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Phosphoryl Transferring Activity was Revealed from F_1-ATPase of *Escherichia coli* by ^{31}P NMR Investigation

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^{31}PNMR spectroscopy revealed the adenylate kinase-like activity and the phosphotransferase activity from F_1-ATPase of *Escherichia coli*. 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 the 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 activity of F_1-ATPase. Both adenylate kinase-like activity and the phosphotransferase activity has not been reported from F_1-ATPase from *Escherichia coli*. ^{31}P NMR proved that it could be a valuable tool for the investigation of phosphorous related enzyme.

Key Words: ^{31}P NMR spectroscopy, F_1-ATPase of *Escherichia coli*, Adenylate kinase activity, Methyl phosphate, Phosphoryl transferring 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 (Senior and Wise, 1983; Senior, 1988; Futai et al., 1989, Penefsky and Cross, 1991; Senior, 1992; Boyer, 1993). 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 $\alpha_3\beta_3\gamma\delta\varepsilon$.

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 (Boyer, 1993). In general, it is considered that this type of enzyme has six sites of which half are catalytic and half have some other non-catalytic function. This is confirmed by the recent structural study of F_1 (Abrahams et al., 1994). 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 (Cross and Nalin, 1982; Senior, 1988; 1992). In contrast, the catalytic sites are not adenine-specific and rapidly exchange bound ligand with ligand in the medium ($t_{1/2}$ ~ minutes) (Cross and Nalin, 1982; Perlin et al., 1984; Wise et al., 1983).

The inherent adenylate kinase activity of F_1-ATPase was so far only reported from F_1-ATPase of chloroplast (Mouudrianakis and Tiffert, 1976, Carr et al., 1990). 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

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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 activity of F_1-ATPase from *E. coli*.

MATERIALS AND METHODS

1. Growth of cells

E. coli strain SWM1, which is an overproducer of F_1-ATPases, was obtained from Dr. A. Senior (University of Rochester). Glyphosate, [N-(phosphonomethyl)glycine], was a gift from Dongbuhannong Chemical. 5-fluorotryptophan was purchased from Sigma. All other chemicals were reagent grade from commercial grade. 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 (approx. 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℃ 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.

2. Preparation of enzyme

F_1-ATPase was prepared as described previously (Jung and Kim, 1998; Lee et al., 2000). Enzyme was stored at -20℃ 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 Ting and Wang (1980). Protein was determined by the Bradford microassay procedure (Bradford, 1976) using heat denatured F_1-ATPase as a protein standard. All the chemicals used were reagent grade from commercial sources.

3. 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℃. ^{31}P NMR spectra were obtained at 338.79 MHz (360 MHz ^1H). All spectra were taken with 2.5 ml samples in a 10 mm diameter tube. A capillary insert containing D_2O was used as an internal field frequency lock. All the spectra were obtained using pulse-and-collect sequence with 50℃ pulse and interpulse delay of 2 s at 25℃ 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

Fig. 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 resonances at 3 ppm and 4.2 ppm to appear. Comparison with the ^{31}P NMR spectrum of the standard mixture of ADP, ATP, AMP and *Pi* shows that the resonances at 3 ppm and 4.2 ppm corresponds to the ^{31}P resonance from AMP and *Pi*. The intensity of both resonances increased very slowly as incubation continued up to 20 hours. A very tiny resonance also appeared at -18.3 ppm which is the position of the resonance of 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 hydrolysis of ATP produced. The incubation of ADP with F_1-ATPase in the absence of $MgCl_2$ showed only very tiny resonances at 3 ppm and 4.2 ppm even after 20 hours of incubation, indicating adenlyate kinase-like

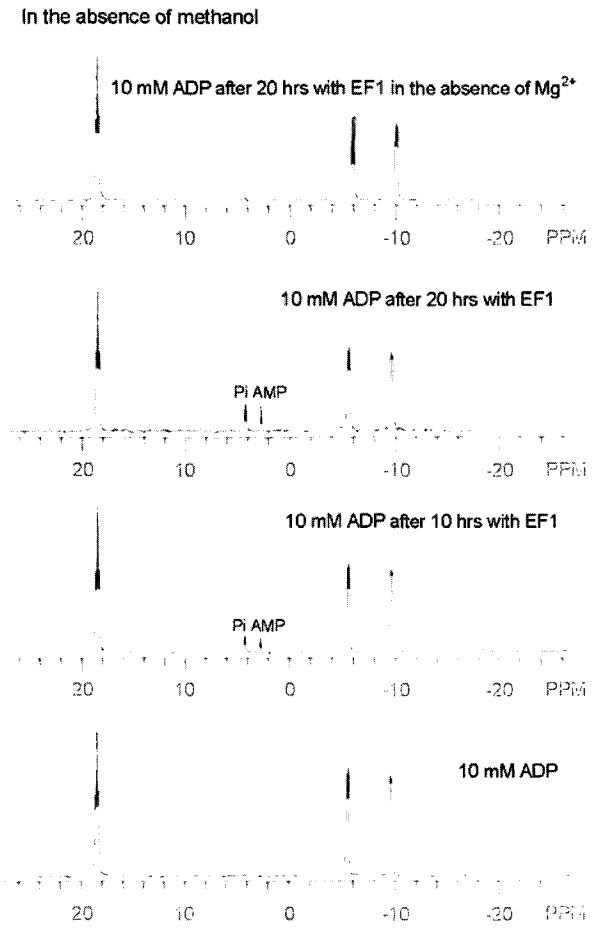

Fig. 1. ADP (10 mM) was incubated with 1 mg of F_1-ATPase in the absence of methanol. ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 50° excitation pulse and an interpulse delay of 2 sec. 500 scans were accumulated for each spectrum.

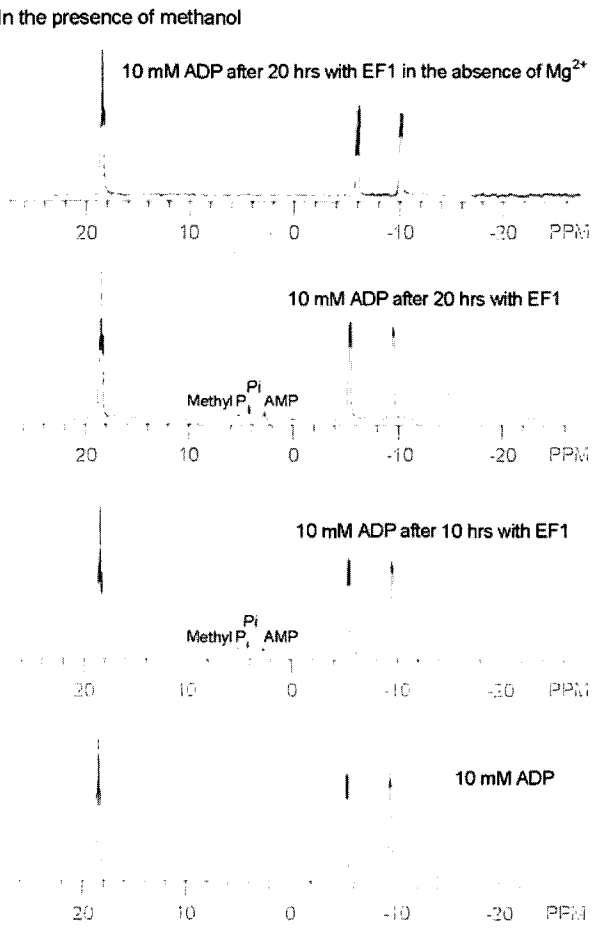

Fig. 2. ADP (10 mM) was incubated with 1 mg of F_1-ATPase in the presence of 10% methanol (v/v). ^{31}P spectra were acquired with a simple pulse-and-collect sequence using 50° excitation pulse and an interpulse delay of 2 sec. 500 scans were accumulated for each spectrum.

activity of F_1-ATPase requires Mg^{2+}.

The experiment was repeated with the addition of 10% methanol, normally added as a stabilizing agent of F_1-ATPase (Fig. 2). Incubation of ADP with F_1-ATPase from *E. coli* in the presence methanol caused a third resonance at 5.2 ppm as well as the resonances of AMP and *Pi* to appear. The resonance at 5.2 ppm is must originate from a transfer of phosphoryl group to a possible acceptor molecule in the medium, which is added methanol. Thus, the resonance at 5.2 ppm could be identified as the resonance from methyl phosphate. The appearance of new resonance at 5.2 ppm shows that the phosphoryl group of ADP was enzymatically transferred to methanol producing methyl phosphate. Like adenylate-kinase like activity, phosphoryl transferring activity of F_1-ATPase generating methyl phosphate could not be observed in the absence of Mg^{2+}.

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 (Fig. 1). However, the relative peak height of *Pi* was higher than that of AMP and methyl phosphate (methyl phosphate at 5.2 ppm: *Pi* at 4.2 ppm: AMP at 3.0 ppm = 1:2:1) when F_1-ATPase was incubated with ADP in the presence of methanol (Fig. 2). This may suggest each AMP and methyl phosphate was produced equally with production of ATP which is re-hydrolyzed to phosphate. The transphorylaton activity to ethanol and glycerol had been previously observed from acid phosphatases

of a *Citrobacter* sp., but never been reported in F_1-ATPases from various sources (Jeong et al., 1994).

Considering extremely slow generation of AMP which is an end product, the appearance of the resonance from β-phosphate of ATP at -18.3 ppm suggests that F_1-ATPase must be inhibited very strongly by ADP in the presence of Mg^{2+}. Although F_1-ATPase from various sources showed strong inhibition following binding of ADP in the presence of Mg^{2+} (Drobindskaya et al., 1985; Yoshida and Allison, 1986), 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+} was not detected in the steady state coupled assay with pyruvate kinase and lactate dehydrogenase. The inhibition may be very weak and reversed quickly during hydrolysis in the presence of excess ATP in the assay condition.

The significance of the finding that F_1-ATPase can transfer the terminal phosphoryl group form 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 (Vogel and Cross, 1991). 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 transphosphorylation reaction (Webb et al., 1980), the slow transphosphorylation reaction between sites observed in the present investigation may play a regulatory role.

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

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