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^{19}F NMR Investigation of F_1-ATPase of *Escherichia coli* Using Fluorinated Ligands

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Asymmetry amongst nucleotide binding sites of *Escherichia coli* F_1-ATPase was examined using ^{19}F NMR signals from fluorinated analogs of adenine nucleotides bound to nucleotide binding sites. ADP-CF_2-PO_3^{2-} showed no inhibitory effect to F_1-ATPase. But ADP-CHF-PO_3^{2-} (racemic mixture) showed competitive inhibition of F_1-ATPase with K_i of 60 μm. ADP-CHF-PO_3^{2-} shows only negligible binding to EF_1 in the absence of Mg^{2+}. With the addition of Mg^{2+} to the medium, the ^{19}F resonance of free ADP-CHF-PO_3^{2-} disappeared and the new broad resonances appeared. Appearance of more than two new asymmetric resonances following the binding of ADP-CHF-PO_3^{2-} to EF_1 may indicate that at least one of the isomers showed split resonances. This may suggest that the region between α- and β-phosphate of ADP-CHF-PO_3^{2-} which is bound to catalytic sites is experiencing a different environment at different sites.

Keywords: *Escherichia coli*, F_1-ATPase, ^{19}F NMR, Fluorinated Ligands.

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, has 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; 1990). 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; Wise *et al.*, 1983; Perlin *et al.*, 1984).

The intrinsically asymmetric structure of the enzyme, together with a variety of experimental investigations, indicates that the properties of the putative catalytic sites may be distinct from one another at any instant in a catalytic cycle. Direct evidence for such distinct properties has, however, been lacking. In the case of *E. coli*, it has been established that the enzyme conforms to the general pattern of nucleotide binding and that nucleotides can be removed from all binding sites. In the present investigation, heterogeneity amongst nucleotide binding sites was examined using ^{19}F NMR signals from fluorinated analogs of adenine nucleotides bound to *E. coli* F_1-ATPase (EF_1). Fluorinated analogs of nucleotide have been used because the variations in chemical shift of the fluorine residues are sufficiently large to permit

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identification of individual resonances, even in relatively large proteins (Post *et al.*, 1984; Rule *et al.*, 1987; Kim *et al.*, 1990). The other advantageous features of the fluorine nucleus are that ^{19}F occurs at 100% natural abundance and the sensitivity is close to that for the proton.

Materials and Methods

Growth of cells *E. coli* strain SWM1, which is an overproducer of F_1-ATPases, was obtained from Dr. D. Parsonage and Dr. A. Senior (University of Rochester). Fluorinated adenine nucleotide analogs, ADP-CHF-PO_3^{2-} and ADP-CF_2-PO_3^{2-} (15,400 $M^{-1} \cdot cm^{-1}$ at 259 nm) were obtained from Dr. Blackburn at University of Sheffield (Blackburn *et al.*, 1984). 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. Since strain SWM1 is, unlike the wild type, resistant to chloramphenicol, this antibiotic (60 μg/ml in ethanol) was added just before inoculation to a final concentration of 60 μg/l. One liter cultures in L-broth were grown overnight and used to inoculate 25 l of medium in a New Brunswick Scientific Pilot Fermentor. The cell 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 ATPase was prepared as described by Senior *et al.* (1979). 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 percent glycerol. Activity was measured using a steady state coupled assay with pyruvate kinase and lactate dehydrogenase as in Ting and Wang (1980). Protein was determined by the Bradford (1976) microassay procedure 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 ^{19}F 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 0.6 s at 25°C without sample spinning and with a sweep width of 8000 Hz with 4K data points. For each spectrum 10K scans were accumulated and the mixture was incubated at room temperature for at least half an hour to allow binding of fluorinated nucleotide analogs to nucleotide-depleted EF_1.

Preparation of nucleotide-depleted EF_1 Rather than using the procedures of Garrett and Penefsky (1975), which involves a long desalting column at a very low rate (1 ml/h) to prepare nucleotide-depleted EF_1, a new method using an ultrafiltration membrane was used here. F_1-ATPase samples were precipitated with 67 percent saturated ammonium sulfate, collected by centrifugation and dissolved in 50 mm Tris/HCl, 2 mM EDTA, 50 percent glycerol (v/v), pH 8.0. Such samples were concentrated in an Amicon cell fitted with a PM-30 membrane using the same buffer until an A_{280}/A_{260} ratio higher than 1.8 was obtained. The A_{280}/A_{260} ration obtained by this method is similar to that for the peak fraction obtained from the long desalting column in the conventional method. Nucleotide depleted EF_1 in this method contained about 0.4 mole of ADP per mole of enzyme.

Results

The effects of fluorinated analogs of ATP on the F_1-ATPase ADP-CF_2-PO_3^{2-} showed no inhibitory effect when added to F_1-ATPase. However, ADP-CHF-PO_3^{2-} showed competitive inhibition of F_1-ATPase. K_i was 60 μM, when assayed with pyruvate kinase and lactate dehydrogenase coupled method. Pyruvate kinase was not inhibited by the concentrations of ADP-CHF-PO_3^{2-} used. Considering that ADP-CHF-PO_3^{2-} exists as a racemic mixture, K_i could be 30 M for one of the diasteroisomers with the other being noninhibitory.

Titration of ADP-CHF-PO_3^{2-} with nucleotide-depleted *E. coli* F_1-ATPase Fluorinated ATP analog ADP-CHF-PO_3^{2-} was added sequentially to the nucleotide-depleted EF_1. The addition of one equivalent of ADP-CHF-PO_3^{2-} to the nucleotide-depleted EF_1 in the absence of $MgCl_2$ (Fig. 1A) showed a sharp resonance at the chemical shift position of ADP-CHF-PO_3^{2-}, indicating that most of ADP-CHF-PO_3^{2-} added existed as a free form. The addition of $MgCl_2$ to the medium caused the resonance of free ADP-CHF-PO_3^{2-} to disappear (Fig. 1B). The lack of a narrow resonance indicates that both diastereoisomers bind to nucleotide-depleted EF_1. When two equivalents of ADP-CHF-PO_3^{2-} were added to the nucleotide-depleted EF_1, both resonances at −2 ppm and −6 ppm increased. The sharp resonance at −4.5 ppm which is the same chemical shift as that of free ADP-CHF-PO_3^{2-} in the presence of $MgCl_2$ also appeared (Fig. 1C). The third addition of ADP-CHF-PO_3^{2-} to the nucleotide-depleted EF_1 seemed to cause one more resonance at about 0 ppm to appear (Fig. 1D). However, low signal to noise ratio does not allow positve identification. As more equivalents of ADP-CHF-PO_3^{2-} were added to the nucleotide-depleted EF_1, the resonance at −6 ppm increased together with sharp resonance of free ADP-CHF-PO_3^{2-} at −4.5 ppm (Fig. 1E).

Titration of ADP-CF_2-PO_3^{2-} with nucleotide-depleted EF_1 The addition of one equivalent ADP-CF_2-PO_3^{2-} to the nucleotide-depleted EF_1 in the absence of $MgCl_2$ (Fig. 2A) showed a sharp resonance at the chemical shift position of free ADP-CF_2-PO_3^{2-}, indicating that ADP-CF_2-PO_3^{2-} did not bind at all to F_1-ATPase in the absence of

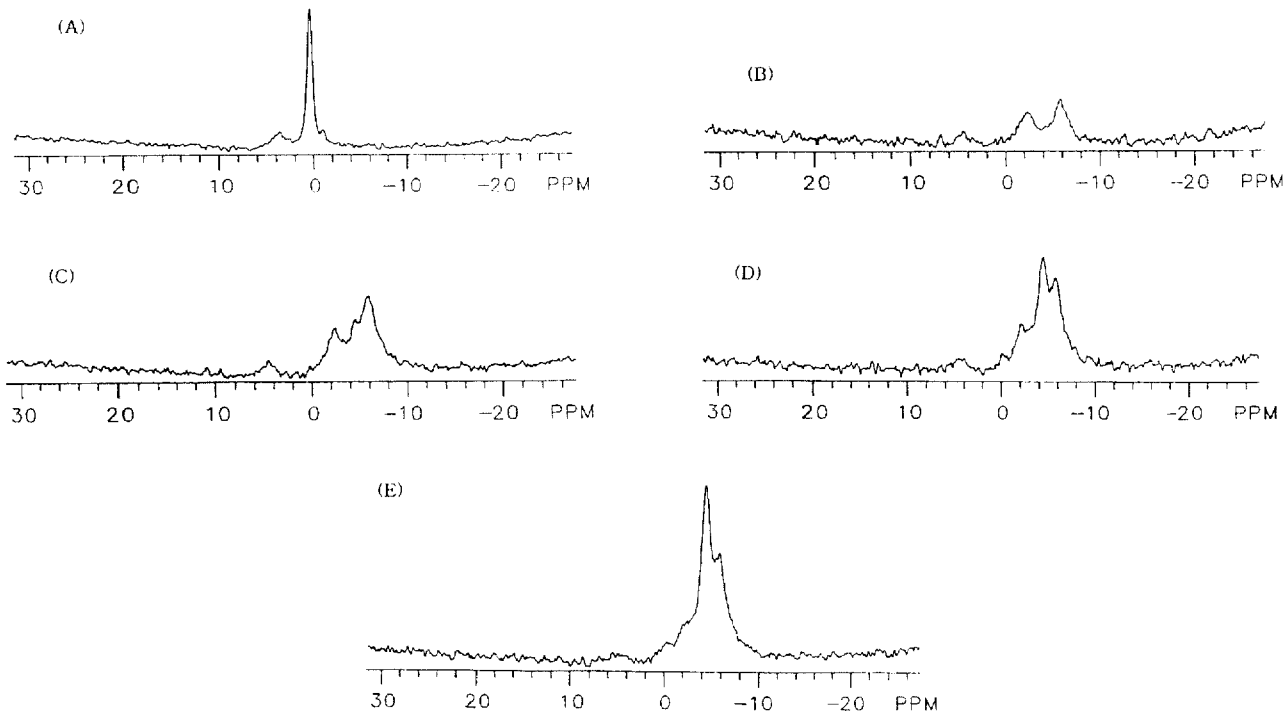

Fig. 1. Titration of ADP-CHF-PO_3^{2-} with nucleotide-depleted EF_1 (60 mg/ml). The buffer was 50 mM Tris/HCl, 1 mM EDTA, pH 7.4. For each spectrum 10K scans were accumulated; (A) After addition of one equivalent ADP-CHF-PO_3^{2-}, (B) After sequential addition of $MgCl_2$ (5 mM), (C) After sequential addition of two (total) equivalents ADP-CHF-PO_3^{2-} now in the presence of $MgCl_2$, (D) After addition of three equivalents of ADP-CHF-PO_3^{2-} in the presence of $MgCl_2$, (E) After addition of four equivalents of ADP-CHF-PO_3^{2-} in the presence of $MgCl_2$. Chemical shifts of free ADP-CHF-PO_3^{2-} was used as a reference for measuring ^{19}F chemical shifts.

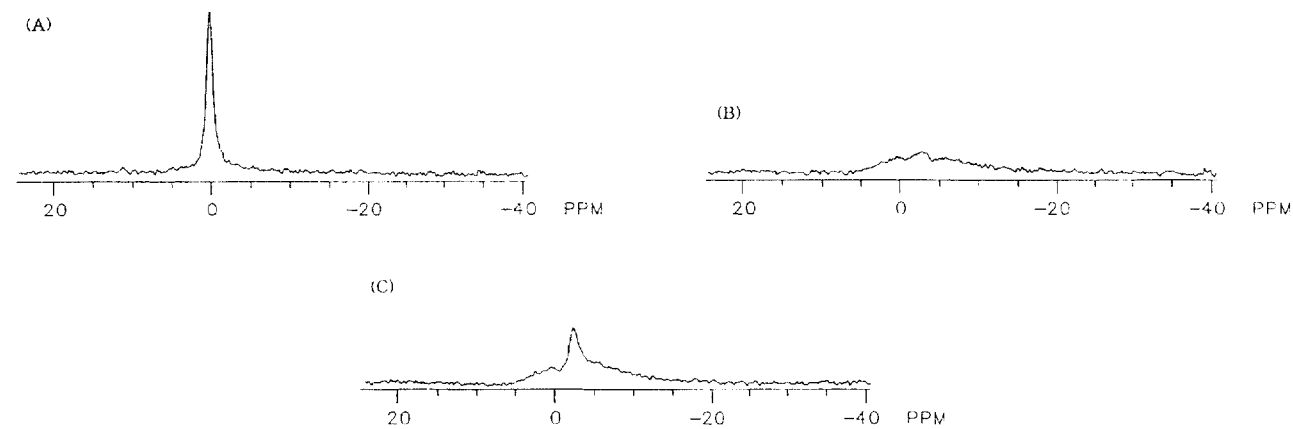

Fig. 2. Titration of ADP-CF_2-PO_3^{2-} with nucleotide-depleted EF_1 (60 mg/ml). The buffer was 50 mM Tris/HCl, 1 mM EDTA, pH 7.4. For each spectrum 10K scans were accumulated; (A) After addition of one equivalent ADP-CF_2-PO_3^{2-}, (B) After sequential addition of $MgCl_2$ (5 mM), (C) After sequential addition of two (total) equivalents ADP-CF_2-PO_3^{2-} now in the presence of $MgCl_2$. Chemical shifts of free ADP-CF_2-PO_3^{2-} was used as a reference for measuring ^{19}F chemical shifts.

$MgCl_2$. After addition of $MgCl_2$ to the medium, all the sharp resonance disappeared and a very broad resonance appeared (Fig. 2B). The second equivalent addition of ADP-CF_2-PO_3^{2-} showed the appearance of the resonance of free ADP-CF_2-PO_3^{2-} (Fig. 2C).

Discussion

The ^{19}F spectrum of nucleotide-depleted EF_1 after addition of one equivalent of a racemic mixture of ADP-CHF-PO_3^{2-} in the absence of $MgCl_2$ showed that most of the added ADP-CHF-PO_3^{2-} exists as a free form (Fig. 1A).

$MgCl_2$ was needed for the binding of ADP-CHF-PO_3^{2-}. This is in contrast to the titration of nucleotide-depleted EF_1 with ADP in which almost four equivalents of ADP could be bound to the enzyme without added Mg^{2+}, and six nucleotides could be bound in the presence of Mg^{2+} (Kim, 1991). As the ADP-CHF-PO_3^{2-} binds to the EF_1 only in the presence of Mg^{2+}, and severely inhibit catalytic activity of EF_1, ADP-CHF-PO_3^{2-} is likely to be a catalytic site specific ATP analog. Generally the catalytic sites have broader nucleotide specificity compared to non-catalytic sites (Cross and Nalin, 1982; Wise *et al.*, 1983; Perlin *et al.*, 1984). The structural difference between ATP and ADP-CHF-PO_3^{2-} suggests that the catalytic site has more flexibility in binding the abnormal structure of ATP analog between β- and γ-phosphate region. In contrast to ADP-CHF-PO_3^{2-} which is a strong inhibitor of F_1-ATPase, the fluorinated analogue ADP-CF_2-PO_3^{2-} did not inhibit F_1-ATPase activity at all. This also suggests the importance of the region between β- and γ-phosphate of nucleotide triphosphate for the binding of nucleotide binding site. The replacement of hydrogen atom by fluorine must have impaired the binding affinity of catalytic site for ADP-CF_2-PO_3^{2-}.

The addition of $MgCl_2$ caused the disappearance of resonances of free ADP-CHF-PO_3^{2-} and caused split broad resonances to appear (Fig. 1B). The further equivalent addition of ADP-CHF-PO_3^{2-} may cause one more resonance to appear (Figs. 1D–1E). It is not clear whether all these resonances appearing during titration of nucleotide-depleted EF_1 in the presence of $MgCl_2$ reflect different nucleotide binding sites or different environments of the fluorine atom in the two isomers at a single binding site. However, the appearance of several asymmetric resonances indicates that at least one of the isomers showed split resonances. This implies that the region between the β- and γ-phosphate of ADP-CHF-PO_3^{2-} which is bound to catalytic sites, is experiencing a different environment at different sites. Further experiments are expected to give more information about nucleotide binding sites when pure isomers of ADP-CHF-PO_3^{2-} are available.

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