^{19}F NMR Investigation of F_1-ATPase of *Escherichia coli* Using Fluoroaluminate Complex

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A number of enzymes or proteins involved in nucleotide binding or phosphate group transfer are sensitive to fluoride anions. Kinases and phosphatases are inhibited, while adenyl cyclases are generally activated.[1] In all cases milimolar concentrations of fluoride are needed. It was also demonstrated that the presence of traces of aluminum or beryllium were required to activated adenyl cyclase with fluoride.[2] In G protein systems it was proposed that AlF_4^- and BeF_3^- might act as phosphate analogues, and that AlF_4^- and BeF_3^- in the form of tetrahedral complexes structurally similar to phosphate, combined with GDP at the position occupied by γ-phosphate of GTP.[3,4] This phosphate analogue model of fluoroaluminate complex has been widely accepted not only for G proteins but also for many other proteins.[5-8] Since the activation was maximal at the fluoride concentration where the proportion of AlF_4^- had been calculated to be maximal,[8] tetrahedral geometry was assumed for the fluoroaluminate complex which would be binding to the active site.

A number of investigations on F_1-ATPases from many sources, bacteria, thylakoids and mitochondria, has established that there are three catalytic sites, and a further set of non-catalytic sites.[9-11] This is confirmed by the recent structural study of F_1-ATPase.[12] The non-catalytic sites have the characteristics that exchange of the bound ligand for ligand in the medium is slow ($t_{1/2}$ ~ hours) and there is considerable specificity for adenine nucleotides.[13,14] In contrast, the catalytic sites are not adenine-specific and rapidly exchange bound ligand with ligand in the medium ($t_{1/2}$ ~ minutes).[13,15,16]

In the case of mitochondrial F_1-ATPase irreversible inhibition was found to occur in the presence of aluminum, fluoride, and ADP, and was revealed to be the result of complexation of AlF_4^- with ADP at the catalytic site.[8] Fluoroberyllate in the presence of ADP was also found to inhibit irreversibly the mitochondrial F_1-ATPase.[17] Taking advantage of the virtually irreversible nature of the inhibition of F_1-ATPase by fluoroaluminate or fluoroberyllate, the ratio of ADP, beryllium or aluminum, and fluoride were measured. In the case of aluminium induced inhibition, the bound inhibiting species was found to be ADP · AlF_4^-.

Despite the large molecular weight of 380 K, F_1-ATPase were investigated by ^{31}P ad ^{19}F NMR spectroscopy.[18-20] ^{31}P NMR signals from chloroplast F_1-ATPase were too broad to be observed in the native form of enzyme, and could only be detected after modification with 4-chloro-7-nitro-benzofurazan.[18] However, ^{19}F NMR signals of *Escherichia coli* F_1-ATPase (EF1) from ^{19}F labelled ligand or internally substituted fluorotryptophan could be resolved.[19,20] The advantageous features of the fluorine nucleus are that ^{19}F occurs at 100% natural abundance and the sensitivity is close to that for the proton. In the present work, ^{19}F NMR signals from fluoroaluminate complex bound to the nucleotide binding sites of EF1 were investigated.

Experimental Section

Growth of cells. *Escherichia coli* strain SWM1, which is an overproducer of F_1-ATPases, was obtained from Dr. A. Senior (University of Rochester). For preparation of the enzyme, cells were grown in large batch culture using M9 media to which was added 1 mL of a concentrated trace element solution (14 mM $ZnSO_4$, 1 mM MnSO4, 4.7 mM $CuSO_4$, 2.5 mM $CaCl_2$ and 1.8 mM $FeCl_3$) per liter. After sterilization, 1 mL of sterile 1 M $MgSO_4$ was added per liter with other growth supplements as follows; 30 mM glucose, 0.2 μM thiamine hydrochloride, 0.8 mM L-arginine hydrochloride and 0.2 mM uracil. Since strain SWM1 is, unlike the wild type, resistant to chloramphenicol, this antibiotic (60 mg/mL in ethanol) was added just before inoculation to a final concentration of 60 μg/L. One liter cultures in L-broth were grown overnight and used to inoculate 25 liters of medium in a New Brunswick Scientific Pilot Fermenter. The cell were grown at 37 °C with vigorous aeration and pH was maintained at 7.2 through controlled addition of 2.5 M NaOH solution. Cell growth was monitored from absorbance at 750 nm. When the mid-exponential phase growth was reached, cells were harvested using an Amicon concentrator.

Preparation of enzyme. F_1-ATPase was prepared as described previously.[21] Enzyme was stored at -20 °C in column buffer which contained Tris/HCl (50 mM, pH 7.4), 1.0

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mM ATP, 1 mM DTT, 2 mM EDTA/Na and 10% glycerol. Activity was measured using a steady state coupled assay with pyruvate kinase and lactate dehydrogenase.[22] Protein was determined by the Bradford microassay procedure[23] using heat denatured F_1-ATPase as a protein standard. All the chemicals used were reagent grade from commercial sources.

NMR spectrometers and operating conditions. ^{19}F NMR spectra were obtained at 338.79 MHz (360 MHz ^1H). All spectra were taken with 2.5 mL samples in a 10 mm diameter tube. A capillary insert containing D_2O was used as an internal field frequency lock. All the spectra were obtained using pulse-and-collect sequence with 50° pulse and interpulse delay of 0.6s at 25 °C without sample spinning and with a sweep width of 8000 Hz with 4 K data points. For each spectrum 10 K scans were accumulated and the mixture was incubated at room temperature for at least for half an hour to allow binding of fluorinated nucleotide analogues to nucleotide-depleted EF1.

Preparation of nucleotide-depleted EF1. Nucleotide-depleted EF1 was prepared by the procedures of Garrett and Penefsky,[24] which involves a long desalting column at a very low rate (1 mL/h). Nucleotide-depleted EF1 in this method contained about 0.4 mole of ADP per mole of enzyme.

Results and Discussion

It was recently shown that mixture of NaF, $AlCl_3$, ADP forms ADP-fluoraluminate complexes in the absence of Mg^{2+}, but ADP-fluoroaluminate complex disappeared in the presence of Mg^{2+}.[25] When NaF (5 mM), $AlCl_3$ (1 mM), and ADP (1 mM) were incubated with nucleotide-depleted EF1, the ^{19}F spectrum (Figure 1A) showed a new tiny resonance at -20.7 ppm from free fluoride (0 ppm) in addition to the fluoroaluminate complexes observed in the absence of Mg^{2+}.[25] The addition of $MgCl_2$ to the medium not only caused a resonance at -29.8 ppm which was assigned to ADP · AlF_4^- [25] to disappear but also caused a resonance at -20.7 ppm to increase greatly (Figure 1B).

F_1-ATPase is known to be irreversibly inhibited by fluoride and aluminum complex in the presence of Mg^{2+}. The inhibitory species was found to be ADP · AlF_4^- bound to the catalytic site of F_1-ATPase.[17,26] As ADP · AlF_4^- complex could not be formed in the presence of Mg^{2+},[25] the inhibition of F_1-ATPase by fluoride and aluminum in the presence of Mg^{2+} is believed due to a binding of AlF_4^-, to a phosphate binding region of a catalytic site at which ADP bound independently. Thus, ^{19}F resonance at -20.7 ppm whose intensity increase greatly after the addition of Mg^{2+} (Figure 1B) must be from AlF_4^- complex, isomorphous to a phosphate, bound to a phosphate binding region (γ-position) of a certain catalytic site.

When the enzyme was freed from unbound reagent by

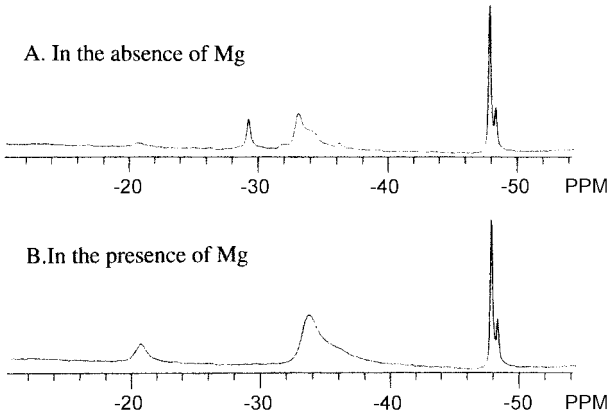

Figure 1. ^{19}F NMR spectra of fluoroaluminate complex binding to nucleotide-depleted EF1 with excess reagent. To the sample of EF1 containing approximately 19 mg/mL, NaF, $AlCl_3$, ADP, and $MgCl_2$ were added from stock solutions to give final solutions of 5 mM NaF. 1 mM $AlCl_3$, 1 mM ADP, and 5 mM $MgCl_2$. A capillary insert containing 1 mM fluorouracil in D_2O was used as an internal field frequency lock (-48 ppm). ^{19}F NMR spectra were taken with a simple pulse-and-collect sequence using 50° pulse and an interpulse delay of 0.6 s. A. Nucleotide-depleted EF1 incubated with NaF, $AlCl_3$, ADP (in the absence of Mg). B. Nucleotide-depleted EF1 incubated with NaF, $AlCl_3$, ADP and $MgCl_2$ (in the presence of Mg).

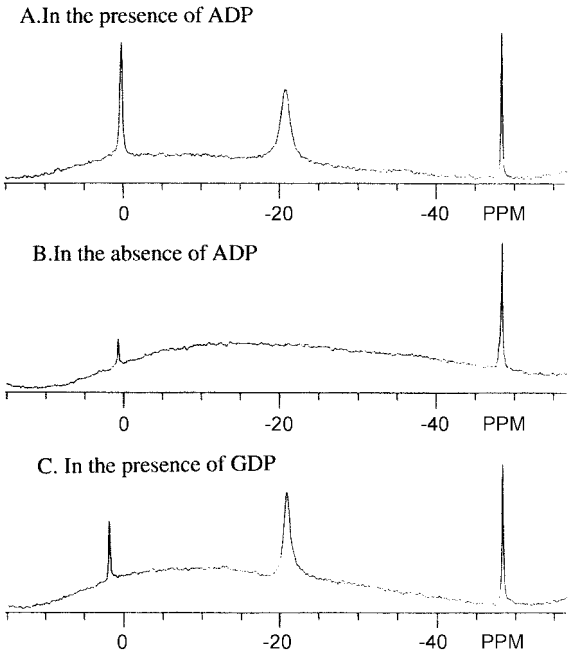

Figure 2. ^{19}F spectra of nucleotide-depleted EF1 after freed from excessive reagent. To the sample of EF1 containing approximately 19 mg/mL, NaF, $AlCl_3$, ADP/GDP, and $MgCl_2$ were added from stock solutions to give final solutions of 5 mM NaF. 1 mM $AlCl_3$, 1 mM ADP/GDP, and 5 mM $MgCl_2$. After standing at room temperature for at least an hour to allow binding, EF1 was freed of excessive reagent by passage through desalting column which had been equilibrated with Tris/HCl (pH 7.5). A capillary insert containing 1 mM fluorouracil in D_2O was used as an internal field frequency lock (-48 ppm). ^{19}F NMR spectra were taken with a simple pulse-and-collect sequence using 50° pulse and an interpulse delay of 0.6 s. A. Nucleotide-depleted EF1 incubated with NaF, $AlCl_3$, $MgCl_2$, and ADP (in the presence of ADP). B. Nucleotide-depleted EF1 incubated with NaF, $AlCl_3$ and $MgCl_2$ (in the absence of ADP). C. Nucleotide-depleted EF1 incubated with NaF, $AlCl_3$, $MgCl_2$, and GDP (in the presence of GDP).

passage through a desalting column, a broad resonance at -20.7 ppm still existed regardless of the presence of Mg^{2+}, suggesting very tight binding (Figure 2A). However, ^{19}F spectrum showed no discernable resonances when enzyme was incubated in the absence of ADP (Figure 2B), suggesting that the presence of ADP is essential for the binding of fluoroaluminate complex to EF1. The binding of fluoroaluminate complex in combination with GDP, catalytic site specific nucleotide, to the nucleotide-depleted EF1 also showed a resonance at the same chemical shift as that of ADP with similar intensity (Figure 2C). This confirms that the binding of fluoroaluminate complex to EF1 is to a catalytic site.[17,26]

Similar ^{19}F NMR studies were carried out to investigate the mechanism of activation of the α subunits of G proteins (G_α) for fluoroaluminate binding.[27,28] Addition of fluoroaluminate comlexes to the GDP-bound form of either of two G_α resulted in the appearance of peaks at -19 or -20 ppm from free fluoride in ^{19}F spectroscopy. Chemical shifts of fluoroaluminate complexes bound to G_α are similar to that of fluoroaluminate complex bound to F_1-ATPase, suggesting similar environment of nucleotide binding sites in both proteins.

Tetrahedral geometry of AlF_4^- which could fit to the nucleotide binding site in combination with ADP was suggested to represent 'precondensed state' of catalysis, which is analogous to (ADP + phosphate) entrapped in a closed catalytic site, or 'pentacoordinated transition state' in the intermediate step of condensation of ADP and phosphate in F_1-ATPase.[17] Since most of the structural information by x-crystallography was based on a unnatural static model, this characteristic of fluorometal complexes representing intermediate state of catalysis in solution could be widely used for the structural investigation of many catalytic pathways involving hydrolysis of nucleotide.[29,30]

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