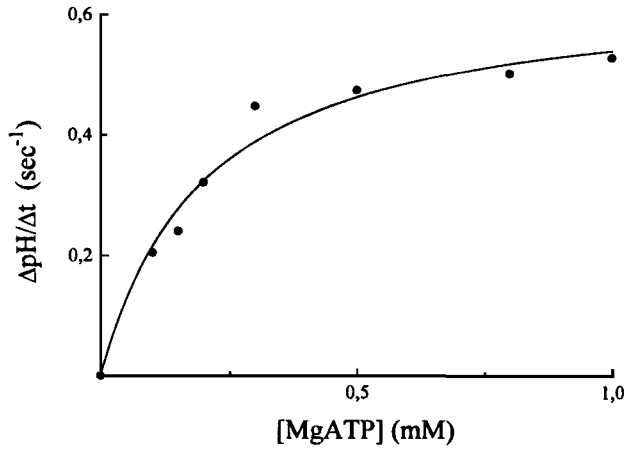

Fig. 4. Kinetic analysis of the proton pumping activity coupled to Mg-ATP hydrolysis in submitochondrial particles. The initial rate of ΔpH formation at increasing Mg-ATP concentrations was calculated from the time course of proton gradient formation, extrapolating the initial rate at $t = 0$. The kinetic constants were determined by nonlinear regression analysis.

ated to a gradient formation across the membrane. In order to verify whether Ca-ATP preincubated with submitochondrial particles could inhibit Mg-ATP driven proton translocation, assays were performed in the presence of both substrates. Thus, keeping Mg^{2+} concentration constant (0.1 m*M*), several concentrations of Ca^{2+} (up to 1 m*M*) were added to the incubation mixture prior to when ATP was added at concentrations equimolar with $Mg^{2+} + Ca^{2+}$. From the time dependence of fluorescence quenching, the initial velocity of the proton-pumping activity was determined and plotted as a function of increasing [Ca-ATP] (Fig. 5). It clearly appears that a decrease of the rate of ΔpH formation as [Ca-ATP] was increased, indicating a competition of the two Me-ATP for the catalytic sites. The experimental points could be fitted to a rate equation for competitive inhibition by Ca-ATP versus Mg-ATP; the inhibition constant thus determined (0.25 m*M*) is in good agreement with the experimental $K_{Ca\text{-}ATP}$ for the hydrolysis reaction. The inhibition by Ca-ATP could be reversed by increased concentrations of Mg-ATP, consistent with a competitive mechanism between two alternative substrates (not shown). This was expected, since both Ca- and Mg-ATP are substrates hydrolyzed by the enzyme and Mg-ATP only can support proton pumping.

Phosphorescence Measurements

We have previously shown that the ϵ subunit of the bovine heart mitochondrial F_1-ATPase is located in the stalk connecting F_0 to F_1 and suggested that it might be involved in the coupling between F_0 and F_1 (Gabellieri *et al.*, 1997). In earlier studies, we demonstrated that loading different nucleotides to the loose nucleotide binding sites of the nucleotide-depleted F_1 determined conformational changes of the ϵ subunit, as evidenced by monitoring changes of the phosphorescence decay parameters of the single Trp located at position 4 of the ϵ subunit (Baracca *et al.*, 1995).

Since Ca-ATP appeared unable to couple ATP hydrolysis catalyzed by F_1 to proton translocation through F_0, we hypothesized that if the ϵ subunit N-terminal segment were involved in transmitting conformational changes from F_1 to F_0, then different conformational changes might distinguish the binding of either Mg-ATP or Ca-ATP to the enzyme. Here we repeated the earlier experiments, in the presence of either Me-nucleotide. Both Mg-ATP and Ca-ATP could enhance the Trp phosphorescence decay rate to the same extent (Fig. 6).

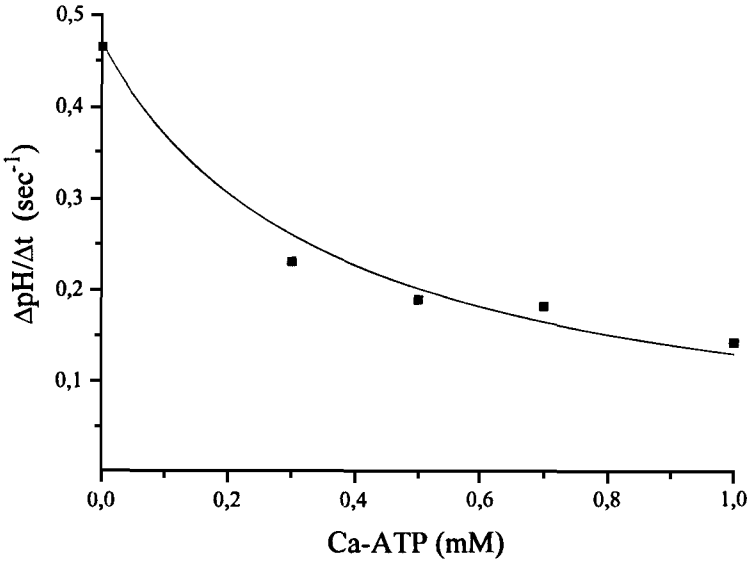

Fig. 5. Inhibition of Mg-ATP driven ΔpH formation by Ca-ATP. The assay medium contained 0.5 μ*M* ACMA, 0.4 mg submitochondrial particles, 0.1 m*M* Mg^{2+}, and increasing amounts of Ca^{2+}. The reaction was started by adding ATP at concentrations equimolar to the sum of the two metal cations. Fitting of the data was obtained on the basis of a model of alternative substrate inhibition according to Casadio and Melandri (1997). The $K_{Ca\text{-}ATP}$ calculated (0.25 m*M*) is coincident with that of Ca-ATP hydrolysis reported in Table I.

The average phosphorescence lifetimes (τ_{av}) derived from a biexponential curve fitting were 2.64 ms with Mg-ATP and 2.48 with Ca-ATP added to F_1 (Table II). These results suggest that both the magnesium and the calcium nucleotide bound to the catalytic sites of the enzyme can induce similar conformational changes on the N-terminal segment of the ε subunit, therefore suggesting an exclusion of the N-terminal segment of the ε subunit from the direct coupling of ATP hydrolysis to proton pumping. Results similar to that measured in the presence of Mg-ATP (not shown) were found from the phosphorescence decay rates of F_1 in the presence of the remaining Me-nucleotides tested, Zn-, Co-, Ni-, and Mn-.

DISCUSSION

The experiments described in this paper show, for the first time, that the beef heart mitochondrial F_0F_1-ATPase can not couple ATP hydrolysis to proton transport when some divalent cations, including Ca^{2+}, are bound to ATP. In this respect, the mitochondrial enzyme behaves similarly to homologous F_0F_1-ATPases from both bacterial photosynthetic membranes (Strid and Nyrèn, 1989; Casadio and Melandri, 1996) and chloroplast thylakoids (Pick and Weiss, 1988). Moreover, our results agree and supply a direct experimental support to early observations on beef heart submitochondrial particles, showing that some metal–nucleoside triphosphates did not participate in energy-linked reactions even though they were hydrolyzed by the proton ATPase. This implies, but does not demonstate, that hydrolysis of nucleoside triphosphate was not necessarily coupled to proton transport (Harris *et al.*, 1978). It is surprising that the only related system claimed to be capable of coupling Ca-ATP hydrolysis with transmembrane proton transport is the F_0F_1-ATPase of *E. coli* (Perlin *et al.*, 1984.)

Our results indicate that the ATPase activity of uncoupling Me-ATP is an intrinsic property of the mitochondrial F_0F_1-ATPase: the similarity of the V_{max}/K_m values for the metal-ATP complexes as hydrolysis substrates, as seen in Table I, suggests that the enzyme *in situ* could similarly utilize several metal-ATP complexes as substrates. Indeed, in chromatophores, the Ca-ATP behaves as a competitive substrate with respect to Mg-ATP (Casadio and Melandri, 1996). However, in mammalian cells, the concentration of the divalent cation assayed is about three orders of

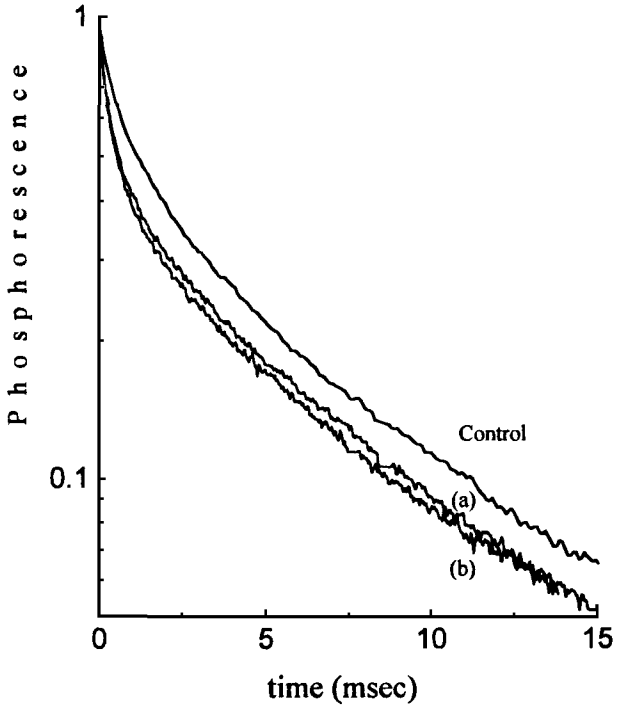

Fig. 6. Decay of phosphorescence intensity of F_i in the presence of Mg-ATP and Ca-ATP. The measurements were performed at 283K exciting the samples at 292 nm and detecting the emission at 440 nm. In all samples, F_i was present at a final concentration of 3 μM. Control: F_i; (a), in the presence of 0.8 mM Mg-ATP; (b), in the presence of 0.8 mM Ca-ATP. The decay parameters obtained are reported in Table II.

magnitude lower than that of Mg^{2+}. Therefore, the uncoupling effect induced on the mitochondrial F_0F_1-ATPase by several divalent metal tested, including Ca^{2+}, seems without any physiological meaning.

Table II. Kinetic Parameters of F_i Phosphorescence Decay (λ_{ex} = 292 nm) Measured at 283K[a]

	Phosphorescence decay				
Sample	τ_1 (ms)	α_1	τ_2 (ms)	α_2	τ_{av} (ms)
F_i	0.80	0.64	7.76	0.36	3.32
F_i + Mg-ATP	0.44	0.65	6.70	0.35	2.64
F_i + Ca-ATP	0.50	0.67	6.50	0.33	2.48

[a] The enzyme (3 μM) was dissolved in 0.15 M sucrose, 10 mM Hepes, 1 mM KH_2PO_4, pH 8, and 10% glycerol. Me-ATP was added to a final concentration of 0.8 mM. Triplet state lifetimes (τ_i) and preexponential terms (α_i) are derived from a biexponential fitting of the phosphorescence decay $P(t) = \alpha_1 e^{-t/\tau_1} + \alpha_2 e^{-t/\tau_2}$. τ_{av} is the average lifetime ($\tau_{av} = \alpha_1\tau_1 + \alpha_2\tau_2$).

The absence of proton translocation by some metal–ATP complexes does not appear to be the consequence of a structural dissociation of F_0 from F_1, since both oligomycin and DCCD, specific inhibitors of F_0, could inhibit the Me-ATPase activity. This also implies that a partial functional coupling between the two sectors is operative during these Me-ATP turnovers. On the other hand, the possibility that the Me-ATP could induce some artifactual proton leak in submitochondrial particles has to be considered unlikely, since in either the presence or the absence of Ca-ATP in the incubation medium, the respiratory chain of mitochondria sustained the formation of ΔpH equally well (not shown). The possibility remains that the uncoupling Me-ATP complexes differ from Mg-ATP since the metal-nucleotide might bind to the catalytic sites of the enzyme differently, suggesting that this could induce different conformational changes from the nucleotide-binding sites to the proton-transporting moiety of the enzyme.

Divalent cations are essential for nucleotides to be substrates of ATPases. They form coordinated complexes with phosphate oxygens of nucleotides and with protein residues (Abrahams *et al.*, 1994; Story and Steitz, 1992; Fry *et al.*, 1986), thereby contributing to the reactions at the catalytic sites. The behavior of Mg^{2+} and Ca^{2+} at the catalytic sites during the catalytic process appears rather similar; the lack of proton translocation coupled to the hydrolysis of Ca-ATP (or Ni-ATP) is possibly due to basic differences in the coordination chemistry of the cation (e.g., coordination bonds and angles), which do not allow that conformational changes associated with the γ-phosphate bond cleavage are efficiently transmitted to the protein moiety. This problem has been exhaustively discussed by Casadio and Melandri (1996) for the photosynthetic chromatophore H^+-ATPase and by Sondek *et al.* (1994) for transducin-α that hydrolyzes GTP. However, if one considers the ATP synthase as a rotating molecular machine (Junge and Engelbrecht 1997), then it can reasonably be added that the different behavior of the two cations could be related to the coupling mechanism within the F_1F_0-ATPase. Thus (assuming the nomenclature of the *E. coli* enzyme) during catalysis, the $\gamma\epsilon$ unit, which is firmly bound to the c subunit ring of F_0, rotates with respect to the $\alpha_3\beta_3$–ab unit, inducing the proton translocation through F_0 by transmitting conformational changes of the subunits. Therefore, one might speculate that Ca-ATP hydrolysis cannot induce a steady rotation of the $\gamma\epsilon$ unit, since it does not allow the correct conformational changes of the protein at the basis of the β-$\gamma\epsilon$ interaction leading to proton

translocation through F_0. Alternatively, rotation of the $\gamma\epsilon$ unit might arise from Ca-ATP hydrolysis, but the conformational changes of the same subunits, with this substrate, might not be capable of inducing proton flux through the enzyme.

Finally, an intriguing problem in the study of the mitochondrial F_1-ATPases concerns the understanding of the functional role of the smaller subunits. In particular, it was evidenced that a functional uncoupling occurred between F_0 and F_1 of a yeast mutant lacking the ϵ subunit (Guèlin *et al.*, 1993), which suggests that the subunit might be involved in the coupling of the two ATPase sectors. Moreover, the subunit is located in the stalk of the F_0F_1 complex (Gabellieri *et al.*, 1997), and, therefore, in a domain of the protein involved in the coupling between F_0 and F_1. Since the binding of different nucleotides to the catalytic sites of F_1 could induce different conformational changes of the ϵ subunit, as monitored by intrinsic phosphorescence emission (Baracca *et al.*, 1995), we assayed whether the binding of the uncoupling Ca-ATP, could induce different conformational changes of the ϵ subunit with respect to Mg-ATP. As shown in Table II, this was not the case and, in general, for any other Me-ATP tested (not shown), suggesting that the Trp phosphorescence changes are reporting the binding step of Me-ATP to the catalytic site, rather than intra- or intersubunit conformational changes related to ATP-induced proton translocation. Therefore, the ϵ subunit or at least its N-terminal segment, where Trp is located (Walker *et al.*, 1985; Solaini *et al.*, 1993), seems not to be involved in the coupling between catalytic and proton transport processes in the mitochondrial F_0F_1 complex.

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