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^{19}F NMR detection of a fluorine-labelled enzyme in vivo

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Phosphoglycerate kinase was fluorine-labelled in the yeast *Saccharomyces cerevisiae* by inducing enzyme synthesis in the presence of 5-fluorotryptophan. ^{19}F NMR measurements on the intact cells showed two resonances from the two tryptophan residues in the protein. The chemical shifts of these resonances were sensitive to the metabolic status of the cells.

Phosphoglycerate kinase; Yeast; ^{19}F NMR

1. INTRODUCTION

High resolution ^{1}H NMR has been used extensively to study the conformation, ligand binding and ionisation properties of proteins in solution [1,2]. The non-invasive nature of NMR has also made it a widely used technique for studying metabolism in vivo [3]. Although these studies have concentrated largely on monitoring the concentrations and spatial distribution of small molecules in vivo, the technique is also capable of detecting signals from macromolecules in the cell. ^{13}C NMR has been used to monitor the concentration and ^{13}C enrichment of glycogen in a number of systems [3] and ^{1}H NMR spectra of cells and tissues show resonances both from small metabolite molecules and macromolecules such as proteins [4]. However, the proton resonances from proteins are generally broad and unresolved. With the exception of hemoglobin in erythrocytes [5], there appear to be no cases where assigned proton resonances from a specific protein in a cell can be observed in vivo. Clearly in cells where there is no predominant protein, observation of a specific protein by NMR will require some form of labelling. Furthermore, label detection must be very sensitive since the labelled protein concentration is likely to be low. Fluorine is an ideal label in this respect. There are no interfering background signals, the detection sensitivity is close to that of proton and its large chemical shift range makes it very sensitive to its environment. Fluorine labels have already been used to study relatively large proteins by ^{19}F NMR in vitro [6–8]. We describe here a methodology which has allowed us to fluorine label a specific enzyme in vivo and detect the ^{19}F NMR signals from this protein in the intact cell.

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2. MATERIALS AND METHODS

Yeast growth media were obtained from Difco USA Ltd. DL-5-fluorotryptophan and low-gelling-temperature agarose were from Sigma. All other reagents were of analytical grade.

Cells of the *Saccharomyces cerevisiae* strain FY3-1 (*α, leu2-3, leu2-112, trp4*), transformed with the plasmid pKV43 [9] were grown aerobically at 30°C. Cells were inoculated at a cell density of 4×10^5 cells/ml into a medium containing 2% glucose, 2% bactopeptone and 1% yeast extract. After 14 h of growth, the cells were harvested, washed and then transferred to a medium containing 6.7 g/l yeast nitrogen base, 1% galactose and an amino acid mixture lacking leucine and containing 0.1 μg/l tryptophan. The inoculation cell density on this medium was 2×10^7 cells/ml. After a further 12 h, the point at which enzyme assays on cell extracts showed that phosphoglycerate kinase (PGK) induction was just beginning, 5-fluorotryptophan was added to the medium to give a concentration of 0.2 g/l. The incubation was continued for a further 36 h before harvesting the cells. The cells (4 g wet wt) were immobilised in low-gelling-temperature agarose and perfused as described in [10,11].

NMR experiments were performed using a Bruker AM-300 spectrometer. The fluorine and phosphorus resonance frequencies at this field were 282.38 MHz and 121.47 MHz, respective-

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ly. The ^{19}F NMR spectra were acquired into 4K data points with a sweep width of 6 kHz using 70° pulses and an interpulse delay of 1.34 s. Experiments on cell extracts showed that under these conditions the protein resonances were fully relaxed. An exponential line broadening of 30 Hz was applied to the free induction decays prior to Fourier transformation. The chemical shifts were referenced to *p*-fluorophenylalanine in water at 0.0 ppm. Phosphorus spectra were acquired into 2K data points with a sweep width of 8 kHz using a 90° pulse and an interpulse delay of 5.127 s.

Fluorinated PGK was isolated from yeast as described in [12]. PGK activity was assayed spectrophotometrically in cell extracts [11].

3. RESULTS AND DISCUSSION

PGK was fluorine labelled by inducing enzyme synthesis in the presence of 5-fluorotryptophan in cells which were auxotrophic for tryptophan. Enzyme induction was obtained by transforming the cells with a plasmid in which the PGK coding sequence was under the control of a galactose-inducible promoter [9]. Incubation of the cells with galactose in the presence of 5-fluorotryptophan resulted in the production of fluorine-labelled PGK. Fig.1 shows ^{19}F NMR spectra from immobilised, perfused cells in which PGK had been fluorine labelled. The PGK activity in these cells was four times greater than in the non-induced controls. The resonances at -7.65 ppm and -8.00 ppm in fig.1A and at -7.73 ppm and -8.19 ppm in fig.1B have been assigned to the two tryptophan residues in the protein [13] on the basis of experiments with the purified enzyme and cell extracts (see below). The peak at -9.15 ppm in both spectra resonates at the frequency of free 5-fluorotryptophan. Cells which had been transformed with a control plasmid in which the PGK coding sequence had been replaced with an interferon coding sequence [9] and which had been taken through the same induction protocol, showed no detectable ^{19}F NMR resonances. The concentration of interferon in these cells is considerably less than that of PGK [9]. Addition of glucose to the cell perfusion buffer resulted in a significant upfield shift of the PGK resonance at -8.00 ppm and a decrease in intensity of the peak at -9.15 ppm. ^{31}P NMR spectra acquired before glucose addition showed resonances predominantly from P_i and polyphosphates. Addition of glucose resulted in the production of nucleoside triphosphates, a decrease in the P_i concentration and an

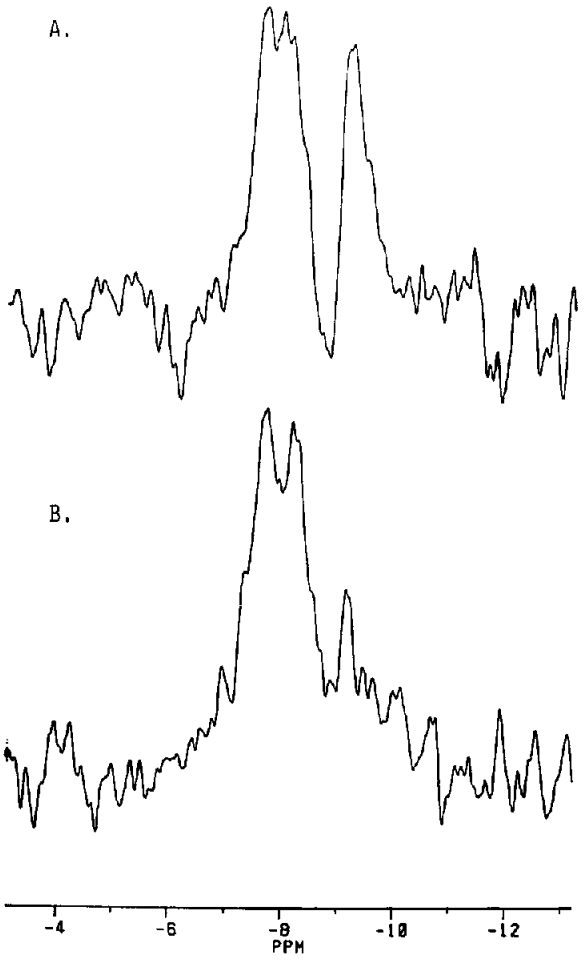

Fig.1. ^{19}F NMR spectra of cells containing fluorine-labelled PGK. (A) Spectrum (24 576 scans) from cells perfused without any substrate. (B) Spectrum (24 576 scans) from the same cells perfused with 50 mM glucose.

alkalinisation of the cytoplasm. The changes in the ^{19}F NMR spectra appear to be reversible since similar changes were observed in cells initially perfused with glucose and then under substrate-free conditions. The mean shifts for the resonances in vivo were -7.78 ± 0.10 ppm, -8.06 ± 0.06 ppm and -9.18 ± 0.03 ppm ($n = 3$, $\pm$ SD) under substrate-free conditions (fig.1A) and -7.76 ± 0.06 ppm, -8.25 ± 0.07 ppm and -9.16 ± 0.01 ppm ($n = 5$) in cells perfused with glucose (fig.1B). In order to understand the factors influencing the shifts of the protein resonances, experiments were carried out on the isolated enzyme and on the enzyme in cell extracts.

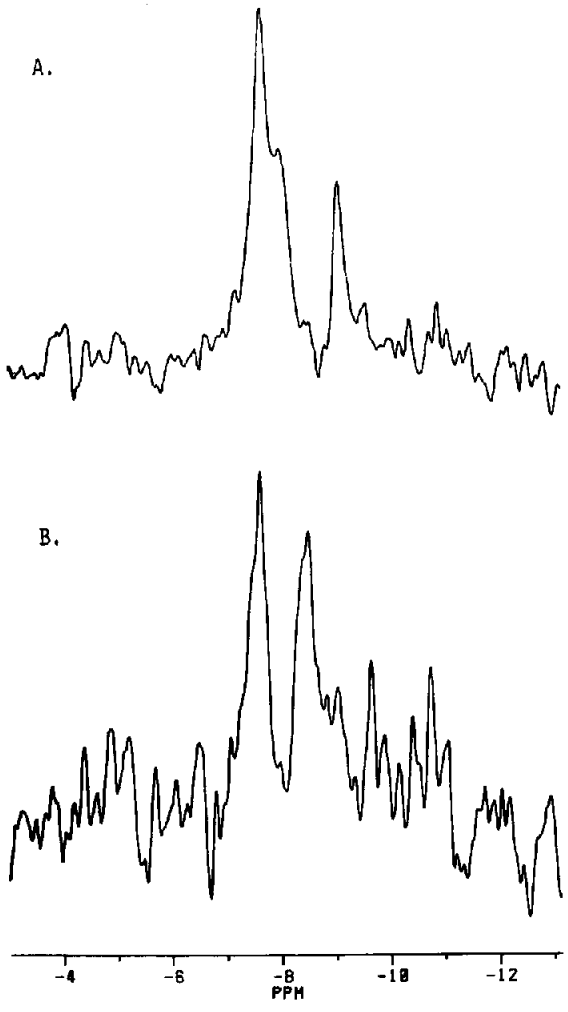

Fig.2. ^{19}F NMR spectrum of a cell lysate prepared from cells containing fluorine-labelled PGK. (A) The cells had been perfused in the absence of substrate for 6 h prior to extraction. (B) Spectrum from the same lysate following dilution and ultrafiltration through an Amicon PM-10 membrane. This procedure gave a 14 × dilution of molecules smaller than 10 kDa in the lysate.

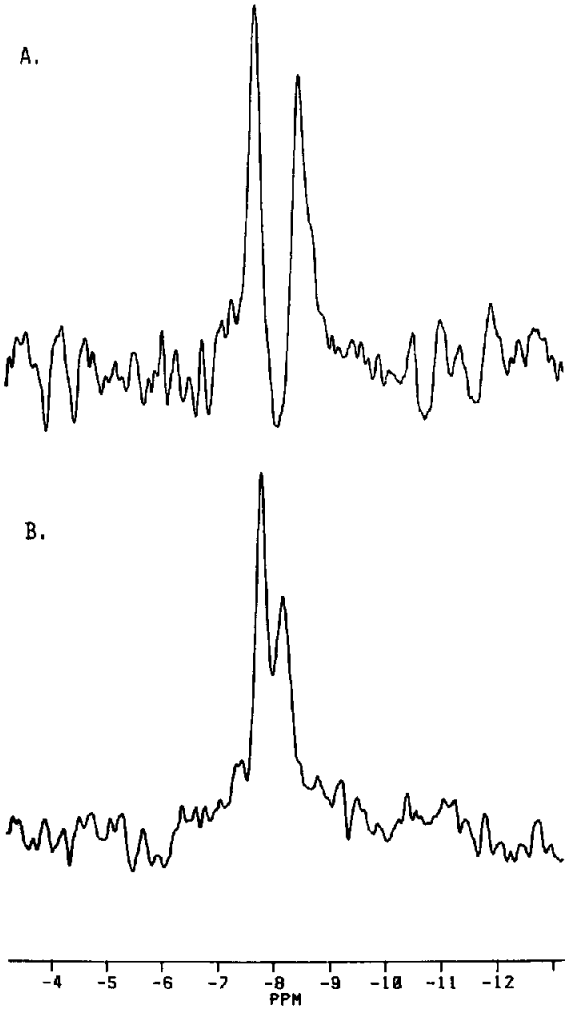

Fig.3. ^{19}F NMR spectra of the purified enzyme in the presence (B) and absence (A) of 2 mM ATP and 4 mM Mg^{2+}. Quantification of the fluorine signal and determination of the protein concentration indicated that the protein was approximately 60% fluorinated.

A cell lysate prepared from cells perfused under substrate-free conditions gave a ^{19}F NMR spectrum which was similar to that observed in the intact cell (fig.2A). The chemical shifts of the resonances in this spectrum are −7.78 ppm, −8.11 ppm and −9.17 ppm. Removal of small molecules from this extract by dilution and subsequent ultrafiltration gave the spectrum shown in fig.2B. The peak resonating at the frequency of free 5-fluorotryptophan has disappeared and the chemical shift difference between the protein resonances has increased. The chemical shifts of these resonances (−7.63 ppm and −8.50 ppm) are similar to those observed for the purified enzyme at −7.66 ppm and −8.43 ppm, respectively (fig.3A). Addition of 2 mM ATP to the purified enzyme, a concentration similar to that found in vivo [11], resulted in a broadening and significant downfield shift of the upfield resonance (fig.3B). The chemical shifts of the resonances in this spectrum are −7.81 ppm

and −8.19 ppm. Addition of higher concentrations of ATP resulted in a further downfield shift of the upfield resonance (data not shown). The chemical shifts of the protein resonances at 2 mM ATP are similar to those observed in cells perfused with glucose (fig.1B). ATP binding could account, therefore, for the chemical shift observed in vivo. However, ATP binding is not the only factor influencing the shifts since perfusion of cells under substrate-free conditions, when there is no ^{31}P NMR detectable ATP in the cells, results in a further downfield shift of the upfield resonance. This is the opposite to what would be expected if the shifts were only determined by ATP binding. The experiments on the cell extract prepared from cells perfused under substrate-free conditions show that other small molecules must also be affecting the shifts in vivo.

The shift observed in the upfield protein resonance on the binding of ATP to the purified enzyme leads to a tentative assignment of this resonance to Trp 308 since this residue is exposed to the solvent at the ATP binding site [13]. However, although the fluorine atom is within 5 Å of the adenine ring [13], the shift cannot be due to a ring current since it is of the opposite sign to that expected [14]. Changes in the hydrophobicity of the binding site may lead to the observed shift.

In conclusion, we have demonstrated that ^{19}F NMR can be used to monitor a fluorine-labelled enzyme in vivo and that the chemical shifts of the fluorine resonances are sensitive to the intracellular conditions. The techniques described here appear to offer the possibility of being able to study some aspects of enzyme-substrate and protein-protein interactions in vivo.

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