Effects of Overexpression of Phosphofructokinase on Glycolysis in the Yeast *Saccharomyces cerevisiae*†

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Received May 28, 1991; Revised Manuscript Received February 19, 1992

ABSTRACT: The influence of 6-phosphofructo-1-kinase on glycolytic flux in the yeast *Saccharomyces cerevisiae* was assessed by measuring the effects of enzyme overexpression on glucose consumption, ethanol production, and glycolytic intermediate levels under aerobic and anaerobic conditions. Enzyme overexpression had no effect on glycolytic flux under anaerobic conditions, but under aerobic conditions, it increased glycolytic flux up to the anaerobic level. The Pasteur effect was thus abolished in these cells. The increased glycolytic flux was accompanied by a compensatory decrease in flux in oxidative phosphorylation. The concentrations of the enzyme substrates showed only small or insignificant changes. These data imply that the enzyme has a low flux control coefficient for glycolysis. However, in cells overexpressing the enzyme, there was a compensatory decrease in 6-phosphofructo-2-kinase activity which was accompanied by a corresponding decrease in fructose 2,6-bisphosphate concentration. Measurements in vitro showed that the decrease in the concentration of this positive allosteric effector of 6-phosphofructo-1-kinase could significantly lower its specific activity in the cell and that this could compensate for the increased enzyme concentration in the overproducer.

Measurements of the kinetic properties of the isolated enzyme and analyses of changes in glycolytic intermediate levels in the transition between aerobic and anaerobic and anaerobic conditions have identified 6-phosphofructo-1-kinase (PFK1)[1] (EC 2.7.1.11) as a controlling enzyme in the Pasteur effect (activation of sugar catabolism by anaerobiosis) and as an enzyme with a significant flux control coefficient for glycolysis. Studies of glycolytic oscillations in yeast have also shown that PFK1 has an important flux-regulating role in glycolysis under normal steady-state conditions (Hess & Boiteux, 1971). However, despite these data and the widespread belief that PFK1 is the principal enzyme controlling flux in glycolysis (Stryer, 1988), the primary role of the enzyme in glycolytic flux control has been questioned (Bosca & Corredor, 1984).

We have sought here to reinvestigate the role of PFK1 in glycolytic flux control and the Pasteur effect in yeast by monitoring the effects of PFK1 overexpression on glycolytic flux and glycolytic intermediate levels using a combination of noninvasive NMR techniques and more conventional biochemical assays. In particular, we investigated how the steady-state substrate and effector concentrations of PFK1 responded to changes in the concentration of the enzyme.

The study has shown that the enzyme has no regulatory role in glycolysis under anaerobic conditions and only a relatively small effect under aerobic conditions. However, the cells do show a significant and specific adaptation in response to PFK1 overexpression which acts to reduce the effects of increased enzyme concentration on glycolytic flux.

Materials and Methods

Yeast Transformation and Growth Conditions. *Saccharomyces cerevisiae* strain AH22 (a, *his* 4-519, *leu*2-3, *leu* 2-112) was transformed, as described by Hinnen et al. (1978), with either a control plasmid, pMA3, or the PFK1-expressing plasmid pPFK-D1.2. Plasmid pPFK-D1.2 contains the two genes for the α and β subunits of PFK1 in the yeast multicopy shuttle vector YEp13 (Heinisch, 1986). The plasmid contains the 2μ origin of replication and the LEU2 gene as the selectable marker in yeast. Plasmid pMA3 is also a multicopy vector based on 2μ and which uses the LEU2 gene as the yeast-selectable marker (Dobson et al., 1982). This molecule, however, lacks an expression cassette. Transformants were grown aerobically at 30 °C on a selective medium containing 2% glucose, 6.7 g/L yeast nitrogen base, and an amino acid mixture lacking leucine. Main cultures were inoculated from precultures with 4×10^5 cells/mL and harvested 21 h later in early stationary phase. Cell growth was monitored by periodic sampling and cell counting during the 21-h incubation. Both cell types showed similar growth rates.

Perifusion of cells and ^{31}P NMR Experiments. Four grams wet weight of yeast was immobilized in fine agarose gel threads (Foxall & Cohen, 1983; Brindle & Krikler, 1985), placed in a 20-mm-diameter NMR tube with a total sample volume of 17 mL, and perifused at 30 °C with 1 L of buffer containing 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.0, 2 mM $MgSO_4$, 1.7 mM NaCl, 2 mM KCl, and 50 mM glucose as described previously (Brindle & Krikler, 1985). The buffer reservoir was bubbled with either O_2 or N_2 for aerobic and anaerobic measurements, respectively. The buffer was sampled for glucose consumption and ethanol production measurements. Oxygen consumption measurements were carried out during aerobic perifusion by measuring dissolved oxygen in the influent and effluent buffer.

NMR measurements were made using a Bruker AM-300 spectrometer operating at 121.5 MHz for phosphorus, using

† This work was supported by the Wellcome Trust and by the Royal Society. K.M.B. was the recipient of a Royal Society University Research Fellowship.

[1] Abbreviations: Fru-6-P, fructose 6-phosphate; Fru-1,6-BP, fructose 1,6-bisphosphate; Fru-1,6-BPase, fructose-1,6-bisphosphatase; Fru-2,6-BP, fructose 2,6-bisphosphate; Glu-6-P, glucose 6-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MDP, methylenediphosphonate; MES, 2-(*N*-morpholino)ethanesulfonic acid; PCA, perchloric acid; PFK1, 6-phosphofructo-1-kinase; PFK2, 6-phosphofructo-2-kinase.

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home-built probes. Experiments on perifused cells were carried out in a 20-mm probe at 30 °C. Spectra were acquired into 2048 data points, with a sweep width of 8 kHz using a 90° pulse and an interpulse recycle delay of 5.127 s. Free induction decays were collected in blocks of 256 scans. Intracellular phosphate metabolite concentrations were determined by comparing their resonance intensities with that of a methylenediphosphonate (MDP) standard contained in a coaxial capillary tube suspended in the center of the NMR tube (Brindle & Krikler, 1985; Brindle, 1988). Intracellular concentrations were calculated assuming 1.67 g wet weight yeast contains 1 mL of cell water (Gancedo & Gancedo, 1973). Intracellular pH was calculated from the chemical shift difference between P_i and the MDP standard (Brindle & Krikler, 1985). Perchloric acid (PCA) extracts were made of the cells after 4–5-h perifusion as described previously (Brindle et al., 1990). ^{31}P NMR experiments on cell extracts were carried out using a 10-mm probe. Spectra were acquired into 8192 data points, with a sweep width of 8 kHz using a 60° pulse and an interpulse recycle delay of 4.127 s (Brindle, 1988). Free induction decays were acquired with composite pulse decoupling during the acquisition period and collected in blocks of 1024 scans. The concentrations of ATP, ADP, glucose 6-phosphate (Glu-6-P), and fructose 1,6-bisphosphate (Fru-1,6-BP) were determined by reference to an MDP standard contained in a coaxial capillary.

Metabolite and Enzyme Assays. Glucose and ethanol concentrations in the buffer reservoir were assayed according to Bergmeyer (1974). Glu-6-P, fructose 6-phosphate (Fru-6-P), and citrate in PCA extracts were assayed as described by Bergmeyer (1974). The cAMP concentration was measured according to Tovey et al. (1974). The fructose 2,6-bisphosphate (Fru-2,6-BP) concentration was measured in alkaline extracts, as described by van Schaftingen et al. (1982). One gram wet weight of cells was immobilized in agarose threads as described above and perifused for 1 h with 50 mL of MES buffer, pH 6.0, containing 50 mM glucose either anaerobically or aerobically. The threads were tipped quickly into 10 mL of 0.1 M NaOH at 80 °C and maintained at this temperature for 10 min. Cell debris was removed by centrifugation and the supernatant stored at −20 °C until analysis. Prior to analysis, the extract was neutralized with acetic acid in the presence of 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) and made up to 25 mL. Fru-2,6-BP was measured by its ability to activate pyrophosphate-dependent PFK (van Schaftingen et al., 1982).

Enzymes activities were assayed spectrophotometrically at room temperature in cell extracts. Fifty milligrams wet weight of cells was washed in water and resuspended in 0.2 mL of extraction buffer containing 50 mM P_i, pH 7.0, 1 mM EDTA, and 1 mM dithiothreitol. Cell extracts for PFK1 activity measurements also contained 2 mM Fru-6-P, to prevent loss of enzyme activity (Reibstein et al., 1986). The cells were freeze–thawed in liquid nitrogen, and an equal volume of 40-mesh siliconized glass beads was added. This mixture was vortexed for 2 min and refrozen, and the process was repeated. Cell debris was removed by centrifugation at 4 °C. Pyruvate kinase, hexokinase, and citrate synthase were assayed according to Bergmeyer (1974), cytochrome *c* oxidase according to Wharton and Tzagaloff (1967), and PFK1 according to Racker (1947), as modified by Reibstein et al. (1986). 6-Phosphofructo-2-kinase (PFK2) was assayed by the production of Fru-2,6-BP according to François et al. (1984), with the following modifications. The Fru-6-P concentration was increased to 4 mM and Glu-6-P concentration to 16 mM to overcome any increased utilization of substrate by the presence of increased PFK1 in the overproducer. Fru-6-P was measured in the assay buffer at the end of the 10-min incubation period and was not found to be significantly reduced in overproducer assays compared to control assays. The rate of Fru-2,6-BP production was constant throughout this 10-min incubation period.

Table I: Enzyme Activities[a]

	act. [units (mL of cell water)$^{-1}$]	
	control (n = 8)	overproducer (n = 8)
hexokinase	89 ± 29	77 ± 32
6-phosphofructo-1-kinase	35 ± 8	162 ± 44
pyruvate kinase	636 ± 241	733 ± 234
citrate synthase	7 ± 1	7 ± 2
cytochrome *c* oxidase	6 ± 2	5 ± 2
6-phosphofructo-2-kinase[b]	0.33 ± 0.13[c]	0.17 ± 0.08[c]

[a] Enzyme activities measured in cell extracts; data shown are the means with standard deviations. The numbers in parentheses represent the number of cell batches on which the assays were performed. [b] The 6-phosphofructo-2-kinase activity quoted is in milliunits per milliliter of cell water and represents the means of determinations on five different batches of cells. [c] The activities for the control and overproducer cells were compared, and the statistical significance of the difference between the two values was assessed using a two-tailed Student's *t*-test for unpaired samples ($p < 0.05$).

Purification of PFK1 and Kinetic Measurements in Vitro. PFK1 was purified from the overproducer according to Welch and Scopes (1981), adapted to a smaller scale, starting with 25 g wet weight of yeast. The purification was approximately 50-fold, to give a specific activity of 55 μmol min^{-1} (mg of protein)$^{-1}$. PFK1 activity was measured spectrophotometrically at 30 °C under conditions designed to mimic intracellular conditions in terms of pH, ionic strength, and substrate and effector concentrations. The assay mixture, in a total volume of 1 mL, contained 50 mM HEPES, pH 7.3, 100 mM potassium acetate, 8 mM $MgCl_2$, 10 mM NH_4Cl, 2.5 mM dithiothreitol, 0.2 mM NADH, 2.0 mM ATP, and 0.5 mM phosphoenolpyruvate. The concentrations of AMP, Fru-6-P, Fru-1,6-BP, and Fru-2,6-BP are indicated in the appropriate figure legends. Auxiliary enzymes were desalted on a Sephadex G-25M column; the assay mixture contained 2 units/mL lactate dehydrogenase and 2 units/mL pyruvate kinase. Protein concentrations were determined by the method of Bradford (1976) using an assay kit from Bio-Rad.

Statistics. Measurements are expressed as the mean ± standard deviation from the mean. The numbers in parentheses represent the number of determinations. Statistical significance was determined using a two-tailed Student's *t*-test for unpaired samples.

Results

Enzyme Levels. Transformation of the *Saccharomyces cerevisiae* strain AH22 with the plasmid pPFK-D1.2 resulted in an approximately 5-fold increase in PFK1 concentration in the cells when compared to cells transformed with plasmid pMA3 (Table I). The latter cells are hereafter referred to as control cells and cells transformed with pPFK-D1.2 as overproducer cells. Measurements of other enzyme activities in cell extracts showed that there were no significant changes in the concentrations of the cytoplasmic glycolytic enzymes pyruvate kinase and hexokinase nor were there any changes in the concentration of the mitochondrial enzyme cytochrome oxidase or the concentration of citrate synthase, which is found in both the cytoplasm and the mitochondria (Rickey & Lewin, 1986). The lack of change in citrate synthase and cytochrome

Table II: Fluxes in Yeast Modified to Overproduce 6-phosphofructo-1-kinase[a]

	μmol s^{-1} (mL of cell water)$^{-1}$			
	anaerobic		aerobic	
	control (n = 6)	overproducer (n = 6)	control (n = 9)	overproducer (n = 10)
glucose consumption	0.55 ± 0.07	0.59 ± 0.11	0.44 ± 0.04[b]	0.58 ± 0.05[b]
ethanol production	0.72 ± 0.10	0.76 ± 0.09	0.53 ± 0.11[b]	0.69 ± 0.09[b]
oxygen consumption[c]			0.11 ± 0.02[b]	0.07 ± 0.02[b]

[a] Fluxes measured in vivo from 4 g wet weight of yeast perifused either anaerobically or aerobically with 50 mM glucose. The fluxes shown are the means with standard deviations. The numbers in parentheses represent the number of different batches of cells used for the measurements. Glucose consumption and ethanol production were determined by sampling the buffer reservoir hourly for 4–5 h. The rates were linear over this time period (r = 0.99). Oxygen consumption measurements were carried out as described under Materials and Methods. [b] The rates in the control and overproducer cells were compared, and the statistical significance of the difference between the two values was assessed using a two-tailed Student's t-test for unpaired samples ($p < 0.01$). [c] The oxygen consumption measurements shown are the means of determinations on five batches of cells.

oxidase activities indicated that there were no significant differences in mitochondrial content in the overproducer cells. The activity of PFK2, however, was significantly decreased ($p < 0.05$) in the overproducer cells compared to the control cells.

Metabolite Fluxes. Immobilized cell preparations (see Materials and Methods) were perifused either anaerobically or aerobically with 50 mM glucose for 4–5 h. The rates of glucose consumption and ethanol production measured during this period in the control and overproducer cells under aerobic and anaerobic conditions are shown in Table II. The data show that under anaerobic conditions there were no significant differences in glucose consumption and ethanol production rates between the control and overproducer cells. However, under aerobic conditions, the rates of glucose consumption and ethanol production were significantly higher ($p < 0.01$) in the overproducer than in the control and, furthermore, similar to the rates observed in both the overproducer and control cells under anaerobic conditions. The oxygen consumption rate in the overproducer was significantly lower ($p < 0.01$) than in the control cells; however, the calculated ATP turnover rates were similar (Figure 1).

Decreasing the glucose concentration in the reservoir to 20 mM decreased glycolytic flux and abolished both the Pasteur effect and the effect of PFK1 overexpression on aerobic glycolysis. Abolition of the Pasteur effect at low glucose concentrations has been observed previously although at much

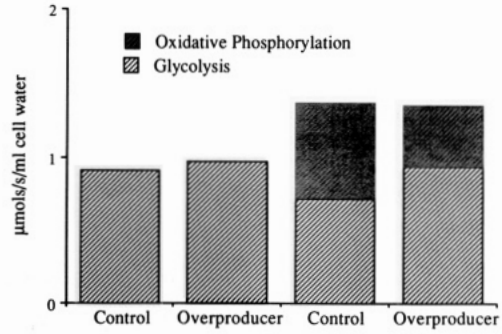

FIGURE 1: Calculated ATP turnover during aerobic and anaerobic perifusion in control and overproducer cells. ATP turnover was calculated by assuming that there are 2 mol of ATP produced/mol of glucose consumed and assuming a P/O ratio for oxidative phosphorylation of 3. This is similar to values measured in vivo in yeast using ^{31}P NMR magnetization transfer techniques (Brindle & Krikler, 1985; Cambell-Burk et al., 1987). (Dark-hatched boxes) Calculated ATP production due to oxidative phosphorylation; (light-hatched boxes) calculated ATP production due to glycolysis.

lower glucose concentrations (den Hollander et al., 1986a). The glucose consumption rate was 0.27 ± 0.03 μmol s^{-1} (mL of cell water)$^{-1}$ (n = 4) in anaerobic control cells and 0.27 ± 0.01 μmol s^{-1} (mL of cell water)$^{-1}$ (n = 4) in aerobic control cells. The ethanol production rates were 0.32 ± 0.06 (n = 4) and 0.29 ± 0.01 μmol s^{-1} (mL of cell water)$^{-1}$ (n = 4), respectively. Increasing PFK1 content under these conditions had no effect on glycolytic flux in either aerobic or anaerobic cells. The glucose consumption and ethanol production rates were 0.26 ± 0.09 (n = 4) and 0.31 ± 0.09 (n = 4) μmol s^{-1} (mL of cell water)$^{-1}$, respectively, in anaerobic overproducer cells and 0.30 ± 0.04 μmol s^{-1} (mL of cell water)$^{-1}$ (n = 4) and 0.35 ± 0.02 (n = 4) μmol s^{-1} (mL of cell water)$^{-1}$, respectively, in aerobic overproducer cells.

Glycolytic Intermediate and PFK1 Effector Concentrations. ^{31}P NMR measurements in vivo showed there were no significant differences in the intensities of the phosphomonoester (which includes the sugar phosphates), P_i, nucleoside triphosphate(s), and NAD(H) resonances between the control and overproducer cells under both aerobic and anaerobic conditions (data not shown). The cytoplasmic pHs, calculated from the chemical shifts of the P_i resonance, also showed no significant differences between control and overproducer cells (data not shown). Measurements made on cell extracts showed that with the exception of the Fru-6-P/Glu-6-P ratio and the concentration of Fru-2,6-BP, there were no significant differences between control and overproducer cells in the concentration of the product of the PFK1 reaction, Fru-1,6-BP, or in the concentrations of known effectors of the enzyme,

Table III: Metabolite Concentrations in Cell Extracts[a]

	μmol (mL of cell water)$^{-1}$			
	anaerobic		aerobic	
	control	overproducer	control	overproducer
ATP/ADP ratio	5.79 ± 1.94 (n = 5)	4.81 ± 1.40 (n = 6)	9.66 ± 1.11 (n = 5)	8.36 ± 2.07 (n = 6)
AMP	0.06 ± 0.03 (n = 5)	0.09 ± 0.06 (n = 6)	0.02 ± 0.01 (n = 5)	0.05 ± 0.03 (n = 6)
Fru-6-P/Glu-6-P ratio	0.117 ± 0.017[b] (n = 5)	0.092 ± 0.013[b,c] (n = 6)	0.156 ± 0.045 (n = 6)	0.132 ± 0.012[c] (n = 8)
Fru-2,6-BP [nmol (mL of cell water)$^{-1}$]	5.24 ± 1.63[d] (n = 4)	2.70 ± 0.74[d] (n = 4)	4.71 ± 1.15[e] (n = 4)	2.93 ± 0.67[e] (n = 4)
cAMP [nmol (mL of cell water)$^{-1}$]	0.324 ± 0.083 (n = 5)	0.295 ± 0.046 (n = 5)	0.378 ± 0.070 (n = 5)	0.327 ± 0.076 (n = 5)
citrate	15.21 ± 6.32 (n = 5)	10.43 ± 3.33 (n = 7)	16.20 ± 2.49 (n = 5)	14.74 ± 3.04 (n = 8)
Fru-1,6-BP	1.31 ± 0.25 (n = 5)	1.96 ± 0.51 (n = 6)	1.18 ± 0.15 (n = 5)	1.27 ± 0.28 (n = 6)

[a] ATP, ADP, and Fru-1,6-BP concentrations were determined from proton-decoupled ^{31}P NMR spectra of cell extracts (Brindle et al., 1990). AMP was calculated from the ATP and ADP concentrations assuming an equilibrium constant for the adenylate kinase reaction of 1.0 (Veech et al., 1979). Other metabolite concentrations were measured in cell extracts using standard enzymatic and chemical assays, as described under Materials and Methods. Means with standard deviations from the means are shown. The numbers in parentheses represent the number of different cell extracts used. The statistical significance of the difference between the pairs of values labeled *b–e* was assessed using a two-tailed Student's t-test for unpaired samples. In all cases, $p < 0.05$.

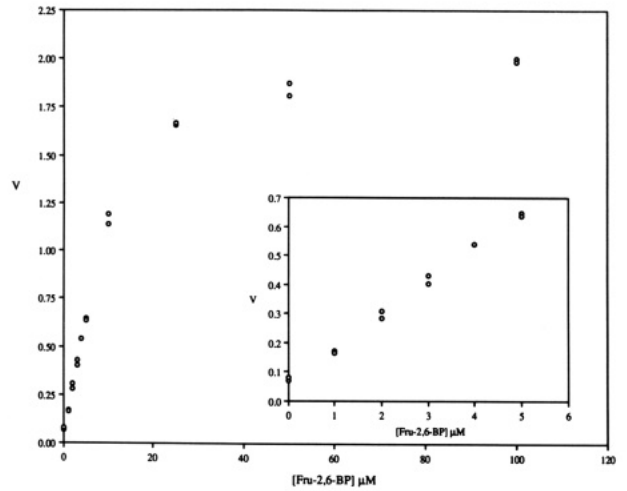

FIGURE 2: Effect of Fru-2,6-BP concentration on flux in the PFK1-catalyzed reaction in vitro. Purified PFK1 was assayed in the presence of substrate and effector concentrations similar to those measured in vivo, this is, 2.0 mM ATP, 0.05 mM AMP, 0.08 mM Fru-6-P, and 1.5 mM Fru-1,6-BP. The effect of varying the concentration of Fru-2,6-BP over the concentration range measured in vivo (see inset) and up to 100 μM is presented. The measured flux, *V*, is expressed as micromoles of Fru-6-P converted per minute per unit of enzyme activity, where enzyme activity was measured using the same set of standard assay conditions used for measurement of PFK1 activities in cell extracts. Each point was measured in duplicate.

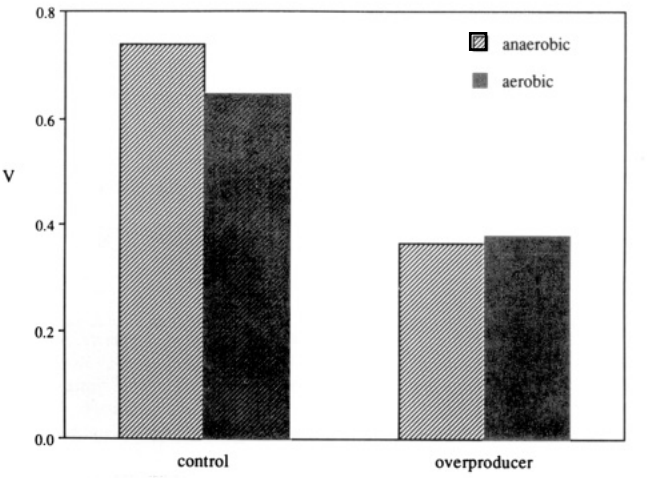

FIGURE 3: Flux in the PFK1-catalyzed reaction in vitro under conditions designed to mimic those found in vivo with respect to substrate and effector concentrations. Metabolite concentrations were as follows: control anaerobic, 2.0 mM ATP, 0.08 mM AMP, 0.08 mM Fru-6-P, 1.65 mM Fru-1,6-BP, and 5 μM Fru-2,6-BP; overproducer anaerobic, 2.0 mM ATP, 0.08 mM AMP, 0.06 mM Fru-6-P, 1.65 mM Fru-1,6-BP, and 3 μM Fru-2,6-BP; control aerobic, 2.0 mM ATP, 0.04 mM AMP, 0.08 mM Fru-6-P, 1.2 mM Fru-1,6-BP, and 5 μM Fru-2,6-BP; overproducer aerobic, 2.0 mM ATP, 0.04 mM AMP, 0.08 mM Fru-6-P, 1.2 mM Fru-1,6-BP, and 3 μM Fru-2,6-BP. The activity measured, *V*, is expressed as micromoles of Fru-6-P converted per minute per unit of enzyme activity, where enzyme activity was measured using the same set of standard assay conditions used for the measurement of PFK1 activities in cell extracts. The mean data from two experiments are shown. (Light-hatched boxes) Calculated flux under anaerobic conditions; (dark-hatched boxes) calculated flux under aerobic conditions.

cAMP, citrate, AMP, ADP, and ATP (see Table III). cAMP is an indirect effector of the enzyme via its effect on PFK2 activity (see below). The Fru-6-P/Glu-6-P ratio was slightly lower in the overproducer compared to the control under anaerobic conditions and was lower in the overproducer under anaerobic conditions as compared to aerobic conditions. In previous studies (Reibstein et al., 1986; Brindle, 1988), it has been assumed that the Fru-6-P and Glu-6-P concentrations are near-to-equilibrium in the cell in the reaction catalyzed by phosphoglucose isomerase. The data presented here and in a previous study of Lagunas and Gancedo (1983) show that this is not the case. The most striking result shown in Table III, however, is the marked decrease in the Fru-2,6-BP concentration in the PFK1 overproducer compared to the control under both aerobic and anaerobic conditions. The level of this metabolite in each cell type, however, did not appear to change between aerobic and anaerobic conditions. The decrease in the Fru-2,6-BP concentration correlated well with the decrease in the activity of PFK2 measured in cell extracts (Table I).

Kinetic Measurements on PFK1 in Vitro. The results of steady-state kinetic measurements on purified PFK1 in vitro, under conditions designed to mimic those in the cell with respect to effector and substrate concentrations, are shown in Figures 2 and 3. The effect of Fru-2,6-BP on the enzyme's activity under conditions designed to mimic those in vivo is shown in Figure 2. The fluxes quoted are specific activities expressed as micromoles per minute per unit of enzyme activity, where the units of activity were determined spectrophotometrically under a standard set of assay conditions (see Materials and Methods and Table I).

Measurements of flux in the enzyme-catalyzed reaction in vitro, with substrate and effector concentrations similar to those found in the overproducer and control cells under aerobic and anaerobic conditions, are shown in Figure 3. The data show that the specific activity of the enzyme is significantly higher under conditions designed to mimic those in the control cells under anaerobic conditions compared to the same cells under aerobic conditions. The specific activities under control conditions are also significantly higher than under the conditions

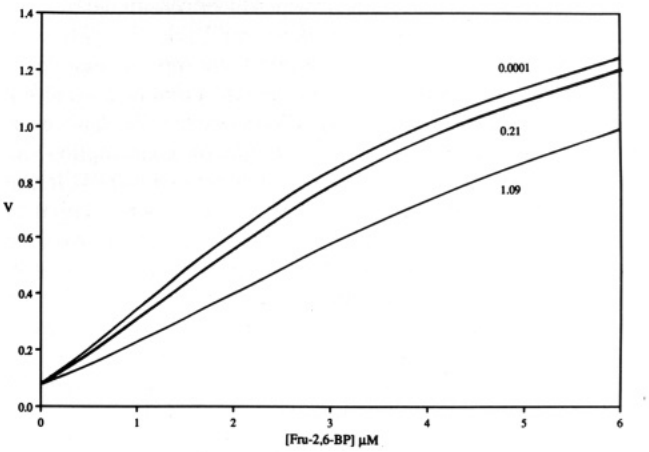

FIGURE 4: Theoretical curves showing the effects of enzyme and Fru-2,6-BP concentrations on the specific activity of PFK1. The calculated flux, *V*, is expressed as micromoles of Fru-6-P converted per minute per unit of enzyme activity. The enzyme concentrations in the control and overproducer cells were calculated from the activities measured in cell extracts using a specific activity of 180 units/mg (Welsh & Scopes, 1981) and a molecular weight of 850 292 (Heinisch et al., 1989). This gives an enzyme concentration in the overproducer of 1.09 μM and in the control cells of 0.21 μM. Calculation of the theoretical curves is described in the text.

found in the overproducer cells. The specific activity of the enzyme under conditions similar to those found in the overproducer cells shows no significant change between aerobic and anaerobic conditions.

The extrapolation of flux measurements made in vitro to the cell assumes that there are no effects of enzyme concentration on the specific activity of the enzyme. Figure 4 shows the theoretical effect of PFK1 concentration on the specific activity of the enzyme as a function of total Fru-2,6-BP concentration. The theoretical curves shown in this figure were

derived using the Monod–Wyman–Changeux-type model used by Kessler et al. (1988) to describe binding of Fru-2,6-BP and Fru-1,6-BP to the enzyme. The binding and other constants used in this calculation were those of Kessler et al. (1988). The specific activities of the fully Fru-2,6-BP-bound and unbound forms of the enzyme were taken from the data shown in Figure 2. The activity of the enzyme between these two limits was assumed to be directly proportional to the calculated occupancy of the Fru-2,6-BP binding sites.

Discussion

Increasing the PFK1 concentration by a factor of 5 resulted in a significant increase in glycolytic flux under aerobic conditions up to a value similar to that observed in the same cells under anaerobic conditions. Thus, in the cells overexpressing PFK1, the Pasteur effect was abolished. Under anaerobic conditions, however, increasing the PFK1 concentration had no effect on glycolytic flux. Examination of the results shown in Table II shows that while glycolytic flux is raised under aerobic conditions by increases in PFK1 concentration, the oxygen consumption is lowered. This is despite the fact that the mitochondrial content, as determined by assay of mitochondrial marker enzymes (see Table I), is unchanged. Furthermore, if the rate of ATP turnover is calculated from the glucose consumption, ethanol production, and oxygen consumption data, then it can be seen that the decreased oxygen consumption in the PFK1 overproducer quantitatively compensates for the increased ATP production via glycolysis (Figure 1). Thus, PFK1 overexpression can only increase glycolytic flux and ATP production if it does so at the expense of ATP production via mitochondrial oxidative phosphorylation. This could explain why, in two previous studies (Heinisch, 1986; Schaaff et al., 1989), no effect of PFK1 overexpression on glycolytic flux could be observed. These studies were carried out on growing cells, where respiration makes only a small contribution to cellular ATP production and the Pasteur effect is absent (Lagunas, 1979; Lagunas et al., 1982). In contrast, in resting cells, such as those used here, up to 100% of the sugar can be respired (Lagunas et al., 1982).

The reciprocal relationship between ATP generation via glycolysis and oxidative phosphorylation, illustrated in Figure 1, implies tight coordinate control of these two processes to meet a similar ATP demand in the overproducer and control cells. What the intracellular signal is that mediates this coordinate control is not clear. Possible candidates include P_i and ADP, which are thought to have a controlling influence on the rates of both oxidative phosphorylation and glycolysis (Racker, 1974). However, these do not appear to change significantly in concentration between the control and overproducer cells. The ADP concentration, however, is the total cellular ADP concentration measured in cell extracts rather than the free ADP concentration in the cytoplasm, which is the relevant species in this case.

The smaller or insignificant effects of PFK1 overexpression on glycolytic flux and intermediate levels suggest that PFK1 has a very low flux control coefficient for aerobic glycolysis and a flux control coefficient of near-zero for anaerobic glycolysis. However, some caution is required in interpretation of these data since the cells clearly show an adaptive response to PFK1 overexpression which counteracts the effects of increased PFK1 concentration on glycolytic flux (see below). Adaptation of this sort must be considered in any study which utilizes a chronic and long-term overexpression of an enzyme in order to determine its influence on metabolic flux. However, despite this caveat, the data confirm PFK1 as the principal regulatory site for the control of the Pasteur effect. If the flux control coefficient of the enzyme is lowered, either by raising the enzyme concentration or by lowering the glucose concentration, then the Pasteur effect is abolished. Abolition of the effect at low glucose concentrations occurs presumably because the flux control coefficients of steps earlier in the glycolytic pathway, such as glucose transport, are raised by decreasing the glucose concentration, and thus the flux control coefficient of PFK1 is lowered.

The absence of a change in glucose consumption or ethanol production rates in anaerobic cells despite a 5-fold increase in PFK1 content implies that there must be some compensatory changes in the enzyme's substrate concentrations in vivo, such that flux in the reaction is unchanged. Among the enzyme's substrates and effectors, the only metabolite which showed a significant change in concentration between control and overproducer cells was Fru-2,6-BP (see Table III). The concentration of Fru-6-P appeared to be slightly lower in the overproducer compared to the control under anaerobic conditions. However, this difference was not statistically significant (data not shown). Further evidence that the Fru-6-P concentration may be lowered in the overproducer came from a comparison of the Fru-6-P/Glu-6-P ratios in the control and overproducer cells which showed that there was a small but statistically significant decrease in the Fru-6-P/Glu-6-P ratio in the overproducer under anaerobic conditions (see Table III). A decrease in the Fru-6-P concentration with an increase in PFK1 content in the cell is predictable from earlier studies (den Hollander et al., 1986b; Reibstein et al., 1986). The decrease in the Fru-2,6-BP concentration, however, was unexpected and is discussed below. The question now arises as to whether these changes in the effector concentrations of PFK1 can compensate for the increased enzyme concentration in the overproducer such that flux in the enzyme-catalyzed reaction is unchanged.

The effect of Fru-2,6-BP concentration on the specific activity of PFK1 under conditions designed to mimic those in vivo is shown in Figure 2. This shows that a decrease in Fru-2,6-BP concentration from 5 to 3 μM decreases the specific activity of the enzyme by 35%. The decrease in Fru-6-P concentration under anaerobic conditions causes an additional decrease in the specific activity (see Figure 3) such that the total decrease in specific activity is approximately 50%. Extrapolation of these fluxes to cellular enzyme concentrations gives a PFK1 flux under conditions found in anaerobic control cells of 0.41 μmol s^{-1} (mL of cell water)$^{-1}$, a flux in aerobic control cells of 0.36 μmol s^{-1} (mL of cell water)$^{-1}$, a flux in anaerobic overproducer cells of 1.02 μmol s^{-1} (mL of cell water)$^{-1}$, and a flux in aerobic overproducer cells of 1.06 μmol s^{-1} (mL of cell water)$^{-1}$. These calculated values for the PFK1 flux in vivo in control cells are comparable with the glucose consumption and ethanol production rates measured in control cells. In comparing PFK1 fluxes measured in vitro with those measured in vivo, we are assuming that the latter closely reflect flux through the PFK1 reaction in the cell. This need not be the case since significant quantities of glucose carbon can also flow into the pentose phosphate pathway and into trehalose and storage polysaccharides as well as into ethanol (den Hollander et al., 1986a). Furthermore, in aerobic cells, there can be significant flux in the fructose-1,6-bisphosphatase (Fru-1,6-BPase) reaction, which is effectively the reverse of the PFK1 reaction in the glycolytic pathway. Thus, flux in the PFK1-catalyzed reaction can significantly exceed net glycolytic flux as determined from measurements of glucose consumption and ethanol production. However, at the high glucose concentrations used in this study, Fru-1,6-BPase is

rapidly inactivated by proteolysis (Funayama, 1980). The enzyme's activity had decreased to undetectable levels within the first hour of cell perifusion (data not shown).

Although there is good agreement between the PFK1 flux in control cells calculated from the kinetic measurements in vitro and the glucose consumption and ethanol production rates actually measured in these cells, the calculated fluxes in the overproducer cells appear to be higher than those measured. Clearly, the decrease in specific activity of the enzyme observed in vitro under conditions designed to mimic those in the overproducer cells cannot explain why flux through the PFK1 reaction apparently remains unchanged despite a 5-fold increase in enzyme concentration; i.e., the specific activity would need to decrease by 80% to compensate for this increase in enzyme concentration. This extrapolation of enzyme kinetic data to the cell assumes, however, that the Fru-2,6-BP concentration measured in cell extracts represents the free effector concentration available to the enzyme in vivo. This is clearly not the case. Assuming a specific activity of 180 units/mg (Welsh & Scopes, 1981), the enzyme concentration in the control cells is 0.21 μM (assuming a molecular weight of 850 292) (Heinisch et al., 1989) and the Fru-2,6-BP binding site concentration is 0.84 μM (Kessler et al., 1988). In the overproducer, this is increased to 4 μM; i.e., the Fru-2,6-BP binding site concentration on PFK1 alone is comparable to the total extractable Fru-2,6-BP concentration in the cell. The influence of enzyme concentration on the specific activity of the enzyme at Fru-2,6-BP and PFK1 concentrations comparable to those found in the cell is illustrated in Figure 4. The theoretical curves shown in this figure were derived from the Monod–Wyman–Changeux-type model used to describe binding of Fru-2,6-BP and Fru-1,6-BP to the enzyme (Kessler et al., 1988). The data shown in Figure 4 show that at very low enzyme concentrations, comparable to those used in the kinetic measurements in vitro, the specific activity of the enzyme, at a given total Fru-2,6-BP concentration, is significantly higher than that found at enzyme concentrations comparable to those measured in the cell. Using these theoretical curves, a comparison can be made of the expected PFK1 fluxes in the control and overproducer cells which takes into account both the Fru-2,6-BP and enzyme concentrations. Thus, at 3 μM Fru-2,6-BP and 1.09 μM enzyme, the concentrations measured in the PFK1 overproducer, the specific activity is 53% of that found at 5 μM Fru-2,6-BP and 0.21 μM enzyme, which are the concentrations measured in the control cells. Thus, the higher enzyme concentration found in the overproducer accentuates the decrease in specific activity brought about by the decrease in Fru-2,6-BP concentration. Although the agreement between the experimental data obtained here and the model, based on previously published binding data, is not close, it nevertheless serves to illustrate the point that enzyme concentration will have a profound effect on the specific activity of the enzyme.

The kinetic measurements in vitro, as in the previous study of Reibstein et al. (1986), also show that the increased flux in the PFK1 reaction in anaerobic cells can be accounted for, at least semiquantitatively, by changes in the enzyme's effector concentrations. This is illustrated by the data shown in Figure 3. The specific activity of the enzyme observed in vitro under conditions designed to simulate those in anaerobic control cells is significantly higher than the specific activity measured under conditions designed to simulate those in aerobic control cells. Similarly, simulation of the conditions found in the overproducer under aerobic and anaerobic conditions produced no change in the specific activity of the enzyme. This is in agreement with the absence of an observable Pasteur effect in the overproducer. The increased flux under anaerobic control conditions as compared to aerobic control conditions is due to the increase in AMP concentration. AMP has been shown previously to activate the enzyme (Nissler et al., 1983; Przybylski et al., 1985; Otto et al., 1986). The effect of an increase in AMP concentration in the overproducer under anaerobic conditions is offset by a decrease in the Fru-6-P concentration such that the specific activity of the enzyme is unchanged (see Figure 3 and the legend to this figure).

Possibly the most interesting finding made in this study was the observation that the Fru-2,6-BP concentration is markedly decreased by overproduction of PFK1. Fru-2,6-BP plays a key role in the regulation of carbohydrate metabolism in yeast (van Schaftingen, 1987). It is a potent activator of PFK1, as already shown, and a powerful inhibitor of Fru-1,6-BPase (Lederer et al., 1981; Bartrons et al., 1982; Nissler et al., 1983; Przybylski et al., 1985; Otto et al., 1986). The compound is synthesized from Fru-6-P by PFK2, which can be phosphorylated by a cyclic AMP-dependent protein kinase, which results in its activation (François et al., 1984; Yamashoji & Hess, 1984a,b). There has been some debate as to how the level of Fru-2,6-BP in yeast is controlled. Initially it was believed that the rise in the Fru-2,6-BP concentration was secondary to a rise in the Fru-6-P concentration following glucose addition (Lederer et al., 1981). Clifton and Fraenkel (1983) showed that in PFK1 mutants strains, which had low PFK1 activity, Fru-2,6-BP concentrations were markedly increased and that this correlated with increased Fru-6-P concentrations. This study has interesting parallels with the work presented here in that both studies show a reciprocal relationship between PFK1 activity and Fru-2,6-BP levels. Françoise et al. (1984) subsequently showed, however, that Fru-2,6-BP levels are controlled primarily through cAMP-dependent phosphorylation and activation of PFK2.

The data presented here show that the Fru-2,6-BP concentration does not depend on the Fru-6-P concentration but correlates directly with the PFK2 activity measured in cell extracts. The activity of this enzyme showed a statistically significant ($p < 0.05$) decrease in activity in the PFK1 overproduced to a value which was 52% of the activity found in control cells. The Fru-2,6-BP concentration in the overproduced showed a proportionately similar decrease in concentration, the Fru-2,6-BP concentration being approximately 60% of the value found in the control cells (Table III). A correlation between PFK2 activity and Fru-2,6-BP concentrations has also been observed in a number of previous studies (Clifton & Fraenkel, 1983; François et al., 1984, 1987). Whether this decrease in PFK2 activity represents a change in the phosphorylation state or a change in enzyme concentration is not clear. The absence of a difference in cAMP concentration between control and overproducer cells (Table III) suggests that there may be a change in enzyme concentration. However, this could also indicate that there is a considerable delay between changes in cAMP levels and changes in the phosphorylation state of PFK2. For example, during cell growth the levels of cAMP in the control and overproducer cells may have been different. Françoise et al. (1984) have also observed changes in PFK2 activity which were not paralleled by changes in cAMP concentration.

A change in PFK2 concentration, in response to a change in the concentration of PFK1, could indicate a higher level of glycolytic flux control operating at the level of gene expression. Thus, changes in PFK1 concentration would be compensated for by reciprocal changes in the concentration

of PFK2 and thus the potent activator of PFK1, Fru-2,6-BP. Support for this type of regulation can be found in the recent study of Moore et al. (1990), who reported that the translation of the mRNA for the β subunit of PFK1 can be inhibited by excess pyruvate kinase mRNA.

In summary, we have shown that PFK1 overexpression can only increase glycolytic flux under aerobic conditions, where it does so at the expense of flux in oxidative phosphorylation. The cells adapt to overexpression of PFK1 by reducing the specific activity of the enzyme by lowering the concentration of Fru-2,6-BP through a decrease in the activity of PFK2.

ACKNOWLEDGMENTS

We thank Dr. Alan Kingsman for the plasmid pMA3, Dr. Jürgen Heinisch for the plasmid pPFK-D1.2, and Dr. Keith Elliott for the cAMP binding protein and [8-^{3}H]cAMP. We also thank Dr. Sandra Fulton for her help and advice. The initial stages of this work were carried out at the Department of Biochemistry, University of Oxford, and we thank Prof. George Radda and the MRC for the use of their NMR facilities.

Registry No. Fru-2,6-BP, 77164-51-3; PFK1, 9001-80-3; PFK2, 78689-77-7.

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