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# $^{31}$P NMR Magnetization-Transfer Measurements of Flux between Inorganic Phosphate and Adenosine 5′-Triphosphate in Yeast Cells Genetically Modified To Overproduce Phosphoglycerate Kinase†

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ABSTRACT: $^{31}$P NMR magnetization-transfer measurements were used to measure flux between inorganic phosphate and ATP in the reactions catalyzed by phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase in anaerobic cells of the yeast *Saccharomyces cerevisiae*. Flux between ATP and $P_i$ and glucose consumption and ethanol production were measured in cells expressing different levels of phosphoglycerate kinase activity. Overexpression of the enzyme was obtained by transforming the cells with a multicopy plasmid containing the phosphoglycerate kinase coding sequence and portions of the promoter element. Fluxes were also measured in cells in which the glyceraldehyde-3-phosphate dehydrogenase activity had been lowered by limited incubation with iodoacetate. These measurements showed that both enzymes have low flux control coefficients for glycolysis but that phosphoglycerate kinase has a relatively high flux control coefficient for the ATP ↔ $P_i$ exchange catalyzed by the two enzymes. The $P_i$ ↔ ATP exchange velocities observed in the cell were shown to be similar to those displayed by the isolated enzymes in vitro under conditions designed to mimic those in the cell with respect to the enzyme substrate concentrations.

Extrapolation of enzyme kinetic data obtained in vitro to the intact cell assumes a detailed knowledge of the intracellular environment in terms of the enzyme's substrate concentrations and the levels of various effectors and other factors which may influence the enzyme's activity. An important feature of the intracellular environment is the relatively high protein concentration. This can have a number of consequences; for example, the free concentration of an enzyme's substrates may be much less than the total extractable concentration if there is binding of the substrate to other enzymes and cell proteins [see Sols and Marco (1970) for review]. High enzyme concentrations may also result in enzyme–enzyme interactions which are not observed at the relatively low concentrations used for determination of steady-state kinetics in vitro (Srivastava & Bernhard, 1986a,b). There is continued interest, for example, in the hypothesis that the enzymes of a metabolic pathway may associate to form a multienzyme complex [see Srere (1987) for review]. Complex formation, it is suggested, can result in "channeling" of substrates within the complex leading to an increase in the rate of the coupled reactions. Indeed, it has been shown that coimmobilization of the glycolytic enzymes can result in an enormous increase in the rate of reaction compared with that observed with the same concentrations of enzymes free in solution (DeLuca & Krika, 1983). Some of the best evidence for complex formation among the glycolytic enzymes in vivo has come from studies showing specific interactions between sequential pairs of glycolytic enzymes and interactions between the glycolytic enzymes and other cellular proteins (Srivastava & Bernhard, 1986a,b; Srere, 1987). An interaction has been observed, for example, between glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase which results in direct transfer of 1,3-diphosphoglycerate from one enzyme active site to the other, resulting in a substantial increase in the rate of the coupled reaction (Weber & Bernhard, 1982). There have also been numerous studies on the association of glycolytic enzymes with the band 3 protein of the erythrocyte membrane. Whether this interaction occurs in vivo is not clear since binding is only observed under conditions of low ionic strength in vitro. We investigated the binding of glyceraldehyde-3-phosphate dehydrogenase to the band 3 protein in the human erythrocyte by comparing the activity expressed by the enzyme in the cell with the activity expressed by the isolated enzyme in vitro, when free and when bound to the band 3 protein (Brindle et al., 1982). Enzyme activity in vivo was measured by using $^1$H NMR to monitor noninvasively a coupled $^1$H/$^2$H exchange reaction in which the enzyme was involved. The study showed that the enzyme was totally inhibited when bound to band 3 and that the activity expressed by the enzyme in the cell was too high for there to be any significant binding of the enzyme to the membrane. This strategy of comparing the kinetic properties expressed by an enzyme in vivo and in vitro has been used here to seek evidence for glycolytic enzyme complex formation in the yeast *Saccharomyces cerevisiae*. Since glycolytic flux can be very high in yeast, we would expect a glycolytic enzyme complex, if its exists, to be evident in this organism.

The enzymes investigated in this study, phosphoglycerate kinase (PGK)[1] and glyceraldehyde-3-phosphate dehydrogenase

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(GAPDH), catalyze an exchange between inorganic phosphate and ATP (eq 1 and 2) which can be measured in the intact

$$\text{GAP} + \text{P}_\text{i} + \text{NAD}^+ \overset{\text{GAPDH}}{\rightleftharpoons} \text{1,3-DPG} + \text{NADH} + \text{H}^+ \quad (1)$$

$$\text{1,3-DPG} + \text{ADP} \overset{\text{PGK}}{\rightleftharpoons} \text{3PGA} + \text{ATP} \quad (2)$$

cell and in vitro by $^{31}$P NMR magnetization-transfer measurements. NMR methods and in particular NMR magnetization-transfer techniques are well suited to the measurement of enzyme kinetics in vivo since they are noninvasive and, in the case of magnetization-transfer techniques, they allow measurements of relatively rapid exchanges which are inaccessible to other methods [reviewed in Brindle and Campbell (1987)]. Although $^{31}$P NMR saturation-transfer measurements of exchange between $P_i$ and ATP in a number of different systems showed that the flux was due solely to the activity of the mitochondrial $F_1F_0$ ATP synthase (Matthews et al., 1981; Freeman et al., 1983; Roberts et al., 1984), subsequent work has shown that, in some systems and under certain metabolic conditions, there can be substantial involvement of the glycolytic enzymes GAPDH and PGK (Brindle & Krikler, 1985; Campbell et al., 1985; Campbell-Burk et al., 1987a; Brindle & Radda, 1987; Kingsley-Hickman et al., 1987).

In order to determine the relative contribution of these enzymes to the $P_i \leftrightarrow$ ATP exchange observed under anaerobic conditions in yeast and in order to better define their intracellular environment, we have adopted an approach, initially proposed by Kacser and Burns (1973) and Heinrich and Rappoport (1974), for determining the contribution of an individual enzyme to the overall flux in a metabolic pathway. Experimentally this involves changing the intracellular activity of an enzyme either by the use of a specific irreversible inhibitor [see, for example, Groen et al. (1982)] or by genetic manipulation of the enzyme concentration. The latter approach was adopted by Walsh and Koshland (1985) to determine the influence of citrate synthase activity on flux in the Krebs cycle in *Escherichia coli*. The intracellular concentration of the enzyme was varied by inserting the gene for the enzyme into a plasmid, where it was under the control of a synthetic adjustable promoter. The contribution of an individual enzyme to the overall flux can be expressed quantitatively as its flux control coefficient ($C_i$), where this is defined as

$$C_i = (\delta J/J)/(\delta e_i/e_i)$$

where $J$ is the steady-state flux through the pathway and $e_i$ is the concentration of the enzyme (Burns et al., 1985). In the experiments described here we have investigated the influence of PGK and GAPDH activity on net glycolytic flux and $P_i \leftrightarrow$ ATP exchange by (a) titrating GAPDH activity with iodoacetate and (b) elevating PGK activity by genetic modification. Intracellular PGK concentration was increased by transforming the cells with a multicopy plasmid containing the PGK coding sequence and promoter element. A preliminary account of related work has been published (Brindle et al., 1986). These experiments have shown that both the enzymes have low flux control coefficients for glycolysis but that PGK has a relatively high flux control coefficient for $P_i \leftrightarrow$ ATP exchange. Comparison with measurements on the isolated enzymes in vitro has shown that the activities expressed by the enzymes in the cell are similar to those measured in vitro, under conditions designed to mimic those in the cell with respect to substrate concentrations, pH, and ionic strength.

## Materials and Methods

Glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and alcohol dehydrogenase (EC 1.1.1.1) (from yeast) and aldolase (EC 4.1.2.13), triosephosphate isomerase (EC 5.3.1.1), and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) (from rabbit muscle) were obtained from Boehringer Mannheim. $NAD^+$, 3-phosphoglycerate, ATP, ADP, fructose 1,6-bisphosphate, and triethanolamine hydrochloride were from Boehringer Mannheim. Low gelling temperature agarose, Mes, Hepes, amino acids, NADH (in preweighed vials), and a glucose assay kit using glucose oxidase and *o*-dianisidine were obtained from Sigma. Yeast nitrogen base was from Difco. Glass beads (40 mesh) were from BDH. All other reagents were of analytical grade.

*Preparation of Cells.* Cells of the *Saccharomyces cerevisiae* strain AH22 (a, *his* 3-52, *leu* 2-3, *leu* 2-112) were grown aerobically at 30 °C on a synthetic medium containing 6.7 g/L yeast nitrogen base, 2% glucose, and an amino acid mixture lacking leucine. The main cultures were inoculated from precultures at $4 \times 10^5$ cells/mL and harvested 19 or 24 h later when the cells were in late-log or stationary phase, respectively. The cells were transformed with one of three multicopy plasmids, each of which complemented the *leu2* mutations. Plasmid pMA27 (Mellor et al., 1983) contained the PGK promoter element and coding sequence. The other two plasmids, which were derived from pMA27, contained a deletion in the PGK promoter (pMA766) (Ogden et al., 1986) and replacement of the PGK coding sequence with an interferon coding sequence (pMA301-1) (Mellor et al., 1985). The plasmids were kindly provided by Dr. A. J. Kingsman, Oxford.

Cell harvest, immobilization in agarose, and perifusion in the NMR spectrometer were carried out as described previously (Brindle & Krikler, 1985). The cells, a total of 4 g wet wt, were perifused at 30 °C in a total sample volume of 17 mL with a buffer containing 2 mM $MgSO_4$, 1.7 mM NaCl, 2 mM KCl, and 50 mM Mes. The pH was adjusted to 6.0 with NaOH.

*Enzyme and Metabolite Assays.* Glucose consumption and ethanol and acetaldehyde production were determined by sampling the cell perifusate. Fructose 1,6-bisphosphate, glucose 6-phosphate, dihydroxyacetone phosphate + glyceraldehyde 3-phosphate, 3-phosphoglyceric acid, and ATP were assayed in cell extracts. The metabolites were assayed by enzymatic methods described in Bergmeyer (1974a). Cell extracts were prepared by emptying the contents of the NMR tube into a mortar cooled with liquid nitrogen. The frozen cells were then ground to a powder, and 5 mL of 30% ice-cold perchloric acid was added. The mixture was then reground and freeze-thawed 3 times in an ethanol/dry ice bath. The resulting precipitate was removed by centrifugation at 4 °C. The supernatant was adjusted to pH 6.0 with $K_2CO_3$ and the precipitate removed by centrifugation at 4 °C. This procedure is similar to methods described previously (den Hollander et al., 1986; Sáez & Lagunas, 1976). The extract was poured down a Chelex column and lyophilized for NMR experiments or assayed immediately for cellular metabolites as described

[1] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12); PGK, phosphoglycerate kinase (EC 2.7.2.3); GAP, glyceraldehyde 3-phosphate; 1,3-DPG, 1,3-diphosphoglycerate; 3PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; $C_i$, flux control coefficient; $J$, pathway flux; $e_i$, concentration of enzyme; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $T_1$, spin–lattice relaxation time; $M_z$, observed $z$ magnetization; $M_0$, equilibrium $z$ magnetization.

above. For the NMR experiments the lyophilized material was redissolved in 3 mL of a buffer containing 50 mM triethanolamine buffer, pH 8.0, and 1 mM EDTA.

GAPDH and PGK were assayed spectrophotometrically at 25 °C as described previously (Brindle & Radda, 1987). Cell extracts containing the enzymes were prepared by freeze-thawing the cells in liquid nitrogen at a cell density of 0.2 g/mL and then vortexing them for 2 min with 40-mesh siliconized glass beads. Cell debris was removed by centrifugation on an Eppendorf microcentrifuge for 10 min at 4 °C. Protein concentrations were determined with either the biuret method (Gornall et al., 1949) or an assay available from Bio-Rad Laboratories.

*Exchange Measurements on the Isolated Enzymes.* Equilibrium mixtures of the enzymes and their substrates were prepared for NMR experiments by adding ATP, $P_i$, NADH, $NAD^+$, 3-phosphoglycerate, ADP, and fructose 1,6-bisphosphate to a mixture of GAPDH, PGK, aldolase, and triosephosphate isomerase. The enzymes, which were obtained as suspensions in ammonium sulfate, were dialyzed twice against 500 volumes of 50 mM Hepes (pH 7.2), 1 mM EDTA, and 2 mM dithiothreitol and then once against 250 volumes of 50 mM Hepes (pH 7.20), 100 mM potassium acetate, 1 mM EDTA, and 2 mM dithiothreitol. The latter buffer was used in all the exchange measurements and was designed to mimic the intracellular conditions with respect to pH (see Results) and ionic strength (Gancedo & Gancedo, 1973). Glyceraldehyde 3-phosphate and 1,3-diphosphoglycerate were generated endogenously from the added substrates. The concentrations of added substrates needed to give the equilibrium concentrations required were calculated with an equilibrium constant for the aldolase reaction ($K_{ALD}$) of $1 \times 10^{-4}$ M (Rutter, 1961), an equilibrium constant for the triosephosphate isomerase reaction ($K_{TIM}$) of 22 (Veech et al., 1969), and a combined equilibrium constant for the reactions catalyzed by GAPDH and PGK of $1.83 \times 10^{-4}$ (Veech et al., 1979), where these equilibrium constants are defined as

$$K_{ALD} = \frac{[DHAP][GAP]}{[FDP]} \qquad K_{TIM} = \frac{[DHAP]}{[GAP]}$$

$$K_{GAPDH/PGK} = \frac{[\Sigma 3PGA][\Sigma ATP][NADH][H^+]}{[\Sigma GAP][\Sigma ADP][\Sigma P_i][NAD^+]}$$

The value for the coupled GAPDH/PGK reaction was obtained at pH 7.0 for a free $Mg^{2+}$ concentration of 1 mM at 38 °C and an ionic strength of 0.25. The free $Mg^{2+}$ concentration in the experiments described here was approximately 3 mM. No correction was made for the effect of the free $Mg^{2+}$ concentration on the combined equilibrium constant. This effect, however, is small (Veech et al., 1979). The validity of these calculations and the stability of the mixture with respect to the equilibrium concentrations of the substrates were checked experimentally by monitoring spectrophotometrically the NADH absorbance at 340 nm (see Table III). On completion of all additions, the equilibrium saturation-transfer measurement was made immediately. This involved acquisition of a 32-scan spectrum with selective saturation of the $\gamma$-phosphate resonance of ATP followed by a 32-scan spectrum with control irradiation. The time taken for acquisition of each spectrum was approximately 30 min. Samples taken before and after the measurement showed that during this period there was no significant loss of activity of either enzyme. However, with prolonged incubation there were both a significant loss of GAPDH activity and a change in the equilibrium substrate concentrations (see Table III). This made $T_1$ measurements in the presence of exchange impossible, since the exchange rate would be changing during the measurement. $T_1$ measurements were made, therefore, in mixtures which had only very low activities of the enzymes and, therefore, negligible exchange activity.

*NMR Measurements.* The experiments were performed on a home-built spectrometer, operating at a $^{31}$P NMR frequency of 73.84 MHz, using a Nicolet 1180 computer and 293B pulse programmer interfaced to an Oxford Instruments 4.7-T magnet. In the later stages of this work the spectrometer was replaced with a Bruker Biospec I console. All measurements were made at 30 °C. Saturation-transfer measurements on the cells and on the isolated enzymes were carried out as described previously (Brindle & Krikler, 1985; Brindle & Radda, 1987). The steady-state $P_i$ $z$ magnetization ($M_z$) was measured in the presence of selective saturation of the $\gamma$-phosphate resonance of ATP. The ratio of $M_z$ to the equilibrium magnetization ($M_0$) measured in the presence of irradiation of the $\gamma$-phosphate resonance of ATP is given by (Forsen & Hoffman, 1963)

$$M_z/M_0 = 1/(1 + kT_{1p})$$

where $k$ is a first-order rate constant describing loss of $z$ magnetization from $P_i$ and $T_{1p}$ is the spin–lattice relaxation time for the phosphorus nucleus in $P_i$. The selectivity of the irradiation was checked as described in Figure 1. The spectra used to obtain values for $M_z$ and $M_0$ were acquired with a 90° pulse and for the cell experiments were collected concurrently in interleaved blocks of 16 scans to give a total of either 256 or 512 scans for each spectrum. The total delay between aquisitions was 5.512 s. Under these conditions all cell resonances were fully relaxed. In experiments on the isolated enzymes the two spectra were collected sequentially and were the sum of 32 scans with a total delay between acquisitions of 50.512 s. The relaxation rate of the $P_i$ $z$ magnetization to its equilibrium value following inversion, in the presence of selective saturation of the $\gamma$-phosphate resonance of ATP, is given by $1/T_{1p} + k$ (Mann, 1977). For the cell experiments this value was determined by measuring the $P_i$ $z$ magnetization at 9–13 different delay times ($\tau$) following its inversion, with the pulse sequence 180°–$\tau$–90°–acquire. (see Figure 2). The spectra obtained at each different delay were collected in blocks of 16 scans. Four cycles through the delay list gave a total of 64 scans for each spectrum. In experiments on the isolated enzymes the $P_i$ $T_1$ was obtained by measuring the $P_i$ $z$ magnetization at 20 different delay values following its inversion. The individual spectra were the sum of 16 or 32 scans. In all of the experiments selective saturation of the $\gamma$-phosphate resonance of ATP was obtained with an auxiliary frequency source and amplifier. The lowest power compatible with complete saturation of the ATP resonance was used, and the irradiation was applied continuously except during acquisition.

Combination of the $T_1$ measurement with a determination of $M_z/M_0$ yields a value for $k$. Multiplication of $k$ by the $P_i$ concentration gives the exchange velocity. For the cell experiments the intracellular $P_i$ concentrations and those of the other metabolites were determined by comparing the resonance intensities with that of a methylenediphosphonate standard contained in a coaxial capillary tube suspended in the center of the NMR tube, as described previously (Brindle & Krikler, 1985). Resonance assignments in the cell spectra were made by comparing them with previously published spectra (den Hollander et al., 1981) and in cell extracts by adding the geniune compound to the extract. Resonance intensities were determined by cutting the peaks out of the plotted spectra and weighing them or by use of the integration routine in the computer software. Intracellular concentrations were calcu-

Table I: Metabolite Concentrations and Fluxes in Cells Overproducing Phosphoglycerate Kinase[a]

| | (A) cells transformed with plasmid pMA27 | (B) cells transformed with plasmid pMA301-1 | (C) cells transformed with plasmid pMA766 | (D) cells transformed with plasmid pMA301-1 |
|---|---|---|---|---|
| | Enzyme Activities (units/mL of Cell Water) | | | |
| enzyme | | | | |
| GAPDH | 765 ± 74 ($n$ = 4) | 1174 | 972 ± 34 ($n$ = 4) | 864 ± 24 ($n$ = 2) |
| PGK | 20172 ± 2670 ($n$ = 4) | 725 | 2790 ± 613 ($n$ = 4) | 644 ± 189 ($n$ = 4) |
| | Concentrations in Vivo ($\mu$mol/mL of Cell Water) | | | |
| metabolite | | | | |
| sugar phosphate | 14.2 ± 3.5 ($n$ = 20) | 10.0 ± 2.1 ($n$ = 20) | 10.0 ± 1.7 ($n$ = 14) | 9.3 ± 1.5 ($n$ = 10) |
| $P_i$ | 3.6 ± 0.9 ($n$ = 20) | 3.2 ± 1.0 ($n$ = 20) | 3.2 ± 0.9 ($n$ = 14) | 2.7 ± 0.6 ($n$ = 10) |
| NTP | 5.3 ± 0.9 ($n$ = 20) | 4.5 ± 0.6 ($n$ = 20) | 4.9 ± 0.6 ($n$ = 14) | 4.1 ± 0.7 ($n$ = 10) |
| $NAD^+$ | 2.6 ± 0.5 ($n$ = 20) | 2.4 ± 0.5 ($n$ = 20) | 2.6 ± 0.4 ($n$ = 14) | 2.4 ± 0.2 ($n$ = 10) |
| NADH | 0.121 ± 0.042 ($n$ = 9) | 0.059 ± 0.029 ($n$ = 3) | 0.107 ± 0.018 ($n$ = 3) | 0.081 ± 0.020 ($n$ = 10) |
| pH | 7.17 ± 0.04 ($n$ = 20) | 7.20 ± 0.05 ($n$ = 20) | 7.18 ± 0.05 ($n$ = 14) | 7.16 ± 0.04 ($n$ = 10) |
| | Concentrations Measured in Cell Extracts ($\mu$mol/mL of Cell Water) | | | |
| glucose 6-phosphate | 6.9 ± 0.5 ($n$ = 2) | 3.7 | 3.6 ± 1.2 ($n$ = 2) | 4.4 ± 0.3 ($n$ = 2) |
| fructose 6-phosphate | 2.8 | 1.5 | 1.4 | 1.8 |
| fructose 1,6-bisphosphate | 2.4 ± 0.5 ($n$ = 2) | 2.1 | 2.2 ± 0.1 ($n$ = 2) | 2.2 ± 0.2 ($n$ = 2) |
| DHAP plus GAP | 1.9 ± 0.1 ($n$ = 2) | 0.8 | 0.9 ± 0.1 ($n$ = 2) | 0.9 ± 0.1 ($n$ = 2) |
| 3-phosphoglyceric acid | 0.75 | | 0.4 ± 0.05 ($n$ = 2) | 0.3 |
| ATP | 1.4 | | 1.9 ± 0.5 ($n$ = 2) | 1.2 |
| ADP | 1.7 | | 1.4 ± 0.1 ($n$ = 2) | 1.4 |
| AMP | 1.6 | | 0.9 ± 0.0 ($n$ = 2) | 1.6 |
| $NAD^+$ | 1.3 | | 1.2 ± 0.3 ($n$ = 2) | 1.2 |
| | Fluxes [$\mu$mol $s^{-1}$ (mL of Cell Water)$^{-1}$] | | | |
| glucose consumption | 0.53 ± 0.04 ($n$ = 9) | 0.50 ± 0.09 ($n$ = 6) | 0.59 ± 0.05 ($n$ = 5) | 0.57 ± 0.04 ($n$ = 3) |
| ethanol production | 0.75 ± 0.08 ($n$ = 9) | 0.82 ± 0.19 ($n$ = 5) | 0.83 ± 0.06 ($n$ = 5) | 0.83 ± 0.07 ($n$ = 3) |
| calculated net $P_i$ consumption | 0.92 ± 0.09 ($n$ = 9) | 0.92 ± 0.17 ($n$ = 6) | 1.1 ± 0.13 ($n$ = 5) | 0.99 ± 0.07 ($n$ = 3) |
| $P_i \rightarrow$ ATP flux | 3.5 ± 1.5 ($n$ = 20) | 1.2 ± 0.5 ($n$ = 20) | 3.1 ± 1.2 ($n$ = 14) | 1.7 ± 1.0 ($n$ = 10) |

[a] The cells in parts A and B were grown for 24 h, and the cells in parts C and D were grown for 19 h. The concentrations of metabolites in vivo were estimated by comparing their resonance intensities with that of a methylenediphosphonate standard contained in a coaxial capillary (Brindle & Krikler, 1985). The intracellular pH was estimated from the chemical shift difference between this standard and the intracellular $P_i$ resonance (Brindle & Krikler, 1985). The estimates of NTP and $NAD^+$ concentrations are only approximate since their resonances overlap those of the polyphosphates and the $\alpha$-phosphates of the NTPs, respectively. The intracellular NADH concentration was calculated by assuming that the reaction catalyzed by alcohol dehydrogenase is near to equilibrium in the cell and that ethanol and acetaldehyde are rapidly transported across the cell membrane. The NADH concentration was estimated, therefore, from the measured intracellular $NAD^+$ concentration and pH and the ethanol/acetaldehyde ratio in the perifusate leaving the NMR tube. The equilibrium constant for the reaction was assumed to be $1.19 \times 10^{-11}$ M (Sund & Theorell, 1963). The sugar phosphate resonance is due to a number of compounds including glucose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate, and glyceralde 3-phosphate. These compounds were assayed enzymatically in cell extracts as described under Materials and Methods. The concentration of fructose 6-phosphate was derived from the glucose 6-phosphate concentration by assuming that glucose 6-phosphate isomerase is catalyzing a reaction that is near to equilibrium in the cell (Johnson, 1960). The concentration of 3-phosphoglyceric acid, ATP, ADP, and AMP were determined from proton-decoupled $^{31}P$ NMR measurements on concentrated cell extracts. Peak assignments were made by adding the genuine compounds to the extract. The concentrations of the compounds in the extract were determined by comparing their resonance intensities with the resonances of compounds present in known amount, e.g., fructose 1,6-bisphosphate and glucose 6-phosphate. Extract spectra obtained at pulse repetition times of 4 and 8 s showed no significant differences. The metabolite concentrations in the extracts are average values obtained from repeat measurements at 73.836 and 121.494 MHz. Preparation of extracts for enzyme assay and the details of the assays are given under Materials and Methods. The enzyme activities shown were determined from extracts made prior to perifusion of the cells (see text and Table II). Glucose consumption and ethanol production were determined by sampling the buffer reservoir at hourly intervals for at least 5 h. Over this period both rates were linear (correlation coefficient of 1.00). The calculated net $P_i$ consumption due to glycolysis is equal to (2 × glucose consumption rate + ethanol production rate)/2 (see Discussion). The $P_i \rightarrow$ ATP flux was measured by magnetization transfer. All values are quoted per milliliter of cell water by assuming that 1.67 g of wet yeast contains 1 mL of cell water (Gancedo & Gancedo, 1973). The errors quoted are standard deviations from the mean. The numbers in parentheses represent the number of determinations. For the concentration measurements in vivo these numbers represent the number of 256- or 512-scan spectra from which the data were taken. The numbers in the case of the NADH concentrations represent the number of determinations of ethanol and acetaldehyde in the cell perifusate. The numbers in the case of the magnetization-transfer measurements represent the number of individual determinations of $M_z/M_0$, and in the case of the glucose consumption and ethanol production rates they represent the number of different cell batches used.

lated by assuming that 1.67 g of wet yeast contains 1 mL of cell water (Gancedo & Gancedo, 1973). Intracellular pH was estimated from the chemical shift difference between the $P_i$ and methylenediphosphonate resonances (Brindle & Krikler, 1985).

## Results

Steady-state saturation-transfer measurements on the plasmid-transformed cell preparations used in this study are shown in Figure 1. Exchange between $P_i$ and the $\gamma$-phosphate of ATP results in a reduction in intensity of the $P_i$ resonance following selective saturation of the ATP resonance, as shown by the difference spectra [Figure 1: (A) II − I; (B) II − I; (C) II − I; (D) II − I]. Cells transformed with plasmid pMA27 also show a significant exchange between the sugar phosphate resonances and the $\gamma$-phosphate of ATP. This exchange, which has been observed previously (Campbell-Burk et al., 1987b), has been attributed to exchange between ATP and glucose 6-phosphate and fructose 1,6-bisphosphate. The difference spectra show that the percentage reduction in the $P_i$ resonance following selective saturation of the $\gamma$-phosphate resonance of ATP is larger in the cells overexpressing PGK (Figure 1A,C) than in the control cells expressing interferon (Figure 1B,D). At the signal-to-noise ratios obtained in these spectra, there was no detectable exchange between the $\beta$-phosphate of ATP and the $\beta$-phosphate of ADP (Brindle & Radda, 1987). The results of repeated measurements are shown in Table I. Combination of the steady-state saturation-transfer measurements with measurements of the $P_i$ $T_1$ in the presence of selective saturation of the $\gamma$-phosphate resonance of ATP

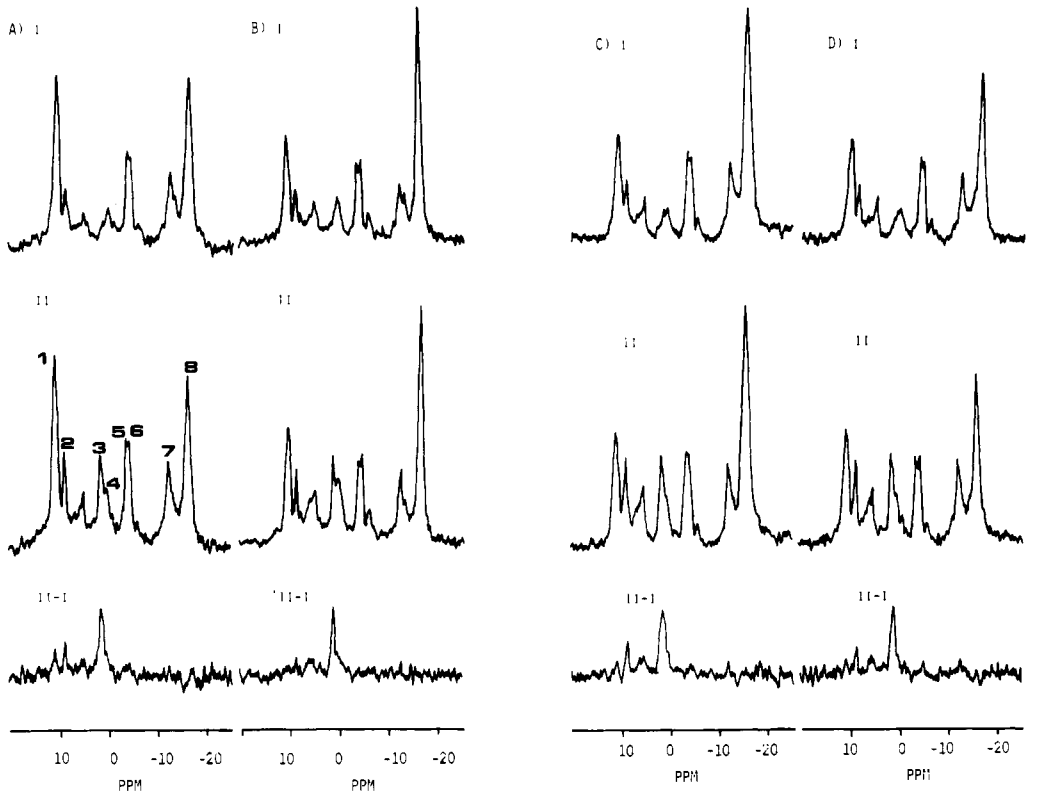



FIGURE 1: Steady-state saturation-transfer measurements of ATP ↔ $P_i$ exchange in cells with different concentrations of phosphoglycerate kinase. The spectra are each the sum of 512 scans, collected in blocks of 16 scans with an interpulse delay of 5.512 s and a sweep width of ±2000 Hz (see Materials and Methods). The chemical shift scale is referenced to the transmitter offset at 0.0 ppm. An exponential line broadening of 15 Hz was applied. The resonances were assigned by comparing them with previously published spectra (den Hollander et al., 1981). The assignments are as follows (see spectrum A-II): 1, sugar phosphates; 2, cytosolic orthophosphate; 3, γ-phosphate of nucleoside triphosphates and β-phosphate of nucleoside diphosphates; 4, terminal phosphates of polyphosphates; 5, α-phosphates of nucleoside di- and triphosphates; 6, NAD(H); 7, β-phosphate of nucleoside triphosphates and penultimate phosphates of polyphosphates; 8, inner phosphates of long-chain polyphosphates. Spectra A, I and II, were from cells containing plasmid pMA27 which were grown for 24 h; spectra B, I and II, were from cells containing plasmid pMA301-1 which were grown for 24 h; spectra C, I and II, were from cells containing plasmid pMA766 which were grown for 19 h; spectra D, I and II, were from cells containing plasmid pMA301-1 which were grown for 19 h. The PGK activities in these cells are given in Table I. Spectra A-I, B-I, C-I, and D-I were collected with selective saturation of the γ-phosphate resonance of ATP. Spectra A-II, B-II, C-II, and D-II are control spectra in which the irradiating field was applied at a frequency downfield of the $P_i$ resonance equal to the frequency difference between the resonances of $P_i$ and the γ-phosphate of ATP.

can be used to estimate the $P_i \rightarrow$ ATP flux (see Materials and Methods). All flux measurements were calculated by assuming that a simple two-site exchange model can be used to represent the exchange between ATP and $P_i$ (see Discussion). A typical inversion recovery $T_1$ measurement on the $P_i$ resonance is shown in Figure 2. Four $T_1$ measurements, three on cells containing plasmid pMA301-1 and one on cells containing plasmid pMA27, gave an intrinsic $T_1$ for the $P_i$ resonance (the $T_1$ which would be observed in the absence of exchange) of 1.05 ± 0.11s. This $T_1$ was used in all calculations of the $P_i \rightarrow$ ATP flux from the steady-state saturation-transfer measurements.

Measurements of magnetization transfer, glucose consumption, ethanol production, and the cellular metabolite concentrations in the four different cell preparations used in the study are shown in Table I. With the exception of the enzyme activities, the measurements shown in the table were made during the 5 h following the start of cell perifusion at 30 °C. During the 5-h incubation the observed intracellular concentrations of the cell metabolites and the rates of glucose consumption and ethanol production remained nearly constant, as was observed previously (Brindle & Krikler, 1985). However, there were small increases in the ethanol/acetaldehyde ratio and decreases in intracellular enzyme activities. For example in an experiment on cells transformed with plasmid pMA27, the ethanol/acetaldehyde ratio increased from 390 after 78 min to 550 after 314 min. In an experiment on cells transformed with plasmid pMA766, the GAPDH activity dropped from 987 to 662 units/mL of cell water and the PGK activity decreased from 2361 to 1938 units/mL of cell water after 246 min of cell perifusion. The data shown in Table I, with the exception of the enzyme activities and glucose consumption and ethanol production rates, are averages of data not only from different cell batches but from different times within the 5-h perifusion period. A single experiment was also carried out on cells transformed with plasmid pMA 301-1 in which the GAPDH activity had been selectively reduced by limited incubation with iodoacetate (see Table II).

The total concentrations of sugar phosphate determined from the peak areas in vivo (see Figure 1) show reasonable agreement with the concentrations of glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-bisphosphate, dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate, and 3-phosphoglyceric acid determined in cell extracts (Table I). Note that the fructose 1,6-bisphosphate contributes 2 phosphate equiv to the peak observed in vivo. The concentrations of these metabolites fall within the range of published values for cells incubated anaerobically with glucose (Gancedo & Gancedo, 1973). The glyceraldehyde 3-phosphate concentration in vivo was calculated from the measured dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate concentration, assuming that the reaction catalyzed by triosephosphate isomerase was near to equilibrium. $^{13}$C NMR measurements have shown that in glucose-grown cells incu-

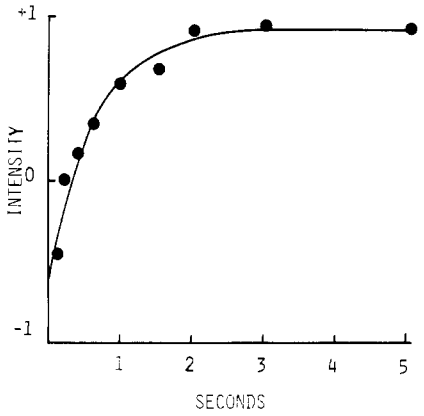



FIGURE 2: Inversion recovery $T_1$ measurement on the cytosolic $P_i$ resonance in the presence of selective saturation of the γ-phosphate resonance of ATP. Plot of peak intensity versus the time between the 180° pulse and the 90° acquisition pulse in an inversion recovery $T_1$ measurement. The spectra obtained at each different delay following inversion were collected in blocks of 16 scans. Four cycles through the delay list gave a total of 64 scans for each spectrum. The delay between the end of the acquisition period and the next 180° pulse was 5 s. Peak intensities were determined by measuring peak heights. The solid line is a least-squares fit of the data to the function $y = A_1(1 - A_2e^{-(1/T_{1P}+k)t})$, where $y$ is the $P_i$ $z$ magnetization, $t$ is the delay between the 180° and 90° pulses, $T_{1p}$ is the intrinsic $T_1$ of the $P_i$ resonance, and $k$ is the exchange rate constant (see Materials and Methods).

Table II: Metabolite Concentrations and Fluxes in Cells Treated with Iodoacetate[a]

| enzyme | enzyme act. (units/mL of cell water) |
|---|---|
| glyceraldehyde-3-phosphate dehydrogenase at $t$ = 0 min | 198 |
| glyceraldehyde-3-phosphate dehydrogenase at $t$ = 262 min | 180 |
| phosphoglycerate kinase at $t$ = 0 min | 784 |
| phosphoglycerate kinase at $t$ = 262 min | 456 |

| metabolite | concn in vivo (μmol/mL of cell water) |
|---|---|
| sugar phosphate | 10.7 ± 1.0 ($n$ = 2) |
| $P_i$ | 3.7 ± 0.1 ($n$ = 2) |
| NTP | 4.3 ± 0.8 ($n$ = 2) |
| $NAD^+$ | 2.3 ± 0.1 ($n$ = 2) |
| pH | 7.17 ± 0.04 |

| | fluxes [μmol $s^{-1}$ (mL of cell water)$^{-1}$] |
|---|---|
| glucose consumption | 0.54 |
| ethanol production | 0.79 |
| calcd net $P_i$ consumption | 0.94 |
| $P_i \rightarrow$ ATP | 1.8 ± 0.8 ($n$ = 2) |

[a] The cells were transformed with plasmid pMA301-1 and grown for 19 h on 2% glucose as described under Materials and Methods. Inhibition of GAPDH activity was obtained by incubating the cells with 0.5 mM iodoacetate for 60 min at room temperature prior to immobilization in agarose. The cells were thoroughly washed after iodoacetate treatment. Measurements of enzyme activities, metabolite concentrations, intracellular pH, and fluxes were made as described in the legend to Table I and under Materials and Methods.

bated anaerobically with [1-$^{13}$C]glucose there is considerable scrambling of the label between the 1- and 6-positions of fructose 1,6-bisphosphate, indicating that the reactions catalyzed by aldolase and triosephosphate isomerase are near to equilibrium (den Hollander et al., 1979). Calculations show that, in the cells transformed with plasmid pMA27, the measured concentrations of fructose 1,6-bisphosphate and dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate are close to those expected if aldolase and triosephosphate isomerase are catalyzing reactions which are near to equilibrium. The calculated equilibrium concentrations are 2.2 mM fructose 1,6-bisphosphate and 2.3 mM dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate. However, for the other cells there is a discrepancy between the observed and calculated equilibrium concentrations. In these cases the expected equilibrium concentrations are 1.7 mM fructose 1,6-bisphosphate and 2.0 mM dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate. This discrepancy indicates that in these cells there may be some disequilibrium in the reactions catalyzed by aldolase and triosephosphate isomerase.

The measured $P_i$ concentrations are slightly lower than a value of 5 mM reported for glucose-grown derepressed cells during anaerobic steady-state glycolysis (den Hollander et al., 1981) and similar to a value of 3.9 mM that we reported previously for ethanol-grown yeast perifused aerobically with glucose (Brindle & Krikler, 1985). The $NAD^+$ concentrations determined in cell extracts are similar to values reported for cells growing aerobically on glucose (Sáez & Lagunas, 1976). The higher values determined in vivo here probably reflect overlap of the $NAD^+$ resonances with other cellular resonances (see Figure 1). The NADH concentrations shown in Table I were estimated from the ethanol/acetaldehyde ratio in the perifusate, the intracellular $NAD^+$ concentration, and the intracellular pH, assuming that ethanol and acetaldehyde rapidly cross the cell membrane and that the reaction catalyzed by alcohol dehydrogenase is near to equilibrium in the cell. Holzer et al. (1956) used this approach in a previous study of the $NAD^+$/NADH ratio in yeast and have provided evidence for these assumptions. The ethanol/acetaldehyde ratios measured in this previous study were similar to those measured here. However, the calculated $NAD^+$/NADH ratio was much higher at 400:1 since an intracellular pH of 6.0 was assumed. The calculated $NAD^+$/NADH ratio in this study, with an intracellular pH of 7.2, was approximately 12:1 (Table I). The NADH concentrations shown in Table I were calculated with the $NAD^+$ concentrations determined in cell extracts. The estimated intracellular pHs are similar to a previously reported value of 7.23 for glucose-derepressed cells incubated anaerobically with glucose (den Hollander et al., 1981).

Analysis of cell extracts using HPLC showed that the adenine nucleosides comprised approximately 80% of the total nucleoside pool. If it is assumed that the AMP concentration measured in the cell extracts is artifactually high and is derived from the ATP and ADP pool, then there is good agreement between the concentrations of ATP and ADP observed in vivo and the concentrations of adenine nucleosides determined in cell extracts (see Table I). The high ADP and AMP concentrations measured in cell extracts are probably an artifact of the extraction procedure. Sáez and Lagunas (1976) reported values for cells grown aerobically on glucose of approximately 1.3 mM for ATP, 0.3 mM for ADP, and 0.1 mM for AMP. Reibstein et al. (1986) reported values for glucose-grown derepressed cells incubated anaerobically on glucose of 2.8 mM for ATP, 0.68 mM for ADP, and 0.16 mM for AMP. Delaying transfer of the cells to liquid nitrogen prior to perchloric acid extraction has been shown to result in a rapid loss of ATP and an increase in ADP and AMP (Sáez & Lagunas, 1976). Since the contents of the NMR tube were rapidly transferred to a liquid nitrogen cooled mortar (10 s) in the experiments described here, this suggests that extraction of ATP from cells embedded in agarose may be poor.

Magnetization-transfer measurements on the isolated enzymes, under conditions designed to simulate those in the cell with respect to the concentrations of the enzymes substrates, are shown in Table III. The range of substrate concentrations

Table III: Exchange Velocity Measurements in Vitro[a]

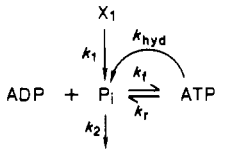
| part | equilibrium substrate concn (mM) | | | | | | | measured NADH concn (mM) | | enzyme act. (units/mL) | | measd exchange velocity (mM $s^{-1}$) | calcd exchange velocity (mM $s^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ATP | $P_i$ | NAD | NADH | GAP | 3PGA | ADP | at $t$ = 0 | at $t$ = 30 min | GAPDH | PGK | | |
| A | 4 | 4 | 1 | 0.20 | 0.09 | 0.9 | 0.7 | 0.200 | 0.151 | 35 | 27 | 0.16 | 4.2 |
| | 4 | 4 | 1 | 0.20 | 0.09 | 0.9 | 0.7 | | | 35 | 547 | 0.19 | |
| | 4 | 4 | 1 | 0.20 | 0.09 | 0.09 | 0.07 | | | 35 | 25 | 0.11 | 2.9 |
| | 4 | 4 | 1 | 0.20 | 0.09 | 0.09 | 0.07 | | | 35 | 595 | 0.12 | |
| B | 4 | 4 | 1 | 1.30 | 0.09 | 0.10 | 0.50 | | | 25 | 26 | 0.06 | |
| | 4 | 4 | 1 | 1.30 | 0.09 | 0.10 | 0.50 | | | 20 | 579 | 0.06 | |
| | 4 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.019 | 0.074 | 0.111 | 20 | 26 | 0.02 | |
| | 4 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.019 | | | 20 | 579 | 0.12 | |
| C | 4 | 4 | 1 | 0.05 | 0.01 | 0.029 | 0.05 | 0.055 | 0.072 | 40 | 21 | 0.05 | |
| | 4 | 4 | 1 | 0.05 | 0.01 | 0.029 | 0.05 | | | 40 | 788 | 0.10 | |
| D | 1 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.005 | 0.076 | 0.130 | 44 | 18 | 0.06 | |
| | 1 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.005 | | | 44 | 111 | 0.18 | |
| | 1 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.005 | | | 44 | 777 | 0.15 | |
| E | 1 | 4 | 1 | 0.05 | 0.09 | 1.00 | 0.05 | 0.052 | 0.080 | 44 | 12 | 0.07 | |
| | 1 | 4 | 1 | 0.05 | 0.09 | 1.00 | 0.05 | | | 41 | 68 | 0.19 | |
| | 1 | 4 | 1 | 0.20 | 0.09 | 1.00 | 0.194 | | | 39 | 12 | 0.04 | |
| | 1 | 4 | 1 | 0.20 | 0.09 | 1.00 | 0.194 | | | 39 | 68 | 0.10 | |
| F | 4 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.019 | | | 42 | 23 | 0.05 | |
| | 4 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.019 | | | 42 | 27 | 0.08 | 2.1 |
| | 4 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.019 | | | 42 | 136 | 0.15 | 3.3 |
| | 4 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.019 | | | 42 | 276 | 0.11 | |
| | 4 | 4 | 1 | 0.05 | 0.09 | 0.10 | 0.019 | | | 11 | 31 | 0.06 | |
| | 4 | 4 | 1 | 0.05 | 0.09 | 1.0 | 0.19 | | | 42 | 27 | 0.14 | 3.6 |
| | 4 | 4 | 1 | 0.05 | 0.09 | 1.0 | 0.19 | | | 42 | 136 | 0.20 | 4.4 |
| G | 4 | 4 | 1 | 0.06 | 0.04 | 0.096 | 0.050 | | | 29 | 25 | 0.08 | 2.2 |
| | 4 | 4 | 1 | 0.06 | 0.04 | 0.096 | 0.050 | | | 33 | 120 | 0.23 | 6.2 |
| | 4 | 4 | 1 | 0.06 | 0.04 | 0.096 | 0.050 | | | 33 | 240 | 0.16 | |
| | 4 | 4 | 1 | 0.06 | 0.04 | 0.96 | 0.50 | | | 33 | 25 | 0.18 | 5.0 |
| | 4 | 4 | 1 | 0.06 | 0.04 | 0.96 | 0.50 | | | 33 | 120 | 0.24 | 7.4 |

[a] Sample preparation and the measurement of exchange velocities are described under Materials and Methods. The substrate concentrations were calculated from the known added substrate concentrations and the equilibrium constants for the reactions catalyzed by GAPDH, PGK, triosephosphate isomerase, and aldolase. The activities of GAPDH and PGK were assayed immediately prior to the NMR measurements. Loss of enzyme activity during the exchange measurements was negligible. The measured NADH concentrations at the beginning and end of each experiment were determined spectrophotometrically in parallel incubations outside the magnet. The calculated exchange velocities in vivo were obtained by multiplying the measured exchange velocities by the ratio of the enzyme activities in vivo and in vitro. At low PGK concentrations in vitro, simulating conditions in the cells transformed with plasmid pMA301-1, the calculated exchange velocity in vivo was obtained by multiplying the measured exchange velocity by the ratio of the PGK and GAPDH activities in vivo, 700 and 900 units/mL of cell water, respectively (see Table I), with their activities in vitro. At higher PGK concentrations, simulating conditions in cells transformed with plasmid pMA766, the calculated exchange velocity was obtained by multiplying the measured exchange velocity by the ratio of the PGK and GAPDH activities in these cells, 3000 and 900 units/mL of cell water, respectively, with their activities in vitro.

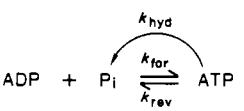
used in these experiments was based on the measured cellular metabolite concentrations (Table I) and on the values given in the literature (see above). The measurements shown in Table III (part A) give calculated exchange velocities for the concentrations of GAPDH and PGK found in the interferon control cells that were 3–4 times higher than those actually measured in vivo. Furthermore, unlike the cell, there was no significant increase in the exchange velocity with increasing PGK concentration. Increasing the NADH concentration to 1.3 mM, which is similar to the total extractable NADH concentration measured in cell extracts (Sâez & Lagunas, 1976), lowered the exchange velocity, but there was still no increase in exchange velocity with increases in PGK concentration. Lowering both the NADH and ADP concentrations lowered the exchange velocity and made it sensitive to the concentration of PGK (Table III, part B). Further experiments, under a variety of conditions, showed that if the ADP and/or 3-phosphoglyceric acid concentrations were relatively low, then there was an increase in the exchange flux with increasing PGK concentration. Furthermore, in some cases, these fluxes and the increase in flux with increasing PGK concentrations were quantitatively similar to those found in vivo. For example in the experiments shown in Table III, part F, the calculated exchange velocities for the concentrations of GAPDH and PGK found in the interferon control cells and the cells transformed with plasmid pMA766 are very similar to the exchange velocities measured in vivo. Further increases in PGK concentration produced no further increase in the exchange velocity, similar to the situation observed in vivo where the exchange velocities in the cells overproducing PGK 5- and 20-fold were very similar (see Table III). Increasing the ADP and 3-phosphoglyceric acid concentrations increases the exchange flux to values in excess of those observed in vivo (Table III, parts A and G) and diminishes the increase in flux observed with increases in PGK concentration. These data suggest, therefore, that in vivo the 3-phosphoglyceric acid and ADP concentrations are relatively low. The ADP concentrations measured in extracts, as already discussed, are artifactually high. However, assuming that the measurements of all the other substrates for the coupled GAPDH and PGK reactions are reliable, then the calculated equilibrium ADP concentration in the cells is between 0.1 and 0.2 mM. If this assumption is correct, then there is reasonably good agreement between the exchange velocities measured in vitro and those measured in the cell [see Tables I and III (part F)]. Decreasing the GAPDH activity by a factor of 4 in vitro (Table III, part F) has little effect on the exchange velocity and further demonstrates the low flux control coefficient of this enzyme for the exchange. This is similar to the situation observed in vivo where a reduction in GAPDH activity, through iodoacetate inhibition, had little effect on the exchange velocity (Table II).

## Discussion

The magnetization-transfer measurements have been analyzed by assuming that the following model can be used to describe the exchange between $P_i$ and ATP (Alger et al., 1982; Brindle & Krikler, 1985):




where the rate constants are apparent first order. Thus $k_f[P_i]$ describes flux from inorganic phosphate to ATP in the coupled reactions catalyzed by GAPDH and PGK, $k_r$[ATP] describes the reverse flux, and $k_{hyd}$[ATP] describes the hydrolysis of ATP in other cell reactions. Phosphate consumption in reactions other than that catalyzed by the glycolytic enzymes is described by $k_2[P_i]$. In the aerobic yeast cell the principle reaction consuming $P_i$ is that catalyzed by the mitochondrial $F_1F_0$ ATP synthase (Brindle & Krikler, 1985). However in the anaerobic cell, we have assumed that this reaction is negligible since there is no evidence for an exchange reaction catalyzed by the synthase. $^{31}P$ NMR magnetization-transfer measurements in a variety of systems including yeast (Brindle & Krikler, 1985; Campbell et al., 1985; Campbell-Burk et al., 1987a), heart (Matthews et al., 1981), kidney (Freeman et al., 1983), and maize root tips (Roberts et al., 1984) have shown that flux in the mitochondrial ATP synthase reaction is unidirectional in vivo. $X_1$ represents a pool of phosphate from which the production of free cytosolic $P_i$ is relatively slow on the time scale of the saturation-transfer experiment. This pool includes vacuolar $P_i$ and compounds which are phosphorylated by ATP but are broken down only very slowly in phosphatase reactions to regenerate $P_i$. If flux from ATP to $P_i$ via $X_1$ were relatively fast and the exchanging molecule in $X_1$ were present in concentrations comparable to those of ATP and $P_i$, then the exchange between ATP and $P_i$ could no longer be regarded as a simple two-site exchange. Under these conditions the calculated flux between ATP and $P_i$ could be underestimated. An example of such a reaction in the yeast cell is the reaction catalyzed by fructose 1,6-bisphosphatase. The cyclic reactions catalyzed by phosphofructokinase and fructose 1,6-bisphosphatase could catalyze an exchange between ATP and $P_i$ in which the intermediate, fructose 1,6-bisphosphate, is present at comparable concentrations to those of ATP and $P_i$ (Table I). However, fructose 1,6-bisphosphatase activity is relatively low compared to the fluxes measured here and is absent in cells grown on glucose (Maitra & Lobo, 1978; Campbell-Burk et al., 1987b). If the molecule in $X_1$ were very low in concentration and therefore turning over very rapidly, the exchange between ATP and $P_i$ could still be treated as two-site exchange (Brindle & Radda, 1985). Under these conditions the intermediate in $X_1$ is kinetically equivalent to the enzyme–substrate intermediates in the ATP hydrolysis reactions described by $k_{hyd}$[ATP] and can be ignored. An example of such an intermediate is 1,3-diphosphoglycerate, which is an intermediate in the ATP ↔ $P_i$ exchange reaction catalyzed by GAPDH and PGK (Brindle & Krikler, 1985; Brindle & Radda, 1987). The model can therefore be reduced to




The observed intracellular ATP concentrations in vivo are in a steady state, and therefore, $k_{for}[P_i] = (k_{hyd} + k_{rev})$[ATP]. The rate of ATP hydrolysis will be equal to the rate of net ATP synthesis, and in the anaerobic cell we have assumed that this is equal to twice the rate of glucose consumption or equal to the rate of ethanol production. The value shown in Table I is an average value derived from the glucose consumption and ethanol production data. Therefore, the flux measured by magnetization transfer, i.e., $k_{for}[P_i]$, will be equal to the net $P_i$ consumption in glycolysis if $k_{rev}$ is negligible, that is, if the coupled GAPDH and PGK reactions are effectively unidirectional, and will exceed the net $P_i$ consumption if the enzymes catalyze an exchange reaction (see below).

Cells transformed with plasmid pMA27 showed a 20-fold increase in PGK activity compared to cells transformed with plasmid pMA301-1, in which the PGK coding sequence was replaced with an interferon coding sequence. However, despite this marked increase in PGK concentration, there was no significant difference in glycolytic flux, as determined by glucose consumption or ethanol production (Table I). The flux control coefficient of this enzyme for glycolytic flux is, therefore, very low. Magnetization-transfer measurements of $P_i \rightarrow$ ATP flux, however, showed a significant increase in the cells overproducing PGK. The $P_i \rightarrow$ ATP flux was $3.5 \pm 1.5$ $\mu$mol $s^{-1}$ (mL of cell water)$^{-1}$ in the overproducer compared to $1.2 \pm 0.5$ $\mu$mol $s^{-1}$ (mL of cell water)$^{-1}$ in the cells producing interferon (Table I). These measurements indicate, therefore, that the observed exchange is limited to some extent by PGK activity; i.e., the flux control coefficient for the exchange is relatively high. This conclusion requires, however, that there are no significant differences in the substrate concentrations of GAPDH and PGK in the overproducer and interferon control cells. The NMR measurements on the intact cells showed that cellular pH and the concentrations of $P_i$, nucleoside triphosphates, and NAD(H) were similar but that the sugar phosphate peak was significantly larger in the PGK overproducer. The observed peak in the cell is composed of several resonances including those of fructose 1,6-bisphosphate and glucose 6-phosphate. Enzymatic analysis of cell extracts showed that the overproducer contained significantly more glucose 6-phosphate and dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate than the control cells producing interferon (Table I). The difference in the $P_i \rightarrow$ ATP flux between the two different cell types may therefore reflect a difference in substrate concentrations rather than a change in PGK concentration. The reason for this difference has not been established; however, the PGK overproducer grew more slowly and achieved a lower cell density in stationary-phase than the interferon-producing cells. This presumably reflects the metabolic stress placed on the cell by the massive overproduction of the enzyme, which constitutes up to 40% of total cell protein. Another notable difference between the overproducer and control cells is the presence of significant exchange between ATP and the sugar phosphates in the overproducer. This has been observed previously and attributed to flux between ATP and fructose 1,6-bisphosphate and glucose 6-phosphate (Campbell-Burk et al., 1987b).

Cells transformed with plasmid pMA766 show a more moderate increase in PGK concentration with a 5-fold increase in PGK activity compared to the control cells producing interferon (Table I). Again there were no significant differences in glycolytic flux, but the magnetization-transfer measurements showed an increase in $P_i \rightarrow$ ATP flux similar to that seen in the cells overproducing PGK 20-fold. The $P_i \rightarrow$ ATP flux was $3.1 \pm 1.2$ $\mu$mol $s^{-1}$ (mL of cell water)$^{-1}$ in the PGK overproducer compared to $1.7 \pm 1.0$ $\mu$mol $s^{-1}$ (mL of cell water)$^{-1}$ in

the control cells producing interferon. The measured fluxes, enzyme activities, and substrate concentrations observed in these cells are summarized in Table I. These data show no significant differences in cell metabolite concentrations between the two cell types. Furthermore, unlike the cells transformed with plasmid pMA27, these pMA766-transformed cells grow at the same rate and achieve the same cell densities in stationary phase as the control cells.

Reducing the activity of GAPDH 4-fold, by limited incubation with iodoacetate, has no significant effect on glycolytic flux or the intracellular metabolite concentrations (Table II), indicating that the flux control coefficient of this enzyme for glycolysis is also relatively low. Similarly, the reduction of GAPDH activity has no significant effect on the measured $P_i \rightarrow$ ATP flux, indicating that the enzyme also has a low flux control coefficient for the exchange reaction. This is consistent with the relatively high flux control coefficient of PGK for the exchange.

The absence of any significant change in glycolytic flux or metabolite concentrations following moderate increases in PGK concentration or decreases in GAPDH activity indicates that both enzymes have low flux control coefficients for glycolysis. The marked increase in $P_i \rightarrow$ ATP flux in the cells overproducing PGK 5-fold and the absence of any significant change in the exchange velocity in cells with lower GAPDH activity indicate that PGK activity limits the exchange flux in the control cells. The differences in metabolite concentrations in cells overproducing PGK 20-fold may reflect changes in the activities of other enzymes due to the slower growth rate of these cells. This latter observation shows that it may be important to limit the overproduction of an enzyme when its contribution to flux in a metabolic pathway is investigated since the effects of overproduction may extend outside a direct kinetic effect of the enzyme on the pathway itself. Walsh and Koshland (1985) in their study on *E. coli* noted that acetate inhibited growth in cells in which there was a 50-fold overproduction of citrate synthase but had little effect in cells with wild-type levels of the enzyme.

Although GAPDH and PGK seem to be present in catalytic excess, PGK does not appear to be catalyzing a reaction which is considerably faster than the net glycolytic flux since in the control cells the measured $P_i \rightarrow$ ATP flux is, at most, only a factor of 2 greater than the net $P_i$ consumption in glycolysis, estimated from the glucose consumption and ethanol production data (see Table I). In order to investigate the relatively low exchange velocity expressed by the enzyme in the cell and to determine whether its kinetic properties in vivo could be predicted from the kinetic properties expressed by the isolated enzyme in vitro, a series of measurements were made on the isolated enzymes in vitro under conditions designed to mimic those in the cell. These measurements have shown that the exchange velocities, when corrected for the difference in spectrophotometrically assayed enzyme activities, are similar to those measured in vivo. Furthermore, the changes in flux with increases in PGK or decreases in GAPDH activity are similar to those observed in the cell. This provides further support for the conclusion that the flux through the reaction catalyzed by PGK in the control cells is not in considerable excess of the glycolytic flux. However, a linear extrapolation of the data obtained in vitro to the intact cell, based on the measured activities of the enzymes, assumes that the higher enzyme concentrations in vivo have no other effects on the kinetic properties expressed by the enzymes. Assuming a specific activity for GAPDH of 100 units/mg and for PGK of 450 units/mg (Bergmeyer, 1974b), the concentrations of GAPDH and PGK in control cells are approximately 0.24 $\mu$mol/mL of cell water and 0.035 $\mu$mol/mL of cell water, respectively. In the cells transformed with plasmid pMA766, the PGK concentration is approximately 0.15 $\mu$mol/mL of cell water, and in the cells transformed with plasmid pMA27, it is approximately 1.0 $\mu$mol/mL of cell water. In contrast, the enzyme concentrations used in vitro are between 1 and 40 $\mu$M. The difference in enzyme concentrations can have two important consequences: (1) there may be enzyme–enzyme interactions at the higher enzyme concentrations found in vivo but not at the lower concentrations used in vitro, and (2) a significant proportion of the enzymes' substrates may be bound to the enzymes in vivo so that the measured extractable substrate concentrations may be very much greater than the free concentrations.

The active site concentrations of adolase, triosephosphate isomerase (Pette, 1965), GAPDH, and PGK (in control and pMA766-transformed cells) are relatively small in comparison to the measured concentrations of $P_i$, ATP, $NAD^+$, fructose 1,6-bisphosphate, and dihydroxyacetone phosphate plus glyceraldehyde 3-phosphate (Table I). The measured concentrations of 3-phosphoglyceric acid also do not appear to be significantly affected by moderate increases in PGK concentration. Therefore, the measured total concentrations of these substrates can be assumed to represent the free substrate concentrations available to the enzymes in the cell. The similarity of the exchange velocities observed in the cell and in vitro, at the estimated free substrate concentrations in vivo, and the changes in these fluxes with decreases in GAPDH activity or increases in PGK activity support this assumption. The NADH concentrations shown in Table I are the calculated free concentrations based on the ethanol/acetaldehyde ratio and the measured $NAD^+$ concentration.

The enzyme concentrations used in vitro are similar to those used in a previous study which demonstrated a kinetic interaction between GAPDH and PGK in vitro (Weber & Bernhard, 1982). Thus, the enzyme concentrations used here in vitro are sufficiently high to give an enzyme–enzyme interaction. The similarity of the fluxes measured in the cell and in vitro implies that this interaction also occurs in vivo and that the higher protein concentrations in vivo have no further significant effect on this interaction.

The similarity of the results obtained in the cell and in vitro suggests that the intracellular environment of these enzymes is relatively well-defined in terms of their substrate concentrations. If there is a glycolytic enzyme complex, with compartmentation of glycolytic intermediates, then its influence on the environment and the kinetic properties expressed by PGK in the cell would appear to be relatively small. However when increasing the concentration of PGK in vivo, we may only be increasing the concentration of the free uncomplexed form of the enzyme, and therefore, the activity observed at high enzyme concentration will reflect predominantly the activity of this form. In order to further investigate the proposal that PGK is complexed to other enzymes in the cell and to better define the flux control coefficient of the enzyme for both glycolysis and the exchange reaction will require studies on cells in which the PGK activity has been lowered below the normal expression level of the chromosomal PGK gene (Walsh & Koshland, 1985). This could be achieved, for example, by replacing the chromosomal PGK gene with one in which the promoter had been partially deleted (Rothstein, 1983).

Molecular genetics has been used here to determine the contribution of an enzyme to the flux in a coupled enzyme reaction in vivo. This is a powerful technique which has been

proposed and exploited by a number of workers (Flint et al., 1981; Middleton & Kacser, 1983; Walsh & Koshland, 1985). Combination of this methodology with NMR spectroscopy should be a useful approach for the investigation of metabolic control, not only in microorganisms but also in isolated cells of higher organisms and in inact animals, where the value of NMR in the noninvasive investigation of cellular metabolism and enzyme kinetics is already well established (Gadian & Radda, 1981; Avison et al., 1986; Brindle & Campbell, 1987).

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**Registry No.** $P_i$, 14265-44-2; PGK, 9001-83-6; GAPDH, 9001-50-7; ATP, 56-65-5; D-glucose, 50-99-7; ethanol, 64-17-5.

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