Estimation of the Intracellular Free ADP Concentration by ^{19}F NMR Studies of Fluorine-Labeled Yeast Phosphoglycerate Kinase in Vivo†

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ABSTRACT: Yeast phosphoglycerate kinase was selectively fluorine-labeled in vivo by inducing enzyme synthesis in stationary phase cells in the presence of 5-fluorotryptophan. Inducible expression was obtained using a galactose-inducible expression vector containing the yeast phosphoglycerate kinase coding sequence. ^{19}F NMR measurements on intact cells showed two resolved resonances, from the two tryptophan residues in the protein, which underwent reversible changes in chemical shift under different metabolic conditions. Measurements in vitro showed that the difference in the chemical shifts of these two resonances was dependent on the adenine nucleotide concentration, in particular the MgADP concentration. A comparison of the spectra obtained in vitro with those obtained from the intact cell indicated that in glucose-fed cells the cytosolic free MgADP concentration was less than 50 μM, which is significantly lower than the concentrations measured in whole-cell extracts.

Nuclear magnetic resonance (NMR) has been widely used to monitor small metabolites in vivo (Iles et al., 1982), but only rarely have resonances assigned to specific proteins been detected in intact cells and tissues. For example, in the human erythrocyte ^1H resonances from surface histidines of hemoglobin have been observed and in muscle hyperfine- and ring-current-shifted ^1H resonances from myoglobin have been detected. The chemical shifts of these resonances titrate with pH (Brown et al., 1977) and pO_2 (Kreutzer et al., 1992), respectively, and thus could be used to estimate these parameters in the intact cell. ^{13}C-labeled hemoglobin has also been detected in vivo in erythrocytes prepared from mice fed with L-[2-^{13}C]histidine (London et al., 1975). In all these cases, the protein observed was naturally present at high concentration and had some serendipitous property that allowed unambiguous resonance assignment. Specific observation by NMR of a chosen protein in a more typical cell requires some form of protein labeling. Fluorine-19, the 100% naturally abundant isotope, is an excellent label in this respect since it is a spin-$^1/_2$ nucleus with a high receptivity to detection by NMR and a wide chemical shift dispersion and it is absent from most living tissue, avoiding interference from background signals (Gerig, 1989).

Fluorine labels have successfully been introduced into many different proteins using biosynthetic incorporation of fluoroamino acids (Sykes & Weiner, 1980). The purified proteins have then been studied in vitro using ^{19}F NMR. We have shown that in the yeast *Saccharomyces cerevisiae* a single protein species can be selectively fluorine-labeled in the intact cell by using an inducible expression vector containing the coding sequence of the protein of interest. When induced in stationary phase cells, the target protein is the predominant protein synthesized in the cell, and a fluorine-labeled amino acid added to the medium at this point is incorporated essentially into a single protein. The labeled protein can then be observed in the intact cell using ^{19}F NMR. We have used this technique previously to fluorine-label the two tryptophan residues in yeast 3-phosphoglycerate kinase (PGK)[1] by biosynthetic incorporation of 5-fluorotryptophan (Brindle et al., 1989). We show here that the ^{19}F NMR chemical shifts of these tryptophan resonances in PGK are sensitive primarily to the MgADP concentration.

The free cytosolic MgADP concentration is an important parameter to determine in studies of cellular energy metabolism since it represents the metabolically relevant free concentration of the molecule in the cytosol. In contrast, measurement of the total cellular ADP concentration in cell extracts also includes compartmentalized and macromolecular-bound forms of the nucleotide which are not available to participate in cytosolic reactions. The fluorinated PGK can be used in the cell as a probe of the free cytosolic MgADP concentration in the same way that hemoglobin and myoglobin were used as probes of pH and pO_2, respectively (Brown et al., 1977; Kreutzer et al., 1992). This work has suggested a more general strategy for producing ^{19}F-labeled protein and peptide NMR probes for the free concentrations of a variety of small molecules in the intact cell.

MATERIALS AND METHODS

General. The yeast strain used was *S. cerevisiae* BJ2168 (**a**, ura3–52, leu2–3, leu2–112, trp1, pep4–3, prb1–1122, prc1–407) (Jones, 1990). Yeast growth media were obtained from Difco Laboratories. Low-gelling-temperature agarose, 3-phosphoglycerate, 5-fluoro-DL-tryptophan, and protease inhibitors were obtained from Sigma Chemical Co. Rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase, dithiothreitol, and the sodium salts of AMP, ADP, and ATP were obtained from Boehringer Mannheim. Protein concentrations were determined using a dye-binding assay (Bradford, 1976) kit from Bio-Rad Laboratories with bovine serum albumin as the standard. Chelex resin was obtained from Bio-Rad Laboratories. Other chemicals used were of reagent grade from various suppliers.

Cell Growth and Induction. The plasmid pKV43 contains the PGK coding sequence under the control of a galactose-

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; PGK, 3-phosphoglycerate kinase (EC 2.7.2.3); GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); SDS–PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate; NMP, NDP, NTP, nucleoside 5′-mono-, di-, and triphosphate; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; 1,3-BPG, 1,3-bisphosphoglycerate.

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inducible version of the strong PGK promoter, the GAL1-10 UAS replacing the PGK UAS (Johnston, 1987; Cousens et al., 1990; Kingsman et al., 1990). The synthesis of PGK from this vector occurs only in the presence of galactose and absence of glucose, and it can thus be regulated by simple manipulation of the growth medium.

BJ2168 cells were transformed with the plasmid pKV43 by the method of Hinnen et al. (1978). The transformed cells were grown on defined medium containing 2% w/v glucose, 0.67% w/v yeast nitrogen base, 0.002% w/v uracil, and 0.002% w/v tryptophan. The leucine auxotrophy in BJ2168 cells was complemented in transformed cells by the presence of the LEU2 gene in the vector pKV43. Induction of PGK synthesis was carried out using a protocol adapted from Broker et al. (1991).

A single colony was inoculated into 50 mL of minimal medium and incubated aerobically in an orbital incubator at 30 °C. After 24 h, 1 mL of this culture was used to inoculate 100 mL of the same medium, and this culture was then grown for a further 48 h. The 100-mL starter culture was added to 1 L of complete medium, which comprised 2% w/v glucose, 2% w/v Bacto-Peptone, and 1% w/v yeast extract. Each liter of culture was incubated aerobically in an orbital incubator in a 5-L conical flask at 30 °C. After growth for 20 h, when the culture had reached stationary phase, galactose was added to the medium to 2% w/v. Six hours later, when the induction process was established, 0.2 g of filter-sterilized 5-fluoro-DL-tryptophan in 50 mL of water was added to each liter of culture. The cultures were incubated for a further 66 h before chilling and harvesting by centrifugation.

Cell Immobilization and Perifusion. Cells were washed twice at 4 °C in a buffer containing 50 mM MES, 2 mM $MgSO_4$, 2 mM KCl, and 1.7 mM NaCl at pH 6.0. Four grams of cells were briefly warmed to 37 °C and mixed with 4 mL of a solution of 1.8% w/v low-gelling-temperature agarose dissolved in the same buffer. The mixture was held at 37 °C and extruded through 0.5-mm i.d. tubing under ice into a 20-mm diameter NMR tube containing chilled buffer to form fine agarose gel threads which entrapped the cells (Foxall et al., 1984; Brindle & Krikler, 1985). The threads were confined to a volume of 16.5 mL in the NMR tube by a perforated vortex plug 65 mm above the bottom of the tube.

The immobilized cells were perifused at 25–30 mL/min with 1 L of recirculating buffer at 30 °C. For anaerobic experiments, the medium was sparged with nitrogen, and an oxygen electrode was used to confirm that the medium arriving at the thread bed was free of oxygen. For aerobic experiments, the medium was sparged with oxygen. Glucose (50 mM) or 2% v/v ethanol was added to the medium when appropriate. Perifusions typically lasted 5 h. Cell extracts were prepared by perchloric acid extraction following rapid freezing and grinding in liquid nitrogen. Cell extracts were neutralized, treated with Chelex resin, and lyophilized prior to reconstitution in 50 mM triethanolamine and 5 mM EDTA, pH 8.0. The intracellular concentrations of the nucleotides were measured in perchloric acid cell extracts by ^{31}P NMR. The NTP in similar glucose-fed cells has been shown by HPLC to be approximately 80% ATP and 20% GTP (Brindle et al., 1990).

Purification of PGK. The purified labeled PGK used for the in vitro studies was prepared from the same batches of cells that were used for the in vivo experiments. Cell lysates were prepared by mechanical disruption of the cells with glass beads at 4 °C in 50 mM sodium phosphate, 5 mM EDTA, 1 mM 1,10-phenanthroline, and 2 mM dithiothreitol, pH 7.5. A protease inhibitor cocktail was added to provide 1 μM pepstatin A, 10 μM E64, and 100 μM 3,4-dichloroisocoumarin. PGK was purified from the lysates according to Fifis and Scopes (1978). The purified PGK appeared homogeneous under SDS–PAGE. PGK activity was assayed at room temperature in the coupled reverse reaction with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) (Bücher, 1947) under conditions described in Wilson et al. (1988) but using rabbit muscle GAPDH as the coupling enzyme. The specific activity of both fluorinated and nonfluorinated PGK prepared by this method was around 400–450 units/mg. PGK was stored at 4 °C as an ammonium sulfate precipitate prior to use. For NMR studies, PGK was desalted and resuspended to approximately 25 mg/mL (approximately 0.6 mM) in 50 mM HEPES, 130 mM potassium acetate, and 2 mM dithiothreitol, pH 7.2. Samples contained 10% v/v ^2H_2O as a field frequency lock. pH measurements were not corrected for any deuterium isotope effect.

NMR Measurements. NMR experiments were performed using an 89-mm bore 9.4-T Oxford Instruments magnet and Varian Unity 400 spectrometer. The resonant frequencies of ^1H, ^{19}F, and ^{31}P at this field were 399.95, 376.29, and 161.90 MHz, respectively. ^{19}F spectra of cells and purified PGK were collected without ^1H decoupling with Varian 25-mm $^1H/^{19}F$ and 5-mm $^1H/^{19}F$ probes, respectively. ^{31}P spectra of cells and cell extracts were collected with a home-built 20-mm probe and a Varian 10-mm broad-band probe, respectively. Spectra were processed and analyzed with standard Varian VNMR software; the integration routines were validated by weighing peaks cut from paper plots of NMR spectra. Extract spectra were acquired with broad-band ^1H decoupling. The acquisition conditions used to acquire particular spectra are indicated in the appropriate figure legends.

^{31}P NMR spectra obtained from perifused cells were used to determine the pH (Moon & Richards, 1973; Seo et al., 1983) and estimate the free magnesium ion concentrations (Gupta et al., 1978, 1983a,b) in the same cells used for the ^{19}F NMR studies. The ^{31}P NMR orthophosphate chemical shift vs pH titration curve used in this work was determined according to the method of den Hollander et al. (1981).

RESULTS

Induction and Fluorine Labeling of PGK. The PGK content of wild-type cells was approximately 1000 units/mL of cell water. After galactose-induced expression of PGK from the vector pKV43 in the presence of 5-fluorotryptophan, the PGK content was elevated to approximately 10 000 units/mL of cell water, around 25–30 mg of PGK/mL of cell water. Comparison of the protein and fluorine concentrations in samples of purified labeled PGK showed that the fractional replacement of tryptophan by fluorotryptophan was 25–30%. Higher fractional replacement values (approximately 60%) could be obtained using other induction protocols (Williams, 1992) but at the expense of much lower protein yield.

Previous work has shown that changes in PGK concentration in the cell over a range from one-twentieth to twenty times wild-type levels have very little effect on glycolytic flux and cell growth rates (Brindle, 1988; Williams, 1992). The glucose consumption rates of the glucose-fed cells used in this work were comparable to those measured in other strains in this laboratory (Brindle, 1988; Davies & Brindle, 1992) (data not shown). The similarity of the ^{31}P NMR spectra (see below) and glucose consumption rates showed that the cells were not

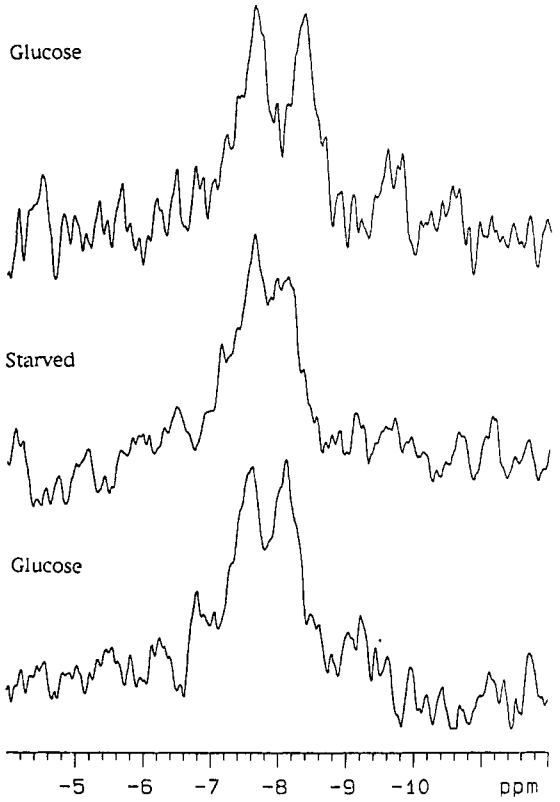

FIGURE 1: ^{19}F NMR spectra of intact cells containing 5-fluorotryptophan-labeled PGK perifused first with glucose (bottom), then starved (middle), and then with glucose again (top). Four grams of cells containing approximately 150 μM 5-fluorotryptophan-labeled 3-phosphoglycerate kinase in the cell water were perifused with 100 mM glucose or substrate-free while each spectrum was collected. A 1-h equilibration period was allowed before collecting each spectrum. The spectra are the summation of 10 000 transients with a pulse repetition time of 0.67 s and a 65° pulse width. The chemical shift axis is referenced to aqueous fluorophenylalanine in a coaxial capillary at 0.0 ppm. All the spectra are presented on the same scale and were processed with a 25-Hz exponential line broadening.

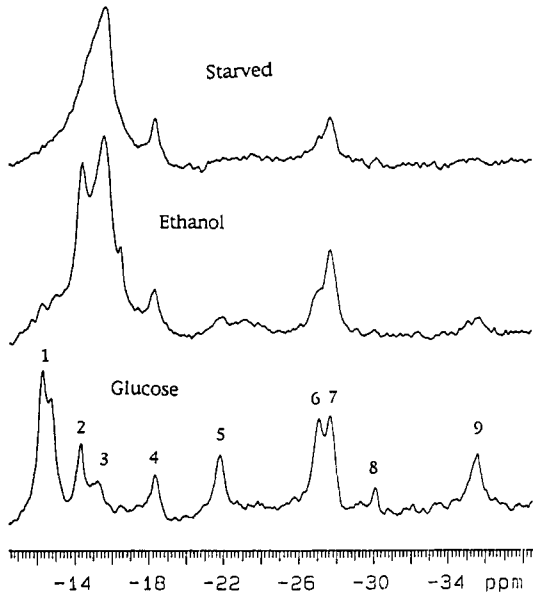

FIGURE 2: ^{31}P NMR spectra of intact yeast cells containing 5-fluorotryptophan-labeled PGK under different metabolic conditions. Four grams of cells containing approximately 150 μM 5-fluorotryptophan-labeled 3-phosphoglycerate kinase in the cell water were perifused with a medium containing glucose (bottom), ethanol (middle), or no substrate (top). The spectra were acquired with an interpulse delay of 5.4 s and a spectral width of 10 000 Hz. A total of 256 scans were collected over 23 min for each spectrum. All the spectra are presented on the same scale and processed with a 25-Hz exponential line broadening. The chemical shift values are quoted with reference to MDP at 0.0 ppm. The ethanol-fed cells were aerobic, the others anaerobic. The numbered peaks were assigned by reference to previous work [Brindle, K. M. (1988) *Biochemistry 27*, 6187–6196, and references therein]: 1, phosphomonoesters (particularly sugar phosphates); 2, cytoplasmic phosphate; 3, vacuolar phosphate; 4, phosphodiesters; 5, γ-NTP and β-NDP; 6, α-NTP and α-NDP; 7, NAD(P)(H); 8, NDP-sugars; 9, β-NTP.

significantly perturbed by the presence of the fluorinated protein.

NMR Spectra of Intact Cells Containing Fluorine-Labeled PGK. ^{19}F and ^{31}P NMR spectra of perifused cells under different metabolic conditions are presented in Figures 1 and 2, respectively. The ^{19}F spectra clearly show two distinct resonances, one from each of the two tryptophans in PGK. No resonances could be detected in control cells which were incubated with fluorotryptophan but in which PGK induction had not taken place. The ^{31}P NMR spectra of the glucose-fed cells are typical of those obtained from other strains of *S. cerevisiae* in this laboratory (Brindle, 1988) and others (Navon et al., 1979; den Hollander et al., 1981; Campbell-Burk et al., 1987). With the growth protocol described here, the cells are depleted of vacuolar polyphosphates and do not show the corresponding resonances.

When glucose-fed cells were starved, the upfield resonance in the ^{19}F NMR spectra moved downfield and broadened; these trends were reversed when the cells were re-fed glucose (see Figure 1 and Table I). Table I lists the ^{19}F chemical shift differences between the two resonances in cells under different metabolic conditions and the corresponding changes in nucleoside phosphate concentrations. There was a substantial decrease in the concentration of the nucleoside triphosphates upon starvation, seen both in the ^{31}P NMR spectra of intact cells and in perchloric acid extracts prepared from these cells (see Figure 2 and Table I). The concentration of NTP, predominantly ATP, in glucose-fed cells was approximately 3 mM. The extracts were prepared after 3 h of cell perifusion, i.e., to the midpoint of a typical cell perifusion used for collecting data from the intact cells.

The ^{19}F NMR spectra obtained in this work were very similar to those obtained in a different cell type and at a lower magnetic field strength (Brindle et al., 1989), but with higher resolution, as illustrated in Figure 3. There is approximately a 0.1 ppm difference in the absolute chemical shift values recorded in the two cell types, but the chemical shift differences ($\Delta\delta$) between the resonances observed were the same. The difference in the absolute values of the chemical shifts is probably the result of the two cell types having different magnetic susceptibilities, possibly the result of the polyphosphate depletion in BJ2168 causing high levels of paramagnetic ions in the cytoplasm (Klionsky et al., 1990).

Because the strongly field-dependent chemical shift anisotropy component of ^{19}F relaxation is large, one might expect that increases in the magnetic field strength would lead to poorer spectral resolution, especially in a large molecule. This is not the case, and the improvement in resolution obtained at 9.4 T compared with 7 T is, in fact, in accord with calculations of the expected line widths and peak separations at the different fields for 5-fluorotryptophan held rigidly in an isotropically tumbling molecule the size of PGK (Williams, 1992).

The cell line used for the 7-T studies, FY3-1, appeared to degrade the fluorinated PGK on a time scale consistent with its being proteolytically turned over by the normal degradative pathways, i.e., with a half-time of about a day when glucose-

Table I: Intracellular Nucleotide Concentrations and the Chemical Shift Positions of the Two Peaks in the ^{19}F NMR Spectrum of 5-Fluorotryptophan-Labeled PGK in Intact Yeast Cells[a]

perifusion condition	[NMP] (mM)	[NDP] (mM)	[NTP] (mM)	chemical shift of the downfield F-PGK peak (ppm)	chemical shift of the upfield F-PGK peak (ppm)	chemical shift difference (ppm)	*n*
glucose (anaerobic)	0.1	0.36	2.96	−7.51 ± 0.07	−8.06 ± 0.04	0.55 ± 0.09	6
glucose (aerobic)	0.1	0.26	2.56	−7.51 ± 0.02	−8.08 ± 0.02	0.56 ± 0.02	3
ethanol (aerobic)	0.83	1.24	0.67	−7.57 ± 0.04	−7.98 ± 0.05	0.41 ± 0.06	4
starved (anaerobic)	1.46	1.44	0.74	−7.59 ± 0.03	−7.93 ± 0.03	0.34 ± 0.04	10

[a] Four grams of yeast cells immobilized in agarose threads were perifused under the conditions indicated. The nucleotide concentrations were determined from ^{1}H-decoupled ^{31}P NMR spectra of perchloric acid extracts of the cells. The nucleotides of different bases cannot be individually quantitated by this technique, but HPLC analysis showed that the adenine nucleotides predominate. The values of the nucleotide concentrations are the means of two determinations, which were not significantly different from each other. The ^{19}F NMR spectra were acquired as described in the legend to Figure 1. Chemical shifts are upfield from an external *p*-fluorophenylalanine standard at 0.0 ppm. For the chemical shift data, *n* is the number of experiments performed, and the figures are means quoted with the standard deviations.

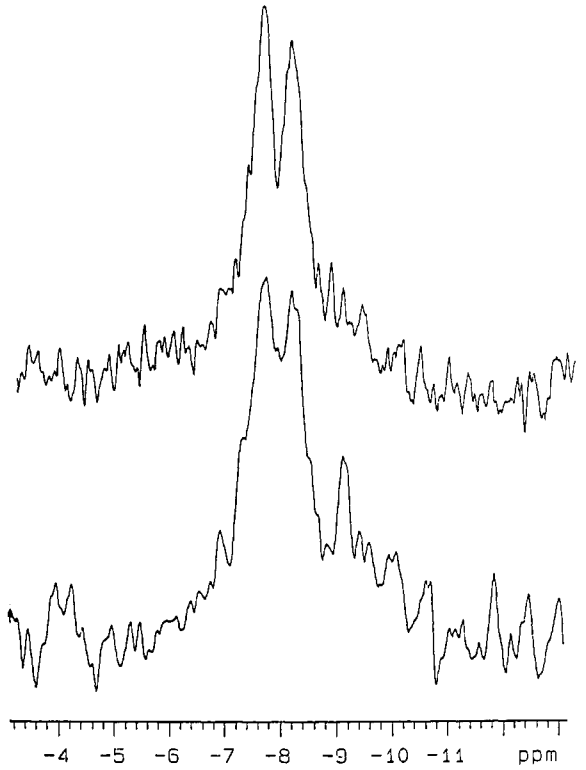

FIGURE 3: ^{19}F NMR spectra of intact yeast cells containing 3-phosphoglycerate kinase labeled with 5-fluorotryptophan. The spectra were acquired from 4 g of cells perifused anaerobically at 30 °C with a 50 mM glucose solution. The interpulse delay was 0.67 s, the spectral width 12 000 Hz, and the pulse angle 65° (typically 80–100 μs). Both spectra are presented on the same scale and processed with a 25-Hz exponential line broadening. The 9.4-T spectrum is, however, displaced approximately 0.1 ppm to allow a better comparison of the spectra. This is to compensate for an apparent difference in the bulk magnetic susceptibility of the two cell types (see text). The chemical shift scale is referenced to aqueous fluorophenylalanine in a coaxial capillary at 0.0 ppm. The top spectrum was obtained at 9.4 T (^{19}F resonance frequency 376 MHz) from cells of the BJ2168 strain. A total of 2000 scans were accumulated over 22 min. The cells contained approximately 300 μM labeled PGK in the cell water. The bottom spectrum was obtained at 7 T (^{19}F resonance frequency 282 MHz) from cells of the FY3-1 strain. A total of 24 576 scans were accumulated over 4.5 h. The cells contained approximately 100 μM labeled PGK in the cell water.

fed and approximately 10 times more rapidly when starved (Bachmair et al., 1986; Dice, 1987; Chiang & Schekman, 1991). The degradation was evident as a loss of signal intensity at approximately −8 ppm and a commensurate increase in signal intensity at approximately −9.2 ppm, the chemical shift position of fluorotryptophan free in aqueous solution. The studies at 9.4 T employed a cell line, BJ2168, lacking functional pathways of general proteolytic degradation (Jones, 1990), and these cells showed very little accumulation of material at −9.2 ppm even when starved.

The visibility of the fluorinated PGK in vivo was assessed by comparing the signal intensities of fully relaxed spectra from cells and from extracts prepared from the same cells by mechanical disruption with glass beads (data not shown). Within the error of the measurements, the fluorinated PGK was wholly visible, suggesting that there was no large pool of PGK tightly bound to cellular structures rendering it NMR-invisible. The spectra obtained from intact cells were deconvoluted and could be approximated well as the sum of two Lorentzian lines, but the signal-to-noise ratio of the spectra prevents ruling out altogether the presence of a very broad component under the two principal peaks. However, the high visibility of the fluorinated PGK in the cell makes it unlikely that there is a large immobile component which could give rise to a very broad resonance.

The line widths of fluorinated PGK measured in glucose-fed cells were approximately 110–130 Hz, measured by fitting Lorentzian lines to the spectra with standard Varian VNMR software. The line widths of purified PGK studied at 30 °C in a HEPES/acetate buffer (pH 7.15, $I = 0.15$ M) were also measured. In the absence of nucleotide, the line widths were 35 Hz, or 50 Hz if the viscosity relative to water was raised to 1.9 by including glycerol in the buffer. These line widths are in excellent agreement with those calculated for the relaxation of an immobile fluorine atom in an isotropically tumbling sphere with a rotational correlation time similar to that expected for PGK (Harris, 1986; Williams, 1992). In the absence of glycerol but in the presence of approximately 1 mM MgATP and 50 μM MgADP, the upfield peak line width was broadened by fast ligand-binding exchange processes to 120 Hz, comparable to that seen in vivo. These data indicate that the fluorinated PGK is tumbling in a cytoplasmic medium of near-aqueous viscosity, without significant motional restriction from binding to cellular components (Williams, 1992). This low estimate of the cytoplasmic viscosity is in good agreement with recent measurements in mammalian cells using picosecond polarization decay fluorescence (Dix & Verkman, 1990; Fushimi & Verkman, 1991).

Effects of Ligand Binding on the ^{19}F NMR Spectra of Fluorotryptophan-Labeled PGK. The binding properties of the adenine nucleotides to PGK are relatively complex and depend on the distribution of the various ionic forms (Bishop et al., 1990). The influence of pH, ionic strength, and free Mg^{2+} concentration on the equilibrium constants and kinetic properties of phosphate-transfer reactions has been measured in several systems, including the PGK–GAPDH couple (Cornell et al., 1979; Lawson & Veech, 1979; Veech et al., 1979). In the experiments described here, the ionic strength,

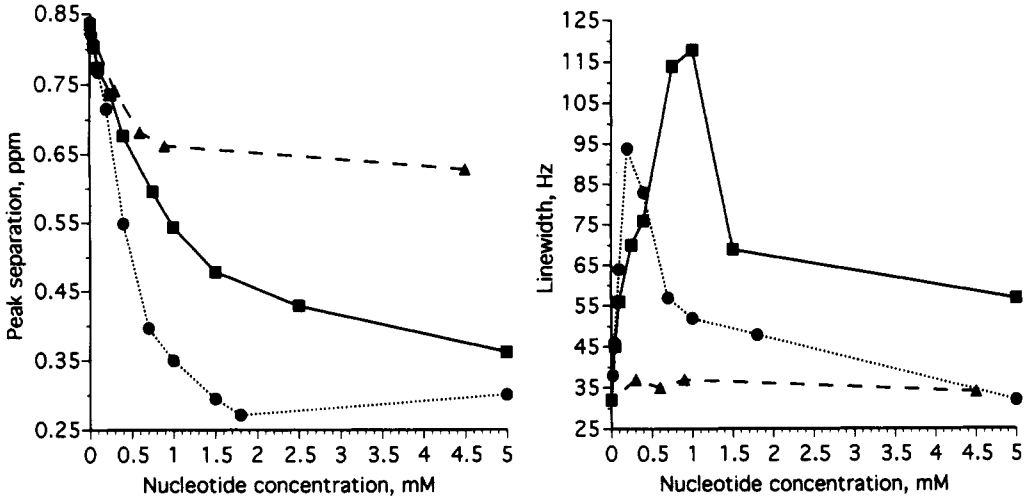

FIGURE 4: Data from ^{19}F NMR spectra of fluorotryptophan-labeled PGK in vitro, showing (A, left) the peak separation, $\Delta\delta$ (ppm), and (B, right) the line width of the upfield peak, as functions of the magnesium nucleotide concentration. The magnesium concentration was 0.5 mM in excess of the nucleotide concentration. The line widths were determined by iterative fitting to a Lorentzian line using Varian's VNMR software. The spectra were collected at 30 °C and 9.4 T using the same acquisition parameters as described in Figure 3. ATP (■), ADP (●), AMP (▲).

pH, and free Mg^{2+} concentrations were similar to those measured in the intact cell. The ionic strength was taken to be 0.15 M (Gancedo & Gancedo, 1973). The cytoplasmic pH, measured from the chemical shift of the cytoplasmic P_i resonance, was 7.2. The free magnesium concentration measured from the chemical shift difference between the NTP-α and -β resonances was approximately 3 mM. The concentration measured in other cell types in this laboratory is somewhat less than this, typically 0.5–1 mM. The high free magnesium ion concentration seen in these cells may reflect the fact that they have very little polyphosphate to sequester magnesium in the vacuole (Okorokov et al., 1980; Klionsky et al., 1990). The measurement is subject to a large error since the K_d for the Mg–ATP interaction [50 μM (Gupta et al., 1983a)] is very much less than the free magnesium ion concentration (London, 1991). Consequently experiments were performed in vitro at both high (3 mM) and low (0.5 mM) free magnesium ion concentrations. The total Mg^{2+} concentration required to give the designated free Mg^{2+} concentration was calculated from the pH-corrected equilibrium constants (Lawson & Veech, 1979). These were quoted for a temperature of 38 °C and were not corrected for the temperature used in this study of 30 °C.

The Effect of pH. The pH was varied between 6 and 8 with no significant effects on the chemical shifts of the two tryptophan resonances.

The Effect of the Triose Phosphates. The crystal structure suggested that the triose ligands could only have indirect effects on the tryptophan residues since the triose site is much farther away from the two tryptophans than the nucleotide site (Watson et al., 1982). Titrations with 3-phosphoglycerate up to a concentration of 1 mM gave only a small change in the chemical shift difference between the two peaks, of around 0.1 ppm. Since the steady-state concentration of 3-phosphoglycerate in vivo is around 300 μM (Brindle, 1988), it was concluded that physiological changes in the level of this ligand were unlikely to significantly influence the spectra recorded from intact cells.

1,3-Bisphosphoglycerate is unstable and was therefore generated in situ in the presence of ATP and 3-phosphoglycerate. The equilibrium mixture was established from starting concentrations of 4 mM MgATP and 10 mM 3-phosphoglycerate and thus contained about 1.7 mM 1,3-bisphosphoglycerate (1,3-BPG) and ADP, since the equilibrium constant is approximately 3.6×10^3 (Cornell et al., 1979). The chemical shift difference between the two resonances was 0.93 ppm in the absence of ligands, 0.46 ppm in the presence of MgATP alone, and 0.34 ppm in the presence of the equilibrium mixture. Since the K_d for 1,3-BPG binding is very tight, around 50–60 nM (Scopes, 1978; Fairbrother et al., 1990a), the 0.6 mM PGK used in these experiments should be fully saturated with 1,3-BPG. The small incremental change in chemical shift difference in the presence of 1,3-BPG can be accounted for, at least qualitatively, by the presence of MgADP (which has a large effect on the chemical shifts; see below), and so the presence of the 1,3-BPG was thought to be unimportant in determining the appearance of the spectra obtained from the intact cell.

The Effect of the Adenine Nucleotides. The PGK concentration used was 25–30 mg/mL (about 0.6 mM), the same as measured in the cells used for this work. The pH was 7.15.

Typical titration curves are shown in Figure 4a, which shows the chemical shift difference between the two peaks as a function of nucleotide concentration. The total magnesium concentration was maintained at 0.5 mM in excess of the total nucleotide concentration. The line widths showed a pronounced variation with the nucleotide concentration, being maximal at intermediate ligand concentration. This is what would be expected from exchange broadening in which the two species of labeled PGK (ligand-free and ligand-bound) are in fast exchange, and the "average" resonance is observed (Harris, 1986). The line width of the upfield peak as a function of nucleotide concentration is plotted in Figure 4b.

These data have only been treated in a qualitative way, because the analysis of the situation is complicated by the existence of a second, nonindependent binding site for ATP (Larsson-Raznikiewicz, 1973; Schierbeck & Larsson-Raznikiewicz, 1979; Fairbrother et al., 1990b). As the Mg–nucleotide concentrations are increased beyond those shown in Figure 4, some reversal of the trend toward smaller peak separations occurs. This is evident for ADP binding in Figure 4a but also occurs for ATP at MgATP concentrations in excess of around 10 mM. This effect may arise from the nonspecific interaction of high concentrations of ATP or ADP at the general anion binding site (Fairbrother et al., 1990a). The data show that the affinities of the nucleotides for PGK are

Table II: Comparison of the ^{19}F NMR Chemical Shift Differences between the Two Peaks of 5-Fluorotryptophan-Labeled PGK at 30 °C either in the Intact Cell or in Vitro[a]

	^{19}F NMR chemical shift difference (ppm)	
metabolic condition	in vitro	in vivo
starved (anaerobic)	0.28	0.34
ethanol-fed (aerobic)	0.28	0.41
glucose-fed (anaerobic)	0.28	0.55

[a] In vitro, the pH and ionic strength and the concentrations of enzyme, nucleotides, and free magnesium ions were similar to those measured in the intact cell under the specified conditions. The intact cell data are taken from Table I. The samples included approximately 0.6 mM labeled PGK in 50 mM HEPES, 130 mM potassium acetate, and 2 mM dithiothreitol (pH 7.2, I = 0.15 M). The free magnesium ion concentration, determined from the chemical shift difference of the α and β peaks of NTP in the ^{31}P NMR spectra of intact cells, was approximately 3 mM. The nucleotide concentrations used were similar to those measured in perchloric acid extracts of the cells, assuming that all the nucleotides present were adenine nucleotides. The concentrations used were as follows: starved, 1.46 mM AMP, 1.44 mM ADP, and 0.74 mM ATP; ethanol-fed, 0.83 mM AMP, 1.24 mM ADP, and 0.67 mM ATP; glucose-fed 0.1 mM AMP, 0.36 mM ADP, and 2.96 mM ATP. The experiments were performed twice, and the mean results are shown. There was no significant difference between the repeats.

Table III: Chemical Shift Difference, $\Delta\delta$, between the Two Peaks of 5-Fluorotryptophan-Labeled PGK at Different ATP, ADP, and Free Mg Concentrations[a]

	[ATP] (mM)	[ADP] (mM)	[free Mg^{2+}] (mM)	$\Delta\delta$ (ppm)
1	0	0	0	0.86
2	2.9	0.05	0	0.82
3	2.9	0.05	0.5	0.62
4	2.9	0.1	0.5	0.53
5	2.9	0.05	3.0	0.50
6	2.9	0.3	0.5	0.46
7	2.9	0.1	3.0	0.42
8	2.9	0.3	3.0	0.36
9	2.9	0.3	>7	0.33

[a] Approximately 0.6 mM labeled PGK was studied at 30 °C in a buffer containing 50 mM HEPES, 130 mM potassium acetate, and 2 mM dithiothreitol (pH 7.2, I = 0.15 M). The ADP concentration includes 50 μM from the ATP solution. ^{31}P NMR spectra of the sample showed broad signals from the enzyme-bound nucleotides and little inorganic phosphate, consistent with there being no significant hydrolysis of the ATP to ADP on the time scale of the measurements.

different, with ADP > ATP ≫ AMP. This is in agreement with previous measurements by gel filtration studies (Scopes, 1978) and inhibitor titrations (Larsson-Raznikiewicz & Arvidsson, 1971).

The Effects of Mixtures of the Adenine Nucleotides. Samples of 5-fluorotryptophan-labeled PGK were studied in buffers containing combinations of nucleotides at the same concentrations as those found in the starved, ethanol-fed, and anaerobic glucose-fed cells. The free magnesium concentration was set at 3 mM. The results are shown in Table II. The chemical shift differences between the two peaks in ^{19}F spectra obtained in vitro were significantly different from spectra obtained from labeled PGK in the intact cell.

The differences between the ^{19}F spectra obtained in vitro and in vivo were characterized with further studies of labeled PGK in vitro, using different combinations of free magnesium ion and ADP concentrations. The experiments were performed so that combinations of high ADP/low magnesium and high magnesium/low ADP were present. The data are presented in Table III.

Inspection of lines 2, 3, and 5 of Table III emphasizes the importance of the free magnesium ion concentration. Note that the chemical shift difference in vitro most closely resembles that seen in the glucose-fed cells in vivo (0.55 ppm) when the ADP concentration is very low; see lines 3, 4, and 5. The chemical shift differences in vivo are presented in Table I.

DISCUSSION

The concentration of free cytosolic ADP (i.e., ADP and MgADP free in solution in the cytosol and available to participate in metabolic reactions) in the cell is an important parameter to determine in studies of energy metabolism (Atkinson, 1977). It can regulate ATP generation directly and indirectly through the generation of AMP in the adenylate kinase reaction. Differences between the concentrations of free and total ADP measured by ^{31}P NMR have been reported previously (Gadian, 1982; Iles et al., 1982). In muscle, actin-binding appears to sequester ADP, but in kidney and liver cellular compartmentation may be more important (Stubbs et al., 1984).

The work reported here shows that the free cytosolic MgADP concentration can be estimated directly from ^{19}F NMR spectra of labeled PGK in intact yeast cells. Assuming that the concentration of ATP to which the labeled enzyme is exposed in the cell is the same as that measured in ^{31}P NMR spectra of intact cells or in cell extracts, then limits can be placed on the free MgADP concentration available to the enzyme in vivo. These limits depend on the intracellular free magnesium ion concentration. If the free magnesium ion concentration is 3 mM, i.e., the apparent concentration in the cells used in this work, then the available MgADP concentration would be approximately 50 μM (line 5 of Table III). Since the estimate of free magnesium is subject to a relatively large error, an upper limit for the available ADP concentration was obtained using a free magnesium ion concentration of 0.5 mM, a value typical in some other yeast strains used in this laboratory. The intracellular ADP concentration would then be somewhat higher, approximately 100 μM (line 4 of Table III). These values for the available ADP concentration are 3–7 times lower than the total cellular concentration measured in cell extracts (0.36 mM).

Of the ADP apparent to the PGK probe, the fraction which is bound to the relatively high concentration of PGK itself in the cell can be estimated from the K_d for MgADP binding to PGK. Scopes gives a value for this K_d of 40 μM (Scopes, 1978). The K_d can also be estimated from the ^{19}F NMR spectra of the enzyme–nucleotide mixtures. The apparent line width, made up of contributions from ligand-free and ligand-bound PGK, is greatest when the contributions (and hence populations) are equal, i.e., when the MgADP concentration is equal to the K_d (Harris, 1986). Measured by this technique, the apparent K_d was approximately 300 μM (see Figure 4b). These values set an upper limit of approximately 40 μM on the free cytosolic MgADP concentration in the intact yeast cell, a value comparable to that tentatively derived from measurements of the near-equilibrium concentrations of the substrates of PGK and GAPDH (Brindle, 1988).

Estimates of the free cytosolic ADP concentration have been made previously in other systems by measuring the near-equilibrium concentrations of the substrates of creatine kinase (EC 2.7.3.2). For example, in brain and muscle the ADP concentration measured in cell extracts was up to 20 times greater than the free ADP concentration estimated in vivo (Veech et al., 1979). Similarly, creatine kinase has been successfully expressed in the livers of transgenic mice and used in conjunction with noninvasive ^{31}P NMR measurements as a probe of the free ADP concentration, where the results suggested a similarly low value of approximately 50 μmol/g wet weight (Brosnan et al., 1990).

The problems associated with calibrating the intracellular response of the labeled PGK to MgADP concentrations is a feature of any probe-based study. The empirical calibration of the labeled protein with model solutions employed in this study is also necessary with other probes observed in vivo, such as the fluorescent probes of cellular viscosity (Dix & Verkman, 1990), luminescent aequorin-based calcium probes (Rizzuto et al., 1992), and NMR ion probes (Kirschenlohr et al., 1988). PGK is not an ideal probe for the intracellular free MgADP concentration because of the relatively complex interaction between its nucleotide ligands and magnesium ion concentration. However, the low K_d of the enzyme for MgADP, which is significantly lower than that for MgATP (Scopes, 1978) (also evident in Figure 4b), means that it does show reasonable discrimination against MgATP and makes it responsive primarily to the MgADP concentration. This is clear from Table III.

The results obtained in this study suggest a general strategy for measuring the free concentration of almost any small molecule in the cell by introducing a fluorinated protein "probe" which binds the molecule of interest. This approach has a precedent in the development of protein probes which can be detected optically. For example, intracellular calcium ion transients have been monitored with high sensitivity using the jellyfish-derived bioluminescent protein aequorin as the probe molecule (Rizzuto et al., 1992). Engineering aequorin to make it responsive to ligands other than calcium has been suggested as a general way of investigating intracellular metabolite concentrations (Campbell, 1990). The complementary approach, to derivatize a ligand-binding protein to give it the desired optical properties, has been illustrated with cAMP-dependent protein kinase and, more recently, with calmodulin. Both of these proteins have been fluorescently tagged, microinjected, and studied in single cells. Fluorescence ratio imaging of labeled calmodulin allowed investigations of its activation within the cell (Hahn et al., 1992), while resonance energy transfer studies of the kinase allowed the measurement of cAMP levels within single cells (Adams et al., 1991).

Although they can be monitored with high sensitivity, optical probes have a number of disadvantages, in some situations, when compared to the less sensitive NMR protein probes proposed here. This may make the latter more suitable for some purposes. Tailoring a bioluminescent protein such as aequorin to monitor a given ligand requires that the probe is functionally coupled to a ligand-binding element. Optically tagging a preexisting protein probe, as with calmodulin, requires the subsequent microinjection of the probe into the cells examined. The NMR approach described here is, in principle, much more flexible since it only requires the selection of an appropriate cloned protein which binds the molecule of interest and whose ^{19}F NMR spectrum changes in response to ligand binding. This may require that the labeled amino acids are close to the binding site. If there were no suitable residues, then these could be introduced using site-directed mutagenesis. The possibility of engineering proteins for ligand-binding studies has been illustrated recently in calmodulin, where tryptophan residues introduced by mutagenesis close to the calcium binding sites were used as fluorescence probes of the mechanism of the Ca^{2+}–calmodulin interaction (Kilhoffer et al., 1992). Luminescent or fluorescent probes are necessarily limited to systems where they can be optically monitored, precluding their use in many tissues and animal organs. The methods described here should be applicable in these situations, although studies in higher organisms will clearly depend on the development of appropriate systems for the rapid induction of a particular protein species. Fluoroamino acids, although toxic, can be readily introduced into higher organisms, for example hemoglobin has been fluorinated by feeding rabbits *p*-fluorophenylalanine (Gerig et al., 1983).

Although the intracellular concentration of the PGK probe molecule used here was approximately 0.6 mM, it should be noted that it was only 25–30% labeled, and similar results could, therefore, be obtained with much lower levels of more highly fluorinated enzyme (Williams, 1992), especially if the technique were made more sensitive through the use of relatively small protein domains as probes. These would give rise to narrower line widths in the NMR spectrum and consequently could be observed with better signal-to-noise ratios. Indeed, in many cases it may be desirable to use an isolated ligand-binding domain as a probe that is catalytically inert. For example, the isolated ADP-binding domain of PGK has native structure and simplified ligand-binding properties (Fairbrother et al., 1989), and could, therefore, be used as a more sensitive probe of the free ADP concentration.

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