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^{31}P NMR Spectroscopy Revealed Adenylate kinase-like Activity and Phosphotransferase-like Activity from F_1-ATPase of *Escherichia coli*

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Adenylate kinase-like activity and phosphotransferase-like activity from F_1-ATPase of *Escherichia coli* was revealed by ^{31}P NMR spectroscopy. Incubation of F_1-ATPase with ADP in the presence of Mg^{2+} shows the appearance of ^{31}P resonances from AMP and *Pi*, suggesting generation of AMP and ATP by adenylate kinase-like activity and the subsequent hydrolysis to *Pi*. Incubation of F_1-ATPase with ADP in the presence of methanol shows additional peak from methyl phosphate, suggesting phosphotransferase-like activity of F_1-ATPase. Both adenylate kinase-like activity and phosphotransferase-like activity has not been reported from F_1-ATPase of *Escherichia coli*. ^{31}P NMR could be a valuable tool for the investigation of phosphorous related enzyme.

Key Words: ^{31}P NMR, F_1-ATPase, *Escherichia coli*, Adenylate kinase-like activity, Phosphotransferase-like activity

Introduction

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 to that found 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, and (ii) F_1 sector which contains five polypeptide chains with 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, have established that there are at least two and plausibly three catalytic sites, and a further set of non-catalytic sites.[7] In general, it is considered that this type of enzyme has six sites of which half are catalytic and the other half are non-catalytic. This is confirmed by the recent x-ray structural study of F_1.[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 inherent adenylate kinase-like activity of F_1-ATPase was so far only reported from F_1-ATPase of chloroplast.[11,12] The significance of the finding that F_1-ATPase can transfer the terminal phosphoryl group from one bound nucleoside diphosphate to another lies in the implication of similarities in nucleotide binding and possible mechanism between F_1-ATPase and adenylate kinase. In the present investigation ^{31}P NMP spectroscopy was used to find the adenylate kinase-like activity and the phosphotransferase-like activity of F_1-ATPase from *E. coli*.

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 and 0.2 mM uracil. Glyphosate (1 g/L), chloramphenicol (60 mg/mL), 10 μM p-benzoic acid, tyrosine (50 mg/L), phenylalanine (50 mg/L) and 5-fluorotryptophan (36 mg/L) 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. 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.[13,14] 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.[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.

NMR Spectrometers and Operating Conditions. ADP (10 mM) was incubated with 1mg of F_1-ATPase in the absence or in the presence of methanol at 25 ^oC. ^{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 50^o pulse and interpulse delay of 2 seconds 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

Figure 1 shows ^{31}P resonances from the incubation of ADP with F_1-ATPase from *E. coli*. The incubation of ADP with F_1-ATPase in the presence of $MgCl_2$ caused two resonances to appear at around 3 to 4 ppm. Comparison with the ^{31}P NMR spectrum of AMP and *Pi* shows that these ^{31}P resonances are from AMP and *Pi*, respectively. The intensity of both resonances increased very slowly during NMR merasurement. A very tiny resonance also appeared at around 18 ppm. Comparison with the ^{31}P NMR spectrum of ATP could confirm that this resonance is from β-phosphate of ATP. As there were initially only ADP molecules in the medium, these results suggest that the resonances of AMP and ATP were produced by adenylate kinase-like activity of F_1-ATPase. *Pi* must be produced by the re-hydrolysis of ATP produced. The incubation of ADP with F_1-ATPase in the absence of $MgCl_2$ showed very tiny resonances of AMP and *Pi*, indicating adenlyate kinase-like activity of F_1-ATPase requires Mg^{2+}. 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 experiment was repeated with the addition of 10% methanol, normally added as a stabilizing agent of F_1-ATPase (Figure 2). Incubation of ADP with F_1-ATPase from *E. coli* in the presence methanol caused a third resonance at around 5 ppm to appear as well as the resonances of AMP and *Pi*. The resonance at around 5ppm must be originated from a transfer of phosphoryl group of ADP to a possible acceptor molecule in the medium, which is added methanol. Comparison with the ^{31}P NMR spectrum of alkyl phosphate could confirm the presence

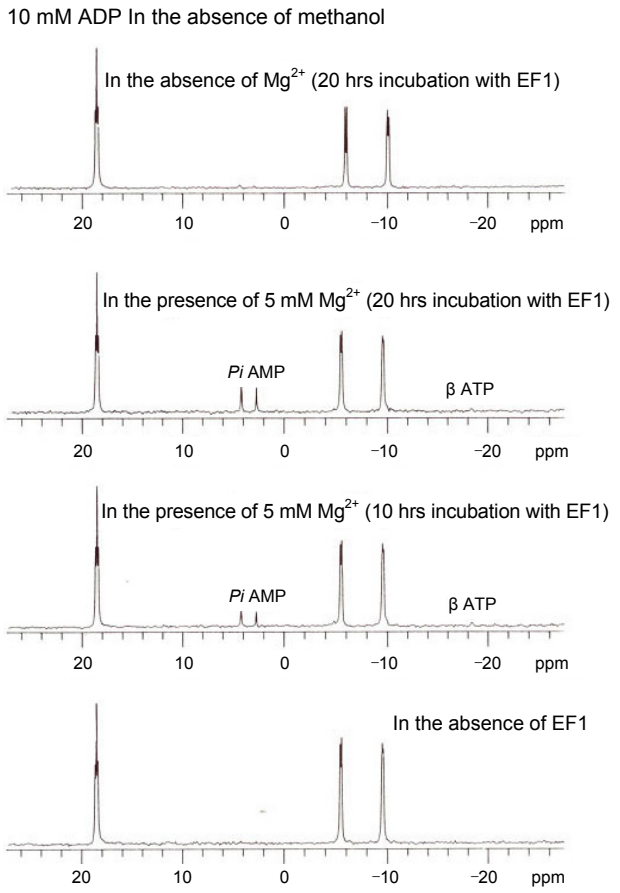

Figure 1. ADP (10 mM) was incubated with 1 mg of F_1-ATPase in the absence of methanol. ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 50^o excitation pulse and an interpulse delay of 2 sec. 500 scans were accumulated for each spectrum.

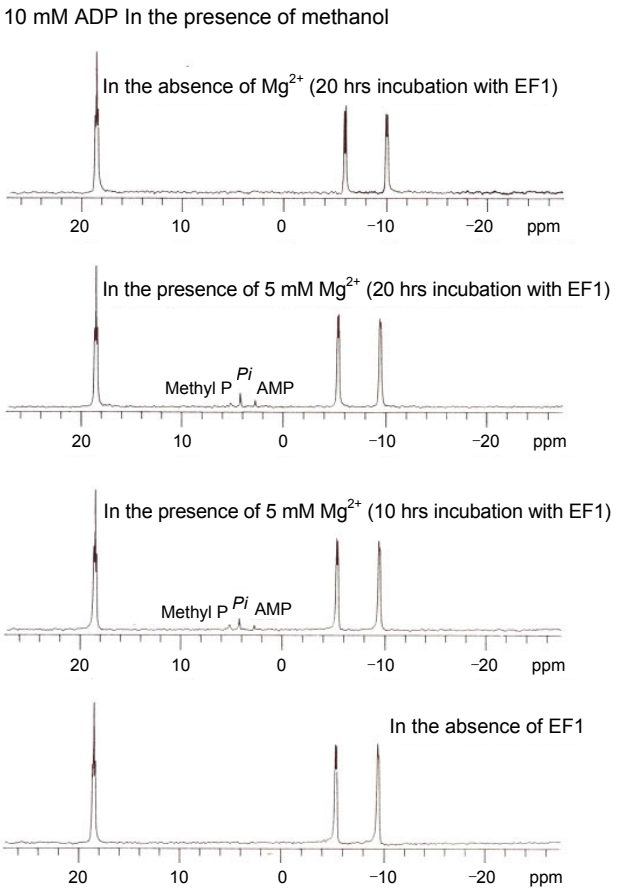

Figure 2. ADP (10 mM) was incubated with 1 mg of F_1-ATPase in the presence of 10% methanol (v/v). ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 50^o excitation pulse and an interpulse delay of 2 sec. 500 scans were accumulated for each spectrum.

of methyl phosphate.[19] ^{31}P resonance of methyl phosphate indicates that the phosphoryl group of ADP was enzymatically transferred to methanol. Both Adeynlate kinase-like activity and phosphotransferase-like activity of F_1-ATPase could be also observed when titration of nucleotide-depleted F_1-ATPase with ADP was monitored with ^{31}P NMR spectroscopy (unpublished data).

Discussion

The peak height of the ^{31}P resonances of AMP and *Pi* was almost same when F_1-ATPase was incubated with ADP in the absence of methanol (Figure 1). However, the relative peak height of *Pi* was higher than that of AMP and methyl phosphate (methyl phosphate:*Pi*:AMP = 1:2:1) when F_1-ATPase was incubated with ADP in the presence of methanol (Figure 2). This may suggest each AMP and methyl phosphate was produced equally with production of ATP which is re-hydrolyzed to phosphate by F_1-ATPase activity. The slow transphorylation activity to ethanol and glycerol had been previously observed from acid phosphatases of a *Citrobacter* sp,[19] but never been reported in F_1-ATPases.

Observance of the very tiny resonance from β-phosphate of ATP even in the presence of F_1-ATPase suggests that ATP hydrolyzing activity was very slow in the presence of ADP and Mg^{2+}. Although F_1-ATPase from various sources showed strong inhibition following binding of ADP in the presence of Mg^{2+},[20,21] it has not been so far reported in F_1-ATPase from *E. coli*. The inhibition of F_1-ATPase from *E. coli* by incubation with ADP in the presence of Mg^{2+} may be very weak and reversed quickly during hydrolysis in the presence of excess ATP in the in the steady state coupled assay with pyruvate kinase and lactate dehydrogenase.[17]

The significance of the finding that F_1-ATPase can transfer the terminal phosphoryl group from one bound nucleoside diphospahte to another lies in the implication of similarities in nucleotide binding and possible mechanism between F_1-ATPase and adenylate kinase. Di(adenosine-5')pentaphosphate which is an inhibitor of adenylate kinase also inhibit mitochondrial F_1-ATPase.[22] This supports a model for the structure of nucleotide binding sites on F_1-ATPase which places catalytic site and non-catalytic site in close proximity in an orientation analogous to ATP and AMP binding sites on adenylate kinase. As the normal catalytic reaction pathway does not include a phosphotransferase-like reaction,[23] the slow transphosphorylation reaction between sites observed in the present investigation may play a regulatory role.

The observed adenlyate kinase-like activity and phosphotransferase-like activity of F_1-ATPase from *E. coli* was so slow that it could not be detected with ordinary enzyme assay system. ^{31}P NMR spectroscopy proved that it could be a valuable tool for the investigation of phosphorous related enzyme.

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