Volume 272, number 1,2, 34–36 FEBS 08941 October 1990

The specific incorporation of labelled aromatic amino acids into proteins through growth of bacteria in the presence of glyphosate

Application to fluorotryptophan labelling to the H^+-ATPase of *Escherichia coli* and NMR studies

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Received 27 July 1990

Growth of *Escherichia coli* in the presence of glyphosate, an inhibitor of aromatic amino acid biosynthesis, has permitted the production of proton translocating ATPase that is specifically labelled with 5-fluorotryptophan. Five sets of ^{19}F nuclear magnetic resonances are resolved. The use of glyphosphate should be of wide applicability in the preparation of proteins labelled in aromatic amino acid residues for NMR studies.

NMR; Glyphosate; ATP synthase; ATPase; Fluorotryptophan; *Escherichia coli*

1. INTRODUCTION

NMR studies of polypeptides and proteins can be aided by the selective labelling of one or more types of amino acid. For example, selective deuteration of certain amino acids can aid in the assignment of resonances to amino acids, especially for larger proteins [1,2]. For proteins that are larger than approx. 25 kDa the detailed study and sequential assignment of proton resonances is technically beyond reach even with the aid of isotope labelling. However, such larger proteins can be studied by NMR methods if the resonances from selectively introduced nuclei are analysed. For example, fluorinated analogues of aromatic amino acids have been used because the variations in chemical shift of the fluorine residues are sufficiently large to permit identification of individual resonances, even in relatively large proteins [3–7]. 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.

The application of NMR methods to the F_1 part of the proton translocating ATPase of *E. coli* could answer a central question concerning this enzyme. This is whether the α and β chains within the $\alpha_3\beta_3\gamma\delta\epsilon$ structure are asymmetric. Here we introduce a new method of preparing proteins labelled on aromatic amino acids and show that, even for a protein as large as the ATPase (M_r 380000), it is possible to resolve clearly resonances from fluorotryptophans in five environments in this enzyme. Labelling with fluorotryptophan was chosen because this enzyme has only a low tryptophan content, with the distribution amongst the five polypeptides being α-1, β-1, γ-2, δ-1, ϵ-0 [8].

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2. MATERIALS AND METHODS

E. coli strain SWM1, which is an overproducer of F_1-ATPase, was obtained from Dr D. Parsonage (University of Rochester). For preparation of the enzyme, cells were grown in large batch culture M9 media to which was added 1 ml of concentrated trace element solution (4 mM $ZnSO_4$, 1 mM $MnSO_4$, 4.7 mM H_3BO_3, 0.7 mM $CuSO_4$, 2.5 mM $CaCl_2$ and 1.8 mM $FeCl_3$) per liter. After sterilization, 1 ml of sterile 1 M $MgSO_4$ solution was added per liter, together with other growth supplements as required, 30 mM glucose, 0.2 mM thiamine hydrochloride, 0.8 mM arginine hydrochloride and 0.2 mM uracil. Glyphosate (1 g/l), chloramphenicol (60 mg/l), 10 μM *p*-aminobenzoic acid, 10 μM *p*-hydroxybenzoic acid, tyrosine (50 mg/l), phenylalanine (50 mg/l) and 5-fluorotryptophan (36 mg/l) 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 medium in a New Brunswick Fermenter. From growth curves 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 for growth. Although 5-fluorotryptophan is toxic to the cell, sufficient growth could be obtained in 25 liter cultures with the combination of the tryptophan from the starter culture (1.5 liter, which meant that the final tryptophan concentration was approx. 6 mg/l) and the added analogue. The cells were grown at 37°C, and pH was maintained at 7.0 through controlled addition of 2.5 M NaOH solution. Cell growth was monitored from the absorbance at 750 nm. When the mid-exponential phase of growth was reached cells were harvested using an Amicon concentrator.

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00145793/90/$3.50

The preparation of ATPase from *E. coli* was as described by Senior et al. [9]. Activity was measured using a steady-state coupled assay with pyruvate kinase and lactate dehydrogenase as in [10]. Protein was determined by the Bradford microassay [11] procedure using heat denatured F_1-ATPase as a protein standard.

^{19}F-NMR spectra were obtained at 338.8 MHz. All spectra were taken with 2.5 ml samples in 10 mm diameter NMR tubes fitted with a capillary insert containing D_2O to act as a field frequency lock. Data acquisition, 40000 transients, was with a spectral width of 15 kHz, 75° (16 μs) pulse width, pulse delay of 0.5 s. Proton decoupling was not used. Free 5-fluorotryptophan was used as an external reference for measuring the ^{19}F chemical shifts. The fluorotryptophan content of labelled enzyme was estimated by unfolding a sample of the enzyme with 1% SDS. The area of the resulting 5-fluorotryptophan peak was standardized by reference to the area for a known amount of 5-fluorotryptophan. The ratio of 5-fluorotryptophan to tryptophan was calculated to be 4:1 from knowledge of the total number of tryptophan residues in the protein [8] and the protein concentration.

Glyphosate in the free acid form was a gift from Dr G. Jacob of the Monsanto Corporation and was dissolved in 1 M NaOH. 5-Fluorotryptophan was purchased from Sigma. All other chemicals were reagent grade from commercial sources.

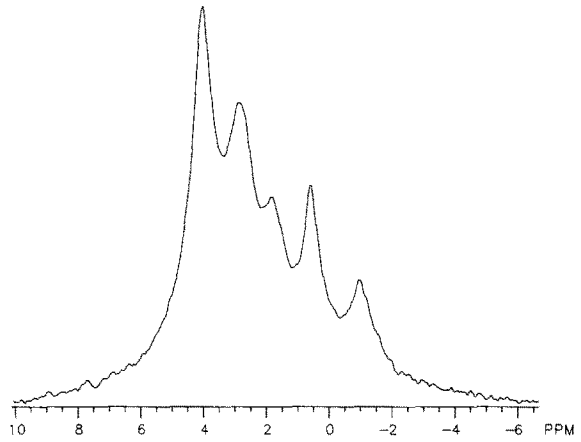

Fig. 1. ^{19}F-NMR spectrum of F_1-ATPase from *E. coli* grown in the presence of 5-fluorotryptophan. The protein concentration was 60 mg/ml and the temperature 20°C. The buffer was 50 mM Tris-HCl, 1 mM EDTA, pH 7.4. The spectrum was accumulated for 4 h (40000 scans). The chemical shifts are given with reference to the ^{19}F signal from free 5-fluorotryptophan.

3. RESULTS

Fluorotryptophan has been introduced previously into proteins through inclusion of the amino acid analogue in the growth medium, of a strain auxotrophic for tryptophan [12–14]. This approach has been used because fluorotryptophan is toxic to strains that are wild-type with respect to tryptophan biosynthesis [12]. In principle, added fluorotryptophan could also be competed out of proteins by endogenously produced tryptophan. As an alternative to using auxotrophic strains, tryptophan biosynthesis was, in the present work, blocked by the inclusion of glyphosate, an inhibitor [15,16] of the 5-*enol*pyruvylshikimic acid-3-phosphate synthase reaction of aromatic amino acid biosynthesis. Glyphosate at 1 g/l was found to inhibit growth of cells of *E. coli* unless the three aromatic amino acids were included in the medium. When tryptophan was entirely replaced by 5-fluorotryptophan growth was prevented. But growth in the presence of glyphosate was sustained by 5-fluorotryptophan when it was supplemented by 6 mg/l tryptophan (see section 2). A lower concentration of tryptophan severely limited growth in the presence of glyphosate.

The F_1-ATPase could be isolated from cells grown in the presence of glyphosate plus 5-fluorotryptophan. The enzyme had a specific activity that was very similar to the enzyme prepared from cells grown on tryptophan. The ^{19}F-NMR spectrum of the labelled enzyme is shown in Fig. 1. There are clearly five resolved resonances. Analysis showed that the content of fluorotryptophan was 80% of the total tryptophan content. Thus the incorporation of the analogue appears to occur without discrimination between the fluorotryptophan and tryptophan added to the medium.

The linewidths (≈350–500 Hz) are as expected for a protein of the size of the F_1-ATPase and the chemical shift values are within the range reported previously for other proteins [6,14]. The assignment of the resonances to the total of nine tryptophans in the molecule is required. If all tryptophans are labelled equally and the sets of α- and β-chain residues are each in equivalent environments then five separate resonances would be expected with relative intensities 3:3:1:1:1. As a step towards full assignment, which will require reconstitution of enzyme with specifically labelled subunits or tryptophan replacement by site-directed mutagenesis, carboxypeptidase treatment was undertaken because the tryptophan on the α-chain is at the C-terminus [8]. This procedure led to loss of the signal at 4 ppm and the appearance of a relatively intense and sharp resonance at the chemical shift value expected for free fluorotryptophan. As this is one of the most intense signals, it is reasonable to assign it to the three tryptophans of the α-chains.

There is evidence that addition of ATP and Mg^{2+} causes conformational changes in the F_1-ATPase [17–19]. However, in experiments so far the presence of ATP and Mg^{2+} in the medium did not cause observable changes in the ^{19}F spectrum.

4. DISCUSSION

The study of structural and functional aspects of proteins using NMR can be facilitated by selective labelling of certain amino acids. This is frequently achieved by adding the labelled amino acid to the growth medium of a microbial cell that is an auxotroph for that amino acid. An alternative method is proposed here which is applicable to any one of, or combination of, the three aromatic amino acids. Inclusion of glyphosate, a specific inhibitor of a key step in

aromatic amino acid biosynthesis, in growth media permits the specific incorporation of a labelled aromatic amino acid. This has been demonstrated for the incorporation of fluorotryptophan into the F_1 sector of the proton translocating ATPase from *E. coli*. The advantages for specific labelling of proteins via growth of cells in the presence of glyphosate are: (i) it dispenses with the need to isolate auxotrophs for an organism which synthesises the protein of interest; (ii) in instances where products of cloned genes are being studied it is not necessary to transfer the genes to auxotrophic strains; (iii) one, two or all three of the aromatic amino acids can be labelled, depending upon omission of the normal amino acid(s) from the medium and addition of the labelled amino acid(s).

In the case of the F_1-ATPase studied here the ^{19}F-NMR spectra permit some conclusions. The selective removal of the resonance at 4 ppm following treatment with carboxypeptidase together with its relative intensity strongly suggests that this corresponds to the C-terminal tryptophan of the α-chain. The single resonance for this amino acid suggests that all three carboxy-termini are in essentially identical environments. More experiments are being conducted to assign the resonance and characterise their relationship to conformational changes. The methodology established here shows that it is possible to use fluorotryptophan as a probe of environment, even in proteins as large as the F_1-ATPase. The ability to introduce and delete tryptophans by site-directed mutagenesis, as shown for lactate dehydrogenase [20–22], will allow the method to be used in a variety of problems.

Acknowledgements: We thank the SERC for a grant awarded to I.D.C. and S.J.F., the ORS scheme and Oxford University for awards to H.-W.K. and Dr G. Jacob of the Monsanto Corporation for the generous provision of glyphosate. Helpful discussions with Dr Paul Barlow and Dr Steve Rigby are gratefully acknowledged.

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