Model of 2,3-bisphosphoglycerate metabolism in the human erythrocyte based on detailed enzyme kinetic equations¹: *in vivo* kinetic characterization of 2,3-bisphosphoglycerate synthase/phosphatase using ¹³C and ³¹P NMR

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This is the first in a series of three papers [see also Mulquiney and Kuchel (1999) *Biochem. J.* **342**, 579–594; Mulquiney and Kuchel (1999) *Biochem. J.* **342**, 595–602] that present a detailed mathematical model of erythrocyte metabolism which explains the regulation and control of 2,3-bisphosphoglycerate (2,3-BPG) metabolism. 2,3-BPG is a modulator of haemoglobin oxygen affinity and hence plays an important role in blood oxygen transport and delivery. This paper presents an *in vivo* kinetic characterization of 2,3-BPG synthase/phosphatase (BPGS/P), the enzyme that catalyses both the synthesis and degradation of 2,3-BPG. Much previous work had indicated that the behaviour of this enzyme *in vitro* is markedly different from that *in vivo*. ¹³C and ³¹P NMR were used to monitor the time courses of selected metabolites when erythrocytes were incubated with or without [U-¹³C]glucose. Simulations of the experimental time courses were then made. By iteratively changing the parameters of the BPGS/P part of the model until a good match between the NMR-derived data and simulations were achieved, it was possible

to characterize BPGS/P kinetically *in vivo*. This work revealed that: (1) the pH-dependence of the synthase activity results largely from a strong co-operative inhibition of the synthase activity by protons; (2) 3-phosphoglycerate and 2-phosphoglycerate are much weaker inhibitors of 2,3-BPG phosphatase *in vivo* than *in vitro*; (3) the K_m of BPGS/P for 2,3-BPG is significantly higher than that measured *in vitro*; (4) the maximal activity of the phosphatase *in vivo* is approximately twice that *in vitro*, when P_i is the sole activator (second substrate); and (5) 2-phosphoglycollate appears to play no role in the activation of the phosphatase *in vivo*. Using the newly determined kinetic parameters, the percentage of glycolytic carbon flux that passes through the 2,3-BPG shunt in the normal *in vivo* steady state was estimated to be 19%.

Key words: erythrocyte enzymes, glycolysis, intracellular pH, membrane potential, nuclear magnetic resonance.

INTRODUCTION

2,3-Bisphosphoglycerate (2,3-BPG) plays an important role in human erythrocytes by regulating their blood oxygen transport and delivery. This is exemplified by the fact that, under clinical and environmental conditions where oxygen transport has been compromised, such as anaemia, congenital heart disease and high altitude, the concentration of 2,3-BPG is elevated above normal values [1,2]. 2,3-BPG is important in oxygen transport because it is a heterotropic allosteric effector of oxygen binding by haemoglobin (Hb); by binding preferentially to the deoxygenated form of Hb, it decreases the apparent affinity of Hb for O₂. While the binding of 2,3-BPG to Hb has been known for over 30 years [3,4], the precise mechanism(s) by which 2,3-BPG concentration is regulated has continued to be the subject of debate.

In this and the following two papers [5,6], a comprehensive and realistic model of human erythrocyte metabolism is presented that can explain the regulation and control of 2,3-BPG metabolism. In the present paper, an *in vivo* kinetic characterization of

2,3-BPG synthase/phosphatase (BPGS/P), the enzyme that catalyses both the synthesis and degradation of 2,3-BPG, is made. Much previous work has indicated that the behaviour of this enzyme *in vivo* is markedly different from that *in vitro*. In the second paper [5] the comprehensive model of erythrocyte metabolism is described. Although the human erythrocyte has been the subject of a number of extensive mathematical models [7,8], those presented so far have ignored a number of processes that are significant in the description of 2,3-BPG metabolism. And, in the final paper [6], a computer simulation and Metabolic Control Analysis of 2,3-BPG metabolism is given. This analysis was performed using the model that is presented here and in the second paper [6]. The analysis yielded a number of new insights into the regulation and control of 2,3-BPG metabolism.

The enzyme that catalyses both the synthesis and degradation of 2,3-BPG, BPGS/P, has been the object of several detailed kinetic studies [9]. These studies were, typically, performed with preparations of purified enzyme. There is evidence to suggest, however, that the behaviour of this enzyme *in vivo* is markedly different from that *in vitro*. For example, it has been claimed that

Abbreviations used: 1,3-BPG, 1,3-bisphosphoglycerate; 2,3-BPG, 2,3-bisphosphoglycerate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; 2-PGly, 2-phosphoglycollate; BPGS/P, 2,3-BPG synthase/phosphatase; Hct, haematocrit; HPA, hypophosphorous acid (H₃PO₂); Glc, glucose; Lac, lactate; MeP, methylphosphonate; PGM, phosphoglycerate mutase; SCF, signal correction factor; TeP, triethyl phosphate; TR, recycle time; pH_i, intracellular pH; ΔpH, transmembrane pH difference; nOe, nuclear Overhauser effect; f.i.d., free induction decay; Glc(1,6)P₂, glucose 1,6-bisphosphate.

¹ This is the first of a series of three papers on this topic (the other two papers are [5,6]).

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the synthase shows a significant pH-dependence that has not been observed *in vitro* [10]. In addition, the *in vitro* kinetic parameters for the phosphatase appear to give estimates of activity considerably less than those observed in intact erythrocytes [11]. This could be due to the presence of the potent phosphatase activator, 2-phosphoglycollate (2-PGly), *in vivo* [9,12] or it could be due to the inhibition of the phosphatase by 3-phosphoglycerate (3-PGA) being much weaker than that observed *in vitro* [13,14].

A problem in comparing predicted activities with *in vivo* activities for these reactions is that the determination of their *in vivo* activities is non-trivial. The activity of the phosphatase reaction is usually determined by inhibiting the synthase activity. Synthase activity can be inhibited by stopping 1,3-bisphosphoglycerate (1,3-BPG) production; this is achieved by incubating the cells in the absence of glucose (Glc) or by inhibiting glycolysis at the glyceraldehyde-3-phosphate dehydrogenase step, or at proximal reactions in the pathway [10,15,16]. Alternatively the synthase activity can be inhibited by decreasing the pH; this inhibition may be due to a decreased supply of 1,3-BPG [13] or pH-dependent inhibition of the synthase [10]. The K_m of the phosphatase for 2,3-BPG is significantly lower than the normal *in vivo* concentration of 2,3-BPG [11] and hence determination of the phosphatase activity should be independent of 2,3-BPG concentration. A problem with this method is, however, that 2,3-BPG depletion causes the accumulation of P_i ; and P_i has been shown to activate the phosphatase both *in vitro* [11] and *in vivo* [13]. Thus it has been claimed that the phosphatase activities determined by this method (20–25% of the glycolytic flux [10,15,16]) are in excess of the normal *in vivo* activity [13]. Furthermore, this method ignores the inhibition of the phosphatase by 3-phosphoglycerate (3-PGA); in the absence of glucose, 3-PGA has a concentration significantly lower than its *in vivo* value. This will also contribute to an overestimate of flux via the phosphatase.

Determination of the synthase activity *in vivo* is even more problematic. It has long been recognized that measurements of the synthase activity will be overestimated by isotopic tracer methods due to 2,3-BPG label exchange in the phosphoglycerate mutase (PGM) reaction [17,18]. This is partly because 2,3-BPG is involved in the reaction mechanism of PGM [19,20]. Despite this, two relatively recent measurements of the synthase activity have been made using ^{13}C -labelled Glc and ^{13}C NMR spectroscopy [21,22]. Not surprisingly, those authors obtained estimates of synthase activity that were higher than the above-mentioned overestimates of phosphatase activity. It is noteworthy that, under normal *in vivo* steady-state conditions, the flux through the synthase and phosphatase reactions should be equal.

Despite the problems of data interpretation associated with the NMR method of Oxley et al. [21] for the estimation of synthase activity, the work was valuable for demonstrating the utility of multinuclear NMR techniques for studying metabolic fluxes. By using NMR to measure simultaneously ^{13}C , ^{31}P and ^1H nuclei in a suspension of erythrocytes, incubated with $[1-^{13}\text{C}]\text{Glc}$, those authors were able to monitor the concentrations of $[1-^{13}\text{C}]\text{Glc}$, $[3-^{13}\text{C}]2,3\text{-BPG}$, total 2,3-BPG, $[3-^{13}\text{C}]\text{lactate}$ (Lac), unlabelled Lac, P_i and ATP in a single experiment in real time.

In the present work, ^{13}C and ^{31}P NMR were used to follow the time course of selected metabolites when erythrocytes were incubated with or without $[\text{U}-^{13}\text{C}]\text{Glc}$. Simulations of the experimental time courses were then made using the model described in the second paper of this series [5]. By iteratively changing the parameters of the BPGS/P part of the model until a good match between the NMR-derived data and simulations was achieved, it was possible to characterize kinetically BPGS/P

in vivo. This method of iteratively refining the parameters of a mathematical model by reference to NMR-derived data has been used previously (see, e.g., [23]). In using the detailed metabolic model to interpret the results, account was taken of the isotope exchange at the PGM reaction. In addition, since with ^{31}P NMR it was possible to monitor both 2,3-BPG decline and P_i accumulation, account could be taken of the effects of P_i on phosphatase activity. The effect of pH on the synthase activity was also determined by incubating the cells at a number of different initial pH values. Finally, it was possible to determine the effects of 3-PGA on the phosphatase reaction, since cells incubated without Glc contained low concentrations of this compound in comparison with cells incubated with Glc. Thus it was possible to use the newly determined kinetic parameters to estimate the normal *in vivo* activity of 2,3-BPG synthase and 2,3-BPG phosphatase and hence to estimate the percentage of glycolytic flux that passes via the 2,3-BPG shunt.

EXPERIMENTAL

Sample preparation and incubation details

Erythrocyte incubations with $[\text{U}-^{13}\text{C}]\text{Glc}$

Samples (2.5 ml) of freshly prepared saline-washed and carbon-monooxygenated leucocyte-depleted erythrocyte suspensions from a single donor (P.J.M.) {final haematocrit (Hct) ≈ 0.75 ; prepared as described in [24]} were incubated with 9.78 mM $[\text{U}-^{13}\text{C}]\text{Glc}$, 10 mM hypophosphorous acid (H_3PO_2) (HPA); pH adjusted to 7.4 with 10 M HCl, 4.35 mM methylphosphonic acid and 1.92 mM triethyl phosphate, at 37 °C in a 10-mm-outer-diameter NMR tube. In some time courses erythrocytes were washed with 40 mM PBS, pH 7.4. All time-course and calibration samples contained penicillin G (27.0 mg/l), streptomycin sulphate (50 mg/l), and amphotericin B (50 mg/l). In some experiments the external pH of the erythrocyte suspensions was altered by washing with isotonic solutions containing added HCl or NaOH.

The time courses were monitored by sequentially recording sets of three different NMR spectra. Each set of three spectra took ≈ 22 min to record and was obtained from a 'rapidly pulsed' ^{13}C -NMR experiment, a 'rapidly pulsed' ^{31}P NMR experiment and a 'fully relaxed' ^{31}P NMR experiment. The 'rapidly pulsed' spectra were acquired in order to obtain the maximum signal-to-noise ratio in the time available. The fully relaxed spectrum (relaxation delay $> 5 \times T_1$ of HPA which was 5×5.1 s) was recorded in order to obtain the relative intra- and extra-cellular concentrations of HPA; this information was used for the quantification of [Lac] and $[P_i]$. The time taken to load the sample into the spectrometer and to obtain field-frequency locking meant that the first transient was not acquired until ≈ 3 min after sample mixing.

Erythrocyte incubations in the absence of Glc

^{31}P NMR was used to monitor the decline in 2,3-BPG in erythrocytes incubated without Glc. Samples were prepared as described above, except that no Glc was added. The time course was monitored by sequentially recording a rapidly pulsed and a fully relaxed ^{31}P NMR spectrum.

Label-exchange experiments

Experiments were performed to investigate the exchange of ^{13}C label between 3-PGA and 2,3-BPG *in vivo*. Saline-washed erythrocytes were suspended in 50 mM Hepes, pH 7.4, at a final

Hct of ≈ 0.2 and incubated with 4 mM [$U\text{-}^{13}\text{C}$]Glc at 37 °C for 9 h in order to label the endogenous pool of 2,3-BPG. To decrease the rate of 2,3-BPG degradation during this period, the cells were washed and resuspended in the incubation mixture after ≈ 4.5 h. A concentrated haemolysate (Hct > 0.98) was then prepared from the cell suspension by centrifugation at 7710 *g* for 20 min (4 °C) using a fixed angle rotor (SS34; Sorvall, DuPont Instruments, Newtown, CT, U.S.A.). The supernatant was removed by aspiration and the cell suspension lysed by repeated freeze–thaw cycles (–20 °C/37 °C). The concentrated haemolysate was then incubated at 37 °C and the time course of [$U\text{-}^{13}\text{C}$]2,3-BPG and total 2,3-BPG metabolism monitored by sequentially recording a rapidly pulsed ^{13}C NMR spectrum and a rapidly pulsed ^{31}P NMR spectrum. After ≈ 2 h, 10 mM 3-PGA was added to the incubation mixture.

NMR details

All NMR spectra were acquired using a Bruker AMX-600 spectrometer (Bruker, Karlsruhe, Germany) with a 14.1 T superconducting magnet (Oxford Instruments, Oxford, UK) using a 10-mm triple-resonance probe, with the inner coil tuned to the ^{13}C and ^{31}P frequencies, and the outer coil to the ^1H and ^2H frequencies. The signal from [^2H]water was used for field/frequency locking. [^2H]water was kept ‘external’ to the sample in most cases by placing it in a coaxial capillary insert (Wilma, Buena, NJ, U.S.A.). The $\beta\text{-Glc C-1}$ resonance was used as a chemical-shift (δ) reference (δ 97.4 p.p.m. relative to TSP-d4 (sodium 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate) at 0.000 p.p.m. [25]) in ^{13}C NMR experiments, while triethyl phosphate (TeP) was added to all samples as a ^{31}P chemical-shift (δ) reference (δ 0.44 p.p.m. relative to 85% H_3PO_4 at 0.000 p.p.m. [26]).

^{13}C -NMR acquisition and processing parameters

Parameters were as follows: spectrometer frequency, 150.92 MHz; number of transients per spectrum, 1200; acquisition time, 0.6 s; pulse angle, $\pi/3$; recycle time (TR), 0.6 s; spectral width, 27780 Hz; broadband proton decoupling (WALTZ-16) was applied continuously; free induction decays (f.i.d.) were zero-filled with 32 k data points, and a 6 Hz exponential multiplication was applied before Fourier transformation.

Fully relaxed ^{31}P NMR acquisition and processing parameters

Parameters were as follows: spectrometer frequency, 242.94 MHz; number of transients per spectrum, 8; acquisition time, 0.6 s; pulse angle, $\pi/2$; TR, 28.1 s ($> 5 \times T_1$ of HPA); spectral width, 12500 Hz; broadband proton decoupling (WALTZ-16) was applied continuously; f.i.d. were zero-filled with 16000 data points and 6 Hz exponential multiplication was applied before Fourier transformation.

Rapidly pulsed ^{31}P NMR acquisition and processing parameters

These were as for the fully relaxed ^{31}P NMR experiments except that the number of transients per spectrum was 512, the pulse angle was $\pi/3$, and TR was 0.6 s.

Note that pulse angles in the rapidly pulsed experiments were chosen so as to maximize the signal-to-noise ratio of the C-3 2,3-BPG resonance in the ^{13}C experiment and the 3P 2,3-BPG resonance in the ^{31}P experiment. The variable-temperature unit was set to 33 °C to give a sample temperature of 37 °C due to

heating associated with broadband decoupling; relatively high decoupling power was required to decouple fully the HPA resonances ($J_{\text{H-P}} \approx 520$ Hz [27]). No attempt was made to adjust the temperature control to 37 °C during the acquisition of the fully relaxed ^{31}P spectra, since the time for the sample to reach thermal equilibrium (≈ 8 min) was much greater than the time required to obtain the ^{31}P spectrum (≈ 4 min).

Measurement of pH and membrane potential

The intracellular pH (pH_i) of cell suspensions was monitored during NMR time-course experiments by measuring the ^{31}P NMR chemical shift of methylphosphonate [28]. The Donnan ratio, membrane potential, and the transmembrane pH difference (ΔpH) were calculated from the added HPA [27].

Calibration methods

In fully relaxed spectra, and in the absence of the nuclear Overhauser effect (nOe), the intensity of each NMR resonance is directly proportional to the concentration of contributing nuclei. In the present work, NMR acquisition parameters were chosen so as to optimize the signal-to-noise ratio in the time available. This involved radiofrequency pulsing almost immediately after signal acquisition. Such rapid pulsing meant that the magnetization of most nuclear populations became partially saturated. In addition, since the ^{31}P and ^{13}C spectra were obtained with continuous broadband proton decoupling, the resonances were subject to various degrees of nOe. Thus to relate signal intensity to concentration, it was necessary to correct for the effects of partial saturation and nOe. The signal correction factor (SCF) was defined as

$$\text{SCF} = S/S_c \quad (1)$$

where S is the intensity of the resonance from the rapidly pulsed spectrum and S_c is the correct intensity. Measurements of S_c and S were made by interleaving the acquisition of an inverse-gated proton-decoupled spectrum (relaxation delay 10 times maximum T_1) with the rapidly pulsed time-course spectrum. A relaxation delay of 10 times the maximum T_1 was needed for the nOe to fully dissipate between the acquisition of transients [29].

Since the extent of partial saturation during rapid pulsing is a function of T_1 [30], and given that T_1 is very sensitive to variables such as salt concentration and the concentration of paramagnetic ions and solutes (such as O_2 , deoxy-Hb, and met-Hb), for the determination of SCF the calibration samples were as similar as possible to the time-course samples. In addition, the resonances from nuclei from metabolites which exchanged across the cell membrane (Glc, Lac and P_i) required two SCF each, owing to differences in T_1 between the intra- and extra-cellular spaces [31].

For a metabolite in slow exchange (on the NMR timescale) across the cell membrane, and assuming a homogeneous magnetic field across the sample, from eqn. (1) above:

$$\text{SCF} = \left(\frac{p_i}{\text{SCF}_i} + \frac{p_e}{\text{SCF}_e} \right)^{-1} \quad (2)$$

where p_i and p_e are the relative proportions of the nuclei in the intra- and extra-cellular environments, and SCF_i and SCF_e are the corresponding SCFs. Eqn. (2) was also assumed to apply to nuclei of metabolites in intermediate and fast exchange across the cell membrane. In applying eqn. (2) it was assumed that [Glc] was equal inside (in the cell water) and outside the cell and hence:

$$p_i = \frac{\alpha \text{Hct}}{1 - \text{Hct}(1 - \alpha)} \quad (3)$$

where α is the fraction of cell volume available to water and solutes dissolved in it.

The HPA ion was assumed to distribute passively and in accordance with the Donnan ratio (r ; [27]), hence the intra-/extra-cellular ratio of Lac was assumed to be equal to the distribution ratio of the added HPA. Thus, for Lac:

$$p_1 = \frac{S_{c,HPA,I}}{S_{c,HPA,I} + S_{c,HPA,E}} \quad (4)$$

where $S_{c,HPA,I}$ and $S_{c,HPA,E}$ are the intra- and extra-cellular signals from the hypophosphate ion in the fully relaxed spectra respectively. Phosphate was assumed to distribute according to:

$$\frac{p_{1I}}{p_{1E}} = \left(\frac{1 + 10^{pH_I - pK_{a2}}}{r^{-1} + 10^{pH_I - pK_{a2}r^{-2}}} \right) \left(\frac{\alpha Hct}{1 - Hct} \right) \quad (5)$$

where $pK_{a2} = 6.75$ (cf. [32]).

Intracellular SCF were measured for metabolites in concentrated haemolysates ($Hct > 0.98$). The concentrations of the metabolites of interest in these haemolysates were found to be stable or to change only at low linear rates. Thus, by interleaving the acquisition of inverse-gated proton-decoupled spectra (relaxation delays: 40 s, ^{31}P ; 120 s, ^{13}C) with the acquisition of the usual 'rapidly pulsed' spectra, it was possible to interpolate S_c values at the times that the rapidly pulsed spectra were obtained.

Intracellular ^{31}P SCF were measured on the concentrated haemolysate of an erythrocyte suspension that had been incubated with 10 mM Glc, 15 mM ouabain, 10 mM HPA, and 5 mM P_i , for 0.5 h, at 37 °C, and then 5 mM NaF and 3 mM iodoacetate for a further 30 min. In order to calibrate the α -ATP resonance it was necessary to supplement the haemolysate with 10 mM ATP, 10 mM Mg^{2+} and 2 mM EGTA. Intracellular ^{31}P SCF values (mean $\pm$ S.E.M.) relative to the SCF of intracellular HPA at 37 °C were: HPA, 1.00 ± 0.01 ($n = 6$); 2,3-BPG (3P), 0.92 ± 0.01 ($n = 6$); 2,3-BPG (2P), 0.87 ± 0.02 ($n = 6$); P_i , 0.95 ± 0.04 ($n = 6$); ATP (αP), 0.92 ± 0.03 ($n = 4$). Intracellular ^{13}C calibration factors were measured on the concentrated haemolysate of an erythrocyte suspension that had been incubated with 10 mM [U - ^{13}C]Glc, for 3 h, at 37 °C and then with added 5 mM NaF and 3 mM iodoacetate for a further 30 min. Intracellular ^{13}C SCF values (mean $\pm$ S.E.M., $n = 8$) relative to the SCF of intracellular 2,3-BPG at 37 °C were: 2,3-BPG (C-3), 1.00 ± 0.05 ; α -Glc (C-1), 1.59 ± 0.06 ; β -Glc (C-1), 1.72 ± 0.05 ; Lac (C-3), 3.29 ± 0.18 .

Extracellular calibration factors were determined on samples of the metabolites dissolved in saline (pH 7.2, osmolality adjusted to ≈ 280 mOsmol $\cdot$ kg $^{-1}$). Extracellular ^{31}P SCF values (mean $\pm$ S.E.M., $n = 6$) relative to the SCF of extracellular HPA at 37 °C were: HPA, 1.00 ± 0.01 ; P_i , 1.02 ± 0.01 . Extracellular ^{13}C SCF values (mean $\pm$ S.E.M., $n = 8$) relative to the SCF of intracellular 2,3-BPG at 37 °C were: α -Glc (C-1), 2.13 ± 0.12 ; β -Glc (C-1), 2.25 ± 0.12 ; Lac (C-3), 3.44 ± 0.26 . Extra- and intra-cellular ^{13}C SCF values were related to each other by including 5 mM [2 - ^{13}C]acetate in calibration samples.

Corrected signal intensities were calibrated to absolute concentrations in two ways. For ^{13}C resonances a proportionality constant was chosen so that the concentration given by the Glc C-1 resonance, extrapolated to zero time, was equivalent to the concentration of added [U - ^{13}C]Glc. For ^{31}P resonances, the proportionality constant was determined for each time course from the measured distribution of HPA between the intra- and

extra-cellular compartments. The average ratio of extra- and intra-cellular SCF values for HPA was 1.08 ± 0.05 ($n = 12$).

Numerical methods

^{13}C NMR spectra were integrated using the Bruker integration subroutine in UXNMR (version 930601). Linear-baseline correction was applied prior to integration. Due to the overlap of resonances in the ^{31}P spectra, it was necessary to determine integrals using a non-linear peak-fitting routine (UXNMR) that assumes a mixed Lorentzian–Gaussian lineshape. Again linear-baseline correction was applied prior to integration.

Kinetic description used to model BPGS/P and PGM

Detailed kinetic models of BPGS/P and PGM were required in order to apply isotopic-tracer methods to measure flux into and out of the 2,3-BPG shunt. These equations formed submodels of the whole model of erythrocyte metabolism [5] and were significantly different from those that have been presented previously.

In the human erythrocyte, the three enzyme activities, 2,3-BPG synthase (BPGS), 2,3-BPG phosphatase (BPGP) and phosphoglycerate mutase, reside in three isoenzymes in differing proportions [33–35]. One of the isoenzymes (BPGS/P) accounts for most of the total cellular activity of the synthase and phosphatase reactions. Another (PGM) functions primarily as a phosphoglycerate mutase, accounting for the majority of the total cellular mutase activity. The three different reactions are catalysed at a common active site in both BPGS/P and PGM [36–39]. The mechanisms of all reactions involve phosphorylation of the enzyme, and it has been shown that the free enzyme is phosphorylated at a single site in each subunit by both 1,3-BPG and 2,3-BPG (see [9,40] for reviews).

Steady-state kinetics of BPGS/P

Synthase activity

The stoichiometry of the synthase activity of BPGS/P has been verified as [41]:

The enzyme operates via an irreversible substituted-enzyme mechanism in both horse [42] and human erythrocytes [9], the substituted enzyme being the phosphorylated form. An earlier report of the human erythrocyte enzyme operating via an ordered-ternary-complex mechanism [41] was subsequently retracted as being in error [9]. 2,3-BPG is a strong inhibitor of the reaction, being competitive with respect to 1,3-BPG. 2-PGly stimulates the hydrolysis of 1,3-BPG to 3-PGA. 2-PGA can also act as the phosphoryl group acceptor instead of 3-PGA to produce 2,3-BPG. However, even at low concentrations of 3-PGA (≈ 0.36 μ M) and high concentrations of 2-PGA (≈ 1330 μ M), 3-PGA is preferentially phosphorylated over 2-PGA [41]. P_i is also known to be a competitive inhibitor with respect to 3-PGA.

Phosphatase activity

The phosphatase activity of the horse enzyme has an absolute requirement for an activator. The low level of phosphatase activity, previously reported in the absence of an activator, was

Table 1 Comparison of the reported kinetic parameters of human erythrocyte BPGS/P with those predicted (at pH 7.2) from the model described in Scheme 1

The justification for parameter values used in the model is given in the Results and discussion section. The k_i values that yielded steady-state parameters consistent with the literature values were: ($M^{-1} \cdot s^{-1} \times 10^3$), $k_1 = 0.8$, $k_4 = 1.85$, $k_{14(H-2-PGly)} = k_{14(H-P_i)} = 10$, $k_6 = 1 \times 10^{-5}$, $k_{13} = 0.018$; (s^{-1}), $k_2 = 4.0 \times 10^2$, $k_3 = 9.9$, $k_5 = 1.0 \times 10^3$, $k_7 = 1 \times 10^8$, $k_8 = 1 \times 10^4$, $k_9 = 0.55$, $k_{10} = 1.98 \times 10^3$, $k_{11} = 1 \times 10^{-2}$, $k_{12} = 1 \times 10^3$, $k_{15(H-2-PGly)} = 8.9 \times 10^4$, $k_{15(H-P_i)} = 6.1 \times 10^5$, $k_{16(H-2-PGly)} = 4.03$ and $k_{16(H-P_i)} = 0.19$. Notes: ^a[61], human erythrocytes, pH 7.24, 25 °C, 0.1 M KCl; ^b[41] human erythrocytes, pH 7.5, 25 °C; ^c[9], human erythrocytes, pH 7.5, 25 °C, 50 mM sodium Tes buffer; ^d $k_{cat,f}$ for the synthase activity from the purified (2000 $\times$) human erythrocyte enzyme was calculated from the data obtained by Sasaki et al. [34] (9.78 units/mg, 37 °C, pH 7.8). The M_r of the dimer was taken to be 60 000 [34,44]; $k_{cat,f}$ for the phosphatase activity was reported to be five times smaller than $k_{cat,f}$ for the synthase activity [9,34]; $k_{cat,f}$ for the mutase activity is reported to be ≈ 7.4 times lower than the synthase activity in the rabbit muscle enzyme [37] and equal in human erythrocytes [34]; ^e[11] human erythrocytes, pH 7.5, 25 °C, 0.1 M KCl. Note value reported for K_{m,P_i} is an activation constant; ^fcalculated by assuming that BPGS/P is responsible for all physiologically relevant phosphatase activity (see [34] and that the maximal activity of the fully phosphate-activated phosphatase in human erythrocytes is 0.84 mmol $\cdot$ litre of erythrocytes⁻¹ $\cdot$ h⁻¹ [11]); ^g[36] human erythrocytes, pH 7.65, 25 °C, 0.1 M KCl for lower value, 0.13 M KCl/0.02 M NaCl for higher value; ^h[20], pH 7.5, 25 °C, $K_{eq} = c/b$; ⁱ[62]; ^jestimated from the measured BPGS activity in human erythrocytes (1577 units/litre of erythrocytes, pH 7.6, 37 °C; [63]) and the value of $k_{cat,f}$ for the synthase reaction; ^k2-PGA and 3-PGA give the same maximal rate for the synthase reaction [9]; ^l[10] determined *in vivo* from human erythrocytes, pH_o 7.41, 37 °C. e_0 , initial enzyme concentration.

Activity	Parameter	Reported value	Predicted value
Synthase	$K_{m,1,3BPG}$	3.1 μM^a , 0.42 μM^c	5.1 μM
	$K_{m,3-PGA}$	0.7 μM^a , 1–1.5 μM^b , 0.48 μM^c	530 μM
	$K_{m,2-PGA}$	$\gg K_{m,3-PGA}^a$	15 mM
	$K_{i,2,3-BPG}$	20 μM^a , 12 μM^c	0.56 mM
	K_{i,P_i}	0.6 mM ^a	0.6 mM
	$K_{i,2-Pgly}$		63 μM
	$k_{cat,f}$	9.8 s ^{-1d}	9.8 s ⁻¹
	$K_{m,2,3-BPG}$ (2-PGly-activated)	2.5 μM^c , 25 μM^c	49 μM
	$K_{m,2,3-BPG}$ (P _i -activated)	0.08 μM^e , 384 μM^f	142 μM
	$K_{m,2-Pgly}$	12.5 μM^c	10.7 μM
Phosphatase	K_{m,P_i}	700 μM^e	454 μM
	$K_{i,3-PGA}$, 2-PGA)	2.0–3.0 μM^e	35 μM
	$k_{cat,f}$ (2-PGly-activated)	1.8 s ^{-1d}	0.49 s ⁻¹
	$k_{cat,f}$ (P _i -activated)	0.09 s ^{-1f}	0.14 s ⁻¹
	$K_{m,3-PGA}$		30 μM
	$K_{m,2-PGA}$		0.83 mM
	$K_{m,2,3-BPG}$	80–200 μM^g	0.56 mM
	$k_{cat,f}$		0.0034 s ⁻¹
	$k_{cat,r}$	1.2–9.8 s ^{-1d}	0.56 s ⁻¹
	K_{eq}	0.08 ^h , 0.17 ⁱ	0.17
Mutase	e_0	3.8 μM^l	3.8 μM

an artifact of the assay method used [42]. Of the activators, 2-PGly and P_i are probably the most important *in vivo* [11,20]. The steady-state kinetic behaviour of the phosphatase is consistent with a substituted-enzyme mechanism [42] in which the activator is treated as the second substrate. The products of the reaction in human erythrocytes are P_i and 3-PGA, and no 2-PGA is produced [11]. The monophosphoglycerates are competitive inhibitors with respect to the activator/second substrate [11,42], and P_i is an inhibitor with respect to 2-PGly [11].

Mutase activity

The steady-state kinetics of the mutase reaction of BPGS/P have not been studied in detail. It was assumed that the mutase reaction operates by the same mechanism as that of PGM.

Table 2 Comparison of the reported kinetic parameters of human erythrocyte PGM with those predicted (at pH 7.2) from the model described in Scheme 1

The k_i values that yielded steady-state parameters consistent with the literature values were: ($M^{-1} \cdot s^{-1} \times 10^3$), $k_1 = k_4 = k_6 = k_{14(H-2-PGly)} = k_{14(H-P_i)} = 1.0$, $k_{13} = 0.083$; (s^{-1}), $k_2 = 3.1 \times 10^3$, $k_3 = 1.1$, $k_5 = 4.2 \times 10^3$, $k_7 = 1.4 \times 10^3$, $k_8 = 4.1 \times 10^4$, $k_9 = 880$, $k_{10} = 8 \times 10^3$, $k_{11} = 880$, $k_{12} = 1.4 \times 10^1$, $k_{15(H-2-PGly)} = 4.8 \times 10^5$, $k_{15(H-P_i)} = 3.5 \times 10^6$, 8.7×10^5 , $k_{16(H-2-PGly)} = 0.3$, $k_{16(H-P_i)} = 6.7 \times 10^{-4}$. Notes: ^a $k_{cat,f}$ for the mutase activity was calculated from the specific activity of the purified human erythrocyte enzyme (714 units/mg, pH 7.5, 37 °C; [64]); the PGM dimer has a subunit M_r of 30 000 [64]; $k_{cat,f}$ is $\approx 1.11 k_{cat,f}$ [37] (rabbit muscle enzyme); $k_{cat,f}$ for the phosphatase activity is 2700 times smaller than that for the mutase activity in human erythrocyte PGM [34] and 480 times smaller in the rabbit muscle enzyme [37]; the synthase is ≈ 760 times lower in the human, and 3300 times smaller in the rabbit enzyme; ^bchicken muscle, pH 7.5, 50 mM triethanolamine/HCl buffer [20]; ^cchicken muscle, pH 7.5, 50 mM sodium Tes buffer/0.1 M KCl [20]; ^dthe activity of the phosphatase is ≈ 450 times smaller when activated by phosphate rather than 2-PGly [20]; ^epH 7.5, 25 °C; $K_{eq} = c/b$ [20]; ^f[62]; ^gcalculated from the normal human erythrocyte activity in the reverse direction (12 444 units/litre of erythrocytes [63]) and the value of $k_{cat,f}$ for the mutase reaction. e_0 , initial enzyme concentration.

Activity	Parameter	Reported value	Predicted value
Synthase	$k_{cat,f}$	1.0 s ⁻¹ , 0.24 s ^{-1a}	1.0 s ⁻¹
Phosphatase	$K_{m,2,3-BPG}$ (2-PGly activated)	0.065 μM^b	0.004 μM
	$K_{m,2-Pgly}$	479 μM^b	480 μM
	$k_{cat,f}$ (2-PGly-activated)	1.6 s ⁻¹ , 0.3 s ^{-1a}	0.3 s ⁻¹
Mutase	$K_{m,3-PGA}$	168 μM^c	170 μM
	$K_{m,2-PGA}$	14 μM^c	26 μM
	$K_{m,2,3-BPG}$	0.069 μM^c	0.14–0.16 μM
	K_{i,P_i}	3.5 mM	3.5 mM
	$k_{cat,f}$	795 s ^{-1a}	795 s ⁻¹
	$k_{cat,r}$	714 s ^{-1a}	714 s ⁻¹
	K_{eq}	0.08 ^e , 0.17 ^f	0.17
	e_0	410 nM ^g	410 nM

Steady-state kinetics of PGM

The mechanisms of the three activities of PGM in rabbit muscle have been comprehensively presented [20]; in the present work it was assumed that the human erythrocyte enzyme operates via the mechanisms proposed by those authors.

Mutase activity

The mutase activity of PGM in chicken breast muscle is consistent with a substituted-enzyme intermediate [19,20] where the first substrate is 2,3-BPG and the second is a monophosphoglycerate. The mechanism of the mutase activity presented by Rose and Dube [20] also accounts for the finding that 2,3-BPG does not participate in every turnover cycle of the enzyme [43].

Phosphatase activity

The mechanism of the phosphatase reaction is consistent with a substituted-enzyme mechanism in which the second substrate (the activator) is a competitive inhibitor of the first (2,3-BPG), and the first substrate is a competitive inhibitor of the second [20]; the monophosphoglycerates and P_i were shown to be inhibitors of the stimulatory effect of 2-PGly [20].

Scheme 1 A minimal model of human erythrocyte BPGS/P and PGM

Symbols: E, free enzyme; F, phosphorylated enzyme; A, 1,3-BPG; B, 3-PGA; C, 2-PGA; D, 2,3-BPG; H, 2-PGly or P_i . Expressions for the steady-state kinetic parameters in terms of the rate constants are: (1) synthase: $k_{cat,f} = (N_1b + N_2c)/(D_6b + D_7c)$, $K_{m,A} = (D_2b + D_3c)/(D_6b + D_7c)$, $K_{m,B}$ (in absence of C) = D_1/D_6 , $K_{m,C}$ (in absence of B) = D_1/D_7 , $K_{i,D} = (D_2b + D_3c)/(D_6b + D_{10}c)$ and $K_{i,H} = D_1/D_6$; (2) phosphatase: $k_{cat,f} = N_4/D_{11}$, $K_{m,D} = D_5/D_{11}$, $K_{m,H} = D_4/D_{11}$ and $K_{i,eq}$ (mixture of B and C) = $[D_4(1 + K_{eq})]/(D_9 + D_{10}K_{eq})$; (3) mutase: $k_{cat,f} = N_6/D_9$, $k_{cat,r} = N_9/D_{10}$, $K_{m,B} = D_4/D_9$, $K_{m,C} = D_4/D_{10}$, $K_{m,D} = D_2/D_9$ and D_3/D_{10} , and $K_{eq} = c/b = N_8/N_9$.

Synthase activity

The synthase reaction has an irreversible substituted-enzyme mechanism [20] that is similar to that described above for BPGS/P.

The known Michaelis constants for the three activities of BPGS/P and PGM are given in Tables 1 and 2.

Model of BPGS/P and PGM

The mechanisms of BPGS/P and PGM that are consistent with the kinetic behaviour of the individual reactions, and the existence of a single catalytic site, are shown in Scheme 1.

BPGS/P

Scheme 1 accounts for most features of the individual enzyme activities. However, the activation of 3-PGA phosphorylation by 2-PGA or 2-PGly [9,41] was neglected, as these effects have not been well characterized kinetically. The inhibition of the synthase reaction at high concentrations of 2-PGly (above 0.4 mM; [41]), 3-PGA (K_i 375 μ M; [9]) and 2-PGA (K_i 2 mM; [36]) due to competition with 1,3-BPG for the free enzyme was also ignored due to the low cellular concentrations of these metabolites relative to their inhibition constants.

PGM

The model accounts for most features of the individual enzyme activities. However, it neglects the competitive inhibition by 2-PGA, P_i , and 2-PGly with respect to 2,3-BPG in the mutase reaction, and the competitive inhibition by 2,3-BPG with respect to P_i or 2-PGly reported for the muscle enzyme [20], as there have been no reports of these effects on the erythrocyte enzyme.

Derivation of steady-state rate equation

The general steady-state rate equation for the mechanism shown in Scheme 1 is:

$$\frac{dp}{dt} = \frac{e_0 N}{(D_1a + D_2b + D_3c + D_4d + D_5h + D_6ab + D_7ac + D_8ah + D_9bd + D_{10}cd + D_{11}dh)} \quad (7)$$

where N depends on the identity of p such that:

If $p = d$, then $N = N_1ab + N_2ac - N_4dh$

If $p = a$, then $N = -N_1ab - N_2ac - N_3ah$

If $p = [P_i]$, then $N = N_3ah + N_4dh$

if $p = b$, then $N = -N_5ab + N_6ac + N_3ah + N_{10}dh - N_8bd + N_9cd$

if $p = c$, then $N = N_5ab - N_6ac + N_7dh + N_8bd - N_9cd$

(The expressions for the D_i can be obtained by e-mail from P. W. K. on request)

Determination of unitary rate constants

Unitary rate constants were determined from the Michaelis parameters as described in [5]. However, when determining the values of the unitary rate constants, consideration was given to the data of Rose and Dube [37], in which continuous-flow and rapid-quench techniques were used to determine the kinetics of enzymic phosphorylation and phosphoryl transfer. It was found that for BPGS/P, $k_3 \approx k_{cat,f}$ (synthase), $k_9 \approx k_{cat,f}$ (mutase), and $k_{16} \approx 6.2 \times k_{cat,f}$ (phosphatase), and for PGM, $k_3 \approx k_{cat,f}$ (synthase), $k_{16} \approx k_{cat,f}$ (phosphatase), k_9 and/or $k_{11} > 100 \text{ s}^{-1}$, and $k_8 > 100 \text{ s}^{-1}$.

Table 3 Initial values* and rates of change for the external parameters used in the simulations shown in Figures 2 and 5–7

Parameter	Initial pH _i ...	Value				
		7.3	7.2	7.1	7.0	6.8
Rate of change of pH _i		−0.050	−0.040	−0.030	−0.021	−0.011
Hct		0.76	0.75	0.76	0.76	0.77
Donnan ratio (r)		0.68	0.72	1.06	0.90	1.05
Rate of change of $r^\dagger$		0.043	0.036	0.05	0.010	0.030

* Note each value was determined from an average of two experiments, except for the data obtained at pH 7.2 which was an average of four experiments.

† Rate has units of h^{-1} .

pH-dependence of kinetic parameters

BPGS/P

The pH-dependence of the synthase activity was studied by Rose and Whalen [44]. However, it has been suggested that the *in vitro* pH-dependence of the enzyme is insufficient to explain the rapid decline in 2,3-BPG steady-state concentrations at pH values between 7.4 and 6.9 and that H^+ may be a strong co-operative inhibitor of the synthase [10]. Rose and Whalen [44] found that, between pH 6.75 and 7.75, $K_{m,1,3\text{-BPG}}$, $K_{i,2,3\text{-BPG}}$, and $k_{cat,f}$ increased, while $K_{m,3\text{-PG}}$ was independent of pH. The pH dependence of $K_{m,1,3\text{-BPG}}$ and $K_{i,2,3\text{-BPG}}$ were modelled well by replacing k_1 and k_{13} with:

$$k \propto \frac{1}{\left(1 + \frac{10^{-pK_a}}{10^{-\text{pH}}}\right)} \quad (8)$$

where $pK_a = 6.8$. However, because of the uncertainty in the pH-dependence of the synthase activity, k_3 was modelled with:

$$k_3 \propto \frac{1}{\left[1 + \left(\frac{10^{-pH}}{10^{-pK_a}}\right)^n\right]} \quad (9)$$

where $pK_a = 7.17$ and $n = 4$; these values were chosen to give the best fit to the experimental data (see the Results and discussion section). It was necessary to change k_4 and k_6 by the same fraction as k_3 in order to keep the K_m values for 3-PGA and 2-PGA independent of pH. The phosphatase has a pH optimum about 6.8–7.5 [11,45]. This indicates that pH changes near physiological values would not greatly affect $k_{cat,f}$ for the phosphatase reaction. Also, the activation constants for P_i and 2-PGly are independent of pH [11]. The changes in apparent $K_{m,2,3-BPG}$ as a function of pH that were measured *in vivo* [10] were ignored as a first approximation, since, under most conditions, $[2,3-BPG] \gg K_{m,2,3-BPG}$.

PGM

The pH-dependence of the kinetic parameters of PGM were assumed to be independent of pH because of a lack of information on it and because of the low control coefficients determined by simulations for this enzyme under most conditions.

Assumptions used for simulations

Simulations of the experimental time courses were made using the model described in [5]. The model included the kinetic descriptions of BPGS/P and PGM described above and was modified to account for the existence of both unlabelled and ^{13}C -labelled metabolite pools. In accounting for labelled and unlabelled pools, it was assumed that the concentrations of unlabelled pentose phosphate pathway intermediates were negligible at the beginning of the time courses and that insignificant amounts of unlabelled carbon from 2,3-BPG breakdown went into the pentose phosphate pathway. This latter assumption was justified because the concentration of unlabelled glycolytic intermediates in the pathway above 1,3-BPG were predicted to be minimal in experiments which involved incubations with $[\text{U-}^{13}\text{C}]\text{Glc}$.

When performing simulations, most external parameters were kept at the constant values reported in Table 3 [5], with the following exceptions. In all simulations extracellular Lac, pyruvate, and phosphate were treated as 'internal parameters' with initial concentrations of zero. $[\text{Glc}]$ was also treated as an internal parameter with the initial concentration dependent on the experimental conditions. Initial values of pH_i , the Donnan ratio, and Hct were determined in each experiment (Table 3). In addition, the values of pH_i and the Donnan ratio were assumed to change at the experimentally determined rates (Table 3). In the simulations, the initial concentrations of all other intermediates were set to zero, while the initial concentrations of 2,3-BPG were specified in each simulation.

For simulations of the experiments in which erythrocytes were incubated in the absence of glucose, it was necessary to account for the catabolism of AMP [10]. This was modelled by assuming a constant rate of AMP decline of $100 \mu\text{M} \cdot \text{h}^{-1}$.

As noted above, the concentration of glucose 1,6-bisphosphate $[\text{Glc}(1,6)\text{P}_2]$ was assumed to be constant over the entire time course. Since the time courses were simulated for periods of ≈ 10 h and the turnover time of this compound is ≈ 20 h [46] this assumption needs more justification. Thus, Gerber et al. [46] found that, at physiological pH, and in the presence of glucose,

there was little change in $\text{Glc}(1,6)\text{P}_2$ concentration in a 5 h period. At lower values of pH, the rate of $\text{Glc}(1,6)\text{P}_2$ degradation is even slower; without Glc there is a $\approx 20\%$ decrease over 5 h.

Another assumption implicit in applying the model in [5] is that changes in cell volume and haemolysis were minimal over the course of the experiment. Simultaneous bench experiments (P. J. Mulquiney and P. W. Kuchel, unpublished work) showed that the mean cell volume increased by $\approx 3\%$ during the time course and that the cell count decreased by $\approx 5\%$. These values were considered to be negligible from the point of view of the simulations.

RESULTS AND DISCUSSION

Incubations of erythrocytes with $[\text{U-}^{13}\text{C}]\text{Glc}$

Erythrocytes incubated with $[\text{U-}^{13}\text{C}]\text{Glc}$ at 37°C , initial pH_i 7.2, metabolized glucose at an approximately linear rate until the Glc was nearly exhausted (Figures 1A and 2A). The initial rate of Glc decline, $1.49 \text{ mmol} \cdot \text{litre of erythrocytes}^{-1} \cdot \text{h}^{-1}$, is in agreement with other measurements made at similar pH values [$1.44 \text{ mmol} \cdot \text{litre of erythrocytes}^{-1} \cdot \text{h}^{-1}$, [47]; $1.35 \text{ mmol} \cdot \text{litre of erythrocytes}^{-1} \cdot \text{h}^{-1}$, [13]]. Metabolism of the $[\text{U-}^{13}\text{C}]\text{Glc}$ led to the production of Lac labelled in the C-1, C-2 and C-3 positions. Analysis of the multiplet patterns at each Lac ^{13}C resonance indicated that these resonances arose predominantly from $[\text{U-}^{13}\text{C}]\text{Lac}$. The Lac production rate was constant after an initial lag phase of 1–2 h. During the time course, significant resonances from the three carbon nuclei of 2,3-BPG were also seen. The time courses of these resonances had a characteristic shape (Figure 2B and Figure 3), namely, after an initial influx of label into 2,3-BPG the concentration of labelled 2,3-BPG reached a maximum and then declined.

Total 2,3-BPG, ATP and P_i were monitored during the time course by the acquisition of ^{31}P NMR spectra (Figure 1C). These spectra indicated that, during the time course, total 2,3-BPG declined with a consequent accumulation of P_i (Figure 2B). The ATP concentration was approximately constant over the entire time course (Figure 2A). The addition of HPA enabled the determination of the Donnan ratio [27] (Figure 1B and Figure 4C), while the addition of MeP enabled the monitoring of intra- and extra-cellular pH [28] (Figure 4A). The Donnan ratio increased at an approximately constant rate during the decline of 2,3-BPG. This was consistent with the important role that 2,3-BPG plays in determining the Donnan equilibrium [48]. The pH decreased during the time course at a rate of $\approx 0.04 \text{ pH unit} \cdot \text{h}^{-1}$. This was consistent with the accumulation of Lac in the incubation vessel.

The decline of total 2,3-BPG during the incubation indicated that the relative rate of 2,3-BPG synthase to 2,3-BPG phosphatase decreased dramatically during the time course. This outcome is in accordance with the findings that the activity of 2,3-BPG synthase declines dramatically with a decline in pH, whereas the 2,3-BPG phosphatase reaction is insensitive to pH in the physiological domain of pH values [10]. Since the initial rate of production of ^{13}C -labelled 2,3-BPG is a direct measure of 2,3-BPG synthase activity (albeit complicated by isotope mixing at PGM; see below), the $[\text{U-}^{13}\text{C}]\text{Glc}$ incubations were repeated at a variety of different initial pH values. In so doing, direct evidence was obtained for the pH-dependence of the synthase activity (Figure 5B).

Incubations of erythrocytes without glucose

Erythrocytes were also incubated without Glc at 37°C to investigate the *in vivo* kinetics of 2,3-BPG phosphatase under

Figure 1 A multinuclear NMR time course obtained from the incubation of human erythrocytes with 9.78 mM [$U\text{-}^{13}\text{C}$]Glc and 10 mM HPA at 37 °C

Sets of three different NMR spectral series were recorded sequentially over a 10 h period. Each set of three spectra took ≈ 22 min to acquire. **(A)** ^{13}C NMR spectra showing the metabolism of [$U\text{-}^{13}\text{C}$]Glc to 2,3-BPG and Lac. Unannotated resonances between 70 and 80 p.p.m. are C-2–C-5 from α - and β -Glc and 2,3-BPG (C-2). **(B)** Fully relaxed ^{31}P NMR spectra showing the intra- and

Figure 2 Concentrations of (A) Glc C-1 (○), Lac C-3 (△), and ATP (□) and (B) 2,3-BPG 2P (○), P_i (△), and 2,3-BPG C-3 (□) as a function of time from incubations of human erythrocytes with 9.78 mM [$U\text{-}^{13}\text{C}$]Glc and 10 mM HPA at 37 °C, and initial pH_i 7.2

Each data point is the mean ($n = 4$) $\pm$ S.D. For Glc and Lac, the concentrations are expressed with respect to the total sample volume, while for ATP, 2,3-BPG, and P_i the concentration is expressed with respect to total intracellular water. The solid curves are the results of simulations of the corresponding metabolite concentrations as described in the Experimental section.

conditions where 2,3-BPG synthase activity was minimal. ^{31}P NMR was used to monitor the concentration of 2,3-BPG, P_i and total ATP during incubations (Figure 6). The initial upwardly concave nature of the total 2,3-BPG curve was consistent with the activation by P_i of the phosphatase and was similar to that found by Momsen and Vestergaard-Bogind [13]. The gradual decline in total ATP that occurred during the time course was in conformity with the findings of Rapoport et al. [10].

Cells were also incubated with a high (≈ 5 mM) concentration of P_i to determine whether phosphate activation occurred *in vivo* (Figure 7). The pH-independence of the phosphatase [10] was also verified by incubating cells without Glc at pH_i 6.8 (Figure 7). There was the possibility that HPA, present at high concen-

Figure 3 Time course of the C-3 resonance of 2,3-BPG from the ^{13}C NMR spectra shown in Figure 1

trations in all incubations, could have been responsible for activation of the phosphatase. However incubations performed with and without HPA showed no significant differences in the rate of 2,3-BPG decline (Figure 7).

Simulation of metabolite time courses

Simulations of the experimental time courses were made using the model described in [5]. Initially, using parameter values for BPGS/P that were consistent with the available experimental parameters (Table 1), it was not possible to produce simulations that matched the NMR-derived data. Hence a process of iterative parameter refinement was undertaken to estimate the parameters for BPGS/P which would be valid descriptors of the situation *in vivo*. The simulations that are shown in Figures 2 and 5–7 are the resulting ‘best fit’ simulations. These were made using the parameter values that are reported in [5] and in Tables 1–3. In fitting simulated time courses to the data, only the time courses shown in Figures 2 and 6 were considered. However, the reasonable fit between simulations and the data shown in Figures 5 and 7 indicated the wider applicability of these parameter values. (The assumptions underlying all the simulations are given in the Experimental section.) It is important to note that the values of the Michaelis–Menten parameters were refined by making changes to the unitary rate constants. A change made to one unitary parameter would affect the values of a number of the Michaelis–Menten parameters because of their highly inter-dependent nature.

Synthase activity

It was not possible to simulate the decrease in total 2,3-BPG concentration that occurred during the incubation with [$U\text{-}^{13}\text{C}$]Glc unless a large co-operative inhibition by protons was included in the model of the synthase activity (see the Experimental section). As noted above, there is some controversy

extra-cellular distribution of HPA as a function of time. The ratio of the intra- and extra-cellular signals is a measure of the Donnan ratio. (C) Rapidly pulsed ^{31}P -NMR spectra showing the decline in total 2,3-BPG, the production of P_i , and the maintenance of ATP concentration during the time course. Full experimental details and NMR acquisition parameters are given in the Experimental section. The time given beside each spectrum refers to the time at which half of the transients had been acquired for each spectrum.

Figure 4 (A) pH_i , (B) ΔpH and (C) the Donnan ratio (r) as a function of time from the mean time course shown in Figure 2

Each data point is the mean ($n = 4$) $\pm$ S.D.

about the pH-dependence of this enzyme. Rapoport et al. [10] reported that the decline in 2,3-BPG with pH is due to a strong co-operative inhibition of the synthase activity by protons. They based their argument on the pH-independence of the phosphatase activity, together with a report that 1,3-BPG concentrations were not diminished at lower pH values in rabbit erythrocytes [49]. However, in response to this work, Momsen and Vestergaard-Bogind [13] measured 1,3-BPG concentrations in human red cells as a function of pH. They did find a rapid decline in 1,3-BPG with a decline in pH and claimed that this was sufficient to

Figure 5 Concentrations of Glc C-1 (A) and 2,3-BPG C-3 (B) as a function of time and pH

The initial pH values for each time course were as follows: $\square$, pH_i 6.8; $\blacktriangle$, pH_i 7.0; $\triangle$, pH_i 7.1; $\bullet$, pH_i 7.2; $\circ$, pH_i 7.3. The continuous lines are the results of simulations of the corresponding metabolite concentrations as described in the Experimental section.

account for the pH-dependent decline in 2,3-BPG. The cause of this discrepancy is unclear; however, the fact that 1,3-BPG is involved in reactions which involve P_i , H^+ , NAD, NADH, MgATP, and MgADP, indicates that its concentration will most probably be sensitive to slight changes in intracellular conditions. Simulation of the experimental conditions used in the present study indicated that [1,3-BPG] increased during the time course. With this constraint, the only way to emulate the 2,3-BPG decline was to assume a co-operative inhibition of the synthase by H^+ . It is acknowledged that the validity of this finding depends entirely on the validity of the glycolytic model. In favour of the model is its ability to predict erythrocyte behaviour under a wide range of conditions [5]. In addition, with the assumed pH-dependence, the model predicts the steady-state concentrations of 2,3-BPG as a function of pH that were observed by others ([10]; Figure 2 in [6]). Also, there was a favourable match between simulations and experimental time courses for incubations starting at different pH values (Figure 6).

Phosphatase activity

The incubation of erythrocytes in the absence of Glc allowed the study of the activity of the phosphatase under conditions where

Figure 6 Concentration of 2,3-BPG 2P (○), ATP (□), and P_i (△) as a function of time, from incubations of human erythrocytes without Glc at 37 °C, pH_i 7.2, in the presence of 10 mM HPA

Error bars represent $\pm$ S.D. ($n = 4$). The continuous lines are the results of simulations of the corresponding metabolite concentrations as described in the Experimental section.

Figure 7 Effect of P_i , pH, and HPA on the depletion of 2,3-BPG in human erythrocytes incubated without glucose at 37 °C

●, pH_i 7.2, 10 mM HPA; ○, pH_i 6.8, 10 mM HPA; □, pH_i 7.2, 0 mM HPA; and ■, pH_i 7.2, 0 mM HPA, $[P_i]_i = 5.0$ mM. Each point was the mean value from at least two time courses. Error bars are shown for ● ($n = 4$, $\pm$ S.D.) and ■ ($n = 2$, $\pm$ maximum deviation) time courses only. The time courses were calibrated so that $[2,3\text{-BPG}] = 7.03$ mM at the first time point. The continuous lines are the results of simulations of the corresponding metabolite concentrations as described in the Experimental section.

synthase activity was minimal. The initial upwardly concave time course of total 2,3-BPG agreed well with the *in vitro* behaviour of the enzyme, which indicated that the enzyme was activated by P_i (or more correctly, the enzyme requires P_i as the second

substrate). By considering this time course alone, it was not possible to refine the values of $K_{m,2,3\text{-BPG}}$, $K_{i(3\text{-PGA},2\text{-PGA})}$ and $k_{cat,r}$. However, to match time courses of 2,3-BPG in the presence of Glc as well, it was necessary to decrease the inhibitory effect of 2-PGA and 3-PGA in the simulations. The possibility of weaker inhibition by 3-PGA *in vivo* has been suggested by other authors [13,14]. And, by requiring the model to balance the rates of the synthase and phosphatase reactions in the normal *in vivo* steady state, it was necessary to increase the value of $K_{m,1,3\text{-BPG}}$ relative to $K_{m,2,3\text{-BPG}}$. The higher value that was found for $K_{m,2,3\text{-BPG}}$ was, however, in agreement with the apparent $K_{m,2,3\text{-BPG}}$ measured *in vivo* by Rapoport et al. [10]. It was also necessary to increase the $k_{cat,f}$ value for the phosphatase by a factor of ≈ 1.6 in order to match the simulation to the real data.

2-PGly as an *in vivo* activator of the phosphatase reaction

The refinement of the phosphatase parameters discussed in the previous section assumed the absence of 2-PGly *in vivo*, but the importance of 2-PGly as a regulator of 2,3-BPG concentration *in vivo* must be considered. There is debate about whether 2-PGly is actually present in erythrocytes; levels of $\approx 5 \mu\text{mol}\cdot\text{litre}^{-1}$ of erythrocytes⁻¹ [50,51] have been reported, while none was detected in other studies [52,53]. It has been noted that the reported concentration is very small and hence verification of its presence is difficult [54]. Attempts to discover the metabolic source of the detected 2-PGly have been unsuccessful. Although pyruvate kinase catalyses the production of 2-PGly from glycolate, its activity is insufficient to account for the reported concentrations of 2-PGly, given the activity of erythrocyte 2-phosphoglycolate phosphatase [55]. Also, the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, which catalyses the synthesis of 2-PGly in the presence of oxygen in plants, is absent in erythrocytes [54]. The existence of the 2-PGly phosphatase in the human erythrocyte [56] supports the notion that 2-PGly does have metabolic effects, including affecting the concentration of 2,3-BPG [9]. However, a study of both healthy and anaemic blood donors failed to find any correlation between 2-PGly phosphatase activity and 2,3-BPG concentrations. This suggests that the phosphatase is not a regulator of 2-PGly concentration [57]. Therefore, it seems most likely that 2-PGly phosphatase has been 'naturally selected' to protect the cell against any 2-PGly that transiently and inadvertently forms via pyruvate kinase and glycolate [54,56] or is ingested in the diet from plant material.

From the simulations it seemed unlikely that 2-PGly plays a role in activating the phosphatase *in vivo* under the conditions of the present experiments. With the finding that the value of $K_{i(3\text{-PGA},2\text{-PGA})}$ is much lower *in vivo*, it was only necessary to increase the reported *in vitro* value for $k_{cat,r}$ by a small amount to simulate successfully the experimental results. If 2-PGly was included in the calculations, the simulated time courses did not match the experimental ones at all well; e.g., by decreasing the value of K_{m,P_i} it was possible to simulate rates of 2,3-BPG hydrolysis that were similar to the experimental ones by assuming that 2-PGly was present at micromolar concentrations. This change caused the initial part of the simulated 2,3-BPG decline curve to have less curvature. This was considered to be due to the effect of decreasing the phosphate activation of the enzyme.

Contribution of PGM to the rate of production of ¹³C-labelled 2,3-BPG

As discussed in the Introduction, it has long been known that the rate of production of labelled 2,3-BPG that results from the incubation of human erythrocytes with labelled glucose is not

Scheme 2 Four possible paths (A–D) of ^{13}C -label exchange between 2,3-BPG and 2- and 3-PGA that can occur via the reaction mechanisms for PGM and BPGS/P (see Scheme 1)

*Denotes the ^{13}C -labelled metabolite.

solely related to the activity of 2,3-BPG synthase. In the present work, the initial rate of production of labelled 2,3-BPG was about twice that of the synthase reaction at pH 7.2. It has previously been assumed that PGM is responsible for this effect [17,18]. However, given the fact that BPGS/P has mutase activity as well, and that the phosphatase and synthase activities share part of the mutase mechanism, BPGS/P may catalyse the exchange of label between 2,3-BPG and 3-PGA or 2-PGA as well. In other words, BPGS/P can catalyse the production of ^{13}C -labelled 2,3-BPG via a mechanism other than the synthase (Scheme 2).

In order to investigate the relative contributions of PGM and BPGS/P to the non-synthase production of labelled 2,3-BPG it was initially assumed that PGM operates via a simple reversible Michaelis–Menten mechanism in which 2,3-BPG is not involved (see [5]). Specifically, it was initially assumed that PGM does not participate in label exchange. In order for the rate of labelled 2,3-BPG production not to be overestimated, the rate constant defining the rate of phosphorylation of BPGS/P by 2,3-BPG had to be decreased to 0.65 s^{-1} . This rate constant is approximately equal to $k_{\text{cat,r}}$ for the mutase activity, and the value of 0.65 s^{-1} is slightly lower than the lowest experimentally determined value (Table 1). This indicated that PGM may not play a significant role in label exchange. Indeed when the full PGM reaction was included in the model (Scheme 1), it was possible to find a set of unitary rate constants that were consistent with the Michaelis–Menten parameters and which predicted minimal label exchange at PGM (Table 2).

To provide additional evidence for label exchange between 2,3-BPG and 3-PGA *in vivo*, haemolysates were prepared from

erythrocytes preincubated with $[\text{U-}^{13}\text{C}]\text{Glc}$ so that they contained pools of 2,3- $[\text{U-}^{13}\text{C}]\text{BPG}$. The time course of 2,3-BPG decline was then monitored by both ^{13}C and ^{31}P NMR spectroscopy. After $\approx 2\text{ h}$, 3-PGA was added and the label exchange between 2,3-BPG and 3-PGA was monitored (Figure 8). Figure 8 shows a gradual decline in the concentration of 2,3-BPG as the haemolysate was incubated in the absence of substrates. On addition of 3-PGA the concentration of total 2,3-BPG increased, whereas the concentration of labelled 2,3-BPG declined rapidly and then gradually increased. The total 2,3-BPG time course is explained by the synthesis of 2,3-BPG from 3-PGA. For this to occur, a molecule of 3-PGA must form 1,3-BPG via phosphoglycerate kinase, consuming an ATP in the process. It is surmised that the initial rapid decline in labelled 2,3-BPG is due to label exchange between 3-PGA and 2,3-BPG catalysed by the mutase reactions of BPGS/P, and possibly PGM. After this initial rapid exchange, the concentration of labelled 2,3-BPG begins to rise again as 2,3-BPG continues to be synthesized from the pools of labelled and unlabelled 3-PGA.

Changes to parameters other than those from the 2,3-BPGS/P model

Phosphofructokinase

Given that the values of the binding (dissociation) constants of phosphofructokinase for the allosteric effectors P_i and $\text{Glc}(1,6)\text{P}_2$ appear to be much larger *in vivo* than *in vitro* [5], there was the possibility that the other allosteric effectors would bind more weakly as well. It was found that, if the relevant allosteric

Figure 8 Time course of ^{13}C -labelled 2,3-BPG (○) and total 2,3-BPG (△) in a haemolysate incubated at 37 °C that had been prepared by preincubating erythrocytes with $[\text{U-}^{13}\text{C}]$ glucose

Unlabelled 3-PGA (10 mM) was added at $t = 120$ min. The sharp decrease in labelled 2,3-BPG concentration upon the addition of 3-PGA was due to label exchange between 2,3-BPG and 3-PGA. A time course for labelled 2,3-BPG in an incubation in which no 3-PGA was added is also shown (●). Note that both time courses were representative of the data from several experiments.

binding constants were increased by an order of magnitude, the model was able to match more closely the Glc decline at various initial pH values. Hence the larger values were chosen for all simulations. However, these parameter values had little influence on the choice of the final 'consensus' parameters of BPGS/P.

Pyruvate kinase

The importance of pyruvate kinase in regulating 2,3-BPG levels has long been recognized [58]. For example, an abnormally high activity of pyruvate kinase causes 2,3-BPG concentrations to fall. It was possible, then, that a marked increase in the activity of pyruvate kinase during the time course (Figures 1–3) caused the decline in 2,3-BPG rather than the co-operative H^+ inhibition of 2,3-BPG synthase. Two possible allosteric effectors that were ignored in the model of pyruvate kinase [5] were P_i and 2,3-BPG. P_i is an activator [59], while 2,3-BPG is an inhibitor [60], of pyruvate kinase. Thus the combination of the accumulation of P_i and the decline of 2,3-BPG during the time course may increase the activity of pyruvate kinase. However, the model that was used for pyruvate kinase was one in which the enzyme is predominantly present in the relaxed or R state at the beginning of the time course, and hence any additional allosteric activation would be ineffectual in lowering 2,3-BPG concentrations. These possible effectors were therefore ignored.

ATPase

It was found that, in order to simulate the data shown in Figure 2, k_{ATPase} had to be increased by 80% from the value initially chosen. This same high rate was then used for all the simulations shown in Figure 5. At lower values of k_{ATPase} there was a 'phosphate catastrophe'. In other words, the rate of phosphate

production was not sufficient to meet the rate of phosphate utilization at glyceraldehyde-3-phosphate dehydrogenase. This resulted in an incessant decline in $[\text{P}_i]$ with a concomitant shut-down of glycolysis. Whether the increased rate of ATP consumption is real, or an artifact of the model, is not known. It was, however, not possible to simulate the results unless this increase was made. In support of this assumption, however, is the fact that total ATP concentrations predicted by the model still favourably matched the experimental ones.

For simulations of metabolite time courses in erythrocytes incubated without Glc, k_{ATPase} was initially chosen to be $\approx 30\%$ lower than the normal *in vivo* value. This is in agreement with a previous finding that cells incubated in the absence of glucose consume ATP at a lower rate than cells incubated with glucose [10]. By increasing k_{ATPase} at a rate of 10% of the initial value/h, the time courses of ATP and P_i became more closely matched. This modification had little effect on the time course of total 2,3-BPG concentration, and hence it had little effect on the parameter values determined for 2,3-BPGS/P.

Lag phase of labelled Lac production

When erythrocytes were incubated with labelled Glc, there was a lag phase of $\approx 1\text{--}2$ h before an approximately linear time course of labelled Lac occurred. In simulations it was not possible to reproduce this lag phase. This lag phase was observed in both the Lac C-1 and Lac C-3 resonances, indicating that it was unlikely to be an artifact due to spectral-peak overlap.

Percentage of the glycolytic flux that passes through the 2,3-BPG shunt

From the set of parameters for BPGS/P (Table 1) that were found to be consistent with the experimental data presented above, and in conjunction with the metabolic model presented in [5], it was possible to estimate the percentage of glycolytic flux that passes through the 2,3-BPG shunt under normal *in vivo* conditions. The calculated value was 19%; this value is on the low end of the range found by previous authors using estimates of 2,3-BPG phosphatase activity, without accounting for phosphate activation or 3-PGA inhibition (20–25% of the glycolytic flux [10,15,16]). Moreover, the value is significantly lower than those estimates of 2,3-BPG synthase activity made without correction for 2,3-BPG label exchange (38% [22]; 27% [21]).

CONCLUSIONS

By using NMR spectroscopy, the concentrations of ^{13}C -labelled glucose, Lac, and 2,3-BPG, as well as total concentrations of 2,3-BPG, P_i , and ATP, that resulted from the incubation of erythrocytes with and without $[\text{U-}^{13}\text{C}]$ Glc, were monitored. These experiments verified previous observations that the rate of the 2,3-BPG phosphatase reaction is virtually independent of pH [10] and that the reaction is activated by P_i *in vivo* [13]. Also, by altering the initial pH of the glucose incubation mixtures, it was shown directly that the 2,3-BPG synthase activity is highly dependent on pH.

Using the detailed metabolic model presented in [5], simulations of the NMR-derived data were made. By iteratively adjusting the parameter values of the BPGS/P model, it was possible to characterize kinetically this enzyme *in vivo*. It was found that: (1) the pH-dependence of the synthase activity results largely from a strong co-operative inhibition of the synthase activity by protons; (2) 3-PGA and 2-PGA are much weaker inhibitors of 2,3-BPG phosphatase *in vivo* than *in vitro*;

(3) The K_m of 2,3-BPGS/P for 2,3-BPG is significantly higher than that measured *in vitro*; (4) the maximal activity of the phosphatase *in vivo* is approximately twice as high as the maximal activity of the phosphatase *in vitro* when P_i is the sole activator (second substrate); (5) 2-PGly appears to play no role in the activation of the phosphatase *in vivo*.

Using these newly determined kinetic parameters, it was estimated that the percentage of glycolytic carbon flux that passes through the 2,3-BPG shunt is 19% in the normal *in vivo* steady state.

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