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

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A number of enzymes or proteins involved in nucleotide binding or phosphate group transfer are sensitive to fluoride anions. Kinase and phosphate 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 tetrahedral complexes structurally similar to phophate, 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 site.[9-11] This is confirmed by the 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($t_{1/2}$-minutes).[13,14]

In the case of mitochondrial F_1-ATPases 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. The bound inhibiting species was found to be ADP·AlF_4^- for aluminum induced inhibition and ADP·BeF_3^- or ADP·BeF_2 for beryllium induced inhibition.[17,18]

Despite the large molecular weight of 380 K, F_1-ATPase could be investigated by ^{31}P and ^{19}F NMR spectroscopy.[19-21] ^{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-benzo-furazan. However, ^{19}F NMR signals of *Escherichia coli* F_1-ATPase(EF1) from ^{19}F labelled ligand or internally substituted fluorotryptophan could be resolved.[20,21] Recently the fluoroaluminate complex binding to EF1 could be investigated by ^{19}F NMR spectroscopy.[22] The advantageous features of the fluorine nucleus are that ^{19}F occur at 100% natural abundance and the sensitivity is close to that for the proton. In the present work, ^{19}F NMR signals from fluoroberyllate complex bound to the nucleotide binding sites of EF1 were investigated.

Materials and Methods

Growth of cells. *E. 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 $MnSO_4$, 4.7 mM $CuSO_4$, 2.5 mM $CaCl_2$ and 1.8 mM $FeCl_3$) per liter. After sterilization, 1ml 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. Chloramphenicol (60 mg/mL) and 10 μM *p*-benzoic acid were added as filter-sterilized solutions just before inoculation. 1.5 liter cultures in L-broth were grown overnight and used to inoculate 25 liters of medium in a New Brunswick Scientific Pilot Fermentor. From the growth curve it was determined that the tryptophan content (approximately 150 mg) in 1.5 liters of L-broth was the lowest quantity of tryptophan that provided non-limiting concentration from growth. Cells 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.[20-23] Enzyme was stored at −20 °C in column buffer which contained Tris/HCl (50 mM, pH 7.4), 1.0 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.[24] Protein was determined by the Bradford microassay proce-

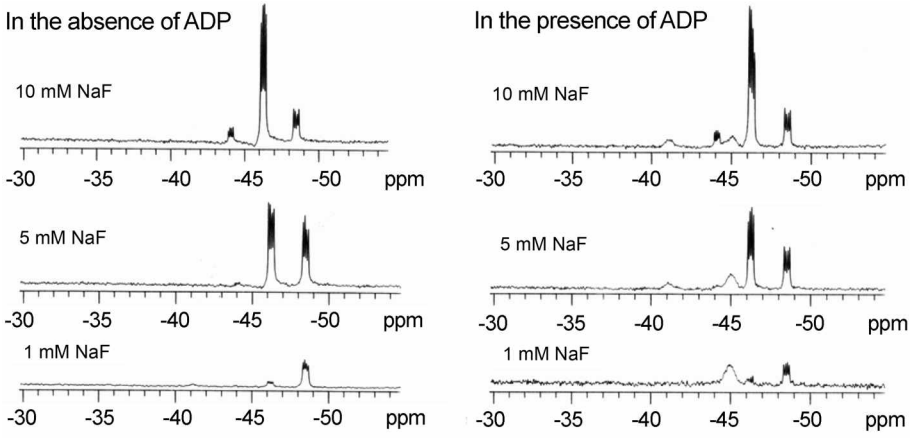

Figure 1. To a solution of 1 mM $BeCl_2$ in Tris/HCl buffer (pH 7.5) NaF was added form a 500 mM stock solution to make solutions of 1 mM, 5 mM and 10 mM. ^{19}F spectra were acquired with a simple pulse-and-collect sequence using 50° excitation pulse and an interpulse delay of 0.6 sec. 2 K scans were accumulated for each spectrum.

dure[25] 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. Chemical shifts of free fluoride was taken as 0 ppm. All the spectra were obtained using pulse-and-collect sequence with 50° pulse and interpulse delay of 0.6 s at 25 °C without sample spinning and with a sweep width of 8000 Hz with 4 K data points.

Preparation of nucleotide-depleted EF1. Nucleotide-depleted EF1 was prepared by the procedures of Garett and Penefsky,[26] which involves a long desalting column at a very low rate (1 mL/h).

Results and Discussion

By varying the concentration of NaF at a fixed concentration of the $BeCl_2$ (1 mM), formation of fluoroberyllate complexes were investigated. There appeared 3 detectable ^{19}F resonances at −44 ppm, −46.3 ppm, and −48.5 ppm from free fluoride (Figure 1). By comparing with the distribution curve of fluoroberyllate complexes depending on the fluoride concentration,[27] 3 resonances could be reasonably assigned to BeF_4^{2-}, BeF_3^-, and BeF_2, respectively. The addition of ADP to fluoroberyllate complexes caused 2 more resonances to appear. Both resonances are presumed to be from ADP-fluoroberyllate complex.

After incubating the fluoroberyllate complexes (10 mM NaF) with the native EF1 for 30 minutes, excess reagent was removed by passage through a desalting column. The ^{19}F spectrum showed two resonances at −32 ppm and −37 ppm only when binding was carried out in the presence of Mg^{2+} (Figure 2), suggesting that the binding of fluoroberyllate to EF1 was to the phosphate binding region of a certain catalytic site.

To find out whether presence of two ^{19}F resonances represents asymmetric nature of nucleotide binding sites or just different fluoroberyllate complex species, binding was carried out with nucleotide depleted EF1 with ADP. Dependency of the relative intensity of ^{19}F resonances on the concentration of NaF (Figure 3) may suggest that the two ^{19}F resonances represent the different fluoroberyllate complexes. By the dependency of fluoroberyllate complexes on the concentration of NaF, the resonance at −32 ppm and −37 ppm could be assigned to be BeF_3^- and BeF_2, respectively. The analysis of bound fluoride and beryllate in beef heart

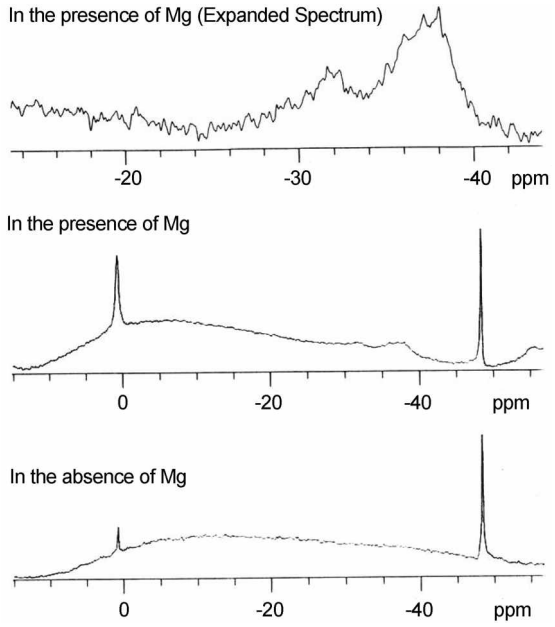

Figure 2. To samples of native EF1 (19 mg/mL) NaF, $BeCl_2$, and $MgCl_2$ were added from stock solutions to give final concentrations of 10 mM NaF and 1 mM $BeCl_2$ and 5 mM $MgCl_2$. After standing at room temperature for 30 minutes to allow binding, EF1 was freed of extra reagent by passage through a desalting column which had been equilibrated with Tris/HCl (pH 7.5). ^{19}F spectra were obtained at room temperature using pulse-and-collect sequence using 50° excitation pulse and an interpulse delay of 0.6 sec. 20 K scans were accumulated for each spectrum.

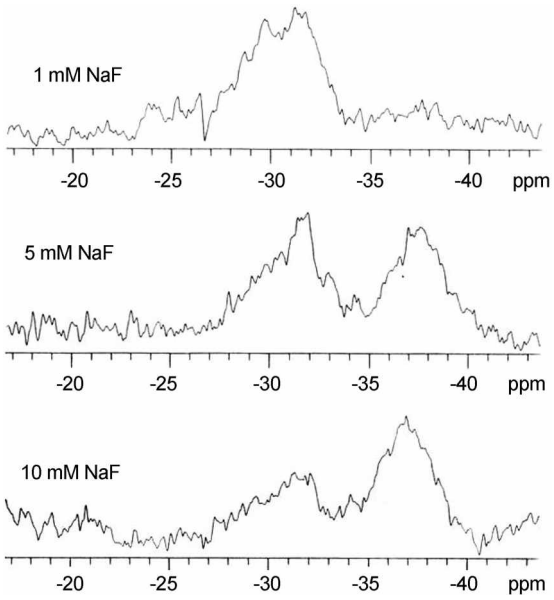

Figure 3. To samples of nucleotide depleted EF1 (19 mg/mL) NaF, $BeCl_2$ and $MgCl_2$ and ADP were added from stock solutions to give final concentrations of 10 mM, 5 mM and 1 mM of NaF, respectively, and 1 mM $BeCl_2$ 5 mM $MgCl_2$ and 1 mM ADP After standing at room temperature for 30 minutes to allow binding, EF1 was freed of extra reagent by passage through a desalting column which had been equilibrated with Tris/HCl (pH 7.5). ^{19}F spectra were obtained at room temperature using pulse-and-collect sequence using 50° excitation pulse and an interpulse delay of 0.6 sec. 20 K scans were accumulated for each spectrum.

mitochondrial enzyme also showed that BeF_3^- and BeF_2 are the species which are bound to EF1.[17]

Strict dependence of fluoroberyllate complexe binding to EF1 to Mg^{2+} is in contrast to that of the binding of fluoroaluminate complexes to EF1 of which single ^{19}F resonance appeared regardless the presence of Mg^{2+}.[22] The difference in the requirement of Mg^{2+} to the binding of each fluorometal complex to the EF1 may suggest that each inhibited EF1 which is blocked by fluoroaluminate and fluoroberyllate represent different catalytic states.

Tetrahedreal geometry of AlF_4^- which fits to the nucleotide binding site in combination with ADP was suggested to represent 'precondensed state' or 'intermediate 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.[17] Appearance of ^{19}F resonance at the same chemical shift in the absence Mg^{2+} suggests[22] that fluoroaluminate complex binding is likely to be 'precondensed state' which may not be differentiated with magnesium independent non-catalytic site. On the other hand the inhibited state blocked by fluoroberyllate with strict dependence of Mg^{2+} could represent a 'condensed state' in the pathway of ATP synthesis which is specific to the catalytic sites.

Most of the structural information of EF1 was gathered with x-ray crystallography based on unnatural static model. ^{19}F NMR spectroscopy of fluorometal complex binding to EF1 suggests that the 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.[28-31]

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References

1. Hewitt, E. J.; Nicholas, D. J. D. In *Metabolic Inhibitors*; Hochester, R. M., Quastrel, J. H., Eds.; Academic press: New York, U.S.A., 1963; Vol. 2, p 31.
2. Sternweis, P. C.; Gilman, A. G. *Proc. Natl. Acad. Sci. USA.* **1982**, *79*, 4888.
3. Bigay, J.; Deterre, P.; Pfister, C.; Charbre, M. *FEBS Lett.* **1985**, *191*, 181.
4. Bigay, J.; Deterre, P.; Pfister, C.; Charbre, M. *EMBO J.* **1987**, *6*, 2907.
5. Carlier, M. F.; Didry, D.; Melki, R.; Chabre, M.; Casteels, T. *Biochemistry* **1988**, *27*, 3555.
6. Combeau, C.; Carlier, M. F. *J. Biol. Chem.* **1988**, *27*, 17429.
7. Lange, A. J.; Arion, W. J.; Burchell, A.; Burcjell, B. *J. Biol. Chem.* **1986**, *261*, 101.
8. Lunardi, J.; Dupuis, A.; Garin, J.; Issartel, J. P.; Michel, L.; Chabre, M.; Vignais, P. V. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 8958.
9. Boyer, P. D. *Biochim. Biophys. Acta* **1993**, *1140*, 215.
10. Penefsky, H. S.; Cross, R. *Adv. Enzymol.* **1991**, *64*, 173.
11. Weber, J.; Senior, A. E. *Biochim. Biophys. Acta* **1997**, *1319*, 19.
12. Abrahams, J. P.; Leslie, A. G. W.; Lutter, R.; Walker, J. E. *Nature* **1994**, *370*, 621.
13. Cross, R. L.; Nalin, C. M. *J. Biol. Chem.* **1982**, *257*, 2874.
14. Senior, A. E. *Physiol. Revs.* **1988**, *68*, 177.
15. Perlin, D. S.; Latchney, L. R.; Wise, J. G; Senior, A. E. *Biochemistry* **1984**, *23*, 4998.
16. Wise, J. G.; Duncan, T. M.; Latchney, L. R.; Cox, D. N.; Senior, A. E. *Biochemical J.* **1983**, *214*, 343.
17. Dupuis, A.; Issartel, J. P.; Vignais, P. *FEBS Lett.* **1991**, *255*, 47.
18. Isaartel, J. P.; Dupuis, A.; Unardi, J.; Vignais, P. V. *Biochemistry* **1991**, *30*, 4726.
19. Carr, M.; Mulvey, D.; Willis, A.; Ferguson, S. J.; Campbell, I. D. *Biochim. Biophys. Acta* **1990**, *1015*, 79.
20. Jung, S.; Kim, H.-W. *J. Biochem. Mol. Biol.* **1998**, *31*, 44.
21. Lee, H.-W.; Sohn, J. H.; Yeh, B.-I.; Choi, J.-W.; Jung, S.; Kim, H.-W. *J. Biochem.* **2000**, *127*, 1053.
22. Yoon, J.; Sohn, J. H.; Lee, H.-W.; Yeh, B.-I.; Choi, J.-W.; Jung, S.; Kim, H.-W. *Bull. Korean Chem. Soc.* **2001**, *22*, 90.
23. Senior, A. E.; Falyle, D. R. H.; Downie, J. A.; Gibson, F.; Cox, G. B. *Biochemical. J.* **1979**, *180*, 111.
24. Ting, L. P.; Wang, J. J. *Biochemistry* **1980**, *19*, 5665.
25. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
26. Garett, N. E.; Penefsky, H. S. *J. Biol. Chem.* **1975**, *256*, 6640.
27. Martin, R. B. *Biochim. Biophys. Res. Commun.* **1988**, *155*, 1194.
28. Hazlett, T. L.; Higashijima, T.; Jameson, D. M. *FEBS Lett.* **1991**, *278*, 225.
29. Higashijima, T.; Graziano, M. P.; Suga, H. S.; Kaiosho, M.; Gilman, A. G. *J. Biol. Chem.* **1991**, *266*, 3396.
30. Schindelin, H.; Kisler, C.; Schlessman, J. L.; Howard, J. B.; Rees, D. C. *Nature* **1997**, *387*, 370.
31. Sondek, J.; Lambright, D. G.; Noel, J. P.; Hamm, H. E.; Sigler, P. B. *Nature* **1994**, *372*, 276.