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Catalytic Sites of F_1-ATPase from *Escherichia coli* was Investigated with GTP and GDP Titration Using ^{31}P NMR Spectroscopy

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

Many investigations on F_1-ATPases from diverse origins, bacteria, thylakoids and mitochondria, established that the composition of F_1-ATPases comprised of three catalytic sites and a further set of non-catalytic sites.[7,8] The non-catalytic sites had the characteristics of slow exchange of the bound ligand for ligands in the medium ($t_{1/2}$ ~ hours) with considerable specificity for adenine nucleotides.[2,5,6] In contrast, the catalytic sites were not adenine-specific and revealed rapid exchanges of bound ligand with ligands in the medium ($t_{1/2}$ ~ minutes).[5,9,10]

The intrinsic asymmetric structure of the enzyme ($\alpha_3\beta_3\gamma\delta\varepsilon$), together with variety of experimental investigations, indicated that the properties of the putative catalytic sites may be distinct from one another at any instant in a catalytic cycle. The participating catalytic sites are in principle identical, but having different affinities for substrate and/or products at any instant.[5] Direct evidence for such distinct properties in physiological condition, however, was absent, although 3D structure of F_1-ATPase was revealed, and suggested the cooperative mechanism involving all three catalytic sites.[8]

In principle ^{31}P NMR should be able to discriminate different nucleotide binding sites. ^{31}P NMR studies were 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 wide range of experimental conditions were tried. ^{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 could be ascribed to chemical shift anisotropy, producing a very broad line width.

In the case of *E. coli*, it was established that the enzyme conformed to the general pattern of nucleotide binding, and that nucleotides could 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.

Recently, ^{31}P NMR spectroscopy was tried to investigate F_1-ATPase of *E. coli*.[17-19] In contrast to CF1, ^{31}P resonances from 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, ^{31}P NMR spectroscopy was employed to monitor the conformational change amongst nucleotide binding sites with addition of catalytic site specific guanosine nucleotides to nucleotide-depleted EF1.

Experimental Section

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

Abbreviations: EF1: F_1-ATPase from *Escherichia coli*, CF1: F_1-ATPase from thylakoids, GDP: Guanosine Nucleotide Di-Phosphate, GTP: Guanosine Nucleotide Tri-Phosphate, GMP: Guanosine Nucleotide Mono-Phosphate, ADP: Adenosine Nucleotide Di-Phosphate, ATP: Adenosine Nucleotide Tri-Phosphate, Pi: Phosphate, NMR: Nuclear Magnetic Resonance

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.[12-21] Enzyme was stored at −20 °C 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.[20] Protein concentration was determined by the Bradford microassay procedure[21] 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. Nucleotide-depleted EF1 was prepared using an ultrafiltration membrane.[13-19] 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.[22]

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 75° pulse and inter-pulse delay of 3 s at 25 °C 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

A ^{31}P spectrum of a mixture of GTP, GDP, GMP and Pi (1 mM each) indicated that the phosphorous chemical shifts of guanosine nucleotides were same as those of adenosine nucleotides[17] (Figure 1).

A GTP titration of nucleotide-depleted EF1 was shown in Figure 2. When one equivalent GTP was added to nucleotide-depleted EF1 in the absence of Mg^{2+}, the ^{31}P NMR spectrum revealed sharp resonances of free phosphate and α- and β-phosphate of free GDP. The presence of free phosphate and GDP indicated the hydrolysis of GTP with nucleotide-depleted EF1 in the absence of added Mg^{2+}. The

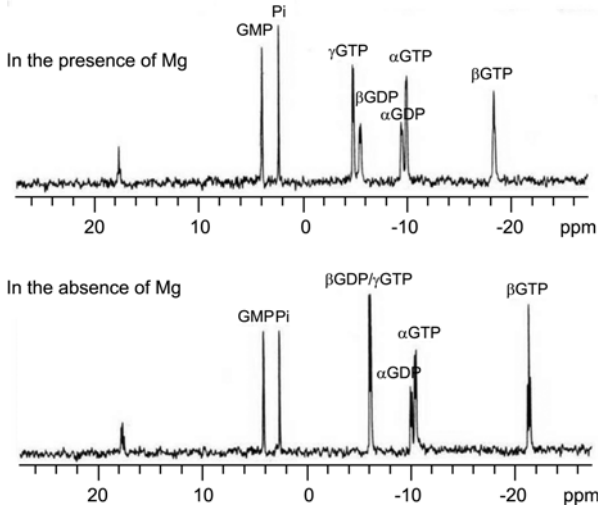

Figure 1. ^{31}P NMR spectrum of GTP, GDP, GMP and Pi (each 1 mM). ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 50° excitation pulse and inter-pulse delay of 2 seconds at 25 °C without sample spinning. 1000 scans were accumulated for standard mixture. 85% Phosphoric acid was used as an external reference for measuring the ^{31}P chemical shifts.

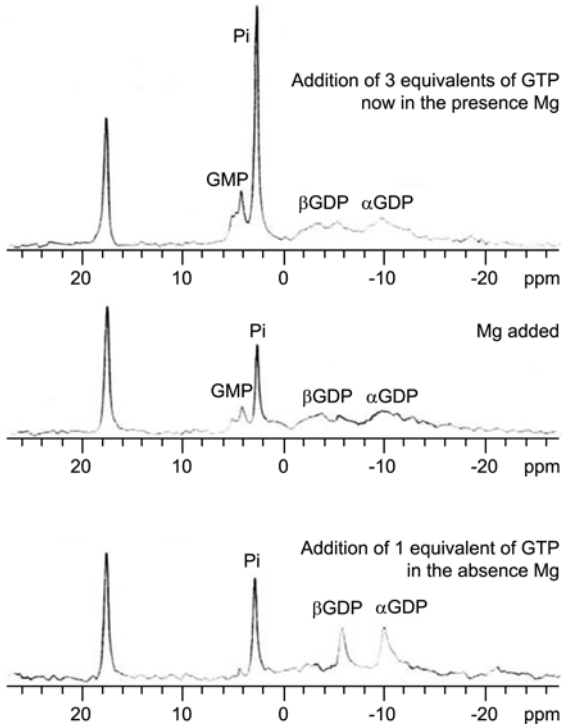

Figure 2. ^{31}P Spectra of nucleotide-depleted EF1 with sequential addition of GTP. GTP titration was performed on sample containing 100 mg/mL of EF1. ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 75° excitation pulse and inter-pulse delay of 3 seconds at 25 °C without sample spinning. 20 K scans were accumulated. 85% Phosphoric acid was used as an external reference for measuring the ^{31}P chemical shifts.

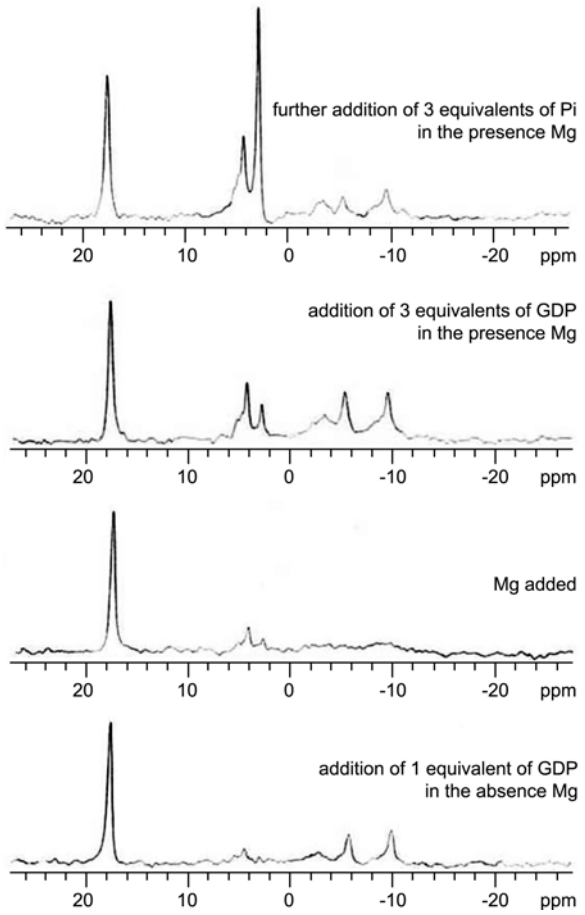

Figure 3. ^{31}P Spectra of nucleotide-depleted EF1 with sequential addition of GDP. GDP titration was performed on sample containing 100 mg/mL of EF1. ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 75° excitation pulse and inter-pulse delay of 3 seconds at 25 °C without sample spinning. 20 K scans were accumulated. 85% Phosphoric acid was used as an external reference for measuring the ^{31}P chemical shifts.

presence of free GDP after addition of one equivalent of GTP to nucleotide-depleted EF1 suggested that the binding of the resultant GDP was weak in the absence of Mg^{2+}.

After the addition of $MgCl_2$, the sharp resonances from α- and β-phosphate of free GDP disappeared, indicating the binding of GDP to EF1. Resonance of α-phosphate of GDP broadened after binding to EF1, but chemical shift position did not change, which was also observed during the titration of EF1 with ADP.[19] However, two broad resonances appeared at the chemical shift positions expected for the β-phosphate of GDP. As the binding of GDP to EF1 was specific to its catalytic site, the presence of two resonances in the region of GDP suggested the presence of two distinct environments at the catalytic sites, which enabled distinct interactions with the β-phosphate group of GDP. The presence of two resonances could be evidence for the asymmetry between catalytic sites of EF1.

Addition of two equivalent concentrations of GTP (in total of 3 X concentrations) enhanced the resonances of the bound GDP. After further addition of GTP (in total of more than 3 X concentrations), sharper characteristic resonances of free GDP appeared (data not shown). As guanosine nucleotide was known to bind only to the catalytic site of EF1, maximal binding of three equivalents of GDP was expected.

Nucleotide-depleted EF1 was also titrated with GDP (Figure 3). At one equivalent addition of GDP in the absence of Mg^{2+}, a β-phosphate resonance of free GDP was observed. After addition of Mg^{2+}, the sharp resonance from free GDP disappeared. After addition of three equivalents of GDP, resonances from β-phosphate of free GDP were observed. This observation would be in contrast with the GTP titration of nucleotide-depleted EF1, which indicated that the enzyme could bind three equivalents of guanosine nucleotides (Figure 2). Subsequent addition of three equivalents of phosphate, which was believed to be the difference between GDP and GTP titrations, caused the disappearance of most of the resonances from β-phosphate of free GDP. Unlike the binding of ADP to nucleotide-depleted EF1, which could bind four equivalents of ADP[19] in the absence of Mg^{2+} and almost six equivalents of ADP[19] in the presence of Mg^{2+}, the binding of GDP to a nucleotide-depleted EF1 (presumable catalytic site) needed the presence of Pi for its complete binding, suggesting that Pi caused higher affinity nucleotide binding to catalytic sites.

^{31}P resonance of GMP could be monitored for the GTP and GDP titrations, measuring guanylate kinase-like activity of EF1. For GDP titration without addition of Pi, the both resonance of free GMP and Pi could be observed suggesting guanylate kinase-like activity. Adenylate kinase-like activity and phosphotransferase-like activity of EF1 was reported previously.[17-19] In GTP and GDP titration ^{31}P resonance of methyl phosphate, in which phosphoryl group of GDP was enzymatically transferred to methanol (added as stabilizer) also appeared, but was relatively weak compared to the ADP titration of nucleotide-depleted EF1.[19] As guanylate nucleotide was binding to its specific catalytic site,[9,10] guanylate and adenylate kinase-like activity and phosphotransferase-like activity of EF1 could be the distinct property at the catalytic site. ^{31}P NMR spectroscopy proved that it could be a valuable tool for investigating the catalytic activities of phosphorous related enzyme in real time.

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