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Conformational Changes of Nucleotide Binding Sites Following Sequential Addition of ADP to Nucleotide-depleted F_1-ATPase of *Escherichia coli* Investigated with ^{31}P NMR Spectroscopy

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Oxidative phosphorylation in *Escherichia coli* is catalyzed by an electron transport system that generates a proton electrochemical gradient across the cytoplasmic membrane and an ATP synthase enzyme that catalyzes the conversion of ADP and Pi to ATP at the expense of a gradient of sufficient magnitude. The ATP synthase of this organism is essentially identical in other bacteria, the mitochondria of eukaryotes and the thylakoids of green plants.[1-7] Two functionally distinct parts of the protein can be distinguished. These are: (i) the F_0 sector, which, in the case of *E. coli*, comprises three polypeptide chains known as a, b and c, which together form a transmembrane proton channel: (ii) F_1 sector which contains five polypeptide chains with relative stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$.

A considerable number of investigations on F_1-ATPases from many sources, bacteria, thylakoids and mitochondria, have established that there are three catalytic sites and a further set of non-catalytic sites.[7,8] 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.[2,5,6] In contrast, the catalytic sites are not adenine-specific and rapidly exchange bound ligand with ligand in the medium ($t_{1/2}$ ~minutes).[5,9,10]

The intrinsic asymmetric structure of the enzyme, together with a variety of experimental investigations, indicates that the properties of the putative catalytic sites may be distinct from one another at any instant in a catalytic cycle. Direct evidence for such distinct properties, however, has been lacking. In principle ^{31}P NMR should be able to discriminate amongst different sites. ^{31}P NMR studies have been performed to characterize the nucleotide binding sites of CF.[11] Although the latent CF1 was found to have tightly bound non-dissociable nucleotide per enzyme molecule, no ^{31}P NMR signals from these bound signals could be detected despite a wide range of experimental conditions. ^{31}P NMR signals arising from the α and β phosphate groups of bound ADP molecules could be observed only after modification with *N*-ethylmalemide and 4-chloro-7-nitrobenzo-furazan.[11] The failure to detect ^{31}P NMR signals in native CF1 was ascribed to chemical shift anisotropy, which could produce a very broad line width.

In the case of *E. coli*, it has been established that the enzyme conforms to the general pattern of nucleotide binding and that nucleotides can be removed from all binding sites. Many ^{19}F NMR studies of EF1 using fluorinated ligands,[12] fluoroaluminate complex,[13] fluoroberyllate complex[14] and internal fluoro-tryptophan labeling[15,16] were performed to characterize nucleotide binding sites of F_1-ATPase of *E. coli* in the physiological condition.

Very recently ^{31}P NMR spectroscopy was tried to investigate F_1-ATPase of *E. coli*.[17] In contrast to CF1, ^{31}P resonances form enzyme bound nucleotides were clearly visible. ^{31}P resonances from nucleotide tightly bound to EF1 showed asymmetry amongst nucleotide binding sites in physiological condition. In this investigation we have used ^{31}P NMR spectroscopy to monitor the conformational change amongst nucleotide binding sites with addition of ADP to nucleotide-depleted EF1.

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, 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, 0.2 mM uracil; chloramphenicol (60 mg/mL) and 10 μM *p*-benzoic acid was 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. 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.[12-16] Enzyme was stored at −20 ºC in column buffer which contained Tris/HCl (50 mM, pH 7.4), 1 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.[18] Protein concentration was determined by the Bradford microassay procedure[19] using heat denatured F_1-ATPase as a protein standard. All the chemicals used were reagent grade from commercial sources.

Removal of Unbound Nucleotide from EF1. This was achieved by passing purified EF1 through a Sephadex G25-100 gel filtration column (1 × 15 cm) which was equilibrated with buffer containing Tris/HCl (50 mM, pH 7.4). EDTA (2 mM) and 10% methanol, or the same buffer containing 5 mM $MgCl_2$. Typically less than 2.5 mL of protein sample was passed down through a desalting column. Pooled fractions were concentrated by an Amicon filtration system using a PM-30 membrane.

Preparation of Nucleotide-Depleted EF1. Rather than using the procedure of Garrett and Penefsky[20] which involves a along desalting column running at a very low rate (1 mL/h) to prepare nucleotide-depleted F1, a new method using an ultrafiltration membrane was used. EF1 samples were precipitated with 67% saturated ammonium sulfate, collected by centrifugation and dissolved in 50 mM Tris/HCl, 2 mM EDTA, 50% glycerol(V/V), pH 8.0. Such samples were concentrated in an Amino cell fitted with a PM-30 membrane using the same buffer until A280/A260 ratio higher than 1.8 was obtained. The A280/A260 ratio obtained by this method is similar to that for the peak fraction obtained from the long desalting column in the conventional method.

NMR Spectrometers and Operating Conditions. ^{31}P NMR spectra were obtained at 145.8 MHz (360 MHz ^1H). All spectra were taken with 2.5 ml samples in a 10 mm diameter tube. A capillary insert containing methylendiphonic acid (resonance at 18 ppm) dissolved in D_2O was used as an internal field frequency lock and size control. All the spectra were obtained using pulse-and-collect sequence with 75º pulse and inter-pulse delay of 3 s at 25 ºC without sample spinning and with a sweep width of 8000 Hz with 500 data points. 85% Phosphoric acid was used as an external reference for measuring the ^{31}P chemical shifts.

Results and Discussion

^{31}P spectra of nucleotide-depleted EF1 with sequential additions of ADP are shown in Figure 1-3. ^{31}P spectrum of nucleotide-depleted EF1 showed no detectable resonance (Figure 1). When one equivalent of ADP was added in the absence of Mg^{2+}, a single resonance appeared at around −10 ppm which is the position of α-phosphate of ADP, and the unresolved resonances were observed in the region of the β-phosphate of ADP (−3 to −7 ppm) (Figure 1). Chemical shift position of α-phosphate of ADP did not change at all even after many equivlalents of ADP were added. However,

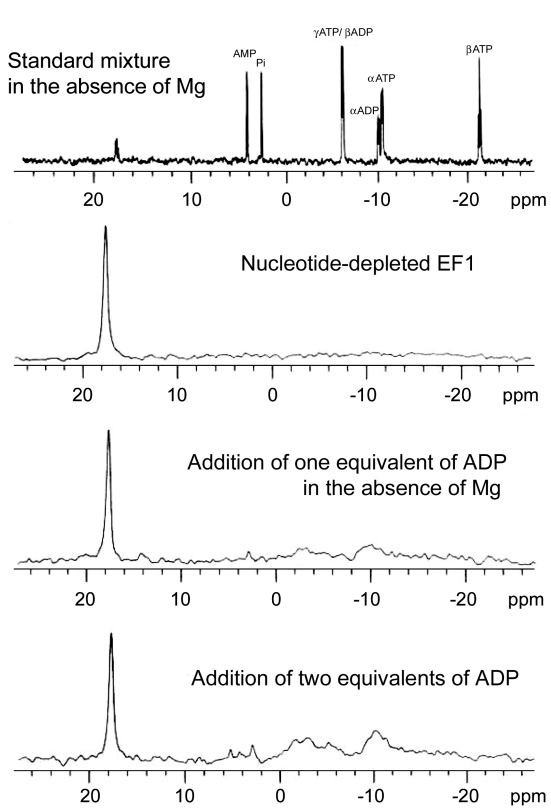

Figure 1. ^{31}P Spectra of nucleotide-depleted EF1 with sequential addition of ADP (1-2 equivalents). ADP titration (1-2 equivalents) was performed on sample containing 100 mg/mL of EF1 in the absence of Mg^{2+}. ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 75º excitation pulse and inter-pulse delay of 3 s at 25ºC without sample spinning. 20 K scans were accumulated for protein and 1000 scans were accumulated for standard mixture in the absence of Mg^{2+}.

chemical shift position of the β-phosphate region of ADP kept changing with further addition of ADP.

When two equivalents of ADP were added (Figure 1), ^{31}P spectrum in the region of the β-phosphate of ADP showed multiple resonances of which the pattern is similar to that of native EF1.[17] The native EF1 prepared in the absence of Mg^{2+} also contained about 2 equivalents of ADP.[17] The presence of multiple resonances at the region of the β-phosphate may suggest the asymmetry amongst nucleotide binding sites.

When three and four equivalents of ADP were added (Figure 2), multiple ^{31}P resonances also appeared, but the chemical shift and relative intensities changed depending on the amount of ADP added, suggesting conformational changes as more nucleotides are bound to the enzyme. No free β-phosphate of ADP was observed even after addition of four equivalents of ADP, indicating four equivalents of ADP could be bound to EF1 in the absence of Mg^{2+}.

When five equivalents of ADP were added, free ^{31}P resonance from unbound β-phosphate of ADP could be observed (Figure 2). However, the subsequent addition of $MgCl_2$ to the medium resulted in disappearance of all the free the β-phosphate of ADP (Figure 3), implying a decrease

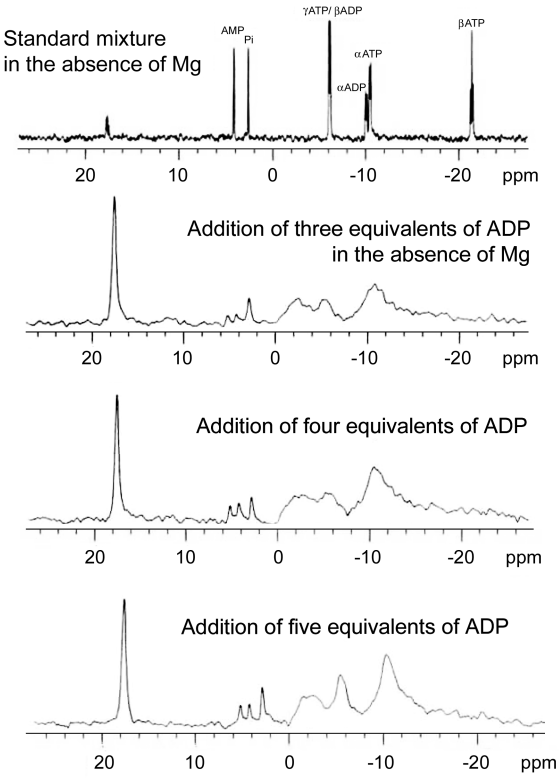

Figure 2. ^{31}P Spectra of nucleotide-depleted EF1 with sequential addition of ADP (3-5 equivalents) in the absence of Mg^{2+}. ADP titration (3-5 equivalents) was performed on sample containing 100 mg/ml of EF1 in the absence of Mg^{2+}. ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 75° excitation pulse and inter-pulse delay of 3 s at 25 °C without sample spinning. 20 K scans were accumulated for protein and 1000 scans were accumulated for standard mixture in the absence of Mg^{2+}.

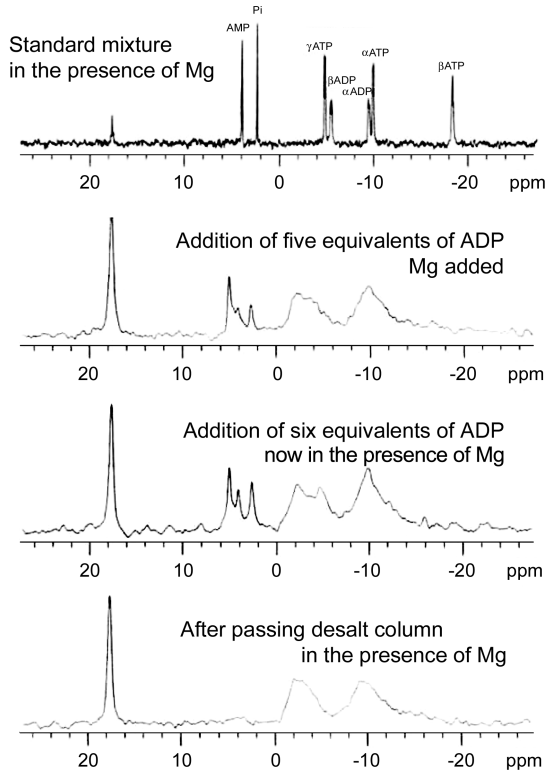

Figure 3. ^{31}P Spectra of nucleotide-depleted EF1 with sequential addition of ADP (5-6 equivalents) in the presence of Mg^{2+}. ADP titration (5-6 equivalents) was performed on sample containing 100 mg/ml of EF1 in the presence of Mg^{2+}. ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 75° excitation pulse and inter-pulse delay of 3 s at 25 °C without sample spinning. 20 K scans were accumulated for protein and 1000 scans were accumulated for standard mixture in the presence of Mg^{2+}.

of Kd for ADP for fifth nucleotide binding site in the presence of Mg^{2+}. Even after six equivalents of ADP, most of the ADP added seemed to be bound to EF1 (Figure 3). When free ADP was removed by passage through a desalting column, ^{31}P spectrum of the β-phosphate region of enzyme bound ADP was similar to that with five equivalents of ADP (Figure 3).

These titration results showed that fifth and sixth nucleotide binding sites of EF1 are Mg-dependent. Recent ^{31}P spectroscopy of native EF1 and titration of nucleotide depleted EF1 with ATP showed that the Mg-dependent tightly bound nucleotide binding site is non-catalytic.[17] The appearance of Mg-dependent non-catalytic site only after addition of five equivalents of ADP to EF1 suggests asymmetry amongst non-catalytic sites, indicating that the nucleotide binding to one of the non-catalytic sites is Mg-independent.

As titration continued, resonances at 3 ppm, 4.2 ppm and 5 ppm also increased. Comparison with the ^{31}P NMR spectrum of AMP and *Pi* shows that the resonances at 3 ppm and 4.2 ppm are ^{31}P resonance from AMP and *Pi*, respectively. As titration was carried out with only ADP molecules in the medium, these results suggest that the resonances of AMP and *Pi* were produced by adenylate kinase-like activity of F_1-ATPase. Adenylate kinase-like activity produced both ATP and AMP from ADP, ATP is then rehydrolyzed to produce *Pi*. As molecular weight of F_1-ATPase (380 K) is very large compared to that of adenylate kinase (32 K), adenylate kinase-like activity of F_1-ATPase cannot be from contaminated adenylate kinase after gel filtration in the preaparation.[13-16]

The resonance at around 5 ppm must be originated from a transfer of phosphoryl group of ADP to a possible acceptor molecule in the medium, which is methanol added as a stabilizer of F_1-ATPase. Comparison with the ^{31}P NMR spectrum of alkyl phosphate could confirm the presence of methyl phosphate.[21,22] ^{31}P resonance of methyl phosphate indicates that the phosphoryl group of ADP was enzymatically transferred to methanol.

Both adenylate kinase-like activity and phosphotransferase could be observed in the absence of Mg^{2+}, of which the resonance of AMP at around 3 ppm is the largest. This is in contrast to ^{31}P spectra of ADP with small amount of EF1,[22] of which both adenylate kinase-like activity and phosphotransferase was negligible in the absence of Mg^{2+}. The addition of Mg^{2+} caused the resonances of *Pi* and methyl phosphophosphate to increase. Especially, ^{31}P resonance of methyl phosphate increased greatly with addition of Mg^{2+},

indicating the relative increase of phosphotransferase-like activity compared to adenylate kinase-like activity.

^{31}P NMR spectroscopy could discriminate between different binding sites on a protein as large as *E. coli* F_1-ATPase. The observation of ^{31}P resonances contrasted with a previous study of F_1-ATPase from thylakoids in which the resonances form the native form of the enzyme were too broad to detect.[11] The detection of multiple resonances from β-phosphates of ADP bound to EF1 suggests the asymmetry amongst nucleotide binding sites at physiological condition.

^{31}P NMR spectroscopy could also detect the appearance of *Pi*, AMP, and methyl phosphate which could not be detected with normal assay condition, showing phosphotransferase-like activity compared to adenylate kinase-like activity of EF1. ^{31}P NMR spectroscopy proved that it could be a valuable tool for the investigation of phosphorous related enzyme.

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