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^{31}P Nuclear Magnetic Resonance Spectroscopy proves the interaction of methanol and nucleotide binding sites of F_1-ATPase

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Abstract: The ^{31}P NMR spectroscopy of native *Escherichia* coli F_1-ATPAse (EF1) showed ^{31}P resonance from free AMP, indicating adenylate kinase-like activity of F_1-ATPase. However, ^{31}P resonance from free AMP did not appear in the presence of the methanol which is known to stabilize F_1-ATPase. The presence of methanol could have affected on the interaction with nucleotides bound to EF1 and inhibited adenylate kinase-like activity of EF1. Monitoring of very slow adenylate-kinase like activity is only possible from ^{31}P NMR spectroscopy. ^{31}P resonances of EF1 bound nucleotides were more clearly resolved in the absence of methanol than those of in the presence of methanol, which demonstrates the interaction of methanol with nucleotides binding sites of EF1. The separated resonances in the region of β phosphate of ADP in the absence of methanol might suggest structural asymmetry amongst nucleotide binding sites of EF1 in the various conditions. ^{31}P NMR proved to be a valuable tool for investigating phosphorous related enzymes.

Key words: ^{31}P NMR spectroscopy, F_1-ATPase, *Escherichia coli*, methanol, nucleotide binding site, adenylate-kinase like activity

1. Introduction

Oxidative phosphorylation 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. ATP generating system is very essential for life, as life uses ATP as energy source. Two functionally unique parts of the protein can be distinguished(1-7). 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 (F_1-ATPase), 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 (1-7). 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,

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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 in physiological condition, however, was lacking. In principle, ^{31}P NMR should be able to discriminate the specific nucleotide 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 by inhibitor binding nucleotide binding site, N-ethylmalemide and 4-chloro-7-nitrobenzo-furazan (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. *Escherichia* coli F_1-ATPAse (EF1) could be first investigated with ^{31}P NMR spectroscopy very recently (17-19). In contrast to CF1, bound nucleotides to EF1 were could be observed in ^{31}P spectroscopy.

In the previous studies, ^{31}P spectroscopy was tried mostly in the presence of methanol added as a stabilizer (17-19).^{31}P spectroscopy with EF1 could reveal phsphotransferase-like activity as well as adenylate kinase-like activity (20). Although methanol did not affect activity of EF1, it is needed to investigate the effect of methanol on ^{31}P spectroscopy of EF1, as methanol could be involved for the transphosphorylation activity of EF1. In the present investigation we have compared ^{31}P NMR spectroscopy of native EF1 in the presence and in the absence of methanol.

2. Material and Methods

EF1 overproducing *E. coli* strain, SWM1, was obtained from Dr. A. Senior (University of Rochester). For preparing EF1, cells were grown as previously reported (17-20). F_1-ATPase was prepared as described previously (12-16). Enzyme was stored at -20℃ in column buffer, which contained Tris/HCl (50 mM, pH 7.4), 1 mM ATP, 1mM DTT, 2 mM EDTA/Na and 10% glycerol. Activity was measured using a steady state coupled assay with pyruvate kinase and lactate dehydrogenase (21). Protein concentration was determined by the Bradford microassay procedure (22) using heat denatured F_1-ATPase, as a protein standard. All the chemicals used were reagent grade from commercial sources.

^{31}P NMR spectra were obtained at 145.8 MHz (360 MHz ^{1}H). All spectra were taken with 2.5ml 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 a size control. All the spectra were obtained using pulse-and-collect sequence with 50°pulse and inter-pulse delay of 2 seconds 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 (0 ppm) for measuring the ^{31}P chemical shifts.

3. Results

Purified EF1 stored with ATP (1 mM) was freed of loosely bound ATP and Mg^{2+} by two passages through a desalting column equilibrated with the appropriate buffer. ^{31}P NMR spectroscopy of EF1

shows the ^{31}P resonance of free AMP at around 4 ppm in the absence of methanol, indicating very slow adenylate-kinase like activity (Fig.1). However, ^{31}P spectroscopy of EF1 in the presence of methanol did not show ^{31}P resonance of free AMP (17). As phosphotransferase-like activity as well as adenylate kinase-like activity was reported from EF1 (18, 20), it is possible that the presence of methanol affected nucleotide binding site of EF1 and inhibited adenylate kinase-like activity of EF1.

In the absence of methanol, ^{31}P NMR spectroscopy of EF1 prepared showed broad peaks from enzyme bound phosphate of EF1, at around -3ppm, -4ppm, -6 ppm, -10 ppm, -17 ppm. Comparison with mixture of free nucleotides could assign a peak at -10 ppm as a resonance of the enzyme bound α -phosphate of ADP/ATP and a peak at -17 ppm as a resonance from the enzyme-bound β-phosphate of ATP (Fig. 1). By comparison with the previous ^{31}P resonances of EF1 consists of only ADP (17, 18), the separated peak at about -3ppm and -4 ppm could be assigned as resonances from β phosphate of ADP, and thus the peak at about -6 ppm could be assigned as a resonance from γ phosphate of ATP. This separated resonances in the region of β phosphate of

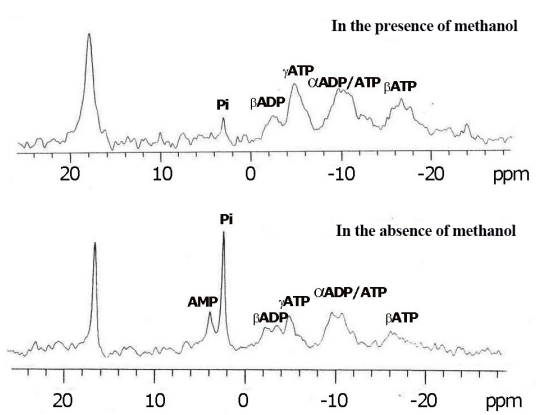

Fig. 1 ^{31}P NMR Spectra of native EF1 in the presence and in the absence of methanol

ADP could not be observed from native EF1 in the presence of methanol.

^{31}P NMR spectroscopy proved to be a valuable tool for monitoring subtle structural changes in nucleotides bound to enzyme and for monitoring very slow enzymatic reaction related with phosphorous.

4. Discussion

The resonance of enzyme-bound β-phosphate of ATP at -17 ppm appears only when enzyme was prepared in the presence of ATP and Mg^{2+} and ATP binding to EF1 is very tight that it could not be exchanged with other nucleotides (17), suggesting that this Mg^{2+} dependent ATP binding site would be non-catalytic. Compared to the resonance of free ATP, a resonance of enzyme-bound β-phosphate of ATP at -17 ppm was downfield shifted by about 1.5 ppm. This was similar to the downfield shift change of the β-phosphate resonance of MgATP upon binding to adenylate kinase (23). Previously, the downfield shift of ATP in enzyme bound state was unique for adenylate kinase among all examined kinases (24).

Adenylate kinase-like and phosphotransferase-like activity was recently reported from EF1 (18, 20). Adenylate kinase-like of F_1-ATPase was so far only reported from F_1-ATPase of chloroplast. The transphosphorylation activity to alcohol for phosphorous related enzyme had been previously observed from acid phosphatase of a *Citobacter* sp (25, 26)) and from EF1 using ^{31}P NMR spectroscopy (18, 20). The transphosphorylation activity to alcohol for phosphorous related enzyme is so slow that it could be observed only with ^{31}P NMR spectroscopy.

Current ^{31}P spectroscopy suggests that the presence of methanol affected the resonances of β

phosphate of ADP and γ phosphate of ATP bound to nucleotide binding sites of EF1, indicating the possible interaction between methanol and nucleotide binding sites of EF1.

Transphosphorylation activity of EF1 to methanol was observed when nucleotide was depleted from EF1 and reloaded with ADP in the presence of methanol (18). The peak of methyl phosphate increased with addition of Mg^{2+}, indicating dependence of transphosphorylation activity of EF1 on the Mg^{2+} (18, 20). When nucleotide-depleted EF1 was reloaded with GDP which is catalytic site specific nucleotide in the presence of methanol, ^{31}P resonance of methyl phosphate did not appear and only the ^{31}P resonance of free GMP and phosphate appeared (19). This suggests that the adenosine nucleotide bound to non-catalytic site of EF1 might be essential for transphosphorylation activity of EF1.

The presence of adenylate kinase-like activity and phosphotransferase-like activity from EF1 supports a model for the structure of nucleotide binding sites on F_1-ATPase which places catalytic site and no-catalytic sites in close proximity in an orientation analogue to ATP and AMP binding sites on adenylate kinase. As the normal catalytic reaction pathway does not include a phosphotransferase-like reaction, the interaction of methanol with nucleotide binding sites might play a role involving non-catalytic sites.

This separated resonances in the region of β phosphate of ADP in the absence of methanol suggest that the investigation of structural asymmetry amongst nucleotide binding sites of EF1 in the various conditions could be possible by ^{31}P spectroscopy, especially in the absence of methanol. ^{31}P NMR spectroscopy proved it could be a valuable tool for the investigation of phosphorous related enzyme.

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