A mutant phosphofructokinase produces a futile cycle during gluconeogenesis in *Escherichia coli*

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Strains of *Escherichia coli* bearing different forms of phosphofructokinase were used to assess the occurrence of futile cycling in cell resuspensions supplied with glycerol as gluconeogenic carbon source. A model was used to simulate results of different kinds of experiments for different levels of futile cycle. The main predictions of the model were experimentally confirmed in a strain with a mutant phosphofructokinase-2 (phosphofructokinase-2*) which is not inhibited by MgATP. The intracellular fructose 1,6-bisphosphate concentration reaches significantly higher levels in the mutant-bearing strain than in strains with either phosphofructokinase-1 or -2. Also, this strain showed a higher rate and level of *in vivo* radioactive labelling of fructose 1,6-bisphosphate, from a trace of [U-¹⁴C]glucose supplied during gluconeogenesis, indicating higher kinase activity in these conditions. Cell resuspensions of the mutant-bearing strain produced higher levels of radioactively labelled CO₂ when supplied with [U-¹⁴C]glycerol as the only carbon source. Simultaneously, fewer glycerol carbons were incorporated into HClO₄-insoluble macromolecules. Finally, radioactive CO₂ output was measured in resuspensions supplied

with glycerol as the major carbon source with traces of either [1-¹⁴C]glucose or [6-¹⁴C]glucose. It was found that, whereas in the strains with either of the wild-type phosphofructokinase isoenzymes, radioactive CO₂ output from [1-¹⁴C]glucose was higher than with [6-¹⁴C]glucose, the reverse is found for the strain with phosphofructokinase-2*. This result also agrees with the corresponding prediction of the model. Using the radioactivity flux rates predicted by the model, an explanation linking the futile cycle to the differential labelling of CO₂ is advanced. Finally, on the basis of these results it is proposed that strains bearing phosphofructokinase-2* sustain higher rates of futile cycling during gluconeogenesis than strains bearing either of the wild-type isoforms of phosphofructokinase. The kinetic equations and parameter values used for the model simulations are given in Supplementary Publication SUP 50183 (8 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1997) 321, 8.

INTRODUCTION

The kinetic and regulatory properties of enzymes, mostly established *in vitro*, are believed to play a crucial role in the behaviour of the metabolic networks to which they belong [1]. However, there are not many studies showing a link between such properties and actual *in vivo* metabolic fluxes. Also, it appears that the question of which aspects of metabolism are actually affected by such regulatory properties is still unclear [2]. We have found an unusual situation in *Escherichia coli* where this problem can be approached from two sides: the enzymological *in vitro* characterization of kinetically different forms of an enzyme, and the *in vivo* study of the effects that the loss of a regulatory property by one of them may have on carbon metabolism and, ultimately, on cell growth rate.

Two isoenzymes of fructose-6-phosphate 1-kinase (Pfk; ATP-D-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) have been found in *E. coli*. Pfk-1 exhibits sigmoidal kinetics with respect to fructose 6-phosphate (F6P), allosteric activation by ADP or GDP and allosteric inhibition by phosphoenolpyruvate [3,4]. The minor form, Pfk-2, exhibits hyperbolic kinetics with respect to F6P but it is inhibited by MgATP at low concentrations of F6P [4,5]. Strains with similar levels of either Pfk-1 or Pfk-2 grow at comparable rates on both glycolytic and gluconeogenic carbon sources [6].

A mutant form of Pfk-2 (Pfk-2*) has been isolated, characterized, cloned and sequenced [5–10]. Pfk-2* is not inhibited by MgATP at low concentrations of F6P [5]. Strains with Pfk-2* have a somewhat puzzling phenotype: on glycolytic carbon sources they grow at the same rate as the Pfk-1- and Pfk-2-bearing strains (doubling times of 90–95 min) but on gluconeogenic carbon sources they grow three or four times more slowly (doubling times of 350–400 min [6]). A simple hypothesis accounting for this phenotype was advanced by Daldal et al. [6]: that the lack of sensitivity of Pfk-2* to inhibition by MgATP would result in futile cycling during gluconeogenesis. The negative impact of the cycle on either the intracellular pool of ATP or the flux of carbon skeletons to anabolic pathways, or both, would eventually result in slower growth. On complete medium, all strains grow at similar wild-type rates.

Using an *in vitro* model in which *E. coli* fructose bisphosphatase (FBPA) and Pfk-1, Pfk-2 or Pfk-2* were combined in a simulated *in vitro* gluconeogenic flux system, we have shown that Pfk-2* could lead to higher rates of substrate cycling than either of the two other enzymes [11]. Here, we use a mathematical model of glycolysis and gluconeogenesis to generate predictions on the effect of different futile cycle rates at the phosphofructokinase (PFK)/FBPA steps on the metabolite levels and the metabolic fate of gluconeogenic (glycerol) carbons. We assessed some of the predictions in cell resuspensions of *E. coli* strains with

Abbreviations used: HMS, hexose monophosphate shunt; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; RU5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; X5P, xylulose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; Pfk-1, phosphofructokinase-1; Pfk-2, phosphofructokinase-2; Pfk-2*, phosphofructokinase-2*; PFK, generic phosphofructokinase; FBPA, fructose bisphosphatase; further abbreviations are defined in the legend to Scheme 1.

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different PFKs supplied with glycerol as the sole carbon source. The agreement between the predictions of the model and the experimental results supports the hypothesis of higher futile-cycle rates during gluconeogenesis in the Pfk-2*-bearing strains than in strains bearing either Pfk-1 or Pfk-2.

MATERIALS AND METHODS

Mathematical model

The mathematical model comprises a simplified version of the central pathways of carbon metabolism, including reactions of glycolysis, gluconeogenesis and the hexose monophosphate shunt (HMS) (Scheme 1). We chose to focus on the flux of carbon skeletons, so all aspects of energy and redox metabolism were excluded. Also, in several cases, reactions have been lumped into one step: glycogen synthesis; all output reactions from R5P, glycolytic reactions from GOP (this and some other abbreviations are defined in the legend to Scheme 1) to pyruvate; all output reactions from acetyl-CoA that lead to CO₂; glycerol uptake and conversion into DHAP. All these reactions have been made 'practically irreversible'. Of the 18 metabolites of the model, two act as sources of the variables mass and radioactivity: glucose and glycerol. Another three are sinks: CO₂, glycogen and ribose phosphate products. The remaining 13 are intermediates: G6P, 6PG, F6P, FBP, DHAP, GOP, pyruvate, acetyl-CoA, RU5P, R5P, X5P, S7P and E4P. The mass and radioactivity variables associated with each of these intermediates will change with time depending on the rates of inputs to and outputs from each intermediate. Also, each reaction has an associated mass rate (v_{ei} ; mass per unit time) and one or more radioactivity flux rates (radioactivity per unit time) depending on the number of reactions associated with the metabolite.

The mathematical model is based on a set of differential equations that describe the temporal behaviour of each variable. The equations governing the mass are as follows:

$$[\text{Glucose}] = -v_{\text{IPT2}}$$

$$[\text{Glucose 6-phosphate}] = v_{\text{IPT2}} - v_{\text{G6PD}} - v_{\text{GPI}} - v_{\text{OPT2}}$$

$$[\text{Fructose 6-phosphate}] = v_{\text{GPI}} + v_{\text{FBPA}} - v_{\text{PFK}} + v_{\text{TK2}} + v_{\text{TA}}$$

$$[\text{Fructose 1,6-bisphosphate}] = v_{\text{PFK}} - v_{\text{FBPA}} - v_{\text{ALD}}$$

$$[\text{Dihydroxyacetone phosphate}] = v_{\text{IPT1}} - v_{\text{TPI}} + v_{\text{ALD}}$$

$$[\text{Glyceraldehyde 3-phosphate}]$$

$$= v_{\text{ALD}} + v_{\text{TPI}} - v_{\text{GLY1}} + v_{\text{TK1}} + v_{\text{TK2}} - v_{\text{TA}}$$

$$[\text{Glycerol}] = -v_{\text{IPT1}}$$

$$[\text{Pyruvate}] = v_{\text{GLY1}} - v_{\text{PDC}}$$

$$[\text{6-phosphogluconate}] = v_{\text{G6PD}} - v_{\text{6PGD}}$$

$$[\text{Ribulose 5-phosphate}] = v_{\text{6PGD}} - v_{\text{EP}} - v_{\text{KI}}$$

$$[\text{Ribose 5-phosphate}] = v_{\text{KI}} - v_{\text{TK1}} - v_{\text{OPT1}}$$

$$[\text{Xylulose 5-phosphate}] = v_{\text{EP}} - v_{\text{TK1}} - v_{\text{TK2}}$$

$$[\text{Sedoheptulose 7-phosphate}] = v_{\text{TK1}} - v_{\text{TA}}$$

$$[\text{Erythrose 4-phosphate}] = v_{\text{TA}} - v_{\text{TK2}}$$

$$[\text{CO}_2] = v_{\text{6PGD}} + v_{\text{PDC}} + 2v_{\text{OPT3}}$$

$$[\text{Glycogen}] = v_{\text{OPT2}}$$

$$[\text{RPP}] = v_{\text{OPT1}}$$

The behaviour of most of the reactions is determined by equations and parameters taken from Schuster and Holzhütter

[12], and correspond to those of the glycolytic enzymes of red blood cells. This was done in view of the paucity of kinetic data for the *E. coli* enzymes. The equations used are given in Supplementary Publication SUP 50183 (8 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1997) 321, 8.

We have found that the main properties of the model are quite insensitive to the parameter values and equations used, at least in terms of the predicted effects of the PFK–FBPA futile cycle on the various rates. In fact, when wholly arbitrary and uniform parameters were used, the results obtained were qualitatively identical (results not shown). Since the model predictions were used only to compare situations with different rates of futile cycle, the actual magnitudes of the variables and parameters are irrelevant.

Using the formalism of Schuster and Holzhütter [12], the general form of the equations is the following:

$$v = V_m r(X) \left(\prod_i x_i^{c_{ij}^+} - \frac{1}{q} \prod_i x_i^{c_{ij}^-} \right)$$

where V_m is the maximum activity, $r(X)$ a regulatory function including non-linearities due to saturation, allostery, etc., q the equilibrium constant and c^+ and c^- positive and negative elements of the stoichiometric matrix.

On the other hand, the variable radioactivity is independently associated with each carbon position of each metabolite. The change of this variable with time is determined by a set of 76 differential equations. To illustrate the general form of such equations, the following is the equation governing the change of radioactivity with time for carbon 1 of E4P:

$$S_{C_1^{E4P}}^* = (v_{\text{TA}} R_{C_1}^{S7P} - v_{\text{TA}} R_{C_4}^{E4P}) - (v_{\text{TK2}} R_{C_1}^{E4P} - v_{\text{TK2}} R_{C_3}^{F6P})$$

where $S_{C_1^{E4P}}^*$ is the change in radioactivity of carbon 1 of E4P with time, v_{fi} and v_{ri} are the forward and reverse components of the rate of the reaction catalysed by enzyme i , and $R_{C_j}^{E4P}$, $R_{C_j}^{S7P}$ and $R_{C_j}^{F6P}$ are the specific radioactivities of carbon j of E4P, S7P and F6P respectively. The forward and reverse velocities are easily obtained from the general rate equations. As illustrated in this case, these equations incorporate the known positional fates of each carbon in each reaction: carbon 1 of E4P comes from carbon 3 of F6P in the transketolase reaction 2 (TK2 in Scheme 1) and from carbon 4 of S7P in the transaldolase reaction (TA). The specific radioactivities are obtained by dividing the value of the variable radioactivity associated with each carbon by the variable mass associated with the corresponding metabolite.

To obtain the time courses of mass, radioactivity, mass flux rate and radioactivity flux rate, the two systems of equations were simultaneously solved by numerical integration from defined initial conditions using the fourth order Runge-Kutta method [13] implemented through a computer program. This allowed the analysis of both transient and steady-state situations, depending on whether the carbon source mass was variable or constant respectively. The model also allows simulation of situations in which the sources of mass and radioactivity are different.

To simulate different futile-cycle rates of the reactions catalysed by PFK and FBPA, the maximum activity of PFK was varied while all other maximum activities were kept constant at a value of 0.5 (or varied if so stated). We define the futile-cycling percentage as:

$$\text{FC \%} = \frac{v_{\text{PFK}}}{v_{\text{FBPA}}} 100$$

Scheme 1 Model reactions and their relationships

Reversible reactions are indicated by arrows at the two ends of the line. Metabolite abbreviations are as follows: GLU, glucose; G6P, glucose 6-phosphate; 6PG, 6-phosphogluconate; F6P, fructose 6-phosphate; FBP, fructose 1-6-bisphosphate; DHA, dihydroxyacetone phosphate; GOP, glyceraldehyde 3-phosphate; GLY, glyceral; Pyr, pyruvate; RU5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; X5P, xylulose 5-phosphate; RPP, output products of ribose 5-phosphate; E4P, erythrose 4-phosphate; GLYC, glycogen; ACoA, acetyl-CoA. Enzyme abbreviations are as follows: IPT1, input reaction 1 (from glyceral to dihydroxyacetone phosphate); IPT2, input reaction 2 (entry of glucose to glucose 6-phosphate); OPT1, output reaction 1 (from ribose 5-phosphate to unspecified products); OPT2, output reaction 2 (from glucose 6-phosphate to glycogen); OPT3, output reaction 3 (from acetyl-CoA to CO₂); GPI, glucose-6-phosphate isomerase (EC 5.3.1.9); PFK, generic phosphofructokinase (EC 2.7.1.11); FBPA, fructose-1,6-bisphosphatase (EC 3.1.3.11); ALD, fructose-1,6-bisphosphate aldolase (EC 4.1.2.13); TPI, triose phosphate isomerase (EC 5.3.1.1); GLY1, glycolytic reactions of trioses; PDC, pyruvate dehydrogenase complex; G6PD, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); 6PGD, 6-phosphogluconate dehydrogenase (EC 1.1.1.44); EP, epimerase; KI, ribose phosphate isomerase (EC 5.3.1.6); TK1, transketolase 1 (EC 2.2.1.1); TK2, transketolase 2; TA, transaldolase (EC 2.2.1.2).

Bacterial strains, media and growth

The genotypes, construction and origin of the strains used can be found elsewhere [6]. Minimal medium M63 [14] was supplemented with 1 µg/ml thiamin hydrochloride and variable concentrations of either glucose or glycerol. Routine maintenance of strains was in Luria–Bertholdi growth medium and plates. Growth was monitored by measuring A_{580} of the cultures in a spectrophotometer. The M63 medium contained (per litre): 13.6 g of KH₂PO₄, 2 g of (NH₄)₂SO₄, 0.5 mg of FeSO₄·7H₂O and 0.25 g of MgSO₄·7H₂O (pH adjusted to 7.0 with KOH).

PFK activity in extracts of the different strains was measured as described elsewhere [4]. Periodically, the kinetic properties of the enzymes were monitored by appropriate kinetic assays. The strains with either type of PFK all had similar levels of total PFK activity in extracts.

Metabolite assays

To determine the time course of the intracellular concentration of metabolites, first an aliquot of a Luria–Bertholdi culture was diluted in fresh M63 minimal medium supplied with the corresponding carbon source and grown at 37 °C to saturation. From this, an aliquot was diluted in the same fresh medium

supplied with the same carbon source, the culture was grown at 30 °C, the cells collected by centrifugation when the A_{580} reached 0.5–0.7, and resuspended in M63 minimal medium to a final A_{580} of approx. 15.0. Chloramphenicol and glycerol were added to the cell resuspensions to final concentrations of 34 µg/ml and 0.1 % respectively. They were kept at 30 °C in a shaker, and samples were taken at 5–10 min intervals, added to ice-cold HClO₄ to a final concentration of 3 % and briefly mixed with a vortex mixer. After 5 min on ice, extracts were neutralized with one equivalent of KHCO₃ and centrifuged for 30 min at 4 °C in an Eppendorf centrifuge. The supernatant fractions were recovered and kept overnight at –20 °C.

FBP was measured by the standard coupled assay method using FBPA, aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase [15]. The intracellular concentration (mM) was calculated from the A_{580} as described by others [16]. All enzymes used were from Sigma (St. Louis, MO, U.S.A.).

Measurement of reverse-flux labelling of FBP

We have recently described a method to assess rates of futile cycle, during both glycolytic and gluconeogenic net carbon flux

[17]. Reverse-flux labelling is defined as the radioactive labelling of a metabolite when the direction of the net flux of radioactive label is opposed to the net flux of mass. In this case, the method involves establishing a net gluconeogenic carbon flux, by supplying the cells with glycerol as their only carbon source, and then applying a trace of $[U-^{14}C]$ glucose of high specific radioactivity (negligible in terms of mass). Then the time course of radioactive labelling of FBP is assessed.

Resting cell resuspensions were incubated with glycerol, as described above, and 10 min later $2 \mu\text{Ci}$ of $[U-^{14}C]$ glucose (specific radioactivity 304 mCi/mmol) was added. At various times thereafter, samples were extracted with HClO_4 as described. Radioactivity in the HClO_4 -soluble fraction was determined, and aliquots containing a similar amount of radioactivity were loaded on the chromatographic column.

The radioactively labelled metabolites present in the HClO_4 extracts were separated by HPLC in a Mono Q ion-exchange column. The column was first equilibrated in buffer A: 10 mM Tris/HCl, pH 8.0. A sample of each HClO_4 extract was injected and the column was eluted with a gradient between buffer A and buffer B (20 mM Tris/HCl, pH 8.0, 0.5 M NaCl). The gradient was as follows: 0–20 min, from 0 to 20 % of B; 30–60 min, from 20 to 100 % of B. The eluate went directly to a Beckman on-line liquid-scintillation counter, which fed the radioactivity data to a computer. The FBP peak was identified by its elution time and by selected enzyme treatment.

Determination of $^{14}\text{CO}_2$ produced by cell resuspensions

Cell resuspensions were prepared as already indicated, except that the final A_{580} was 2.0. $[U-^{14}C]$ Glycerol (specific radioactivity 130 mCi/mmol) was added to a final concentration of 0.01 % (final specific radioactivity 10^3 c.p.m./nmol). The resuspension was kept at 25°C , pressurized O_2 was bubbled through the culture, and the labelled CO_2 was trapped by bubbling the outgoing gases through a 0.3 M NaOH solution containing Triton X-100. At selected time points, scintillation mixture was added to the vial and the radioactivity was measured in a Packard liquid-scintillation analyser. Finally, the cell resuspension was extracted with HClO_4 , as indicated above. A part of the HClO_4 -soluble fraction was used to determine the incorporation of radioactivity into glycogen. Glycogen was precipitated by adding the following to the indicated final concentrations: 0.25 mg/ml carrier glycogen, 0.2% Na_2SO_4 and 50% ethanol. After a brief spin, the sediment was washed with ethanol and resuspended in warm water. An aliquot was used to determine the radioactivity.

In other experiments, cell resuspensions were supplied with a mixture of unlabelled glycerol (0.01 %) and a trace (around 50000 c.p.m.) of either $[1-^{14}C]$ glucose or $[6-^{14}C]$ glucose. Then the radioactively labelled CO_2 was trapped and determined as already described.

RESULTS

Overview of the results of the simulations

In this section we show some general characteristics of the predictions of the model for the simulation of steady-state gluconeogenic situations, i.e. when glycerol was the only and constant source of mass. Figure 1 summarizes the major characteristics of the simulation results. Figure 1(A) shows the effect of the PFK/FBPA activity ratio on the steady-state concentration of some metabolites. Figure 1(B) shows the same for the steady-state reaction rate of some model reactions. The

results are summarized in Scheme 2, where the direction of the arrows indicates the direction of the flux through each reaction during gluconeogenesis. The thin and thick arrows indicate reactions in which rates decrease and increase respectively with increased futile cycle. Metabolites printed in bold exhibit steady-state increases with futile cycle, and the remainder exhibit decreased concentration with increased futile cycle. Thus the model predicts that an increased PFK–FBPA futile cycle will result in increased concentrations of metabolites that are located after the PFK–FBPA cycle in the glycolytic direction, and decreased concentrations of those located before the cycle as well as those participating in the HMS, but not for E4P. The highest increase is predicted for FBP (see dotted line in Figure 1A). As to reaction rates, the model predicts increased rates for the enzymes involved in the cycle (PFK and FBPA) and for the enzymes that directly link glycerol input to CO_2 output from the citric acid cycle (Output 3). The remaining reactions will have decreased rates.

Let us consider for a moment that the input flux of glycerol carbons is divided in two major directions or branches. One, the gluconeogenic branch, feeds G6P and its products, glycogen, HMS intermediates and final products, and CO_2 . The other, the glycolytic branch, feeds the citric acid cycle reactions, resulting in CO_2 output. By adding all steady-state outputs to the sinks for each branch, we can inspect the effects of the cycle on the general distribution of fluxes to either branch. Thus, in Figure 2, we show the effect of increased PFK/FBPA activity ratio on the output fluxes of those two branches. The general effect is illustrated in Scheme 3. When the PFK–FBPA futile-cycle percentage is increased from 0 to 70, the flux through the gluconeogenic branch decreases from 84 to 60 % of the glycerol carbon input, and that through the glycolytic branch increases from 16 to 40 %. Thus the cycle acts as a shunting device, which increases the glycolytic fate of carbons at the expense of their gluconeogenic fate. We emphasize this effect because it appears to be the major predictive feature of the model, and the one that survives different kinetic equations and parameters.

The simulations of transient situations, in which the carbon source was exhausted, gave results that followed the pattern observed in the steady-state simulations with the difference that all metabolite levels decreased with the level of the carbon source before a steady state was reached. Some of those results will be analysed below.

Intracellular levels of FBP

Since the most dramatic effect of increased cycle rate in the simulations was on FBP levels, we measured the time course of the intracellular concentration of FBP in cell resuspensions of strains with different PFKs in the presence of glycerol as the only carbon source. Figure 3(A) shows the time course of the intracellular concentration of FBP for strains with Pfk-1, Pfk-2 or Pfk-2* with glycerol as only carbon source. In the strain with Pfk-1, intracellular levels of FBP had similar time courses and reached a maximum level of around 3 mM . In the strain with Pfk-2, intracellular FBP levels increased faster, to reach a shorter quasi-steady-state level of around 5 mM . Finally, in the strain with Pfk-2* FBP levels increased to over 8 mM , without any apparent steady state being reached before the intracellular concentration decreased back to basal levels as the result of carbon source exhaustion. As shown in the corresponding inset, the rate of carbon source uptake was approximately the same for the three strains. These results are compared in Figure 3(B) with the qualitative predictions of the model when applied to a situation where the carbon source (glycerol) is exhausted (see

Figure 1 Simulation results

(A) Effect of increased futile cycle on steady-state metabolite concentrations. According to the model, the effect of increased PFK/FBPA activity ratio on the steady-state concentration of selected model metabolites is shown. The concentration ratio is the corresponding concentration divided by the concentration of the metabolite when the PFK to FBPA ratio is zero. In the right-hand panel, the dotted line and right axis correspond to FBP. (B) Effect of increased futile cycle on steady-state reaction rates during gluconeogenesis. According to the model, the effect of increased PFK/FBPA activity ratio on the steady-state reaction rate of selected model enzymes is shown. The reaction rate ratio is the corresponding reaction rate divided by the rate of the reaction when the PFK/FBPA ratio is zero.

inset). The five time courses represent simulations with increasing PFK/FBPA ratios. Although there are differences in the shape of the curves between the model predictions and the experimental results, it is clear that the experimental results are consistent with the hypothesis of higher cycle rates in the Pfk-2*-bearing strain.

Reverse-flux labelling of FBP from [U-¹⁴C]glucose

The model was also used to simulate what we have called reverse-flux labelling [17], i.e. the radioactive labelling of FBP from trace amounts of [U-¹⁴C]glucose when the carbon source is glycerol. First, gluconeogenic steady states were obtained as indicated, and using these as a starting point, a small value of mass and radioactivity was associated with each carbon position of the glucose (GLU) in the model. The mass variable underwent a slight and transient perturbation to then return to the initial steady-state value (results not shown). The transient labelling of FBP was obtained from the corresponding radioactivity associated with FBP in the model. In what follows we compare model predictions with experimental results obtained in cell resuspensions of strains with different PFKs.

Figure 4(A) shows the time course of labelling of FBP,

expressed as the percentage of label associated with FBP. Clearly, the strain with Pfk-2* shows the highest level of FBP labelling, reaching as much as 40 % compared with approx. 27 % for the Pfk-2- and 21 % for the Pfk-1-bearing strain respectively. The latter reaches labelling levels similar to those of the strain without any PFK. Figure 4(B) shows three simulated reverse-labelling curves for different levels of the PFK/FBPA ratio, ranging from 0.2 to 4.0. As should be evident from the increased PFK activity with higher PFK/FBPA ratios, there is an increased flux of glucose radioactive label towards FBP as the ratio increases. Again, notwithstanding the simplified assumptions used to design the reaction scheme, the results strongly support our hypothesis.

Finally, results did not differ significantly when we used either [1-¹⁴C]glucose or [6-¹⁴C]glucose as label source instead of [U-¹⁴C]glucose (results not shown).

Production of CO₂ in strains with different PFKs during gluconeogenesis

Often a higher respiratory rate, i.e. a higher rate of CO₂ output, has been found associated with the occurrence of a futile cycle

Scheme 2 Model scheme showing the effect of increased futile cycle on concentrations and reaction rates

The thin and thick arrows indicate reactions the rates of which decrease and increase respectively with increased futile cycle. The names in bold face indicate metabolites that increase with increased futile cycle. The others correspond to metabolites that decrease in concentration. Abbreviations as in legend to Scheme 1.

Figure 2 Steady-state output rates (expressed as a percentage of constant input rate from glycerol) as a function of the PFK/FBPA ratio

—, 'Gluconeogenic' output rate, obtained by adding outputs through output reactions 1 and 2 (OPT1 and OPT2) and the CO_2 output at the 6-phosphogluconate dehydrogenase reaction (6PGD); ····, glycolytic output rate, which includes CO_2 outputs at output 3 (OPT3) and the pyruvate dehydrogenase complex reaction.

[18]. As shown in Figure 5(A), our model also predicts higher rates of CO_2 production with increased futile-cycle rates. The results correspond to simulations in which the mass and label source is glycerol and it is exhausted (transient situation).

Therefore we measured $^{14}\text{CO}_2$ production by cell resuspensions of strains with different PFKs supplied with $[\text{U-}^{14}\text{C}]$ glycerol as the only carbon and label source. The results are shown in Figure 5(B) for the strains DF920 (only Pfk-1), DF903 (Pfk-2) and DF905 (Pfk-2*). The three strains exhibited somewhat sigmoidal CO_2 production time courses that reached a plateau at around 3 h after the start of the incubation with glycerol. Whereas the strains with Pfk-1 or Pfk-2 had similar initial rates of labelled CO_2 production, and reached identical final levels of CO_2 production, the strain with Pfk-2* produced as much as 30 % more $^{14}\text{CO}_2$ than the other two strains at the end of the incubation period.

To determine the differing fates of the labelled carbons of glycerol in the three strains, once the CO_2 levels reached a plateau, the resuspended cells were treated with HClO_4 , and the amount of ^{14}C radioactivity in glycogen, HClO_4 -soluble material and HClO_4 -insoluble material was measured. The results are shown in Table 1. In the strain with Pfk-2*, approx. 73 % of the label ends up as CO_2 , compared with 61 and 60 % for DF920 (Pfk-1) and DF903 (Pfk-2) respectively. This increased CO_2 production is associated with a decrease in the number of carbon atoms that end up in both the HClO_4 -insoluble fraction (6–9 % decrease) and the HClO_4 -soluble material (3–5 % decrease). Little overall incorporation into glycogen was observed in the three strains.

Radioactive labelling of CO_2 from $[\text{1-}^{14}\text{C}]$ glucose and $[\text{6-}^{14}\text{C}]$ glucose during gluconeogenic metabolism

The model was also used to simulate the time course of radioactive CO_2 output when the carbon source was gluconeogenic (glycerol)

Scheme 3 Effect of increased cycle rates on the 'gluconeogenic' and 'glycolytic' output rates for two rates of futile cycle

The glycerol input is constant at 100 units. The arrows pointing upwards and to the right represent the 'gluconeogenic' and 'glycolytic' output rates respectively. The widths of the arrows are proportional to the rate. The percentage inside the circle represents the futile-cycle rate.

Figure 3 Time course of intracellular FBP concentration in strains with different PFKs during gluconeogenesis: comparison with model predictions

(A) Intracellular concentration of FBP (mM) at various times after provision of glycerol as the only carbon source to cell resuspensions. The inset shows the time course of glycerol concentration in the medium (%). The strains were DF905 (Pfk-2⁺; ○), DF903 (Pfk-2; □) and DF920 (Pfk-1; △). Each point represents the mean value of four or five measurements conducted in resuspensions prepared from independent colonies. The means and standard errors for the concentrations (mM) at the 25 min points were: Pfk-2⁺, 8.83 ± 0.673 ; Pfk-2, 2.20 ± 0.855 ; Pfk-1, 4.87 ± 0.403 . (B) Simulated time course of FBP concentration. Curves were obtained by applying the model to a transient gluconeogenic situation for various PFK/FBPA ratios (indicated above each curve).

and the label source was a trace amount of glucose radioactively labelled in either carbon 1 or 6. Let us call ΔCO_2^* the difference between the radioactive CO_2 output from $[1\text{-}^{14}\text{C}]\text{glucose}$ and that from $[6\text{-}^{14}\text{C}]\text{glucose}$ during a given time period. When ΔCO_2^* is plotted against time for variable futile-cycle rates, a family of curves of the type shown in Figure 6(D) is obtained. When there is no futile cycle (top curve) the ΔCO_2^* increases with time and is always positive. As the cycle rate is increased there is a transient flattening of the curve, up to a point at which the curve goes through a minimum that is negative at the higher cycle rates, to then return to positive values. In other words, the model predicts decreasing transient values of ΔCO_2^* with increasing futile-cycle rates. The magnitude of the effect was found to be a function of the maximum activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the

model, i.e. the oxidative branch of the HMS. The curves of Figure 6(D) were obtained using maximum velocities for the equations corresponding to those reactions that were one-fifth of those of the other enzymes in the model.

On the basis of this rather unexpected prediction, we designed experiments in which cell resuspensions were supplied with glycerol as the only carbon source and a trace amount of either $[1\text{-}^{14}\text{C}]\text{glucose}$ or $[6\text{-}^{14}\text{C}]\text{glucose}$. This trace was small enough in terms of mass not to affect the net gluconeogenic flux significantly (in terms of carbon mass, since the added glucose carbons were one-hundredth the mass of glycerol carbons). Again, as in the experiments of the previous section, the CO_2 produced was captured in an NaOH trap and the radioactivity associated with it was determined. The results are shown in Figure 6(A) to (C) for the three strains with different PFK types. First, with glucose

Figure 4 Time course of reverse-flux labelling of FBP

(A) Experimental results. Cell resuspensions of strains with different PFKs were supplied with glycerol as carbon source and a trace of $[U-^{14}C]$ glucose as label source. The graphs show the percentage of the total radioactivity in the chromatogram that is found associated with the FBP peak. The strains were DF920 (Pfk-1; $\square$), DF902 (No Pfk; $\triangle$), DF903 (Pfk-2; ∇) and DF905 (Pfk-2*; $\circ$). (B) Model predictions. Simulated time courses of radioactivity in FBP when the carbon source was glycerol, which was kept constant, and the radioactivity was associated with the six carbons of glucose, which was transient. Above each curve the PFK/FBPA ratio used in each simulation is shown.

Figure 5 Simulated and experimental time courses of $^{14}CO_2$ production from $[U-^{14}C]$ glycerol

(A) Model predictions. Simulated time courses of CO_2 production when the mass source in the model was a transient amount of glycerol. The PFK/FBPA ratios used were 0.2 (—), 2.0 (---) and 4.0 (····). (B) Experimental results. Experimental time courses of production by cell resuspensions of strains with different PFKs when the carbon source was $[U-^{14}C]$ glycerol. The strains were DF905 (Pfk-2*; $\square$), DF903 (Pfk-2; $\circ$) and DF920 (Pfk-1; $\triangle$). Results are means $\pm$ S.E.M. ($n = 4$).

labelled in either position, the strain with Pfk-2* produces higher levels of radioactive CO_2 than the other strains, just as we had seen when the source of both mass and label was glycerol (Figure 5B). However, some noteworthy features appear in this case. The strains with either Pfk-1 or Pfk-2 both had similar $^{14}CO_2$ outputs from $[1-^{14}C]$ glucose, but the strain with Pfk-1 had a higher $^{14}CO_2$ output from $[6-^{14}C]$ glucose than the strain with Pfk-2. The positive ΔCO_2^* persists to the end of the incubation period (Figure 6E, circles and triangles). In the strain with Pfk-2*, on the other hand, during most of the incubation period $^{14}CO_2$ output from $[6-^{14}C]$ glucose was higher than that from $[1-$

$^{14}C]$ glucose, hence negative values of ΔCO_2^* are obtained (Figure 6E, squares). By the end of the incubation period, however, both outputs reached the same final level, i.e. ΔCO_2^* returns to the zero basal level.

DISCUSSION

In a previous report we showed, using an *in vitro* assay model, that under a certain set of conditions, the different PFKs of *E. coli* led to different rates of futile cycling between F6P and FBP.

Table 1 Distribution of [U-¹⁴C]glycerol radioactivity in various end products in strains of *E. coli* with different PFKs

Cell resuspensions of the different strains were prepared as indicated in the text. [U-¹⁴C]glycerol was supplied as the only carbon source and cells were incubated for 3 h. The CO₂ produced was captured throughout the incubation period. At the end of the incubation, cells were extracted with HClO₄, and a HClO₄-soluble and HClO₄-insoluble fraction were obtained. Glycogen was precipitated from the HClO₄-soluble fraction. The radioactivity in each fraction was determined. Results are means ± S.E.M. (*n* = 4).

Strain	PFK in strain	[U- ¹⁴ C]glycerol radioactivity in fraction (%)			
		CO ₂	Glycogen	HClO ₄ -soluble material	HClO ₄ -insoluble material
DF920	Pfk-1	61.2 ± 1.10	3.0 ± 0.40	21.2 ± 0.50	14.0 ± 1.30
DF903	Pfk-2	60.2 ± 1.20	3.0 ± 0.05	19.0 ± 0.70	17.2 ± 0.80
DF905	Pfk-2*	73.0 ± 0.70	2.0 ± 0.40	16.2 ± 1.00	8.5 ± 0.50

Figure 6 Simulated and experimental time courses of radioactive CO₂ output from [1-¹⁴C]glucose and [6-¹⁴C]glucose during gluconeogenesis

(A) to (C) Experimental results. Time courses of ¹⁴CO₂ outputs from [1-¹⁴C]glucose (○) or [6-¹⁴C]glucose (□) for strains with Pfk-2 (A), Pfk-2* (B) or Pfk-1 (C). Glycerol concentration was approx. 1 mM (0.01 %) and labelled glucose concentration was approx. 4 × 10⁻⁴ mM (specific radioactivity 55 mCi/mmol). Data points are the mean of four experiments. Standard errors are not shown (but see E). (D) Model predictions. Simulated time courses of ΔCO₂* (the difference between the radioactive CO₂ output from glucose labelled in carbon 1 minus that from glucose labelled in carbon 6) for three Pfk/FBPA ratios, 0.0, 1.0 and 4.0, as indicated. The mass source was a transient amount of glycerol. In all these cases the amounts of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were reduced to one-fifth of the amount of the other enzymes in the model. (E) Experimental time courses of ΔCO₂* (see the text) for gluconeogenesis from glycerol and label supplied by glucose labelled in carbons 1 or 6 for strains with different PFKs. The strains were DF903 (Pfk-2; ○), DF905 (Pfk-2*; □) and DF920 (Pfk-1; △). The curves were obtained by subtracting the corresponding curves of (A) to (C). The values shown are the means ± S.E.M. for four experiments.

Pfk-2* was the enzyme that produced the highest rates in all the conditions assayed [11]. We suggested that this effect could be the basis for the phenotype of slow growth on gluconeogenic carbon sources of Pfk-2*-bearing strains, since high futile-cycle rates

could be expected to have an impact on either ATP levels or flux of carbon skeletons (or both).

In this study we have looked for evidence that *in vivo* the different PFKs also sustain different futile-cycle rates. Since direct and unequivocal evidence for the occurrence of futile cycle *in vivo* is not possible, we have relied on a number of indirect experimental pieces of evidence to support our hypothesis. We have used a mathematical model to generate predictions on the expected effects of the futile cycle on metabolite levels and reaction rates. Several predictions of our model show a remarkable degree of agreement with the experimental results.

The main effect predicted by the model is that the PFK-FBPA futile cycle shunts carbon flux from a gluconeogenic to a glycolytic fate. This is intuitively appealing, since PFK activity results in a backflow of hexose phosphate molecules, which will tend to reduce the net flux of hexose skeletons from FBP to F6P. In fact, all the predictions of the model are accounted for by this effect, as we will see in each of the following cases.

The Pfk-2* strain, when supplied with glycerol as sole carbon source, develops intracellular FBP concentrations that are significantly higher than strains with the other PFK types. In fact, the level of this metabolite increases throughout the incubation period to then decrease without reaching the quasi-steady state observed in the other strains. The same behaviour was exhibited by FBP in the model at increased futile-cycle rates. In this case, the increase results from the relative increases in the inputs and outputs to FBP. The increased input through PFK outweighs the increased output through FBPA and the decreased input through aldolase.

Previously we have presented a method to estimate futile-cycle rates by means of reverse-flux labelling [17]. Using the model, we have simulated such types of experiments in which the mass source is glycerol and the label source is glucose with radioactively labelled carbons. As we found *in vitro*, the model predicts higher labelling of FBP with higher cycling rates. On the other hand, the same type of experiment in cell resuspensions showed that FBP is labelled to significantly higher levels in the Pfk-2*-bearing strain. The fact that some labels did reach FBP in a strain lacking either PFK type argues for some activity of the HMS in at least that strain (reverse activity of FBPA should be negligible in gluconeogenesis). We do not know to what extent that pathway contributes to the labelling of FBP in the other strains. The model predicts that at higher cycle rates the HMS activity will be decreased, i.e. if that holds true *in vivo* and, as we hope to show, the Pfk-2* strain has higher cycle rates, then the fraction of label in FBP that reached it via HMS is higher in the Pfk-1 and Pfk-2 strains. Anyhow, the flux of glucose carbons downwards through the HMS should be quite small given the net gluconeogenic supply of mass. This latter statement is further supported by the ¹⁴CO₂ outputs from [1-¹⁴C]glucose and [6-¹⁴C]glucose during gluconeogenesis (see below) and by the fact that the same differences between strains are obtained if [1-¹⁴C]glucose or [6-¹⁴C]glucose are used instead of [U-¹⁴C]glucose. In conclusion, although we cannot specify the role of flux through the HMS in these results, all the evidence supports a role that ratifies rather than contradicts our interpretation.

One of the possible fates of carbons entering glycolysis and gluconeogenesis is CO₂, released at the oxidative entry to the HMS, at the pyruvate dehydrogenase step or during the citric acid cycle. We consulted our model regarding the effect of increased cycle rates on the fraction of glycerol carbons that end up as CO₂. As should be obvious from Scheme 1, the shunting effect of the cycle results in more glycerol carbons being directed towards a glycolytic fate and, in the model, to CO₂ production.

Therefore higher cycle rates should lead to higher final levels of CO_2 (Figure 5A). This was borne out by cell resuspensions of the Pfk-2* strain, supplied with $[\text{U-}^{14}\text{C}]$ glycerol, developing significantly higher levels of $^{14}\text{CO}_2$ (Figure 5B). As shown in Table 1, this increase was made at the expense of mainly HClO_4 -insoluble cell material and, to a lesser degree, of small HClO_4 -soluble molecules. Often, increased O_2 consumption is taken as evidence of the occurrence of a futile cycle [18–21]. The rationale is as follows: the cycle depletes the ATP pool, and the decreased ATP/ADP ratio triggers a regulatory response by which more carbons are respired to restore the ratio [18]. Neither ATP nor regulatory signalling mechanisms are included in our model, but it does associate higher cycle rates with higher CO_2 production. The latter results merely from the deviation of carbons towards CO_2 -releasing reactions that is caused by the shunting effect of the cycle. Clearly, the Pfk-2*-bearing strain does not restore the levels of whatever is limiting its growth by increasing its respiratory rate. In fact, on the basis of the final distribution of glycerol carbons, it could be suggested that the shunting of more carbons to CO_2 is decreasing the supply of some precursor for macromolecular biosynthesis.

Finally, simulations of radioactive CO_2 production from glucose labelled in carbons 1 or 6 during gluconeogenesis generated a prediction that is less evident from a simple inspection of the reaction scheme. At higher cycle rates, the model predicts more negative transient minima for ΔCO_2^* , whereas experimentally we found a markedly negative transient minimum. Inspection of the time courses of the rates of the various radioactivity fluxes reveals that this results from the effect of the gluconeogenic to glycolytic shunting acting on the differential circulation of carbons 1 and 6 of glucose in the aldolase and triose phosphate isomerase reactions.

The simulated time courses of $^{14}\text{CO}_2$ output shown in Figure 6(D) also result from the shunting effect of the cycle. As more 1 and 6 carbons are shunted towards a glycolytic fate, the difference between CO_2 output from carbon 1 and that from 6, which is always negative after the peak corresponding to the entry to the HMS (carbon 1), is magnified, resulting in an increasingly negative ΔCO_2^* .

Experimentally, we found first that the Pfk-2* strain produced higher levels of $^{14}\text{CO}_2$ output with either of the labelled glucoses, as it did with labelled glycerol, thus confirming that the glucose carbons have the same fate as the glycerol carbons. Secondly, we found that the $^{14}\text{CO}_2$ output differential, ΔCO_2^* , in the Pfk-2* strain goes through a more negative transient peak, returning to zero only at the end of the incubation period. The shape of the curves for the differential are quite similar to those produced by the model, taking into account that the reaction scheme is highly qualitative and that we do not know the magnitude of the actual fluxes through the HMS involved.

We here give experimental evidence that supports the hypothesis that a Pfk-2*-bearing strain sustains higher futile-cycle

rates during gluconeogenesis on glycerol than strains with the other PFK types. Demonstrating the occurrence of a futile cycle is not as straightforward as others have assumed [18,19,21]. In the absence of accurate information on the activity of all the metabolic pathways involved, a given result is always open to alternative interpretations. This is further complicated for organisms, such as bacteria or yeast, in which metabolic changes can be fast enough to obscure during experimental manipulation the situation *in vivo*. Our approach has been to generate a set of predictions that can be put to the experimental test. Although any of the results could by themselves be subjected to a different interpretation, we find it difficult to imagine a mechanism as simple as the futile cycle that would simultaneously account for all the results presented here. Finally, although we have shown that a Pfk-2*-bearing strain has higher cycle rates during gluconeogenesis, it remains an unsolved issue whether the link between the cycle and the growth phenotype is established through either ATP or precursor limitations, or both.

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