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Observation of Asymmetry amongst Nucleotide Binding Sites of F_1-ATPase of *Escherichia coli* by ^{31}P NMR Spectroscopy

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It was regarded that the ^{31}P resonances of inherent nucleotides in F_1-ATPase (EF1), as large as 380KDa, could not be observed by ^{31}P NMR spectroscopy. However, our ^{31}P NMR spectroscopy could differentiate between different nucleotide binding sites on EF1 from *Escherichia coli*. When EF1 was prepared in the absence of Mg^{2+}, EF1 contained only ADP. Multiple ^{31}P resonances from β-phosphates of ADP bound to the EF1 were observed from the enzyme prepared without Mg^{2+}, suggesting asymmetry or flexibility amongst nucleotide binding sites. ^{31}P resonances from enzyme bound ATP could be observed only from EF1, when the enzyme was prepared in the presence of Mg^{2+}. This Mg^{2+} dependent ATP binding was very tight that, once bound, nucleotide could not be removed even after removal of Mg^{2+}. ^{31}P NMR proved to be a valuable tool for investigating phosphorous related enzymes.

Key Words: ^{31}P NMR spectroscopy, F_1-ATPase of *Escherichia coli*, Asymmetry, Nucleotide binding site

Introduction

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

A considerable number of investigations on F_1-ATPases from many sources, bacteria, thylakoids and mitochondria, established least two and plausibly three catalytic sites with non-catalytic sites for binding interactions.[7] In general, it was considered for this type of enzyme to have six sites with half for the catalytic and the other half for some other non-catalytic functions, as confirmed by the structural study of F_1.[8] The non-catalytic sites revealed the slow ligand-exchange characteristics ($t_{1/2}$ ~ hours) for the bound and unbound ligands the medium with considerable specificity towards the adenine nucleotides.[2,5,6] In contrast, the catalytic sites were not adenine-specific with rapidly exchanges ($t_{1/2}$ ~ minutes) between bound and unbound ligands in the medium.[5,9,10]

The intrinsic asymmetric structure of the enzyme, together with a variety of experimental investigations, indicated that the properties of the putative catalytic sites may be distinct from one another by any perturbation or interaction in a catalytic cycle. Direct evidence for such distinct properties, however, was lacking. In principle ^{31}P NMR should be able to discriminate the specific binding site(s) from numerous potential sites. Previously, ^{31}P NMR studies characterized the nucleotide binding sites of the chloroplast F_1-ATPase (CF1).[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 the modification with NEM and NBD-Cl.[11] The failure to detect ^{31}P NMR signals in native CF1 was ascribed to the chemical shift anisotropy, which could result a very broad line width.

In the case of *E.coli*, it was established that F_1-ATPase (EF1) conformed to the general pattern of nucleotide binding, where 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 fluorotryptophan labeling[15,16] characterized the nucleotide binding sites in the physiological condition. However, EF1 was not previously characterized with ^{31}P NMR.

In this research, EF1 was investigated with ^{31}P NMR spectroscopy. In contrast to CF1, bound nucleotides to EF1 were clearly visible in ^{31}P resonances. EF1 in its native form seemed to bind tightly to the nucleotides: its nucleotide-depleted form may bind up to 6 mol of nucleotide per mol of enzyme. ^{31}P resonances of tightly bound nucleotide to EF1 could provide valuable information on both nucleotide binding sites.

Materials and Methods

Growth of Cells. EF1 overproducing *E. coli* strain, SWM1, was obtained from Dr. A. Senior (University of Rochester). For preparing EF1, cells were grown in large batch culture using M9 media with 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 the sterilization, 1 mL of sterile 1 M $MgSO_4$ was added per liter with other growth supplements as following; 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, as filter-sterilized solutions just before the inoculation. Overnight cultures of 1.5 liter in L-broth were used to inoculate into 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 additions of 2.5 M NaOH solution. Cell growth was monitored with absorbance at 750 nm. When the mid-exponential growth phase was reached, cells were harvested using an Amicon concentrator.

Preparation of EF1. F_1-ATPase was prepared as described previously.[12-16] 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.[17] Protein concentration was determined by the Bradford microassay procedure[18] using heat denatured F_1-ATPase, as a protein standard. All the chemicals used were reagent grade from commercial sources.

Removal of Unbound Nucleotides from EF1. Unbound nucleotides were removed 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 samples were passed through a desalting column. Pooled fractions were concentrated by an Amicon filtration system using a PM-30 membrane.

Quantitation of EF1 Bound Nucleotides. EF1 (19 mg) was denatured by adding an equal volume of 20% TCA (w/v) solution. After mixing vigorously, denatured protein was pelleted by centrifugation (20000 rpm) for 5 minutes. The pH of supernatant was adjusted to 7.4 with 4M NaOH. After diluting to 2 mL with Tris/HCl (50 mM, pH 7.4) buffer, 10 μL of 1 M $MgCl_2$ were added to make final concentration of 5 mM $MgCl_2$.

Preparation of Nucleotide-depleted EF1. Rather than using the procedure from Garrett and Penefsky,[19] which involved a desalting column with very low running rate (1 mL/h) to prepare nucleotide-depleted EF1, a new method using an ultrafiltration membrane was introduced. EF1 samples were precipitated with saturated ammonium sulfate (67%), collected by centrifugation, and dissolved in 50 mM Tris/HCl, 2 mM EDTA, 50% glycerol (V/V), pH 8.0. Such samples were concentrated with an Amicon cell, fitted with a PM-30 membrane, using the same buffer until reaching A280/A260 ratio of 1.8 or higher. The similar concentrations of EF1 were obtained from both methods, Amicon cell concentrator with A280/A260 ratio, and the peak fractions 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 ^{1}H). All spectra were taken with 2.5 mL samples in a 10 mm diameter tube. A capillary insert containing methylendiphonic acid (resonance at 18ppm) 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 50° pulse and inter-pulse delay of 2 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

^{31}P NMR Spectra of Native EF1 Prepared in the Absence of Mg^{2+}. Purified EF1 stored with ATP (1 mM) was freed of loosely bound ATP by two passages through a desalting column equilibrated using buffer without Mg^{2+} (Tris/HCl pH 7.5, 2 mM EDTA and 10% methanol added as a stabilizer). ^{31}P NMR spectroscopy could show the resonances from bound phosphate in the presence of EF1 (Figure 1A). The ^{31}P resonances of phosphate groups could be assigned by comparison with ^{31}P spectrum of a mixture of ATP, ADP, AMP, Pi (Figure 1B&1C). ^{31}P NMR spectroscopy. After 20% (W/V) trichloroacetic acid extraction, the spectrum showed only 2 mol of ADP and very small amount of Pi and AMP per mol of enzyme, but without ATP (Figure 2).

The sharp resonances at 3 ppm and the broad resonance at −10 ppm were assigned to free Pi and α-phosphate of enzyme bound-ADP, respectively, according to the same chemical shift

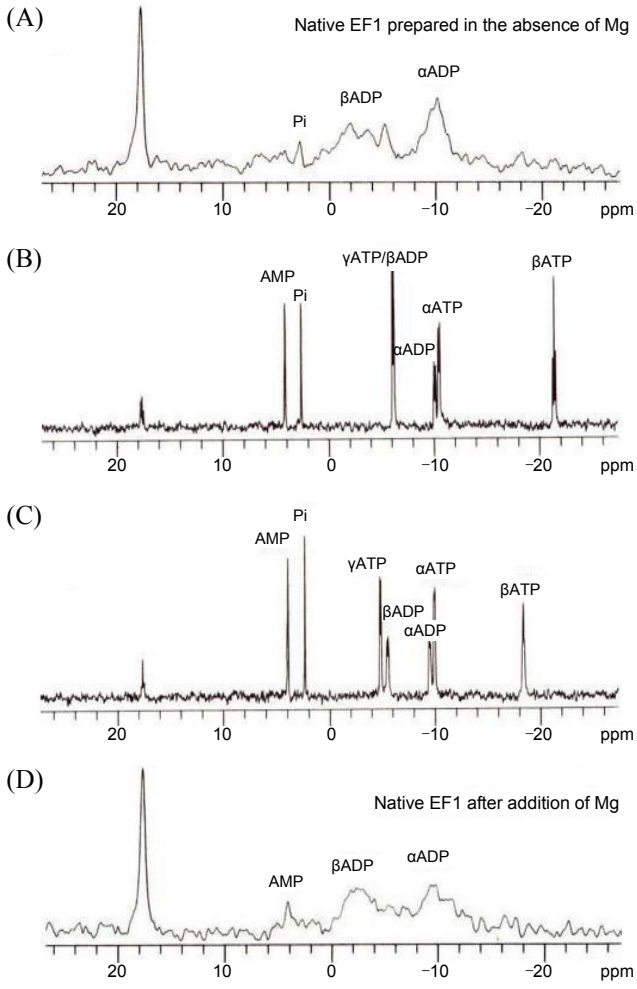

Figure 1. ^{31}P Spectra of native EF1 prepared in the absence of Mg^{2+} 31P spectra were acquired with a simple pulse-and collect sequence using 50° excitation pulse (16 μs) and interpulse delay of 2 seconds. 20 K scans were accumulated for protein, and 1000 scans were accumulated for the standard mixture. (A) Native EF1 (60 mg/mL) prepared in the absence of Mg^{2+}, (B) Mixture of ATP, ADP, AMP, and Pi (1 mM each) in the absence of Mg^{2+}, (C) Mixture of ATP, ADP, AMP, and Pi (1 mM each) in the presence of 5 mM of $MgCl_2$, (D) Native EF1 after addition of 5 mM of $MgCl_2$.

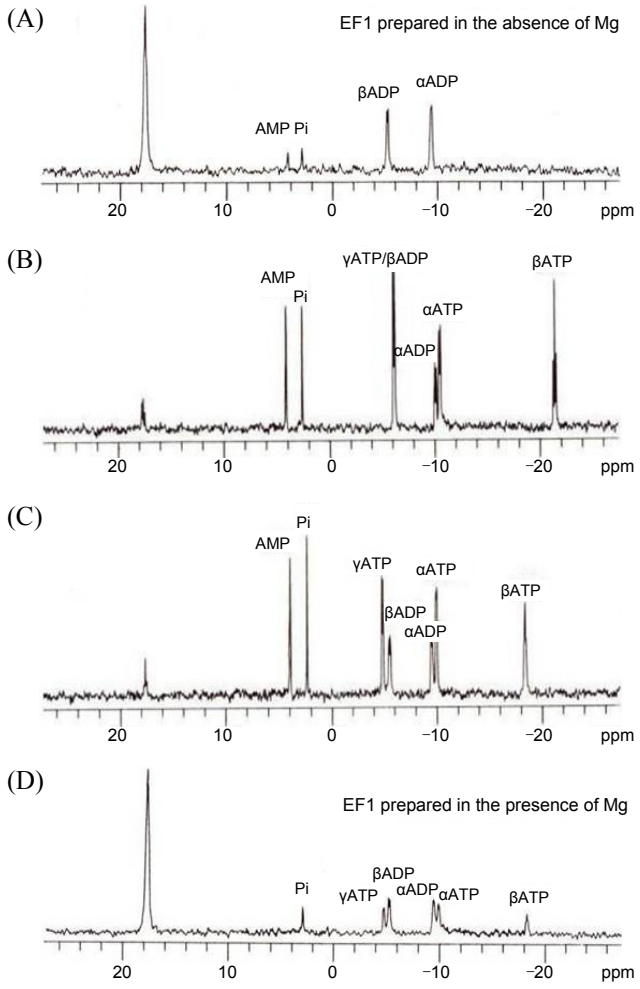

Figure 2. ^{31}P spectra of denatured EF1 Samples of EF1 (19 mg) were denatured by TCA treatment (20%, W/V). ^{31}P spectra were acquired with a simple pulse-and collect sequence using 50^{o} excitation pulse (26 μs) and interpulse delay of 1 second. 30 K scans were accumulated for protein. (A) Denatured EF1 prepared in the absence of Mg^{2+}, (B) Denatured EF1 prepared in the presence of 5 mM of $MgCl_2$.

values as free Pi and α-phosphate of ADP (and/or ATP).

The resonances from −2 to −5 ppm needed more consideration, because the unambiguous assignment of all the resonances from high noise level of the spectrum would be very difficult. However, since only ADP was present in this preparation (Figure 2), this region (−2 to −5 ppm) near the free β-phosphate of ADP, could be reasonably assigned to the β-phosphate of enzyme bound ADP.

The presence of multiple β-phosphate resonances of ADP suggested that the tightly enzyme-bound ADP molecules were distributed amongst different binding sites, which could be differentiated by ^{31}P NMR spectroscopy. All these resonances were downfield-shifted in comparison to the free β-phosphate of ADP. In particular, the resonance at −2 ppm was downfield shifted by about 4 ppm from free β-phosphate of ATP. Generally, the changed values of less than 2 ppm were observed.[20,21]

The later addition of $MgCl_2$ (5 mM) to the samples of EF1 caused the appearance of a broad single resonance (Figure 1D). Interestingly, the resonances of P_i at 3 ppm also disappeared

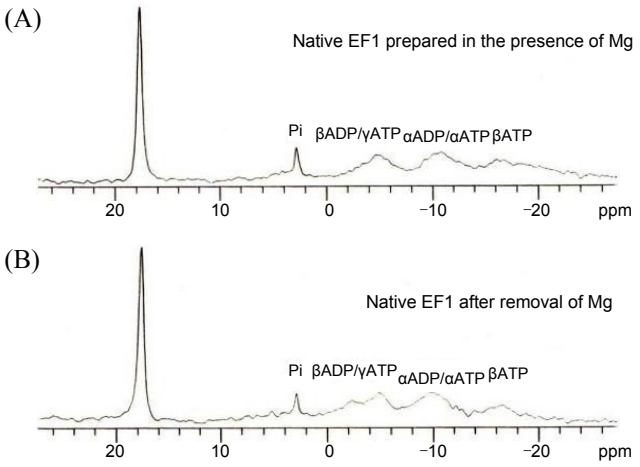

Figure 3. ^{31}P Spectra of native EF1 prepared in the presence of Mg^{2+} ^{31}P spectra were acquired with a simple pulse-and collect sequence using 50^{o} excitation pulse (16 μs) and interpulse delay of 2 seconds. 20 K scans were accumulated for protein and 1000 scans were accumulated for standard mixture. (A) Native EF1 (60 mg/mL) prepared in the presence of 5 mM $MgCl_2$, (B) Native EF1 after removal of $MgCl_2$ through desalting column.

and the new resonance at 4.2 ppm appeared. By comparison with the ^{31}P spectra of standard mixture (Figure 1B&1C), the resonance at 4.2 ppm was assigned as the resonance to AMP. The results may suggest that addition of $MgCl_2$ facilitated inherent adenylate kinase-like activity of EF1, which was only reported from CF1.[11,22]

^{31}P Spectra of Native EF1 Prepared in the Presence of Mg^{2+}. When EF1 without loosely bound ATP was prepared using desalting column equilibrated with buffer with Mg^{2+} (Tris/HCl pH 7.5, 2 mM EDTA, 5 mM $MgCl_2$ and 10% methanol), ^{31}P spectroscopy showed quite different resonances (Figure 3) from the native enzyme, which was prepared in the absence of Mg^{2+} (Figure 1A). Three broad ^{31}P resonances at around −5 ppm, −10 ppm and about −16 ppm appeared. By comparison with a ^{31}P spectrum of standard mixture of ATP, ADP, AMP, Pi in the presence of Mg^{2+} (Figure1C), the broad resonances at about −10 ppm could be assigned as a resonance of the α-phosphate of ADP (and/or ATP). The broad resonance near −5 ppm could be the peak of β-phosphate of ADP or γ-phosphate of ATP. ^{31}P spectrum of an extract of EF1 that had been denatured showed that this preparation contained approximately 0.8 mol of ATP per mol of enzyme as well as 1 mol of ADP (Figure 2B). Since the broad resonance neat −16 ppm appeared only in the presence of ATP in enzyme, it was therefore assigned as a resonance from the enzyme-bound β-phosphate, although it was downfield shifted by about 1.5 ppm. This was similar to the downfield shift change (1.5 ppm) of the β-phosphate resonance of MgATP upon binding to adenylate kinase.[23] Previously, the downfield shift of ATP in enzyme bound form was unique for with adenylate kinase among all examined kinases.[20]

When $MgCl_2$ was removed from EF1 by two passage through a desalting column equilibrated with buffer without Mg^{2+} (Tris/HCl pH 7.5, 2 mM EDTA and 10% methanol), the ^{31}P spectrum of EF1 still showed a resonance in the region of β-phosphate

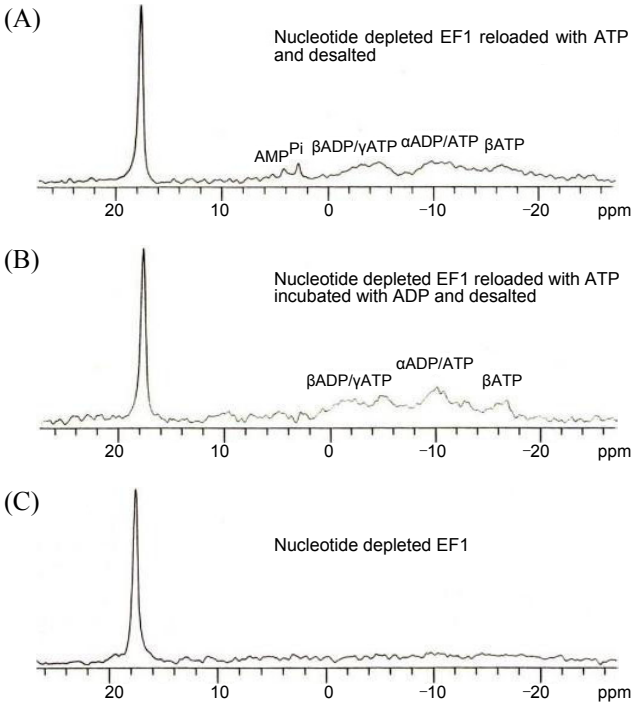

Figure 4. ^{31}P Spectra of nucleotide-depleted EF1 reloaded with ATP ^{31}P spectra of EF1 (60 mg/mL) were acquired with a simple pulse-and collect sequence using 50^{o} excitation pulse (16 μs) and interpulse delay of 2 seconds. 20 K scans were accumulated for each spectrum. (A) Nucleotide-depleted EF1 reloaded with 1 mM of ATP and desalted in the presence of 5 mM $MgCl_2$, (B) Sample in A was incubated with 10 mM ADP in the presence of 5 mM $MgCl_2$. ADP and $MgCl_2$ were removed afterwards through a desalting column. (C) Nucleotide-depleted EF1 without the addition of nucleotides.

of ATP. In addition, single broad resonance near −5 ppm was splitted into two resonances (Figure 3B). This could be due to the differentiation of γ-phosphate of ATP and β-phosphate of ADP.

^{31}P Spectrum of Nucleotide-depleted EF1 after Reloading ATP in the Presence of Mg^{2+}. Nucleotide-depleted EF1 was prepared and reloaded with ATP in the presence of $MgCl_2$: loosely bound ATP was removed by two passages through desalting column. When the native EF1 prepared in the presence of Mg^{2+} was prepared, the ^{31}P spectrum revealed three broad resonances (Figure 4A). A broad resonance near −16 ppm, characteristic resonance of β-phosphate of tightly bound ATP, was clearly visible. To determine whether this ATP was exchangeable with other nucleotides in the medium, EF1 was incubated with excess ADP in the presence of $MgCl_2$. A ^{31}P spectrum (Figuare 4B) after removal of ADP and Mg^{2+} by two passages through desalting column still showed the bound ATP, implying that this ATP at the binding site was not exchangeable with other nucleotides in the medium. Appearance of two resonances after removal of Mg^{2+} was also observed.

Discussion

Although the endogenous nucleotide content for EF1 was variable depending on strain and different preparation method, generally, binding of 2 - 3 mol of nucleotides per mol of EF1 was reported.[24-26] In this study, ^{31}P spectroscopy showed that the presence of Mg^{2+} might be the main reason for the difference in nucleotide contents. In this preparation, 2 mol of ADP per mol of EF1 were observed with EF1 prepared in the absence of Mg^{2+}, while 1 mol of ADP and 0.8 mol of ATP per mol of EF1 were observed with EF1 prepared in the presence of Mg^{2+}. The presence of tightly bound ATP was repeatedly observed from EF1 in the presence of Mg^{2+}, although the proportion of ADP and ATP in EF1 was somewhat variable depending on the preparation (data not shown).

Tight binding of ATP could be observed only from the native form of EF1, which was prepared in the presence of Mg^{2+}, and also from reloaded nucleotide-depleted EF1 with ATP in the presence of Mg^{2+} (Figure 3A & Figur 4A). In the presence of Mg^{2+}, this bound ATP was not exchangeable with other nucleotides in medium, suggesting that Mg^{2+} dependent ATP binding site would be non-catalytic.

The multiple ^{31}P resonances of the EF1 prepared in the absence of Mg^{2+} suggest asymmetry or flexibility amongst nucleotide binding sites. The EF1 in this preparation could bind fluoroaluminate and fluoroberyllate, which were isomorphous to Pi and could bind to catalytic site only in the presence of preexisting ADP.[13,14] Thus, the distribution of tightly bound nucleotides in EF1 seemed to include one nucleotide at a catalytic site, supporting the previous findings with the bovine mitochondrial F_1-ATPase.[30]

Regardless of different sample preparations, multiple ^{31}P resonances could be observed in the absence of Mg^{2+}. However, the presence of Mg^{2+} revealed only single broad resonance. This could be due to the chemical exchange between multiple and conversions of the specific nucleotide binding sites in the presence of Mg^{2+}, suggesting actual physiological condition at the catalytic site.

In conclusion, we have shown that ^{31}P NMR could discriminate between different nucleotide 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, where 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 might suggest the asymmetry amongst nucleotide binding sites in physiological conditions. ^{31}P NMR proved to be a valuable tool for the investigation of phosphorous related enzyme.

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