

# HONEYBEE FLIGHT MUSCLE PHOSPHOGLUCOSE ISOMERASE: MATCHING ENZYME CAPACITIES TO FLUX REQUIREMENTS AT A NEAR-EQUILIBRIUM REACTION

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## Summary

In honeybee flight muscle, there are close matches between physiological flux rates and the maximal activities ( $V_{\max}$ ; determined using crude homogenates) of key enzymes catalyzing non-equilibrium reactions in carbohydrate oxidation. In contrast, phosphoglucose isomerase (PGI), which catalyzes a reaction believed to be close to equilibrium, occurs at  $V_{\max}$  values greatly in excess of glycolytic flux rates. In this study, we measure the  $V_{\max}$  of flight muscle PGI, the kinetic parameters of the purified enzyme, the apparent equilibrium constants for the reaction and the tissue concentrations of substrate and product. Using the Haldane equation, we estimate that the forward flux capacity ( $V_f$ ) for PGI required to achieve physiological glycolytic flux rates is between 800 and

1070 units  $\text{ml}^{-1}$  cell water, approximately 45–60% of the empirically measured  $V_{\max}$  of 1770 units  $\text{ml}^{-1}$  cell water at optimal pH (8.0) and low ionic strength (no added KCl). When measured at physiological pH (7.0) and ionic strength (120  $\text{mmol l}^{-1}$  KCl) with saturating levels of substrate, PGI activity is 1130 units  $\text{ml}^{-1}$  cell water, a value close to the calculated  $V_f$ . These results reveal a very close match between predicted and measured PGI flux capacities, and support the concept of an economical design of muscle metabolism in systems working at very high metabolic rates.

Key words: honeybee, insect, *Apis mellifera*, muscle metabolism, phosphoglucose isomerase, near-equilibrium.

## Introduction

Over the last 15 years there has been considerable interest regarding the way in which natural selection may influence the design of biological systems. One theory suggests that selection would favour economical designs, minimizing excess structures that are expensive in terms of materials, energy and space. Some researchers have investigated this concept by measuring how closely maximal flux capacities match maximal physiological flux rates in the mammalian respiratory and digestive systems (Weibel *et al.* 1981; Taylor *et al.* 1987; Hammond and Diamond, 1992, 1994).

We have applied this strategy to the design of the glycolytic pathway in muscles by examining the relationship between maximal glycolytic flux rates ( $v$ ) and maximal enzyme activities ( $V_{\max}$ ; determined using crude homogenates) in various tissues of different animals. We find that the fractional velocities ( $v/V_{\max}$ ) of key glycolytic enzymes vary directly with the glycolytic flux rate, such that the fractional velocity of hexokinase (HK) is less than 1% in low-flux systems, such as trout cardiac muscle, while in honeybee flight muscle working at very high glycolytic rates, it may be as high as 98% (Suarez *et al.* 1996). This relatively close match between

pathway flux rates and HK capacity in honeybee flight muscle appears to contrast with the  $V_{\max}$  of phosphoglucose isomerase (PGI), which is 23-fold higher than the glycolytic flux rate (Suarez *et al.* 1996).

During flight, insects sustain some of the highest mass-specific oxygen consumption rates ( $\dot{V}_{\text{O}_2}$ ) of any animals (Sacktor, 1976). Honeybee  $\dot{V}_{\text{O}_2}$  increases approximately 100-fold in the transition from rest to flight (Rothe and Nachtigall, 1989), and a discrete mass of thoracic flight muscle consisting of a single fibre type is estimated to account for more than 90% of this metabolic rate. The high ATP turnover rate is fuelled by carbohydrate oxidation (Rothe and Nachtigall, 1989) and, as insect flight muscle has no gluconeogenic capacity and very low activities of pentose phosphate shunt enzymes (Newsholme *et al.* 1972; Clark *et al.* 1973), the function of glycolytic enzymes is almost exclusively to supply pyruvate for mitochondrial oxidation. This system provides an excellent model for quantifying the flux through the glycolytic pathway and estimating how closely glycolytic enzyme activities are matched to this flux.

Phosphoglucose isomerase (EC 5.3.1.9) catalyzes the

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Fig. 1. Initial steps of the glycolytic pathway. Phosphoglucose isomerase (PGI) catalyses the reversible aldose to ketose isomerization of glucose 6-phosphate (G6P) to fructose 6-phosphate (F6P). HK, hexokinase; PFK, phosphofructokinase; F1,6BP, fructose 1,6-bisphosphate.

reversible aldose to ketose isomerization of glucose 6-phosphate to fructose 6-phosphate (Fig. 1), a reaction believed to be close to equilibrium *in vivo* (Kashiwaya *et al.* 1994). Enzymes catalyzing near-equilibrium reactions have been considered unimportant in terms of metabolic regulation (Brooks, 1996). However, it has long been recognized that  $V_{\max}$  at near-equilibrium steps must be much greater than net flux through pathways (Haldane, 1930; Veech *et al.* 1969) and that the closer a reaction is to equilibrium, the greater is the forward flux capacity ( $V_f$ ) required to maintain a given pathway flux rate (Suarez, 1994, 1996; Brooks, 1996).

In this study, we test the hypothesis that, like honeybee flight muscle HK and phosphofructokinase (PFK), the  $V_{\max}$  of PGI is not greatly in excess of the flux capacity required to support physiological glycolytic flux rates. To investigate this, we measure the kinetic parameters of purified PGI, the apparent equilibrium constant ( $K_{\text{eq}}$ ) of the reaction and the tissue concentrations of its substrate and product. Using this information, along with estimates of glycolytic flux rates derived from respirometry of flying honeybees (Suarez *et al.* 1996), we estimate  $V_f$  using the Haldane equation (Haldane, 1930) and compare it with maximal PGI activities.

## Materials and methods

### Animals

Several thousand swarming honeybees (*Apis mellifera* L.) were collected from a eucalyptus tree in Santa Barbara, CA, USA, and stored in a styrofoam box lined with plastic at  $-75^\circ\text{C}$  until used for enzyme purification. For metabolite measurements, live unladen workers were caught, using plastic bags perforated with air holes, as they left the hive.

### Enzyme purification

Frozen whole honeybees were placed in a ceramic mortar cooled on dry ice. Gentle grinding with a chilled pestle separated the abdomens and heads from the thoraces, which were then cleaned of adhering wings and legs. This procedure allowed for the rapid isolation of large quantities of thoraces.

The PGI purification was based on the affinity-substrate elution method of Phillips *et al.* (1976). Approximately 40 g of thoraces were pooled in 4 vols of ice-cold buffer consisting of  $75\text{ mmol l}^{-1}$  Tris (pH 8.7 at  $5^\circ\text{C}$ ) with  $2\text{ mmol l}^{-1}$  EDTA and 0.1%  $\beta$ -mercaptoethanol ( $\beta$ -ME). The thoraces were homogenized on ice with three 1 min bursts of a Polytron

homogenizer at 85% maximum speed, with 2 min between bursts. The homogenate was then stirred at  $0^\circ\text{C}$  for 30 min, and centrifuged at  $10\,000\text{ g}$  for 30 min. The supernatant was passed through eight layers of cheesecloth, slowly brought to 40% ammonium sulphate saturation and stirred for 1 h at  $4^\circ\text{C}$ . The resulting suspension was centrifuged at  $10\,000\text{ g}$  for 20 min, and the supernatant was slowly brought to 70% ammonium sulphate saturation, stirred for 1 h and centrifuged at  $10\,000\text{ g}$  for 20 min. The supernatant was discarded and the pellet was resuspended in  $20\text{ mmol l}^{-1}$  imidazole (pH 6.6 at  $5^\circ\text{C}$ ) with  $1\text{ mmol l}^{-1}$  EDTA and 0.1%  $\beta$ -ME (buffer A) to 15% of the original homogenate volume. This fraction was dialyzed (15 000 MWCO dialysis tubing; MFP Inc., San Antonio, TX, USA) overnight against two changes of 100 vols of buffer A at  $4^\circ\text{C}$ .

Cellulose phosphate (CP; Sigma) was prepared as in Phillips *et al.* (1976) and equilibrated with buffer A. The CP was poured into a large Buchner funnel through Whatman no. 4 filter paper, rinsed with 1 l of buffer A under mild suction, and dried to dampness. The CP was then stirred with the dialyzed homogenate fraction for 30 min. It was then poured back into the Buchner funnel and rinsed with buffer A. The optical density at 280 nm ( $\text{OD}_{280}$ ) of the effluent was measured as an indicator of protein concentration, and when no further protein was eluted, the CP was again dried to dampness and resuspended in  $20\text{ mmol l}^{-1}$  imidazole (pH 7.2 at  $5^\circ\text{C}$ ) with  $1\text{ mmol l}^{-1}$  EDTA and 0.1%  $\beta$ -ME (buffer B). The CP was again rinsed until no further protein was eluted, resuspended in buffer B and loaded into a glass column (i.d. 2.7 cm, 20 cm long) equipped with a flow regulator. Buffer B with  $3\text{ mmol l}^{-1}$  fructose 6-phosphate (F6P) was pumped through the column at  $50\text{ ml h}^{-1}$ , and 10 ml fractions were collected for analysis of  $\text{OD}_{280}$  and PGI activity. The PGI eluted in a sharp peak after approximately 100 ml of buffer had passed through the column. Fractions containing high PGI activity were pooled and dialyzed overnight against two changes of 100 vols of 70% ammonium sulphate,  $25\text{ mmol l}^{-1}$  Tris (pH 8.0 at  $37^\circ\text{C}$ ),  $1\text{ mmol l}^{-1}$  EDTA and 0.1%  $\beta$ -ME. This precipitated the protein out of solution and it was stored in this buffer at  $4^\circ\text{C}$ . No detectable loss of enzyme activity was noted in preparations stored in this way for at least 2 months. Preparations were dialyzed overnight against 1000 vols of assay buffer with  $1\text{ mmol l}^{-1}$  EDTA and 0.1%  $\beta$ -ME before use in kinetic,  $K_{\text{eq}}$  or pH optimum determinations.

The homogeneity of the purified PGI was confirmed, and the apparent subunit molecular mass was determined using SDS-PAGE.

### Enzyme assays

At an ambient temperature of  $22^\circ\text{C}$ , the thoracic temperature of unrestrained flying honeybee workers is approximately  $37^\circ\text{C}$  (Heinrich, 1980). Therefore, all enzyme assays were performed at  $37^\circ\text{C}$  using an SLM-Aminco DW-2000 spectrophotometer (Rochester, NY) interfaced with an IBM-compatible computer. Substrates and cofactors were dissolved in assay buffer and adjusted to the experimental pH.

All coupling enzymes were dialyzed overnight against 1000 vols of assay buffer (see below) with  $1 \text{ mmol l}^{-1}$  EDTA and 0.1 %  $\beta$ -ME.

The  $V_{\text{max}}$  of PGI was determined in the reverse (F6P→G6P) direction using crude homogenates, as in Suarez *et al.* (1996), with the following exception: approximately 0.5 g of frozen thoraces was pooled (15–20 thoraces) and homogenized in 4 vols of buffer. The  $V_{\text{max}}$  values of PGI in the forward and reverse directions are known to be very similar (Hall, 1985; Zera, 1987). In addition, PGI activity at saturating F6P concentration was determined at pH 7.0 with  $120 \text{ mmol l}^{-1}$  KCl to simulate intracellular pH and ionic composition (Piek *et al.* 1977).

For assays used to determine the kinetic properties of the purified enzyme in the forward (glycolytic) direction,  $0.15 \text{ mmol l}^{-1}$  NADH (Sigma),  $1 \text{ mmol l}^{-1}$  ATP (Sigma), excess PFK, aldolase, triosephosphate isomerase and  $\alpha$ -glycerophosphate dehydrogenase (Sigma) were used. Glucose 6-phosphate (G6P; Sigma) concentrations ranged between 0.2 and  $8.0 \text{ mmol l}^{-1}$ . Assay buffers contained either  $25 \text{ mmol l}^{-1}$  Tris (pH 8.0 at  $37^\circ\text{C}$ ) with  $50 \text{ mmol l}^{-1}$  KCl and  $5 \text{ mmol l}^{-1}$   $\text{MgCl}_2$ , or  $25 \text{ mmol l}^{-1}$  imidazole (pH 7.0 at  $37^\circ\text{C}$ ) with  $120 \text{ mmol l}^{-1}$  KCl and  $5 \text{ mmol l}^{-1}$   $\text{MgCl}_2$ .

For kinetic determinations of purified PGI in the reverse direction,  $1 \text{ mmol l}^{-1}$  NADP<sup>+</sup> (Sigma) and excess glucose-6-phosphate dehydrogenase (G6PDH; Boehringer Mannheim) were used with F6P (Boehringer Mannheim) concentrations ranging from 0.004 to  $2.0 \text{ mmol l}^{-1}$ . Buffers consisted of either  $25 \text{ mmol l}^{-1}$  Tris (pH 8.0 at  $37^\circ\text{C}$ ) or  $25 \text{ mmol l}^{-1}$  imidazole (pH 7.0 at  $37^\circ\text{C}$ ) with  $120 \text{ mmol l}^{-1}$  KCl. All F6P solutions were checked for potential G6P contamination by incubation with NADP<sup>+</sup> and G6PDH.

Assays used to determine pH optima were performed in the reverse direction at  $37^\circ\text{C}$  using the purified enzyme and saturating concentrations of F6P. Buffers were initially at pH 8.0 ( $25 \text{ mmol l}^{-1}$  Tris) or 7.0 ( $25 \text{ mmol l}^{-1}$  imidazole), and pH was altered by additions of  $10 \mu\text{l}$  increments of  $0.1 \text{ mol l}^{-1}$  HCl or  $0.1 \text{ mol l}^{-1}$  NaOH.

Reaction rates were calculated from linear regressions of the initial straight portions of NAD(P)H absorbance curves using Datacan V software (Sable Systems, Las Vegas, NV, USA). A unit of enzyme activity is defined as the amount necessary to catalyze the conversion of  $1 \mu\text{mol}$  of substrate to product (in either direction) per minute.

#### Metabolite measurements

For metabolite measurements in resting animals, honeybees were kept in small perforated plastic bags at  $37^\circ\text{C}$  for 10 min. Honeybees walked vigorously, but the small volume precluded flying, and the warm ambient temperature would have produced a thoracic temperature of approximately  $37^\circ\text{C}$  while minimizing thermoregulatory shivering by flight muscles (Cahill and Lustick, 1976). Honeybees were freeze-clamped directly through the plastic bag with aluminium tongs cooled in liquid nitrogen. The entire bag was then immersed in liquid nitrogen, and the honeybee was removed from the bag and

stored at  $-75^\circ\text{C}$  until metabolites were extracted. For measurements in flying honeybees, individuals were released from perforated holding bags close to a lighted window pane in an otherwise dark room at approximately  $22^\circ\text{C}$ . Honeybees immediately flew to the window pane and hovered against it. This allowed us to freeze-clamp them in mid-flight after at least 30 s of flight.

Thoraces were dissected from whole frozen honeybees in a mortar cooled with liquid nitrogen. Approximately 0.3 g (7–10 thoraces) was pooled, powdered under liquid nitrogen and homogenized on ice in 9 vols of 7% perchloric acid. These extracts were centrifuged at  $10\,000g$  for 20 min at  $4^\circ\text{C}$ , and supernatants were neutralized on ice with  $3 \text{ mol l}^{-1}$   $\text{K}_2\text{CO}_3$ . After centrifugation at  $10\,000g$  for 20 min at  $4^\circ\text{C}$ , neutralized supernatants were aspirated and stored at  $-75^\circ\text{C}$  until assayed.

G6P and F6P were assayed enzymatically as in Passonneau and Lowry (1993).

#### Equilibrium constants

Equilibrium was approached from either the forward or reverse direction using  $0.5 \text{ mmol l}^{-1}$  G6P or F6P respectively. Buffers contained  $25 \text{ mmol l}^{-1}$  Tris (pH 8.0 at  $37^\circ\text{C}$ ) and  $25 \text{ mmol l}^{-1}$  imidazole with  $120 \text{ mmol l}^{-1}$  KCl (pH 7.0 at  $37^\circ\text{C}$ ) with  $5 \text{ mmol l}^{-1}$   $\text{MgCl}_2$  added to buffers for measurements in the forward direction. Samples were taken at 15 min intervals for 1 h by pipetting  $900 \mu\text{l}$  of the reaction mixture into  $100 \mu\text{l}$  of 70% perchloric acid on ice. Samples were centrifuged, neutralized and assayed for G6P and F6P as described above. Apparent equilibrium constants ( $K_{\text{eq}}$ ) were calculated for the forward reaction direction (i.e.  $K_{\text{eq}} = [\text{F6P}]/[\text{G6P}]$ ).

#### Calculations and statistics

Glycolytic flux rates, metabolite measurements and enzyme activities are expressed relative to millilitres of cell water, assuming that flight muscle makes up 75% of thorax mass (Nachtigall *et al.* 1995) and that 63% of flight muscle mass is water (Piek *et al.* 1977). The free energy change ( $\Delta G$ ) of the forward reaction was calculated as in Lehninger *et al.* (1993).  $K_m$  values were determined using unweighted, least-squares non-linear regression of the Michaelis–Menten equation using SigmaStat 1.0 (Jandel Scientific, San Rafael, CA, USA) and Marquardt's algorithm (Brooks, 1992). Calculations and modelling of the Haldane equation were performed by computer using Mathcad 4.0 (Mathsoft, Cambridge, MA, USA).

## Results

In initial experiments (data not shown) using carboxymethyl Sepharose ion-exchange chromatography (Blackburn *et al.* 1972), PGI eluted in one sharp peak in a 9–24  $\text{mmol l}^{-1}$  sodium phosphate gradient. This suggests that in honeybee flight muscle the enzyme occurs in only one form. The purification scheme resulted in a homogeneous preparation with an apparent subunit molecular mass of  $66\,300 \pm 200$  (mean  $\pm$  S.E.M.,  $N=4$ ). The enzyme was purified 570-fold relative to the

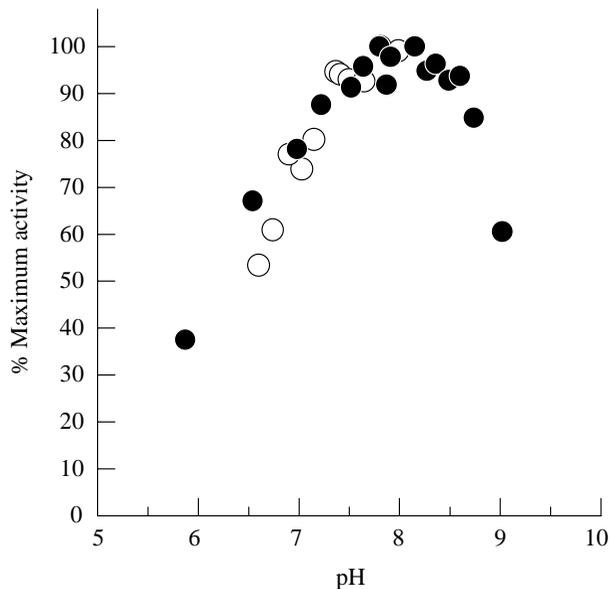


Fig. 2. Effect of pH on the activity of purified phosphoglucose isomerase. Enzyme activity from three separate preparations and pH were measured at 37 °C. The buffers used were 25 mmol l<sup>-1</sup> Tris (filled circles) and 25 mmol l<sup>-1</sup> imidazole (open circles).

crude homogenate with a yield of 44%. The pH optimum for the activity of purified flight muscle PGI is close to pH 8.0 at 37 °C (Fig. 2). The specific activity of the purified enzyme is 2540±179 units mg<sup>-1</sup> protein (mean ± S.E.M., N=4), yielding an apparent turnover number ( $k_{cat}$ ) of 168 000±11 000 min<sup>-1</sup> (mean ± S.E.M., N=4).

The tissue G6P and F6P contents (Table 1) both increase in the transition from rest to flight ( $P=0.004$  for G6P,  $P=0.046$  for F6P; unpaired  $t$ -test). Changes in G6P and F6P contents are proportionately similar, so that the mass-action ratios (MAR; [F6P]/[G6P]),  $\Delta G$  values and disequilibrium ratios (MAR/ $K_{eq}$ ) do not change significantly ( $P>0.05$ ; unpaired  $t$ -test after arcsin square-root transformation; Table 1).

The estimated Michaelis constants and the apparent  $K_{eq}$  values are listed in Table 2. The  $K_m$  of PGI for G6P is approximately 10 times higher than that for F6P. At pH 7.0

Table 1. Honeybee thorax metabolite concentrations

	Resting (N=6)	Flying (N=5)
G6P ( $\mu\text{mol ml}^{-1}$ cell water)	0.400±0.061	0.681±0.059
F6P ( $\mu\text{mol ml}^{-1}$ cell water)	0.091±0.013	0.144±0.019
Mass-action ratio	0.24±0.01	0.21±0.02
Disequilibrium ratio	0.73	0.64
$\Delta G$ (kJ mol <sup>-1</sup> )	-1.9	-2.3

Values for glucose 6-phosphate (G6P), fructose 6-phosphate (F6P) and mass-action ratio ([F6P]/[G6P]) are means ± S.E.M.

The disequilibrium ratio is defined as the mass-action ratio divided by the apparent  $K_{eq}$  of 0.33 (determined at pH 7.0, 120 mmol l<sup>-1</sup> KCl; see Table 2).

Table 2. Kinetic parameters and apparent  $K_{eq}$  estimated using purified honeybee flight muscle phosphoglucose isomerase at 37 °C

	pH 8.0, low ionic strength	pH 7.0, high ionic strength
$K_m$ forward (G6P)	400±9.1	1070±48.5
$K_m$ reverse (F6P)	36.5±3.8	117±4.6
$K_{eq}$	0.30±0.02	0.33±0.02

Values are means ± S.E.M., N=4.  
Units for  $K_m$  values are  $\mu\text{mol l}^{-1}$ .  
G6P, glucose 6-phosphate; F6P, fructose 6-phosphate.

with 120 mmol l<sup>-1</sup> KCl, the  $K_m$  values in either direction are approximately 2.5- to three times higher than those estimated at pH 8.0 and low ionic strength. Under the two assay conditions described, equilibrium was reached within 15 min from both directions, yielding apparent  $K_{eq}$  values that are not significantly different ( $P=0.225$ , paired  $t$ -test).  $V_{max}$  for PGI was 1770±15 units ml<sup>-1</sup> cell water (mean ± S.E.M., N=3). With saturating F6P concentration at pH 7.0 and 120 mmol l<sup>-1</sup> KCl, PGI activity was 1130±42 units ml<sup>-1</sup> cell water (mean ± S.E.M., N=3).

## Discussion

Steady-state concentrations of G6P and F6P in insect flight muscles are established within a few seconds of the initiation of flight and remain constant for several minutes (Sacktor and Wormser-Shavit, 1966; Rowan and Newsholme, 1979; Worm and Beenackers, 1980). Since flying honeybees were freeze-clamped after at least 30 s of free flight, it is reasonable to assume that a steady state had been achieved. Honeybee thorax G6P and F6P concentrations (Table 1) are slightly higher than those reported for bumblebee, locust and blowfly (Sacktor and Wormser-Shavit, 1966; Rowan and Newsholme, 1979; Worm and Beenackers, 1980; Leite *et al.* 1988). Despite differences in absolute concentrations, mass-action ratios in steady-state flight are similar to values obtained from other insect flight muscles (Rowan and Newsholme, 1979; Worm and Beenackers, 1980). The G6P inhibition constants of locust flight muscle HK are 87  $\mu\text{mol l}^{-1}$  (for glucose) and 12  $\mu\text{mol l}^{-1}$  (for ATP; Storey, 1980), so the relatively high G6P concentrations reported here may be expected to inhibit honeybee flight muscle HK significantly. As HK is estimated to work at approximately 98%  $V_{max}$  *in vivo* (Suarez *et al.* 1996), any inhibition by G6P is probably slight and may be overcome by the effects of inorganic phosphate, alanine and glycerol 3-phosphate (Storey, 1980). Detailed kinetic studies of honeybee flight muscle HK are currently in progress.

Kashiwaya *et al.* (1994) suggest that reactions with mass-action ratios greater than 0.5 can be considered to be close to equilibrium. In free-flying honeybees, we obtained a mass-action ratio of 0.64. In addition, calculated values of  $\Delta G$  (Table

1) are quite low. Taken together, these observations indicate that in honeybee flight muscle this reaction is close to equilibrium.

The purified enzyme has a pH optimum (Fig. 2) and an apparent subunit molecular mass similar to those reported for PGI from yeast (Kempe *et al.* 1974) and mammals (Dyson and Noltmann, 1968). The  $K_m$  and apparent  $K_{eq}$  values agree well with those reported in studies using mammals (Dyson and Noltmann, 1968), insects (Watt, 1977; Zera, 1987) and molluscs (Hall, 1985). The  $V_{max}$  of PGI is approximately 15% higher in this study than that reported in Suarez *et al.* (1996). Other than using honeybees from a different population, the only methodological difference between the two studies is that the previous study used individual honeybees, while in this study several thoraces were pooled for each measurement. This allowed for a relatively small dilution during homogenization which may have helped to preserve enzyme activity. The  $V_{max}$  of honeybee flight muscle PGI is approximately twofold higher than values obtained from bumblebee flight muscle (Clark *et al.* 1973; assuming a  $Q_{10}$  of 2).

The maximal activities of enzymes catalyzing reactions presumed to be held near equilibrium greatly exceed net pathway flux rates (Veech *et al.* 1969; Brooks, 1996). The net forward flux ( $J$ ) through PGI represents the difference between forward and reverse flux rates and can be calculated from the equilibrium binding kinetic model for reversible reactions (Haldane, 1930):

$$J = \frac{V_f \times \frac{[G6P]}{K_f} - V_r \times \frac{[F6P]}{K_r}}{1 + \frac{[G6P]}{K_f} + \frac{[F6P]}{K_r}}, \quad (1)$$

where [F6P] and [G6P] are steady-state concentrations of product and substrate,  $V_f$  and  $V_r$  are the maximal enzymatic capacities in the forward and reverse directions, respectively, and  $K_f$  and  $K_r$  are the Michaelis constants for G6P and F6P, respectively. According to the Haldane relationship, the kinetic parameters of the enzyme are related to the equilibrium constant as follows:

$$K_{eq} = \frac{[F6P]_{eq}}{[G6P]_{eq}} = \frac{V_f}{K_f} \times \frac{K_r}{V_r} \quad (2)$$

(Brooks, 1996), where  $[F6P]_{eq}$  and  $[G6P]_{eq}$  are the concentrations of fructose 6-phosphate and glucose 6-phosphate at equilibrium. Rearranging equation 2 to solve for  $V_r$  we obtain:

$$V_r = V_f \times \frac{K_r}{K_{eq} \times K_f} \quad (3)$$

Substituting the right-hand side of equation 3 into equation 1 and simplifying yields:

$$J = V_f \times \frac{K_r}{\{K_{eq} \times (K_f \times K_r + [G6P] \times K_r + [F6P] \times K_f)\}} \times \frac{K_{eq}}{\{K_r \times ([G6P] \times K_{eq} - [F6P])\}} \quad (4)$$

By rearranging and simplifying equation 4, it is possible to solve for  $V_f$ :

$$V_f = J \times (K_f \times K_r + [G6P] \times K_r + [F6P] \times K_f) \times \frac{K_{eq}}{\{K_r \times ([G6P] \times K_{eq} - [F6P])\}} \quad (5)$$

Equation 5 shows that the relationships between product and substrate concentrations *in vivo*, the kinetic properties of the enzyme, the thermodynamic equilibrium constant and the net rate of flux can be used to predict  $V_f$ , the enzymatic activity in the forward direction required *in vivo*. Also, by manipulating [G6P] and [F6P] in equation 5 to yield a range of disequilibrium ratios, the relationship of  $V_f$  to the disequilibrium ratio can be modelled (Fig. 3).

To our knowledge, no data exist regarding intracellular pH (pHi) or ionic composition of honeybee flight muscle. However, pHi (temperature not indicated),  $[K^+]$  and  $[Cl^-]$  of butterfly (*Pieris brassicae*) flight muscle have been measured as pH 7.0, 121 mmol $l^{-1}$   $K^+$  and 78 mmol $l^{-1}$   $Cl^-$  (Piek *et al.* 1977), and pH does not change during steady-state flight in locusts (Wegener *et al.* 1991). We therefore assume that pH 7.0 (at 37 °C) and 120 mmol $l^{-1}$  KCl adequately simulate intracellular conditions of pH and ionic strength in this tissue.

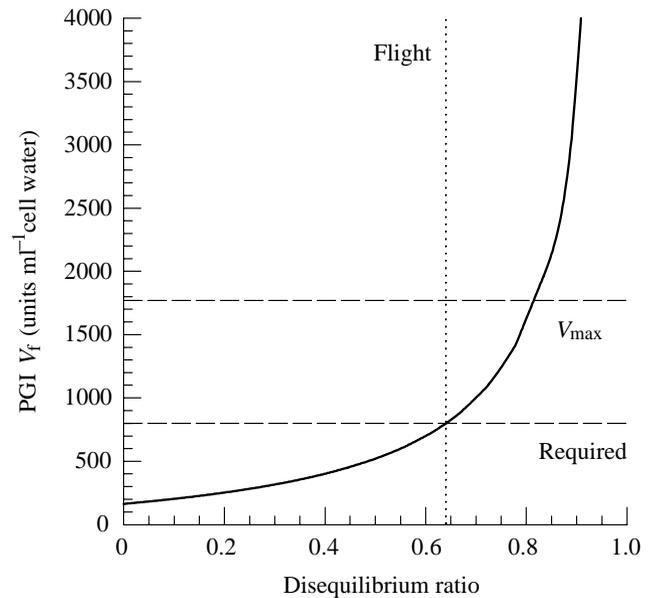


Fig. 3. Model of the phosphoglucose isomerase (PGI) reaction using the Haldane equation (equation 5) with kinetic parameters for the purified enzyme and apparent  $K_{eq}$  measured at pH 7.0 with 120 mmol $l^{-1}$  KCl, and estimates of glycolytic flux rates in unladen flying honeybees (63.5  $\mu$ mol ml $l^{-1}$  cell water min $l^{-1}$ ; Suarez *et al.* 1996). The *in vivo* disequilibrium ratio (determined using tissue F6P and G6P concentrations from flying honeybees, Table 1, and apparent  $K_{eq}$ , Table 2) is indicated (dotted vertical line). Given this ratio, the forward flux capacity ( $V_f$ ) of PGI required to support *in vivo* glycolytic flux rates is 800 units ml $l^{-1}$  cell water (lower dashed horizontal line). The  $V_{max}$  of honeybee flight muscle PGI is 1770 units ml $l^{-1}$  cell water (upper dashed horizontal line).

Values of  $K_m$  and  $K_{eq}$  determined under these conditions (Table 2) were used in solving equation 5.

From Fig. 3, it is evident that, as the reaction approaches equilibrium (disequilibrium ratio=1), the  $V_f$  required to achieve a given rate of net forward flux ( $J$ ) must increase exponentially. We estimate an *in vivo* disequilibrium ratio of 0.64 (Table 1), which would require a  $V_f$  of at least 800 units  $\text{ml}^{-1}$  cell water to accommodate the glycolytic flux rate ( $J$ ) of  $63.5 \mu\text{mol ml}^{-1}$  cell water  $\text{min}^{-1}$  estimated from respirometry of unladen flying honeybees (Suarez *et al.* 1996). Glycolytic rates close to  $84.7 \mu\text{mol ml}^{-1}$  cell water  $\text{min}^{-1}$  have been estimated for free-flying honeybees (Balderrama *et al.* 1992; assuming an average thorax mass of 28.6 mg, Suarez *et al.* 1996, accounting for most of the  $V_{O_2}$ ). Substituting  $84.7 \mu\text{mol ml}^{-1}$  cell water  $\text{min}^{-1}$  for  $J$  into equation 5 yields a  $V_f$  value of 1070 units  $\text{ml}^{-1}$  cell water, assuming that all other parameters remain unchanged. Given the  $V_{max}$  of 1770 units  $\text{ml}^{-1}$  cell water measured here, it appears that the maximal activity of honeybee flight muscle PGI is only approximately 1.7- to 2.2-fold greater than the forward flux capacity required to support physiological flux rates (Fig. 3). This value is much lower than the apparent 23-fold excess estimated solely on the basis of fractional velocities (Suarez *et al.* 1996). By comparison, using kinetic parameters from rabbit skeletal muscle (Dyson and Noltmann, 1968),  $V_{max}$  (Parra and Pette, 1995) and glycolytic flux rate estimates (Spreit, 1989) from rat fast-twitch skeletal muscle, and values of apparent  $K_{eq}$  from Dyson and Noltmann (1968), the  $V_{max}$  of mammalian PGI is estimated to be sevenfold greater than the  $V_f$  required to maintain physiological glycolytic flux rates.

It is possible that the  $[\text{Cl}^-]$  used here overestimates the true intracellular concentration, and high  $[\text{Cl}^-]$  may significantly affect enzyme  $K_m$  values (Hochachka and Somero, 1984). We therefore recalculated  $V_f$  from equation 5 using the  $K_m$  and  $K_{eq}$  values determined at pH 8.0 and low ionic strength (Table 2). At glycolytic flux rates of  $63.5$  and  $84.7 \mu\text{mol ml}^{-1}$  cell water  $\text{min}^{-1}$ , the estimated  $V_f$  values are 840 and 1120 units  $\text{ml}^{-1}$  cell water, which are in close agreement with the values of 800 and 1070 units  $\text{ml}^{-1}$  cell water obtained using  $K_m$  and  $K_{eq}$  values determined at pH 7.0 with  $120 \text{ mmol l}^{-1}$  KCl. Therefore, although the  $K_m$  values in the forward and reverse directions differ by 2.5- to threefold between the two assay conditions, the estimates of  $V_f$  change by only approximately 5%.

Another model has been proposed to describe steady-state flux through reversible Michaelis–Menten reactions such as that catalyzed by PGI (Brooks and Storey, 1992; Brooks, 1996). It differs from the equations we employed (equations 1, 4 and 5) in that it incorporates the kinetic properties of the subsequent enzyme in the pathway, in this case PFK (Fig. 1).

$$\frac{[\text{F6P}]}{[\text{G6P}]} = \frac{K_2 \times K_r \times (V_f - J)}{K_f \times K_r \times (V_2 - J) + K_f \times K_2 \times (V_r + J)} \quad (6)$$

In equation 6,  $K_2$  is the  $K_m$  for PFK,  $V_2$  is the  $V_{max}$  for PFK and the other variables are defined in equations 1–5. Equation

6 can be algebraically rearranged to solve for  $V_f$ . Insect flight muscle PFK is half-saturated ( $S_{0.5}$  value) at F6P concentrations ranging from 25 to  $540 \mu\text{mol l}^{-1}$  (Storey, 1985; Leite *et al.* 1988; Khoja *et al.* 1992). These values depend largely on the conditions of pH and temperature and the concentrations of activators and inhibitors used in the measurements. It has been estimated that honeybee flight muscle PFK works at approximately 50%  $V_{max}$  during flight (Suarez *et al.* 1996), so that tissue F6P concentrations in flight would be close to  $S_{0.5}$  *in vivo*. From Table 1, tissue F6P during flight is  $0.144 \mu\text{mol ml}^{-1}$  cell water, which falls within the range of  $S_{0.5}$  values for PFK quoted above. Using this F6P concentration (substituted for  $K_2$ ) and a  $V_{max}$  for PFK of 144 units  $\text{ml}^{-1}$  cell water (Suarez *et al.* 1996) in equation 6 yields estimated  $V_f$  values for PGI of 874 and 955 units  $\text{ml}^{-1}$  cell water for flying honeybees with glycolytic flux rates of  $63.5$  and  $84.7 \mu\text{mol ml}^{-1}$  cell water  $\text{min}^{-1}$  respectively. These values are in close agreement with our estimates of 800 and 1070 units  $\text{ml}^{-1}$  cell water obtained using equation 5.

It therefore appears that the  $V_{max}$  for PGI is approximately twofold greater than  $V_f$ . Values of  $V_{max}$  are useful in establishing upper limits of enzyme capacities and promoting consistency between studies; however, as assay conditions are optimized (in this case pH 8.0, no added KCl), these values may not be directly applicable to the *in vivo* condition. Indeed, at saturating F6P concentration under physiological conditions of pH and ionic strength (pH 7.0,  $120 \text{ mmol l}^{-1}$  KCl), PGI activity is 1130 units  $\text{ml}^{-1}$  cell water, 36% lower than  $V_{max}$ . This is only 5% greater than the highest  $V_f$  estimates (1070 units  $\text{ml}^{-1}$  cell water; see above) and indicates a very close match between predicted and measured flux capacities.

Obviously a certain minimum level of PGI activity is necessary to maintain carbohydrate metabolism. Mutations in the angiosperm plant *Clarkia xantiana* that decrease maximal PGI activity by as little as 25% result in shifts in *in vivo* mass-action ratios and reduced photosynthetic flux rates (Kruckeberg *et al.* 1989). From this, the authors concluded that there is no excess PGI activity in the normal plant above that required to maintain the reaction close to equilibrium. Despite this, there would appear to be no advantage to having a large excess of the enzyme. A genetically engineered yeast strain that expresses PGI at 11-fold higher levels did not differ from the wild-type in terms of F6P and G6P concentrations and glycolytic flux rate (Benevolensky *et al.* 1994). Given the energetic expense of protein synthesis (Hawkins, 1991) and space constraints within cellular compartments (Fulton, 1982), it is reasonable to predict that needless overexpression of any enzyme would be selected against. Indeed, yeast strains expressing pyruvate kinase at levels fivefold higher than those of the wild type (but not those overexpressing PGI) sporulate poorly (Rosenzweig, 1992). Cellular crowding may be particularly important in muscles working at high metabolic rates, where large volume densities of myofibrils, mitochondria and sarcoplasmic reticulum (in vertebrate and synchronous

insect muscle) within muscle cells may constrain the amount of physical space available for, and the interactions between, cytosolic enzymes (Fulton, 1982).

In summary, by using the Haldane equation with properties of purified honeybee flight muscle PGI and tissue concentrations of G6P and F6P, we estimate that PGI  $V_{\max}$  is 1.7- to 2.2-fold in excess of that required to support the *in vivo* glycolytic flux rates. If measured at physiological pH and ionic strength (pH 7.0, 120 mmol l<sup>-1</sup> KCl), PGI activity is only in excess of that required by 5%. The observation that there is 'enough but not too much' PGI complements data showing close matches between maximal enzyme activities and flux rates for non-equilibrium reactions in this tissue. It also supports the concept that economical designs of muscle metabolism are favoured in systems working at high flux rates.

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