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Fluoroaluminum and Fluoroberyllium Nucleoside Diphosphate Complexes as Probes of the Enzymatic Mechanism of the Mitochondrial F_1-ATPase†

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ABSTRACT: The mechanism by which fluoride and aluminum or beryllium in combination with ADP inhibit beef heart mitochondrial F_1-ATPase was investigated. The kinetics of inhibition depended on the nature of the anion present in the F_1-ATPase assay medium. Inhibition required the presence of Mg^{2+} and developed more rapidly with sulfite and sulfate than with chloride, i.e., with anions which activate F_1-ATPase activity. The ADP-fluorometal complexes were bound quasi-irreversibly to F_1, and each mole of the inhibitory nucleotide-fluorometal complex was tightly associated with 1 mol of Mg^{2+}. One mole of nucleotide-fluorometal complex was able to inhibit the activity of 1 mol of catalytic site in F_1. Direct measurements of bound fluoride, aluminum, beryllium, and ADP indicated that the F_1-bound ADP-fluorometal complexes are of the following types: $ADP_1Al_1F_4$, $ADP_1Be_1F_1$, $ADP_1Be_1F_2$, or $ADP_1Be_1F_3$. Fluoroaluminates or fluoroberyllates are isomorphous to P_i, and the inhibitory nucleotide-fluorometal complexes mimicked transient intermediates of nucleotides that appeared in the course of ATP hydrolysis. On the other hand, each mole of fully inhibited F_1 retained 2 mol of inhibitory complexes. The same stoichiometry was observed when ADP was replaced by GDP, a nucleotide which, unlike ADP, binds only to the catalytic sites of F_1. These results are discussed in terms of a stochastic model in which the three cooperative catalytic sites of F_1 function in interactive pairs.

In the presence of magnesium ions, three out of six nucleotide binding sites that are present in mitochondrial F_1-ATPase[1] exchange their bound ADP or ATP with medium nucleotides, whereas the other three retain their bound nucleotides in a tightly associated form (Cross et al., 1982: Kironde & Cross, 1986). The three exchangeable binding sites are thought to be catalytic and to act in a cooperative manner (Gresser et al., 1982: Cross et al., 1982). Besides the catalytic sites, regulatory sites have been proposed to account for the unusual kinetic behavior of F_1 under specific conditions (Di Pietro et al., 1980; Wang, 1984; Weber et al., 1985). We recently reported that F_1-ATPase is inhibited by aluminum or beryllium in the presence of fluoride and ADP (Lunardi et al., 1988). A possible explanation was that fluoroaluminum and fluoro-

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[1] Abbreviations: F_1-ATPase, catalytic sector (soluble) of the beef heart mitochondrial ATPase complex; p*F*, colog of the free fluoride concentration; AMPPNP, 5'-adenylyl imidodiphosphate.

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beryllium complexes mimic the γ-phosphate group of ATP, a situation similar to that postulated for the guanine nucleotides in the case of the G proteins (Bigay et al., 1985, 1987). Complexation of ADP and fluorometals generates compounds that are structurally similar to nucleoside triphosphate and behave as abortive complexes at the active site(s) of F_1. Inhibition also occurs when IDP, GDP, or CDP is substituted for ADP. As these nucleotides are thought to bind specifically to the catalytic sites of F_1, it was concluded that the catalytic sites of F_1 are targets for the nucleoside diphosphate–fluorometal complexes (Lunardi et al., 1988). In the present study, we have experimentally determined the stoichiometry of the components involved in the complexes formed between Mg^{2+}, nucleotides, and fluoroaluminate or fluoroberyllate. The stoichiometric data concerning the number of bound nucleoside diphosphate–fluorometal complexes required for full inhibition of F_1 are discussed with respect to the nature of the inhibitory process and its relation to the catalytic mechanism of F_1.

Materials and Methods

Beef heart mitochondrial F_1-ATPase was isolated according to Knowles and Penefsky (1972) with the modification introduced by Klein et al. (1982), and stored as an ammonium sulfate precipitate. Prior to use, the enzyme suspension was pelleted. The pellet was rinsed with 60% (w/v) ammonium sulfate in a medium composed of 0.15 M sucrose/50 mM Tris–acetate, pH 8.0, and then solubilized in the same buffer without ammonium sulfate (STA medium). It was immediately subjected to two sequential filtration–centrifugations through 1-mL G50 Sephadex columns. The first column was equilibrated with the STA medium and the second with a medium consisting of 0.15 M sucrose, 30 mM NaCl, 10% (w/v) glycerol, and 50 mM Tris–H_2SO_4, pH 8.0 (STNG medium). This treatment yielded a mitochondrial F_1 preparation essentially free of the loosely bound nucleotides present on the native enzyme, but still containing three tightly bound nucleotides (Issartel et al., 1987).

The ATPase activity was measured spectrophotometrically at pH 8.0 and at 30 °C, using a standard regenerating system containing 4 mM phosphoenolpyruvate, 25 μg/mL pyruvate kinase, 12.5 μg/mL lactate dehydrogenase, 0.12 mM NADH, 30 mM KCl, and 50 mM Tris–H_2SO_4. The inhibited enzyme samples were diluted 200-fold during the assay. Aluminum or beryllium cations and fluoride anions had no effect on the regenerating system under these experimental conditions. Radioactivity was measured by scintillation counting with Ready Value cocktail (Beckman). The protein concentration was estimated by using the Coomassie Blue method (Bradford, 1976). A molecular mass of 371 kDa was used for calculation of the F_1 concentrations.

For measurement of tightly bound ligands, the inhibited F_1 was subjected to filtration–centrifugation through a G50 Sephadex column equilibrated in the STNG medium to remove the free ligands. It was then submitted to a cold chase in the presence of 0.75 mM ATP for 15 min, followed by an ammonium sulfate precipitation. After 30 min at 4 °C, the pellets were recovered by centrifugation, solubilized in 10 mM Tris–H_2SO_4, pH 8.0, and filtered through a second G50 Sephadex centrifugation column equilibrated in the same buffer. The eluted materials were then used for the measurement of the ATPase activity and the determination of the bound species (metals, fluoride, and nucleotides). Before assay, the bound fluoride was extracted by heat treatment of the enzyme (Dupuis et al., 1989). Quantitative extraction was ascertained by reextraction of internal controls with known amounts of fluoride. [^{3}H]ADP radioactivity was measured on a fraction of the thermally denatured enzymic extract used for fluoride determination. Fluoride concentration was measured by using a fluoride-sensitive electrode (Dupuis et al., 1989). Protein-bound adenine nucleotides were measured as described by Issartel et al. (1986). Aluminum assays were performed by atomic absorption spectrometry with a Perkin-Elmer Model 3030 spectrometer equipped with a Zeeman HGA 600 graphite furnace module. Samples of F_1 were diluted to a concentration close to 1 μM with 1% HNO_3 and analyzed directly. Magnesium was analyzed by flame absorption atomic spectrometry using a Perkin-Elmer Model spectrometer 2380 (Paschen & Fuchs, 1971). For the aluminum and magnesium assays, all the vessels used were carefully washed with 1 N HNO_3, and Sephadex G50 was washed and stored in the presence of 2 mM EDTA.

Table I: Anion Effects on the Kinetics of F_1 Inhibition in the Presence of Fluoroberyllium[a]

anion added	concentration (mM)	time required for 50% inhibition (min)
Cl^-	50	45
SO_4^{2-}	20	14
SO_3^{2-}	20	2

[a] Desalted F_1 was incubated, at a concentration of 1 μM, in STNG medium in which sulfate anion was replaced by the indicated anions, in the presence of 2 mM $MgCl_2$, 80 μM ADP, and 2.5 mM sodium fluoride for 15 min at 25 °C. Then beryllium chloride was added to a final concentration of 20 μM, and the kinetics of inhibition were followed by measuring the decrease of ATPase activity.

For kinetic analysis (Figure 2), we took advantage of the quasi-irreversible nature of the inhibition of the ATPase activity by fluorometals. The half-time for the inhibition of an enzyme by a tightly bound inhibitor was shown (Levy et al., 1963) to be related to the inhibitor concentration by the expression $\log (1/t_{1/2}) = n \log I + \log (k_{on}/\ln 2)$, where I is the concentration of the inhibitor, n the number of molecules bound per functional unit required to fully inhibit the enzyme, and k_{on} the kinetic constant relative to the binding of the inhibitor to the enzyme. When plotting $\log (1/t_{1/2})$ vs $\log I$, the concentration of added metal, Al^{3+} or Be^{2+}, was taken to be equal to the concentration of the inhibitor species. This assumption relies on the fact that in the presence of millimolar concentrations of fluoride, largely exceeding the concentrations of Al^{3+} or Be^{2+}, the totality of the metal is complexed with fluoride. Furthermore, even if among the different fluorometal species present in solution only one is inhibitory, this species would correspond to a constant fraction of the total concentration of metal, and the slope of the plots corresponding to the above equation would remain unchanged.

Results

Kinetics of Inhibition of F_1-ATPase by ADP–Fluorometal Complexes. It was previously shown that mitochondrial F_1 is fully inhibited by fluoroaluminum or fluoroberyllium complexes provided the incubation medium contains ADP (Lunardi et al., 1988). A new finding is that the kinetics of inhibition are strongly dependent on the nature of the anions present in the assay medium (Table I). Inhibition developed 3 times more slowly when sulfate was replaced by chloride ($t_{1/2}$, 45 vs 14 min) and was virtually instantaneous in the presence of sulfite ($t_{1/2}$ = 2 min). Interestingly, the anions that promoted inhibition of F_1 by the fluorometals are well-known activators of the ATPase activity catalyzed by the isolated F_1 (Ebel & Lardy, 1975; Penefsky, 1977), and there was a striking analogy between the enhancing effect of a given anion on ATPase

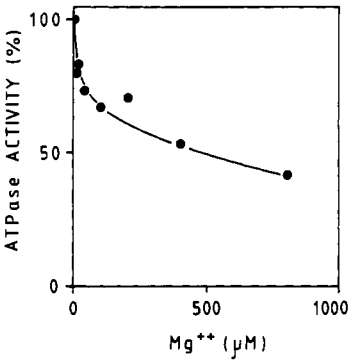

FIGURE 1: Effect of magnesium concentration on the development of the inhibition of F_1 activity induced by fluoroberyllate complexes. F_1, desalted as described under Materials and Methods, was incubated in STNG medium (see Materials and Methods) for 30 min at 25 °C in the presence of 100 μM ADP, 3 mM fluoride, and increasing concentrations of $MgCl_2$ ranging from 0 to 800 μM. Beryllium chloride was further added at a final concentration of 20 μM. After 25 min, samples were assayed for their ATPase activity. Control F_1 was treated in the same way except that beryllium ions were omitted.

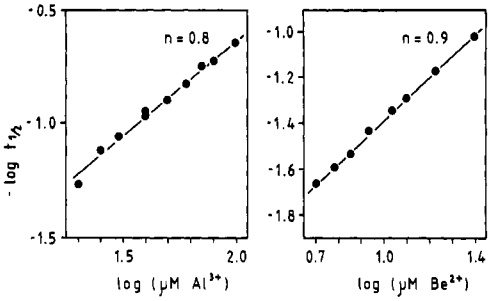

FIGURE 2: Determination of the stoichiometry of bound beryllium or bound aluminum per catalytic site of F_1 by kinetic measurements. Desalted F_1 was incubated, at a concentration of 1 μM, in STNG medium in the presence of 2 mM $MgCl_2$, 80 μM ADP, and 2.5 mM sodium fluoride for 15 min at 25 °C. Indicated amounts of beryllium or aluminum were then added, and kinetics of inhibition were determined by measuring the decrease of the ATPase activity. Half-times of inactivation of F_1 at various concentrations of aluminum or beryllium were determined. The log of the reciprocal of calculated values for $t_{1/2}$ was plotted vs the log of aluminum or beryllium concentrations. The slope of the linear plot (n) is equal to the number of ligands bound per catalytic site in the fully inhibited state of F_1.

inhibition by fluorometals and the stimulating effect of the same anion on the F_1-ATPase activity. For convenience, all the experiments to be described were carried out in a medium containing sulfate anions.

It has been suggested that the biological effects of aluminum (or beryllium) might be mediated by substitution of magnesium by aluminum or beryllium in the magnesium–nucleotide complexes (Martin, 1986). If this hypothesis was valid, one should expect that high concentrations of magnesium would displace beryllium and aluminum from their assocation with ADP and would therefore restore ATPase activity. Just the opposite effect, i.e., enhancement of inhibition, was observed (Figure 1), suggesting that the inhibitory fluorometal species is composed of Mg-ADP associated with fluoroaluminum or fluoroberyllium. That the inhibition was specifically related to the presence of the fluorometals in the solution was ascertained by control experiments in which aluminum or beryllium cations were omitted.

Inhibition of F_1 by fluorometals is quasi-irreversible despite the fact that fluorometals are not covalently bound (Lunardi et al., 1988; this paper). It was therefore justified to apply to the kinetics of inhibition the same mathematical treatment as that used for inactivation reactions. For kinetic studies of inhibition, both metal and ADP were added at concentrations in excess with respect to that of F_1. The concentration of free fluoride was set to its optimal value for full inhibition, and beryllium or aluminum were added at various concentrations. Inhibition of F_1 by fluoroberyllate species followed pseudo-first-order kinetics (data not shown). The same result was obtained when aluminum was substituted for beryllium. The apparent kinetic constants (k_{on}) related to the binding of aluminum or beryllium to F_1 were approximately 30×10^{-6} and 50×10^{-6} $\mu M^{-1} \cdot s^{-1}$, respectively. When the colog of the half-times of inactivation ($-\log t_{1/2}$) was plotted against the log of the inhibitor concentrations (Levy et al., 1963), a straight line was obtained, the slope of which was close to 1 (Figure 2). This indicates that 1 mol of inhibitor reacts with 1 mol of the independent catalytic site of F_1 to form an inactive complex.

ADP–Fluorometals Are Tightly Bound to F_1. The following experiments indicate that the fluorometal complexes bind to F_1 with very high affinity. An F_1 preparation inhibited up to 85% following treatment with fluoroaluminate in the presence of [^{3}H]ADP was filtered through a Sephadex G50 column equilibrated in 100 mM Tris–H_2SO_4, 4 mM EDTA, and 50% (w/v) glycerol, final pH 8.0. Sephadex filtration in the presence of glycerol was originally introduced by Garrett and Penefsky (1975) to deplete F_1 from tightly bound nucleotides. As shown in Table II, glycerol treatment resulted in a modest recovery of the activity (19%) of the inhibited enzyme, which was accompanied by a negligible release of bound [^{3}H]ADP (0.1 mol out of the 1.4 mol of bound [^{3}H]-ADP), contrasting with the relatively efficient release of total bound nucleotides. In fact, out of the 4 mol of ADP bound (including the 1.4 mol of [^{3}H]ADP), 2.6 mol was retained on the inhibited F_1 after glycerol treatment. When submitted to the same treatment, control F_1 was nearly freed of nucleotides (Table II). In brief, inhibition of F_1 induced by the ADP–fluoroaluminate complex is hardly relieved even by a procedure which is efficient in removing tightly bound nucleotides. The inhibited F_1 retained not only the [^{3}H]ADP–fluoroaluminate complex but also part of the originally bound nucleotides which were displaced by the glycerol treatment in control F_1 (Table II, rows 3 and 4 vs rows 1 and 2). In another experiment, two samples of F_1 inhibited respectively by fluoroaluminum and fluoroberyllium ions were carefully desalted, incubated in a glycerol-supplemented medium

Table II: Effect of the Filtration of F_1 through a Sephadex G50 Column Equilibrated in a Medium Containing 50% Glycerol on the Reversion of the Inhibited State of F_1 Obtained by Preincubation in the Presence of Fluoroaluminum[a]

sample	Sephadex filtration	bound [^{3}H]ADP (mol/mol of F_1)	inhibition (%)	total bound nucleotides (mol/mol of F_1)
control F_1	before	1.0 (±0.1)	0	3.1 (±0.2)
	after	0	0	0.6 (±0.1)
inhibited F_1	before	1.4 (±0.2)	85	4.0 (±0.1)
	after	1.3 (±0.1)	66	2.6 (±0.3)

[a] F_1 (13 μM) was inhibited in STNG medium in the presence of 2 mM $MgCl_2$, 80 μM [^{3}H]ADP, 6 mM sodium fluoride, and 100 μM aluminum chloride for 30 min at 25 °C. Control F_1 was incubated in the same medium except that aluminum chloride and sodium fluoride were omitted. F_1 was precipitated in this magnesium-supplemented medium by ammonium sulfate, 60% (w/v). After centrifugation, F_1 was solubilized in a medium composed of 100 mM Tris-H_2SO_4, 4 mM EDTA, and 50% (w/v) glycerol, final pH 8.0, and the ATPase activity and the stoichiometries of bound nucleotides were determined. F_1 was then loaded on a Sephadex G50 column (1 × 25 cm) equilibrated in the glycerol medium described above and eluted at a flow rate of 1 mL/h. Average results from four experiments.

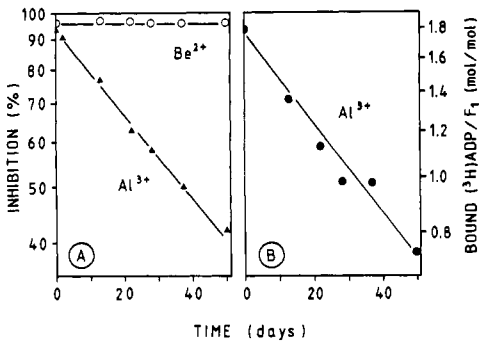

FIGURE 3: Reversibility of the inhibited state of F_1 induced by fluorometals. Desalted F_1 solubilized at a concentration of 4 μM in STNG medium was inhibited by incubation in the presence of 100 μM [^{3}H]ADP, 1 mM $MgCl_2$, 5 mM sodium fluoride, and 100 μM aluminum chloride or 100 μM beryllium chloride. Samples were incubated for 30 min at 25 °C. The fully inhibited enzyme was freed of unbound ligand and submitted to a cold chase with ATP as described under Materials and Methods. At last, F_1 was solubilized in a glycerol medium consisting of 100 mM Tris–H_2SO_4, 4 mM EDTA, and 50% (w/v) glycerol, final pH 8.0. Inhibited and control samples were stored at 4 °C. Under these conditions, the ATPase activity of control F_1 proved to be stable for more than 2 months. At various times, samples were withdrawn, diluted to 15% glycerol, and filtered through a 1-mL G50 Sephadex centrifuge column equilibrated in STNG medium. ATPase activity and bound radioactivity were assayed on the eluates. Percentages of inhibition were calculated by comparing the ATPase activities of the inhibited samples and those of the control samples measured at various times. The log of the inhibition of the beryllium- or aluminum-treated F_1 (panel A) and the log of the [^{3}H]ADP bound to F_1 inhibited in the presence of aluminum (moles per mole of F_1) (panel B) are expressed as a function of the time of incubation (days).

identical with that used in the preceding experiment, and left to stand at 4 °C for several days. Inhibition of the aluminum-treated F_1 was very slowly reversed; in fact, only 50% of the ATPase activity was regained in 1 month. However, during the same period of time, the beryllium-treated enzyme showed no reactivation. It is noteworthy that almost no loss of activity was observed when control F_1 was incubated under the same conditions. Interestingly, the slow recovery of activity by fluoroaluminate-inhibited F_1 paralleled the release of bound radiolabeled ADP; at time zero, the inhibited F_1 contained 1.8 mol of bound [^{3}H]ADP per mole, whereas after 38 days the extent of inhibition had dropped to 50% and the amount of bound [^{3}H]ADP to 0.9 mol/mol of F_1. All these results strengthen the idea that the inhibition caused by the fluorometals is poorly reversible. From the data of Figure 3, a k_{off} value of 0.2×10^{-6} s^{-1} was calculated for the release of the fluoroaluminum from F_1. From this k_{off} value and the k_{on} of 30×10^{-6} $\mu M^{-1} \cdot s^{-1}$ reported above, an apparent k_d value of 6 nM was calculated for the binding of the ADP–fluoroaluminate complex to F_1. In the case of fluoroberyllates, the absence of any detectable reversibility of F_1-ATPase inhibition indicates that the fluoroberyllate species are too tightly bound to F_1 for an accurate measurement of the dissociation constant.

Inhibition of F_1-ATPase Correlates with the Binding of Fluorometal Complexes. As shown in Table II, the inhibition of F_1-ATPase by ADP–fluoroaluminate and ADP–fluoroberyllate complexes is accompanied by the tight binding of radiolabeled ADP to the enzyme. To assess accurately the relationship between inhibition of F_1 and entrapping of ADP, F_1 was incubated with [^{3}H]ADP and either fluoroaluminate or fluoroberyllate for different periods of time, and the incubation was terminated by centrifugation–filtration through Sephadex G50 columns. The eluted F_1 was submitted to a cold chase with ATP, then precipitated by ammonium sulfate, and once more filtrated through Sephadex G50 (see Materials and Methods) to remove any loosely bound [^{3}H]ADP. The

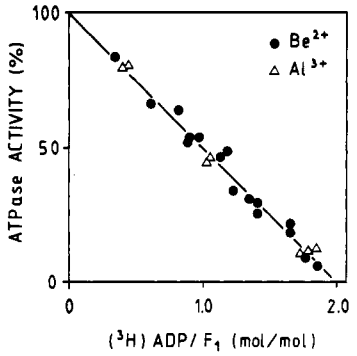

FIGURE 4: Correlation between the tightly bound [^{3}H]ADP and the inhibition by fluoroberyllate or fluoroaluminate. Desalted F_1 was incubated at a concentration of 0.8 μM for 15 min at 25 °C in STNG medium containing 80 μM [^{3}H]ADP, 2 mM $MgCl_2$, and 2.5 mM or 6 mM sodium fluoride, depending on the added metal. Inhibition was initiated by the addition of 20 μM beryllium chloride or 80 μM aluminum chloride. At various times, samples were withdrawn and freed of loosely bound ligands as described under Materials and Methods and assayed for bound ADP and ATPase activity.

Table III: Stoichiometry of Ligands Bound to F_1[a]

sample	inhibition (%)	bound Mg^{2+} (mol/mol of F_1)	bound aluminum (mol/mol of F_1)	endogenous ADP + ATP (mol/mol of F_1)
control	0	1.4 (±0.1)	less than 0.1	1.5 (±0.1)
inhibited F_1	87	3.4 (±0.1)	2.0 (±0.1)	3.1 (±0.2)

[a] F_1 (6 μM) was incubated in STNG medium in the presence of 2 mM $MgCl_2$, 100 μM ADP and 6 mM sodium fluoride for 30 min at 25 °C, then aluminum chloride was added to a final concentration of 100 μM, and inhibition was allowed to proceed for 30 additional min. Samples were desalted in STNG medium, submitted to an ATP chase, and further treated as described under Materials and Methods. Eluates were assayed for ATPase activity, bound nucleotides, aluminum, and magnesium as described under Materials and Methods. Average results from four experiments.

percentage of inhibition of the ATPase activity in the treated F_1 was calculated by reference to a control F_1 treated in the same way, but in the absence of metal, and plotted against the amount of bound [^{3}H]ADP expressed in moles of [^{3}H]-ADP per mole of F_1 (Figure 4). Inhibition of ATPase activity was linearly related to the tight binding of [^{3}H]ADP, full inhibition being attained for 2 mol of bound [^{3}H]ADP per mole of F_1, whatever the cation used, i.e., Al^{3+} or Be^{2+}. As the amount of bound beryllium ions is strictly equal to that of bound [^{3}H]ADP (Dupuis et al., 1989), it may be concluded that full inactivation of F_1 requires the binding of 2 mol of beryllium and 2 mol of [^{3}H]ADP per mole of F_1. When [^{3}H]GDP, a nucleotide which is essentially recognized by catalytic sites, was substituted for [^{3}H]ADP, a strict linear relationship between F_1 inhibition and the binding of [^{3}H]GDP was also obtained, each mole of fully inhibited F_1 retaining 2 mol of [^{3}H]GDP (data not shown). In the absence of added nucleoside diphosphate, and in the presence of fluorometals, maximum inhibition of F_1 was always less than 15%, with no more than 0.3 mol of metal bound per mole of F_1. This small amount of bound metal probably reflected a partial filling of F_1 by some residual ADP remaining in the exchangeable catalytic sites.

Stoichiometric data concerning F_1-bound aluminum, magnesium, and ADP plus ATP are given in Table III. The experimental conditions were such that inhibition of the ATPase activity was subtotal (87% inhibition). About 2 mol of aluminum was bound per mole of F_1. Compared to control F_1, there was an additional entrapping of about 2 mol of

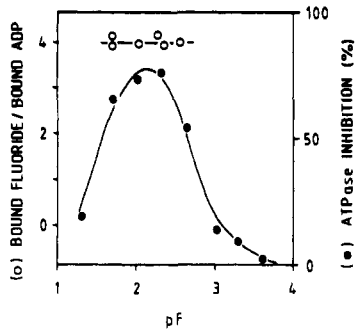

FIGURE 5: Variation of the ratio of bound fluoride to bound ADP in the fluoroaluminate–ADP complex entrapped in the inhibited F_1 as a function of the concentration of free fluoride (pF). F_1 (7 μM) was incubated in STNG medium in the presence of 100 μM [^{3}H]ADP, 2 mM $MgCl_2$, and sodium fluoride for 30 min at 25 °C. The concentration of free fluoride was set between 0.17 mM (pF = 3.7) and 50 mM (pF = 1.3). Inhibition of F_1-ATPase was initiated by the addition of 100 μM aluminum chloride. After 30 min at 25 °C, the inhibited F_1 samples were freed of unbound ligands and submitted to a cold chase with ATP, and finally desalted as described under Materials and Methods. Resulting samples were assayed for protein, ATPase activity (●), and measurement of the bound species (cf. Materials and Methods). The ratios of bound fluoride to bound ADP (○) are reported only for pF values between 1.7 and 2.5, i.e., for amounts of inhibited enzyme sufficient to allow confident determination of bound species.

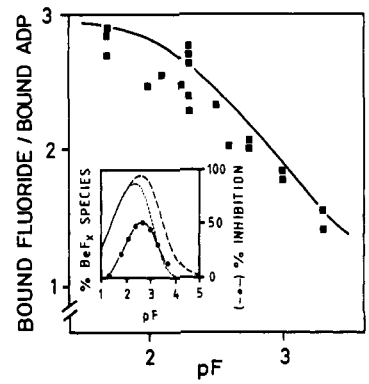

FIGURE 6: Correlation of the ratio of bound fluoride to bound ADP in the fluoroberyllate–ADP complex entrapped in F_1 with the predicted stoichiometry of fluoride in the fluoroberyllate species in solution. F_1 was inhibited and treated as described in Figure 6, except that 20 μM beryllium chloride was substituted for aluminum chloride. The concentration of free fluoride was set between 0.17 mM (pF = 3.7) and 50 mM (pF = 1.3). Experimental data were plotted against pF. The ratios of bound fluoride to bound ADP (■) are reported only for pF values between 1.7 and 3.3. The solid line represents the theoretical average number of fluorides per beryllium in the fluoroberyllate complexes in solution (see text). Insert: Inhibition curve of F_1-ATPase activity (○) as a function of pF is compared to the distribution curve for total concentrations of fluoroberyllate complexes containing two and three fluorides (---) or one, two, and three fluorides (––).

nucleotide per mole of F_1. A similar stoichiometry was recently reported for beryllium and ADP bound to F_1 (Dupuis et al., 1989). Bound magnesium in inhibited F_1 amounted to 3.4 mol/mol of F_1, which corresponds to the additional binding of 2 mol of Mg^{2+}, compared to control F_1. These results taken together make it clear that 2 mol of the following species, ADP, magnesium, beryllium, and aluminum, is required for full inhibition of F_1.

Stoichiometric data illustrated in Figure 5 concern the binding of fluoride to F_1 inhibited in the presence of fluoroaluminum. For fluoride concentrations ranging between 3 (pF = 2.5) and 20 mM (pF = 1.7), 4 mol of fluoride was associated with 1 mol of bound aluminum. When fluoroberyllium was the inhibitory species and the concentration of free fluoride was varied between 0.5 (pF = 3.3) and 20 mM (pF = 1.7), the stoichiometric ratio of bound fluoride to bound ADP (moles per mole) ranged between 1.5 and the values close to 3 (Figure 6). The curve depicting the inhibition of F_1 as a function of the free fluoride concentration in solution is bell-shaped (Figure 6 insert). At pF values lower than 1.5, the apparent loss of efficiency of the inhibitory species might be explained by the formation of the poorly soluble species MgF_2. With this reservation in mind, the inhibition curves were compared to the distribution curves for the fluoroberyllate species in solution calculated according to Martin (1988). The theoretical distribution curve, based on the hypothesis that the fluoroberyllate inhibitory species contains one, or two, or three fluorides, depending on the fluoride concentration, mimicked the shape of the experimental inhibition curve (Figure 6 insert, dashed line). On the contrary, the inhibition curve differed from the theoretical curve calculated on the basis that the inhibitory fluoroberyllate species would contains only two or three fluorides. These results support the idea that the three different fluoroberyllate complexes, namely, BeF_1^+, BeF_2, and BeF_3^- (and not Be^{2+} or BeF_4^{2+}), are the inhibitory species recognized by F_1. The stoichiometric ratio of bound fluoride to bound ADP (or Be) (moles per mole) in inhibited F_1 as a function of the free fluoride concentration was calculated, assuming that the kinetic constants for the binding of BeF_1^+, BeF_2, and BeF_3 to F_1 are similar. This ratio, which is equal to $(3[BeF_3^-] + 2[BeF_2] + [BeF_1^+])/([BeF_3^-] + [BeF_2] + [BeF_1^+])$, was used to draw a curve (solid line in Figure 5) that fitted reasonably well the experimental data, corroborating the hypothesis that BeF_1^+, BeF_2, and BeF_3^- are recognized by F_1 with nearly identical efficiency.

Discussion

Nature and Stoichiometry of the Components of the Inhibitory Fluorometal Species. It has been postulated that the inhibition of F_1-ATPase induced by fluoroaluminate or fluoroberyllate complexes in the presence of ADP results from the formation of an abortive complex, made up of ADP, fluoroaluminate, or fluoroberyllate. This complex is thought to behave as an analogue of ATP, with the fluorometal mimicking the γ-phosphate of ATP (Lunardi et al., 1988). An obligatory requirement for inhibition is the preincubation of F_1 with ADP, aluminum or beryllium, fluoride, and magnesium. A number of questions have arisen recently regarding the nature and mode of action of the active forms of aluminum or beryllium, e.g., are metals the only inhibitory species, and are they associated with fluoride in a binary fluorometal complex, or do fluoride and metals bind to different sites on the enzyme (Stadel & Crook, 1988; Macdonald & Martin, 1988; Jackson, 1988). The present results address some of these questions.

(1) The ATPase activity of F_1 is not affected by preincubation with fluoride, aluminum, and beryllium added simultaneously or separately, in the absence of magnesium, suggesting that aluminum or beryllium does not take the place of magnesium to form a magnesium–ADP-like complex at the nucleotide binding site. In addition to beryllium or aluminum and ADP, magnesium is required for the inhibition of F_1.

(2) Native beef heart mitochondrial F_1 contains 1 mol of tightly bound magnesium per mole of enzyme, the release of which leads to inactivation of the enzyme (Senior et al., 1980). On the basis of this result, one could speculate that Al^{3+} or Be^{2+} decreases the F_1-ATPase activity by competing with and displacing bound Mg^{2+}. This explanation is not valid, however, because F_1 inhibited by fluorometal contains more magnesium than native F_1.

(3) Since fluoroberyllates and fluoroaluminates behave as effectors of numerous enzymes involved in nucleotide binding

or phosphate group transfer, such as G proteins (Blackmore & Exton, 1986; Brandt & Ross, 1986; Paris & Pouyssegur, 1987), hexokinase (Lange et al., 1986), actin (Combeau & Carlier, 1988), tubulin (Carlier et al., 1988), P-type ATPases (Robinson et al., 1986; Missiaen et al., 1988), and mitochondrial F_1 (this work), it is likely that they bind as fluorometals to the catalytic sites of the enzymes mentioned above, and not as metal and fluoride to separate binding sites.

(4) The functional analogy between phosphate and fluoroberyllate or fluoroaluminate is now widely documented. Indeed, the fluorometals are structurally isomorphous with inorganic phosphate, e.g., all fluoroberyllates are tetracoordinated [solubilized form: $BeF_x(H_2O)_y$ with $x + y = 4$]. Their anionic charge depends on the protonated state of the bound water except for BeF_4^{2-}. Fluoroberyllates with a single anionic charge are expected to bind to the P_i binding site of F_1, which specifically recognizes the monovalent anionic form of P_i (Penefsky, 1977).

(5) The tight binding of fluoroberyllates or tetrafluoroaluminate is accompanied by the entrapment of an equistoichiometric amount of nucleoside diphosphate, which suggests that both compounds are bound to the same nucleotide binding site, or in closely related sites.

Atomic absorption analysis of the inhibitory complex bound to F_1 indicated that inhibition of F_1-ATPase developed in parallel with the tight binding of aluminum and magnesium to F_1. Taken together with the titrations of bound ADP, beryllium, and fluoride, these results suggest that two types of complexes with tetracoordinated beryllium or aluminum can occur in the inhibited F_1, namely, $ADP_1Mg_1Al_1F_4$, and $ADPMg_1Be_1F_x(H_2O)_{4-x}$ with $1 < x < 3$. According to the pseudonucleoside triphosphate model formulated by Bigay et al. (1985, 1987), the association of the bound nucleoside diphosphate with the fluorometal in a pseudonucleoside triphosphate requires a maximum of three bound fluorides for the tetracoordinated fluoroberyllium entrapped in F_1, which is confirmed by the experimental results. On the other hand, the association of four fluorides with one aluminum at the active site of F_1 leads to the conclusion that a pentacoordinated fluoroaluminum might be present in the γ-phosphate subsite of F_1. In this case, the ADP–AlF_4 complex would mimic a transient intermediate of ATP hydrolysis or synthesis, with a pentacoordinated γ-phosphate as suggested by stereochemical studies on hydrolysis of ATP by F_1 (Webb et al., 1980). Alternatively, AlF_4^- could bind to the γ-phosphate subsite of the nucleotide binding sites of F_1 as a high-affinity phosphate analogue, without combining with ADP. The above-mentioned complexes are illustrated in Figure 7. In these complexes, $BeF_1(H_2O)_2^+$, $BeF_2(H_2O)$, BeF_3^-, AlF_4^- are suggested to be located close to the β-phosphate group or even to react with it. Magnesium ions are depicted as bridging the two phosphate groups of ADP, this type of bridge being consistent with the mechanism proposed for various kinases and F_1-ATPase (Dunaway-Mariano et al., 1979; Dunaway-Mariano & Cleland, 1980; Cross, 1981). Along this line, the inhibitory effect of fluorometals might result from the locking of an α–β Mg–ADP bidentate, impeding any motion of Mg^{2+} or release of the Mg–ADP complex.

Nature of the Nucleotide Binding Sites of F_1 Occupied by the ADP–Fluorometal Inhibitory Complexes. Six adenine nucleotide binding sites are present in native F_1; three of them exchange readily bound nucleotides during hydrolysis of ATP and are thought to be catalytic, whereas the other three nonexchangeable sites do not (Cross & Nalin, 1982). Desalted F_1 retains three bound adenine nucleotides; two of them are

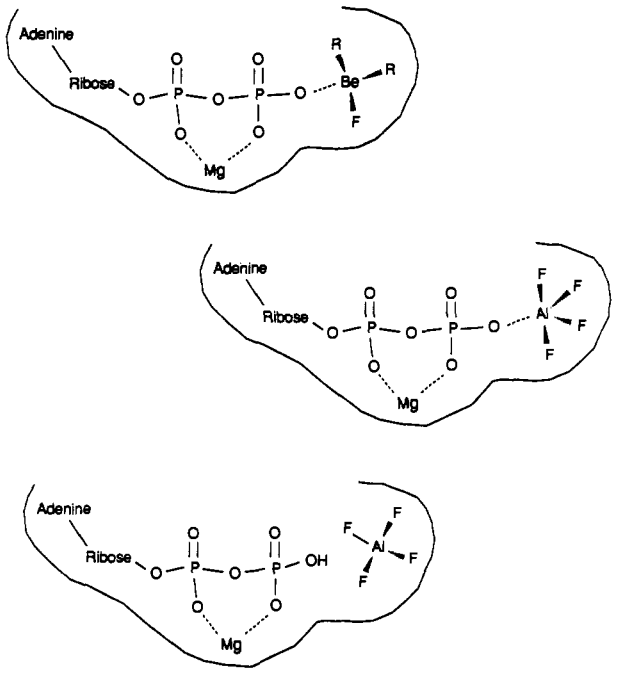

FIGURE 7: Postulated chemical structures of the bound inhibitory complexes (R = OH or F).

bound at nonexchangeable sites and one at an exchangeable site. The vacant nonexchangeable (noncatalytic) site is rapidly filled by ADP during ATP hydrolysis; it is the same site which releases nucleotide when F_1 is desalted (Kironde & Cross, 1986). The following lines of evidence indicate that the catalytic sites of F_1, but not the noncatalytic sites, are involved in inhibition by fluorometals.

(1) The very low amount of beryllium fluoride which is able to bind in the absence of added ADP agrees with the idea that the two nonexchangeable nucleotides present on the noncatalytic sites do not interact with fluorometals.

(2) The homogeneous first-order kinetics of inhibition and the rectilinear relationship of the extent of ATPase inhibition to the amount of bound ADP (Figure 4) make it unlikely that the two immobilized fluoroaluminates are independently bound to two different types of sites.

(3) In photolabeling experiments carried out with 2-azido-[^{32}P]ADP, the catalytic site of the β subunit of F_1 is essentially labeled, and the radioactivity is found to be located at Tyr-345 of the β subunit and in a few amino acid residues adjacent to this residue (Garin et al., 1986). In the presence of fluorometals, a similar photolabeling pattern by 2-azido[^{32}P]ADP is found, with a more predominant labeling of Tyr-345, which is in accordance with the idea of a restricted motion of the amino acid residues at the catalytic site in the presence of fluorometals (Garin, 1989).

These data taken together strongly suggest that the two ADP–fluorometal complexes whose binding is required for complete inhibition of F_1 are entrapped in two catalytic sites. This is consistent with the parallelism found between the properties of inhibition of F_1 by the fluorometals and the catalytic properties of F_1: (1) Fluorometals exhibit shape, geometries, and charges similar to those of phosphate. (2) Inhibition by fluorometals requires magnesium, as does catalysis. (3) Anions, like sulfite and sulfate, known to activate the enzymatic activity of F_1 markedly stimulate inhibition by fluorometals. (4) In submitochondrial particles, the inhibition of F_1 by fluorometals can be reversed by generation of a membrane potential (Lunardi et al., 1988), which is known to promote the release of newly synthesized ATP tightly bound

to F_1 (Penefsky, 1985). The fact that fluorometals behave like high-affinity phosphate analogues readily explains that the concomitant binding of Mg–ADP and fluorometal to catalytic sites of F_1 results in ATPase inhibition. The high affinity of F_1 for fluorometals is attributable in part to the strong electronegativity of the fluorides in these complexes, which leads to the formation of strong hydrogen bonds. Indeed, the apparent K_d for fluoroaluminate binding in the presence of ADP is in the nanomolar range. The affinity of F_1 for fluoroberyllium is even higher since its binding is virtually irreversible.

Workers in a number of laboratories favor the view that all three catalytic sites in F_1 are functional and that strong cooperative interactions occur between these sites. However, the question is still debated as to whether the three sites function in a sequential order (alternative site or sequential model) or whether they function in pairs, in a random fashion (stochastic model). In the alternative site mechanism (Boyer, 1989), each catalytic site in turn goes through three major stages, namely, ATP binding, interconversion of bound ATP to bound ADP and P_i, and release of ADP and P_i. At any given time, the three catalytic sites are present under different conformations. As discussed by Cross (1988), one approach to discriminate between a sequential or random order for a three-site model is to determine the minimum number of catalytic sites which must be derivatized in order to inhibit multisite catalysis. If the order is sequential, modification of a single catalytic site per F_1 should suffice to inhibit all three sites. In contrast, if the process is random, the modification of one site leaves the remaining two sites with the ability to catalyze ATP hydrolysis by an alternative bi-site mechanism, and a second molecule of modifier will definitely inhibit the remaining hydrolysis.

Many results reported in literature concerning the binding stoichiometry of F_1 with respect to chemical modifiers and photolabels have led to ambiguous results. In fact, depending on experimental conditions, the amount of the covalently bound reagent resulting in full inhibition of F_1-ATPase ranged between 1 and 3 [for reviews, see Vignais and Lunardi (1985) and Senior (1988)]. For example, full inhibition of mitochondrial F_1 by photolabeling with 2-azido-ATP (ADP) requires the incorporation of 1 mol of azido derivative (Van Dongen et al., 1986) or 2 mol of azido derivative (Boulay et al., 1985); in the case of chloroplastic F_1, when one-third of the total three potential catalytic sites was modified by 2-azido-ATP, the enzyme still retains catalytic cooperativity (Melese & Boyer, 1985). A factor of complexity in the evaluation of binding data with modifiers and photolabels resides in the fact that their precise localization at a catalytic or noncatalytic site is not always convincingly determined. In this context, an obvious advantage of the ADP–fluorometal complexes is that they bind very tightly, but not covalently, to catalytic sites of F_1. In addition, the binding stoichiometry relative to fluorometal, leading to full inhibition of F_1, could be accurately determined since the extent of F_1 inhibition was rectilinearly related to the amount of bound fluorometal and [^{3}H]ADP; 2 mol of ADP–fluorometal was required for full inactivation of F_1. This stoichiometry is not readily explained by the alternative site mechanism. To fit the results with this mechanism, one should postulate that the binding of one of the two ADP–fluorometal complexes to one catalytic site of F_1 is sufficient to promote full inhibition and that the binding of the second ADP–fluorometal complex results from a concomitantly induced very fast change of conformation of a second catalytic site that was not previously accessible. While this possibility remains to be demonstrated, a more direct

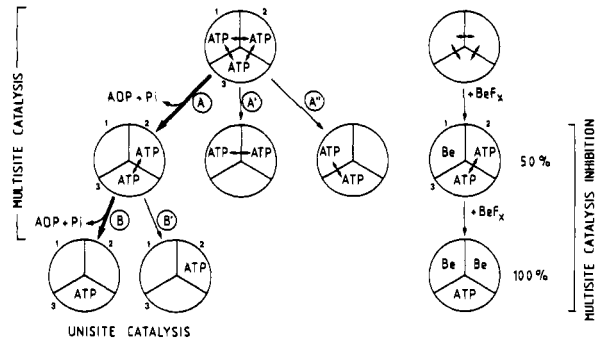

FIGURE 8: Cooperativity between pairs of catalytic subunits according to a stochastic model. The numbers 1, 2, and 3 refer to the catalytic subunits. Double arrows indicate interactions between catalytic subunits. In the bottom row, the single remaining catalytic subunit filled by ATP is postulated to carry out unisite catalysis. The three possible types of subunit–subunit interactions are depicted in A, A′, and A″. On the left-hand-side, the two fast hydrolytic steps, A and B (multisite catalysis), and the slow hydrolytic step (unisite catalysis) are illustrated. For the sake of clarity, only the first fast hydrolytic step (A′ and A″) is shown for the two alternative possibilities of subunit–subunit interactions. On the right-hand-side, sequential binding of 2 mol of fluoroberyllates per mole of F_1 is represented. Multisite catalytic activity of the F_1 molecules tagged by fluoroberyllate is inhibited by 50 and 100% for a stoichiometry of 1 and 2 mol of bound fluoroberyllate per mole of F_1, respectively. "Be" represents inhibitory complexes containing ADP, Mg, Be, and F.

explanation is afforded by the stochastic mechanism which is based on the cooperative interaction of pairs of catalytic subunits at random. In this mechanism, at any given time, for a saturating concentration of substrate, three possible interactive pairs of catalytic subunits (double arrows in the F_1 molecule in the first row of Figure 8) are operating in a fully cooperative manner, which corresponds to multisite catalysis (Grubmeyer et al., 1982; Cross et al., 1982). As illustrated in Figure 8, the catalytic pathway of ATP hydrolysis might consist of two fast steps and a slow one. In the first two steps denoted by thick arrows A and B, hydrolysis of the first molecule of ATP (arrow A) is performed by subunit 1 under the positive control of subunit 2 or subunit 3, and hydrolysis of a second molecule of ATP by subunit 2 (arrow B) is promoted by subunit 3. Finally, subunit 3 catalyzes the hydrolysis of the third bound molecule of ATP by the slow unisite catalysis process [cf. Grubmeyer et al. (1982) and Cross et al. (1982)], unless refilling of subunits 1 or 2 with ATP initiates another multisite catalytic cycle. In summary, under steady-state conditions, one multisite catalytic cycle results in the fast hydrolysis of two ATP per F_1. In the case of inhibition of F_1 by fluorometals, the experimental observations might be rationalized as follows: for a 50% inhibition corresponding to the binding of one molecule of fluorometal to subunit 1, for example (see Figure 8), only the pair 2,3 would remain operational to perform fast hydrolysis of one ATP per F_1 since refilling of subunit 1 with ATP is hampered by the presence of bound fluorometal in this subunit. Finally, the binding of a second mole of fluorometal to one out of the two untagged catalytic subunits, namely, subunits 2 and 3, and would lead to a nearly complete inhibition of F_1 since the enzyme with one remaining unmodified catalytic subunit is unable to promote fast hydrolysis of ATP. A stochastic model for the functioning of F_1 was proposed by Lübben et al. (1984) to explain results of photolabeling of mitochondrial F_1 by azidonaphthoyl-ADP. Also supporting the stochastic model is a recently reported experiment in which thermophilic bacterium F_1 reconstituted with one mutated β subunit incorporated in a $\alpha_3\beta_3\gamma$ complex exhibited 46% of the activity of the wild-type complex (Miwa et al., 1989).

Whereas the requirement of two bound fluorometals per F_1 for full inhibition of ATPase activity is a property shared by a number of ligands, including 2-azido-ADP, 8-azido-ADP, 3′-arylazido-ADP, 3′-arylazido-AMPPNP, dicyclohexylcarbodiimide, and fluorometals, there are ligands such as nitrobenzofurazan, phenylglyoxal, azidonitrophenyl phosphate, and the natural ATPase inhibitor whose binding to only one β subunit of F_1 suffices to inhibit the enzyme activity [for a review, see Vignais and Lunardi (1985)]. The one to one stoichiometry observed with the latter category of ligands fits well the alternative turnstile site mechanism; yet, this stoichiometry could be explained as well by the stochastic model, provided that one postulates that the binding of one of these ligands to one β subunit of F_1 is able to induce an asymmetric arrangement of the three β subunits. This induced asymmetry might consist in a strong interaction of one of the two remaining β subunits with the small γ, δ, and ϵ subunits, preventing its participation in catalytic events.

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