A ^1H n.m.r. study of the kinetic properties expressed by glyceraldehyde phosphate dehydrogenase in the intact human erythrocyte

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(Received 4 May 1982/Accepted 13 September 1982)

Glyceraldehyde phosphate dehydrogenase is one of four glycolytic enzymes in the human erythrocyte that together can catalyse exchange of isotope between the C-2 position of lactate and solvent. Detailed measurements of the exchange can be made by using a non-invasive spin-echo p.m.r. method that has been described previously [Brindle, Brown, Campbell, Foxall & Simpson (1982) *Biochem. J.* **202**, 589–602]. By studying the dependence of the exchange on the activity of an individual enzyme, the specific isotope-exchange equilibrium velocity of the enzyme can be measured. Suggestions that glyceraldehyde phosphate dehydrogenase is bound to the membrane in the intact cell, and that it may, under certain conditions, be rate-limiting for glycolytic flux, were examined in the present study by comparing the exchange properties expressed by the enzyme *in situ* and *in vitro*. It is concluded that glyceraldehyde phosphate dehydrogenase is not rate-limiting for glycolytic flux and that it is unlikely that a significant fraction of the enzyme is bound to the erythrocyte membrane *in situ*.

Measurements of glyceraldehyde phosphate dehydrogenase binding to erythrocyte 'ghosts' have revealed a single high-affinity binding site located on the cytoplasmic pole of the integral membrane protein, 'band-3 protein' (Yu & Steck, 1975). Fructose biphosphate aldolase (EC 4.1.2.13) (Strapazon & Steck, 1977), phosphofructokinase (EC 2.7.1.11) (Higashi *et al.*, 1979), haemoglobin (Salhany *et al.*, 1980) and catalase (EC 1.11.1.6) (Aviram & Shaklai, 1981) have also been shown to bind at this site. The affinity of these enzymes for the membrane is modulated by their substrates. The effect of this on the enzymes' kinetic properties has led to speculation that membrane binding may be important to cellular metabolism (Kant & Steck, 1973; Strapazon & Steck, 1977; Karadsheh & Uyeda, 1977; Higashi *et al.*, 1979; Gillies, 1982; Tsai *et al.*, 1982). A consistent observation, however, is that all of these proteins are almost totally eluted from the membrane at physiological ionic strength. This has led some workers to suggest that membrane binding is simply an artifact of the hypo-osmotic conditions used to prepare the membranes (Maretzki *et al.*, 1974).

In order to assess better the possible physiological relevance of membrane binding observed *in*

vitro, attempts have been made to determine the amount of glyceraldehyde phosphate dehydrogenase bound to the membrane in the intact cell. These studies have included an electron-microscopic investigation of the distribution of radiolabelled enzyme (Solti *et al.*, 1981), cross-linking of the enzyme to the 'band-3-protein' site *in situ* (Keokitichai & Wrigglesworth, 1980) and investigation of the kinetics of enzyme release from detergent-lysed cells (Kliman & Steck, 1980). In the two last-mentioned studies, between 50 and 60% of the enzyme was estimated to be bound to the erythrocyte membrane *in situ*.

Another suggestion concerning glyceraldehyde phosphate dehydrogenase is that it may be catalysing a reaction that is rate-determining for glycolytic flux and therefore a possible control point in erythrocyte glycolysis (Mills & Hill, 1971; Wang & Alaupovic, 1980). These proposals, however, which were based on studies of the enzyme *in vitro*, do not meet with universal agreement (Rose & Warms, 1970; Maretzki *et al.*, 1973).

We have been using ^1H n.m.r. to study $^1\text{H}/^2\text{H}$ isotope-exchange reactions catalysed by human erythrocyte enzymes. By comparing the properties expressed by the enzymes *in situ* and *in vitro*, the intracellular environment can be investigated (Simpson *et al.*, 1982). Solvent isotope exchange at the lactate C-2 position, which is catalysed by a coupled system of four glycolytic enzymes, can be used to

Abbreviations used: V_{C2X} , measured equilibrium velocity for the exchange of isotope at the lactate C-2 position; t_{null} , time at which peak inversion occurs in an isotope-exchange time course followed by ^1H n.m.r.

measure the kinetic properties of the individual enzymes *in vitro* (Brindle *et al.*, 1982). In the present paper we show that the technique can be used to measure the isotope-exchange properties of glyceraldehyde phosphate dehydrogenase *in situ*. The likelihood of membrane binding in the cell and the role of the enzyme in the control of erythrocyte glycolysis are assessed on the basis of its observed kinetic properties.

Experimental

Materials and general methods

Details of the materials used and the preparation of L-[2-³H]lactate are given in Brindle *et al.* (1982).

Human erythrocytes were prepared as described previously (Brindle *et al.*, 1982). All buffers were made with ¹H₂O and contained 10 mM-glucose. Cell washes were performed at room temperature.

Determinations of enzyme activities were made as described by Beutler (1975), except that the assays were performed at pH 7.4. Enzyme activities are expressed in terms of units (μmol of substrate consumed/min). Measurements of erythrocyte metabolite concentrations were performed as described by Beutler (1975), except for assays of lactate and pyruvate. The latter were determined in cell supernatants prepared by diluting the cells in an equal volume of Krebs-Ringer buffer. Contaminating haemoglobin present in the supernatants was removed by precipitation with 2% (w/v) HClO₄. For the pyruvate assays the non-neutralized extract (0.5 ml) was added to 0.4 ml of 0.5 M-imidazole/HCl buffer, pH 7.0, and incubated for 2 min at 37°C. NADH (0.1 ml of a 2 mM solution) in the same buffer was then added, and an initial absorbance reading was recorded at 340 nm. Approx. 10 units of bovine heart lactate dehydrogenase were then added, and the absorbance was recorded after incubation for 5 min at 37°C. The change in absorbance was corrected for the changes observed with blank samples. The final pH in the assay system was approx. 6.3. The lactate assays were performed with a Sigma lactate assay kit. The non-neutralized extract was diluted so that approx. 25 nmol of lactate was added to the assay system. Absorbance measurements were made with a Unicam SP.800A u.v. spectrophotometer. All assays were performed at 37°C.

Rabbit muscle fructose bisphosphate aldolase and triose phosphate isomerase and bovine heart lactate dehydrogenase, obtained as suspensions in (NH₄)₂SO₄ solution, were dialysed against 0.1% EDTA/1 mM-2-mercaptoethanol before use. Unless otherwise specified, human erythrocyte glyceraldehyde phosphate dehydrogenase was used as a suspension in (NH₄)₂SO₄ solution. In the exchange system studied *in vitro*, when the concentration of

glyceraldehyde phosphate dehydrogenase was varied, the concentration of (NH₄)₂SO₄ was also varied so that all samples contained the same (NH₄)₂SO₄ concentration.

P.m.r. methods

N.m.r. experiments were performed as described previously (Brindle *et al.*, 1982). In mixtures of the enzymes in ¹H₂O, the water resonance was suppressed by applying a saturating pulse at the water resonance frequency between successive repetitions of the 90°-τ-180°-τ pulse sequence. All experiments were performed at 37°C.

Preparation of human erythrocyte glyceraldehyde phosphate dehydrogenase

The enzyme was prepared as described by Eby & Kirtley (1979). Membranes, which were freed of fructose bisphosphate aldolase activity by incubation with fructose 1,6-bisphosphate (Strapazon & Steck, 1977), were used as the starting material in the preparation of glyceraldehyde phosphate dehydrogenase and for studies on the kinetic properties of this enzyme when bound to the membrane (see the Results and discussion section). Column fractions containing the enzyme were pooled and concentrated by ultrafiltration, and the enzyme was resuspended in 2.6 M-(NH₄)₂SO₄.

Measurement of isotope-exchange equilibrium velocities

Exchange of isotope at the lactate C-2 position with solvent was monitored by following the inversion of the spin-coupled lactate methyl resonance in the spin-echo spectrum (Brindle *et al.*, 1982). In an exchange system studied *in vitro*, where there is no change in the lactate concentration, the first-order rate constant for the exchange was obtained from a least-squares fit of the exchange time course. In cells, where there is endogenous lactate production, V_{C2X} was estimated from the point of peak inversion (see Brindle *et al.*, 1982). At this point 50% of the lactate is protonated at the C-2 position and 50% is deuterated.

V_{C2X} is related to the equilibrium velocities of the four enzymes catalysing the exchange by a double-reciprocal relationship (Brindle *et al.*, 1982). For glyceraldehyde phosphate dehydrogenase, one of the enzymes involved in the exchange:

$$1/V_{C2X} = 1/V_R + 2/\alpha \cdot A$$

where $1/V_R$ is the sum of the reciprocals of the equilibrium velocities of the other enzymes involved in the exchange and A is the concentration of glyceraldehyde phosphate dehydrogenase expressed in units of activity measured spectrophotometrically. If V_{C2X} is measured at different concentrations of the enzyme, the specific equilibrium velocity of the

enzyme, α , can be obtained from the slope of a plot of $1/V_{C2X}$ versus $1/A$ (Brindle *et al.*, 1982). In the studies described in the present paper, however, α was estimated from a direct plot analysis (Eisenthal & Cornish-Bowden, 1974, 1978; also see Fig. 1), since this yields a more reliable estimate of its value than does the double-reciprocal plot. The median intercepts on the x - and y -axes of the direct plot were estimated numerically by using a computer program that was also used to estimate an error in α . The errors were estimated by the procedure described by Cleland (1967) and are quoted, not as true standard errors, but as an index of the relative precision of a particular determination.

Titration of glyceraldehyde phosphate dehydrogenase activity in the erythrocyte

Packed-cell suspensions (70–80% haematocrit) were incubated for 2 to 15 min at 37°C with 20–100 μ M-iodoacetate and then washed five times with 5 vol. of ice-cold Krebs–Ringer buffer (Krebs & Henseleit, 1932) containing 1 mM-2-mercaptoethanol to remove excess iodoacetate. The cells were then assayed for their glyceraldehyde phosphate dehydrogenase activity. By varying the incubation conditions, cell populations could be prepared with a range of glyceraldehyde phosphate dehydrogenase activities. Before use in isotope-exchange experiments, the cells were stored for 2–3 days at 4°C in order to raise the concentrations of the triose phosphates. Assays of enzyme activity at the completion of isotope-exchange determinations showed that there had been no loss of enzyme activity during incubation at 4°C. Assays of the other enzymes involved in the exchange showed that iodoacetate treatment inhibited only glyceraldehyde phosphate dehydrogenase (Brindle, 1982). This is consistent with previous studies of this metabolic inhibitor (Webb, 1966). Lactate transport across the cell membrane is not inhibited by iodoacetate (Deuticke *et al.*, 1978).

Titration of glyceraldehyde phosphate dehydrogenase activity in vitro

Exchange systems studied *in vitro* containing 15 units of glyceraldehyde phosphate dehydrogenase/ml were incubated for 1 h at 37°C with iodoacetate concentrations in the range 1–10 μ M. Control incubations lasting 3 h showed that there was no further loss of activity after 1 h. By this method systems were prepared *in vitro* containing glyceraldehyde phosphate dehydrogenase activities in the range 2–15 units/ml.

Results and discussion

In a previous paper (Brindle *et al.*, 1982) we showed that ^1H spin-echo n.m.r. could be used to monitor

exchange of isotope at the lactate C-2 position. The exchange is catalysed by a coupled system of four glycolytic enzymes: fructose biphosphate aldolase, triose phosphate isomerase (EC 5.3.1.1), glyceraldehyde phosphate dehydrogenase and lactate dehydrogenase (EC 1.1.1.27). In the erythrocyte the exchange requires the presence of relatively high triose phosphate and fructose 1,6-bisphosphate concentrations. The specific isotope-exchange equilibrium velocity of an individual enzyme can be measured in the intact erythrocyte if the active enzyme concentration is changed by specific irreversible inhibition. In the case of glyceraldehyde phosphate dehydrogenase the concentration of the active enzyme in the human erythrocyte can be varied by incubation of the cells for various periods of time with low concentrations (20–100 μ M) of iodoacetate. Preparation of the erythrocytes and titration of their glyceraldehyde phosphate dehydrogenase activity are described in the Experimental section.

Determinations of the equilibrium velocity of glyceraldehyde phosphate dehydrogenase in vitro

The determinations were performed as described previously (Brindle *et al.*, 1982). The results in Table 1 show that the specific equilibrium velocity of the enzyme is determined predominantly by the $[\text{NAD}^+]/[\text{NADH}]$ ratio. The total concentration of coenzyme used corresponds to the estimated free concentration in the erythrocyte (Simpson *et al.*, 1982). Performing the determinations in the presence of 1 mM- P_i , changing the solvent from $^1\text{H}_2\text{O}$ to $^2\text{H}_2\text{O}$ (and using L-[U- ^3H]lactate), lowering the salt concentration and increasing the added NAD^+ concentration up to 80 μ M had no significant effect on the equilibrium velocity of the enzyme (Brindle, 1982). Titration of the enzyme activity with iodoacetate in the system studied *in vitro* (Table 2) gave similar results to those obtained by changing the added enzyme concentration.

In a simulation of the intracellular environment possible protein–protein interactions should also be considered. It has been suggested, for example, that the enzyme may be membrane-bound in the intact cell.

Effect of membrane binding

Membranes containing the bound enzyme were prepared as described in the Experimental section and used *in vitro* in a system that had a low ionic strength. Under these conditions a significant fraction of the enzyme was bound to the membrane, but there was negligible binding of rabbit muscle fructose biphosphate aldolase that was added to the system with 1 mM-fructose 1,6-bisphosphate. Addition of 80 mM-KCl to the system resulted in a large increase in V_{C2X} and an increase in the fraction of

Table 1. *Glyceraldehyde phosphate dehydrogenase equilibrium-velocity determinations in vitro*

The exchange system studied *in vitro* contained 100 mM-Tris/HCl buffer, pH 7.4, 0.5 mM-EDTA, 80 mM-KCl and 3 mM-dithiothreitol in H_2O . In part (a), with the exception of the result obtained after addition of NADH, the system contained approx. 100 units of triose phosphate isomerase/ml, 30 units of lactate dehydrogenase/ml and 1 unit of fructose biphosphate aldolase/ml. The added fructose 1,6-bisphosphate concentration was 700 μM and the L-[2- ^3H]lactate concentration was 12 mM. In the case where NADH was added there were unknown concentrations of the enzymes and the lactate concentration was 6 mM. In system (b) there were approx. 100 units of triose phosphate isomerase/ml, 1–2 units of fructose biphosphate aldolase/ml and 100 units of lactate dehydrogenase/ml. The added NAD^+ concentration was 15 μM and the added lactate concentration was 12 mM. The $[\text{NAD}^+]/[\text{NADH}]$ ratio was calculated by assuming an equilibrium constant for the reaction catalysed by lactate dehydrogenase of 1.11×10^{-11} (Williamson *et al.*, 1967). The reaction catalysed by glyceraldehyde phosphate dehydrogenase has a negligible effect on the ratio. The equilibrium concentrations of the triose phosphates were calculated by assuming an equilibrium constant for the fructose biphosphate aldolase reaction of 1.2×10^{-4} (Herbert *et al.*, 1940) and an equilibrium constant for the triose phosphate isomerase reaction of 22 (Veech *et al.*, 1969). Equilibrium-velocity determinations were performed as described in the Experimental section and in Brindle *et al.* (1982). V_{C2X} was measured at four different enzyme concentrations. Exchange at the lactate C-2 position was monitored until 30–50% of the lactate had exchanged.

(a) Effect of NAD^+/NADH concentration

Added NAD^+ concentration (μM)	Added pyruvate concentration (μM)	$[\text{NAD}^+]/[\text{NADH}]$ ratio (calculated)	Glyceraldehyde phosphate dehydrogenase equilibrium velocity ($\mu\text{mol}/\text{min}$ per unit of enzyme)
10	—	1.3	0.067 ± 0.014
20	—	2.0	0.070 ± 0.008
10	15	5.0	0.031 ± 0.002
Added NADH			
concentration (μM)			
10	10	0.6	0.176 ± 0.034

(b) Effect of triose phosphate concentration

Added fructose 1,6-bisphosphate concentration (mM)	Estimated total triose phosphate concentration (mM)	Glyceraldehyde phosphate dehydrogenase equilibrium velocity ($\mu\text{mol}/\text{min}$ per unit of enzyme)
0.4	0.57	0.062 ± 0.018
0.6	0.78	0.072 ± 0.012
1.0	1.12	0.074 ± 0.008
2.0	1.79	0.074 ± 0.001
3.0	2.31	0.066 ± 0.002

unbound glyceraldehyde phosphate dehydrogenase. This suggested that the enzyme is inhibited in the bound state. This was confirmed by measuring V_{C2X} in the presence of various amounts of membrane preparation. If it is assumed that the bound enzyme shows no activity and the measured V_{C2X} is plotted against the free enzyme concentration, then the direct plot analysis yields a value for the specific equilibrium velocity of $0.057 \pm 0.009 \mu\text{mol}/\text{min}$ per unit of free enzyme. This is similar to that measured for the enzyme under identical conditions in the absence of membranes, i.e. $0.065 \pm 0.021 \mu\text{mol}/\text{min}$ per unit. This suggests that the assumption made was correct, i.e. that the enzyme is completely inhibited when bound to the membrane. Complete inhibition of activity when the enzyme is bound to the membrane has been observed by Tsai *et al.* (1982). If NADH is added to the system [part (B) of Table 3] the fraction of unbound enzyme is

increased, and an equilibrium-velocity determination under these conditions, which again assumes that the bound enzyme is inactive, yields an equilibrium velocity for the free enzyme of $0.170 \pm 0.001 \mu\text{mol}/\text{min}$ per unit. Elution of the enzyme by NADH suggests that inhibition on membrane binding is the result of occlusion of the NADH-binding site.

Determinations of the equilibrium velocity of glyceraldehyde phosphate dehydrogenase in situ

The system studied *in vitro* is at chemical equilibrium, and the equilibrium concentrations of the intermediates involved in the exchange can be predicted from the published values for the equilibrium constants of the enzyme-catalysed reactions (Brindle, 1982). The system in the erythrocyte is not at equilibrium, but in a near-equilibrium steady state. It is essential that, during exchange measurements,

Table 2. Iodoacetate titration of glyceraldehyde phosphate dehydrogenase activity *in vitro*

Systems (a) and (b) contained 100 mM-Tris/HCl buffer, at the specified pH, 0.5 mM-EDTA, 80 mM-KCl and 3 mM-dithiothreitol. System (c) contained 100 mM-glycylglycine/imidazole buffer, pH 7.4, 0.5 mM-EDTA, 3 mM-dithiothreitol and 0.15 M-KCl. All the systems contained approx. 100 units of triose phosphate isomerase/ml, approx. 100 units of lactate dehydrogenase/ml and 1 unit of fructose biphosphate aldolase/ml. In systems (a) and (b) glyceraldehyde phosphate dehydrogenase was added as a suspension in $(\text{NH}_4)_2\text{SO}_4$ solution, and in system (c) the enzyme was dialysed before use. The added fructose 1,6-bisphosphate, NAD^+ and lactate concentrations in all three systems were 1 mM, 15 μM and 12 mM respectively. The solvent was $^1\text{H}_2\text{O}$. Details of the iodoacetate titrations are given in the Experimental section. Equilibrium-velocity determinations were made by measuring V_{C2X} at four different active enzyme concentrations.

pH of the exchange system	Glyceraldehyde phosphate dehydrogenase equilibrium velocity ($\mu\text{mol}/\text{min}$ per unit of enzyme)
(a) 7.2	0.095 ± 0.022
(b) 7.4	0.082 ± 0.005
(c) 7.4	0.116 ± 0.031

the labelled intermediates are either retained near their steady-state concentrations or that, if there are variations, these cause negligible changes in the equilibrium velocities of the enzymes. Metabolite assays during exchange measurements showed that there were no significant changes in the concentrations of the triose phosphates (Table 4) or in the $[\text{NAD}^+]/[\text{NADH}]$ ratio (Table 5).

The equilibrium velocity of the enzyme was estimated from a direct plot of V_{C2X} , corrected for the effect of endogenous lactate present at $t = 0$ (see Brindle *et al.*, 1982), versus enzyme activity (see Table 4 and Fig. 1). The ratio of the median intercepts on the x - and y -axes of the points of line intersections on the direct plot gives the specific equilibrium velocity of the enzyme. The results are shown in Table 4.

Measurements of lactate production (Table 4) show that, despite a 10-fold decrease in the activity of glyceraldehyde phosphate dehydrogenase, there is no significant change in the rate of lactate production. This indicates that the enzyme cannot be rate-determining for glycolytic flux in the human erythrocyte under these conditions.

Comparison of the kinetic properties expressed by the enzyme *in situ* and *in vitro*

Studies on the enzyme *in vitro* (Table 1) show that the activity of the enzyme is largely determined

Table 3. Effect of binding to membrane on the equilibrium velocity of glyceraldehyde phosphate dehydrogenase

The membranes, depleted of fructose 1,6-bisphosphate aldolase activity, were prepared as described in the Experimental section. The membranes were washed in the exchange-system buffer before use. This buffer had the following composition: 30 mM-imidazole/HCl buffer, pH 7.2, 3 mM-dithiothreitol and 1.5 mM-EDTA in $^1\text{H}_2\text{O}$. The exchange system contained this buffer plus approx. 1 unit of fructose biphosphate aldolase/ml, 100 units of triose phosphate isomerase/ml, 100 units of lactate dehydrogenase/ml, 20 mM-nicotinamide and 1 mM-fructose 1,6-bisphosphate. In part (A) 15 μM - NAD^+ was added to the exchange system and in part (B) 10 μM - NAD^+ plus 10 μM - NADH . Exchange time courses were initiated by the addition of 12 mM-L-[2- ^3H]-lactate. The fractions of enzyme free and bound were assessed, after an exchange measurement, by separating the free and the bound forms by centrifugation for 5 min at 37°C in an Eppendorf 5412 micro-centrifuge. The glyceraldehyde phosphate dehydrogenase activity measured in the supernatant was taken to represent the concentration of free enzyme. The concentration of bound enzyme was assessed by resuspending the membrane pellet, assaying the total activity present and subtracting from this the activity measured in the supernatant.

	Glyceraldehyde phosphate dehydrogenase activity (units/ml)		Fraction bound	V_{C2X} ($\mu\text{mol}/\text{min}$ per ml)
	Free	Bound		
(A)	1.3	3.8	0.75	0.029
	2.2	8.9	0.80	0.049
	3.5	12.1	0.78	0.067
	4.3	15.5	0.78	0.070
(B)	2.4	2.7	0.47	0.083
	5.6	5.6	0.50	0.108*
	9.9	9.9	0.50	0.120

* The fractions of enzyme free and bound were in this case estimated from the volume of membrane preparation added to the exchange system and the results of the other two assays.

by the $[\text{NAD}^+]/[\text{NADH}]$ ratio. Between a ratio of 1 and 2 the enzyme shows an equilibrium velocity of about 0.07–0.08 $\mu\text{mol}/\text{min}$ per unit. This increases to 0.18 $\mu\text{mol}/\text{min}$ per unit at a ratio of 0.6, and decreases to 0.03 $\mu\text{mol}/\text{min}$ per unit as the ratio increases between 2 and 5. Equilibrium-velocity determinations on the enzyme *in situ* yield values in the range 0.1–0.2 $\mu\text{mol}/\text{min}$ per unit (Table 4) at an estimated $[\text{NAD}^+]/[\text{NADH}]$ ratio of about 4 (Table 5). The values found *in situ* are therefore slightly higher than those found *in vitro*. There are a number of possible explanations for this discrepancy.

Estimations of the equilibrium velocity of the

Table 4. *Glyceraldehyde phosphate dehydrogenase equilibrium-velocity determinations in situ*

Sections (A), (B) and (C) show the results from three different cell preparations. The estimated equilibrium velocities for the enzyme (see the text) were $0.160 \pm 0.018 \mu\text{mol/min}$ per unit (section A), $0.204 \pm 0.020 \mu\text{mol/min}$ per unit (section B) and $0.110 \pm 0.017 \mu\text{mol/min}$ per unit (section C). Exchange time courses were initiated by the addition of $12 \text{ mM-L-[2-}^3\text{H]lactate}$ to an erythrocyte suspension prewarmed to 37°C . The concentration of endogenous solvent-labelled lactate present at $t=0$ was estimated by accumulating a spectrum before the addition of the labelled lactate. The exchange was monitored for up to 90 min by accumulating successive 112-scan 2 min spectra. The rate of lactate production was measured by monitoring the growth in the lactate peak height in cells to which no labelled lactate had been added. The peak intensity was calibrated in terms of lactate concentration by adding a known concentration of $\text{L-[U-}^3\text{H]lactate}$. Growth in the lactate peak height, which was observed to be linear, was monitored before and after addition of the standard lactate solution. Triose phosphate assays were performed on parallel incubations as described in the Experimental section. Apart from the triose phosphate concentrations, the concentrations of lactate and the rates and activities shown are quoted per ml of solvent water. This was calculated by assuming that 72% of the erythrocyte volume is solvent water (Eilam & Stein, 1974). The haematocrit of the cell preparations was in the range 70–74%.

	Glyceraldehyde phosphate dehydrogenase activity (units/ml of solvent water)	Estimated total triose phosphate concentration during exchange measurements ($\mu\text{mol/ml}$ of cell water)	Rate of lactate production ($\mu\text{mol/min}$ per ml of solvent water)	Estimated V_{C2X} (corrected for effect of endogenous lactate present at $t=0$) ($\mu\text{mol/min}$ per ml of solvent water)
(A)	1.4		0.065	0.083
	4	0.9	—	0.199
	5	0.8	0.063	0.218
	9	0.8	0.060	0.297
	33	0.9	—	0.383
(B)	6	0.9	0.066	0.273
	11	0.8	0.063	0.342
	20	0.9	0.058	0.390
	64	0.6	0.058	0.478
(C)	5	0.2	0.073	0.098
	6	0.4	—	0.214
	13	0.5	—	0.315
	14	0.5	—	0.360
	56	0.5	0.068	0.459

enzyme from exchange time courses predicted by a model of the exchange system (see the Appendix and Table 6), which is based on the kinetic properties of the isolated enzymes *in vitro* (Brindle, 1982), indicate that chemical flux *in situ* will lead to a 15–20% overestimate of the enzyme's equilibrium velocity in the cell.

Additional pathways for exchange *in situ* could affect the estimated equilibrium velocity of the enzyme if they exchange isotope with the $\text{NAD}^+ + \text{NADH}$ pool. However, the basic characteristics of the exchange, i.e. total inhibition after iodoacetate inactivation of glyceraldehyde phosphate dehydrogenase and loss of exchange after depletion of the triose phosphates (Brindle *et al.*, 1982), indicate that the exchange pathway, originally proposed by Rose & Warms (1969), is correct.

Membrane binding in the cell, in a manner similar to that observed *in vitro*, clearly cannot explain the higher equilibrium velocities observed *in situ*, since binding *in vitro* results in inhibition of the enzyme. These results in fact indicate that it is unlikely that a significant fraction of the enzyme is bound to the

membrane *in situ*. Iodoacetate modification does not affect the membrane-binding properties of the enzyme (Kant & Steck, 1973; Solti & Freidrich, 1976).

Restricted diffusion in the cell could explain a higher equilibrium velocity *in situ*. The possibility that the rates of enzyme and substrate diffusion could limit the rates of certain cellular reactions to values lower than those actually observed has led to suggestions that there may be organization of enzymes in the cytosolic compartment of some cells [see, e.g., Ottaway & Mowbray (1977) and references cited therein]. It has been estimated that there are between 1.4 and 2.4×10^5 molecules of glyceraldehyde phosphate dehydrogenase per erythrocyte (Kant & Steck, 1973). If the erythrocyte volume is taken to be 0.086 pl (Weinstein, 1974) and 72% is solvent water (Eilam & Stein, 1974) then there are approximately 3×10^{15} molecules of enzyme/ml of cell water. Assuming an intracellular free NADH concentration of $2 \mu\text{M}$, diffusion coefficients for NADH and the enzyme of 10^{-5} and $10^{-7} \text{ cm}^2 \cdot \text{s}^{-1}$ respectively and radii of 0.3 and 4 nm

Table 5. *Lactate and pyruvate assays on cells used for isotope-exchange experiments*

Lactate (10 mM) was added to packed cells (80% haematocrit) in Krebs–Ringer buffer, pH 7.4, at 37°C. The cells had been previously incubated at 4°C in order to raise the concentrations of the triose phosphates. At the specified times, after addition of lactate, the metabolites were assayed in cell-free supernatants, prepared by diluting a sample of the cells with Krebs–Ringer buffer at room temperature and then centrifuging in an Eppendorf 5412 centrifuge for 2 min. The assays were performed as described in the Experimental section. The concentrations quoted are the averages of two determinations and were corrected for the excluded volume in the erythrocyte suspension by assuming that 72% of the erythrocyte volume is solvent water (Eilam & Stein, 1974). The $[NAD^+]/[NADH]$ ratio was calculated by assuming that lactate dehydrogenase catalyses a reaction at equilibrium in the human erythrocyte with an equilibrium constant of 1.11×10^{-11} (Williamson *et al.*, 1967). The intracellular pH was assumed to be 7.4 (Brown *et al.*, 1977).

Time (min)	Lactate concentration ($\mu\text{mol/ml}$ of solvent water)	Pyruvate concentration (nmol/ml of solvent water)	$[NAD^+]/[NADH]$ ratio (calculated)
10	10.5	9	3.1
20	11.1	12	3.9
30	10.9	12	3.9
40	13.6	14	3.7

respectively, then we can estimate an enzyme–substrate collision frequency, by using the equation shown by Metzler (1977), of approx. 55 molecules/ms per molecule of enzyme. An isotope-exchange equilibrium velocity of 0.1 mM/min per unit of enzyme is equivalent to an enzyme–substrate collision frequency, assuming all collisions are successful, of approx. 30 molecules/ms per molecule of enzyme. Although there are many uncertainties inherent in this type of calculation, these results suggest that the reaction may be diffusion-limited. Although the calculated collision frequency is apparently sufficiently high to support the observed isotope-exchange velocity, the possibility of differences in enzyme and substrate diffusion, between the environments *in situ* and *in vitro*, leading to differences in the equilibrium velocities observed in the two systems cannot be denied on the basis of these calculations.

Conclusions

Although the equilibrium velocities observed *in vitro* are lower than those observed *in situ*, we do

Table 6. *Model simulations of the effect of lactate production on the observed point of peak inversion in an exchange time course*

The correction for endogenous solvent-labelled lactate present at $t=0$ has been described previously (Brindle *et al.*, 1982). The correction was applied to the observed points of peak inversion in the presence of lactate production. The conditions used in the model were as follows: solvent, 110 mM; dihydroxyacetone phosphate, 1 mM; glyceraldehyde 3-phosphate, 45 μM ; NAD^+ , 10 μM ; $NADH$, 10 μM ; fructose 1,6-bisphosphate, 3 mM; lactate, 13 mM. The initial concentrations of the deuterated intermediates were all zero apart from the lactate, of which 12 mM was deuterated. The equilibrium velocities were given the following values: fructose bisphosphate aldolase, 1.0 mM/min; the recondensation reaction catalysed by fructose bisphosphate aldolase, 0.0 mM/min; triose phosphate isomerase, 15.0 mM/min; lactate dehydrogenase, 1.5 mM/min. The rate of lactate production was set at 0.06 mM/min, and the equilibrium velocity of glyceraldehyde phosphate dehydrogenase was varied as shown in the Table. The value of t_{null} represents the time at which peak inversion occurs in an exchange time course. Setting the equilibrium velocity for the recondensation reaction at 1.0 mM/min had no effect on the predictions of the model.

Equilibrium velocity of glyceraldehyde phosphate dehydrogenase	Observed t_{null} (min) without lactate production	Observed t_{null} (min) with lactate production	t_{null} corrected for effects of endogenous solvent-labelled lactate present at $t=0$
10	21	20	21
5	22	21	22
2	27	25	26
1	35	32	33
0.75	40	37	39
0.50	51	45	47
0.25	82	66	69

not believe that this represents a fundamental difference between the environment *in situ* and that *in vitro*. The most likely explanations for the discrepancy are the effect of glycolytic flux on equilibrium-velocity determinations *in situ* and possibly an overestimate of the cellular $[NAD^+]/[NADH]$ ratio. Membrane binding in the cell clearly cannot explain the discrepancy, since this would be expected to result in inhibition of the enzyme. It is therefore unlikely that a significant fraction of the enzyme is bound to the membrane *in situ*, at least under the conditions used here of high triose phosphate concentrations and a high $[NAD^+]/[NADH]$ ratio.

Failure to inhibit lactate production despite a 10-fold decrease in the activity of the enzyme shows

Fig. 1. Direct plot of V_{C2X} versus glyceraldehyde phosphate dehydrogenase activity in the intact erythrocyte. The exchange data were taken from section (A) in Table 4. The point obtained at the lowest enzyme activity is an outlier (Eisenthal & Cornish-Bowden, 1974) and it is not shown in the Figure.

that it cannot be rate-determining for glycolytic flux in the human erythrocyte.

This is a contribution from the Oxford Enzyme Group, which is supported by the Science and Engineering Research Council. K. M. B. is supported by the Science and Engineering Research Council, and R. J. S. by the Wellcome Trust. We thank Mr. Rod Porteous and Mr. Nick Soffe for their expert technical assistance with regard to construction and maintenance of the high-field n.m.r. spectrometers.

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APPENDIX

Kinetic model of the exchange system

In the following equations g represents the rate of lactate production or rate of chemical flux through the C-2 exchange system in C_3 equivalents. The V terms represent the isotope-exchange equilibrium velocities of the denoted enzymes, and the X terms the fractional labelling of denoted metabolites. The enzymes and metabolites are represented by subscripted abbreviations. The abbreviations are as follows: LDH, lactate dehydrogenase; GAPDH, glyceraldehyde phosphate dehydrogenase; TIM, triose phosphate isomerase; ALD, fructose biphosphate aldolase; ALD(RECON), the recondensation reaction between dihydroxyacetone phosphate and glyceraldehyde 3-phosphate catalysed by fructose biphosphate aldolase, which results in labelling at the 4-position of the fructose 1,6-bisphosphate molecule; SOL, solvent (1H_2O or 2H_2O); FDP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; LAC, lactate. $NADH_{(A)}$ represents NADH labelled on the A-face of the nicotinamide

ring and is produced by reaction of labelled lactate with unlabelled NAD^+ in the reaction catalysed by lactate dehydrogenase. $NADH_{(B)}$ represents B-face-labelled NADH and is produced by reaction of labelled glyceraldehyde 3-phosphate with unlabelled NAD^+ in the reaction catalysed by glyceraldehyde phosphate dehydrogenase. $NADH_{(A,B)}$ represents the double-labelled species. Label can be regarded as either hydrogen or deuterium. $[FDP]_0$ and $[LAC]_0$ are the concentrations, at $t=0$, of fructose 1,6-bisphosphate and lactate respectively.

The exchange system can be described by the following set of simultaneous non-linear differential equations (Foxall, 1981; Brindle, 1982). The equations were solved by numerical integration. The computer program employed, which was written in FORTRAN IV, was run on an Oxford University VAX computer. The program called a routine, available on the Oxford system, that integrates a 'stiff' system of differential equations by using a variable-order variable-step Gear method (Garfinkel *et al.*, 1977).

$$\begin{aligned}
 ([FDP]_0 - gt/2) \cdot dX_{FDP}/dt &= V_{ALD(RECON)}(X_{GAP} - X_{FDP}) - gX_{FDP}/2 \\
 [DHAP] \cdot dX_{DHAP}/dt &= V_{TIM}(X_{GAP} - X_{DHAP}) + V_{ALD}(X_{SOL} - X_{DHAP}) + gX_{SOL}/2 \\
 &\quad - gX_{DHAP}/2 \\
 [GAP] \cdot dX_{GAP}/dt &= V_{TIM}(X_{DHAP} - X_{GAP}) + V_{GAPDH}(X_{NADH(A,B)} + X_{NADH(0,A)} - X_{GAP}) \\
 &\quad + V_{ALD(RECON)}(X_{FDP} - X_{GAP}) + gX_{DHAP}/2 + gX_{FDP}/2 - gX_{GAP} \\
 [SOL] \cdot dX_{SOL}/dt &= V_{ALD}(X_{DHAP} - X_{SOL}) \\
 [NAD^+] \cdot dX_{NAD}/dt &= V_{LDH}(X_{NADH(0,A)} + X_{NADH(A,B)} - X_{NAD^+}) \\
 &\quad + V_{GAPDH}(X_{NADH(A,0)} + X_{NADH(A,B)} - X_{NAD^+}) \\
 &\quad + g(X_{NADH(A,B)} + X_{NADH(0,A)} - X_{NAD^+})
 \end{aligned}$$

$$\begin{aligned}
 [\text{NADH}] \cdot dX_{\text{NADH}(*,0)}/dt = & V_{\text{LDH}}[X_{\text{LAC}}(1 - X_{\text{NAD}^+}) - X_{\text{NADH}(*,0)}] \\
 & + V_{\text{GAPDH}}[X_{\text{NAD}^+}(1 - X_{\text{GAP}}) - X_{\text{NADH}(*,0)}] \\
 & + g[X_{\text{NAD}^+}(1 - X_{\text{GAP}}) - X_{\text{NADH}(*,0)}]
 \end{aligned}$$

$$\begin{aligned}
 [\text{NADH}] \cdot dX_{\text{NADH}(*,*)}/dt = & V_{\text{LDH}}(X_{\text{LAC}}X_{\text{NAD}^+} - X_{\text{NADH}(*,*)}) \\
 & + V_{\text{GAPDH}}(X_{\text{NAD}^+}X_{\text{GAP}} - X_{\text{NADH}(*,*)}) \\
 & + g(X_{\text{NAD}^+}X_{\text{GAP}} - X_{\text{NADH}(*,*)})
 \end{aligned}$$

$$\begin{aligned}
 [\text{NADH}] \cdot dX_{\text{NADH}(0,*)}/dt = & V_{\text{LDH}}[X_{\text{NAD}^+}(1 - X_{\text{LAC}}) - X_{\text{NADH}(0,*)}] \\
 & + V_{\text{GAPDH}}[X_{\text{GAP}}(1 - X_{\text{NAD}^+}) - X_{\text{NADH}(0,*)}] \\
 & + g[X_{\text{GAP}}(1 - X_{\text{NAD}^+}) - X_{\text{NADH}(0,*)}]
 \end{aligned}$$

$$([\text{LAC}]_0 + gt) \cdot dX_{\text{LAC}}/dt = (V_{\text{LDH}} + g)(X_{\text{NADH}(*,0)} + X_{\text{NADH}(*,*)}) - V_{\text{LDH}}X_{\text{LAC}}$$

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