Substrate modulation of aldolase B binding in hepatocytes

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The binding properties of hepatic aldolase (B) were determined in digitonin-permeabilized rat hepatocytes after the cells had been preincubated with either glycolytic or gluconeogenic substrates. In hepatocytes that had been preincubated in medium containing 5 mM glucose as sole carbohydrate substrate, binding of aldolase to the hepatocyte matrix was maximal at low KCl concentrations (20 mM) or bivalent cation concentrations (1 mM Mg^{2+}) and half-maximal dissociation occurred at 50 mM KCl. Preincubation of hepatocytes (for 10–30 min) with glucose or mannose (10–40 mM), fructose, sorbitol, dihydroxyacetone or glycerol (1–10 mM), caused a leftward shift of the salt dissociation curve (maximum binding at 10 mM KCl; half-maximum dissociation at 35 mM KCl) but did not affect the proportion of bound enzyme at low or high KCl concentrations. Galactose and 2-deoxyglucose had no effect on aldolase binding. Inhibitors of glucokinase (mannoheptulose and glucosamine) suppressed the effects of glucose but not the effects of sorbitol, glycerol or dihydroxyacetone. Glucagon suppressed the effects of glucose, fructose and dihydroxyacetone but not glycerol. Poly(ethylene glycol) (PEG) (2–10%), added to the permeabilization medium, increased aldolase binding and caused a rightward shift in the salt dissociation curve. In the presence of PEG (6–8%), the

effects of substrates on aldolase dissociation were shifted to higher salt concentrations (50–100 mM versus 35 mM KCl). The effects of substrates (added to the intact cell) on aldolase binding to the permeabilized cell could be mimicked by addition of the phosphorylated derivatives of these substrates to the permeabilized cell. Of the intermediates tested dihydroxyacetone phosphate and fructose 1,6-bisphosphate were the most effective at dissociating aldolase (A_{50} values of 20 μ M and 40 μ M respectively). Other effective intermediates in order of decreasing potency were fructose 1-phosphate, glycerol 3-phosphate, glucose 1,6-bisphosphate/fructose 2,6-bisphosphate. These results show that aldolase B binds to the hepatocyte matrix by a salt-dependent mechanism that is influenced by macromolecular crowding and metabolic intermediates. Maximum binding occurs when hepatocytes are incubated in the absence of glycolytic and gluconeogenic substrates and minimum binding occurs in the presence of substrates that are precursors of either fructose 1,6-bisphosphate or triose phosphates. Since the bound form of aldolase represents a kinetically less active state it is proposed that aldolase binding and dissociation may be a mechanism for buffering the concentrations of metabolic intermediates.

INTRODUCTION

Many glycolytic enzymes show various degrees of intracellular compartmentation. This has been studied by histochemical and immunocytochemical techniques, sedimentation studies with subcellular organelles, binding to cytoskeletal proteins, enzymes and permeabilized cells, and other techniques [1–4]. Aldolase was one of the first actin-binding proteins to be described [5,6]. Three aldolase isoenzymes are expressed in vertebrate tissues encoded by different genes [7]. Aldolase A is the ubiquitous form and the predominant isoenzyme in muscle; aldolase B is the predominant isoenzyme in liver and is also expressed in kidney; aldolase C is expressed in brain and certain other tissues. Aldolase B differs from the other isoenzymes in that it catalyses the hydrolysis of fructose 1-phosphate as well as fructose 1,6-bisphosphate [7]. Muscle aldolase binds strongly and reversibly to filamentous actin and other cytoskeletal proteins [8,9] and it also binds to glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) [10] and glycerophosphate dehydrogenase (EC 1.1.1.8) [11]. The A-isoenzyme in erythrocytes binds to band-3 on the plasma membrane [12]. The C-isoenzyme binds to brain homogenates [13,14] and the B-isoenzyme binds to the nucleus [15] and endoplasmic reticulum [16,17]. Binding of aldolase to different subcellular structures or proteins is of interest because the affinity of the enzyme for its substrate is altered by binding to filamentous actin and glyceraldehyde-3-phosphate dehydrogenase [9,10]. Furthermore fructose 1,6-bisphosphate causes dissociation of aldolase from filamentous actin but enhances association with

glyceraldehyde-3-phosphate dehydrogenase and glycerophosphate dehydrogenase [9–11]. Despite this evidence from studies with purified proteins which argues in favour of a role for changes in binding/compartmentation in the regulation of the kinetics of the enzyme and therefore of metabolic flux, this work is sometimes criticized because binding studies are performed under unphysiological conditions and may therefore not reflect the situation in the intact cell [18]. Permeabilized cells are a useful model to investigate the binding properties of enzymes under conditions where the cellular cytoskeleton is maintained intact. Furthermore by preculturing the cells in different metabolic conditions it is possible to investigate to what extent ‘physiological’ changes in the metabolic status of the cell alter either the distribution of an enzyme between different binding sites or the binding affinity of the enzyme to a particular site. This approach has recently been used to study the translocation by hormones, growth factors or substrates of glucose-6-phosphate dehydrogenase [19,20], phosphatidate phosphohydrolase [21], epoxide hydrolase [22] and glucokinase (hexokinase IV) [23–25]. The aim of the present study was to investigate the binding properties of hepatic aldolase (isoenzyme B) in hepatocytes under different metabolic conditions. The hepatic isoenzyme is of interest because in addition to its role in glycolysis it has important functions in gluconeogenesis and fructose metabolism. Consequently the study of metabolic conditions which alter either the intracellular distribution of this enzyme or its binding properties may help elucidate the physiological role of enzyme binding in relation to metabolic control. We used a similar

approach to characterize the binding properties of aldolase to permeabilized hepatocytes as we used in our previous studies on the binding and translocation of glucokinase [23].

MATERIALS AND METHODS

Chemicals

Digitonin was from BDH (Poole, Dorset, U.K.). Glycerol-3-phosphate dehydrogenase and triose-phosphate isomerase (EC 5.3.1.1) were from Boehringer Mannheim (Germany). Other enzymes and all cofactors, substrates and inhibitors were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sources of other materials were as described previously [23].

Hepatocyte isolation and monolayer culture

Hepatocytes were isolated by collagenase perfusion of the liver from male Wistar rats (body wt. 220–260 g) fed *ad libitum* [26]. They were suspended in Minimum Essential Medium containing 5% (v/v) neonatal calf serum, inoculated in 24-well plates at a density of 4×10^4 cells/cm² and incubated at 37 °C in a humidified atmosphere equilibrated with 5% CO₂/air. After cell attachment (approximately 4 h) the medium was replaced by serum-free Minimum Essential Medium containing 10 nM dexamethasone. Incubations with digitonin for determination of aldolase binding were performed after between 18 h and 24 h of culture. During the initial 30 h culture there was little change in total aldolase activity. However, after this interval some experiments showed a rapid decline in total activity (by about 50% within 2–3 h). All experiments reported in this paper were performed in conditions where there was a negligible decline in total activity.

Incubation of hepatocyte monolayers with substrates and inhibitors

Unless indicated otherwise the standard Minimum Essential Medium contained 5 mM glucose. Where indicated, hepatocyte monolayers were incubated in medium supplemented with a higher glucose concentration or with substrates and inhibitors for 30–40 min before permeabilization with digitonin.

Permeabilization of hepatocytes with digitonin

The hepatocyte monolayers were washed once with 150 mM NaCl and then permeabilized in medium (250 µl per well) containing 0.075 mg/ml digitonin, 2 mM dithiothreitol, 3 mM Hepes, pH 7.2, at 20 °C, and either 300 mM sucrose or 150 mM KCl or various mixtures to give the KCl concentrations indicated [23]. Incubations with the digitonin permeabilization medium were for 6 min (unless indicated otherwise) in unstirred conditions at 20 °C [23]. On termination of the permeabilization the plate was swirled gently and the digitonin eluate was aspirated for determination of enzyme activity. The residual cell matrix of the hepatocyte monolayer was extracted in 100 mM KCl/25 mM Hepes/7.5 mM MgCl₂/4 mM dithiothreitol/0.05% (w/v) Triton X-100, pH 7.2, by sonication for 4 s at maximum intensity (Mistral MSE sonicator with 3 mm diam. probe) for determination of the residual enzyme activity [23].

Determination of enzyme activity

Aldolase activity was determined from the decrease in absorbance of NADH in an assay cocktail containing 50 mM Tris/HCl, pH 7.5, 0.15 mM NADH, glycerol-3-phosphate dehydrogenase (1 m-unit/ml), triose-phosphate isomerase (3 m-units/ml) and

2 mM fructose 1,6-bisphosphate (or where indicated 20 mM fructose 1-phosphate) at 30 °C. Phosphoglucose isomerase and lactate dehydrogenase were assayed on the same extracts as in [23].

Expression of results

The changes in enzyme activity in the digitonin eluate following incubation of the hepatocytes with substrates or inhibitors were associated with equal inverse changes in activity extracted from the cell matrix. The enzyme activity in the digitonin eluate is expressed as a percentage of the total activity in the digitonin eluate and cell matrix. Throughout this study the total aldolase activity was between 30 and 50 m-units/mg of cell protein, where 1 m-unit is the amount of enzyme that catalyses conversion of 1 nmol of substrate per min at 30 °C. Protein was assayed by a Lowry method [27]. All incubation conditions were performed in duplicate wells in each experiment and results are expressed as means $\pm$ S.E.M. for the numbers of experiments indicated. Statistical analysis was by the Student's paired *t*-test.

RESULTS

Effects of KCl concentration on aldolase release from digitonin-permeabilized hepatocytes

When hepatocytes are permeabilized with low concentrations of digitonin (0.03–0.075 mg/ml) the release of some cytoplasmic enzymes (e.g. phosphoglucose isomerase and phosphoglucose mutase [23]) is independent of the ionic composition of the medium, whereas release of other enzymes (e.g. lactate dehydrogenase and glucokinase) is dependent on ionic strength and bivalent ion composition [23]. Figure 1 shows the effects of KCl concentration on enzyme release from digitonin-permeabilized hepatocytes that had been maintained either in basal medium containing 5 mM glucose or in medium supplemented with 20 mM glucose. Release of phosphoglucose isomerase was independent of KCl concentration, whereas release of lactate dehydrogenase was minimal at low salt concentrations (Figure 1A). Release of both enzymes was unaffected by supplementation of the medium with 20 mM glucose (*n* = 4, results not shown). The release of aldolase

Figure 1 Effects of KCl concentration on enzyme release during permeabilization of hepatocytes with digitonin

Hepatocytes were preincubated for 30 min either in basal medium (open symbols) or in medium supplemented with 20 mM glucose (closed symbols). They were then permeabilized for 6 min in medium containing 0.075 mg/ml digitonin, 2 mM dithiothreitol, 3 mM Hepes and various KCl concentrations prepared by mixing appropriate volumes of 150 mM KCl and 300 mM sucrose. The enzyme activity released in the digitonin eluate is expressed as a percentage of total activity in the digitonin eluate plus cell matrix. (A) Phosphoglucose isomerase (PGI, Δ) and lactate dehydrogenase (LDH, $\square$); (B) aldolase. Results are means $\pm$ S.E.M. for four experiments.

Figure 2 Time course of enzyme release during permeabilization of hepatocytes with digitonin

Hepatocytes were preincubated for 30 min either in basal medium (open symbols) or in medium supplemented with 20 mM glucose (closed symbols). They were then permeabilized for the time intervals indicated in medium containing 0.075 mg/ml digitonin, 2 mM dithiothreitol, 3 mM Hepes and various KCl concentrations. (A) Phosphoglucosomerase (PGI); (B) aldolase at 20 mM (∇), 150 mM ($\square$) and 2 M ($\triangle$) KCl. (C) Aldolase at 35 mM KCl. Enzyme activity released in the digitonin eluate is expressed as a percentage of total activity. Values are from one (A and B) or three (C) experiments.

Figure 3 Effects of PEG on enzyme release during permeabilization of hepatocytes with digitonin

Hepatocytes were preincubated either in basal medium (open symbols) or in medium supplemented with 20 mM glucose (closed symbols). They were then permeabilized for 6 min as described in the legend to Figure 1 with the concentrations of KCl and PEG indicated: (A) and (B) 100 mM KCl and various PEG concentrations; (C) 7% PEG and various KCl concentrations. Values are means $\pm$ S.E.M. for three experiments.

differed from that of lactate dehydrogenase in that maximum binding occurred at 20 mM KCl in cells that were maintained in the basal medium. In hepatocytes preincubated for 30 min in medium supplemented with 20 mM glucose there was a leftward shift of the aldolase dissociation curve, resulting in increased aldolase release at KCl concentrations ranging from 20 mM to 70 mM (Figure 1B). When KCl was replaced by sodium isethionate, the release of aldolase showed a similar biphasic response with minimal release at 20 mM sodium isethionate and lactate dehydrogenase release also showed a similar response as in KCl (results not shown), suggesting that the binding curve shown in Figure 1(B) is not specific for either K^+ or Cl^- but represents an effect of monovalent salt. Figure 2 shows the time course of enzyme release during permeabilization with digitonin. The rate of release of phosphoglucosomerase was similar at 20 mM, 150 mM and 2 M KCl (Figure 2A), whereas release of aldolase was slower at 20 mM KCl than at 150 mM or 2 M KCl (Figure 2B). At 35 mM KCl, aldolase release was faster from

cells preincubated in medium supplemented with 20 mM glucose than in basal medium (Figure 2C).

Effects of PEG on enzyme release from digitonin-permeabilized hepatocytes

The above experiments show binding of aldolase to digitonin-permeabilized hepatocytes at a KCl concentration (20–50 mM) that is lower than the cytoplasmic K^+ concentration (~ 140 mM K^+) but within the range of the cytoplasmic Cl^- concentration (20–50 mM). The question arises whether binding of aldolase to digitonin-permeabilized hepatocytes is of physiological significance. In the intact cell, binding of enzymes to subcellular structures or cytoskeletal proteins is influenced by protein concentration through water exclusion or macromolecular crowding [5]. This has been demonstrated previously for binding of aldolase to purified tubulin or actin using PEG (7–14%) to increase protein–protein interaction by water exclusion [28,29].

Figure 4 Recovery of aldolase in supernatant fractions of detergent-free cell homogenates

Hepatocytes were preincubated for 30 min either in basal medium (open symbols) or in medium supplemented with 20 mM glucose (closed symbols). The hepatocyte monolayers were then washed and sonicated in medium without digitonin containing the KCl concentration indicated and without (□, ■) or with 7% PEG (○, ●). The extracts were centrifuged at 13 000 *g* for 10 min and the activity was determined in the supernatant and pellet (extracted as described in the Materials and methods section for the cell matrix). Aldolase activity in the supernatant is expressed as a percentage of the total activity.

The effects of PEG on aldolase binding are shown in Figure 3. Increasing the PEG concentration (0–10%) markedly diminished the release of aldolase, lactate dehydrogenase and phosphoglucoisomerase during permeabilization in the presence of 100 mM KCl/3 mM Hepes. At this salt concentration there was a significantly greater release of aldolase from glucose-pretreated cells compared with control cells at 6–8% PEG ($P < 0.01$) but not at lower PEG concentrations (Figure 3B). Similar results were obtained with 150 mM KCl/3 mM Hepes (results not shown). When the effects of 7% PEG were examined over a range of KCl concentrations, binding of aldolase was markedly enhanced (Figure 3C versus Figure 1B) and the difference in aldolase release between glucose-pretreated and control cells was observed over a wider KCl concentration range. These results (Figure 1B versus Figure 3C) together establish that the differences in binding properties of aldolase between control cells and cells supplemented with 20 mM glucose are functions of both salt concentration and macromolecular crowding and that in the presence of 7% PEG the substrate effect can be demonstrated over a more extensive KCl concentration range.

Effects of KCl concentration and PEG on distribution of aldolase in supernatant and pellet fractions of sonicated cell extracts

Previous studies on the binding of aldolase A and C isoenzymes were performed on the distribution of enzyme between supernatant and pellet fractions of tissue homogenates [9,13]. In the experiments in Figure 4 the distribution of aldolase was determined in supernatant and pellet fractions of hepatocyte extracts prepared by sonication in detergent-free medium at various KCl concentrations with or without 7% PEG. In the absence of PEG, aldolase was recovered predominantly in the supernatant fraction over the range of KCl concentrations examined (Figure 4). However, in the presence of 7% PEG, there was significant binding of aldolase to the pellet fraction at intermediate KCl concentrations (20–60 mM) and greater bind-

ing in control cells than in cells supplemented with 20 mM glucose.

Effects of MgCl_2 concentration on aldolase release from digitonin-permeabilized hepatocytes

When hepatocytes were permeabilized with increasing MgCl_2 concentrations (0–5 mM) in 300 mM sucrose/3 mM Hepes, lactate dehydrogenase release increased with increasing MgCl_2 concentration (Figure 5A) whereas aldolase showed a biphasic response with minimum release at 1 mM MgCl_2 . Hepatocytes preincubated in medium supplemented with 20 mM glucose showed a shift in the aldolase binding curve with increased enzyme release at 1–5 mM MgCl_2 (Figure 5B). When the permeabilization medium was supplemented with 7% PEG, aldolase release with increasing Mg^{2+} concentration was markedly diminished (Figure 5C).

When the effects of MgCl_2 (0–5 mM) on aldolase release were determined in the presence of KCl (50–150 mM) instead of sucrose, the release of aldolase was significantly increased ($P < 0.05$) by 5 mM MgCl_2 at 50 mM KCl (control, $43 \pm 3\%$; + 5 mM MgCl_2 , $66 \pm 4\%$; mean $\pm$ S.E.M., $n = 3$) but not at 100 mM KCl (control, $68 \pm 3\%$; + MgCl_2 , $77 \pm 3\%$) or 150 mM KCl (control, $74 \pm 3\%$; + 5 mM MgCl_2 , $74 \pm 4\%$), indicating that the effects of Mg^{2+} are additive with the effects of submaximally effective concentrations of KCl.

Effects of digitonin concentration on aldolase release

The release of certain cytoplasmic enzymes from digitonin-permeabilized cells is very sensitive to digitonin concentration [23], presumably because concentrations beyond those that permeabilize the plasma membrane release proteins bound to intracellular membrane-bound organelles. Measurement of enzyme release at increasing digitonin concentrations may therefore be indicative of whether enzymes are bound to detergent-sensitive sites. The biphasic release of aldolase with increasing concentrations of KCl or MgCl_2 could be due either to presence of aldolase in different binding sites with different ionic interactions and/or to different aldolase isoenzymes. To distinguish between these possibilities, the effects of increasing concentrations of digitonin were determined in the absence or presence of 5 mM MgCl_2 (Table 1) and aldolase activity was assayed with both fructose 1,6-bisphosphate and fructose 1-phosphate as substrate, since the latter is a substrate for aldolase B but not for the other isoenzymes [7]. Increasing digitonin concentrations caused a much greater increase in aldolase release in the presence of Mg^{2+} than in its absence, suggesting that the activity released in the presence of Mg^{2+} represents enzyme at a detergent-sensitive site. The similar changes in activity assayed with fructose 1,6-bisphosphate and fructose 1-phosphate suggest that the enzyme released in the absence and presence of 5 mM MgCl_2 is unlikely to represent different isoenzymes. This is consistent with evidence that aldolase B is the only isoenzyme expressed in liver [17].

Effects of glycolytic and gluconeogenic precursors on aldolase binding

As shown in Figures 1 and 5, the dissociation curves for aldolase as a function of either KCl or Mg^{2+} concentration are shifted to the left in hepatocytes supplemented with 20 mM glucose. Similar results were obtained when hepatocytes were incubated with

Figure 5 Effects of Mg^{2+} concentration on enzyme release during permeabilization of hepatocytes with digitonin

Hepatocytes were preincubated either in basal medium (open symbols) or in medium supplemented with 20 mM glucose (closed symbols). They were then permeabilized for 6 min in medium containing 0.075 mg/ml digitonin, 2 mM dithiothreitol, 3 mM Hepes, 300 mM sucrose and the $MgCl_2$ concentration indicated either without PEG (**A** and **B**) or with 7% PEG (**C**). The enzyme activity released in the digitonin eluate is expressed as a percentage of total activity. (**A**) Lactate dehydrogenase (LDH); (**B**) and (**C**) aldolase. Results are means $\pm$ S.E.M. for four (**A** and **B**) or three (**C**) experiments.

Table 1 Effects of digitonin concentration and Mg^{2+} on aldolase release assayed with fructose 1,6-bisphosphate or fructose 1-phosphate as substrates

Hepatocytes were preincubated either in basal medium or in medium supplemented with 20 mM glucose. They were then permeabilized in medium containing 300 mM sucrose, 3 mM Hepes and various concentrations of digitonin in the absence or presence of 5 mM $MgCl_2$. Aldolase activity in the digitonin eluate and cell matrix was assayed with both fructose 1,6-bisphosphate and fructose 1-phosphate as substrates and the activity in the digitonin eluate is expressed as a percentage of total activity. Values are means $\pm$ S.E.M. for three experiments.

Permeabilization medium	Aldolase release (%)			
	Fructose 1,6-bisphosphate		Fructose 1-phosphate	
	Control	+ 20 mM Glucose	Control	+ 20 mM Glucose
0.025 mg/ml Digitonin	18 $\pm$ 1	16 $\pm$ 1	16 $\pm$ 1	14 $\pm$ 2
0.05 mg/ml Digitonin	21 $\pm$ 1	20 $\pm$ 1 ^a	18 $\pm$ 1	18 $\pm$ 1
0.075 mg/ml Digitonin	24 $\pm$ 1 ^a	22 $\pm$ 1 ^a	23 $\pm$ 1 ^a	20 $\pm$ 1 ^a
0.025 mg/ml Digitonin + 5 mM $MgCl_2$	13 $\pm$ 1	24 $\pm$ 4 ^b	13 $\pm$ 2	24 $\pm$ 4 ^b
0.05 mg/ml Digitonin + 5 mM $MgCl_2$	33 $\pm$ 1 ^a	50 $\pm$ 4 ^{a,b}	33 $\pm$ 1 ^a	47 $\pm$ 4 ^{a,b}
0.075 mg/ml Digitonin + 5 mM $MgCl_2$	39 $\pm$ 1 ^a	59 $\pm$ 4 ^{a,b}	39 $\pm$ 1 ^a	55 $\pm$ 3 ^{a,b}

^a $P < 0.05$, relative to corresponding values at 0.025 mg/ml digitonin.

^b $P < 0.05$, + 20 mM glucose versus controls.

Table 2 Effects of metabolic inhibitors on substrate modulation of aldolase binding

Hepatocyte monolayers were incubated for 10 min without or with 30 mM mannoheptulose (MH), 30 mM D-glucosamine (GCS) or 5 mM 3,3'-tetramethyleneglutaric acid (TMG). The substrates indicated were then added and the incubations continued for a further 30 min. The hepatocytes were then washed and permeabilized in 300 mM sucrose/3 mM Hepes/2 mM $MgCl_2$ /0.075 mg/ml digitonin for 6 min and the activity of aldolase in the digitonin eluate was expressed as a percentage of the total activity in the digitonin eluate and cell matrix. Values are means $\pm$ S.E.M. for *n* experiments.

Substrates		Aldolase release (%)						
		Expt. 1		Expt. 2		Expt. 3		
		Inhibitors (<i>n</i>)...	None (4)	MH (4)	None (3)	GCS (3)	None (3)	TMG (3)
	None		18 ± 1	16 ± 1 ^a	18 ± 1	14 ± 1	16 ± 1	14 ± 1
	20 mM Glucose		23 ± 1	17 ± 1 ^b	24 ± 2	18 ± 1 ^b	23 ± 1	23 ± 2
	40 mM Glucose		29 ± 2	21 ± 2 ^b	—	—	—	—
	10 mM Fructose		30 ± 2	25 ± 3 ^a	31 ± 2	29 ± 2	29 ± 1	29 ± 2
	10 mM Sorbitol		32 ± 2	31 ± 3	32 ± 2	34 ± 2	33 ± 2	35 ± 2
	10 mM Dihydroxyacetone		37 ± 3	35 ± 2	35 ± 1	35 ± 1	38 ± 1	37 ± 1
	10 mM Glycerol		41 ± 3	38 ± 3	40 ± 2	41 ± 1	41 ± 2	45 ± 2

^a $P < 0.05$; ^b $P < 0.005$, relative to corresponding control without inhibitor.

Table 3 Effects of glucagon on substrate modulation of aldolase binding

Hepatocyte monolayers were incubated for 30 min with the substrates indicated in the absence or presence of 100 nM glucagon. The hepatocytes were then washed and permeabilized in 300 mM sucrose/3 mM Hepes/2 mM MgCl_2 /0.075 mg/ml digitonin for 6 min and the activity of aldolase in the digitonin eluate was expressed as a percentage of the total activity in the digitonin eluate and cell matrix. Values are means $\pm$ S.E.M. for five experiments.

Substrates	Aldolase release (%)	
	Control	100 nM Glucagon
None	16 $\pm$ 1	16 $\pm$ 1
20 mM Glucose	26 $\pm$ 1	16 $\pm$ 1 ^a
40 mM Glucose	41 $\pm$ 1	26 $\pm$ 1 ^a
10 mM Fructose	34 $\pm$ 2	28 $\pm$ 1 ^a
10 mM Sorbitol	39 $\pm$ 2	36 $\pm$ 3 ^a
10 mM Dihydroxyacetone	45 $\pm$ 3	36 $\pm$ 2 ^a
10 mM Glycerol	45 $\pm$ 3	43 $\pm$ 3

^a $P < 0.005$, relative to corresponding control.

5 mM glycerol or dihydroxyacetone instead of 20 mM glucose (results not shown). To investigate further the metabolic basis of substrate-induced modulation of aldolase binding, the effects of a variety of glycolytic or gluconeogenic substrates on the release of aldolase were determined by preincubation of hepatocytes with the substrates for 30 min. The monolayers were then washed and permeabilized with digitonin in the presence of 2 mM MgCl_2 . This Mg^{2+} concentration was selected because it was associated with a large percentage increase in aldolase release after incubation with substrates (Figure 5B). Similar substrate effects were obtained when 35 mM KCl was used instead of 2 mM MgCl_2 (results not shown), indicating that the substrate effect is independent of whether binding is measured in the presence of KCl or MgCl_2 .

Glucose (10–40 mM), mannose (10–40 mM), fructose (1–10 mM), sorbitol (0.2–10 mM), dihydroxyacetone (1–10 mM) and glycerol (1–10 mM) significantly increased ($P < 0.05$) release of aldolase in comparison with control incubations with 5 mM glucose alone (Tables 2 and 3). Galactose, 3-*O*-methylglucose and 2-deoxyglucose at concentrations up to 30 mM had no effect

on aldolase release, and lactate and pyruvate had small effects that were not statistically significant (results not shown). Figure 6 shows that whereas aldolase release increased with increasing glucose concentration, mannose increased enzyme release up to 10 mM but caused no further increase at higher concentrations. Thus at high concentrations, mannose was less effective than glucose (Figure 6A). The concentrations of fructose and sorbitol that caused a half-maximal effect on aldolase release were respectively > 5 mM and 1 mM (Figure 6B). The concentrations of glycerol and dihydroxyacetone that caused half-maximal dissociation were 2–4 mM (results not shown). Time-course experiments showed that the effect of these substrates on enzyme release was maximal within 10 min and remained constant for at least 2 h. Two competitive inhibitors of glucokinase (D-mannoheptulose and D-glucosamine) partially counteracted the effects of glucose on aldolase dissociation, whereas 3,3'-trimethylene-glutaric acid, an inhibitor of aldose reductase, did not counteract the effect of glucose (Table 2). Mannoheptulose, but not glucosamine, also suppressed the fructose effect. Since mannoheptulose is a substrate for fructokinase it may compete with fructose [24]. The glucokinase inhibitors also counteracted the effect of mannose (results not shown). Ethanol (10 mM) suppressed the effects of glucose (Figure 6C) and glucagon (100 nM) partially suppressed the effects of glucose, fructose, sorbitol and dihydroxyacetone but not glycerol (Table 3).

Effects of intermediary metabolites on aldolase dissociation in permeabilized hepatocytes

To investigate the mechanism by which substrates modulate aldolase binding, hepatocytes were preincubated with 5 mM glucose and then permeabilized with digitonin and 2 mM MgCl_2 with various additions of phosphorylated intermediates (Figure 7). Of the intermediates tested, dihydroxyacetone phosphate and fructose 1,6-bisphosphate were the most potent at dissociating aldolase with half-maximally effective concentrations (A_{50}) of 20 μM and 40 μM respectively. The effectiveness of other intermediates in order of decreasing potency was: fructose 1-phosphate (100 μM); glycerol 3-phosphate (110 μM); glucose 1,6-bisphosphate (130 μM); fructose 2,6-bisphosphate (180 μM); glucose 1-phosphate (> 400 μM). Fructose 6-phosphate, glucose 6-phosphate and glyceraldehyde were ineffective at concen-

Figure 6 Effects of preincubation of hepatocytes with substrates on aldolase release during permeabilization of hepatocytes with digitonin

Hepatocytes were preincubated for 30 min in basal medium supplemented with the concentrations of (A) glucose (○), mannose (□); (B) fructose (△), sorbitol (▽); and (C) glucose without (○) or with (●) 10 mM ethanol. They were permeabilized for 6 min in medium containing 0.075 mg/ml digitonin, 2 mM dithiothreitol, 3 mM Hepes, 300 mM sucrose, 2 mM MgCl_2 . Aldolase activity in the digitonin eluate is expressed as a percentage of total activity. Results are means $\pm$ S.E.M. for four experiments.

Figure 7 Effects of metabolic intermediates on aldolase release from digitonin-permeabilized cells

Hepatocytes precultured in basal medium containing 5 mM glucose were permeabilized for 6 min in medium containing 0.075 mg/ml digitonin, 2 mM dithiothreitol, 3 mM Hepes, 300 mM sucrose, 2 mM MgCl_2 and the concentrations of the following intermediates indicated: (A) $\circ$, fructose 1,6-bisphosphate; $\bullet$, glucose 1,6-bisphosphate; $\square$, fructose 2,6-bisphosphate; (B) $\blacksquare$, fructose 1-phosphate; $\triangle$, dihydroxyacetone phosphate; $\blacktriangle$, glycerophosphate. (C) Hepatocytes preincubated without ($\diamond$) or with ($\blacklozenge$) 10 mM glycerol were permeabilized in the presence of various concentrations of fructose 1,6-bisphosphate. Aldolase activity released in the digitonin eluate is expressed as a percentage of total activity in the digitonin eluate plus cell matrix. Values are means $\pm$ S.E.M. for 4–6 experiments for (A) and (B), and one experiment out of two for (C).

trations up to 400 μM . At maximally effective concentrations of fructose 1,6-bisphosphate (0.4–1 mM) there was no further dissociation by glucose 1,6-bisphosphate, dihydroxyacetone phosphate or glycerol 3-phosphate, indicating that these intermediates do not release different fractions of enzyme (results not shown). When hepatocytes were preincubated without or with 10 mM glycerol and then permeabilized with digitonin without or with fructose 1,6-bisphosphate, the release of aldolase by preincubation with glycerol was not additive with release by fructose 1,6-bisphosphate (Figure 7C). This indicates that the effect of substrates added to the intact cell before permeabilization is mediated by the same mechanism as the effect of metabolic intermediates added to the permeabilized cell.

DISCUSSION

Previous studies have shown that aldolase A binds maximally to muscle and brain homogenates and to filamentous actin at low ionic strength. Complete dissociation occurs at 150 mM KCl and half-maximal release at 80 mM KCl. Aldolase C shows similar binding to brain homogenates but with lower affinity [9,13]. The present study shows that aldolase B binds to the matrix of digitonin-permeabilized hepatocytes with similar ionic binding characteristics (KCl and Mg^{2+}) as reported for aldolases A and C. The concentration of KCl that caused half-maximal dissociation of aldolase varied between 35 and 50 mM depending on the concentrations of glucose or other substrates with which the hepatocytes had been incubated before permeabilization. The proportion of enzyme that was present in a bound state at low and high salt concentrations was unaffected by the substrates. It appears therefore that the metabolic status of the cell affects the binding affinity of aldolase.

The finding that PEG enhances aldolase binding to digitonin-permeabilized hepatocytes and to sonicated extracts of cells is consistent with previous findings on binding of muscle aldolase to cytoskeletal proteins [28,29]. It has been argued that since binding of aldolase to the cytoskeleton and dissociation by fructose 1,6-bisphosphate can be demonstrated experimentally only at very low salt concentrations, it is unlikely to be of physiological significance [18]. However, the present data show that in the presence of PEG the substrate effect on aldolase

binding to the hepatocyte matrix is shifted to salt concentrations within the physiological range (50–100 mM versus 35 mM), thus supporting a physiological role for aldolase binding and dissociation.

It is not possible from the present data to estimate what proportion of aldolase is present in free and bound states in the intact cell. Such an estimate would require knowledge of the intracellular ionic activities and protein concentration or chemical potential. However, since most proteins are not freely diffusible an estimate of the concentration of soluble protein is not possible. Likewise K^+ may also not be freely diffusible [30]. On the basis of the finding that the binding of aldolase shows a leftward shift in substrate-treated cells as compared with control cells (incubated with 5 mM glucose), it can be predicted that a higher fraction of aldolase would be bound in cells exposed to 5 mM glucose as sole carbohydrate substrate than in cells exposed to higher glucose concentrations or fructose, sorbitol, glycerol or dihydroxyacetone as substrates.

The inhibitory effect of the competitive glucokinase inhibitors on the effects of glucose and mannose on aldolase dissociation is suggestive of a metabolite(s) distal to glucokinase. Mannose is a substrate for glucokinase and is as effective as glucose at causing glucokinase translocation [23]. However, mannose is less effective than glucose (Figure 6) at modulating aldolase binding. Since mannose 6-phosphate is a much less effective substrate than glucose 6-phosphate for phosphoglucosomerase [31], it seems likely that a metabolite distal to phosphoglucosomerase is involved. The effects of fructose and sorbitol on aldolase dissociation are elicited at concentrations (A_{50} 5 mM and 1 mM, respectively) that are about 100-fold higher than the concentrations that cause glucokinase translocation [24]. This suggests that the effects of these substrates are more likely to be due to a direct effect of fructose 1-phosphate than to glucokinase translocation.

The modulation of aldolase binding by substrates added to the intact cells could be mimicked by metabolic derivatives of these substrates added to digitonin-permeabilized cells. Thus the effects of glycerol and dihydroxyacetone added to intact cells are consistent with the effects of glycerol 3-phosphate and dihydroxyacetone phosphate on the digitonin-permeabilized cells and the effects of glucose, fructose and sorbitol are consistent with the effects of fructose 1,6-bisphosphate, fructose 2,6-

bisphosphate and fructose 1-phosphate. The observation that the maximal effect of substrates added to the intact cell (glucose or glycerol) is not additive with the effect of metabolic intermediates added to the permeabilized hepatocytes supports a common mechanism. Of the metabolites tested, dihydroxyacetone phosphate and fructose 1,6-bisphosphate were the most effective at dissociating aldolase (A_{50} 20 μ M and 40 μ M, respectively). This is consistent with a previous study on the muscle isoenzyme [9] but not with findings on the binding of liver aldolase to the microsomal fraction, where 11 mM dihydroxyacetone did not cause dissociation [17]. The lack of additive effects of fructose 1,6-bisphosphate and triose phosphates on aldolase dissociation from permeabilized hepatocytes suggests that even if there are different pools of bound aldolase, the total bound fraction appears to be dissociated by both metabolites. The similar effects of dihydroxyacetone and glycerol (which are precursors of triose phosphates) by comparison with glucose, fructose and sorbitol, which are precursors of fructose 1,6-bisphosphate, fructose 2,6-bisphosphate and fructose 1-phosphate, suggests that substrate modulation of aldolase binding is not related to the direction of metabolic flux through the aldolase-catalysed reaction. The suppression by glucagon of the effects of glucose, fructose and sorbitol could be due to suppression of the concentrations of metabolic intermediates such as fructose 1,6-bisphosphate, fructose 1-phosphate and fructose 2,6-bisphosphate [32] or to a more reduced cytoplasmic NAD/NADH redox state as suggested also from the effects of ethanol.

Possible locations of aldolase binding and physiological role

Binding of aldolase B to the hepatocyte matrix can be demonstrated more easily in digitonin-permeabilized hepatocytes than in supernatant fractions of sonicated extracts of hepatocytes. Thus aldolase binding may be dependent on preservation of a subcellular structure that is disrupted by sonication but preserved by detergent-permeabilization [33,34] or alternatively, aldolase may be loosely bound to membrane-bound organelles and this binding may be disrupted by sonication of the cells. The increase in aldolase release with increasing digitonin concentration in the presence of Mg^{2+} (Table 1) is suggestive of binding to a detergent-sensitive site.

There is evidence that hepatic aldolase binds to the nucleus [15] and endoplasmic reticulum [16,17]. Studies on Swiss 3T3 fibroblasts microinjected with aldolase A showed that there was an 'immobile' fraction in the perinuclear region and a mobile or free phase throughout the cytoplasm but with a lower diffusion coefficient in the perinuclear region than in the cell periphery, which is consistent with a higher cytoplasmic viscosity in the perinuclear region [35,36]. Kinetic studies on muscle [9] and liver [17] aldolase have shown that binding to F-actin [9] or the microsomal fraction [17] markedly decreases the affinity of the enzyme for fructose 1,6-bisphosphate. A tentative hypothesis, based on the finding that glycolytic and gluconeogenic substrates cause a similar decrease in binding affinity, and presumably therefore in the fraction of bound enzyme in the intact cell, is that in metabolic conditions associated with low concentrations of glycolytic substrates or glycerol, aldolase may be sequestered in an 'inactive' bound state in a central or other location. An increase in substrate availability (glucose, fructose, sorbitol or glycerol) would result in elevated concentrations of metabolic intermediates which would cause a decrease in binding affinity and thereby dissociation, with a consequent increase in activity

[9,17]. Binding and dissociation may therefore be a mechanism for buffering the concentrations of pathway intermediates, since enzyme binding would enable accumulation of intermediates because of the low substrate affinity of the bound enzyme as compared with the soluble enzyme (300 μ M versus 6 μ M) [17].

It is of interest that fructose, a major regulator of glucokinase translocation [24,25], is metabolized by fructokinase to fructose 1-phosphate, which in turn is metabolized by aldolase. During incubation of hepatocytes with either fructose or sorbitol, the concentration of fructose 1-phosphate rises rapidly up to concentrations in the millimolar range, but the increase is transient and declines very rapidly [37]. Since fructose and sorbitol cause a leftward shift of aldolase binding and fructose 1-phosphate also causes dissociation of aldolase at a half-maximal concentration of about 100 μ M, it may be speculated that the rise in fructose 1-phosphate which reaches a peak by 5 min [37] causes dissociation of aldolase which accounts for the rapid decline in fructose 1-phosphate concentration.

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