

³¹P NMR Spectroscopy Revealed Adenylate kinase-like Activity and Phosphotransferase-like Activity from F₁-ATPase of *Escherichia coli*

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Adenylate kinase-like activity and phosphotransferase-like activity from F₁-ATPase of *Escherichia coli* was revealed by ³¹P NMR spectroscopy. Incubation of F₁-ATPase with ADP in the presence of Mg²⁺ shows the appearance of ³¹P resonances from AMP and Pi, suggesting generation of AMP and ATP by adenylate kinase-like activity and the subsequent hydrolysis to Pi. Incubation of F₁-ATPase with ADP in the presence of methanol shows additional peak from methyl phosphate, suggesting phosphotransferase-like activity of F₁-ATPase. Both adenylate kinase-like activity and phosphotransferase-like activity has not been reported from F₁-ATPase of *Escherichia coli*. ³¹P NMR could be a valuable tool for the investigation of phosphorous related enzyme.

Key Words: ³¹P NMR, F₁-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 F₀ 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) F₁ sector which contains five polypeptide chains with relative stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$.

A considerable number of investigations on F₁-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 F₁.⁸ 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 F₁-ATPase was so far only reported from F₁-ATPase of chloroplast.^{11,12} The significance of the finding that F₁-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 F₁-ATPase and adenylate kinase. In the present investigation ³¹P NMR spectro-

scopy was used to find the adenylate kinase-like activity and the phosphotransferase-like activity of F₁-ATPase from *E. coli*.

Materials and Methods

Growth of Cells. *E. coli* strain SWM1, which is an over-producer of F₁-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 ZnSO₄, 1 mM MnSO₄, 4.7 mM CuSO₄, 2.5 mM CaCl₂ and 1.8 mM FeCl₃) per liter. After sterilization, 1 mL of sterile 1 M MgSO₄ was added per liter with other growth supplements as follows; 30 mM glucose, 0.2 μ M thiamine hydrochloride, 0.8 mM L-arginine hydrochloride and 0.2 mM uracil. Glyphosate (1 g/L), chloramphenicol (60 mg/mL), 10 μ M 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 for 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. F₁-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 procedure¹⁸ using heat denatured F₁-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 1 mg of F₁-ATPase in the absence or in the presence of methanol at 25 °C. ³¹P 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 methylenediphonic acid (resonance at 18 ppm) dissolved in D₂O 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 interpulse delay of 2 seconds 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 ³¹P chemical shifts.

Results

Figure 1 shows ³¹P resonances from the incubation of ADP with F₁-ATPase from *E. coli*. The incubation of ADP with F₁-ATPase in the presence of MgCl₂ caused two resonances to appear at around 3 to 4 ppm. Comparison with the ³¹P NMR spectrum of AMP and Pi shows that these ³¹P resonances are from AMP and Pi, respectively. The intensity of both resonances

increased very slowly during NMR measurement. A very tiny resonance also appeared at around 18 ppm. Comparison with the ³¹P 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 F₁-ATPase. Pi must be produced by the re-hydrolysis of ATP produced. The incubation of ADP with F₁-ATPase in the absence of MgCl₂ showed very tiny resonances of AMP and Pi, indicating adenylate kinase-like activity of F₁-ATPase requires Mg²⁺. As molecular weight of F₁-ATPase (380 K) is very large compared to that of adenylate kinase (32 K), adenylate kinase-like activity of F₁-ATPase cannot be from contaminated adenylate kinase after gel filtration in the preparation.¹³⁻¹⁶

The experiment was repeated with the addition of 10% methanol, normally added as a stabilizing agent of F₁-ATPase (Figure 2). Incubation of ADP with F₁-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 5 ppm 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 ³¹P NMR spectrum of alkyl phosphate could confirm the presence

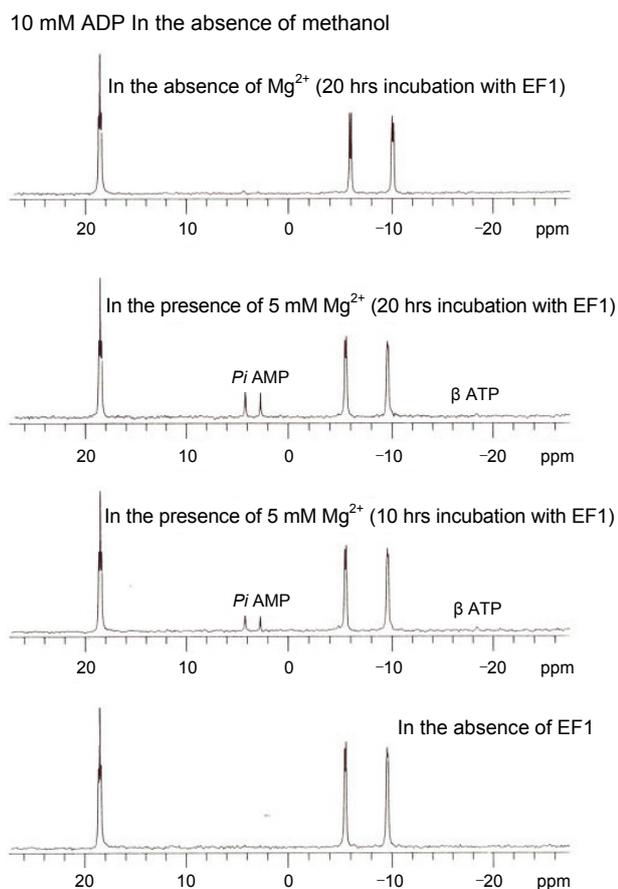


Figure 1. ADP (10 mM) was incubated with 1 mg of F₁-ATPase in the absence of methanol. ³¹P spectra were acquired with a simple pulse-and-collect sequence using 50° excitation pulse and an interpulse delay of 2 sec. 500 scans were accumulated for each spectrum.

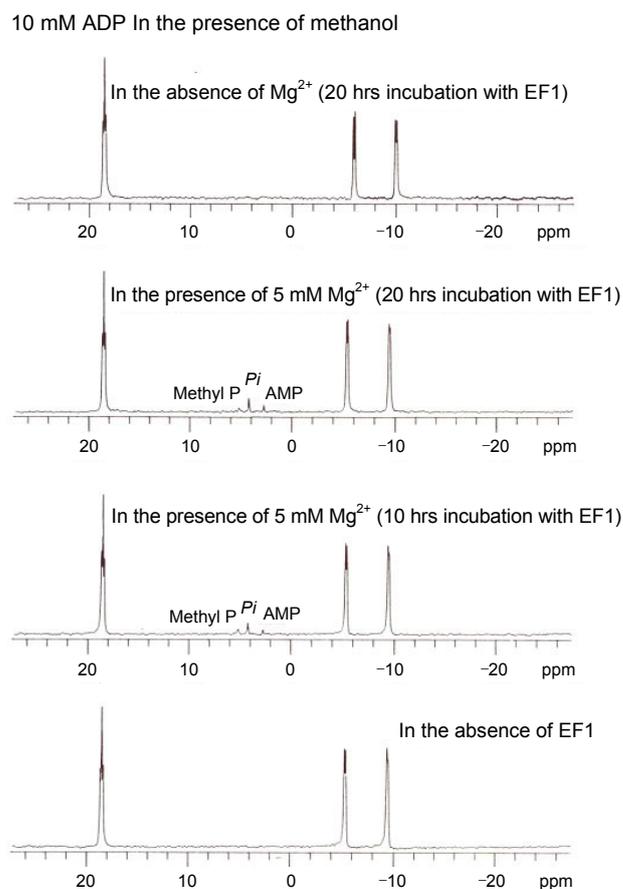


Figure 2. ADP (10 mM) was incubated with 1 mg of F₁-ATPase in the presence of 10% methanol (v/v). ³¹P spectra were acquired with a simple pulse-and-collect sequence using 50° excitation pulse and an interpulse delay of 2 sec. 500 scans were accumulated for each spectrum.

of methyl phosphate.¹⁹ ³¹P resonance of methyl phosphate indicates that the phosphoryl group of ADP was enzymatically transferred to methanol. Both Adenylate kinase-like activity and phosphotransferase-like activity of F₁-ATPase could be also observed when titration of nucleotide-depleted F₁-ATPase with ADP was monitored with ³¹P NMR spectroscopy (unpublished data).

Discussion

The peak height of the ³¹P resonances of AMP and Pi was almost same when F₁-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 F₁-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 F₁-ATPase activity. The slow transphosphorylation activity to ethanol and glycerol had been previously observed from acid phosphatases of a *Citrobacter* sp,¹⁹ but never been reported in F₁-ATPases.

Observance of the very tiny resonance from β-phosphate of ATP even in the presence of F₁-ATPase suggests that ATP hydrolyzing activity was very slow in the presence of ADP and Mg²⁺. Although F₁-ATPase from various sources showed strong inhibition following binding of ADP in the presence of Mg²⁺,^{20,21} it has not been so far reported in F₁-ATPase from *E. coli*. The inhibition of F₁-ATPase from *E. coli* by incubation with ADP in the presence of Mg²⁺ may be very weak and reversed quickly during hydrolysis in the presence of excess ATP in the steady state coupled assay with pyruvate kinase and lactate dehydrogenase.¹⁷

The significance of the finding that F₁-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 F₁-ATPase and adenylate kinase. Di(adenosine-5')pentaphosphate which is an inhibitor of adenylate kinase also inhibit mitochondrial F₁-ATPase.²² This supports a model for the structure of nucleotide binding sites on F₁-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 nor-

mal 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 adenylate kinase-like activity and phosphotransferase-like activity of F₁-ATPase from *E. coli* was so slow that it could not be detected with ordinary enzyme assay system. ³¹P NMR spectroscopy proved that it could be a valuable tool for the investigation of phosphorous related enzyme.

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