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^{19}F NMR Investigation of F_1-ATPase of *Escherichia coli* Using Fluorotryptophan Labeling[1]

Hyean-Woo Lee,[*] Joon Hyung Sohn,[*] Byung-Il Yeh,[*] Jong-Whan Choi,[*] Seunho Jung,[†] and Hyun-Won Kim[*,2]

[*]*Department of Biochemistry and Institute of Basic Medical Science, Yonsei University Wonju College of Medicine, Wonju 220-701, Korea; and* [†]*Department of Microbiological Engineering, College of Engineering, Konkug University, Seoul 134-701, Korea*

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Growth of *Escherichia coli* in the presence of glyphosate, an inhibitor of aromatic amino acid biosynthesis, has permitted the production of proton-dislocating ATPase that is specifically labeled with 5-fluorotryptophan. Five sets of ^{19}F resonances could be assigned to each tryptophan residue by lauryldimethylamine oxide and carboxypeptidase treatment. On labeling with 4-chloro-7-nitro-benzofurazan, the label attached to β155Lys, which is known to be in the catalytic site, which caused one of the residues, β108Trp, to become nonequivalent. ^{19}F NMR spectroscopic investigation of internally fluorotryptophan-labeled F_1-ATPase will provide valuable information about the asymmetric nature of F_1-ATPase and the conformational changes induced by ligand binding.

Key words: F_1-ATPase, ^{19}F NMR, 4-chloro-7-nitro-benzofurazan, *Escherichia coli*, glyphosate.

A number of attempts have been made to incorporate fluorinated amino acid analogues into bacterial proteins (*1–3*). Although such analogues are generally highly toxic to cells, limited growth and incorporation of these analogues into proteins can take place. In a number of cases, a lowered specific activity is found. But in some cases active enzymes are also synthesized. Advantageous features of the fluorine nucleus are that ^{19}F occurs at 100% natural abundance and the sensitivity is close to that for proteins.

The incorporation of fluorinated amino acid analogues into bacterial proteins is generally achieved by adding the labeled amino acid to the growth medium for microbial cells that are auxotrophic for the required amino acid. An alternative method has been presented which is applicable to any one of, or combination of, the three aromatic amino acids (*4*). The inclusion of glyphosate, a specific inhibitor of a key step in aromatic amino acid biosynthesis (*5*, *6*), in growth media permits the specific incorporation of a labeled aromatic amino acid. This has been demonstrated for the F_1 sector of the proton-translocating ATPase (F_1-ATPase) from *Escherichia coli* into which fluorotryptophan was successfully incorporated (*4*).

The application of NMR for the F_1-ATPase was expected to answer a central question concerning the enzyme. This is whether or not the α and β chains within the $\alpha_3\beta_3\gamma\delta\varepsilon$ structure are asymmetric (*7*, *8*). As this enzyme has only a low tryptophan content, the distribution amongst the five polypeptides being α-1, β-1, γ-2, δ-1, ε-0 (*9*), labeling with fluorotryptophan was chosen. Despite the large molecular weight (380K), it was possible to clearly resolve resonances from fluorotryptophan in five environments in the enzyme.

In the present investigation we assigned each of the ^{19}F resonances of F_1-ATPase of *E. coli* (EF1), and investigated the asymmetric nature of the enzyme by means of 4-chloro-7-nitro-benzofurazan (NBD-Cl) labeling.

EXPERIMENTAL PROCEDURES

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 of reagent grade from commercial sources. For preparation of the enzyme, cells were grown in a large batch culture using M9 medium 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/liter), chloramphenicol (60 mg/ml), 10 μM p-benzoic acid, tyrosine (50 mg/liter), phenylalanine (50 mg/liter), and 5-fluorotryptophan (36 mg/liter) were 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 a non-limiting concentration from growth. Cells were grown at 37°C with vigorous aeration and the pH was

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[2] To whom correspondence should be addressed. Tel: +82-371-741-0283, Fax: +82-371-743-0411, E-mail: kimhwbio@wonju.yonsei.ac.kr

Abbreviations: EF1, F_1-ATPase of *Escherichia coli*; LDAO, lauryldimethylamine oxide; NBD-Cl, 4-chloro-7-nitro-benzofurazan.

maintained at 7.2 through controlled addition of a 2.5 M NaOH solution. Cell growth was monitored as the absorbance at 750 nm. When the mid-exponential phase growth was reached, cells were harvested using an Amicon concentrator.

Enzyme Preparation—F_1-ATPase was prepared as described previously (*8*, *10*). The enzyme was stored at −20°C in column buffer which contained Tris/HCl (50 mM, pH 7.4), 1.0 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 (*11*). Protein was determined by the Bradford microassay procedure (*12*) using heat denatured F_1-ATPase as a protein standard. All the chemicals used were of reagent grade from commercial sources.

NBD-Cl Modification—NBD-Cl modification of EF1 was carried out using a method based on that described for the mitochondrial enzyme by Ferguson *et al.* (*13*). The reaction with NBD-Cl was initiated by the addition of a five molar excess of reagent from a 100 mM stock solution in ethanol. The progress of the reaction at room temperature was monitored as the increase in the absorbance at 385 nm. When there was no further increase in the absorbance at 385 nm (at least 2 h incubation), the enzyme was separated from excess reagent using a Sephadex G25-100 column. The fractions containing modified EF1 could be easily identified visually because of their yellow color.

NBD Group Transfer to βLys155—A 1 M Tris solution was added to the EF1 sample modified with NBD-Cl until the pH reached 9.0. After overnight accumulation, some cloudiness was observed. The enzyme was freed of denatured protein by centrifugation, and the pH was readjusted to 7.4 by passage through a Sephadex G25-100 column.

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 (72 mg/ml) 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 a pulse-and-collect sequence with a 50° pulse an interpulse delay of 0.9 s at 25°C without sample spinning and with a sweep width of 15 kHz. Free 5-fluorotryptphan was used as an external reference to measure the ^{19}F chemical shifts.

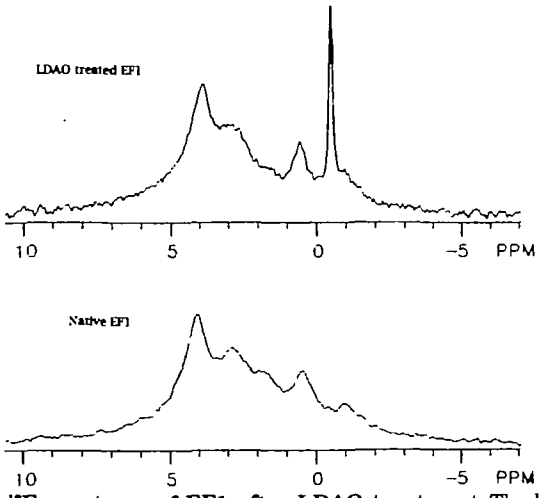

Fig. 1. **^{19}F spectrum of EF1 after LDAO treatment.** The buffer was 50 mM Tris/HCl, 1 mM EDTA, pH 7.4. All the spectra were obtained using a pulse-and-collect sequence with a 50° pulse and an interpulse delay of 0.9 s at 25°C without sample spinning. For each spectrum, 10K scans were collected.

RESULTS

As an alternative to using auxotrophic strains, tryptophan biosynthesis was, in the present work, blocked by the inclusion of glyphosate, an inhibitor of the 5-enol pyruvylshikimic acid–3-phosphatesynthase reaction of aromatic amino acid biosynthesis (*5*, *6*). Glyphosate, 1 g/liter was found to inhibit the growth of cells of *E. coli* unless the three aromatic amino acids were included in the medium. But growth in the presence of glyphosate was sustained by 5-fluorotryptophan when 6 mg/liter tryptophan was also added. Growth was severely limited in the presence of glyphosate and 5-fluorotryptophan if a lower concentration of tryptophan was used. The isolated enzyme exhibited a specific activity that was similar to that of the enzyme prepared from cells grown on tryptophan. The content of fluo-

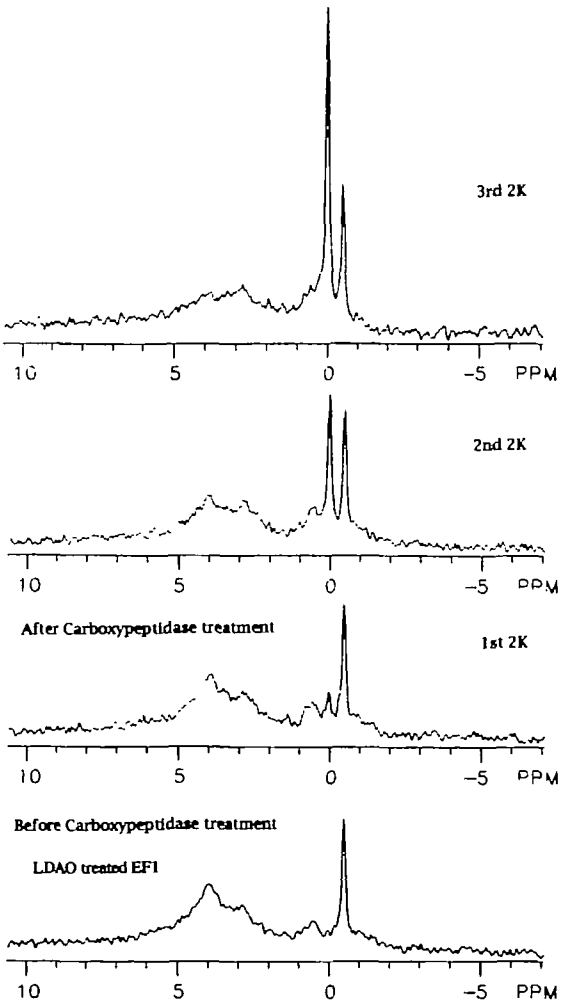

Fig. 2. **^{19}F spectra of EF1 after carboxypeptidase treatment following LDAO treatment.** All the spectra were obtained using a pulse-and-collect sequence with a 50° pulse and an interpulse delay of 0.9 s at 25°C without sample spinning. Each 2K scan of the ^{19}F spectrum was collected sequentially.

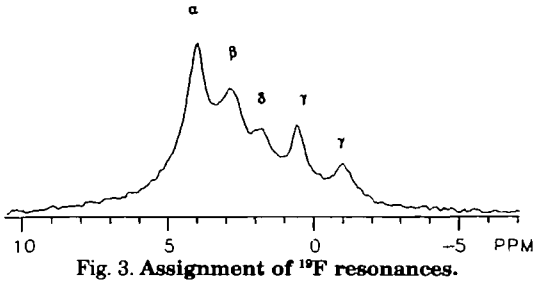

Fig. 3. **Assignment of ^{19}F resonances.**

rotryptophan was 80% of the total tryptophan. Thus, the incorporation of the analogue appears to occur without discrimination between the flurotryptophan and tryptophan added to the medium.

On treatment of lauryldimethylamine oxide (LDAO) with F_1-ATPase, the third resonance of the ^{19}F spectrum at 2 ppm disappeared, being replaced by a relatively sharp and intense resonance at –0.5 ppm (Fig. 1). As the δ subunit is known to be released by LDAO (*14*), the resonance in the ^{19}F spectrum which had been lost can be reasonably assigned to a signal from the δ subunit (δTrp28). The appearance of the very sharp resonance would then correspond to the resonance of the released δ subunit. This sharp resonance from the released δ subunit is contrast with the broad resonance from the δ subunit when it forms part of the F_1-ATPase. Carboxypeptidase treatment of LDAO-treated EF1 led to a gradual loss of the signal at 4 ppm, and to the appearance of a very sharp and intense resonance at the chemical shift expected for free 5-flurotryptophan (0 ppm) (Fig. 2). As the tryptophan in the α subunit is at the carboxy-terminal (αTrp513) (*9*), it may be possible to assign the intense signal at 4 ppm to the resonance from the three carboxy-terminal tryptophans of the α chains.

Following the assignment of resonances from the α and δ subunits, the other three resonances could be also assigned. As the intense signal at 4 ppm was a tryptophan resonance from the α subuint, the other intense resonance at 2.8 ppm can be assigned to the signal from the β subunit, considering the tryptophan distribution amongst the five polypeptides is α-1, β-1, γ-2, δ-1, ε-0 within the $\alpha_3\beta_3\gamma\delta\varepsilon$ structure. The remaining three resonances must therefore originate from small subunits. A resonance at about 2 ppm is assigned to a tryptophan resonance from the δ subunit, thus the resonances appearing 0.5 ppm and –1 ppm could be assigned to signals from the γ subunit (Fig. 3).

The reagent NBD-Cl has been widely used as a potent inhibitor of F_1-ATPase. When F_1-ATPAse was labeled with NBD-Cl in the dark at pH 7, the NBD-label was exclusively attached to a specific tyrosine residue (βTyr297), but with only one label covalently attached per F_1 molecule (*13*, *15*). This NBD label can be transferred spontaneously to Lys155, on incubation at pH 9, *via* an intramolecular process (*13*, *16*). A ^{19}F spectrum of NBD-EF1 did not show any significant difference from the spectrum of the native enzyme (Fig. 4). Subsequently, the pH of the solution of EF1 modified at tyrosine by NBD was raised to pH 9.0 by the addition of a 1 M Tris solution. After 24 h incubation at pH 9.0, the pH of the solution was lowered again to 7.4 by passage through a desalting column. This ^{19}F spectrum of this EF1 in which the NBD label is bound to βLys155 is

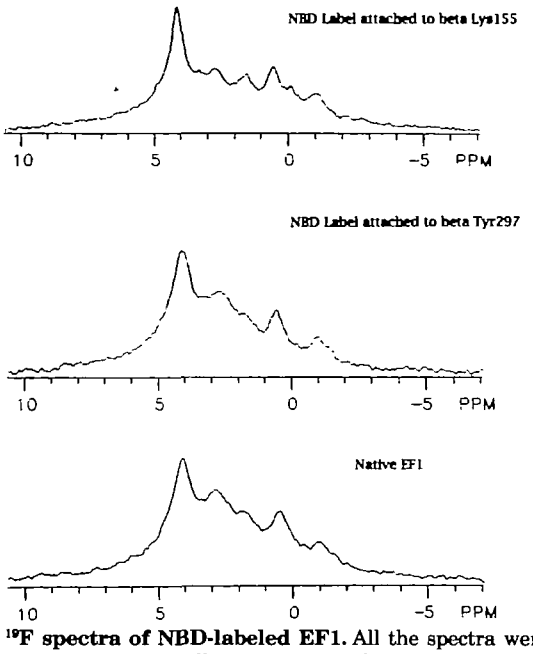

Fig. 4. **^{19}F spectra of NBD-labeled EF1.** All the spectra were obtained using a pulse-and-collect sequence with a 50° pulse and an interpulse delay of 0.9 s at 25°C without sample spinning. Each spectrum was collected with 10 K scans. For the spectrum of the NBD label attached to β155 Lys, the pH was raised to pH 9.0 overnight, and then lowered back to 7.4.

shown in Fig. 4. The ^{19}F spectrum showed six resonances. Although the relative areas seemed to be different from those in the case of the native enzyme, all five resonances which appeared in the spectrum of the native enzyme were at the same chemical shift positions as in that of the native EF1. The sixth resonance appeared at –0.2 ppm, accompanying the decrease in the resonance from β108Trp. This may suggest that the NBD label attached to β155 caused one of the residues, β108Trp, to become nonequivalent.

DISCUSSION

5-Fluorotryptophan was introduced previously into proteins through inclusion of the amino acid analogue in the growth medium for a strain auxotrophic for tryptophan. This approach has been used because fluorotryptophan is toxic to strains that are the wild type with respect to tryptophan biosynthesis. In principle, added fluorotryptophan could also be competed out of proteins by endogenously produced typtophans. The growth of *E. coli* with fluorotryptophan in the presence of glyphosate needed a trytophan concentration of at least 20%, implying that fluorotryptophan cannot replace tryptophans for some critical amino acid positions. However, a fluorotyptophan incorporation ratio of 80% suggests that the incorporation of analogues into F_1-ATPase occurs without discrimination between the flurotryptophan and tryptophan added to the medium.

If all tryptophans are labeled equally within the $\alpha_3\beta_3\gamma\delta\varepsilon$ structure of EF1, and the sets of α- and β-chain residues are each in equivalent environments, then five separate resonances would be expected with relative intensities of 3:3:1:1:1, which in the case of the present investigation. The selective removal of the low field resonance at 4 ppm

following treatment with carboxypeptidase and the appearance of a very narrow resonance at the position of free fluorotryptophan allowed the assignment of the resonance at 4 ppm to the α chain tryptophan, which is at the carboxy terminal. The single resonance for this amino acid suggests that all three carboxy termini are in essentially identical environments. The other intense resonance at 4 ppm can be reasonably assigned to the β chain tryptophan. This may suggest that this amino acid residue (βTrp108) also experiences the same environment in each of the β chains, indicating no asymmetry in this region of the polypeptide chain. Of course, this conclusion must be balanced against the fact that none of the tryptophans in the enzyme are conserved in enzymes from other species and thus they must be located in functionally unimportant parts of the constituent polypeptide chains. However, the tryptophan in the β chain (β108Trp) is toward the middle of the chain, in which residues 149–156 are thought to be a catalytic site (*17*), and thus it is unlikely that there could be significant gross conformational changes without β108Trp being affected. This could explain the appearance of the sixth ^{19}F resonance, after the NBD label is moved from β297Tyr to β155Lys *via* an intramolecular transition. The appearance of the sixth resonance may suggest that the NBD label attached to β155Lys caused one of the residues, β108Trp, to become nonequivalent.

The methodology established here demonstrates that it is possible to use fluorotryptophan as a probe of an environment even in proteins as large as the F_1-ATPase (380K). As ^{19}F resonances are assigned for each trpytophan, when combined with site directed mutagenesis, ^{19}F NMR spectroscopic investigation of F_1-ATPase will provide valuable information about the asymmetric nature of F_1-ATPase and the conformational changes induced by ligand binding.

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