Altered metabolic states do not change the intracellular distribution of hexokinase in Zajdela hepatoma ascites cells

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The distribution of hexokinase between bound and soluble forms was studied by digitonin fractionation of Zajdela hepatoma ascites cells maintained under various metabolic conditions. Addition of glucose to Zajdela cells respiring on endogenous substrates induces an immediate inhibition of respiration by 50–60% (Crabtree effect), and a production of acid due to glycolysis. Acid production decreases abruptly after 60 s to 50% of the initial rate. The ATP/ADP ratio is not altered by the addition of glucose or by different rates of glycolysis. The uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone decreases the ATP/ADP ratio by 10-fold in cells respiring on endogenous substrate, but has little effect on cells oxidizing glucose. Rapid fractionation of the cells under these various metabolic conditions revealed no change in the distribution of hexokinase. Approx. 75% of hexokinase is bound in all cases, in contrast with lactate dehydrogenase, 95% of which was in the soluble form. Longer-term incubations (to 20 min) revealed only slight (10–15%) increases in soluble hexokinase in cells incubated with glucose. Various metabolic inhibitors had little additional effect on the subcellular distribution of hexokinase. Thus a rapid release of hexokinase from mitochondrial membrane is not a mechanism by which glycolysis is regulated in rapidly growing Zajdela hepatoma.

It is now well established that hexokinase in most tumour cells (Rose & Warms, 1967; Bustamante *et al.*, 1981; Kurokawa *et al.*, 1979, 1982) and in brain tissue (Rose & Warms, 1967; Kurokawa *et al.*, 1979) is to a great extent bound to mitochondria. However, hexokinase can be released from isolated mitochondria by incubation *in vitro* with ATP or glucose 6-phosphate (Rose & Warms, 1967; Bustamante & Pedersen, 1980; Bustamante *et al.*, 1981; Kurokawa *et al.*, 1982). The latter properties, plus the observation that bound and soluble hexokinase exhibit different kinetic characteristics (Wilson, 1980; Bessman & Geiger, 1980; Bustamante & Pedersen, 1980), have been used to support the proposal (Wilson, 1968) that metabolite-induced release of hexokinase could regulate glycolysis *in vivo* [see Purich *et al.* (1973), Wilson (1980) and Bessman & Geiger (1980) for reviews]. This view has been widely accepted as a general working hypothesis (Kurokawa *et al.*, 1982; Wilson, 1980; Bessman &

Geiger, 1980; Purich *et al.*, 1973; Bustamante & Pedersen, 1980). However, few data are available showing that release of hexokinase in intact tissues or cells is associated with altered rates of glycolysis, much less that it can be considered the primary event in regulating glucose utilization. Knull *et al.* (1973) reported an increase in soluble hexokinase in the brains of ischaemic chicks after 2.5 min of treatment. The significance of the results of these experiments is difficult to judge, however, since isolation of the particulate fraction was by traditional methods requiring long preparation times, and no account was taken of changes in the equilibrium binding of hexokinase that could have occurred (Rose & Warms, 1967). We have therefore reinvestigated the subcellular distribution of hexokinase by subjecting Zajdela hepatoma ascites cells in defined metabolic states to rapid fractionation in the presence of digitonin.

Methods and materials

Zajdela hepatoma cells were maintained in the ascites form in Sprague–Dawley rats. Cells were harvested on days 7–10 after inoculation and were

Abbreviation used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Na/KP_i, sodium/potassium phosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

washed in saline (0.9% NaCl) and suspended in a modified Schrieber's medium containing 71 mM-NaCl, 65 mM-KCl, 6 mM-KP_i and 3.5 mM-MgCl₂, pH 7.4.

Oxygen consumption was measured with a Clark-type electrode in the above buffer at 30°C. The vessel contained 10 mg of whole-cell protein/ml. Acid production was measured with a glass electrode in the same buffer. Acid production is expressed as lactic acid, which was calculated from the pH changes produced by addition of standard lactic acid solution to the reaction vessel containing cells.

The rapid fractionation of cells and measurement of hexokinase and lactate dehydrogenase activities were carried out as follows. Cells were continuously monitored for respiration and acid production as described above. At the appropriate times, 1 ml samples (10 mg of protein) were removed and added to Eppendorf tubes containing digitonin (50 µl maximum volume). The samples were incubated for 30 s at 30°C and then centrifuged for 30 s in an Eppendorf centrifuge. The supernatant and the pellet [containing mitochondria (Fiskum *et al.*, 1980; Kuzela *et al.*, 1981)] were separated, and the pellet was rinsed once in 1 ml of buffer containing 25 mM-Hepes, pH 7.5, 0.25 M-sucrose and 5 mM-MgCl₂. The pellet was then dissolved in 200 µl of the same buffer containing 0.5% Triton X-100. Hexokinase (EC 2.7.1.1) was measured in this Triton X-100-containing buffer as described by Bustamante & Pedersen (1980). For measurement of total cellular ATP and ADP, the rate of glycolysis was continuously monitored with the pH electrode as described above, and at the appropriate times the reaction was stopped by addition of 5% HClO₄/10% ethanol (final concns.). The extracts were neutralized and ATP and ADP were measured as described by Williamson & Corkey (1969).

Results

Rapid fractionation of tumour cells

The outer membrane of rat liver mitochondria was removed by digitonin treatment (Schnaitman & Greenawalt, 1968). Since hexokinase of tumour cells is bound to the outer membrane (Rose & Warms, 1967; Felgner *et al.*, 1979) and specifically to the pore protein (Lindén *et al.*, 1982a; Fiek *et al.*, 1982), it was necessary to establish conditions under which digitonin treatment of intact cells did not influence hexokinase binding. Fig. 1 shows that 40–50 µg of digitonin/mg of cell protein was sufficient to release about 90–95% of the total lactate dehydrogenase, but only about 25% of the hexokinase. Standard fractionation conditions

Fig. 1. Distribution of hexokinase in digitonin-treated *Zajdela hepatoma ascites* cells

Cells (10 mg of protein/ml) were suspended in a buffer containing 50 mM-Tris/HCl, 100 mM-KCl and, when present, 5 mM-MgCl₂ and 6 mM-KP_i, pH 7.5. Samples were incubated for 3 min at 30°C, after which 1 ml portions were removed and added to an Eppendorf tube containing the appropriate concentration of digitonin (max. vol. 50 µl). The samples were incubated for 30 s and centrifuged for 30 s in an Eppendorf centrifuge. The supernatant and pellet were separated, and the pellet was rinsed in 1 ml of buffer containing 25 mM-Hepes, pH 7.5, 10 mM-MgCl₂ and 0.5% Triton X-100, and was assayed for hexokinase (a) and lactate dehydrogenase (b). The mean values for two experiments are shown. The 100% values for hexokinase and lactate dehydrogenase varied between 100 and 150 nmol/min per mg of protein and between 1.0 and 1.5 µmol/min per mg of protein respectively for the different experimental points: ○, Pellet; ●, supernatant; ---, + Mg²⁺; —, - Mg²⁺.

Fig. 2. Effects of glucose addition on the metabolism of Zajdela hepatoma cells

Cells were suspended in buffer containing 65 mM-KCl, 71 mM-NaCl, 6 mM-NaP_i, pH 7.4, and 3.5 mM-MgSO₄ at 30°C. Respiration (a) and pH (b) were measured by using 10 mg of protein/ml and 3 mg of protein/ml respectively. Glucose (1 mM) and CCCP (1 μ M) were added as indicated. Rates of respiration (ΔO_2) are expressed as ng-atoms of O/min per mg of cell protein, and rates of acid production (ΔpH) are expressed as nmol of lactic acid/min per mg of cell protein. The latter was determined from a standard solution of lactic acid added to cells under the conditions described above.

chosen for the following experiments utilize 40 μ g of digitonin/mg of whole-cell protein (Fig. 1). In order to determine if binding or release of hexokinase occurred during the course of the fractionation, similar experiments were performed in which Mg²⁺, P_i or ATP were removed from, or added to, the fractionation media. Mg²⁺ promotes hexokinase binding and ATP promotes release (Rose & Warms, 1967; Bustamante & Pedersen, 1980). P_i prevents the release of hexokinase from mitochondria (Rose & Warms, 1967; Kurokawa *et al.*, 1982). Our experiments gave identical results in the presence or absence of Mg²⁺ (Fig. 1), ATP or P_i (not shown), indicating that binding or release of hexokinase is slower than the fractionation procedure used here. Thus the subcellular distribution of hexokinase shown in Fig. 1 represents that present in the tumour cell before digitonin treatment.

Metabolic shifts produced by glucose

In order to establish the effects of metabolism on the intracellular distribution of hexokinase, a number of experiments were done to define different metabolic states of the cell. Intact Zajdela hepatoma cells respire actively on endogenous substrate in the absence of glucose (Fig. 2a), but do not produce measurable quantities of acid (Fig. 2b). Respiration is completely inhibited by rotenone or antimycin (results not shown), indicating that it is mitochondrial in origin. Oxidation of endogenous substrate is sufficient to maintain high cellular ATP/ADP ratios (Table 1). The ATP/ADP ratio is highly sensitive to CCCP (Table 1), indicating that oxidative phosphorylation is primarily responsible for ATP synthesis in the absence of glucose. Addition of glucose to Zajdela hepatoma cells respiring on endogenous substrate results in an immediate inhibition of oxygen consumption (Fig. 2a) and a rapid production of acid (Fig. 2b). An initial stimulation of respiration (Chance & Hess, 1959; Koobs, 1972) is not observed in Zajdela cells.

Table 1. Effect of various metabolic states on the adenine nucleotides of Zajdela hepatoma cells
Cells were suspended to 3 mg of protein/ml in the buffer described in Fig. 2. The pH was continuously monitored and at the appropriate times the reactions were stopped with 5% HClO₄/10% ethanol (final concns.). The final concentrations of glucose and CCCP were 1 mM and 1 μ M respectively. Initial and inhibited phases refer to the rates of acid production as shown in Fig. 2(b). The values are expressed as means, or means $\pm$ S.E.M., for the numbers of experiments given in parentheses.

Additions	Content (nmol/mg of protein)			
	ATP	ADP	Total	ATP/ADP
None	15.2 $\pm$ 2.0 (5)	2.5 (2)	22.1 (2)	7.7 (2)
CCCP	7.5 $\pm$ 1.1 (4)	8.8 (1)	22.8 (2)	0.6 (2)
Glucose				
Initial phase	17.3 $\pm$ 1.8 (5)	4.7 (2)	22.8 (2)	4.8 (2)
Inhibited phase	18.1 $\pm$ 1.6 (5)	4.8 (2)	23.8 (2)	4.5 (2)
+ CCCP	13.6 $\pm$ 1.7 (5)	4.8 (2)	22.6 (2)	3.9 (2)

Acid production was not inhibited by rotenone or antimycin, and is due to glycolysis. Two phases of acid production are observed, the second of which appears approx. 1 min after the addition of glucose and is about 50% of the initial rate (Fig. 2b). This corresponds to the state of inhibited glucose utilization described by Chance & Hess (1959). The ATP/ADP ratio is the same in both the initial and inhibited phases of acid production (Table 1), indicating that the down-shift in glycolysis is not a direct reflection of the energy state of the cell. The ATP/ADP ratio is only marginally altered by CCCP in the presence of glucose (Table 1), indicating that the ATP requirement of Zajdela hepatoma can be met by glycolysis.

Effects of different metabolic states on the distribution of hexokinase

In order to test the suggestion (Wilson, 1968) that hexokinase binding to mitochondria might be controlled in the intact cell by metabolite shifts, we have subjected cells in the various states shown in Fig. 2 to rapid fractionation with digitonin. Table 2 shows the distribution of hexokinase under these conditions. There is no correlation between hexokinase binding to mitochondria and the metabolic state of the cell, or with the rapid shifts in metabolism which can be observed. Hexokinase binding is not influenced by the presence or absence of glycolysis (presence or absence of glucose), the rate of glycolysis, or by the large decrease in the energy state of the cell brought about by CCCP in the absence of glucose. The results clearly show that the cellular redistribution of hexokinase is not the initial step in the regulation of glycolysis in Zajdela hepatoma.

The effects of longer-term incubation on the binding of hexokinase was also studied. Incubations carried out for up to 20 min showed a slight increase in soluble hexokinase in cells incubated in the presence of glucose (Fig. 3). Release was observed after 5–10 min of incubation and amounted to approx. 15% of the total cellular hexokinase. Thus a small but significant redistribution of hexo-

kinase occurs in intact cells in the presence of glucose. The amount of solubilized hexokinase was not further increased in the presence or absence of glucose by inhibitors of mitochondrial function (Table 3). In summary, these experiments show that extensive changes in the energy state of the cell over long incubation periods have only marginal, if any, effects on the distribution of hexokinase between bound and soluble forms.

Fig. 3. *Effects of glucose on the distribution of hexokinase over longer periods of incubation*

Cells were suspended at 10 mg of protein/ml in the medium described in Fig. 2, buffered to pH 7.4 with 20 mM-Hepes, in either the presence (○) or absence (●) of 20 mM-glucose. The samples were incubated at 30°C and, at the indicated times, 200 μ l (2 mg of protein) portions were removed and added to an Eppendorf tube containing digitonin to give a final concentration of 40 μ g/mg of protein. Samples were incubated for 30 s and centrifuged for 30 s in an Eppendorf centrifuge. The samples were then processed and measured as in Fig. 1. The mean values are shown for two separate experiments. Hexokinase activity (100%) varied from 130 to 160 nmol/min per mg of whole-cell protein for the different time points.

Table 2. *Effect of various metabolic states on the subcellular distribution of hexokinase in Zajdela hepatoma cells*
Cells (10 mg of protein) were incubated under conditions described in Fig. 2. At the appropriate times the cells were treated with digitonin and fractionated as described in Fig. 1. The mean values for two separate experiments are shown.

Incubation conditions	Total hexokinase activity [nmol/min per mg of cell protein (% of total)]		
	Particulate	Soluble	Total
No additions	125 (88.4)	16.1 (12.1)	141 (100)
CCCP (1 μ M)	129 (87.4)	18.5 (13.1)	148 (100)
Glucose (1 mM)			
Inhibited phase	117 (86.4)	17.7 (14.1)	135 (100)
+ CCCP	121 (86.4)	19.3 (14.1)	140 (100)

Table 3. *Influence of metabolic inhibitors on the subcellular distribution of hexokinase in hepatoma cells after prolonged incubation in the presence or absence of glucose*

Cells were incubated for 10 min under the conditions described in Fig. 3, in either the presence or absence of glucose. Cells were rapidly fractionated as described in Fig. 1. Values are means $\pm$ S.E.M. for the numbers of experiments given in parentheses. Hexokinase activity (100%) varied between 100 and 150 nmol/min per mg of cell protein in the different experiments.

Additions	Hexokinase activity (% of total)			
	No glucose		Glucose (20 mM)	
	Particulate	Soluble	Particulate	Soluble
None	64.7 $\pm$ 2.9 (7)	35.3 $\pm$ 2.9 (7)	45.0 $\pm$ 3.8 (7)	54.6 $\pm$ 3.6 (7)
Iodoacetamide (1 mM)	57.0 $\pm$ 4.5 (6)	45.5 $\pm$ 3.6 (6)	50.7 $\pm$ 3.2 (6)	51.3 $\pm$ 4.0 (6)
CCCP (2 μ M)	64.7 $\pm$ 4.0 (6)	35.3 $\pm$ 4.0 (6)	47.4 $\pm$ 4.1 (6)	51.3 $\pm$ 4.0 (6)
Rotenone (5 μ M)	52.3 $\pm$ 4.6 (4)	47.8 $\pm$ 5.3 (4)	51.8 $\pm$ 4.0 (4)	48.3 $\pm$ 4.0 (4)
Antimycin A (5 μ g)	67.0 $\pm$ 0.7 (3)	33.0 $\pm$ 0.7 (3)	57.7 $\pm$ 4.5 (3)	42.3 $\pm$ 4.5 (3)
Carboxyatractyloside (5 μ g)	63.3 $\pm$ 2.3 (3)	36.7 $\pm$ 2.3 (3)	42.3 $\pm$ 3.5 (3)	53.7 $\pm$ 9.4 (3)
Oligomycin (10 μ g)	56.5 $\pm$ 4.9 (4)	43.5 $\pm$ 4.9 (4)	50.5 $\pm$ 5.0 (6)	49.5 $\pm$ 5.0 (6)

Discussion

In spite of the fact that ATP and glucose 6-phosphate release hexokinase from isolated mitochondria, the present study shows that fluctuating metabolic conditions in Zajdela hepatoma cells do not provide an optimum environment for release of hexokinase from mitochondria in the intact cell. This was clearly demonstrated for rapid metabolic shifts which take place in mitochondrial respiration and in glycolysis after glucose addition. Prolonged incubation, on the other hand, did result in a slight increase in soluble hexokinase in cells incubated in the presence of glucose. However, this must be considered a secondary event to the rapid regulation of glycolysis. Whether these slower changes in hexokinase distribution are due, as suggested (Wilson, 1968, 1980), to increased concentrations of ATP and glucose 6-phosphate, or to intracellular pH changes accompanying glucose metabolism (Gillies *et al.*, 1982), is not known. In view of the present finding (Table 1), it seems likely, however, that further increases in the ATP content of the cell cannot be expected after prolonged incubation. It thus seems reasonable that release and binding of hexokinase, and the accompanying kinetic changes in the enzyme (Wilson, 1980; Bessman & Geiger, 1980; Bustamante & Pedersen, 1980), cannot be considered physiological mechanisms by which glycolytic fluxes are regulated in the intact Zajdela hepatoma cell.

The rapid fractionation procedure used in the present experiments is of primary importance. Separation of the particulate and soluble fractions could be achieved in 30 s. The subcellular distribution of hexokinase was identical when fractionation was carried out in either the presence or

absence of Mg^{2+} , ATP or KP_i , indicating that the equilibrium binding reaction (Rose & Warms, 1967) is slower than the separation procedure. Thus the distribution of hexokinase that was measured reflects that within the intact cell under the metabolic conditions studied. This offers a great advantage over previous experiments in which attempts were made to study the effects of energy and metabolites on the distribution *in vivo* of hexokinase (Knull *et al.*, 1973; Gumaa & McLean, 1969a,b; Baquer & McLean, 1969). Often the fractionation procedures used in these studies required nearly 1 h, and no consideration was taken of the possible redistribution of hexokinase which could occur during this time.

Although it is well established that soluble and bound hexokinases have different kinetic characteristics (Bustamante & Pedersen, 1980; Wilson, 1980; Bessman & Geiger, 1980), the present findings cast doubt on the idea that oscillation between these forms can regulate glycolysis. It seems more reasonable, however, that the kinetic characteristics of the bound form, plus the efficiency with which bound hexokinase utilizes ATP from oxidative phosphorylation (Bessman & Geiger, 1980), function to assure a high and constant rate of glucose entry into glycolysis in cells which preferentially utilize glucose. Regulation of glycolysis in these cells probably operates via one or more of the several additional mechanisms which have been proposed [see Pedersen (1978) and Koobs (1972) for review]. A new possibility should also be considered in light of the recent findings that hexokinase binds to porin in the mitochondrial outer membrane (Felgner *et al.*, 1979; Lindén *et al.*, 1982a; Fiek *et al.*, 1982). Since porin is responsible for the broad permeability properties of the mitochondrial outer membrane

(Zalman *et al.*, 1980; Lindén *et al.*, 1982b), it is conceivable that hexokinase can occlude the pore structure. This could result from substrate- or product-induced conformational alterations in hexokinase without affecting the binding of hexokinase. Such a mechanism would not be detected by the present experiments. Thus the interesting possibility arises that penetration of cytoplasmic metabolites into tumour mitochondria could be regulated at the level of the outer membrane rather than the inner membrane.

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