Mitochondrial ATP Synthase: Dramatic Mg^{2+}-Induced Alterations in the Structure and Function of the F_1-ATPase Moiety†

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ABSTRACT: The ATPase activity of the F_1 moiety of rat liver ATP synthase is inactivated when incubated prior to assay at 25 °C in the presence of $MgCl_2$. The concentration of $MgCl_2$ (130 μM) required to induce half-maximal inactivation is over 30 times higher than the apparent K_m ($MgCl_2$) during catalysis. Moreover, the relative efficacy of divalent cations in inducing inactivation during prior incubation follows an order significantly different from that promoting catalysis. Inactivation of F_1-ATPase activity by Mg^{2+} is accompanied by the dramatic dissociation from the F_1 complex of α subunits and part of the γ-subunit population. The latter form a precipitate while the β, δ, and ϵ subunits, and the remaning part of the γ-subunit population, remain soluble. Dissociation is not a sudden "all or none" event but parallels loss of ATPase activity until α subunits have almost completely dissociated together with about 50% of the γ-subunit population. Mg^{2+}-induced loss of F_1-ATPase activity cannot be prevented by including either the hydrolytic substrates ATP, GTP, or ITP in the incubation medium or the product ADP. Ethylenediaminetetraacetic acid, mercaptoethanol, and dithiothreitol are also ineffective in preventing loss of ATPase activity. Significantly, KP_i at high concentration ($\geq$200 mM) is effective in partially protecting F_1 against inactivation. However, the most effective means of preventing Mg^{2+}-induced inactivation of F_1-ATPase activity is to rebind F_1 to its F_0 moiety in F_1-depleted particles. When bound to F_0, F_1 is protected completely against divalent cation induced inactivation. These results indicate that F_1 contains, in addition to the high-affinity divalent cation sites involved in promoting catalysis, additional sites of lower affinity which either are masked in the intact ATP synthase complex (F_0F_1) or interact with the intact complex with a functional purpose other than inactivation.

The mitochondrial ATP synthase molecule consists of two major components, F_1 and F_0 [for reviews, see Fillingame (1981), Cross (1981), Wang (1983), Senior and Wise (1983), Amzel and Pedersen (1983), and Hatefi (1985)]. F_1 is a water-soluble protein of about 360–380 kilodaltons (kDa)[1] which hydrolyzes ATP in the presence of divalent cations. Mg^{2+} is the physiologically important cation, but Mn^{2+}, Zn^{2+}, and Co^{2+} can also support high rates of ATP hydrolysis (Senior et al., 1980; Williams et al., 1987). The F_1 molecule has a complex substructure which has been shown from several species to be $\alpha_3\beta_3\gamma\delta\epsilon$ (Catterall & Pedersen, 1971; Catterall et al., 1973; Senior, 1973; Esch & Allison, 1979; Foster & Fillingame, 1982). F_0 is a complex which presumably functions as a proton channel. It directs protons to F_1, resulting in net ATP synthesis. That is, when F_0 and F_1 are associated in the mitochondrial inner membrane as the native F_0F_1 complex, and then subjected to an electrochemical proton gradient, the F_1 molecule now synthesizes rather than hydrolyzes ATP.

The substructure involved at the interface between F_0 and F_1 has been the subject of investigation in several laboratories (Futai et al., 1974; Hermolin et al., 1983; Hundal et al., 1983; Dupuis et al., 1985; Ovichinnikov et al., 1985; Penin et al., 1986). In bacteria, the "b" protein of F_0 and the δ subunit of F_1 are critical for binding F_1 to F_0 (Hermolin et al., 1983; Futai et al., 1974). In animal systems, a protein called OSCP, conserved in bacteria as the b + δ proteins (Ovichinnikov et al., 1985), also appears to be critical, at least in part, for the binding interaction between F_0 and F_1 (Hundal et al., 1983; Dupuis et al., 1985; Penin et al., 1986). Two other proteins, one called F_6 and a second referred to as the 26.5-kDa protein, have been implicated also in binding F_1 to F_0 in animal systems (Vadineau et al., 1976; Fisher et al., 1981; Liang & Fisher, 1983).

Central to the function of F_1 in its native physiological environment is the divalent cation Mg^{2+}. It is required when F_1 functions either as an ATP synthase or as a coupled ATPase in supporting ATP-dependent functions. The isolated F_1 molecule has been shown to contain a single tight, nonexchangeable, Mg^{2+} binding site whose function remains to be elucidated (Senior, 1979; Daggett et al., 1985; Williams et al., 1987). F_1 also binds three molecules of MgATP (Cross & Nalin, 1982; Wise et al., 1983; Issartel et al., 1986; Williams et al., 1987) which are believed to interact with catalytic sites on β subunits (Khananshvili & Gromet-Elhanan, 1984, 1985) or at interfaces between α and β subunits (Williams & Coleman, 1982; Khananshvili & Gromet-Elhanan, 1984). Significantly, several laboratories have shown that the substrate for ATP hydrolysis is, in fact, MgATP (Selwyn, 1967; Akimenko et al., 1972; Gruys et al., 1985), and others have indicated that both the Mg^{2+} and ATP moieties of this substrate may be bound to F_1 at catalytic sites (Hochman & Carmeli, 1981; Fry & Mildvan, 1986; Williams et al., 1987).

We report here the surprising findings that Mg^{2+} alone induces dramatic alterations in both the structure and function of F_1. Results of experiments summarized below indicate that

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s); HPLC, high-performance liquid chromatography.

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the divalent cation sites on F_1 involved in promoting these structural–functional alterations are distinct from the four Mg^{2+}/MgATP binding sites identified, to date, on eukaryotic F_1–ATPases (Senior, 1979; Cross & Nalin, 1982; Wise et al., 1983; Daggett et al., 1985; Issartel et al., 1986; Williams et al., 1987; Kironde & Cross, 1987).

Experimental Procedures

Materials

Rats were obtained from Charles River Breeding Laboratories. The following compounds were obtained from Sigma Chemical Co.: ADP, GTP, ITP, $MgCl_2$, Tris-HCl, EDTA, D-(+)-mannitol, Hepes, bovine albumin (fraction V), digitonin, urea, and $ZnCl_2$. ATP was purchased from Pharmacia and $CoCl_2$ from Fisher Chemical Co. $CaCl_2$, KP_i, ammonium sulfate, sucrose, and chloroform were purchased from J. T. Baker Chemical Co. Acrylamide, methylenebis(acrylamide), and Coomassie Blue R-250 were obtained from Bio-Rad. All other reagents were of the highest purity commercially available.

Methods

Isolation of Mitochondria. Rat liver mitochondria were isolated by the "high yield" differential centrifugation method developed in this laboratory (Bustamante & Pedersen, 1977). The isolation medium contained 220 mM D-mannitol, 70 mM sucrose, 2 mM Hepes, and 0.5 mg/mL defatted bovine albumin.

Preparation of Inverted Inner Membrane Vesicles. Purified inner membrane vesicles were prepared by a slight modification of the original procedure described earlier from this laboratory by Chan et al. (1970). The modification is essentially that described by Hackenbrock and Hammon (1975) and is as follows: mitoplasts (10 mL, 50 mg of protein/mL) are suspended in 250 mL of distilled water and centrifuged at 10000*g* for 15 min. The supernatant is discarded, and the sediment is suspended in 10 mL of distilled water. The suspension is then subjected to sonic oscillation with the large probe of a Bronwill Biosonik sonic oscillation apparatus. Sonic oscillation is carried out at 80% maximal intensity for a total of 2 min in 15-s intervals, 0–4 °C. Centrifugation is carried out for 10 min at 10000*g*. The sediment is discarded, and the supernatant is sedimented at 100000*g* for 1 h. The final sediment (purified inner membranes, essentially free of outer membrane, intracristal space, and matrix activities) is suspended in the mitochondrial isolation medium at 20 mg of protein/mL, frozen in liquid nitrogen, and stored at 70 K until use.

Preparation of F_1-Depleted Inner Membrane Vesicles. Urea particles were prepared essentially as described previously by Pedersen and Hullihen (1978). One milliliter of 6.4 M urea was added to 60 mg of fresh inner membrane vesicles in 1 mL of isolation medium (see above). The mixture, after sitting on ice for 5 min, was diluted to 10 mL with isolation medium and centrifuged at 48 000 rpm for 30 min in the Spinco 65 rotor. The sediment was suspended in 8.0 mL of isolation medium and centrifuged again at 48 000 rpm. The final sediment was suspended in isolation medium, frozen, and stored in liquid nitrogen.

Preparation of F_1-ATPase. F_1 was prepared from rat liver mitochondria by the procedure of Catterall et al. (1979) with one exception. The terminal Sephadex G-200 step was replaced by chromatography on a 2.1 × 13 cm Sephadex G-25 column packed on top of a 1.5-cm layer of sea sand. The Sephadex G-200 step had little effect in enhancing the specific ATPase activity of F_1 and was replaced with Sephadex G-25 to facilitate the purification as well as to remove any loosely bound nucleotides. After the Sephadex G-25 step, fresh, homogeneous preparations were concentrated by using an Amicon PM 10 filter, then lyophilized from ~100–200 μL of the Sephadex G-25 eluate containing 250 mM KP_i and 5.0 mM EDTA, pH 7.5, and stored at −20 °C until use. Preparations used in this study had an ATPase specific activity of between 20 and 30 μmol min^{-1} mg^{-1} when assayed in the Tris-HCl system described below.

With the exception of the reconstitution studies noted below, lyophilized samples of F_1 were, prior to all other studies, dissolved in 100–200 μL of water at room temperature. They were then precipitated with an ammonium sulfate solution containing 3.5 M ammonium sulfate, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.5. The enzyme was then redissolved in the buffers indicated in the legends to tables and figures.

Reconstitution of F_1 with F_1-Depleted Particles. Reconstitution of F_1 with urea particles was carried out by the procedure of Pedersen and Hullihen (1979) incubating the following components in a final volume of 0.1 mL for 2 h at 25 °C: 0.5 mg of urea particles, indicated amounts of F_1-ATPase, 5 mM ATP, 1 mg of defatted albumin, 88 mM D-mannitol, 28 mM sucrose, 0.8 mM Hepes, 100 mM KP_i, and 2 mM EDTA, pH 7.5. After the mixture is centrifuged in the Spinco 65 rotor (0–4 °C) at 48 000 rpm for 30 min, the sediment (reconstituted membranes) is resuspended in 0.1 mL of isolation medium (0–4 °C). The reconstituted membrane fraction was assayed immediately for ATPase activity.

Assay for ATPase Activity. ATPase activity was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADH via the pyruvate kinase and lactic acid dehydrogenase reactions (Pullman et al., 1960). The reaction mixture contained the following in a volume of 1 mL at pH 7.5 and 25 °C: 4.0 mM ATP, 65 mM Tris-HCl, 4.8 mM $MgCl_2$, 2.5 mM KP_i, 0.40 mM NADH, 0.60 mM phosphoenolpyruvate, 5 mM KCN, 1 unit of lactic acid dehydrogenase, 1 unit of pyruvate kinase, and 20–50 μg of inner membrane vesicles. Initial rates were used in all specific activity calculations.

Where indicated, ATPase activity was assayed also by monitoring the release of P_i in a 1-mL system containing 45 mM Tris-HCl, pH 7.4, and concentrations of ATP, divalent cation, and F_1 specified in the legends to tables and figures. The assay was carried out for 1 min at 25 °C and then terminated with 0.1 mL of 2.5 M $HClO_4$. Aliquots of the reaction mixture were taken for phosphate determination by the colorimetric procedure of Gomori (1962) or Baginski et al. (1974) as specified in the legends to tables or figures. The 1-min time point lies well within the range of linearity of the assay under all conditions used in this study.

Gel Electrophoresis in SDS. SDS–PAGE was carried out by a modification of the Weber and Osborn (1969) procedure in 10% polyacrylamide gels in the presence of 0.1% SDS. The samples were incubated for 2 h in 5% SDS, 5% mercaptoethanol, 9 M urea, and 10 mM NaP_i, pH 7.0. The gels were run at a constant current of 8 mA/tube for 5–6 h at room temperature. The gels were fixed and stained in 25% isopropyl alcohol, 10% acetic acid, and 0.025% Coomassie blue overnight. The staining solution was then changed to 10% isopropyl alcohol, 10% acetic acid, and 0.0025% Coomassie blue for 6–9 h and destained in 10% acetic acid. The gels were stored in 7.5% acetic acid. Stain intensity was determined by scanning the stained gels at 540 nm using a scanning attachment for a Gilford spectrophotometer.

Determination of Protein. Membrane protein was estimated by the biuret reaction in the presence of 0.26% sodium cholate

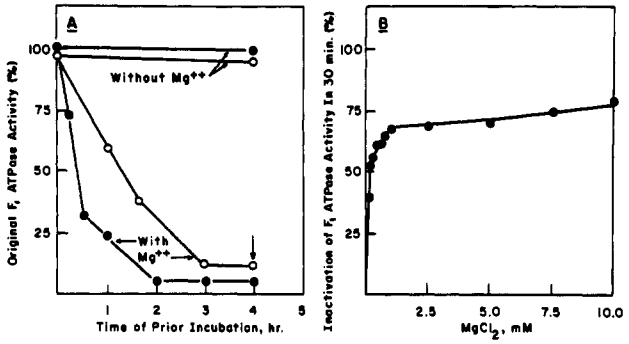

FIGURE 1: Inactivation of F_1 when incubated with the divalent cation Mg^{2+}. (A) F_1 was incubated at 25 °C in a total volume of 0.1 mL containing 50 mM Tris-HCl, pH 7.5, and where indicated 5 mM $MgCl_2$. At the indicated times, aliquots were removed from the incubation mixture and assayed for ATPase activity in the spectrophotometric assay system described under Methods. (●) 0.32 mg/mL; (○) 2.32 mg/mL. At the higher protein concentration, aliquots were taken at 4 h (vertical arrow) for analysis by SDS–PAGE (see Figure 3 and the accompanying legend). (B) Conditions were identical with (A) except a number of different incubations were carried out for 30 min at different $MgCl_2$ concentrations prior to assay. The concentration of F_1 in the prior incubation assay was 0.32 mg/mL. (Note: Half-maximal inactivation occurred at 0.13 mM $MgCl_2$ under these conditions.)

Table I: Comparison of the Relative Capacities of Divalent Cations To Activate the ATPase Activity of F_1 and To Inhibit F_1 If Incubated Prior to Assay

Experiment A[a]	
divalent cation in ATPase act. assay	sp ATPase act. of F_1 (μmol of ATP hydrolyzed min^{-1} mg^{-1})
Zn^{2+}	40.4
Mn^{2+}	25.7
Mg^{2+}	23.0
Co^{2+}	22.8
Ca^{2+}	4.3
Sr^{2+}	0.4
Experiment B[b]	
divalent cation present during prior incubation	loss of ATPase act. (%)
Mn^{2+}	62.9
Zn^{2+}	51.3
Ca^{2+}	51.3
Co^{2+}	48.1
Mg^{2+}	40.3
Sr^{2+}	31.9

[a] ATPase activity of F_1 (13.5 μg) was determined after 1 min in a 1-mL system in the presence of 5 mM ATP/5 mM divalent cation (Cl form) by quantifying the release of P_i at 25 °C by the method of Gomori (1962). [b] Loss of ATPase activity at 25 °C upon 15-min prior incubation of F_1 (67.5 μg) in the presence of 5 mM divalent cation and 50 mM Tris-HCl (final volume = 0.1 mL) was determined by using either the same assay or the spectrophotometric ATPase assay described under Methods. Results are averages of four different experiments carried out in duplicate on different F_1-ATPase preparations.

(Jacobs et al., 1956). Soluble protein was measured by the method of Lowry et al. (1951). The protein was routinely precipitated with 5% trichloroacetic acid and redissolved in 1 N NaOH prior to the determination of protein.

RESULTS

Mg^{2+}-Induced Inactivation of F_1-ATPase Activity. Results of experiments summarized in Figure 1A show that the ATPase activity of purified F_1 is markedly inactivated when the enzyme is placed in the presence of 5 mM $MgCl_2$ and 50 mM Tris-HCl, pH 7.5. Half-maximal inactivation is achieved in less than 30 min when the F_1 protein concentration is 0.32 mg/mL. Increasing the F_1 concentration to 2.32 mg/mL

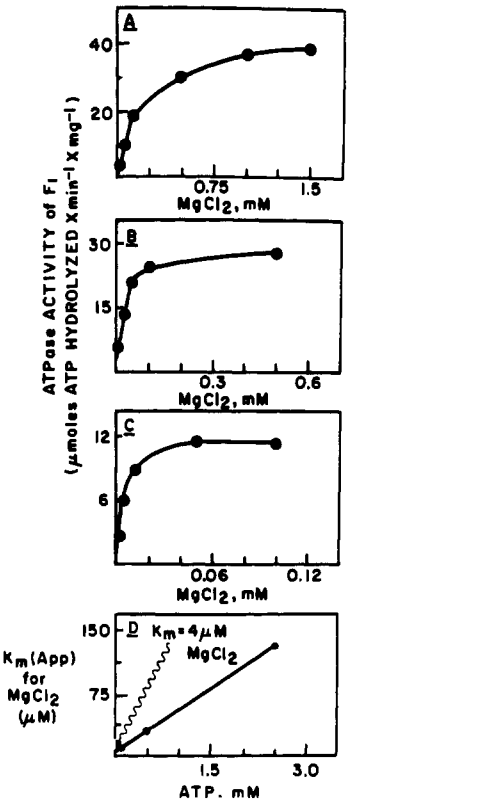

FIGURE 2: Dependence of F_1-ATPase activity on the concentration of $MgCl_2$ at different fixed ATP concentrations. ATPase activity was followed by monitoring the release of P_i after 1 min using the procedure of Baginski et al. (1974). ATP concentrations were 2.5 mM in (A), 0.5 mM in (B), and 0.1 mM in (C). $MgCl_2$ concentrations are specified in the figure. Apparent K_m values for $MgCl_2$ were 0.125 mM in (A), 0.025 mM in (B), and 0.005 mM in (C). An apparent K_m for $MgCl_2$ at "zero" ATP concentration was extrapolated in (D) by plotting apparent K_m values obtained in (A) → (C) vs ATP concentration.

delays the half-inactivation time to about 1.5 h. Significantly, inactivation is induced by Mg^{2+} and not by Cl^-. Thus, in 50 mM Tris-HCl buffer alone, F_1 loses little or no ATPase activity in 4 h at the two protein concentrations tested (Figure 1A).

Results of experiments summarized in Figures 1 and 2 and Table I indicate that the divalent cation site(s) on F_1 involved in the inactivation of ATPase activity of F_1 has (have) kinetic and specificity properties distinctly different from those involved in supporting ATPase activity. Thus, the apparent K_m for $MgCl_2$ of 4 μM (Figure 2D) for sites on F_1 involved in supporting ATPase activity is 33 times less than that required to induce half-maximal inactivation (Figure 1B). Moreover, the specificity of divalent cations in inducing inactivation of F_1-ATPase activity is in the order $Mn^{2+} > Zn^{2+} > Ca^{2+} > Co^{2+} > Mg^{2+} > Sr^{2+}$ (Table IB) whereas the specificity of the same metal cations in supporting ATPase activity is in the order $Zn^{2+} > Mn^{2+} > Mg^{2+} > Co^{2+} > Ca^{2+} > Sr^{2+}$ (Table IA). Significantly, Ca^{2+} and Sr^{2+} have little or no capacity to support ATPase activity during catalytic assays (Table IA). Yet, when incubated with F_1 for 15 min prior to assay, both cations induce more than 30% inactivation of F_1-ATPase activity (Table IB).

Effect of Mg^{2+} on the Subunit Structure of F_1. During prior incubation of F_1 with $MgCl_2$, the solution acquired a distinct turbidity. In order to investigate the nature of this phenomenon, F_1 (2.32 mg/mL) was allowed to incubate at room temperature for 4 h in the presence of 5 mM $MgCl_2$ in Tris-HCl buffer, pH 7.5. At this point, F_1 had lost about 90% of its original ATPase activity. As indicated in Figure 1A,

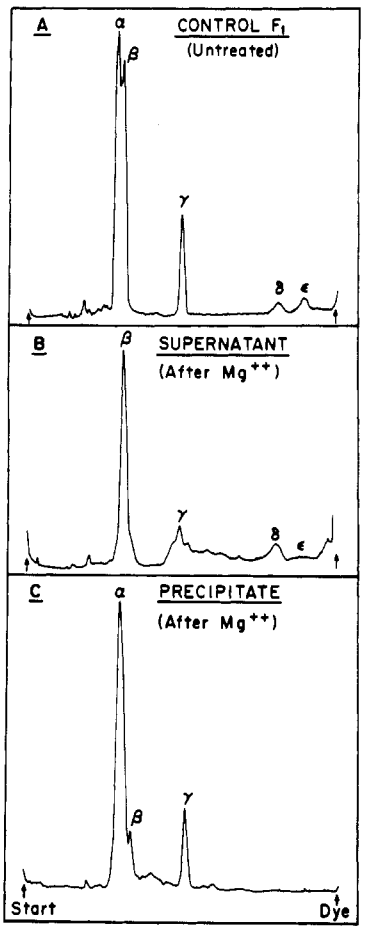

FIGURE 3: SDS–PAGE scans of control F_1 (A) relative to scans of supernatant (B) and pellet (C) fractions of F_1 after incubation with $MgCl_2$ in Tris-HCl buffer. F_1 at 2.32 mg/mL in 50 mM Tris-HCl buffer, pH 7.5, was incubated at 25 °C with 5 mM $MgCl_2$ for 4 h (see Figure 1). The incubation mixture (0.1 mL) was then separated into supernatant and pellet fractions by centrifugation for 5 min in an Eppendorf microfuge. The pellet fraction was dissolved in 0.1 mL of 1% SDS, 1% mercaptoethanol, and 10 mM NaP_i, pH 7.0. SDS–PAGE was then carried out as described under Methods with 10 μL of the pellet and supernatant fractions and with 5 μL (11.6 μg) of untreated F_1.

aliquots were taken then and centrifuged to sediment the precipitate giving rise to the turbidity. Both supernatant and precipitate fractions were analyzed by SDS–PAGE. Results of experiments presented in Figure 3 show clearly that the precipitate contains the α-subunit population and a significant amount of the starting γ-subunit population (Figure 3C). Very little β subunit (<5% of the total) appears in the precipitate. Rather, most of the β-subunit population and that of the δ and ε subunits remain in the supernatant, as does the remaining population of γ subunits (Figure 3B). [The small fraction of active F_1 present after 4 h (Figure 1A) might be expected to give rise in SDS–PAGE gels of the supernatant fraction to a small peak for the α subunit. At the levels of protein used, this small predicted amount of α subunit is not detected, but it is observed upon electrophoresing a 5-fold excess of the supernatant fraction.]

In experiments not summarized here, the supernatant fraction was subjected to HPLC gel permeation chromatography on a Waters I-300 column to establish the aggregation state of F_1 subunits in this fraction. The elution profile was characterized first by a very small peak eluting with the same volume as untreated "native F_1". This small peak was shown to represent that small amount of the F_1 population (~10%) that remained resistant to F_1 inactivation by Mg^{2+} after 4 h

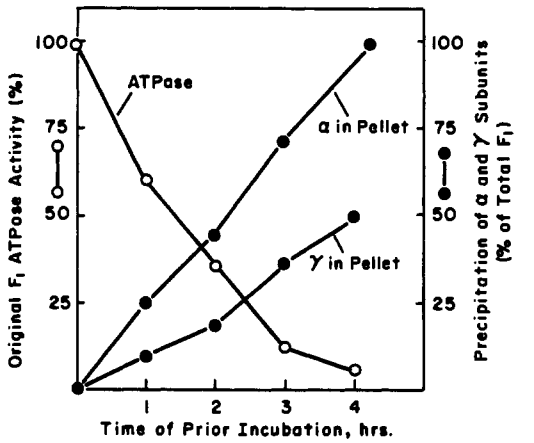

FIGURE 4: Relationship between the loss in F_1-ATPase activity induced by $MgCl_2$ and the appearance of α and γ subunits in the pellet fraction. F_1 was incubated in the presence of 5 mM $MgCl_2$ exactly as described in the legend to Figure 3. At the indicated times, aliquots were removed and assayed for ATPase activity in the spectrophotometric assay described under Methods. Supernatant and pellet fractions were then separated also as described in Figure 3 and subjected to SDS–PAGE as described under Methods. The percent of the total α and γ subunits appearing in the pellet was quantified from scans of Coomassie-stained gels by direct weighing of subunit peaks.

Table II: Inability of Substrates, Products, and EDTA To Protect F_1-ATPase Activity against Inactivation by Mg^{2+}

in prior incubation	ATPase act. (μmol of ATP hydrolyzed min^{-1} mg^{-1})	loss of ATPase (%)
F_1	22.8	0
F_1 + 5 mM $MgCl_2$	8.1	64.4
F_1 + 5 mM $MgCl_2$ + 5 mM ADP	7.4	67.5
F_1 + 5 mM $MgCl_2$ + 5 mM KP_i	7.7	66.2
F_1 + 5 mM $MgCl_2$ + 5 mM ATP	11.4	50.0
F_1 + 5 mM $MgCl_2$ + 5 mM ITP	7.5	67.1
F_1 + 5 mM $MgCl_2$ + 5 mM GTP	4.9	78.0
F_1 + 5 mM $MgCl_2$ + 5 mM EDTA	7.7	66.2

[a] F_1, 41 μg, was incubated for 1 h at 25 °C in a total volume of 0.1 mL containing 50 mM Tris-HCl, pH 7.5. Where indicated, either 5 mM $MgCl_2$ or 5 mM $MgCl_2$ + 5 mM of the specified compounds was included in the incubation mixture. Aliquots were then removed from the incubation mixture and assayed for ATPase activity in the spectrophotometric assay system described under Methods. Results are average values obtained from two different experiments, carried out in duplicate, using two different F_1-ATPase preparations.

(Figure 1A). (It is possible that this Mg^{2+}-resistant fraction represents a population of F_1 containing bound nucleotides, or perhaps more bound nucleotide than the Mg^{2+}-sensitive fraction.) A second much larger peak eluting from the HPLC column exhibited an apparent molecular weight of ~60 000, very near that of individual β subunits. Thus, β subunits remaining in the supernatant after Mg^{2+} treatment appear to be in monomeric form rather than oligomeric form (i.e., as β_2 or β_3 species). Peaks eluting with an apparent molecular weight below 60 000 were not observed, indicating that the smaller subunits either were irreversibly bound to the HPLC column or were not eluted in sufficient quantities to be detected.

Results of experiments presented in Figure 4 show that loss of F_1-ATPase activity upon prior incubation with $MgCl_2$ is accompanied by a corresponding time-dependent increase in the appearance of α and γ subunits in the precipitate fraction. These results show that inactivation of F_1-ATPase activity by Mg^{2+} is related directly to enzyme dissociation.

Inability of Substrates, Products, and EDTA To Protect F_1-ATPase Activity against Inactivation by Mg^{2+}. Results

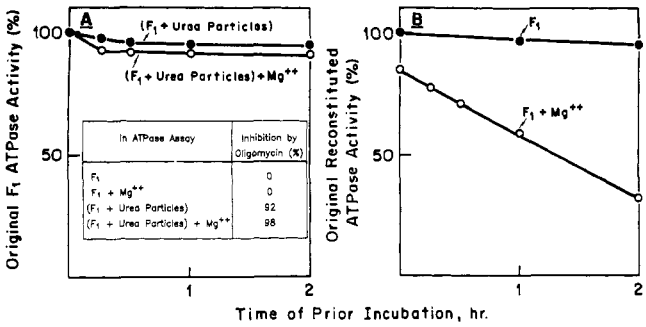

FIGURE 5: Complete protection of F_1-ATPase activity after rebinding to its F_0 moiety in F_1-depleted particles (urea particles). F_1-depleted particles were prepared with urea exactly as described under Methods and reconstituted with purified F_1 also as described under Methods. (A) Urea particles (0.5 mg) reconstituted with 0.15 mg of F_1 (specific activity = 21 μmol of ATP hydrolyzed min^{-1} mg^{-1}) were incubated ±5 mM $MgCl_2$ at 25 °C in 0.1 mL of isolation medium (220 mM mannitol, 70 mM sucrose, 0.5 mg/mL defatted bovine albumin, and 2.0 mM Hepes, pH 7.4). Aliquots were removed at the indicated times and assayed for ATPase activity in the spectrophotometric assay described under Methods. (B) F_1, 490 μg/mL, suspended in 0.1 mL of isolation medium was incubated ±5 mM $MgCl_2$ and assayed at the indicated times exactly as described in (A).

of experiments summarized in Table II show that 5 mM concentrations respectively of ADP, KP_i, ATP, ITP, GTP, and EDTA have little or no effect in protecting F_1 against Mg^{2+} inactivation during prior incubation. Of the compounds tested, only ATP had a slight (~14%) protective effect. In fact, in the presence of Mg^{2+} plus ITP or GTP, both of which are catalytic substrates for F_1, the enzyme lost more activity during prior incubation than in the presence of Mg^{2+} alone. In results not presented here, it was shown that mercaptoethanol and dithiothreitol are also without effect of Mg^{2+}-induced inactivation of F_1, ruling out a role of SH groups in this process.

Partial Protection of F_1 against Mg^{2+} Inactivation by High P_i Concentration and Complete Protection by Rebinding to F_0. Results of additional experiments not presented here showed that KP_i at a concentration of 200 mM protects F_1 partially against inactivation by 5 mM Mg^{2+}. Thus, in 4 h, F_1 (1.86 mg/mL) loses only about 50% of its original ATPase activity when placed in 200 mM KP_i buffer + 5 mM $MgCl_2$ as opposed to 90% when retained in 50 mM Tris-HCl, pH 7.5. This protective effect is reflected in partial retention (~50%) of α subunits in the supernatant fraction. Protection by KP_i could be ascribed to P_i rather than to K^+ as 200 mM KCl was completely ineffective in protecting F_1 against inactivation by Mg^{2+}.

Experimental results presented in Figure 5 show that when F_1 is rebound to its F_0 moiety in F_1-depleted inner membrane vesicles (urea particles) it becomes completely resistant to inactivation by $MgCl_2$. Control experiments also presented in Figure 5A (inset) verify that F_1 has, in fact, bound to F_0, as the enzyme becomes over 90% inhibited by oligomycin. Moreover, Figure 5A shows that the sucrose/mannitol/bovine albumin medium used to conduct the reconstitution studies has no protective capacity. In this medium, F_1-ATPase activity remains markedly inhibited by Mg^{2+}. It seems clear, therefore, that interaction of F_0 with F_1 prevents Mg^{2+} from inducing inactivation of F_1.

Discussion

Results of experiments summarized here provide some new and intriguing information about the interaction of Mg^{2+} with the ATP synthase complex F_0F_1. The data show quite clearly that when the F_1 moiety of this complex is presented with Mg^{2+} either in the presence or in the absence of substrates or products (Figure 1, Table II), it undergoes a dramatic loss of ATPase activity with concomitant dissociation into an $\alpha\gamma$ fraction and a $\beta\gamma\delta\epsilon$ fraction (Figures 3 and 4). The additional findings that the divalent cation specificity and apparent K_m (Mg^{2+}) for the inactivation process (Table I, Figure 2) differ significantly from those for ATP hydrolytic activity (Table I) indicate that F_1 contains separate sites specialized respectively for Mg^{2+}-dependent ATPase activity and Mg^{2+}-induced inactivation. Finally, the experiments showing that rebinding of F_1 to F_0 prevents Mg^{2+}-induced inactivation (Figure 5) suggest either that the "Mg^{2+} inactivation" sites may lie on a region of F_1 which binds to F_0 or that F_0 induces a conformational change in F_1 which completely masks inactivation sites.

The findings that nucleoside triphosphate substrates and EDTA afford little or no protection against Mg^{2+} inactivation of F_1 (Table II) are of interest. Mg^{2+} has a coordination number of 6 and is normally bound to six water molecules, the net charge on the complex being +2 (Stezowski & Hoard, 1984). However, when complexed with EDTA or ATP, the net charge becomes a −2 (Stezowski & Hoard, 1984), as coordination linkages normally involved in binding water are replaced with negative groups (carboxylate groups in the case of EDTA and phosphate groups in the case of ATP). Thus, it seems likely that the metal may lead these individual charged complexes to subunit interacting sites on F_1 in which ionic bonding is a key stabilizing force. Once at these sites, the charges on the incoming metal complexes may disrupt critical salt bridges, leading to subunit dissociation and enzyme inactivation. In the intact, F_0F_1 ATP synthase complex, F_0 must, in some way, stabilize this labile region in F_1 as rebinding of F_1 or F_0 prevents Mg^{2+}-induced inactivation of the enzyme (Figure 5). These observations may be of potential significance to the function of the native F_0F_1 complex, especially if one or more metal binding sites are retained at the interface between F_0 and F_1. Along these lines, it is interesting to note that Abrams and co-workers (Abrams et al., 1976) believe a metal ion site is involved in binding the *Streptococcus facaelis* F_1 to its F_0 moiety, a site localized to the smaller subunits, most likely δ. Moreover, Racker (1977) has speculated that proton translocation through F_0 may displace Mg^{2+} on its path to/through F_1. Finally, Fisher and co-workers (Fisher et al., 1981; Liang & Fisher, 1983) have shown that a stable complex consisting of F_1 and a 26.5-kDa F_0 component can be isolated in the presence of Mg^{2+}.

It is possible also that the Mg^{2+} inactivation site(s) on F_1 may be involved in the degradation of F_1 during mitochondrial turnover. Thus, F_1 may become detached from F_0, exposing Mg^{2+} inactivation sites. In the presence of matrix Mg^{2+} (or MgATP), this would result in the dissociation of F_1 into $\alpha\gamma$ and $\beta\gamma\delta\epsilon$ fractions which may be highly susceptible to the action of proteases. Preparation of F_1 for degradation in this way need not necessarily involve its detachment from F_0, provided that the F_0 component masking Mg^{2+} inactivation sites on F_1 is simply displaced. Significantly, Moyle and Mitchell (1975) have reported in a brief note that Mg^{2+} does induce inactivation of ATPase activity in some submitochondrial preparations.

In addition to providing new information about the interaction of Mg^{2+} with the F_1 moiety of rat liver ATP synthase complex, results presented here are directly relevant also to the structure of F_1. Previous reports from this laboratory (Williams et al., 1984) have shown, using a cold denaturation/heat shock cycle, that the γ subunit is associated with both α and β subunits of F_1. As emphasized in this study,

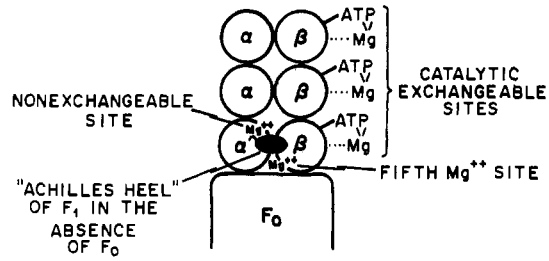

FIGURE 6: Model depicting F_1-ATPases of eukaryotic systems as containing at least three different classes of metal ion binding domains. *Class I* is depicted by the sites for the three exchangeable nucleotide metal complexes (see text for references) now localized to the catalytic β subunit (Kironde & Cross, 1987). *Class II* is depicted by the single, nonexchangeable Mg^{2+} (Senior, 1979; Daggett et al., 1985), bound on either an α or a γ subunit, or at the interface thereof (Williams et al., 1987). *Class III*, characterized by this study, is depicted as a site residing near the interface between F_0 and F_1. The dark region cross-linking the $\alpha\beta$ pair is considered in the model to contain, in addition to the γ subunit, the δ and ϵ subunits which are known to help bind F_1 to F_0. As incubation of rat liver F_1 in the cold followed by heat shock (Williams et al., 1984) or incubation of the enzyme with Mg^{2+} at room temperature (this study) both split F_1 into $\alpha\gamma$ and $\beta\gamma$ fractions, it is suggested that the $\alpha\beta$ pair bridged by the single γ subunit may be the weakest point or "Achilles Heel" of the quaternary structure of F_1. (Please note that the model is not meant to reflect an accurate structural arrangement of α and β subunits which are thought to alternate.)

Mg^{2+}-induced inactivation of F_1 also results in retention of the γ subunit with fractions containing both the α and β subunits (Figures 3 and 4). These results provide additional evidence that the subunit structure of F_1 is characterrized by an "asymmetric center" in which the single γ subunit interacts with at least one of the three $\alpha\beta$ pairs in the enzyme complex (Williams et al., 1987). Moreover, it seems likely that this asymmetric center is the "Achilles heel" of the F_1 structure as the two independent dissociation techniques mentioned above split the enzyme complex into $\alpha\gamma$ and $\beta\gamma\delta\epsilon$ fractions.

Finally, it should be noted that the data summarized here, together with results of recent ligand binding studies of rat liver F_1 (Williams et al., 1987), show that this enzyme complex contains a minimum of five different metal ion binding domains which can be divided into three distinct classes (Figure 6). The first class involves exchangeable sites (three per molecule) in which metal nucleotide triphosphate complexes bind at catalytic sites. The second class involves binding of a single nonexchangeable Mg^{2+} per molecule of F_1, the function of which remains unknown. These two classes of metal ion binding sites have been demonstrated also to be characteristic of bovine heart F_1 (Senior, 1979; Daggett et al., 1985). Finally, the third class defined by this study involves at least one site per molecule of F_1 involved in F_1 inactivation, but not in inactivation of the intact functional F_0F_1 ATP synthase complex.

It is possible that this third type of metal ion binding domain is related to or identical with the inhibitory nucleotide binding site (hysteretic site) described in detail by DiPietro et al. (1980, 1981). These workers have shown that incubation of porcine heart mitochondria with ADP + Mg^{2+} and with ATP + Mg^{2+} markedly inhibits F_1-ATPase. Moreover, we have shown here that F_1 is inhibited not only in the presence of Mg^{2+} but also when ADP + Mg^{2+} and ATP + Mg^{2+} are present in the prior incubation mixture. A key question that arises that will necessitate further experimentation is whether one or two related phenomena are occurring in the presence of adenine nucleotides + Mg^{2+}. Thus, it would be interesting to know whether F_1 first undergoes a conformational change to an inhibitory form followed by subunit dissociation or whether inhibition depends on subunit dissociation. Our results with rat liver F_1 favor the latter explanation as enzyme activity loss is proportional to subunit dissociation (Figure 4) when Mg^{2+} alone is present in the prior incubation.

Future studies will be focused on the identification of the subunit location of these metal ion (or metal nucleotide) binding sites on F_1 and their roles in the function, structure, and turnover of the enzyme complex.

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Probing of Sulfhydryl Groups in the Adenosine 5′-Diphosphate/Adenosine 5′-Triphosphate Carrier by Maleimide Spin-Labels†

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ABSTRACT: Binding of spin-labeled maleimides to the mitochondrial ADP/ATP carrier was investigated both in mitochondria and in the detergent-solubilized carrier protein. In mitochondria, spin-label binding to the carrier was evaluated by preincubation with the inhibitor carboxyatractyloside. The membrane sidedness of SH groups in the carrier molecule was determined by chemical reduction of nitroxides on the cytosolic membrane surface by Fe^{2+} or by pretreatment of the mitochondria with impermeant SH reagents. These experiments suggest that each subunit of the dimeric carrier incorporates one spin-labeled maleimide. Roughly half of the carrier-bound spin-labels were found on either side of the mitochondrial membrane. The detergent-solubilized carrier protein was labeled with a series of maleimide derivatives containing a spacer of increasing length between the maleimide and nitroxide moieties. A total spin-label binding of 2–3 mol/mol of protein dimer, depending on the spin-label length, was found. The electron spin resonance spectra of the spin-labeled protein invariably showed strongly and weakly immobilized components. Increasing the distance of the nitroxide from the maleimide ring resulted in a strong increase of the contribution of the weakly immobilized component. These observations led to the conclusions that the geometrical constraint of spin-label mobility changes at a distance of about 10 Å from the maleimide binding site.

Two conformational states linked to the orientation of the binding center of the mitochondrial ADP/ATP carrier (AAC)[1]—specified as the c and m state, respectively—have been defined by the transport inhibitors atractylate and bongkrekate (Weidemann et al., 1969; Erdelt et al., 1972). Under specific conditions, the ADP/ATP exchange can be

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[1] Abbreviations: AAC, ADP/ATP carrier; ATR, atractylate; CAT, carboxyatractyloside; BKA, bongkrekic acid; NEM, *N*-ethylmaleimide; MOPS, 4-morpholinepropanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; MSL, maleimide spin-label; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PCMB, *p*-(chloromercuri)benzoate; ESR, electron spin resonance.

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