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Decreased-activity mutants of phosphoglucose isomerase in the cytosol and chloroplast of *Clarkia xantiana*

Impact on mass-action ratios and fluxes to sucrose and starch, and estimation of Flux Control Coefficients and Elasticity Coefficients

Arthur L. KRUCKEBERG,* H. Ekkehard NEUHAUS,† Regina FEIL,† Leslie D. GOTTLIEB* and Mark STITT†‡

*Department of Genetics, University of California, Davis, CA 95616, U.S.A.,
and †Lehrstuhl für Pflanzenphysiologie, Universität Bayreuth, 8580 Bayreuth, Federal Republic of Germany

1. Subcellular-compartment-specific decreased-activity mutants of phosphoglucose isomerase in *Clarkia xantiana* were used to analyse the control of sucrose and starch synthesis during photosynthesis. Mutants were available in which the plastid phosphoglucose isomerase complement is decreased to 75% or 50% of the wild-type level, and the cytosol complement to 64%, 36% or 18% of the wild-type level. 2. The effects on the [product]/[substrate] ratio and on fluxes to sucrose or starch and the rate of photosynthesis were studied with the use of saturating or limiting light intensity to impose a high or low flux through these pathways. 3. Removal of a small fraction of either phosphoglucose isomerase leads to a significant shift of the [product]/[substrate] ratio away from equilibrium. We conclude that there is no 'excess' of enzyme over that needed to maintain its reactants reasonably close to equilibrium. 4. Decreased phosphoglucose isomerase activity can also alter the fluxes to starch or sucrose. However, the effect on flux does not correlate with the extent of disequilibrium, and also varies depending on the subcellular compartment and on the conditions. 5. The results were used to estimate Flux Control Coefficients for the chloroplast and cytosolic phosphoglucose isomerases. The chloroplast isoenzyme exerts control on the rate of starch synthesis and on photosynthesis in saturating light intensity and CO_2, but not at low light intensity. The cytosolic enzyme only exerts significant control when its complement is decreased 3–5-fold, and differs from the plastid isoenzyme in exerting more control in low light intensity. It has a positive Control Coefficient for sucrose synthesis, and a negative Control Coefficient for starch synthesis. 6. The Elasticity Coefficients *in vivo* of the cytosolic phosphoglucose isomerase were estimated to lie between 5 and 8 in the wild-type. They decrease in mutants with a lowered complement of cytosolic phosphoglucose isomerase. 7. The implications of these results for regulation and for evolution are discussed.

INTRODUCTION

Carbohydrate metabolism in plants is compartmented between the cytosol and the plastid. The major storage carbohydrate, namely starch, is restricted to the plastid, and the major transport carbohydrate, namely sucrose, is metabolized outside the chloroplast. Glycolysis and gluconeogenesis occur in both of these compartments (ap Rees, 1985; Dennis & Greyson, 1987). In recent years decreased-activity mutants have been developed for the cytosolic and the plastid isoenzymes of phosphoglucose isomerase (PGI, EC 5.3.1.9) in *Clarkia xantiana* (Onagraceae) (Jones *et al.*, 1986*a*,*b*). These mutants provide a novel opportunity to manipulate metabolism selectively in the cytosol or the chloroplast. The present paper characterizes the effect of PGI on fluxes to sucrose and starch. A further paper (Neuhaus *et al.*, 1989) uses these mutants to probe the mechanisms that regulate and co-ordinate metabolism in these two compartments.

PGI is representative of a large group of enzymes that are thought to be present in 'excess', and catalyse reactions lying close to equilibrium. We have therefore asked two questions. (1) Is PGI really present in a large 'excess'? How much of the PGI activity can be removed before the conversion of Fru6*P* into Glc6*P* is restricted and the mass-action ratio ([Glc6*P*]/[Fru6*P*]) starts to decrease? (2) When a restriction is introduced at this site, does it actually lead to a decrease in the flux through the pathway? More precisely, what are the Flux Control Coefficients and Elasticity Coefficients (Kacser & Burns, 1973; Kacser & Porteous, 1987) for PGI in each compartment?

Fig. 1 summarizes the reactions catalysed by the plastid and cytosolic PGIs during photosynthesis. The plastid PGI is essential for starch synthesis, whereas the cytosolic PGI is essential for conversion of triose phosphates into sucrose in the cytosol. The fluxes catalysed by PGI in the plastid and in the cytosol can therefore be estimated by measuring the rates of starch and sucrose synthesis respectively. PGI is not directly required for CO_2 fixation

Abbreviations and symbols used: PGI, phosphoglucose isomerase; Fru6*P*, fructose 6-phosphate; Glc6*P*, glucose 6-phosphate; *C*, Flux Control Coefficient; ϵ, Elasticity Coefficient; *J*, flux *in vivo*; *E*, amount of enzyme; $K_{eq.}$, equilibrium constant; *v*, enzyme activity; Γ, mass-action ratio, [product]/[substrate].

‡ To whom correspondence should be addressed.

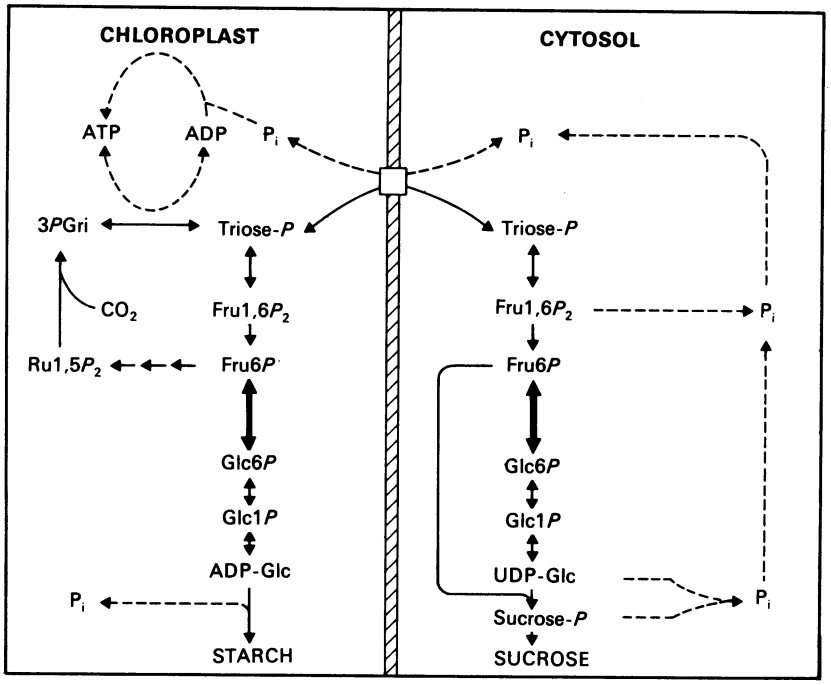

Fig. 1. Metabolic fluxes in the chloroplast and cytosol of leaves during photosynthesis, showing fluxes of carbon (——) and phosphate (----)

and operation of the Calvin cycle. However, there is an indirect dependence because photosynthesis cannot continue unless the phosphorylated intermediates are converted into end products and P_i is recycled.

METHODS

Plant growth and mutant identification

Plants of *Clarkia xantiana* Gray (Onagraceae) were studied. They were derived from a self-pollinating white-flowered population and are highly homozygous (Gottlieb, 1984). Mutations in the nuclear genes encoding plastid and cytosolic PGI isoenzymes were recovered following mutagenesis with ethyl methanesulphonate, as described by Jones *et al.* (1986*a*,*b*). PGI genotypes were determined by electrophoresis of crude plant extracts in starch gels, followed by staining for PGI activity (Gottlieb & Higgins, 1984). The wild-type pattern consists of four bands: the plastid isoenzyme PGI-1 is most anodal, followed by the duplicated cytosolic PGI isoenzymes PGI-2 and PGI-3; the PGI-2/3 heterodimer migrates to an intermediate position between the cytosolic isoenzyme homodimers. The wild-type gentoype is $Pgi1^a1^a$, $Pgi2^b2^b$, $Pgi3^a3^a$.

Homozygous null mutations of $Pgi2^b$ and $Pgi3^a$ do not exhibit cytosolic PGI activity in regions of the gel corresponding to wild-type PGI-2 and PGI-2/3 and wild-type PGI-3 and PGI-2/3 respectively. Mutants that are heterozygous at each locus (i.e. possess a wild-type allele and a null allele) have bands of decreased activity, relative to wild-type, with appropriate mobilities (Jones *et al.*, 1986*a*). The mutant of *Pgi1* has decreased activity and as a homozygote exhibits a weakly staining band after electrophoresis, or no band at all (Jones *et al.*, 1986*b*).

Five genotypes, mutant at either genes encoding cytosolic PGI or at the gene encoding plastid PGI, were examined in this study. All of them have been backcrossed to wild-type for at least five generations and, in principle, are 98.4% wild-type for the remaining genome (see discussion in Jones *et al.*, 1986*a*,*b*). The five genotypes are: (1) $Pgi1*1^a$ (heterozygous plastid mutant); (2) *Pgi1*1** (homozygous plastid mutant); (3) $Pgi2^{nu}2^{nu}$ (homozygous cytosolic null mutant); (4) $Pgi3^{nu}3^{nu}$ (homozygous cytosolic null mutant); (5) $Pgi2^{nu}2^{nu}$, $Pgi3^a3^{nu}$ (a double mutant recovered from a segregating F_2 progeny as described in Jones *et al.*, 1986*a*).

Seeds were germinated in fine vermiculite moistened from below with water. The seeds were covered with 1 cm of vermiculite. Germination occurred after 5–7 days at 12 °C under a 12 h photoperiod. When the seedlings had partially expanded cotyledons and the first leaf pair had emerged (10–14 days), they were transplanted into individual pots (diameter 5 cm) containing a 3:1 mixture of sand and peat, and 1 week later were transferred to a greenhouse with a temperature of 22–28 °C (day) and 15–20 °C (night). Illumination was summer daylight supplemented for 18 h/day with 300 μmol·m^{-2}·s^{-1} tungsten light. Plants were watered with half-strength Hoagland once per week, and each was supplied with controlled-release fertilizer pellets. After genotype identification, selected plants were transferred to 8 cm pots and were used for experiments 3–5 weeks after germination. All experiments were carried out with fully expanded leaves.

Mutants were identified on the basis of starch-gel electrophoresis and PGI activity staining (Gottlieb & Higgins, 1984). The genotype $Pgi2^{nu}2^{nu}$, $Pgi3^a3^{nu}$ displays no PGI-2 or PGI-2/3 band, and diminished staining at PGI-3. Heat treatment was used to confirm the identification of the $Pgi2^{nu}2^{nu}$, $Pgi3^a3^{nu}$ genotype, and to quantify the amount of PGI activity present in the cytosol for each of the other genotypes. Extracts were prepared at 4 °C by homogenizing leaves in 0.2 ml of medium (100 mM-Hepes/KOH buffer, pH 7.5, containing 7 mM-$MgCl_2$, 1 mM-EDTA, 5 mM-2-mercapto-

ethanol, 0.5% bovine serum albumin and 50 μg of phenylmethanesulphonyl fluoride/ml) per mg fresh wt. of leaves. Extracts were centrifuged at 12000 *g* for 3 min and assayed for PGI immediately, or after heating at 56 °C for 10 min. This treatment inactivates the plastid isoenzyme, PGI-1 (Weeden & Gottlieb, 1982). PGI activity was assayed in 100 mM-Tris/HCl buffer, pH 7.8, containing 7 mM-$MgCl_2$, 2 mM-Fru6*P*, 0.4 mM-$NADP^+$, 1 unit of glucose-6-phosphate dehydrogenase/ml and 0.5% bovine serum albumin. These assay conditions are substrate-saturating for the cytosolic PGI.

Plastid PGI was partially purified from wild-type and back-cross-6 *Pgi1*1** plants, which had previously been identified by using starch-gel electrophoresis (see above). Leaves (20 g) were homogenized in 100 ml of 50 mM-Tris/HCl buffer, pH 7.6, containing 42 mM-2-mercaptoethanol, 1 mM-EDTA, 0.1 mg of polyvinylpolypyrollidone/ml and 0.2% (v/v) Triton X-100 at 4 °C, filtered through three layers of Miracloth and one layer of cheesecloth, centrifuged at 16000 *g* for 45 min and loaded on a DE52 DEAE-cellulose column (20 g preswollen weight) pre-equilibrated with 50 mM-Tris/HCl buffer, pH 7.6, containing 50 mM-KCl, 1 mM-EDTA and 42 mM-2-mercaptoethanol. The column was eluted with a 200 ml linear gradient of 50–500 mM-KCl in the pre-equilibration buffer, and 3.3 ml fractions were collected and assayed for PGI spectrophotometrically (in 100 mM-Tris/HCl buffer, pH 8.1, containing 5 mM-$MgCl_2$, 1 mM-$NADP^+$, 1 unit of glucose-6-phosphate dehydrogenase/ml and 5 mM-Fru6*P*) and by starch-gel electrophoresis and activity stain (see above) to identify the fractions containing plastid isoenzyme. These were pooled and dialysed for 12 h against 3 litres of 100 mM-Tris/HCl buffer, pH 8.1, containing 5 mM-$MgCl_2$. The wild-type PGI-1 was assayed at this stage; the enzyme from *Pgi1*1** plants was adjusted to 100 mM-KCl, loaded on a DE52 DEAE-cellulose column (10 g) pre-equilibrated as above and eluted with 80 ml of a linear 50–500 mM-KCl gradient; the plastid-PGI-containing fraction was identified by spectrophotometric and starch-gel-electrophoretic analysis, pooled and dialysed as above, then concentrated 5-fold by centrifugal ultrafiltration (Centricon 10 microconcentrator) and assayed for PGI. Protein was determined with the Bio-Rad protein assay kit. K_m measurements were carried out in conditions as above, with Fru6*P* concentrations of 24, 12, 6, 3, 1.5, 0.75, 0.375, 0.187, 0.094 and 0.049 mM.

Biochemical analysis

Photosynthesis was measured in a leaf-disc oxygen electrode (Hansatech, Kings Lynn, Norfolk, U.K.) as described in Scheibe & Stitt (1988). Material from four leaves (100–180 μg of chlorophyll) was attached to a metal grid with two thin strips of Sellotape. Illumination was provided by a projector, and the intensity was varied with neutral grey filters (Schott, Wiesbaden, Germany). CO_2 was supplied at saturating concentrations from 400 μl of a 2 M-bicarbonate buffer, pH 9.3, containing 5 units of carbonic anhydrase distributed on a felt pad in the base of the electrode. For flux measurements, $H^{14}CO_3^-$ (0.2 μCi/μmol) was included and illumination was continued for 20 min before the leaf material was frozen in liquid N_2. For collection of samples for metabolite analysis, leaves were illuminated for 12 min and then transferred rapidly under continued illumination at the same light intensity into liquid N_2.

Incorporation of ^{14}C into starch was measured by extracting leaves twice in boiling 80% (v/v) ethanol, and once in 50% (v/v) ethanol to remove soluble material, homogenizing the leaves and determining the labelling of insoluble material. The ethanol extracts were combined, dried down and then taken up in 1 ml of water, and the neutral, acidic and basic components were separated by ion-exchange chromatography (Stitt *et al.*, 1985).

Extracts for metabolite measurements were prepared by powdering frozen leaf material in a mortar and pestle precooled to below −100 °C with liquid N_2, and then adding 4.5 ml of an extraction cocktail made up of 3 vol. of methanol, 7 vol. of chloroform and 1 vol. of 20 mM-Hepes buffer, pH 8.8, containing 5 mM-EGTA and 50 mM-NaF. The resulting frozen mixture was thawed with repeated mixing, held for 30 min at 4 °C, vigorously mixed with 4 ml of water and centrifuged at 1000 *g* for 10 min. The lower phase was re-extracted with another 4 ml of water, and the upper phases from both centrifugations were combined, dried down in a rotary evaporator at 36 °C, resuspended in 1 ml of water and stored in liquid N_2 until assay. Fru6*P* and Glc6*P* were assayed as described in Scheibe & Stitt (1988), with the inclusion of 0.5% bovine serum albumin in all the assay buffers. For recoveries of metabolites through the extraction see Neuhaus *et al.* (1989). The [Glc6*P*]/[Fru6*P*] ratio in added biochemicals was not altered during the extraction.

Estimation of Flux Control Coefficients and Elasticity Coefficients

Flux Control Coefficients were estimated from 'adjacent' enzyme pairs by using the equations (Torres *et al.*, 1986):

$$J = \frac{Q_1 \cdot E_i}{Q_2 + E_i} \text{ or } C^J_{E_i} = \frac{Q_2}{Q_2 + E_i}$$

rearranging them to yield the expression:

$$C^J_{E_i} = \frac{e - ep}{p - ep}$$

where $e = E_2/E_1$ and $p = J_2/J_1$, with E_1 and E_2 being two 'adjacent' enzyme activities, J_1 and J_2 the corresponding fluxes, and $C^J_{E_i}$ the Flux Control Coefficient of PGI in the mutant with activity E_i. This calculation assumes a hyperbolic relation of J and E between the two points. Elasticity Coefficients were estimated by using the equations (Groen *et al.*, 1982):

$$\epsilon^{PGI}_{Fru6P} = \left(\frac{1}{1 - \Gamma/K_{eq.}}\right) \cdot (1 - J/V_{max.})$$

$$\epsilon^{PGI}_{Glc6P} = \left(\frac{\Gamma/K_{eq.}}{1 - \Gamma/K_{eq.}}\right) \cdot (1 + J/V_{max.})$$

where Γ is the [Glc6*P*]/[Fru6*P*] ratio measured in the leaves (Table 2), J is the flux to sucrose (Table 4), and $V_{max.}$ is the unidirectional PGI activity measured in the extract with saturating substrate. We also applied an approach suggested by Kacser & Burns (1979), using an equation of the form:

$$\frac{dJ}{J} = \epsilon^{PGI}_{Fru6P} \cdot \frac{d[Fru6P]}{[Fru6P]} + \epsilon^{PGI}_{Glc6P} \cdot \frac{d[Glc6P]}{[Glc6P]} + \frac{d[PGI]}{[PGI]}$$

and data for two independent perturbations of metabolism (changed activity of PGI and changed

light intensity). However, the S.E.M. values for the measured concentrations of Fru6*P* and Glc6*P* were large compared with the change of the mean values for the metabolite concentrations in our experiments. Since this approach is very sensitive to experimental error, our calculations yielded some spurious Elasticity Coefficients, with inverted signs.

RESULTS

Identification and quantification of mutants

Decreased-activity mutants of the cytosolic PGI could be obtained in *Clarkia xantiana* because the coding gene has duplicated in this species and the duplicated isoenzymes are electrophoretically distinct (Gottlieb, 1977; Jones *et al.*, 1986*a*). This allowed the recovery of homozygous mutants in which either PGI-2 or PGI-3 was absent (Jones *et al.*, 1986*a*). Since the expression of the non-mutated cytosolic PGI gene does not change, these plants have a significantly decreased cytosolic PGI activity compared with the wild-type (Jones *et al.*, 1986*a*). A double mutation obtained by crossing $Pgi2^{nu}2^{nu} \times Pgi3^{nu}3^{nu}$ and screening for F_2 segregants with the genotype $Pgi2^{nu}2^{nu}$, $Pgi3^{a}3^{nu}$ had even lower cytosolic PGI activity.

Two criteria were used to identify these various mutants. First, extracts were separated by electrophoresis in starch gels, and stained for PGI activity. The homozygous null cytosolic mutants can be unambiguously identified by the absence of the corresponding isoenzyme band. The genotype $Pgi2^{nu}2^{nu}$, $Pgi3^{a}3^{nu}$ was provisionally identified by absence of staining for PGI-2 and diminished staining for PGI-3. Secondly, advantage was taken of the heat-lability of the plastid isoenzyme, which is totally inactivated after 2 min at 50 °C (Weeden & Gottlieb, 1982; Jones *et al.*, 1986*b*). When wild-type *Clarkia* extracts were heated to 56 °C, there was a 31 ± 1% decrease in activity within 2 min (Fig. 2), and the remaining activity was stable for at least 40 min. This suggests that 31% of the total PGI activity is in the plastid in the wild-type, confirming results obtained by Jones *et al.* (1986*b*). Table 1 summarizes the decrease in activity when each of the cytosolic mutants was heated for 10 min. Genotypes with decreased cytosolic PGI activity lost a larger fraction of their PGI activity during the heat treatment. This is expected, as the heat-labile plastid isoenzyme now contributes a larger proportion of total PGI activity. Assuming that the expression of the plastid isoenzyme has not been modified, the fraction of the wild-type cytosolic PGI activity remaining in the mutants can be estimated (see Table 1 legend) as 64% ($Pgi3^{nu}3^{nu}$), 36% ($Pgi2^{nu}2^{nu}$) and 18% ($Pgi2^{nu}2^{nu}$, $Pgi3^{a}3^{nu}$). This confirms results obtained by Jones *et al.* (1986*a*), who used antibodies to inhibit the cytosolic PGI selectively and estimate the contribution of each isoenzyme. Two further features of the results suggest that our approach is valid. First, the activity estimated for the homozygous nulls for the two cytosolic isoenzymes adds up to 100%. Secondly, the activity estimated for the $Pgi2^{nu}2^{nu}$, $Pgi3^{a}3^{nu}$ genotype is exactly half the activity estimated for the $Pgi2^{nu}2^{nu}$ genotype. This is precisely what is expected if the two isoenzymes are independently expressed (Jones *et al.*, 1986*b*) and there is no compensation when one of the isoenzymes is absent, or when the number of gene copies for an isoenzyme is halved in a heterozygote.

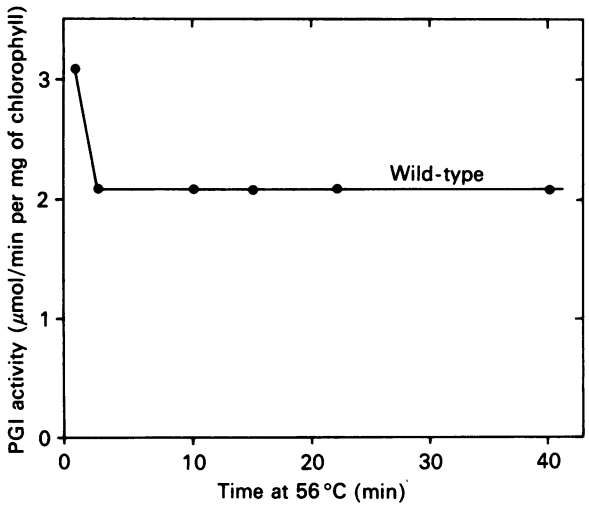

Fig. 2. Influence of heating on PGI activity in extracts

Extracts were prepared as described in the Methods section, heated at 56 °C for various times, cooled to 4 °C, and then assayed at 20 °C for PGI activity.

Table 1. Influence of heating at 56 °C on PGI activity in extracts from different genotypes: estimation of cytosolic PGI activity

The extracts were prepared as described in the Methods section and PGI was assayed immediately or after heating at 56 °C for 10 min. The contribution of the stromal enzyme was revealed as the fraction lost after heating (designated as *y*). The activity of PGI in the cytosol (as a percentage of the activity in the wild-type) was estimated as $100 \times (0.31–0.32y)/0.69y$, assuming that heating has completely inactivated the plastid PGI.

Genotype	Activity (μmol/h per mg of chlorophyll)		Fraction lost (= *y*)	PGI activity (estimated as % of wild-type)	
	Before heating	After heating		Chloroplast	Cytosol
Wild-type	207	142	0.31	100	100
$Pgi3^{nu}3^{nu}$	169	97	0.42	100	64
$Pgi2^{nu}2^{nu}$	105	45	0.57	100	36
$Pgi2^{nu}2^{nu}$, $3^{a}3^{nu}$	98	28	0.72	100	18

A series of mutants was also prepared with decreased plastid PGI activity. Heat inactivation was used to show that the homozygous plastid mutant (*Pgi1*1**) contained 50% of the wild-type PGI-1 activity, confirming results obtained by Jones *et al.* (1986*b*), and that the heterozygote contained 75% of the wild-type activity (results not shown). Since the PGI-1 mutant has a depressed activity of the product of a single gene, we investigated whether it resulted from a decreased affinity for substrate. PGI-1 was partially purified from wild-type and *Pgi1*1** plants to separate it from the cytosolic isoenzymes. The K_m for Fru6*P* for wild-type PGI-1 was 0.26 ± 0.04 mM and for homozygous mutant PGI-1 was 0.28 ± 0.04 mM (means ± S.E.M., $n = 3$). We do not have any direct evidence on whether the decreased activity is due a lower $k_{cat.}$ or a decrease in the amount of protein. However, the observation that PGI-1 from *Pgi1*1** plants was extremely sensitive to warming during extraction and during purification (results not shown) indicates there has been a structural change in the protein.

Mass-action ratios

The reaction catalysed by PGI is usually close to equilibrium *in vivo*, with a [Glc6*P*]/[Fru6*P*] ratio of about 3.3 (Dyson & Noltmann, 1968). The first detectable effect of a decrease in PGI activity would be to shift this ratio further away from equilibrium (Table 2). Glc6*P* and Fru6*P* were measured in leaf material carrying out photosynthesis in saturating light intensity and CO_2. In these conditions the rates of end-product synthesis will be high. There was some variation among the absolute concentrations of these metabolites in samples from different plants (S.E.M. up to 13% of the mean). Nevertheless there was a consistent and significant decrease of the mass-action ratio (S.E.M. 3–4% of the mean) in genotypes with decreased plastid or cytosol PGI. This decrease was already apparent in the mutants with the smallest decrease in PGI activity.

For comparison, we also measured the mass-action ratios during photosynthesis in low light intensity, when the fluxes to end products were lower. The [Glc6*P*]/[Fru6*P*] ratio did not decrease significantly in these conditions in the mutants with lowered plastid PGI activity. In the cytosol the ratio increased, but not as markedly as in high light intensity.

It may reasonably be assumed that the [Glc6*P*]/[Fru6*P*] ratio is increasing in the subcellular compartment where PGI dosage has been decreased. How-

Table 2. Influence of decreased plastid or cytosolic PGI activity on the [Glc6*P*]/[Fru6*P*] ratio

Leaves from four separate plants (120–200 μg of chlorophyll) were illuminated at 1000 μmol·m^{-2}·s^{-1} (saturating) or 125 μmol·m^{-2}·s^{-1} (limiting) in saturating CO_2 at 15 °C for 10 min before they were rapidly transferred into liquid N_2 with illumination being continued at the same light intensity. The results are the means ± S.E.M. for four to eight separate extracts.

PGI activity (as % of wild-type)		Saturating light intensity			Limiting light intensity		
Plastid	Cytosol	[Glc6*P*] (nmol/mg of chlorophyll)	[Fru6*P*] (nmol/mg of chlorophyll)	[Glc6*P*]/[Fru6*P*] ratio	[Glc6*P*] (nmol/mg of chlorophyll)	[Fru6*P*] (nmol/mg of chlorophyll)	[Glc6*P*]/[Fru6*P*] ratio
100	100	564 ± 52	216 ± 11	2.61 ± 0.06 (100)	393 ± 25	137 ± 12	2.87 ± 0.06 (100)
75	100	506 ± 31	233 ± 18	2.17 ± 0.07 (83)	347 ± 25	121 ± 6	2.78 ± 0.08 (97)
50	100	509 ± 58	274 ± 32	1.86 ± 0.08 (71)	394 ± 24	143 ± 8	2.76 ± 0.07 (96)
100	64	562 ± 41	231 ± 21	2.43 ± 0.04 (97)	410 ± 15	140 ± 5	2.92 ± 0.04 (102)
100	36	560 ± 35	251 ± 16	2.23 ± 0.05 (85)	345 ± 10	132 ± 3	2.61 ± 0.06 (91)
100	18	502 ± 65	327 ± 32	1.54 ± 0.09 (59)	385 ± 14	176 ± 8	2.19 ± 0.09 (76)

Table 3. Influence of decreased plastid or cytosolic PGI activity on the rates of starch or sucrose synthesis

Leaves from four separate plants were selected (100–200 μg of chlorophyll) and illuminated in an oxygen electrode at 1000 μmol·m^{-2}·s^{-1} (saturating) or 125 μmol·m^{-2}·s^{-1} (limiting) in the presence of saturating $^{14}CO_2$ for 20 min at 20 °C, before they were quenched in liquid N_2. The results are the means ± S.E.M. for four to six separate samples.

PGI activity (as % of wild-type)		Saturating light intensity			Limiting light intensity		
Plastid	Cytosol	Photosynthesis (μmol of CO_2 fixed/h per mg of chlorophyll)	Sucrose synthesis (μg-atom of C/h per mg of chlorophyll)	Starch synthesis (μg-atom of C/h per mg of chlorophyll)	Photosynthesis (μmol of CO_2 fixed/h per mg of chlorophyll)	Sucrose synthesis (μg-atom of C/h per mg of chlorophyll)	Starch synthesis (μg-atom of C/h per mg of chlorophyll)
100	100	354 ± 16	158 ± 18	134 ± 9	111 ± 5	39 ± 2	40 ± 2
75	100	338 ± 14	156 ± 6	120 ± 5	107 ± 6	32 ± 2	42 ± 2
50	100	275 ± 14	168 ± 10	69 ± 5	100 ± 5	40 ± 2	37 ± 2
100	64	370 ± 31	156 ± 25	136 ± 21	–	–	–
100	36	343 ± 25	148 ± 12	139 ± 11	105 ± 3	37 ± 1	41 ± 2
100	18	348 ± 21	142 ± 9	147 ± 8	112 ± 6	31 ± 2	51 ± 3

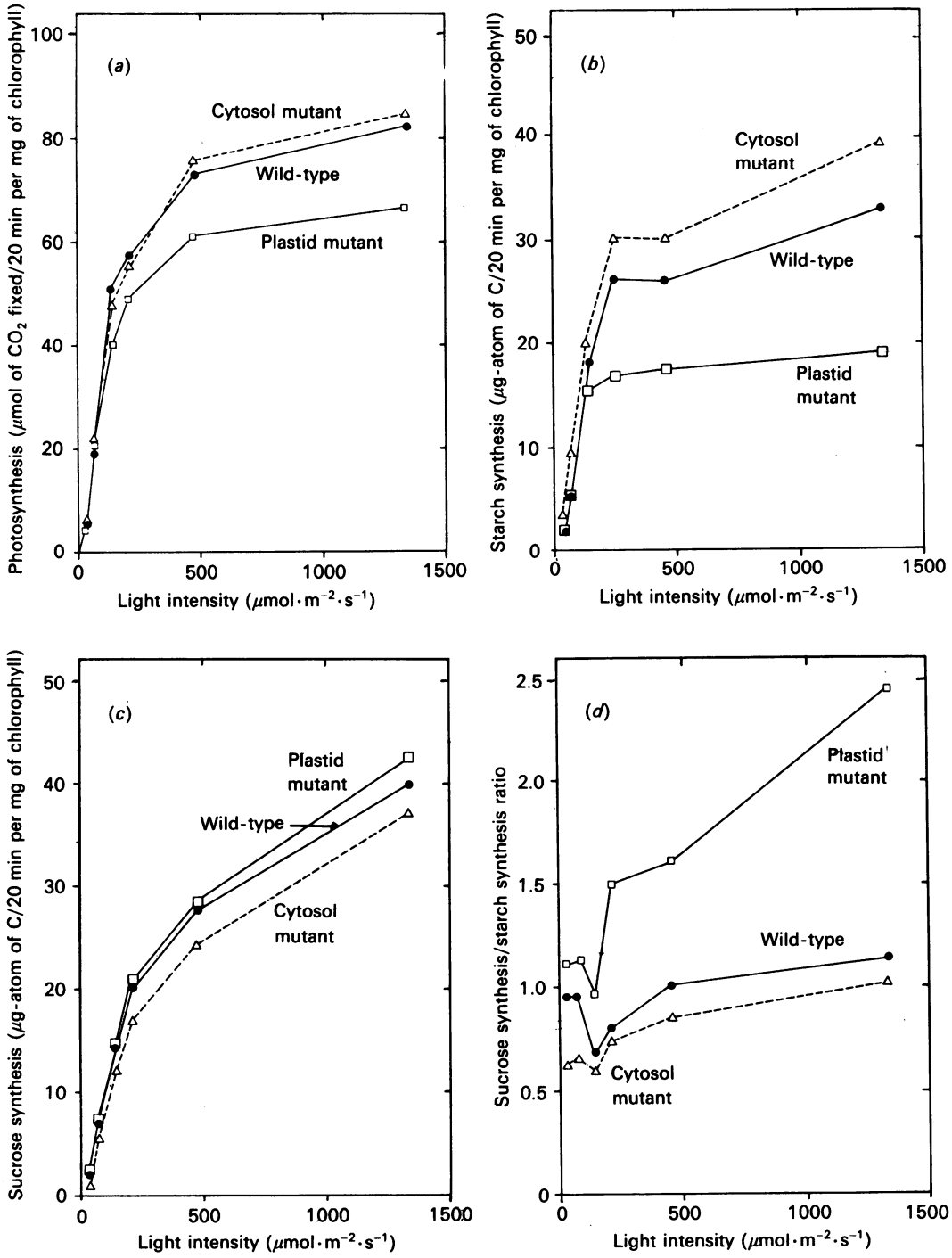

Fig. 3. Influence of light intensity on photosynthesis, sucrose synthesis and starch synthesis in wild-type (●), 18% cytosol PGI mutant (△) and 50% plastid PGI mutant (□)

Four detached leaves were illuminated for 20 min at 15 °C with saturating $^{14}CO_2$ (0.2 μCi/μmol), quenched in liquid N_2 and extracted with boiling ethanol. Insoluble material was taken as starch, and sucrose was separated by ion-exchange chromatography. (*a*) Photosynthesis; (*b*) starch synthesis; (*c*) sucrose synthesis; (*d*) sucrose synthesis/starch synthesis ratio.

ever, subcellular compartmentation complicates quantitative interpretation of these ratios because the overall change will underestimate the extent of the change in the compartment where the PGI activity has been genetically lowered. Previous studies show that 70–90% of the hexose phosphates are located in the cytosol of protoplasts from four different species (Stitt *et al.*, 1985). The corresponding value in spinach leaves varies between 60 and 80% (Gerhardt *et al.*, 1987). In this case the overall measurements will slightly underestimate the extent of the change in the cytosol. However, they probably underestimate the change in the chloroplast by a factor of 2-fold or more.

Influence on fluxes

In saturating light intensity CO_2 uptake was not altered in the plastid mutant heterozygote but was decreased by about 25% in the plastid mutant homozygote (Table 3).

The heterozygote showed a small change in partitioning between sucrose and starch. In the plastid mutant homozygote the rate of starch synthesis was decreased by 50%, and sucrose synthesis increased slightly. These changes were much less marked at low light intensities. The plastid mutant heterozygote showed a small increase of starch synthesis and decrease of sucrose synthesis at low light intensities in this, and other, experiments.

Decreasing cytosolic PGI activity did not have a marked effect on the rate of photosynthesis in saturating light intensity. Partitioning was unaffected in mutants with 65% of the wild-type cytosolic PGI activity, but when the activity was decreased to 36% or 18% there was a slight shift of partitioning in favour of starch. In low light intensity there was a larger effect. Sucrose synthesis was decreased by 22% and starch synthesis increased by 27% in the mutant with 18% of the wild-type cytosolic PGI activity. This shift of partitioning in low light intensity occurred without a decrease of the photosynthetic rate. These changes at low light intensity were not found if cytosolic PGI activity was only decreased to 36%.

The response of the plastid and cytosolic fluxes to increasing rates of photosynthesis was further investigated in Fig. 3. Starch (Fig. 3*b*) and sucrose (Fig. 3*c*) are synthesized at similar rates in the wild-type at the lowest light intensities. As the light intensity is increased sucrose synthesis is decreased relative to starch synthesis, reaching a minimum at light intensities that support about two-thirds of the maximal rate of photosynthesis (Fig. 3*d*). Then, as the light intensity becomes saturating, sucrose synthesis is increased relative to starch synthesis. The individual rates of starch synthesis (Fig. 3*b*) and sucrose synthesis (Fig. 3*c*) show that starch synthesis saturates at a lower light intensity than does sucrose synthesis. Mutants with lowered plastid or cytosolic PGI activity show a different response. The 50% plastid mutant homozygote resembles the wild-type at low light intensity, but starch synthesis saturates at an even lower light intensity. This genotype therefore differs most from the wild-type at high light intensity. The mutant with 18% cytosol PGI activity has enhanced starch synthesis and decreased sucrose synthesis at low light intensity. As the light intensity is increased, the proportional difference from the wild-type becomes smaller.

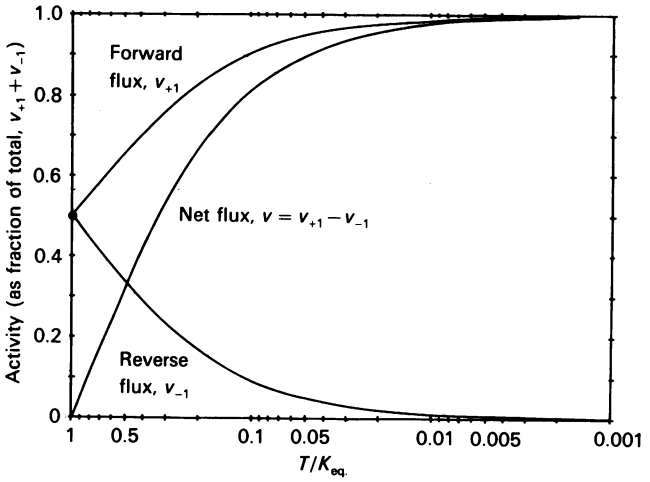

Fig. 4. Relation between the disequilibrium ratio, the rates of the forward reaction and the reverse reaction, and the net flux

The Figure is estimated from the expression $v_{-1}/v_{+1} = \Gamma/K_{eq.}$, after normalizing so that $v_{+1}+v_{-1} = 1$.

DISCUSSION

Application of control analysis

This is a first attempt to apply control analysis experimentally to metabolic control in plants, and although general conclusions can be drawn we are also limited by certain features of the data. First, application of the equations developed by Kacser and co-workers depends, strictly speaking, on experiments in which small perturbations are used. However, the variability in our data means that small changes cannot be reliably quantified. Secondly, the application of this approach requires a series of mutants characterized by small changes in the activity of one enzyme and an identical complement of all the other enzymes, and this ideal is not easy to realize. If material is taken directly from a mutagenesis programme, there will be many other mutagenized sites in the genotype. These can be removed by repeated back-crossing to the wild-type, but this procedure introduces a risk that there may be drift, or even compensatory changes in other enzymes. The mutants studied here were back-crossed five times into a highly homozygous line (Gottlieb, 1984), but we cannot exclude the possibility that some compensation or drift may have occurred. For example, we cannot explain why the heterozygote (1^s1^*) has enhanced starch synthesis in low light intensity compared with the wild-type.

PGI activity, fluxes and disequilibrium

The results demonstrate that a simple concept of 'excess' enzyme activity is inadequate. At high flux rates removal of 36% of the cytosolic PGI already had a measurable influence on the local parameters of the PGI reaction, as revealed by a decreased [Glc6*P*]/[Fru6*P*] ratio. The mass-action ratio decreased even further as cytosolic PGI activity was lowered to 36% and 18% of the wild-type complement. Similarly, removal of 25% of the plastid PGI activity resulted in a decrease in the [Glc6*P*]/[Fru6*P*] ratio. Thus PGI does not seem to be in 'excess' of that required to maintain its reactants close to equilibrium, at least in conditions of rapid flux. In weak light the flux is lower, and more PGI could be removed before there was an appreciable change of the mass-action ratio.

We can arrive at a similar conclusion by comparing the PGI activity with the flux that it supports *in vivo* in each of these genotypes. By using a rate expression based on the Michaelis–Menten kinetics for a hyperbolic reaction and the Haldane relationships, it can be shown that $v_{-1}/v_{+1} = \Gamma/K_{eq.}$ where Γ is the mass-action ratio, $K_{eq.}$ is the equilibrium constant, v_{+1} is the rate of the forward reaction and v_{-1} is the rate of the reverse reaction (Rolleston, 1972). In Fig. 4 this relation has been normalized, by setting the total activity ($v = v_{+1}+v_{-1}$) as unity, to provide a direct plot of v_{+1} and v_{-1} against the disequilibrium ratio ($\Gamma/K_{eq.}$). The disequilibrium ratio can now be directly related to $(v_{+1}-v_{-1})/(v_{+1}+v_{-1})$, the net activity as a fraction of the total activity.

Table 4 summarizes our measurements of the total cytosolic PGI activity and the fluxes required during sucrose synthesis in saturating light intensity in each genotype, taking account of the fact that only half of the hexoses entering sucrose have to be converted through to

Table 4. Comparison of PGI activity, fluxes and the estimated and measured disequilibrium ratios in saturating light intensity and CO_2

Total cytosolic PGI activity was measured in extracts with saturating Fru6*P* concentration in the direction of Glc6*P* formation (Table 1). The net activity needed to catalyse sucrose synthesis *in vivo* is estimated from Table 2, on the basis that one Glc6*P* molecule is needed per molecule of sucrose. The net activity is then expressed as a fraction of the total activity, and is compared with Fig. 4 to estimate the disequilibrium ratio, which is needed to generate the required flux in each genotype. The measured disequilibrium ratio is shown for comparison, estimated from the measured mass-action ratio (Table 2) assuming a $K_{eq.}$ of 3.3 (Dyson & Noltmann, 1968).

Compartment	PGI activity (as % of wild-type)	PGI activity (μmol/h per mg of chlorophyll)	Net activity required *in vivo* (μmol/h per mg of chlorophyll)	Net activity (as fraction of total)	$\Gamma/K_{eq.}$ Predicted	$\Gamma/K_{eq.}$ Measured (overall)
Cytosol	100	142	12.9	0.09	0.82	0.80
	64	97	12.9	0.13	0.76	0.74
	36	45	12.0	0.27	0.60	0.67
	18	28	11.8	0.42	0.42	0.52
Chloroplast	100	71	23.0	0.33	0.47	–
	75	54	21.2	0.39	0.60	–
	50	35	11.5	0.33	0.45	–

Glc6*P* (see Fig. 1). These values are used to estimate the value *in vivo* for $(v_{+1}-v_{-1})/(v_{+1}+v_{-1})$ for each genotype. The relation shown in Fig. 4 can then be used to estimate the expected disequilibrium ratio *in vivo*. This comparison predicts that the cytosolic PGI will be slightly removed from equilibrium even in the wild-type, that removal of even 36% of the activity should lead to a measurable shift away from equilibrium, and that this will become larger if 64% or 82% of the cytosolic PGI activity is removed. Table 4 also summarizes the disequilibrium ratio in each genotype, which we estimate from the measured [Glc6*P*]/[Fru6*P*] ratio (Table 1) assuming an equilibrum constant of 3.3 (Dyson & Noltmann, 1968). There is a reasonable agreement between the predicted and the measured ratios. Although the ratio in leaves does not change as far as predicted, the measured values are likely to be slight underestimates, because the change in the cytosol will be partly masked by the 20–30% of the hexose phosphates that we can expect in the chloroplast (see above).

An analogous estimate can be made for the plastid PGI. In this case every molecule of hexose entering starch has to be converted into Glc6*P*. Since the rates of sucrose and starch synthesis are similar in the wild-type, this implies that the flux over the plastid PGI will be almost twice as high as that over the cytosolic PGI. The plastid PGI, however, is only 31% of the total PGI in *Clarkia xantiana* leaves (Fig. 1). A similar distribution is found in leaves of other plants (see Jones *et al.*, 1986*a*,*b*). Apparently, the next flux to starch in saturating light intensity and CO_2 is already equivalent to 30–40% of the available chloroplast PGI activity, suggesting there will be considerable disequilibrium at this reaction *in vivo*. In fact, two independent studies have already reported that the [Glc6*P*]/[Fru6*P*] ratio in non-aqueously isolated chloroplasts from spinach leaves is near unity, which is well below the expected ratio of about 3 (Dietz, 1985; Gerhardt *et al.*, 1987). The predicted disequilibrium does not increase when the plastid PGI complement is decreased. This could be due to problems in applying these equations once a reaction is far enough removed from equilibrium, and effectors other than the substrate and product become important in determining the net flux. Alternatively, it could reflect subtle changes in the properties of this decreased-activity mutant that complicate application of these kinetic equations.

PGI and the control of flux

Although lower activities of PGI lead to disequilibrium, they do not necessarily lead to the enzyme becoming 'rate-limiting'. For example, the decreased [Glc6*P*]/[Fru6*P*] ratio in high light intensity is not accompanied by a decrease of sucrose synthesis in mutants with 64% cytosolic PGI activity, and there is only a small effect when the PGI activity is lowered to 36% or 18%. The effect on flux is also not simply correlated with increasing disequilibrium. For example, the 18% cytosol mutant showed a larger restriction of sucrose synthesis in low light intensity, but the [Glc6*P*]/[Fru6*P*] ratio decreased more in high light intensity. Clearly, the influence of PGI on flux depends on the interaction between this enzyme and further enzymes in these pathways. This qualitative conclusion can be expressed more precisely by applying the concepts of Kacser and co-workers to quantify (*a*) the contribution that PGI is making to control of flux and (*b*) the influence of shared metabolites on the activity of PGI and of the other enzymes in these pathways.

The contribution of an enzyme to control can be assessed by calculating the Flux Control Coefficient (Kacser & Porteous, 1987). This is done by measuring the fractional change in pathway flux (dJ/J) that results from a fractional change in the enzyme activity (dE/E). When these are plotted, the Flux Control Coefficient is given by the slope $[(dJ/J)/dE/E)]$. The impact of decreasing cytosolic and stromal PGI activity on the fluxes to starch and sucrose is shown in Fig. 5. We have also estimated the Flux Control Coefficients by using an approach summarized in the Methods section, which relates sequential pairs of points (Table 5). The statistical quality of the data (see above) means that small changes of flux cannot be accurately measured and leads to some

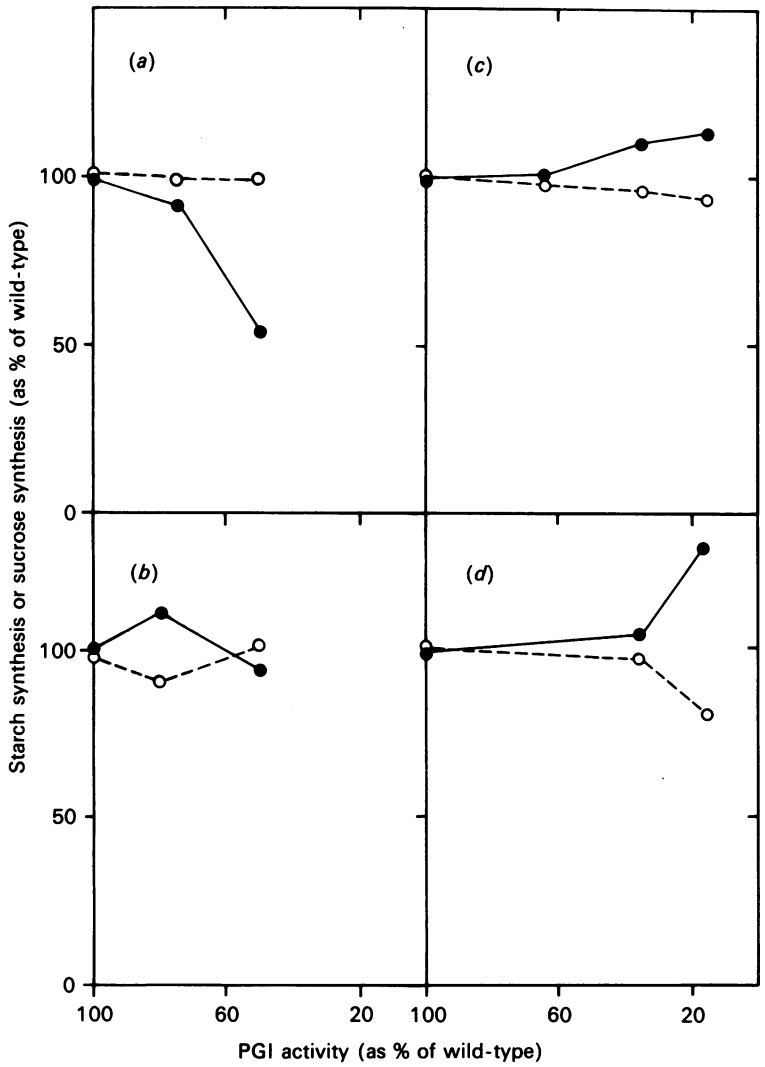

Fig. 5. Enzyme–flux relation for PGI and carbohydrate synthesis

(*a*) Plot of plastid PGI activity versus fluxes at saturating light intensity (1000 μmol·m^{-2}·s^{-1}); (*b*) plot of plastid PGI activity versus fluxes at limiting light intensity (125 μmol·m^{-2}·s^{-1}); (*c*) plot of cytosol PGI activity versus fluxes at saturating light intensity; (*d*) plot of cytosolic PGI activity versus fluxes at limiting light intensity. The results are estimated from Table 3.

spurious Control Coefficients and 'inversion' of signs, for example the alternating positive and negative Control Coefficients of the cytosolic PGI for photosynthesis. Anomalous estimates also appear for the plastid PGI at low light intensity. Nevertheless several general conclusions can be reached where there is a consistent trend, or where the changes of flux are large compared with the statistical variation.

First, the cytosolic PGI has a very low Flux Control Coefficient in the wild-type. However, it does acquire a role as its activity is lowered below the wild-type complement, with a Control Coefficient of about 0.22 for sucrose synthesis in limiting light intensity. Secondly, the plastid PGI has an estimated Control Coefficient of about 0.34 in the wild-type in high light intensity. This result contradicts the widespread assumption that enzymes such as PGI that catalyse near-equilibrium reactions can never exert control. The Flux Control Coefficient rises even further when plastid PGI activity is lowered to 50%. Thirdly, the control strength varies, depending on the conditions. For example, the Control Coefficient of PGI in the mutants with lowered cytosolic PGI complement is 0.22 in limiting light intensity, compared with about 0.05 in saturating light intensity. Fourthly, the Control Coefficients of the cytosolic and plastid PGIs show opposing responses to rising rates of photosynthesis. Plastid PGI has a higher Control Coefficient when photosynthesis is rapid, whereas cytosolic PGI has a higher Control Coefficient at low rates of photosynthesis. Clearly, the Flux Control Coefficient depends on the conditions and on the intracellular milieu rather than on any inherent property of the reaction or the enzyme.

As discussed by Kacser (1983), complex interactions between Control Coefficients can develop in a branched pathway. The cytosolic PGI has a negative Control Coefficient for starch synthesis (i.e. lowered cytosolic PGI activity stimulates starch synthesis) of -0.21 in mutants with decreased cytosolic PGI activity. Interestingly, the plastid PGI only exerted marginal control over sucrose synthesis. This asymmetric relation between the chloroplast and cytosol is reflected in the

Table 5. Estimation of Flux Control Coefficients for the plastid and cytosolic PGI

The Control Coefficients were estimated from pairs of adjacent mutants with PGI activities E_1 and E_2, by using the expression $C^J_{E_1} = (e-ep)/(p-ep)$ where $e = E_2/E_1$ and $p = J_2/J_1$ (see the Methods section). C_{E_c} refers to Flux Control Coefficients for the cytosolic PGI, and C_{E_p} refers to Flux Control Coefficients for the plastid PGI.

Compartment	PGI activity (as % of wild-type)		High light intensity			Low light intensity		
	E_1	E_2	$C^{photosynthesis}_{E_c}$	$C^{sucrose}_{E_c}$	$C^{starch}_{E_c}$	$C^{photosynthesis}_{E_c}$	$C^{sucrose}_{E_c}$	$C^{starch}_{E_c}$
Cytosol	100	64	−0.08	+0.02	−0.03			
	64	36	+0.09	+0.07	−0.03	+0.03	+0.03	−0.01
	36	18	−0.02	+0.04	−0.04	−0.05	+0.22	−0.21
	PGI activity (as % of wild-type)							
	E_1	E_2	$C^{photosynthesis}_{E_p}$	$C^{sucrose}_{E_p}$	$C^{starch}_{E_p}$	$C^{photosynthesis}_{E_p}$	$C^{sucrose}_{E_p}$	$C^{starch}_{E_p}$
Plastid	100	75	+0.14	+0.04	+0.35	+0.16	+0.66	−0.49
	75	50	+0.45	−0.14	+1.17	−0.14	−0.40	−0.27

Control Coefficients of PGI for photosynthetic CO_2 fixation, the pathway preceding the branch point. Although the cytosolic mutant only exerts marginal control, the plastid mutant has a Control Coefficient of 0.14 for photosynthesis in saturating light intensity and CO_2. The presence of a branch point may also explain the Control Coefficient of over 1.0 for starch synthesis in the mutants with decreased plastid PGI activity; the Flux Control Coefficients in a pathway sum to unity (the Summation Theorem; Kacser & Burns, 1973), but Flux Control Coefficients exceeding unity can occur in branched pathways because some of the enzymes in the other branch may have negative Control Coefficients (Kacser & Burns, 1979).

Estimation of Elasticity Coefficients

The effect of metabolites on the activity of an enzyme *in vivo* can be quantified as the Elasticity Coefficient, ϵ, the fractional change in enzyme activity (dv/v) that results from a fractional change in the concentration of a given effector (ds/s) (Kacser & Burns, 1973; Kacser & Porteous, 1987). As these authors have shown, there is an important relation between the Elasticity Coefficients and the Flux Control Coefficient. Broadly, an enzyme with a low Flux Control Coefficient tends to have a high Elasticity Coefficient. More precisely, the ratio of the Flux Control Coefficients of two enzymes is inversely related to the ratio of their Elasticity Coefficients for a shared metabolite (Connectivity Theorem).

Table 6 summarizes estimates for the Elasticity Coefficients of the cytosolic PGI estimated by using the equations developed by Groen *et al.* (1982). These equations only apply to enzymes with Michaelis–Menten kinetics, but this is a reasonable assumption for PGI. This approach allows us to use the [Glc6*P*]/[Fru6*P*] ratios rather than the individual metabolite concentrations, which is useful because the ratios exhibited less statistical variation than did the individual concentrations of Fru6*P* and Glc6*P* (see Table 3). Our data only give the overall changes of metabolite concentrations. This means that the fractional changes of metabolite concentrations will slightly underestimate the probable change in the cytosol (see above), in which case the estimated Elasticity Coefficient will be slightly too high. We did not attempt to estimate Elasticity Coefficients for the plastid PGI mutants because the errors in this case would be larger (see the Results section).

The estimated Elasticity Coefficients for Fru6*P* and Glc6*P* in the wild-type are high, lying between 4.6 and 7.6. They are negative for Glc6*P* because the estimates are for net activity in the direction of Glc6*P* formation, and this will be lowered by rising Glc6*P* concentrations. These values are at least one order of magnitude greater than the Elasticity Coefficients that would be obtained for a hyperbolic enzyme operating at partial substrate saturation in the undirectional mode. These high elasticities *in vivo* are generated because the net flux, v, equals $v_{+1}-v_{-1}$ and, for a near-equilibrium reaction, v_{+1} and v_{-1} are both far larger than v (Fig. 5). Assuming that the substrates are not saturating, v_{+1} will depend on [Fru6*P*] and v_{-1} will depend on [Glc6*P*]. This means that a relatively small change in the concentration of Fru6*P* or Glc6*P* can generate a large change in the net flux v. In other words, these high elasticities are not a property of PGI itself; rather, they emerge when it operates in a pathway (Kacser & Burns, 1979; Kacser, 1983).

The Elasticity Coefficients became progressively smaller in mutants with a lower PGI complement. According to the Connectivity Theorem, PGI could start to exert control as ϵ^{PGI}_{Fru6P} or ϵ^{PGI}_{Glc6P} start to fall into the range of the Elasticity Coefficients of other enzymes that share these metabolites, provided, of course, that these enzymes themselves have significant Flux Control Coefficients. Measurable Flux Control Coefficients for PGI appear as its Elasticity Coefficients fall below about 2 (Table 6). However, there is no simple correlation between the Elasticity Coefficients and the Flux Control Coefficient. For example, the Elasticity Coefficients tend to be lower in saturating light intensity (Table 6), whereas Control Coefficients are larger in limiting light intensity (compare Figs. 5*c* and 5*d*). These results imply (*a*) that other controlling enzymes are likely to have elasticities

Table 6. Estimated Elasticity Coefficients for the cytosolic PGI *in vivo*

The calculations are based on the Fru6*P* and Glc6*P* measurements in Table 2, the fluxes in Table 3 and the estimates of cytosolic PGI activity in Table 1, and use the equations:

$$\epsilon^{PGI}_{Fru6P} = (1-\Gamma/K_{eq.})^{-1}\cdot(1\text{-}J/V_{max.})$$

and

$$\epsilon^{PGI}_{Glc6P} = (\Gamma/K_{eq.})\cdot(1-\Gamma/K_{eq.})^{-1}\cdot(1+J/V_{max.})$$

PGI activity (as % of wild-type)	ϵ^{PGI} For Fru6*P*		ϵ^{PGI} For Glc6*P*	
	High light intensity	Low light intensity	High light intensity	Low light intensity
100	4.6	7.6	−4.6	−6.9
64	3.2	8.4	−3.3	−8.4
36	2.3	5.2	−2.5	−4.9
18	1.2	2.6	−1.5	−2.1

for Fru6*P* and/or Glc6*P* that are around unity and (*b*) that the Elasticity Coefficients and/or the Flux Control Coefficients of these other enzymes have different values in limiting and saturating light intensities. These predictions are examined in more detail in a further paper (Neuhaus *et al.*, 1989).

Implications for evolution

Finally, these results may have implications for evolution. It is clear that a heterozygote between the wild-type and a cytosolic PGI null mutant would phenotypically resemble the wild-type when the pathway flux is analysed as the inherited trait. This follows from the fact that control is shared in a pathway and the decrease in the activity of an enzyme to half of its normal complement often has little influence on the pathway flux (Kacser & Burns, 1981). It might be noted that this is not just because PGI is a 'non-regulatory' enzyme. A decrease in the pyruvate kinase activity in erythrocytes to half of the normal complement also had little influence on the rate of glycolysis (Holzhütter *et al.*, 1985). Similar conclusions were also reached from studies of alcohol dehydrogenase in *Drosophila* (Middleton & Kacser, 1983). These are both enzymes that are generally viewed as being regulated via metabolic or genetic control.

However, although halving PGI activity does not affect fluxes, it does affect the concentrations of its immediate substrates. If these were taken as phenotypic traits, a null mutant of PGI would clearly not be a simple recessive. It is intriguing that, in this sense, there is no large 'excess' of PGI. Indeed, it is difficult to envisage how selection could operate to maintain an enzyme at an activity greatly exceeding that needed to maintain a reaction close to thermodynamic equilibrium. Rather, mutations affecting the efficiency of catalysis or expression would be expected to accumulate and diminish PGI activity. It was previously shown that, following duplication of the gene encoding cytosolic PGI in *Clarkia xantiana*, PGI activity has declined back to a value resembling that in related species where no gene duplication has occurred (Gottlieb & Higgins, 1984). We have now shown that this activity is indeed not greatly above that needed to maintain Fru6*P* and Glc6*P* close to equilibrium during rapid sucrose synthesis. Maybe, selection then starts to operate again, maintaining adequate activities of enzymes such as PGI to keep their reactants close to equilibrium. Why this occurs is not immediately obvious, because natural selection operates on the net phenotype and therefore only indirectly on any one enzyme. However, it is tempting to speculate it may be 'advantageous' to concentrate control at other enzymes with more complex regulatory patterns, which can respond in a more flexible and varied manner to changing physiological or environmental conditions.

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