Use of α -toxin from *Staphylococcus aureus* to test for channelling of intermediates of glycolysis between glucokinase and aldolase in hepatocytes

Marta CASCANTE*, Josep J. CENTELLES* and Lorraine AGIUS†¹

*Departament de Bioquímica i Biologia Molecular, Facultat de Química, Universitat de Barcelona, Martí i Franquès 1, 08028-Barcelona, Spain, and

†Department of Diabetes, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH, U.K.

We investigated whether hepatocytes permeabilized with α -toxin from *Staphylococcus aureus* are a valid model for studying the channelling of intermediates of glycolysis between glucokinase and triosephosphate isomerase. These cells are permeable to 2-aminoisobutyrate, ATP, glucose 6-phosphate (Glc6P) and fructose 2,6-bisphosphate [Fru(2,6)P₂], but maintain cell integrity in the presence of ATP as judged by the retention of cytoplasmic enzymes. During incubation with 25 mM glucose, an ATP-generating system and saturating concentrations of Fru(2,6)P₂, rates of detritiation of [2-³H]glucose and [3-³H]glucose were similar. Exogenous Glc6P (1 mM) and to a lesser extent fructose 6-phosphate, but not Fru(1,6)P₂, decreased the rate of detritiation of [3-³H]glucose. During incubation with

25 mM glucose and Glc6P (0.2–1 mM), with either [3-³H]glucose or [3-³H]Glc6P as labelled substrate, there was dilution of metabolism of [3-³H]glucose with increasing Glc6P but no overall increase in glycolytic flux from glucose and Glc6P, indicating that glycolysis is apparently saturated with Glc6P despite the permeability of the cells to this metabolite. These findings could be explained by partial channelling of Glc6P between glucokinase and glycolysis in the presence of saturating concentrations of Fru(2,6)P₂. They provide an alternative explanation for the concept that there is more than one Glc6P pool.

Key words: aldolase, fructose 2,6-bisphosphate, glucose 6-phosphate.

INTRODUCTION

Hepatic glucokinase (hexokinase IV) exerts strong control on glycogen synthesis and glycolysis [1,2]. This is explained by the reciprocal control from glucokinase in conjunction with a 68 kDa regulatory protein [3,4], and also because there is no feedback inhibition of glucokinase by its product hexose 6-phosphate, either at the level of enzyme inhibition [5] or through increased binding of glucokinase to the regulatory protein [2]. The glucose 6-phosphate [Glc6P] content of hepatocytes increases when glucokinase is overexpressed [6] and also when glucokinase dissociates from the regulatory protein in the presence of precursors of fructose 1-phosphate [Fru1P] [7], and it decreases with glucose 6-phosphatase overexpression [8]. It is established that Glc6P regulates glycogen synthesis by the allosteric activation of glycogen synthase and by enhancing the dephosphorylation of the enzyme [9]; it increases glycolytic flux, at least in part, by increasing the concentration of fructose 2,6-bisphosphate [Fru(2,6)P₂] [10,11]. However, it is unclear whether changes in Glc6P also affect glycolytic flux by a direct substrate effect.

Studies on purified enzymes and cell extracts have provided evidence that various glycolytic enzymes can interact with one another [12,13] or with cytoskeletal proteins [14]. Interactions between consecutive enzymes might enable the transfer of intermediates between enzymes without equilibration with the free pool, a mechanism described as channelling [15,16]. There remains considerable debate on the extent to which channelling occurs *in vivo* and on its physiological advantages [17,18].

Channelling of glycolytic intermediates has been studied in vascular smooth-muscle cells [19] and fibroblast cell lines [20–22] by permeabilization with dextran sulphate, which permits the diffusion of proteins of approx. 400 kDa. This permeabilization technique perturbs the plasma membrane and might therefore interfere with the intracellular organization of enzymes [20].

The aim of this study was to investigate whether there is evidence for channelling of intermediates of glycolysis between glucokinase and triosephosphate isomerase in hepatocytes. We used α -toxin, a 33 kDa protein from *Staphylococcus aureus*, because the pore-forming mechanism of this protein is well characterized. α -Toxin is a hydrophilic protein that inserts into the plasma membrane of mammalian cells and assembles to a heptamer with a transmembrane pore or channel [23,24]. These pores permit the passage of molecules of less than 2–3 kDa, but prevent protein leakage. The toxin has been used to study exocytosis in secretory cells [25] and autophagy [26], triacylglycerol synthesis [27] and channelling of the urea cycle [28] in hepatocytes. We demonstrate, with the use of α -toxin-permeabilized hepatocytes, that during incubation with 25 mM glucose and saturating concentrations of Fru(2,6)P₂, there is partial channelling of Glc6P with apparent saturation of glycolysis by Glc6P, despite the permeability of the cells to metabolic intermediates. These findings support the conclusion that the regulation of glycolysis by changes in Glc6P is largely indirect, through changes in Fru(2,6)P₂ without a substrate effect at the level of Glc6P. They provide an explanation for the apparent presence of more than one pool of Glc6P.

Abbreviations used: Glc6P, glucose 6-phosphate; Fru6P, fructose 6-phosphate; Fru(2,6)P₂, fructose 2,6-bisphosphate; Fru1P, fructose 1-phosphate; Fru(1,6)P₂, fructose 1,6-bisphosphate.

¹ To whom correspondence should be addressed (e-mail lorraine.agius@ncl.ac.uk).

MATERIALS AND METHODS

Materials

[2-³H]Glucose and [3-³H]glucose were from DuPont–NEN (Boston, MA, U.S.A.); 2-amino[1-¹⁴C]isobutyric acid was from Amersham International (Little Chalfont, Bucks., U.K.); bovine heart hexokinase was from Sigma (St Louis, MO, U.S.A.); other enzymes and cofactors were from Roche/Boehringer (Mannheim, Germany). α -Toxin from *S. aureus* [29] was kindly given by Dr E. A. Lehoux and Dr H. O. Spivey (Oklahoma State University, Stillwater, OK, U.S.A.).

Hepatocyte isolation and monolayer culture

Hepatocytes were isolated by collagenase perfusion of the liver of male Wistar rats (body weight 180–240 g) fed *ad libitum*. They were suspended in minimum essential medium containing 7% (v/v) neonatal-calf serum, seeded in 24-well plates at a density of 6×10^4 cells/cm² and cultured at 37 °C equilibrated in air/CO₂ (19:1) [30]. After attachment (approx. 4 h) the medium was replaced by serum-free medium containing 10 nM dexamethasone and 10 nM insulin. Experiments were performed after 16–20 h in culture.

Permeabilization of hepatocyte monolayers with α -toxin

A stock solution of α -toxin (1 mg/ml) was divided into aliquots and stored at –20 °C. Dilutions ranging from 0.5 to 25 μ g/ml were prepared on the day of the experiment in buffer A, which contained potassium glutamate (140 mM), Hepes (20 mM), NaCl (5 mM), MgSO₄ (5 mM), EGTA (1 mM) and BSA (10 mg/ml), pH 7.2. The culture medium in the 24-well plates was aspirated, and the hepatocyte monolayers were washed twice in 150 mM NaCl. They were then incubated for 10 min at 37 °C in buffer A supplemented with 2 mM MgATP²⁻, pH 7.2, and the concentrations of α -toxin indicated. On termination of these incubations the medium was aspirated and the monolayers were incubated in buffer A (without α -toxin) as described below.

Permeability of hepatocytes to 2-aminoisobutyrate

Hepatocyte monolayers were preincubated for 3 h in minimum essential medium containing 0.1 mM 2-amino[1-¹⁴C]isobutyrate (0.5 μ Ci/ml). They were then washed three times in 150 mM NaCl and incubated with α -toxin as described above. After a 10 min incubation with α -toxin the medium was collected for the determination of radioactivity. Additional incubations were performed with 0.04 mg/ml digitonin, for the determination of total radioactivity released. This concentration of digitonin causes the complete release of water-soluble metabolites within 10 min at 37 °C. The radioactivity released during permeabilization with α -toxin was expressed as a percentage of that released with digitonin.

Determination of enzyme release

Lactate dehydrogenase and phosphoglucosomerase were determined in the medium on termination of the 10 min incubation with α -toxin and after a 60 min incubation in buffer A without or with ATP, as indicated. Enzyme activities were determined spectrometrically [31] in the medium and in untreated cells; the activity in the medium was expressed as a percentage of total activity.

Determination of cellular ATP concentration

Hepatocyte monolayers were extracted by sonication in 200 μ l of 0.3 M HClO₄. The extracts were sedimented (13000 g, 15 min) and the supernatants were neutralized with 2 M KOH/0.2 M Hepes. ATP was assayed in the neutralized extracts fluorimetrically. The final assay cocktail contained 50 mM Tris/HCl, pH 8.0, 0.18 mM NADP, 20 mM MgCl₂, 5 mM glucose, 5 units/ml glucose-6-phosphate dehydrogenase and 12.5 units/ml hexokinase.

Preparation of [2-³H]Glc6P and [3-³H]Glc6P

[2-³H]Glc6P and [3-³H]Glc6P were prepared from the corresponding labelled glucose directly before use. The incubation mixture contained, in a final volume of 100 μ l, 0.1 mM glucose, 5 mM MgATP²⁻, 1 mM MgCl₂, 5 mM Hepes, 0.5 unit of bovine heart hexokinase, pH 7.2, and 20 μ Ci of either [2-³H]glucose or [3-³H]glucose. After incubation for 1 h at 30 °C, the assay mixture was centrifuged through concentrator tubes with 10000 kDa cut-off membrane (Vivaspin4; Vivascience, Lincoln, U.K.), to remove the hexokinase, which was washed once with buffer A. The protein-free filtrate was made up to 5 ml in buffer A. It was supplemented with glucose to 25 mM and MgATP²⁻ to 1.5 mM, and other additions as indicated. Complete conversion of glucose into Glc6P was confirmed by determination of the formation of ³H₂O after the addition of phosphoglucosomerase (5 units/ml) to incubations with 2-³H label.

Incubations with 2-³H-labelled or 3-³H-labelled glucose or Glc6P

After incubation for 10 min with α -toxin, the hepatocyte monolayers were incubated for either 30 or 60 min in buffer A containing 25 mM glucose, 1.5 mM ATP, 5 mM phosphocreatine, 5 units/ml creatine kinase and [2-³H]glucose, [3-³H]glucose, [2-³H]Glc6P or [3-³H]Glc6P (4 μ Ci/ml), and with Fru(2,6)P₂ and other additions as indicated. On termination of these incubations, the medium was removed and acidified with HCl to 0.1 M. For the determination of ³H₂O, 50 μ l samples of acidified medium were incubated in 0.5 ml conical tubes placed inside 6 ml mini-vials containing 750 μ l of water. The mini-vials were sealed tightly and incubated at 30 °C for 2 days. The ³H₂O that equilibrated in the 750 μ l of water in the mini-vial was determined and corrected for the recovery of ³H₂O. Rates of detritiation of glucose or Glc6P were linear for 60 min and are expressed as nmol of glucose or Glc6P metabolized/h per mg of cell protein (determined by a Lowry method [32]).

Results are expressed as means $\pm$ S.E.M. for the number of experiments indicated. Statistical analysis was by Student's paired *t* test.

RESULTS

Effects of α -toxin on the permeability to 2-aminoisobutyrate and on cellular ATP concentration

The effect of α -toxin concentration on the permeability of hepatocytes to 2-aminoisobutyrate was determined during a 10 min incubation. The half-maximal effect on the release of 2-aminoisobutyrate was at approx. 0.5 μ g/ml and the maximal effect at approx. 10 μ g/ml α -toxin (Figure 1). The cellular ATP content was determined in hepatocytes that were incubated for 10 min with various α -toxin concentrations in buffer A with 2 mM ATP, followed by a 60 min incubation in buffer A without α -toxin or ATP. The half-maximal effect on ATP depletion was

Figure 1 Release of 2-aminoisobutyrate during the treatment of hepatocytes with α -toxin

Hepatocytes were preincubated for 3 h with 0.1 mM amino[1- 14 C]isobutyrate (AIB). They were then washed and incubated at 37 °C for 10 min with the concentrations of α -toxin indicated. Release of 14 C label is expressed as a percentage of total radioactivity released with digitonin. Results are means $\pm$ S.E.M. for five experiments.

Figure 2 Effects of α -toxin on cellular ATP

Hepatocytes were preincubated for 10 min, with the concentrations of α -toxin indicated, in buffer A containing 2 mM ATP. They were then incubated for 60 min in buffer A without ATP. Results are means $\pm$ S.E.M. for four experiments.

at less than 1 μ g/ml α -toxin; higher concentrations (1–10 μ g/ml) decreased cellular ATP by 77–92% (Figure 2). These results show that α -toxin renders hepatocytes permeable to low-molecular-mass metabolites and that the cells remain permeable to ATP during a 60 min incubation without α -toxin.

Effects of ATP on enzyme release in α -toxin-permeabilized cells

Hepatocytes that had been permeabilized with α -toxin (1–25 μ g/ml) for 10 min did not show any release of either lactate dehydrogenase or phosphoglucosomerase at the end of the 10 min incubation (results not shown). When they were subsequently incubated for 60 min in buffer A with 1.5 mM ATP and an ATP-generating system, there was no significant release of enzyme activity in cells treated with 1–10 μ g/ml α -toxin and there was a small release in cells treated with 25 μ g/ml α -toxin. However, a marked release of both enzymes occurred during incubation for 60 min without ATP (Figure 3).

Figure 3 Effects of ATP on enzyme release from α -toxin-permeabilized hepatocytes

Hepatocytes were incubated for 10 min, with the concentrations of α -toxin indicated, in buffer A with 2 mM ATP. They were then incubated for 60 min in buffer A without (○) or with (●) 1.5 mM ATP, 5 mM phosphocreatine and 5 units/ml creatine phosphokinase. The release of lactate dehydrogenase (LDH) (A) or phosphoglucosomerase (PGI) (B), determined on termination of the 60 min incubation, is expressed as a percentage of total cellular activity. Results are means $\pm$ S.E.M. for five experiments. * $P < 0.05$.

Figure 4 Effects of α -toxin concentration and ATP on the detritiation of [2- 3 H]glucose and [3- 3 H]glucose

Hepatocytes were incubated for 10 min in buffer A containing 2 mM ATP and the α -toxin concentration indicated. They were then incubated for 60 min in buffer A containing 25 mM glucose and 100 μ M Fru(2,6) P_2 without (○, □) or with (●, ■) 1.5 mM ATP, 5 mM phosphocreatine, 5 units/ml creatine phosphokinase and either [2- 3 H]glucose (○, ●) or [3- 3 H]glucose (□, ■). Rates of detritiation are expressed as nmol of glucose/h per mg of protein. Results are means $\pm$ S.E.M. for five experiments.

Figure 5 Effects of Fru(2,6) P_2 on the detritiation of [2- 3 H]glucose and [3- 3 H]glucose

Hepatocytes were incubated for 10 min in buffer A with 2 mM ATP and either 5 μ g/ml (A) or 10 μ g/ml (B) α -toxin. They were incubated for 60 min in buffer A with 25 mM glucose and either [2- 3 H]glucose (●) or [3- 3 H]glucose (■), with 1.5 mM ATP, 5 mM phosphocreatine, 5 units/ml creatine kinase and the indicated concentrations of Fru(2,6) P_2 . Rates of detritiation are expressed as nmol of glucose/h per mg of protein. Arrows indicate the concentration of Fru(2,6) P_2 with half-maximal effect. Results are means $\pm$ S.E.M. for five experiments.

Effects of α -toxin and Fru(2,6) P_2 on glucose metabolism

To determine rates of glycolysis from glucose up to the level of triosephosphate isomerase we used 2- 3 H-labelled and 3- 3 H-labelled glucose (Figure 4). Tritium in the 2-position is lost in the reaction catalysed by phosphoglucose isomerase; tritium in the 3-position is lost at the level of triosephosphate isomerase [33]. Loss of 2- 3 H is incomplete during the conversion of Glc6P into Fru6P as a result of intramolecular transfer of tritium to the 1-position [33]; the detritiation of 2- 3 H might therefore underestimate the flux through phosphoglucose isomerase by up to 50%. In the absence of added Fru(2,6) P_2 , which is a potent activator of phosphofructokinase and inhibitor for fructose biphosphatase [10], rates of detritiation of [3- 3 H]glucose were low in comparison with the detritiation of [2- 3 H]glucose in the permeabilized cells (see Figure 5). The experiments shown in Figure 4 were therefore performed in the presence of 100 μ M Fru(2,6) P_2 .

In the absence of ATP there was a progressive decline in the detritiation of both [2- 3 H]glucose and [3- 3 H]glucose with increasing α -toxin (Figure 4). This is consistent with the depletion

Table 1 Effects of exogenous Glc6P, Fru6P and Fru(1,6) P_2 on the formation of 3 H $_2$ O from [2- 3 H]glucose and [3- 3 H]glucose

Monolayers of hepatocytes were permeabilized with 10 μ g/ml α -toxin and incubated for 60 min with 25 mM [3- 3 H]glucose, 200 μ M Fru(2,6) P_2 , 1.5 mM ATP, 5 mM phosphocreatine and 5 units/ml creatine phosphokinase, together with the additions indicated. Results are means $\pm$ S.E.M. for three or four experiments. Statistical analysis: * P < 0.08; ** P < 0.02 relative to controls with no additions.

Addition	Rate of formation of 3 H $_2$ O (nmol/h per mg of protein)	
	Detritiation of [2- 3 H]glucose	Detritiation of [3- 3 H]glucose
None	283 $\pm$ 20	254 $\pm$ 22
1 mM Glc6P	207 $\pm$ 19**	181 $\pm$ 16**
1 mM Fru6P	215 $\pm$ 19**	212 $\pm$ 22*
1 mM Fru(1,6) P_2	242 $\pm$ 20	252 $\pm$ 9

of ATP (Figure 2). In the presence of 1.5 mM ATP and an ATP-generating system there was an increase in rates of detritiation of glucose with increasing α -toxin. The increase was greater for [3- 3 H]glucose, and at elevated α -toxin the rates of detritiation of 3- 3 H and 2- 3 H were similar.

Figure 5 shows the effects of [Fru(2,6) P_2] on glucose metabolism. There was no effect of Fru(2,6) P_2 on the metabolism of [2- 3 H]glucose, but the detritiation of [3- 3 H]glucose increased with Fru(2,6) P_2 . Half-maximal stimulation by Fru(2,6) P_2 was at 130 or 40 μ M in cells permeabilized with 5 or 10 μ g/ml α -toxin respectively (Figure 5). The Fru(2,6) P_2 content of intact hepatocytes at elevated glucose concentration is between 100–150 pmol/mg protein [7] and corresponds to a concentration of 50–75 μ M, assuming 2 μ l of cell water per mg of cell protein. Consequently the stimulation by Fru(2,6) P_2 in cells permeabilized with 10 μ g/ml α -toxin is within the physiological range. The similar rates of metabolism of [2- 3 H]glucose and [3- 3 H]glucose at saturating concentrations of Fru(2,6) P_2 agree with studies on intact cells incubated in medium with 25 mM glucose [2].

Effects of Glc6P, Fru6P and Fru(1,6) P_2 on α -toxin-treated cells

To investigate whether 3 H-labelled intermediates formed from [2- 3 H]glucose or [3- 3 H]glucose could equilibrate with the free pool we determined the effects of added unlabelled Glc6P, Fru6P and Fru(1,6) P_2 (Table 1). Detritiation of [2- 3 H]glucose was significantly decreased by Glc6P and Fru6P; detritiation of [3- 3 H]glucose was decreased significantly by Glc6P (P < 0.02) and to a smaller extent by Fru6P (P < 0.08), but was unaffected by fructose 1,6-bisphosphate [Fru(1,6) P_2]. The decrease in formation of 3 H $_2$ O from [2- 3 H]glucose in the presence of Glc6P or Fru6P can be explained by the dilution of [2- 3 H]Glc6P and [2- 3 H]Fru6P by the added metabolites. An effect of Fru(1,6) P_2 on the loss of 2- 3 H would be expected if there were significant hydrolysis of Fru(1,6) P_2 to Fru6P (by fructose-1,6-bisphosphatase), in which case Fru(1,6) P_2 would have a similar effect to that of Fru6P. The decrease in formation of 3 H $_2$ O from [3- 3 H]glucose by added Glc6P, and to a smaller extent by Fru6P, suggests leakage at the level of Glc6P and possibly also at Fru6P. The lack of effect of Fru(1,6) P_2 suggests either that there is channelling of this metabolite between phosphofructokinase and aldolase or that flux through aldolase is first-order with respect to the intracellular concentration of Fru(1,6) P_2 .

Table 2 Metabolism of glucose and Glc6P in permeabilized hepatocytes incubated with glucose and Glc6P

Monolayers of hepatocytes were permeabilized with α -toxin (15 μ g/ml) and then incubated in medium containing 25 mM glucose, 200 μ M Fru(2,6) P_2 , 1.5 mM ATP, 5 mM phosphocreatine and 5 units/ml creatine phosphokinase, together with the concentrations of Glc6P indicated and one of [2- 3 H]glucose, [3- 3 H]glucose, [2- 3 H]Glc6P or [3- 3 H]Glc6P. Results are means $\pm$ S.E.M. for five experiments. Statistical analysis: * P < 0.05; ** P < 0.02 relative to controls with 0.02 mM Glc6P.

Glc6P concentration (mM)	Metabolism of glucose or Glc6P (nmol/h per mg of protein)			
	[2- 3 H]Glucose	[3- 3 H]Glucose	[2- 3 H]Glc6P	[3- 3 H]Glc6P
0.02	316 $\pm$ 21	356 $\pm$ 34	4 $\pm$ 1	4 $\pm$ 1
0.2	288 $\pm$ 16*	304 $\pm$ 18*	44 $\pm$ 6**	48 $\pm$ 4**
0.5	232 $\pm$ 22**	256 $\pm$ 16**	102 $\pm$ 11**	96 $\pm$ 8**
1.0	196 $\pm$ 29**	180 $\pm$ 14**	160 $\pm$ 19**	130 $\pm$ 14**

Metabolism of Glc6P by α -toxin-treated cells

To investigate further the effect of added Glc6P on glycolysis in α -toxin-treated cells, experiments were performed with 25 mM glucose and various concentrations of Glc6P in the presence of [3 H]glucose or [3 H]Glc6P to determine the metabolism of both substrates (Table 2). These experiments showed that there was a decrease (P < 0.02) in formation of 3 H $_2$ O from [2- 3 H]glucose or [3- 3 H]glucose with increasing [Glc6P] from 0.2 to 1 mM and that this was associated with an increase (P < 0.02) in the metabolism of [2- 3 H]Glc6P and [3- 3 H]Glc6P. The sum of detritiation of [2- 3 H]glucose and [2- 3 H]Glc6P at 1 mM Glc6P was similar to the rate of metabolism of [2- 3 H]glucose at the lowest concentration of Glc6P (0.02 mM). Similar results were obtained with [3- 3 H]glucose and [3- 3 H]Glc6P (Table 2). The increase in metabolism of [2- 3 H] or [3- 3 H]Glc6P with increasing [Glc6P] (0.2–1 mM) is evidence that α -toxin-treated cells are permeable to Glc6P. The lack of increase in total metabolism of glucose plus Glc6P metabolism with increasing [Glc6P] suggests that, during the incubation of α -toxin-treated cells with 25 mM glucose, phosphoglucose isomerase and glycolysis are apparently saturated with Glc6P, despite the permeability of the cells to phosphorylated intermediates.

DISCUSSION

Studies on purified proteins have provided evidence for specific interactions between enzymes that catalyse consecutive reactions [12,13]. If these interactions occur in the intact cell, there is the possibility of transfer of intermediates between enzymes without complete equilibration with the free pool. This behaviour is described as channelling [16]. The urea cycle is one of the most clearly documented examples of channelling of intermediates [28,34,35]. The urea-cycle enzymes involved in channelling do not show rapid adaptive changes in subcellular compartmentation; this cycle is therefore an example of a channelled pathway in which the component enzymes are statically organized. Channelling is of particular interest in metabolic pathways involving enzymes that partition between free and bound states, such as aldolase [36,37], or that translocate between different subcellular compartments, such as glucokinase [38,39].

The aims of this study were to investigate whether α -toxin from *S. aureus* is a suitable permeabilizing agent with which to study the kinetic properties of enzymes *in situ* and to determine

whether there is evidence for channelling of glycolysis between glucokinase and triosephosphate isomerase in hepatocytes. The following conclusions can be drawn from the first part of the study: first, that the leakage of low-molecular-mass compounds from the cell (aminoisobutyrate and ATP) can be achieved at fairly low concentrations of α -toxin (1–5 μ g/ml); secondly, that the presence of extracellular ATP is essential for maintaining cell integrity; and thirdly, that the affinity of phosphofructokinase for exogenous Fru(2,6) P_2 is a better index of the permeability of the cell to metabolites than the loss of aminoisobutyrate or ATP determined at a single time point. Because the stimulation of detritiation of [3- 3 H]glucose by exogenous Fru(2,6) P_2 in cells treated with 10 μ g/ml α -toxin occurred at 3-fold lower concentrations of regulator than in cells treated with 5 μ g/ml α -toxin, we infer that the permeability of the cells is greater at 10 μ g/ml. Although there was higher permeability at 25 μ g/ml, as shown by the higher rate of metabolism of [2- 3 H]glucose, there was some enzyme release under these conditions. The selection of the α -toxin concentration is therefore a compromise between maximum permeability and minimum disruption of enzyme organization. The main limitation of this experimental system for studying channelling of metabolic intermediates is that it does not allow the accurate determination of the concentrations of intracellular metabolites or the rate of diffusion of metabolites into or out of the permeabilized cells.

In circumstances in which an added extracellular metabolite [e.g. Fru(1,6) P_2] has no effect on the rate of metabolic flux, two explanations are possible. One is that the metabolite is channelled and is therefore not diluted by the exogenous pool. The other is that the rate of entry of the extracellular metabolite is slower than the rate of metabolism, such that the intracellular concentration is lower than the extracellular concentration and flux through the pathway is first-order with respect to the intracellular metabolite concentration. Unless one can exclude the latter possibility, channelling cannot be established unequivocally.

To test whether there is evidence for incomplete equilibration of intermediates with the free pool, we determined the effects of unlabelled intermediates [Glc6P, Fru6P and Fru(1,6) P_2] on the production of 3 H $_2$ O from [3- 3 H]glucose. There was greatest dilution with Glc6P, less dilution with Fru6P and no dilution with Fru(1,6) P_2 (1 mM). Because Fru(2,6) P_2 stimulated glycolysis under the same conditions at 25-fold lower concentrations, we infer that the cells were permeable to sugar biphosphates and that the lack of dilution of label in the presence of Fru(1,6) P_2 suggests channelling of this intermediate between phosphofructokinase and aldolase. An alternative explanation would be that flux through aldolase is first-order with respect to the intracellular concentration of Fru(1,6) P_2 . Although this seems unlikely in view of the permeability of the cells to Glc6P and Fru(2,6) P_2 , we cannot fully exclude this possibility. Isotope dilution techniques provide information on the extent of equilibration of labelled intermediates with the free pool but not on whether channelling occurs by a direct transfer mechanism as opposed to microcompartmentation (proximity effects) [15]. It seems unlikely that the lack of effect of exogenous Fru(1,6) P_2 on the dilution of endogenously formed intermediates is due to microcompartmentation of phosphofructokinase, because exogenous Fru(2,6) P_2 activated the same enzyme in these experiments.

No evidence for the translocation of liver phosphofructokinase between different subcellular compartments in hepatocytes has been reported. However, the enzyme is phosphorylated by cAMP-dependent protein kinase. This covalent modification is apparently not associated with a change in the kinetic properties of the soluble enzyme with regard to affinity for either substrate or

Fru(2,6) P_2 [10,11]. Thus the possibility that phosphorylation might affect its interaction with aldolase merits consideration. Aldolase partitions between a bound and a free state in hepatocytes [36] and in Swiss 3T3 cells [37]. Either of these states might involve association with phosphofructokinase. A possible advantage of direct transfer of Fru(1,6) P_2 between phosphofructokinase and aldolase would be that flux through aldolase during glycolysis can be achieved at a lower free concentration of Fru(1,6) P_2 . Consequently the internal pool of Fru(1,6) P_2 can be smaller and the transient time for the sequence would therefore be shorter [18].

The finding that Glc6P, in contrast with Fru(1,6) P_2 , significantly decreased the detritiation of [3- 3 H]glucose is evidence against the tight channelling of Glc6P between glucokinase and the glycolytic sequence comprising phosphoglucose isomerase, phosphofructokinase and aldolase. The lack of stimulation of detritiation of [2- 3 H]glucose by Fru(2,6) P_2 is also evidence against the tight coupling of flux through glucokinase and the glycolytic sequence. This is consistent with the fact that Glc6P formed from glucokinase is metabolized by pathways other than glycolysis. However, two sets of data are in conflict with a model that does not involve channelling (incomplete leakage) of Glc6P between glucokinase and glycolysis: first, that during metabolism of [3- 3 H]glucose (25 mM) and [3- 3 H]Glc6P (0.20–1 mM) there is apparent saturation of phosphoglucose isomerase with Glc6P, and secondly, the activity of phosphoglucose isomerase in hepatocytes [31] is more than an order of magnitude higher than the activity of phosphofructokinase or glycolytic flux. However, the similar rates of metabolism of [2- 3 H]Glc6P and [3- 3 H]Glc6P in cells permeable to phosphorylated intermediates are not consistent with a model in which phosphoglucose isomerase is much more active than glycolytic flux. A possible explanation for these apparently paradoxical findings is that at saturating concentrations of Fru(2,6) P_2 there is partial channelling of Glc6P between glucokinase and phosphoglucose isomerase and of Fru6P between phosphoglucose isomerase and phosphofructokinase. The established function of Fru(2,6) P_2 in liver is as an allosteric activator of phosphofructokinase 1 and an inhibitor of fructose-1,6-bisphosphatase for switching from gluconeogenesis to glycolysis [10], although other functions have also been proposed [11]. Niemeyer et al. reported that Fru(2,6) P_2 inhibits glucokinase activity determined in liver extracts with the glucose-6-phosphate dehydrogenase coupled assay [40]. This effect was later shown to be due to Fru(2,6) P_2 -dependent metabolism of Glc6P via endogenous phosphoglucose isomerase and phosphofructokinase rather than by the auxiliary enzyme in the coupled assay [41]. This effect was more marked when Fru(2,6) P_2 was present throughout the extraction procedure [40]. These observations are consistent with the present hypothesis that Fru(2,6) P_2 might have a role in promoting or preserving the interaction of glucokinase with phosphoglucose isomerase and phosphofructokinase.

This study supports the conclusion that there is partial channelling of glycolytic intermediates between glucokinase and phosphoglucose isomerase/phosphofructokinase. Several studies have suggested the existence of more than one Glc6P pool [42–44]. If Glc6P is partly channelled through glycolysis as well as through other pathways such as gluconeogenesis, glycogenolysis and glycogen synthesis, then the apparent existence of more than one Glc6P pool can be explained by a single pool of free Glc6P.

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