Finite change analysis of glycolytic intermediates in tuber tissue of lines of transgenic potato (*Solanum tuberosum*) overexpressing phosphofructokinase

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Genetically engineered organisms overexpressing phosphofructokinase (PFK), a supposed 'regulatory' step of glycolysis, often show little or no measurable change in glycolytic or respiratory flux, although the concentrations of glycolytic intermediates may change. We have used the finite change theory of Metabolic Control Analysis (MCA) to analyse the concentrations of glycolytic metabolites in aged disks of tuber tissue from four lines of transgenic potatoes expressing different amounts of PFK that, under aerobic conditions, showed statistically indistinguishable rates of respiration. The constancy of the metabolites' concentration deviation indices for different increases in PFK expression indicated that the metabolite changes form a graded

series, excluding the possibility of anomalous behaviour that might be observed in a single transgenic line. Consequently we were able to use the finite change method to validate the results of an MCA model of tuber glycolysis [Thomas, Mooney, Burrell and Fell (1997) *Biochem. J.* 322, 119–127]. Furthermore the metabolite changes with PFK activity are evidence that near-equilibrium steps do not transmit increased substrate concentrations down the pathway without attenuation. Our results support the view that flux increase by activation of a single enzyme early in the pathway will, contrary to expectations, be of limited effectiveness in achieving flux increases.

INTRODUCTION

Genetic manipulation of steps involved in the energy metabolism of organisms, either as a means of studying metabolic control or in an attempt to generate organisms with specifically altered metabolic characteristics, has frequently been reported. Increased expression of phosphofructokinase (PFK) has been a favoured manipulation, because of, or to test, the widely held view that this step has a high degree of control over respiration and/or glycolysis [1–3]. However, the effects on glycolysis or respiration rates have often been small or non-existent. This is strong evidence for the alternative view that PFK actually has very little flux control over glycolysis or respiration.

A likely explanation for the lack of general flux response to PFK overexpression is that one or more of its effectors changes in concentration in such a way as to oppose the increased activity. This is the case reported by Davies and Brindle [3] for *Saccharomyces cerevisiae*. The only metabolite or important effector of PFK they observed to undergo a significant concentration change was fructose 2,6-bisphosphate (F26BP), which decreased markedly under both aerobic and anaerobic conditions. There was no significant change in fructose 1,6-bisphosphate (F16BP) under either condition, and an increase in glycolytic flux only under aerobic conditions. The lack of a significant change in F16BP is important as, in the absence of changes in ATP, ADP, AMP, cAMP and citrate, it is the only known signal that could transmit the effect of increased PFK activity to the

remainder of the glycolytic chain. Because of the attenuating effect of the downstream glycolytic enzymes [4,5], changes in the glycolytic intermediates downstream of F16BP should also be non-significant. Thus the lack of flux change under anaerobic conditions is explicable. In contrast, the increase in flux under aerobic conditions indicates an additional activation effect downstream of F16BP that has not been considered.

Overexpression of PFK in tuber of potato (*Solanum tuberosum*) similarly caused no increase in glycolytic flux, but did result in dramatic changes in glycolytic intermediate concentrations [6,7]. A range of transgenic plants was produced containing the coding sequence of the *Escherichia coli* *pfkA* gene linked to a patatin promoter. Expression of this gene in tubers resulted in manifold increases in the maximum catalytic activity of PFK, without significantly affecting the activities of the other glycolytic enzymes. However, oxygen uptake and CO₂ production by aged disks of transformed tuber tissue did not differ significantly from controls, indicating no increase in the rate of respiration. Nor did the proportions of ¹⁴CO₂ production from [1-¹⁴C]- and [6-¹⁴C]-glucose vary between control and transformed tissue, making it unlikely that any increase in glycolytic flux in the transgenic plants had been masked by an equal decrease in pentose phosphate pathway flux. Thus PFK appears to exert virtually no control over respiration in potato tuber.

Despite the absence of changes in the respiratory fluxes, most metabolites downstream of PFK increased whereas those upstream decreased in concentration as the expressed PFK activity

Abbreviations used: 2PGA, 2-phosphoglycerate; 3PGA, 3-phosphoglycerate; aldolase, fructose bisphosphate aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13); DHAP, dihydroxyacetone phosphate; enolase, phosphopyruvate hydratase (2-phospho-D-glycerate hydro-lyase, EC 4.2.1.11); F16BP, fructose 1,6-bisphosphate; F26BP, fructose 2,6-bisphosphate; F6P, fructose 6-phosphate; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase [D-glyceraldehyde-3-phosphate-NAD⁺ oxidoreductase (phosphorylating), EC 1.2.1.12]; GUS, β -glucuronidase (β -D-glucuronide glucuronosohydrolase, EC 3.2.1.31); PEP, phosphoenolpyruvate; PFK, 6-phosphofructokinase (ATP-D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11); PFP, pyrophosphate-dependent 6-phosphofructokinase (pyrophosphate-D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.90); PGI, phosphoglucose isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9); PGK, phosphoglycerate kinase (ATP-3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3); PGM, phosphoglucomutase (α -D-glucose-1-phosphate phosphotransferase, EC 5.4.2.2); PGlyM, phosphoglycerate mutase (2-phospho-D-glycerate phosphotransferase, EC 5.4.2.1); PK, pyruvate kinase (ATP-pyruvate 2-O-phosphotransferase, EC 2.7.1.40); TPI, triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, EC 5.3.1.1); MCA, Metabolic Control Analysis.

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increased [7]. These changes are qualitative evidence that the *E. coli* PFK was active in the transgenic tubers *in vivo* to an extent that correlates well with the assayed PFK activity. ADP, ATP and F26BP concentrations were almost unchanged, except for a catastrophic collapse of F26BP at the highest level of PFK expression [7]. The most important inhibitor of potato tuber PFK *in vitro* is phosphoenolpyruvate (PEP) [8], the concentration of which did increase with increasing PFK activity. Although feedback inhibition has the potential to reduce the flux control coefficient of the inhibited enzyme [9], the significance of tuber PFK inhibition by PEP *in vivo* has not previously been determined. The importance of this interaction is assessed quantitatively in the following paper [10].

Despite the absence of any measurable flux change, analysis of the changes in metabolite concentrations in the transgenic tissue can shed light on the behaviour of carbohydrate metabolism in potato tuber. In this paper we analyse the changes in concentrations of glycolytic metabolites [7] using the finite change method of Metabolic Control Analysis (MCA) [11,12]. By analysing the data in this way, we determine to what extent the requisite conditions for applying the finite change method are satisfied in the lines of transgenic plants, and describe possible reasons for the breakdown of the conditions. Also, using 'traditional' (as opposed to finite change) MCA [9,13], we study the effects on metabolite concentrations of pleiotropic changes in expression of the other glycolytic enzymes that accompany overexpression of PFK. This is accomplished using a model of glycolysis in potato tuber described in the following paper [10]. Finally, we discuss the conclusions that can be drawn from the metabolite concentration changes on the nature of flux control in potato tuber glycolysis and respiration.

EXPERIMENTAL

Production of the transgenic plants, plus measurements of PFK activity, metabolite concentrations and gas exchange fluxes in the PFK transgenic lines and a control line expressing β -glucuronidase (GUS) have been described previously [6,7]. The genes were linked to a patatin-specific promoter, so that gene expression was restricted to the tuber. All measurements reported here are the results of experiments performed on disks of tuber (10 mm diameter, 1 mm thick) 'aged' by continuous circulation in 500 ml of aerated distilled water for 16 h. Such aged disks show a very high rate of respiration [14]. O_2 consumption and CO_2 production were measured in one GUS control and four transgenic lines, designated PFK-26, PFK-5, PFK-36 and PFK-22, expressing approximately 15–30, 120, 200, 350 and 940 nmol/min per g fresh weight PFK activity respectively [7]. Metabolite concentrations were measured in all lines except PFK-26.

FINITE CHANGE ANALYSIS

The manyfold change in PFK activity achieved [6,7] cannot be handled accurately by traditional MCA, which is strictly limited to infinitesimal changes in activity [9,13]. The finite change method [11,12] is a branch of MCA that was developed to enable analysis of situations in which large changes in enzyme activity are generated by genetic engineering or other means. The central quantity of the finite change theory is the deviation index, $D_{E_j}^V$, which defines the effect of a finite r -fold change in activity of enzyme E_j (from E_j^0 to E_j^r ; $E_j^r = rE_j^0$) on variable V , the value of which changes from V^0 to V^r :

$$D_{E_j}^V = \frac{\Delta V}{\Delta E_j} \times \frac{E_j^r}{V^r} \quad (1)$$

where $\Delta E_j = E_j^r - E_j^0 = E_j^0(r-1)$, and $\Delta V = V^r - V^0$. V is typically a flux or a metabolite concentration. Thus the deviation index is analogous to the control coefficient of traditional MCA, except that it is scaled at the new, not the original, point, in contrast with the control coefficient [9,13]. Deviation indices show a number of useful properties as long as certain conditions of pathway behaviour are met or, at least, approximated. For the purposes of this paper the most important restriction is that each step of the pathway should show linear kinetics with respect to its substrate(s) and product(s). This amounts to there being no significant change in the saturation of any step during the flux change considered. If this restriction holds, the value of $D_{E_j}^V$ can be shown to be independent of r . Thus it will be constant for different r -fold changes in E_j from the original state. Although this is potentially a very restrictive assumption, it has been shown to be a useful approximation in certain cases [11]. However, the corollary is equally useful: variation in $D_{E_j}^V$ with different r -fold changes in E_i indicates significant deviations from the linear approximation. Thus, with a range of transgenic lines expressing different amounts of PFK activity, the variation in $D_{E_j}^V$ for metabolites and fluxes between the control and each of the transgenic lines will test this assumption. Note that because of the restriction of the definition in eqn. (1), only $n-1$ indices can be calculated for each variable from n different PFK activities if the indices are calculated for deviations from the original point E_j^0 . In this paper deviation indices will refer only to changes between a transgenic line and the control line.

The deviation indices for O_2 consumption and CO_2 production for the four transgenic lines tested are shown in Table 1. Although they show variation between lines, the differences are not significant because of the imprecision of the data. However, the correlation between the rates of O_2 consumption and CO_2 production across the four lines is notable (correlation coefficient = 0.91), giving a strong indication that there is no sizeable change in distribution between anaerobic and aerobic metabolism between the control and the four transgenic lines.

The variation in the concentrations of glycolytic intermediates, especially those downstream of PFK, are particularly interesting. Appreciation of the patterns of changes is simplified by considering their deviation indices (Table 2) and log-log plots of their concentrations against PFK activity (Figure 1, bottom). Log-log plots of the concentrations against PFK activity help visualize the values of the deviation indices, but it must be pointed out that they are not plots of the deviation indices themselves. In traditional MCA the slope of a log-log plot of a flux or a metabolite concentration against enzyme activity gives the control coefficient directly, but this does not strictly apply for deviation indices. The difference is that for infinitesimal changes (r), V^0 and V^r are essentially the same value, and the equalities $dV/V = d\ln V$, and $dE_j/E_j = d\ln E_j$ are mathematically true as therefore is the control coefficient definition $C_{E_j}^V = (dV/V)/(dE_j/E_j) = d\ln V/d\ln E_j$. This is not true for finite changes where V^0 and V^r are different values, and $\Delta V/V \neq \Delta \ln V$, etc. Notwithstanding the strict definition, the gradient of the line of a $\ln V$ versus $\ln E_j$ plot does give a good indication of the value of the deviation index.

The metabolites can be divided into three groups. The members of the first group (G1P, G6P, F6P and 2PGA) show very little change with change in PFK activity. The members of the second, DHAP and PEP, show a monotonic but slowing increase with increasing PFK activity. At all activities the increase in PEP concentration is greater than that in DHAP, even though DHAP is nearer to PFK. The third group (F16BP, GAP, 3PGA and pyruvate) show an abrupt increase between PFK-5 and PFK-36, before a more steady increase to PFK-22. This abrupt change is

Table 1 Deviation indices of CO₂ production and O₂ consumption for PFK overexpression in aged disks of potato tuber

Deviation indices $D_{PFK}^V = (\Delta V / \Delta PFK) (PFK^t / V^t)$, where $\Delta PFK = PFK^t - PFK^c$, $\Delta V = V^t - V^c$ and superscripts t and c represent the transgenic and control lines respectively.

	Deviation index			
	PFK-5	PFK-26	PFK-36	PFK-22
O ₂ consumption	-0.10	-0.02	-0.02	-0.09
CO ₂ production	-0.14	-0.06	-0.08	-0.14

Table 2 Deviation indices of glycolytic metabolites for PFK overexpression in aged disks of potato tuber

Deviation indices $D_{PFK}^V = (\Delta V / \Delta PFK) (PFK^t / V^t)$, where $\Delta PFK = PFK^t - PFK^c$, $\Delta V = V^t - V^c$ and superscripts t and c represent the transgenic and control lines respectively. Standard errors are in parentheses. G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 3PGA, 3-phosphoglycerate; 2PGA, 2-phosphoglycerate.

Glycolytic metabolite	Deviation index		
	PFK-5	PFK-36	PFK-22
ATP	-0.22 (0.26)	-0.49 (0.28)	0.32 (0.18)
ADP	0.11 (0.24)	0.15 (0.25)	0.08 (0.24)
G1P	0.11 (0.35)	-0.22 (0.19)	0.15 (0.17)
G6P	-0.33 (0.22)	-0.45 (0.33)	-0.27 (0.16)
F6P	-0.50 (0.76)	-0.60 (0.75)	-0.89 (1.06)
F16BP	0.75 (0.19)	0.91 (0.03)	0.93 (0.02)
DHAP	0.65 (0.47)	0.79 (0.25)	0.84 (0.16)
GAP	0.21 (0.13)	0.83 (0.16)	0.86 (0.67)
3PGA	0.53 (0.38)	0.90 (0.08)	0.94 (0.04)
2PGA	-0.48 (0.44)	-1.02 (0.76)	-1.51 (0.89)
PEP	0.82 (0.20)	0.86 (0.12)	0.87 (0.09)
Pyruvate	0.17 (0.35)	0.66 (0.05)	0.71 (0.04)

reminiscent of the change in ATP concentration (Figure 1, top), but occurs at a different degree of PFK expression indicating that a different physiological mechanism is the cause. It is not possible to relate the change to flux changes (Table 1), because of the uncertainty in the latter values. The marked changes in the concentrations of F16BP, GAP, 3PGA and pyruvate give rise to markedly different deviation indices for the transitions from the control line of PFK-5 on the one hand, and to PFK-36/PFK-22 on the other (see Table 2).

As described earlier in this section, change in a deviation index for different *r*-fold changes in enzyme activity indicates a breakdown of the assumption of linear kinetics required for application of the finite change method. From the standard deviations of the deviation indices (calculated as described in [11]), the differences in deviation indices for the PFK-5 to PFK-36 transition are significant (*t* test) at the 5 % level for F16BP, 1 % for GAP, 10 % for 3PGA (but 5 % for the PFK-5 to PFK-22 transition) and 0.1 % for pyruvate. Thus, for these metabolites, it appears that breakdown of the linear approximation required for valid application of the finite change method has occurred, so the finite change theory does not apply.

Thus the interesting situation arises of a 'partial failure' of the finite change theory, wherein some metabolites appear to follow the behaviour predicted by linear kinetics, whereas others do not. It is tempting to look at the factors that could influence the concentration of each metabolite, such as a change in saturation

Figure 1 Variation of metabolite concentrations with PFK activity in potato tuber from different transgenic lines

Metabolite concentrations and PFK activities are from [7]. Top, Adenine nucleotides and F26BP: ▲, ADP; ■, ATP; ●, F26BP. Bottom, Glycolytic intermediates: □—□, G1P; △—△, G6P; ■—■, F6P; ▲—▲, F16BP; ●—●, DHAP; □—□, GAP; △—△, 3PGA; ■—■, 2PGA; ▲—▲, PEP; ●—●, pyruvate. gfw, g fresh weight.

at a particular enzyme or the effects of branchpoints, and assign reasons for the differences in behaviour individually for each metabolite. However, this reasoning must be applied with care, remembering that the deviation index is a systemic property (see

the derivation in [11]) and the value for any one metabolite will be influenced by, possibly unexpected, systemic factors. Rather than deciding whether the finite change method is applicable for certain metabolites, it is more meaningful, in view of the systemic nature of the deviation indices, to decide whether or not, overall, the conditions required for the finite change method hold sufficiently well. Considering the conflicting results we have obtained, a certain degree of subjectivity is inevitable. However, in view of the fact that the majority of the metabolites measured show non-significant changes in deviation indices for different *r*-fold changes in PFK activity, we feel justified in maintaining that the conditions required for application of the finite change method appear to have been met, at least as far as can be ascertained with the precision of the data available.

Despite having expressed satisfaction that the conditions of linear kinetics are met sufficiently well to apply the finite change theory, the discontinuities in the concentration changes of F16BP, GAP, 3PGA and pyruvate between PFK-5 and PFK-36 still require explanation. The fact that these significant discontinuities all occur at the same genetic transition is a strong indicator that increasing PFK activity beyond a certain point leads to qualitatively different behaviour. Despite the systemic nature of the deviation indices, and the apparently systemic effect on the four metabolites, it is worthwhile considering the factors that may be responsible for changes in individual metabolites. The behaviour of each metabolite may be affected by different factors, or the changes may be linked and one of the factors listed below may be responsible for the co-ordinated changes in all four compounds. (One likely possibility we cannot investigate, because of the scarcity of kinetic data for potato tuber enzymes [10], is the requirement, specified in the derivation of the finite change equations, that the degrees of saturation of the enzymes involved do not change significantly [11]. This is a shame as it would have removed one source of uncertainty from our application, and added an extra source of verification of the finite change method.)

The case of pyruvate would seem to be the most readily explained. Dephosphorylation of PEP is a strongly exothermic step that is unlikely to approach equilibrium *in vivo* and, although pyruvate kinase (PK) does not appear to be as strongly regulated in plant as it is in mammalian tissues, there is evidence that its kinetics are sensitive to a number of metabolites [15]. Consequently there is freedom for the relative concentrations of PEP and pyruvate to vary. These conclusions are unlikely to be materially altered if PEP is decarboxylated by an alternative enzyme, such as PEP carboxylase [orthophosphate-oxaloacetate carboxy-lyase (phosphorylating), EC 4.1.1.31]. In addition, pyruvate lies outside the major feedback loop in both the control [8] and PFK transgenic potato lines [16,17], namely that of PEP inhibition of PFK, and is therefore likely to respond in a different manner from the other glycolytic intermediates (see the Discussion). Finally, there is the additional partitioning of pyruvate into the mitochondria to consider, although this is likely to be a relatively minor effect as mitochondrial pyruvate is probably a small proportion of the total.

F16BP and GAP could be affected by their positions at junctions with other processes involved in carbohydrate metabolism: as substrate (or products) of pyrophosphate-dependent 6-phosphofructokinase (PFK) and the oxidative pentose phosphate pathway respectively. One, or either, of these processes could serve to maintain metabolite homeostasis until sufficient PFK activity is expressed to overcome their buffering capacity. The question of whether both processes need be invoked in explanation depends on whether or not GAP and F16BP are always in equilibrium *in vivo*. This is an explanation that, until recently, would have been accepted unhesitatingly by most.

Figure 2 Scaled changes of glycolytic metabolites with PFK activity in potato tuber from different transgenic lines

Metabolite concentrations and PFK activities are from [7]. Ordinate and abscissa values were obtained by dividing the metabolite concentrations and PFK activity respectively from the transgenic lines by those from the control line. □—□, G1P; △—△, G6P; ■—■, F6P; ▲—▲, F16BP; ●—●, DHAP; □—□, GAP; △—△, 3PGA; ■—■, 2PGA; ▲—▲, PEP; ●—●, pyruvate.

However, as shown in Table 2 (and in terms of fractional increase plotted in Figure 2), the deviation index in GAP as PFK is increased is consistently less than that in F16BP, which implies that equilibrium is not maintained. Furthermore the qualitatively different behaviour of DHAP and GAP is evidence that the reaction catalysed by triosephosphate isomerase (TPI) is not maintained at equilibrium at all times. Set against this is the good evidence that F16BP and 3PGA are maintained close to equilibrium, as evidenced by their similar deviation indices/fractional changes at all stages of PFK overexpression. Finally, it is worth noting that the deviation indices of the group of consecutive metabolites F16BP, DHAP, GAP and 3PGA become more similar to one another as PFK activity increases, reflecting the fact that the fractional increases for these metabolites for the PFK-36 to PFK-22 transition are more similar than for any other transition. In fact, this transition marks a change from a state in which F16BP and DHAP behave similarly, as do GAP and 3PGA, to one in which F16BP, DHAP and GAP all behave similarly, implying that F16BP, DHAP and GAP move considerably closer to equilibrium in PFK-22. If this is the case, one likely consequence would be a shift of control to the steps after TPI.

The three hexose phosphates show more consistent and less significant changes in deviation indices between the different lines, showing that the linear approximation applies more closely to them. Only G1P shows significant differences in deviation

index at 10 % for the PFK-5 to PFK-36 transition and 5 % for the PFK-36 to PFK-22 transition.

EXPRESSION OF OTHER GLYCOLYTIC ENZYMES

One potential cause of the abrupt changes in metabolite concentrations between lines PFK-5 and PFK-36 is changes in the activities of the other glycolytic enzymes accompanying PFK overexpression in the transgenic plants. The activities for each line were measured [7] and are plotted (logarithmic axes) in Figure 3. It can be seen that there is little difference between any of the lines. Only GAPDH shows a significant difference between the transgenics and the GUS control line, although the difference is not correlated with the extent of PFK expression. However, between PFK-5 and PFK-36 there are apparent increases in the activities of certain enzymes, aldolase, TPI, GAPDH and phosphoglycerate kinase (PGK). Although none of the changes are significant for the precision of the activity data collected, the apparently co-ordinated changes are noteworthy.

The effects of even small changes in an enzyme's activity on the concentrations of its substrates and products should not be dismissed without careful consideration, as metabolite concentrations are often extremely sensitive to enzyme activities. The effect of a change in activity of enzyme E_i on the concentration of metabolite S_j can be calculated from the concentration control coefficient of E_i over S_j , $C_{E_i}^{S_j}$. To a first approximation, the fractional change in activity $\Delta E_i/E_i$ is related to the fractional change in concentration $\Delta S_j/S_j$ by:

$$\frac{\Delta S_j}{S_j} \approx C_{E_i}^{S_j} \times \frac{\Delta E_i}{E_i} \quad (2)$$

The overall change in S_j over the PFK-5 to PFK-36 transition is

Figure 3 Variation of glycolytic enzyme activities with PFK activity in potato tuber from different transgenic lines

Enzyme activities are from [7]. ●—●, PGM; △—△, PGI; ■—■, PFP; ●—●, aldolase; ■—■, TPI; △—△, GAPDH; □—□, PGK; □—□, PGlyM; ▲—▲, enolase; ▲—▲, PK. gfw, g fresh weight.

Table 3 Calculated changes in concentration due to the measured changes in the activities of enzymes other than PFK over the PFK-5 to PFK-36 transition in aged disks of potato tuber

Changes are expressed as a percentage of the calculated change due to changes in expression of all the glycolytic enzymes other than PFK, divided by the change due to overexpression of PFK:

$$\frac{\sum C_{E_i}^{S_j} \times \frac{\Delta E_i}{E_i}}{C_{\text{PFK}}^{S_j} \times \frac{\Delta \text{PFK}}{\text{PFK}}} \times 100$$

where $C_{E_i}^{S_j}$ is the concentration control coefficient of enzyme E_i over S_j , $C_{\text{PFK}}^{S_j}$ is the concentration control coefficient of PFK over S_j , and the summation in the numerator includes all enzymes from PGM to PK, excluding PFK.

Metabolite	Change (%)
G6P	—41
F6P	12
F16BP	—52
DHAP	—41
GAP	—80
3PGA	—41
2PGA	—0.46
PEP	2.3

obtained by summing over all the enzymes that have changed activity. Note that here we have reverted to using traditional, rather than finite change, MCA. Although the changes in enzyme activity are not infinitesimal, they are small enough (between 2 and 17%) not to violate too greatly the assumption of infinitesimal change. Concentration control coefficients can be calculated by applying the matrix method of MCA [18,19]. In the following paper [10] we describe how this approach has been applied to the data being analysed here to calculate concentration control coefficients for the glycolytic enzymes over the glycolytic intermediates. For reasons that are described fully in [10], we have been unable to calculate concentration control coefficients with sufficient precision to give good agreement between the measured metabolite concentration changes and those calculated using eqn. (2). However, the approach is still valid, and the conclusions still informative, merely requiring better estimates of the control coefficients for quantitatively reliable results. Bearing these limitations in mind, the results in Table 3 show that for many metabolites the calculated concentration changes over the PFK-5 to PFK-36 transition could be significantly affected by changes in enzyme activities other than PFK. The important point is that small changes in enzyme activity can generate large changes in metabolite concentrations, which is reflected in the frequent occurrence of relatively large concentration control coefficients. Consequently, when studying metabolite changes in transgenic organisms, it is necessary to be aware that simultaneous pleiotropic changes in activities other than the one intended are likely to affect concentrations.

NATURE OF GLYCOLYTIC AND RESPIRATORY FLUX CONTROL IN POTATO TUBER TISSUE

So far we have analysed metabolite and enzyme activity changes between the control and transgenic lines of tuber, but have not directly addressed where control of respiration and glycolysis reside in potato tuber. If control of respiration lies predominantly within the glycolytic sequence, then control of glycolysis and respiration will be similar. If it lies predominantly after PEP,

then glycolytic and respiratory control will be different. Oxygen consumption measures respiratory flux, whereas CO_2 production can include a contribution from the oxidative pentose phosphate pathway, which can be quite active in tuber tissue [20]. Therefore determination of the relative contributions of mitochondrial oxidation, anaerobic glycolysis and pentose phosphate metabolism to carbohydrate catabolism is not necessarily straightforward, but estimates are necessary for determination of the distribution of flux control. The gas exchange fluxes show a respiratory quotient not significantly greater than 1 in each line, whereas the measured ratio of $^{14}\text{CO}_2$ release from $[6\text{-}^{14}\text{C}]\text{glucose}/[1\text{-}^{14}\text{C}]\text{glucose}$ did not differ significantly between the GUS control and PFK-36 [6,7]. These data are somewhat contradictory. The respiratory quotient of 1 suggests that there is little flow through the oxidative pentose phosphate pathway when endogenous substrate is respired, and little anaerobic catabolism of pyruvate produced by glycolysis. In contrast, the low ratio of C6/C1 CO_2 release indicates a considerable contribution of the oxidative pentose phosphate pathway to catabolism of glucose. The lack of significant differences in respiration between any of the lines implies that PFK has little control over respiration. There was also a slight but insignificant rise in anoxic CO_2 production with increasing PFK activity [6], indicating that PFK also has little control over the glycolytic flux. However, these observations do not show how control over respiration is partitioned between those reactions leading to PEP and those beyond, merely that PFK has little control over either glycolysis or respiration. In the following paper [10], we address this point more fully and, using traditional MCA rather than finite change theory, show that the majority of respiratory control does not lie within the steps we have examined.

Although with the analysis presented here we cannot quantify the distribution of respiratory flux control between the glycolytic and subsequent steps, the pattern of relative changes in the concentrations of the glycolytic intermediates, especially F16BP, PEP and pyruvate (Figure 2) is revealing. F16BP shows very large concentration increases, especially in lines PFK-36 and PFK-22, which is not surprising as it is the product of the overexpressed enzyme. However, PEP (except for line PFK-5) and pyruvate show, in turn, relatively lower proportional increases. In line PFK-36, a 36-fold increase in measured PFK activity has led to a 10.6-fold increase in F16BP, but only a 6.4-fold increase in PEP. Thus the signal (i.e. increased F16BP) has been reduced by 40%. The PEP concentration signal is further reduced by 50% in the production of pyruvate (ignoring partitioning of pyruvate into the mitochondria), which has increased only 3-fold. Although we do not have the data for the tricarboxylic acid cycle intermediate we suggest, by extending the argument above, that this relatively small rise in pyruvate is further attenuated through the tricarboxylic acid cycle and oxidative phosphorylation intermediates, and is consequently insufficient to generate an increased flux.

Finally, the effect of PFK overexpression on the concentrations of effectors of PFK and PFP must be considered. As shown in Figure 1 (top), ADP concentration is effectively unchanged from the control value in all transgenic lines, whereas ATP concentration shows only small changes, except in PFK-22 [7]. These observations argue against changes in ADP and ATP, both known effectors of *S. tuberosum* PFK *in vitro*, having an important role *in vivo* as a homeostatic response to increased PFK activity. F26BP, on the other hand, decreases markedly as the activity of PFK increases, probably as a result of inhibition of PFK2 by 3PGA and triose phosphates [21]. F26BP is thought not to be an activator of either higher plant [22] or *E. coli* PFK [23], but is a potent activator of PFP [24]. As both PFK and PFP

potentially contribute to the glycolytic flux, it is interesting to note that the amount of a known activator of one reaction should decline when the maximum catalytic activity of the other is increased. However, this potential reduction of PFP activity is not sufficient to fully counter the PFK overexpression, as shown by the increase in F16BP.

DISCUSSION

Manyfold overexpression of active *E. coli* PFK has apparently had no significant effect on the fluxes, as measured by gaseous exchange, aerobic respiration or anaerobic glycolysis in aged disks of potato tuber. Nor does it seem to have altered the partitioning of carbohydrate metabolism between the oxidative pentose phosphate and glycolytic pathways. Pleiotropic alterations in the activities of some of the other glycolytic enzymes may have had measurable effects on the concentrations of some of the glycolytic intermediates, but little effect on pathway flux.

The variations in metabolite levels relative to the control, in the series of transgenic plants expressing increasing levels of PFK, occur in a graded manner broadly consistent with the predictions of the finite change theory of MCA. Thus we can have far greater confidence than could be possible by analysis of a single transgenic line that the observations are primarily caused by overexpression of active PFK in the cytoplasm, rather than by some undetected unintended side effect of manipulation and selection.

We have demonstrated how, even in the absence of any usable flux change information, analysis of the glycolytic intermediates is a valuable tool in interpreting the biochemical consequences of enzyme overexpression, especially when used with the finite change theory of MCA. This theory has been able to explain some, but not all, of the features of the transgenic plants' glycolytic metabolism. The likely cause of our inability to explain all the features lies with the interaction of glycolysis with other areas of carbohydrate metabolism: with PFP, via F6P and F16BP; with the oxidative pentose phosphate pathway, via G6P, F6P and GAP; with amyloplastic pathways of carbohydrate catabolism, predominantly via G1P.

The step catalysed by PFK is a classic paradigm for a putative rate-limiting step, located near the start of the pathway whose flux it is supposed to control. Although it has been less well studied from plant than from animal or bacterial sources, the reaction appears to be displaced far from equilibrium *in vivo* [25–28], and the enzyme from plant tissues often shows the properties of a 'regulatory enzyme': sigmoidal kinetics [8,29,30], and regulation by a number of allosteric effectors [31–35] including, in potato, strong feedback inhibition by PEP [8]. All these factors are traditionally held to indicate an important site of metabolic control.

However, such means of identifying putative rate-limiting steps have been shown to be unreliable or even incorrect. In their seminal paper, Kacser and Burns [9] proved both that feedback inhibition serves to move flux control off the inhibited step, downstream of the inhibiting metabolite, and that displacement from equilibrium is not an infallible guide to the magnitude of the flux control coefficient. The implications have been slow to dawn on most biochemists, despite their great importance. Concerning negative feedback, Hofmeyr and Cornish-Bowden [36] went on to show that the homeostatic effect, i.e. the stabilization of the metabolite concentrations within the feedback loop, is a quantitatively far larger effect than flux inhibition. This also helps to explain why flux control by the inhibited enzyme is reduced: increases in the product(s) and downstream metabolites as a result of activating the enzyme are reduced by

the feedback effect, so onward transmission of the flux beyond the activated enzyme is diminished. This effect is analysed quantitatively and at greater length with reference to the inhibition of potato tuber PFK in the following paper [10]. Turning to the effect of disequilibrium, it is easy to show using computer simulation that enzymes with disequilibrium ratios in the region of 0.8 or greater, which would have classified them as 'near-equilibrium' and thus as not important in regulation [37], do not respond passively to activation of an upstream enzyme by increasing their rates and simultaneously transmitting an increased level of metabolites to downstream enzymes without significant attenuation (S. Thomas and D. A. Fell, unpublished work). A succession of such enzymes, e.g. the glycolytic steps from aldolase to enolase, can consequently limit the spread of the perturbation in metabolite concentration so it does not reach the downstream enzymes, which consequently do not increase their rates. This is illustrated in Figure 2 which shows the diminution of the PFK-induced signal in the steps after PFK.

In the specific case of potato tuber, the fact that cold-sweetening of potatoes might be explicable by cold-lability of PFK [38] is not evidence that regulation of PFK has a role in modulating glycolytic flux under other circumstances. Any step, even one with a low degree of control, can become rate-limiting if its activity is inhibited sufficiently. However, the opposite does not hold: even greatly increasing the activity of a step with low flux control will have little effect on the flux. The properties that make PFK a possible candidate for exercising cold-induced flux inhibition do not necessarily make it a candidate for exerting physiological flux control.

The long-standing problems of understanding metabolic control have entered a new era with the huge effort being invested in the production of transgenic organisms in an attempt to generate large non-physiological flux changes. Greater understanding of the complexity of metabolic control is required if real success is to be achieved in such endeavours, especially when attempting to modify pathways of primary metabolism such as glycolysis. Over the past two decades, MCA has shown, not only the fallacy of relying on the identification of rate-limiting steps but, more recently, the small relative flux increases that can be achieved even by manifold overexpression of enzymes with relatively high flux control coefficients [11]. This latter revelation led to the proposal of the Universal Method for achieving desired large flux changes [39], which requires the overexpression of many steps in a pathway. Accordingly, a large increase in tryptophan synthesis in *Saccharomyces cerevisiae* was achieved only by simultaneous overexpression of five steps, when increased expression of one to four enzymes produced relatively poor results [40].

We have previously argued that the Universal Method is a human reinvention of the strategy many organisms apparently use to solve the problem of achieving large physiological flux changes while maintaining metabolite homeostasis, namely the simultaneous modulation of the activity of several enzymes [4]. Cornish-Bowden and co-workers have pointed out that, although overexpression of several enzymes can increase flux, it is inefficient and requires the degree of overexpression of each to be carefully calculated. They propose that large flux changes with good metabolite homeostasis can be achieved efficiently by increasing demand for a pathway product [41]. In potato tuber the demand for PEP comprises its further oxidative metabolism (possibly with a minor contribution from the use of its carbon skeleton), which in turn is linked to the energetic requirements of the cell.

The majority of flux control does appear to reside somewhere amongst these steps [10]. Identification and activation of the controlling step(s) of aerobic metabolism may provide a means of achieving some glycolytic flux increase.

Despite the ability of molecular geneticists to drastically modify an organism's genome, many, probably most, attempts to manipulate fluxes fail because of the application of incorrect strategies of identifying steps for genetic overexpression. As our perception of metabolic control changes, so will proposed strategies for manipulation. One point that does seem certain is that, except perhaps in the simplest of pathways, overexpression of a single enzyme in the supply pathway for a desired metabolite will produce disappointing results.

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