Inhibition of H⁺-transporting ATPase by formation of a tight nucleoside diphosphate–fluoroaluminate complex at the catalytic site

(fluoride/aluminum/beryllium/ATPase activity)

J. LUNARDI*, A. DUPUIS*, J. GARIN*, J.-P. ISSARTEL*, L. MICHEL*, M. CHABRE†, AND P. V. VIGNAIS*

*Laboratoire de Biochimie and †Laboratoire de Biophysique Moléculaire et Cellulaire, Département de Recherche Fondamentale, Centre d'Etudes Nucleaires, BP 85 X 38041 Grenoble Cedex, France

ABSTRACT   Inhibition of the mitochondrial and bacterial F_1-type ATPases [of ATP phosphohydrolase (H⁺-transporting), EC 3.6.1.34] by fluoride was found to depend on the presence of aluminum and ADP at the catalytic site(s) of F_1-type ATPase. AlF_4^- was demonstrated to be the active fluoroaluminate species. The identical pattern of inhibition of F_1-type ATPase activity obtained in the presence of ADP and NaF with beryllium, a metal that forms fluoride complexes strictly tetracoordinated, suggests that aluminum acts through a tetrahedral complex. Inhibition of isolated F_1-type ATPase by AlF_4^- in the presence of ADP cannot be reversed by ADP, ATP, or chelators of aluminum. However, the inhibition of the ATPase activity of the F_1 sector in submitochondrial particles caused by AlF_4^- and ADP was reversed upon addition of an oxidizable substrate. Uncouplers prevented the reversal of inhibition, suggesting that the protonmotive force generated by respiration was responsible for the relief of inhibition. Because of structural similarities between AlF_4^- and PO_4^{3-}, AlF_4^- is postulated to mimic the phosphate group of ATP and form an abortive complex with ADP at the active site(s) of F_1-type ATPase.

Fluoride, an inhibitor of a wide variety of ATP-dependent enzymes including phosphatases, phosphorylases, and kinases (1–3), influences biological systems depending on G proteins (4, 5). In all cases, millimolar concentrations of fluoride are needed. Only recently was the role of aluminum, a usual contaminant of fluoride solutions, recognized. Sternweis and Gilman (6) concluded that activation of adenylate cyclase by fluoride was linked to the presence of trace amounts of aluminum. Bigay *et al.* (7, 8) reported that the fluoride-dependent activation of transducin, a G protein involved in the visual cycle, required traces of aluminum. Transducin activation was mediated by the binding of 1 mol of fluoroaluminate to 1 mol of transducin, provided that GDP was present at the nucleotide-binding site of the protein. A model was proposed in which tetracoordinated aluminum associated with fluoride acted as an analogue of the γ phosphate of GTP by forming a fluoroaluminate complex with GDP at the active site. While studying the mechanism of GTP cleavage and the subsequent P_i release that accompanies microtubule assembly, Carlier *et al.* (9) showed that AlF_4^- exhibited phosphate-like effects, suggesting the binding of fluoroaluminate to the site that interacted with the γ phosphate of GTP. On the other hand, analysis of the mechanism of aluminum toxicity in some physiological processes has led various workers to postulate the formation of rather stable complexes between Al^{3+} and high-energy phosphate components like nucleoside di- and triphosphates (10–12).

Inhibition of proton-translocating ATPases in oral bacteria by fluoride was recently reported by Sutton *et al.* (13). These authors proposed that the targeted site of fluoride was the F_0 sector of the ATPase complex and that the effect of fluoride was mediated through a modification of proton conductivity. In the present work, we demonstrate that inhibition of ATPase activity in mitochondrial and bacterial H⁺-transporting ATPase [ATP phosphohydrolase (H⁺-transporting), EC 3.6.1.34] occurs in the presence of millimolar concentrations of fluoride and micromolar concentrations of both aluminum chloride and ADP and that ATPase inhibition results from the complexation of AlF_4^- with ADP at the catalytic site(s) of the F_1 sector of the ATPase complex. The inhibited fluoroaluminate–ADP-F_1 complex is postulated to mimic a natural intermediate formed during the course of the catalytic cycle of F_1 sector.

MATERIAL AND METHODS

Chemicals were of the highest purity commercially available. Nucleotides and enzymes used for measuring ATPase activity were purchased from Boehringer Mannheim. Aluminum chloride (gold-label compound) came from Aldrich. Beryllium chloride was obtained from Fluka, and ultrapure sodium fluoride was from Riedel de Haen. NaF solutions were made and stored exclusively in plastic containers. All reactions were performed in plastic tubes. Aluminum and fluoride are known to form rather poorly soluble complexes with various ligands. Therefore, light-scattering measurements were performed to check that no insoluble complexes were formed under our different experimental conditions; this was the case under all conditions except for $AlCl_3$ at concentrations >30 μM without NaF.

Beef heart mitochondria were prepared as described by Smith (14). Coupled submitochondrial particles were obtained according to the procedure reported by Beyer (15). Soluble beef heart mitochondrial F_1-type ATPase (MF_1) was prepared as described by Knowles and Penefsky (16) with the modification introduced by Klein *et al.* (17). Before use the enzyme, stored as an ammonium sulfate precipitate, was solubilized in 0.15 M sucrose/50 mM Tris·H_2SO_4, pH 8.0/5% (wt/vol) glycerol medium and immediately subjected to two sequential filtrations through 1-ml Sephadex G-50 columns (18). The first column was equilibrated with the solubilization medium, and the second one was equilibrated with the solubilization medium supplemented with 2 mM $MgCl_2$. This treatment yielded a mitochondrial F_1-type ATPase essentially free of the loose nucleotides present on the native

Abbreviation: MF_1, soluble mitochondrial ATPase from beef heart mitochondria.

enzyme (19). Soluble bacterial ATPase preparations were obtained from *Escherichia coli* K12PP12 (EF₁) (20). Nucleotide-depleted F₁-type ATPase was obtained as described by Garrett and Penefsky (21). Only enzyme preparations with a 280/260 nm OD ratio >1.90 (<0.5 ADP plus ATP per F₁-type ATPase) were used.

ATPase activity was measured spectrophotometrically at pH 8.0 and at 30°C using a standard regenerating system containing 4 mM phospho*enol*pyruvate, pyruvate kinase at 25 μg/ml, lactate dehydrogenase at 12.5 μg/ml, and 0.25 mM NADH. Alternatively, ATPase activity was determined by measuring the phosphate released (19, 22). In both cases, aluminum and fluoride anions had no effect on the regenerating systems under our experimental conditions. Protein was estimated using the Coomassie blue method (23).

RESULTS

Requirement of ADP and AlCl₃ for Inhibition of F₁-Type ATPase by NaF. The ATPase activity of the MF₁ was strongly inhibited when MF₁ was incubated with ADP, AlCl₃, and high concentrations of NaF. In the experiment illustrated in Fig. 1*A*, MF₁ was preincubated with ADP for 30 min. Addition of both AlCl₃ and NaF was required for enzyme inhibition. No inhibition developed when AlCl₃ or NaF was added alone. To ascertain that ADP was required for inhibition, a nucleotide-depleted enzyme was used in a further series of experiments. As shown in Fig. 1*B*, incubation of nucleotide-depleted MF₁ with AlCl₃ and NaF did not significantly inhibit ATPase activity. However, the further addition of ADP elicited strong inhibition. These experiments demonstrate unambiguously that inhibition of MF₁ cannot occur unless ADP, AlCl₃, and NaF are all present in the medium.

The requirement of the presence of a nucleoside diphosphate in the binding site of MF₁ was also suggested by the fact that GDP and IDP, which are the products of GTP and ITP hydrolysis by MF₁, were both able to elicit MF₁ inhibition in the presence of AlCl₃ and NaF, whereas AMP was ineffective. It is noteworthy that not only ATP but also GTP and ITP are hydrolyzed by F₁-type ATPases and that GDP or IDP as well as ADP are ligands for the catalytic sites of F₁-type ATPase (24).

Using the same concentrations of AlCl₃, NaF, and ADP as those used in the experiment illustrated in Fig. 1, a 50% decrease of MF₁ activity was attained only after a 5- to 10-min incubation of the enzyme at 30°C. The fact that inhibition

developed in a time-dependent manner may indicate that binding of inhibitory component(s) is a slow process or, more probably, that inhibition requires a slow transition of the enzyme from an active to an inactive conformation.

The concentration-dependence curves relative to inhibition of MF₁ by AlCl₃ and ADP are shown in Fig. 2. In the presence of 5 mM NaF and 100 μM ADP, half-maximal inhibition was attained with 10 μM AlCl₃. When NaF was omitted, no inhibition occurred, even at the highest concentrations of AlCl₃ used (Fig. 2*A*). Beryllium was reported to be the only metal that could substitute for aluminum to activate adenylate cyclase with fluoride (6). As shown in Fig. 2*A*, F₁-type ATPase activity was inhibited to the same extent in the presence of 5 mM NaF and 100 μM ADP with BeCl₂ added at the same concentrations as AlCl₃. BeCl₂ added with ADP, but without NaF, did not support any inhibition. This result strongly suggests that the inhibition induced by beryllium requires fluoride anions, as was the case for aluminum. Another experiment, illustrated in Fig. 2*B*, clearly showed that the nucleotide-binding sites of MF₁ must be filled with ADP for an inhibition of MF₁ to develop, the half-maximal effect being attained with 2 μM ADP in the presence of 5 mM NaF and 100 μM AlCl₃.

The same pattern of inhibition as that described for MF₁ holds for the soluble *E. coli* F₁-type ATPase. As shown in Table 1, inhibition in the presence of millimolar concentrations of fluoride required micromolar concentrations of both ADP and AlCl₃. This is not surprising, as the structure and functioning of the two enzymes are similar (25, 26).

The AlF₄⁻ Complex Is Responsible for Inhibition of F₁-Type ATPase. Data reported in Fig. 3 indicated that maximal inhibition elicited by AlCl₃ in the presence of ADP was obtained for a concentration of 5 to 10 mM NaF (Fig. 3*A*), a fluoride concentration for which the predominant fluoroaluminate complex was AlF₄⁻. When NaF concentrations >10 mM were used, the inhibition decreased; this is illustrated by the U-shaped curve obtained when ATPase activity was plotted against NaF concentration. This original feature of the inhibition could be explained by the fact that different fluoroaluminate complexes may exist, depending on the fluoride concentration in the medium. Al³⁺ forms relatively strong AlF_x complexes with the fluoride ion F⁻. Distribution curves of the aluminum fluoride-complex species as a function of the molar concentrations of free uncomplexed fluoride ion have been constructed (11, 27). The correlation between percentage of inhibition of F₁-type ATPase activity and
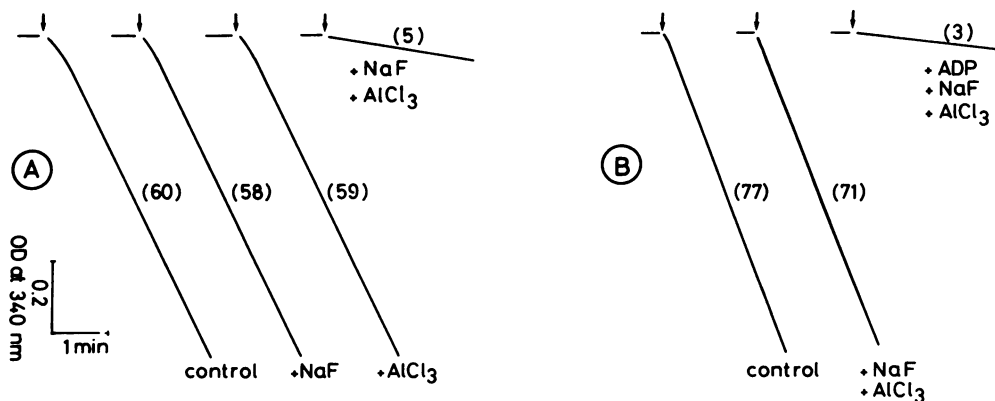

FIG. 1. Effect of AlCl₃ and NaF on ATPase activity of the MF₁. (*A*) ATP hydrolysis rates were recorded after a 30-min preincubation of 0.5 μM native MF₁ at 30°C with 60 μM ADP without (control) or with 30 μM AlCl₃ and/or 5 mM NaF as indicated. (*B*) ATP hydrolysis rates were recorded after a 30-min preincubation of 0.5 μM nucleotide-depleted MF₁ at 30°C without (control) or with 30 μM AlCl₃, 5 mM NaF, and 60 μM ADP as indicated. Preincubation medium was 0.15 M sucrose/50 mM Tris·H₂SO₄/2 mM MgCl₂/5% glycerol (wt/vol), pH 8.0. ATP hydrolysis was initiated by addition (arrows) of 10 μl of the preincubated MF₁ to 2 ml of a regenerating medium containing ATP and MgCl₂ at a final concentration of 3 mM. Values (in parentheses) indicated on the different records corresponded to the ATPase-specific activities at 30°C expressed in μmol of ATP hydrolyzed per min and per mg.
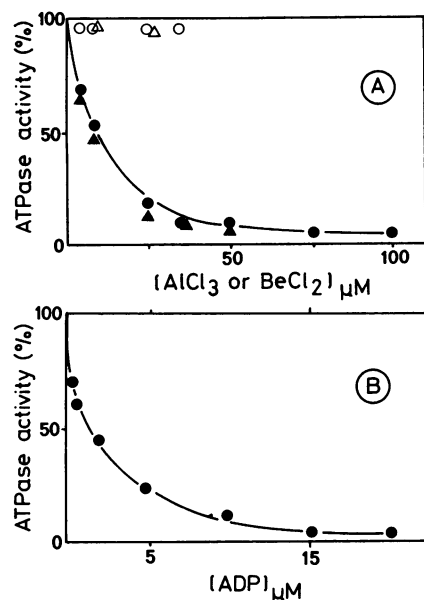
FIG. 2. Dose–effect curves relative to the ATPase inhibition caused by NaF and its dependence on $AlCl_3$ or $BeCl_2$ and ADP. (*A*) MF_1 (0.5 μM) was preincubated with 100 μM ADP with 5 mM NaF (closed symbols) or without NaF (open symbols) at the indicated concentrations of $AlCl_3$ (●, ○) or $BeCl_2$ (▲, △). (*B*) MF_1 (0.5 μM) was preincubated with 100 μM $AlCl_3$ and 5 mM NaF and with the indicated concentrations of ADP. After a 30-min preincubation at 30°C in the same medium as that indicated in Fig. 1, 10-μl aliquots of the MF_1 mixtures were withdrawn to measure enzyme activity as described. Percentage of remaining ATPase activity was calculated using as reference the same enzyme preincubated with ADP alone.

concentration of AlF_x species theoretically present in solution is illustrated in Fig. 3*B*. Clearly, the AlF_4^- species was responsible for the inhibition of ATP hydrolysis by MF_1.

Inhibition of Soluble F_1 by ADP–Fluoroaluminate Is Quasi-Irreversible. When F_1 inhibited by the ADP–fluoroaluminate complex was filtered through a Sephadex G-50 column equilibrated with a medium containing EDTA or citrate, two molecules known to form high-affinity chelates with Al^{3+} (11) or with medium containing a large excess of ADP or ATP in the presence of either EDTA or $MgCl_2$, the enzyme remained inhibited even after standing for 1 hr.

Inhibition of Membrane-Bound F_1-Type ATPase by ADP–Fluoroaluminate Can Be Reversed by Protonmotive Force. The hydrolytic activity of the membrane-bound F_1-type ATPase present in submitochondrial particles was also inhibited after incubation with ADP, $AlCl_3$, and NaF. However, unlike in MF_1, the inhibition of ATPase activity in coupled submitochondrial particles was significantly reversed in 10 min upon addition of succinate. Antimycin, an inhibitor of the respiratory chain, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, an uncoupler that abolishes the protonmotive force, both prevented the succinate-dependent reac-

Table 1. Effect of $AlCl_3$ and NaF on ATPase activity of *E. coli* F_1-type ATPase

ADP, μM	$AlCl_3$, μM	NaF, mM	Inhibition, %
60	0	0	10
60	100	0	6
60	0	5	7
60	100	5	69

E. coli F_1-type ATPase (0.16 μM) was incubated for 30 min at 30°C with ADP, $AlCl_3$, and NaF as indicated before ATPase activity was determined by measuring the P_i released as described in the text. Extent of inhibition was calculated using as control an enzyme incubated without ADP.
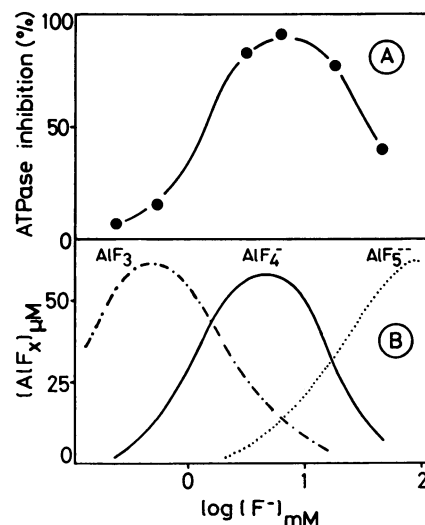

FIG. 3. NaF-dependent inhibition of F_1-type ATPase in the presence of ADP and $AlCl_3$. (*A*) MF_1 (0.5 μM) was preincubated at 30°C with 50 μM ADP, 100 μM $AlCl_3$, and NaF concentrations ranging from 0.3 to 50 mM. After 30 min, 10-μl aliquots of the MF_1 mixture were withdrawn to measure ATPase activity as described. Percentage of inhibition was calculated, using an enzyme incubated with ADP and $AlCl_3$ as reference. (*B*) Concentrations of the different AlF_x species theoretically present in solution. ---, AlF_3; —, AlF_4^-; ····, AlF_5^{2-}. AlF_x concentrations were calculated from data reported in ref. 27.

tivation of F_1-type ATPase in inhibited particles (Table 2). These results indicate that the development of a protonmotive force by respiration in submitochondrial particles influences the conformation of the membrane-bound F_1-type ATPase in such a way that F_1-type ATPase inhibition by the ADP–AlF_4^- complex is relieved.

DISCUSSION

This paper describes a mechanism of inhibition of mitochondrial and bacterial F_1-ATPases by a dead-end product formed

Table 2. Effect of succinate on the inhibition of the ATPase activity of coupled submitochondrial particles generated by ADP and AlF_4^-

Addition	Inhibition of ATPase activity, %
None (inhibited control)	83
EDTA	82
Succinate	23
FCCP	81
Succinate and FCCP	83
Antimycin	84
Succinate and antimycin	81

Particles (0.3 mg) were incubated in 0.35 ml of 0.25 M sucrose/50 mM KCl/20 mM Tris·HCl, pH 8.0/3 mM $MgCl_2$/0.15% fatty acid-free bovine serum albumin (standard medium) with 60 μM ADP, 50 μM $AlCl_3$, and 5 mM NaF. After a 30-min incubation at 30°C, 50 μl of the mixture was withdrawn and diluted 10-fold by addition of standard medium supplemented with 1 mM EDTA, 5 mM succinate, 50 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), or antimycin at 10 μg/ml as indicated; incubation was continued for another 10 min. Control condition corresponded to inhibited particles diluted in the standard medium without any further addition. ATPase activity was then determined as described. Inhibition of ATPase activity was calculated against data from particles processed under similar conditions with 60 μM ADP but without $AlCl_3$ and NaF. Specific activity of these noninhibited particles was 0.3 μmol/min per mg of protein.

by ADP and $AlCl_3$ in the presence of NaF. Experiments carried out with nucleotide-depleted F_1 provided evidence that ADP is required for ATPase inhibition to occur (Fig. 1*B*). It should be noted that IDP or GDP is also effective in supporting the ATPase inhibition in the presence of $AlCl_3$ and NaF, whereas AMP is not. This fact points to the requirement of the diphosphate moiety of the nucleotide for effectiveness of inhibition. Because IDP and GDP are known to bind only to the catalytic site(s) (24, 28), the inhibition of F_1-ATPase obtained with these nucleotides in the presence of NaF and $AlCl_3$ clearly indicates that the NDP–fluoroaluminate inhibitory complexes are formed within the catalytic site(s). By varying the concentration of NaF at a fixed concentration of $AlCl_3$, maximal inhibition of ATPase was found to occur under conditions in which accumulation of the AlF_4^- anion was predominant. Although fluoroaluminate complexes can adopt various coordination geometries, all beryllium fluorides are tetracoordinated and isomorphous to a phosphate group (8, 29). Therefore, the fact that beryllium fluoride inhibits the F_1-type ATPase activity (Fig. 2*A*) strongly suggests that aluminum also acts through a tetrahedral phosphate-like complex.

Aluminum has been reported to directly interact with the metal-binding site(s) of enzymes (12, 30). For MF_1, the inhibition of the ATPase activity is probably not related to the binding of Al^{3+} at the level of a metal-binding site because fluoride and ADP, besides $AlCl_3$, are necessary for inhibition. In other reports (11, 31, 32), the formation of a metal chelate between aluminum and the phosphate groups of ADP has been postulated. Although this possibility cannot be excluded, a more likely explanation is that AlF_4^- mimics the γ phosphate group of ATP at a nucleotide-binding site previously loaded with ADP, as it was postulated in the case of fluoride activation of transducin in the presence of AlF_4^- and GDP (8). In contrast to GDP–transducin complex, which reversibly binds fluoroaluminate (8), quasi-irreversible binding of the ADP–fluoroaluminate complex to isolated MF_1 is suggested by the lack of recovery of ATPase activity upon extensive washing in the presence of chelators.

As illustrated in the scheme of Fig. 4, in F_1-type ATPase the complex formed between AlF_4^- and ADP would substitute for ATP but would differ from ATP by remaining entrapped in an occluded form in the binding site. This would result in the formation of a blocked intermediate state of F_1-type ATPase obtained through a change of conformation of the enzyme upon binding of ADP and AlF_4^- at the catalytic site. That the F_1-type ATPase could undergo conformational changes upon binding of ADP and AlF_4^- is in accordance with the report, based on circular dichroism data, that the simultaneous occupation of catalytic site(s) by nucleotides and phosphate induced an important change in the overall conformation of F_1-type ATPase (33).

Whereas ATPase activity is nearly irreversibly inhibited by the binding of AlF_4^- and ADP to isolated MF_1, significant

recovery of the inhibited ATPase activity was observed when membrane-bound F_1-type ATPase in submitochondrial particles was used and respiration of the particles was induced by addition of succinate. This finding and the fact that uncouplers or oxidative phosphorylation inhibitors prevent the succinate-dependent reversal of ATPase inhibition suggest that the protonmotive force generated by respiration is required for displacement of the ADP–fluoroaluminate complex from its binding site. An analogous situation has been reported for the natural ATPase inhibitor IF_1 and MF_1 (34, 35). Although inhibition of the ATPase activity of isolated MF_1 caused by the natural ATPase inhibitor was virtually irreversible with isolated MF_1, this inhibition could be reversed with membrane-bound F_1-type ATPase in submitochondrial particles after addition of an oxidizable substrate. The respiration-dependent relief of ATPase inhibition caused by adenyl-5'-yl imidodiphosphate in submitochondrial particles (36, 37) is another example of the effect of the protonmotive force on the catalytic site of F_1-type ATPase. In this context, it must be stressed that in oxidative phosphorylation the release of the newly formed ATP still bound at the catalytic site of F_1-type ATPase is thought to be a major energy-requiring step of the catalytic cycle leading to ATP synthesis (38). The displacement or release of the ADP–fluoroaluminate bound to the catalytic site(s) induced upon energization of the submitochondrial particles probably proceeds from the same mechanism. Experiments including kinetic determinations, measurements of binding parameters with radioactive compounds, and the use of NMR are needed to evaluate in more detail the chemical processes by which the binding of ADP and AlF_4^- to F_1 sector blocks ATPase activity.

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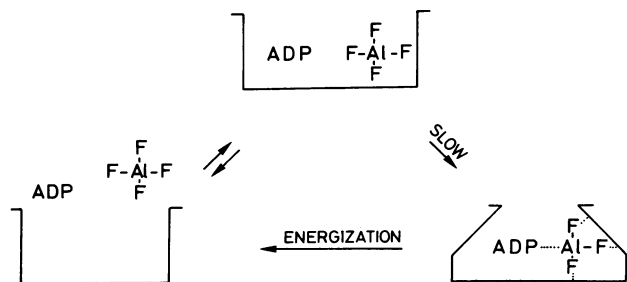20. Issartel, J.-P., Lunardi, J. & Vignais, P. V. (1986) *J. Biol. Chem.* **261**, 895–901.

FIG. 4. Hypothetical model for the F_1-type ATPase inhibition mechanism elicited by formation of the ADP–fluoroaluminate complex at the catalytic site (for details, see text).

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