^{19}F NMR Measurements of the Rotational Mobility of Proteins In Vivo

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ABSTRACT Three glycolytic enzymes, hexokinase, phosphoglycerate kinase, and pyruvate kinase, were fluorine labeled in the yeast *Saccharomyces cerevisiae* by biosynthetic incorporation of 5-fluorotryptophan. ^{19}F NMR longitudinal relaxation time measurements on the labeled enzymes were used to assess their rotational mobility in the intact cell. Comparison with the results obtained from relaxation time measurements of the purified enzymes in vitro and from theoretical calculations showed that two of the labeled enzymes, phosphoglycerate kinase and hexokinase, were tumbling in a cytoplasm that had a viscosity approximately twice that of water. There were no detectable signals from pyruvate kinase in vivo, although it could be detected in diluted cell extracts, indicating that there was some degree of motional restriction of the enzyme in the intact cell.

INTRODUCTION

Studies of the kinetic properties of enzymes in vitro can give important information on the possible roles of these enzymes in the control of metabolism in vivo. This approach presumes, however, a detailed knowledge of their intracellular environments. There is growing evidence for functionally important, but weakly associated, enzyme complexes that do not survive conventional cell extraction procedures and that cannot readily be reconstituted in dilute aqueous solution in vitro (Srivastava and Bernhard, 1986; Srere and Ovadi, 1990; Luby-Phelps, 1994). These complexes are presumed to form only in the unique environment of the cell (Fulton, 1982; Goodsell, 1991; Luby-Phelps, 1994), where the high chemical activities of proteins favor complex formation (Aragon and Sols, 1991; Minton, 1992). In the case of the glycolytic enzymes there has been considerable, though controversial, speculation that these enzymes form organized multienzyme complexes in vivo (Batke, 1989; Beeckmans et al., 1990; Srere and Ovadi, 1990; Brooks and Storey, 1991b). The potential advantages of these complexes include rate enhancement, additional regulatory mechanisms, substrate channeling, and the lowering of pathway intermediate concentrations (Cornish-Bowden, 1991; Mendes et al., 1992). These proposals have been based largely on numerous studies in vitro, which have demonstrated associations between glycolytic enzymes (Srivastava and Bernhard, 1986; Brooks and Storey, 1991b) and between the enzymes and components of the cell cytoskeleton, especially actin and tubulin (Walsh et al., 1989; Shearwin et al., 1990; Knull and Walsh, 1992; Lehotzky et al., 1993). These studies are subject to the criticism that the observed associations are an artifact of the conditions in vitro or even of the models used to interpret the data (Kvassman and Petterson, 1989; Vas and Batke, 1990; Brooks and Storey, 1991a,b; Wu et al., 1991). However, fluorescence microscopy measurements on fibroblasts, microinjected with fluorescently tagged glycolytic enzymes, have demonstrated an association between aldolase and stress fibers in the cell (Pagliaro and Taylor, 1992).

We describe here ^{19}F NMR measurements that were designed to assess the degree of motional restriction of three glycolytic enzymes in intact cells of the yeast *Saccharomyces cerevisiae*. The enzymes, hexokinase (HXK), phosphoglycerate kinase (PGK), and pyruvate kinase (PYK), were selectively fluorine labeled by biosynthetic incorporation of 5-fluorotryptophan by techniques described previously (Brindle et al., 1989; Williams et al., 1993). We determined the extent of enzyme immobilization as the result of tight binding to other cellular macromolecules by comparing the visibility of the ^{19}F resonances in spectra of cells with their visibility in spectra of disrupted cell preparations in which the cell proteins had been diluted. We investigated weaker interactions with cellular constituents by measuring the relaxation times of the ^{19}F resonances from the labeled proteins in the cell and comparing these with the labeled proteins in vitro, in solutions of defined viscosity.

MATERIALS AND METHODS

Materials

The yeast strain used was *Saccharomyces 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 (East Molesey, Surrey, England). Other reagents, all of analytical grade, were obtained from Sigma Chemical Company (Poole, Dorset, England) or from Boehringer Mannheim (Lewes, East Sussex, England). Protein concentrations were determined with a dye-binding assay (Bradford, 1976) kit from Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire, England), with bovine serum albumin as the standard.

Yeast transformation and enzyme induction

Cells were transformed with one of three plasmids, pKV43 (Kingsman et al., 1990), pBF72, or pBF94, by the method of Hinnen et al. (1978). These were constructed by insertion of the coding sequences for yeast PGK (Watson et al., 1982), HXK (B form) (Fröhlich et al., 1985), and PYK

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(McNally et al., 1989), respectively, into the LEU-2-expressing plasmid, pKV49 (Kingsman et al., 1990), where they were under the control of a galactose-inducible version of the PGK promoter. This promoter was constructed by replacing the PGK UAS with the GAL-4 dependent GAL1-10 UAS (Cousens et al., 1990). Expression from this vector occurs only in the presence of galactose and the absence of glucose and thus can be regulated by manipulation of the growth medium. Restriction fragments containing the coding sequences for the enzymes were inserted into the expression site of pKV49 by standard procedures (Sambrook et al., 1989). Some cells were cotransformed with a URA3-containing plasmid, pUG41S, which expresses the GAL4 protein. Coexpression of GAL4 was found to enhance expression of the glycolytic enzymes from the GAL4-dependent pKV49-derived plasmids by 10–50%.

Transformed cells were grown on minimal defined medium containing 2% w/v glucose, 0.67% yeast nitrogen base, and 0.002% w/v tryptophan. Uracil (0.003%) was added to the growth medium of cells lacking the plasmid pUG41S. Induction and fluorine labeling of the enzymes were achieved by the following procedure: A single colony was inoculated into 50 ml of minimal medium and incubated aerobically in an orbital incubator at 30°C. After 36 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 36 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 18 h, when the culture had reached stationary phase, sterile additions of 25 g galactose, in 100 ml water, and 0.25 g 5-fluoro-DL-tryptophan, in 50 ml water, were added to each 1-l culture. The cultures were incubated for a further 48 h before chilling and harvesting by centrifugation.

Cell immobilization and perifusion

Cells were washed twice at 4°C in a buffer containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid, 2 mM $MgSO_4$, 2 mM KCl, and 1.7 mM NaCl at pH 6.0. Four g cells were briefly warmed to 30°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 30°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 (Brindle and 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 2 l of recirculating buffer containing 2% w/v glucose at 30°C. The buffer was sparged with oxygen.

Protein purification

The purified labeled enzymes used for the in vitro studies were prepared from cell lysates, which were obtained by mechanical disruption with glass beads at 4°C. Cells containing overexpressed PGK or HXK were disrupted in 50 mM sodium phosphate, 5 mM EDTA, 2 mM dithiothreitol, and 0.2 mM phenylmethyl sulphonyl fluoride, pH 7.5. PYK-containing cells were disrupted in a similar buffer, which also contained 20% v/v glycerol and 0.1 M sodium chloride. After separation from the glass beads and centrifugation (14,000 g, 4°C, 15 min) to remove cell debris, neutralized protamine sulphate solution was added to a final concentration of 20 mg/g of starting material. The crude lysates with protamine were swirled on ice for 15 min and then centrifuged (14,000 g, 4°C, 15 min) to remove the precipitated nucleic acid.

PGK was purified from the lysate according to Fifis and Scopes (1978), which involves ammonium sulfate fractionation followed by pseudoaffinity elution of PGK from a weak cation-exchange resin by 3-phosphoglycerate. HXK (Jacob et al., 1991) and PYK (Murcott et al., 1991) were purified by salt-gradient elutions from anion exchange resins. The purified enzymes appeared homogeneous under PAGE-SDS with Coomassie Blue staining. PGK activity was assayed in the coupled reaction with glyceraldehyde-3-phosphate dehydrogenase under conditions described in Wilson et al. (1988) but with rabbit muscle GAPDH used as the coupling enzyme. HXK B was assayed in a coupled reaction with glucose-6-phosphate dehydrogenase (Jacob et al., 1991). The HXK activity ratio with fructose versus glucose as substrate was used to confirm the identity of the isoform present (Jacob et al., 1991), which was predominantly the B form. PYK was assayed in a coupled reaction with lactate dehydrogenase (Murcott et al., 1991).

The enzymes were stored at 4°C as ammonium sulfate precipitates with 0.1% w/v sodium azide and 2 mM dithiothreitol. For NMR studies, enzymes were desalted by gel filtration, using Pharmacia (Uppsala, Sweden) NAP-5 or NAP-10 columns (prepacked Sephadex G25), into 50 mM *N*-(2-hydroxyethylpiperazine-*N'*-(2-ethanesulphonic acid), 130 mM K acetate, and 2 mM dithiothreitol, pH 7.2. Samples contained 10% v/v ^2H_2O for a field frequency lock. pH measurements were not corrected for any deuterium isotope effect. Sucrose was added to yield solutions of higher viscosity where required, and the final protein concentration was typically 25 mg/ml.

NMR measurements

NMR experiments were performed with an 89-mm-bore 9.4 T Oxford Instruments (Oxford, England) magnet and a Varian Associates (Palo Alto, CA) UnityPlus 400-MHz spectrometer. The resonant frequency of ^{19}F at this field is 376.29 MHz. ^{19}F spectra of cells and purified enzymes were collected without ^1H decoupling by use of Varian 25-mm $^1H/^{19}F$ and 5-mm $^1H/^{19}F$ probes, respectively. The 5-mm probe was equipped with a *z*-gradient coil capable of producing up to ~30 G/cm. All experiments were performed with sample temperatures controlled at 30°C. The acquisition conditions used to acquire particular spectra are indicated in the appropriate figure and table captions. Fluorine-19 chemical shifts are quoted relative to *p*-fluorophenylalanine standards, either 100 μM internal standard in solutions of isolated enzymes or an external standard contained in a coaxial capillary with cell preparations.

Spectral processing, peak integral determination, and Lorentzian line fitting were performed with Varian VNMR software. Longitudinal relaxation time constants (T_1) were determined in inversion recovery experiments either under fully relaxed conditions or in a faster steady-state variant of the experiment (Canet et al., 1975). Fully relaxed spectra of cells and cell lysates were acquired with 90° pulses and 15-s delays between the pulses. We confirmed full relaxation by acquiring some spectra with 30-s interpulse delays. The visibility of the fluorinated enzyme in the intact cell was compared with that in diluted cell extracts. We prepared the extracts by mechanically disrupting the cells in agarose gel threads and then adding the diluted extract back to the NMR tube so that it occupied exactly the same volume as that previously occupied by the immobilized cells (16.5 ml). The total concentration of fluorine label in the NMR probe was, therefore, the same in the cells and in the cell extracts, even though the cell proteins had been diluted by a factor of 6.9 (this factor was the ratio of the estimated intracellular volume to the total volume occupied by the immobilized cells). We estimated the intracellular volume by assuming that 1.67 g of cells contains 1 ml of cell water (Gancedo and Gancedo, 1973). Cells were disrupted by vortexing with glass beads as for protein purification.

Viscosity measurements of solutions of the isolated enzymes were performed on the same samples as used for the fluorine-19 NMR experiments. The diffusion coefficient of water in the samples was measured from the proton spectra by a pulsed-gradient spin echo technique (Stejskal and Tanner, 1965). The diffusion coefficient was taken to be inversely and linearly proportional to the viscosity of the solution (Cantor and Shimmel, 1980; Freifelder, 1982). The diffusion experiment was calibrated by reference to pure water at 30°C, $D = 2.599\ mm^2\ s^{-1}$ (CRC Handbook, 1978).

Theory of NMR relaxation parameters

The calculations of the expected relaxation parameters are based on the assumptions that 1) the proteins tumble as spheres, 2) the label is held rigidly within the molecule, and 3) quantitatively important relaxation of

the fluorine nucleus is by dipole–dipole interactions with all protons in the protein and by chemical shift anisotropy (CSA). For carbon-13 and proton the dipolar terms are usually sufficient to describe the relaxation, but with fluorine-19 the CSA term can come to dominate (Post et al., 1984; Ho et al., 1989). The CSA term depends on the molecular geometry and can be quite different, even for similar molecules such as 4- and 5-fluorotryptophan. Whereas the proteins are undoubtedly not perfect spheres, the crystal structures of PGK, HXK, and PYK show that they can all reasonably be described as ellipsoids of modest axial ratio (~2). Inasmuch as the difference between a single "average" spherical rotational correlation time and the two needed to describe an ellipsoid properly is small for an axial ratio of 2, this seems a reasonable approximation to make (Woessner, 1962; Tao, 1969; Cantor and Shimmel, 1980). Unlike phenylalanine or tyrosine, the much bigger indole ring of tryptophan does not commonly undergo ring flipping or other rapid motions unless exposed. It is a reasonable assumption, therefore, that the fluorine label on a 5-fluorotryptophan residue will rotate with the same rotational correlation time as the whole protein. This has been verified experimentally by Post et al. (1984), who have successfully used the expressions given here to predict relaxation parameters very close to those actually measured with a 5-fluorotryptophan-labeled protein. The expressions used for the calculations are given in Eqs. 1–6.

Heteronuclear dipole–dipole interactions

$$1/T_1 = Kf(r)[J(\omega_H - \omega_F) + 3J(\omega_F) + 6(\omega_H + \omega_F)], \quad (1)$$

$$1/T_2 = (K/2)f(r)[J(\omega_H - \omega_F) + 3J(\omega_F) + 6(\omega_H + \omega_F) + 4J(0) + 6J(\omega_H)], \quad (2)$$

where

$$J(\omega) = 2\tau_c/(1 + \omega^2\tau_c^2), \quad (3)$$

ω is the resonance frequency of the relaxing nucleus in radians per second. $J(\omega)$ is the spectral density function at frequency ω and is that for an isotropic tumbling motion. K is a constant determined by the magnetic dipoles involved (proton and fluorine-19) and is equal to $\mu_0^2\gamma_H^2\gamma_F^2\hbar/20$, where μ_0 is the permittivity in vacuum, γ_H and γ_F are the gyromagnetic ratios of ^1H and ^{19}F, respectively, and $\hbar$ is Planck's constant divided by 2π. The function $f(r)$ depends on the distance r between the observed fluorine nucleus and the protons with which it is interacting. Considering a sphere starting at distance r from the fluorine (where 2.6 Å is the distance to the nearest protons on the indole ring) stretching to infinity and containing protons at a density ρ gives the expression

$$f(r) = \rho(4/3)\pi r^{-3}. \quad (4)$$

Calculations here have used the proton density value for a typical globular protein of $\rho = 5.73 \times 10^{22}$ ml^{-1}, reported for the 29-kDa J protein (Post et al., 1984).

CSA relaxation

The CSA relaxation terms are given by (Abragam, 1961)

$$1/T_1 = (6/40)\omega_0^2\delta_z^2(1 + \epsilon^2/3)J(\omega_0), \quad (5)$$

$$1/T_2 = (1/40)\omega_0^2\delta_z^2(1 + \epsilon^2/3)[3J(\omega_0) + 4J(0)], \quad (6)$$

where ϵ is the asymmetry parameter of the traceless CSA tensor, given by $(\delta_x - \delta_y)/\delta_z$. $\delta_{x,y,z}$ are the chemical shifts when 5-fluorotryptophan is oriented along the x, y, or z axis, respectively. ϵ was taken to have a value of -1 and δ_z a value of 50 ppm (Hull and Sykes, 1975b; Post et al., 1984).

Correlation times, molecular mass, and viscosity

The hydrated radius, r_{hyd}, for each protein was calculated according to the generalized expression for a solid sphere (Freifelder, 1982):

$$r_{hyd} = (3M_rV/4\pi N_A)^{1/3}, \quad (7)$$

where V is the partial specific volume, N_A is Avogadro's number, and M_r is the relative molecular mass. The partial specific volume was taken to be 0.75, a value typical for globular proteins (Van Holde, 1971). The values obtained from Eq. 7 are for the anhydrous protein. For globular proteins there is typically 0.2–0.3 g of water/g of protein (Tao, 1969). The additional mass caused by this bound water is thus, in the case of PGK, $(44{,}500/6 \times 10^{23}) \times 0.2 = 1.483 \times 10^{-20}$ g, equivalent to an increase in volume of 1.483×10^{-20} ml. The hydrated radii calculated on this basis are 27.0 Å for PGK, 27.8 Å for monomeric and 34.5 Å for dimeric hexokinase, and 43.6 Å for tetrameric PYK.

The rotational correlation time for a spherical molecule, t_c, is given by the Stokes–Einstein relation (Cantor and Shimmel, 1980)

$$\tau_c = 8\pi r^3\eta/6kT, \quad (8)$$

where r is the Stokes radius, η is the viscosity, k is Boltzmann's constant, and T is the temperature in kelvins. (The right side of this equation is equivalent to $V\eta/kT$, where V is the volume of the spherical molecule.)

RESULTS

Enzyme labeling

Previous studies showed that specific enzymes can be fluorine labeled in *Saccharomyces cerevisiae* by induction of enzyme synthesis in the presence of 5-fluorotryptophan (Brindle et al., 1989; Williams et al., 1993). Following the addition of galactose and 5-fluorotryptophan to cells transformed with the galactose-inducible vectors expressing PGK, HXK, or PYK (see Materials and Methods) there was a steady increase in the concentration of these enzymes over the first 24 h of induction, which plateaued at levels ~20-fold greater than those of control cells. These data are summarized in Table 1. A lower level of induction, approximately 10-fold, was obtained in cells that lacked the plasmid expressing the GAL4 protein (pUG41S) and that were given only 1% galactose to initiate the induction process. For all three enzymes the specific activity of the fluorinated and the nonfluorinated enzymes was not measurably different. Comparison of the fluorine-19 resonance intensity from the protein with a concentration standard and the amount of protein present showed that the enzymes were typically 25–30% substituted with 5-fluorotryptophan.

^{19}F NMR spectra of isolated enzymes

The ^{19}F NMR spectra of the labeled enzymes were as expected from their amino acid compositions (see Figs. 1 and 2). Yeast PGK, which is a monomer of molecular mass 45 kDa, has two tryptophans and gave rise to two well-resolved resonances (Brindle et al., 1989; Williams et al., 1993). Yeast HXK B, which is a homodimer of molecular mass 104 kDa (Fröhlich et al., 1985), has four tryptophans per subunit and gave rise to three resolved resonances, one

TABLE 1 Cellular enzyme activities, concentrations, and induction levels

	Enzyme Overexpressed			
			PYK	
	PGK	HXK	Low	High
Activity before induction, *U*/(ml cell water) (n = 6)	949 ± 10	764 ± 42	965 ± 31	
Specific activity, *U*/(mg protein) (*n* = 3)	447 ± 22	312 ± 29	377 ± 24	
Activity after induction, *U*/(ml cell water) (n = 6)	19,440 ± 1844	13,950 ± 1531	7008 ± 541	14,547 ± 680
Concentration of enzyme after induction (mM)	0.96	0.42	0.09	0.18
Concentration of tryptophan in enzyme after induction (mM)	1.9	1.7	0.35	0.73
Mean induction factor	20	18	7	15

The PYK low overexpressor lacked the plasmid expressing the GAL4 protein and was induced with a lower concentration of galactose (1%). Data are given as mean ± SEM for the number of experiments indicated in parentheses in the first column.

of which was double the intensity of the others and is presumably two overlapping resonances. PYK, which is a homotetramer of molecular mass 210 kDa, has a single tryptophan per subunit (Murcott et al., 1991) and gave rise to a single resonance. The chemical shift of this resonance, but not its linewidth, changed significantly (by ~1 ppm) in the presence of millimolar concentrations of fructose 1,6-bisphosphate, an allosteric effector of PYK. This presumably reflects a cooperative reorganization of the subunit interfaces, which is where the tryptophan residues are located (Muirhead et al.; 1986, Murcott et al., 1991). Although other proteins must be labeled during the induction process, only the directed synthesis of the enzymes gave rise to enough material to be detected in the NMR experiment because no signals arose in control experiments in which galactose was omitted from the medium or proteins were expressed only at very low levels (Brindle et al., 1989). Labeled cells induced to express GAL4 alone and not one of

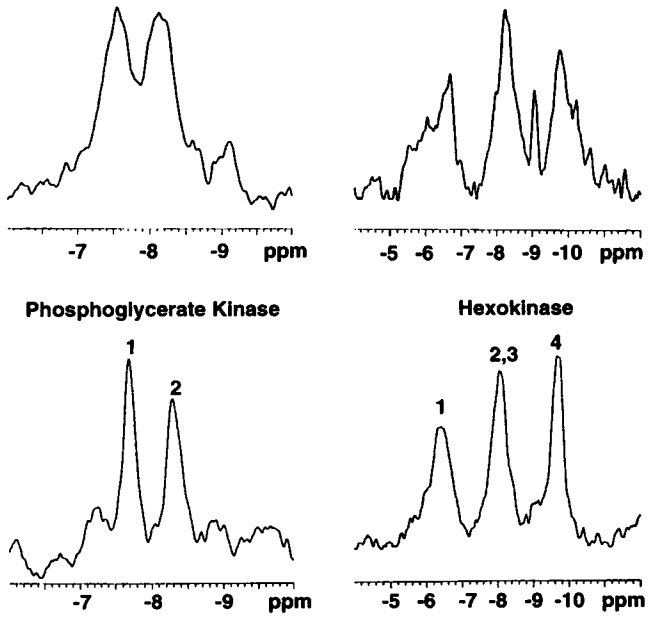

FIGURE 1 ^{19}F NMR spectra of phosphoglycerate kinase and hexokinase containing biosynthetically incorporated 5-fluorotryptophan. Spectra from intact cells (*top*) and purified enzymes (*bottom*). The peak designations in the spectra are used for reference in the text. Spectra were acquired with a spectral window of 12 kHz into 16k data points. The interpulse delay was 0.67 s. Each acquisition was the sum of 2000 transients and took 22 min to acquire. The pulse angles were set at the "Ernst angle" based on anticipated T_1 values of 1 and 3 s, respectively, for PGK and HXK ($\cos \alpha_E = \exp(-T_r/T_1)$), where α_E is the Ernst angle and T_r is the pulse repetition time).

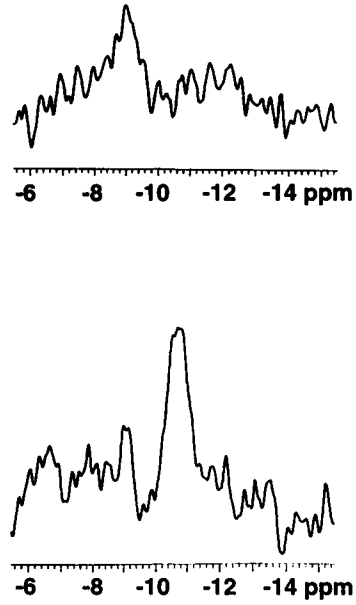

FIGURE 2 ^{19}F NMR spectra of pyruvate kinase containing biosynthetically incorporated 5-fluorotryptophan. The spectra are from intact cells (*top*) and a cell lysate (*bottom*). The lysate was prepared from the cells following their perifusion in the NMR spectrometer. Preparation of the lysate is described in Materials and Methods. Spectra were acquired with a spectral window of 12 kHz into 16k data points. The interpulse delay was 0.67 s. Each acquisition was the sum of 2000 transients and took 22 min to acquire. The pulse angle was set at the "Ernst angle" based on an anticipated T_1 of 5 s ($\cos \alpha_E = \exp(-T_r/T_1)$), where α_E is the Ernst angle and T_r is the pulse repetition time).

the glycolytic enzymes also gave rise to no detectable NMR signals.

^{19}F NMR spectra of the labeled enzymes in the cell

Spectra corresponding to those from the isolated PGK and HXK were also seen in intact cells that had been immobilized in agarose gel threads and maintained in a metabolic steady state by perifusion with oxygenated glucose-containing medium (Fig. 1). In each case, there was an additional resonance at −9 ppm, which had the same chemical shift as unincorporated 5-fluorotryptophan. The signal, which was of low intensity in glucose-fed cells, showed an increase in intensity on glucose starvation that paralleled the decrease in intensity of the protein signals. This presumably reflects protein turnover.

The resonance intensities of labeled PGK and HXK in the intact cell were comparable with those observed in diluted lysates prepared from the same cells following perifusion in the NMR spectrometer (see Materials and Methods and Table 2). Signals from PYK, however, were undetectable in vivo, although they were readily detectable in the postperifusion cell lysates (see Fig. 2 and Table 2). Increasing the number of scans in the experiment shown in Fig. 2 from 2000 to 100,000 scans still did not produce a discernible signal from the labeled enzyme in the cell (data not shown).

^{19}F NMR relaxation time measurements in the cell

The resonances from PGK and HXK were sufficiently intense in vivo that measurements of their T_1 values could be made. The spectra from a typical inversion recovery experiment on HXK in vivo are shown in Fig. 3. These data are summarized in Table 3. For HXK the two overlapping resonances (peaks 2 and 3; see Fig. 1) were treated as one entity, as the behavior of this combined resonance in an inversion recovery experiment showed no detectable biexponential behavior. Peak 1 from HXK, presumably located on a mobile region of the protein, has a much shorter T_1 than the others and was not used as the basis for any viscosity calculations.

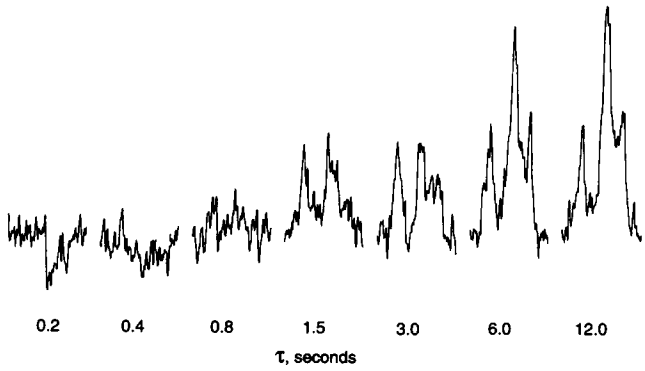

FIGURE 3 Inversion recovery measurement of the longitudinal relaxation time constant T_1 of labeled HXK in vivo. Immobilized cells were perifused aerobically with glucose as described in Materials and Methods. Each spectrum had a spectral window of 12 kHz and was acquired into 12k data points. The delay between the end of one acquisition and the beginning of the next 180° pulse was 3 s. τ was the delay between the 180° and the 90° pulses.

^{19}F NMR relaxation time measurements in vitro

The values of T_1 measured in vitro at different viscosities were compared with measurements made in the intact cell and with theoretical values. These data are presented graphically in Fig. 4 for PGK and in Fig. 5 for HXK. To facilitate comparison with the data obtained in vivo (see Table 2), dashed curves representing the values of T_1 measured in the cell have been drawn across the figures to show where these values lie in relation to the theoretical values and the T_1 values measured in vitro.

Ultracentrifugation studies have shown that HXK exists in a monomer–dimer equilibrium (Derechin et al., 1972; Shill et al., 1974; Hoggett and Kellett, 1976). Knowledge of the dimerization state is clearly important in the prediction of relaxation rates based on correlation times. Therefore the enzyme was examined in vitro under conditions designed to promote monomerization or dimerization. The measured T_1 with 20 mg/ml HXK at pH 9.3, I = 0.6 M, and 18°C, conditions that strongly favor monomerization, was 1.4 ± 0.1s (mean ± 1 SEM, n = 4). This was not significantly different from a value 1.6 ± 0.1s, which was measured at pH 5.5 and I = 25 mM, conditions that favored dimeriza-

TABLE 2 ^{19}F NMR relaxation time constants, line widths, and calculated correlation times for fluorinated PGK and HXK in vivo

	PGK		HXK		
Peak (see Fig. 1)	1	2	1	2, 3	4
T_1 (s) (n = 6)	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	2.2 ± 0.2	2.0 ± 0.2
Linewidth at half-height (Hz) (n = 6)	137 ± 16	110 ± 13	148 ± 19	Peaks overlap	271 ± 44
Apparent T_2 (T_2^*) (ms)	2.4	2.9	2.2		1.2
τ_c calculated from T_1 (ns)	39	35	35		66
τ_c calculated from T_2 (ns)	79	70	90		160

Each T_1 determination involved the acquisition of spectra at seven or eight delay times (see Fig. 3). The peak intensities were fitted to a single exponential function by a three-parameter fit. Linewidths at half-height were measured by fitting Lorentzian lines to the data with Varian's VNMR software. T_2^* was calculated from linewidth = $1/\pi T_2^*$. The data are given as mean ± SEM for the number of experiments indicated in parentheses in the first column.

TABLE 3 Visibility of the labeled enzymes in the cell

	PGK	HXK	PYK	PYK
Enzyme induction factor	20	18	7	15
Percentage visibility of the fluorinated enzyme in the intact cell	95 ± 4 (n = 3)	96 ± 8 (n = 3)	n.d.* (n = 3)	n.d.* (n = 4)

Resonance intensities from fully relaxed spectra of the enzymes observed in the intact cell were compared with those from lysates of the same cells. The spectra were compared by cutting peaks from paper plots and weighing them and by use of Varian integration software (see Materials and Methods). The data are given as mean ± SEM for the number of experiments indicated in parentheses.
* n.d., Not detectable.

tion. These values are also close to the theoretical value expected for the dimer and indicate that at these protein concentrations the protein is predominantly dimeric.

The addition of millimolar concentrations of 3-phosphoglycerate and $MgATP^{2-}$ to PGK and of glucose and $MgATP^{2-}$ to HXK had no measurable effect on the T_1 values.

DISCUSSION

Fluorination has been used to introduce NMR-detectable labels into several proteins to study their structure or function in vitro (Gerig, 1994). The selective labeling procedure described here was used previously by us to monitor ligand binding to yeast PGK in vivo (Williams et al., 1993) and has been used in this study to determine the rotational mobility of three glycolytic enzymes in the intact yeast cell.

NMR relaxation time measurements in vivo and in vitro

The measured values of T_1 of the PGK and HXK resonances in the cell are similar to those measured in vitro at near-aqueous viscosities (see Figs. 4 and 5). The measurements of HXK show that the enzyme is dimerized, because at a relative viscosity of 2 the monomer should report a much shorter T_1 (0.9 s) than the dimer (1.6 s). Despite early interest in the possibility of dimerization as a physiological control mechanism for hexokinase (Derechin et al., 1972;

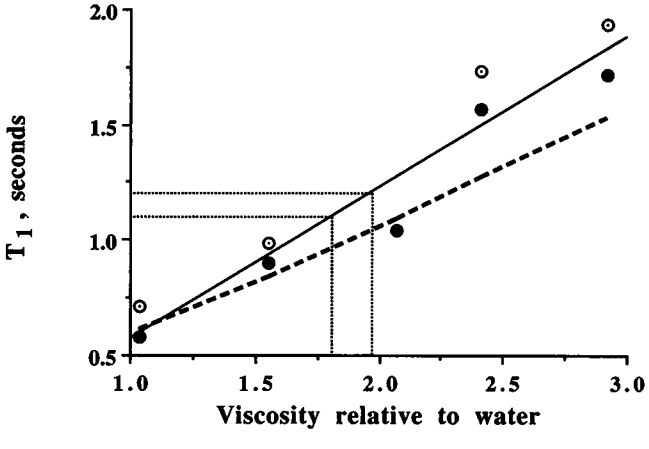

FIGURE 4 Dependence of the T_1 values of the 5-fluorotryptophan resonances from PGK on the viscosity of the medium. The plot symbols show experimentally measured T_1 values for the labeled enzyme in media of specified viscosity, ⊙ for peak 1, ● for peak 2 (see Fig. 1). The values of T_1 of the resonances are expected to be linearly dependent on the viscosity (see Theory), and therefore the solid line through these points was obtained by linear regression. The dashed curve shows the expected variation of T_1 with viscosity based on the relaxation theory described in the text. The model assumes relaxation of the fluorine to a matrix of protons in the protein and includes dipole–dipole and chemical shift anisotropy relaxation terms. The dotted lines across the figure show the T_1 values measured in the intact cell (taken from Table 2, data for peak 1 as an upper limit and peak 2 as a lower limit). The viscosity of the PGK solutions used for the experiments in vitro was increased by the addition of sucrose. The viscosity was checked in each sample by measurement of the diffusion coefficient of the water in a pulsed field gradient experiment (Stejskal and Tanner, 1965)

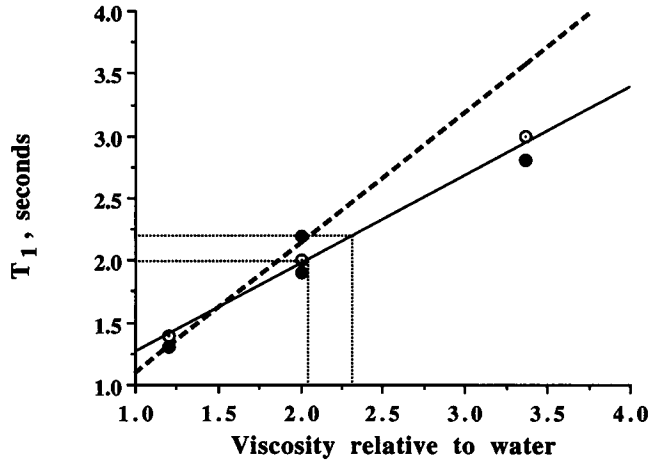

FIGURE 5 Dependence of the values of T_1 of the 5-fluorotryptophan resonances from HXK on the viscosity of the medium. The plot symbols show experimentally measured T_1 values for the labeled enzyme in media of specified viscosity, ⊙ for peaks 2 + 3, ● for peak 4 (see Fig. 1). Three T_1 determinations were made at a relative viscosity of 2.0. All three gave a value of 2.0 s for peaks 2 + 3 and values of 1.9, 1.9, and 2.2 s for peak 4. The T_1 values of the resonances are expected to be linearly dependent on the viscosity (see Theory), and therefore the solid line through these points was obtained by linear regression. The dashed curve shows the expected variation of T_1 with viscosity based on the relaxation theory described in the text. The dotted lines across the figure show the T_1 values measured in the intact cell (taken from Table 2, data for peaks 2 + 3 as an upper limit and peak 4 as a lower limit). The viscosity of the HXK solutions used for the experiments in vitro was increased by the addition of sucrose. The viscosity was checked in each sample by measurement of the diffusion coefficient of the water in a pulsed field gradient experiment (Stejskal and Tanner, 1965) .

Shill et al., 1974; Hoggett and Kellett, 1976), these ultracentrifugation studies were done at very low protein concentrations (tens of micrograms per milliliter). We observe that at physiologically high protein concentrations (20 mg/ml) the enzyme appears to be predominantly dimerized regardless of its ligand concentrations or the surrounding pH or ionic strength. There is evidence that mammalian hexokinases associate in a functionally significant way with porin on the surface of the mitochondria (Lynch et al., 1991), but this phenomenon is thought to be absent in yeast (Kovac et al., 1986), and there is no evidence for it here.

The theoretical relaxation times show good agreement with those measured in vitro, particularly at the lower viscosities. In early experiments with HXK the values of T_1 measured at high viscosities were significantly lower than those shown in Fig. 5. This appeared to be due to higher levels of residual oxygen in the high-viscosity samples as the T_1 values were increased, to the values shown in Fig. 5, by extension of the period of vacuum degassing of the sample from less than 5 min to 30 min (for example, giving T_1 values of 2.2 versus 3.0 s at a relative viscosity of ~3.5). Anaerobic perifusion conditions had no marked effect, however, on the measured values of T_1 in the intact cell. The measured T_1 were similar in cells perifused under aerobic (see Table 2) or anaerobic conditions. In the latter case the T_1 for peak 1 (see Fig. 1) of PGK was 1.2 s ($n = 2$) and for peak 2 + 3 of HXK was 2.0 s ($n = 1$).

Comparison of the relaxation times measured in the cell and in vitro permits estimates of the intracellular viscosity to be made. The cytoplasmic viscosities experienced by both PGK and HXK appear to be ~2.0 times greater than that of water (see Figs. 4 and 5).

The linewidths measured in vitro agreed well with the predictions made from relaxation theory (Abragam, 1961; Hull and Sykes, 1975a; Wittebort and Szabo, 1978; Post et al., 1984; Rule et al., 1987). For example, the linewidth of peak 2 of PGK was measured at 34 Hz at a relative viscosity close to 1, and the calculated value was 36 Hz. In the case of hexokinase the measured linewidth of peak 4 at a relative viscosity of 1.2 was 68 Hz and the calculated value was 84 Hz. However, the linewidths measured in vivo tended to overestimate the correlation time, and hence the viscosity, by a factor of 2 or more compared with the T_1 measurements (see Table 2). This is probably largely the result of line broadening caused by susceptibility discontinuities across the cell sample and is a common problem in NMR studies of biological tissue (Fabry and San George, 1983).

Comparison of the ^{19}F NMR resonance intensities from the labeled enzymes in the cell and diluted cell extracts

We prepared cell extracts by mechanically disrupting the immobilized cells and then adding the diluted extract back to the NMR tube so that it occupied the same volume as that originally occupied by the immobilized cells (see Materials and Methods). The signal intensities from HXK and PGK were the same, within experimental error, in the cell and in the cell extracts (Table 3). However, there was no discernible PYK resonance in cells, although a signal was readily detectable in the diluted cell extracts (see Fig. 2). The calculated T_1 and linewidth of the PYK fluorine resonance were 4 s and 220 Hz, respectively, at a viscosity twice that of water, and therefore the enzyme should have been readily detectable in the cell under the NMR acquisition conditions used to acquire the data shown in both Table 3 and Fig. 2. Although the T_1 of the PYK resonance was not measured in vitro, the measured linewidth of 240 Hz was in good agreement with theory. Loss of PYK signal intensity in the cell could be due to weak binding of the protein to other molecules, resulting in exchange broadening, or to tighter binding to other cellular macromolecules. For example, binding of saturating amounts of fructose-1,6-bisphosphate produced a shift of ~1 ppm in the PYK resonance. However, exchange broadening by fructose-1,6-bisphosphate in the cell is unlikely, given that the concentration that gives 50% binding in vitro is 47 μM (Murcott et al., 1992) and the concentration of fructose-1,6-bisphosphate in the cell is approximately 2 mM (Brindle, 1988). The enzyme may be bound in the cell to actin, which has been shown to bind the muscle enzyme, even at high ionic strength (Shearwin et al., 1990; Knull and Walsh, 1992). Although interactions between yeast pyruvate kinase and yeast actin do not appear to have been studied, yeast PYK is very similar to the mammalian enzyme (Murcott et al., 1991), and yeast actin is homologous in structure and function with the mammalian isoforms (Ng and Abelson, 1980; Nefsky and Bretscher, 1992; Welch et al., 1994).

Early fluorescent measurements of intracellular viscosity in yeast (Burns, 1969) and higher cells (Cerek and Cerek, 1976; Lindmo and Steen, 1977) gave values approximately three to seven times higher than reported here. The intracellular viscosities estimated in this study agree with the more recent estimates obtained from mammalian cells by a variety of independent methods. These include fluorescence polarization measurements of labeled probe molecules (Dix and Verkman, 1990; Fushimi and Verkman, 1991; Bicknese et al., 1993; Luby-Phelps et al., 1993; Luby-Phelps, 1994); ^{13}C NMR relaxation time measurements of ^{13}C-labeled glutathione in erythrocytes (Endre et al., 1983); ^{1}H NMR linewidth measurements of myoglobin in muscle (Livingston et al., 1983); electron spin resonance studies of small-molecule spin-label probes (Mastro et al., 1984); and NMR diffusion measurements of ATP in excised muscle (Hubley et al., 1995). Inasmuch as the measurements described here were made on a minimally derivatized protein, they should be free of artifactual binding problems, which, for example, plagued early estimates of cytoplasmic viscosity based on fluorescence polarization measurements of small probe molecules (Jacobson and Wojcieszyn, 1984; Luby-Phelps et al., 1985).

The intensities of the PGK and HXK resonances in the cell and their relaxation times suggest that there is no

restriction on the rotational motion of these enzymes in the intact yeast cell. However, these enzymes have been overexpressed. Therefore it is possible that these enyzmes are immobilized at their normal concentrations in the cell, but, as this represents such a small fraction of the total enzyme concentration in the overexpressors, the effects of immobilization may not be detectable. In the case of PYK, however, there is evidence that the signal from the labeled enzyme is broadened beyond detection and that this could be due to motional restriction.

One could extend this research by measuring the translational diffusion coefficients of HXK and PGK in the cell, using pulse field gradient techniques (Stejskal and Tanner, 1965). This technique has been used, for example, to measure the diffusion coefficient of hemoglobin in the human erythrocyte (Kuchel and Chapman, 1991) and of ATP in excised muscle (Hubley et al., 1995). Fluorescence recovery after photobleaching studies of labeled proteins and macromolecular polysaccharides, which have been microinjected into mammalian cells, suggest that the diffusion coefficients measured should be much lower than that expected from the cellular viscosity as a result of collisions with and binding to the cellular cytoskeleton (Wojcieszyn et al., 1981; Gershon et al., 1985; Luby-Phelps et al., 1987; Hou et al., 1990; Luby-Phelps, 1994). However, because of the relatively short T_2 valves of the fluorine resonances and the low diffusion coefficients of the labeled proteins, these studies will require exceptionally large pulsed field gradients (several hundred Gauss/cm).

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