SEQUENTIAL CONTROL OF HEXOKINASE IN ASCITES CELLS

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Studies of transient changes in the total rate of glucose phosphorylation compared with the changes in glucose-6-phosphate (G6P), ATP, ADP and fructose diphosphate (FDP) have suggested a sequence of operation of control mechanisms following the addition of glucose to ascites cells. These are: (a) a rapid rate of glucose phosphorylation (50% V_{max}) for 15 sec. which appears to be independent of feedback inhibition by G6P which accumulates to inhibitory concentrations within 3 sec.; (b) a release of bound hexokinase by G6P; (c) a slow rate of glucose phosphorylation under feedback control by G6P; (d) the possible involvement of FDP inhibition; this control may depend on Mg^{++} availability.

It has been shown that glucose phosphorylation by ascites tumour cells in vitro proceeds in three distinct phases, an initial rapid rate of phosphorylation followed by a phase of marked inhibition, and finally a slow steady-state rate about 10-20% of the initial rate (1-4). Gumaa, McLean and Bennette (5) found a 25-fold increase in the G6P content of the Krebs ascites cells 30 seconds after the addition of glucose. This was followed by a decreased rate of accumulation in keeping with previous observations (1-4). That G6P is an inhibitor of hexokinase was first shown by Weil-Malherbe and Bone (6) for the rat brain enzyme, and subsequently the inhibition of ascites cell hexokinase was extensively investigated (3,7). The inhibition of glucose phosphorylation in ascites cells (7) and in brain (8) could not be accounted for solely by the G6P inhibition of hexokinase, and other regulatory factors have been postulated. Among the factors implicated in modulating the hexokinase reaction in vivo, ADP and AMP are inhibitors (7,9), inorganic phosphate is an activator and reverses the inhibition by G6P (3,10), and the reversible binding to particulate structures within the cell raises the apparent inhibitor constant for G6P (3,7,11,12).

In face of the overwhelming amount of evidence for the implication of diverse regulatory mechanisms in the hexokinase reaction, it became pertinent to investigate the interrelationship of some modulators of this reaction during the initial phase of rapid glucose utilisation and correlate the findings with the observed rate of glucose phosphorylation. The study of metabolite levels during the transient state of metabolism lends to the understanding of the state of control of enzymes *in vivo*, and the identification of modifiers of the hexokinase reaction aids in predicting the rate of glucose phosphorylation *in vivo* (7,13).

MATERIALS AND METHODS

Ancillary enzymes were purchased or prepared as previously reported (5). The processing and incubation procedure of the Krebs ascites cells for the study of glucose intermediary metabolites has been described (14). The washed, weighed ascites cells were fractionated by the following procedure: 0.5 volume of 0.154 M NaCl and 6 volumes of ice cold distilled water were added to the ascites cells and the suspension slowly stirred in ice for 10 minutes. Subsequent homogenisation was achieved with 20 strokes in a Potter homogeniser fitted with a teflon plunger, and immediately 2.5 volumes of 1 M sucrose - 20 mM triethanolamine buffer pH 7.4 were added and the homogenate centrifuged for 10 min. at 1600 g. The 1600 g supernatant was centrifuged for 10 min. at 12,000 g and the 12,000 g pellet washed with 0.25 M sucrose - 5 mM triethanolamine pH 7.4 by suspending and centrifuging three times. The 12,000 g supernatant was centrifuged for 45 min. at 100,000 g and the supernatant as well as the washed, suspended 12,000 g particles dialysed against 0.25 M sucrose - 5 mM triethanolamine pH 7.4 for 1 - 2 hr.

For simulating the *in vivo* hexokinase reaction, the 100,000 g supernatant hexokinase was partially purified by the following procedure: An aliquot of the dialysed supernate containing about 10 units of hexokinase and 60 mg protein was mixed with DEAE-cellulose (previously equilibrated with

20 mM Tris-HCl- 5 mM $MgCl_2$ pH 7.4) and the suspension centrifuged at 2000 g for 15 min. The hexokinase-free supernate was discarded and the resin washed with three 10 ml portions of 0.10 M KCl in 20 mM Tris-5 mM $MgCl_2$, and finally eluted with 10 ml of 0.35 M KCl in the same medium, and dialysed against 0.25 M sucrose-5 mM triethanolamine pH 7.4. This preparation was free from ATPase and phosphoglucose isomerase activity.

Hexokinase was assayed by two methods: (a) in a volume of 1.35 ml 40 mM Tris-HCl pH 7.4, 7.5 mM $MgCl_2$, 3.7 mM ATP, 0.7 mM NADP, 10 mM glucose and 0.6 units of glucose-6-phosphate dehydrogenase; (b) 40 mM Tris-HCl pH 7.4, 7.5 mM $MgCl_2$, 3.7 mM ATP, 1 mM PEP, 0.25 mM NADH, 10 mM glucose, 2.5 units pyruvate kinase and 7.0 units of lactic dehydrogenase. Reactions were started by the addition of about 0.01 unit of hexokinase, and followed by the rate of reduction of NADP or reoxidation of NADH at 340 mμ in a Unicam SP 800 recording spectrophotometer fitted with a constant temperature cell housing and a scale-expansion accessory. In both assay systems, a unit of activity is defined as that amount of enzyme producing 1 μmole of product/min. at 25°.

Metabolites were assayed as previously described (5). Mg^{++} was determined in washed ascites cells by emission flame spectrophotometry. Simulation of the in vivo hexokinase reaction was by the use of the partially purified enzyme in assay system (b) in which the ATP and G6P concentrations were adjusted to the levels found in the intact ascites cells at the various times studied. Inorganic phosphate, when used, was maintained at 5 mM concentration.

RESULTS

The total hexokinase activity of Krebs ascites cells is 13.2 units/g. cells at 25° (approx. 26 μmoles/g/min. at 37°) of which 80% is in the high speed supernatant fraction and 20% in the particulate fraction, a distribution in agreement with the values of Rose (3).

The changes in metabolites of Krebs ascites cells during the first

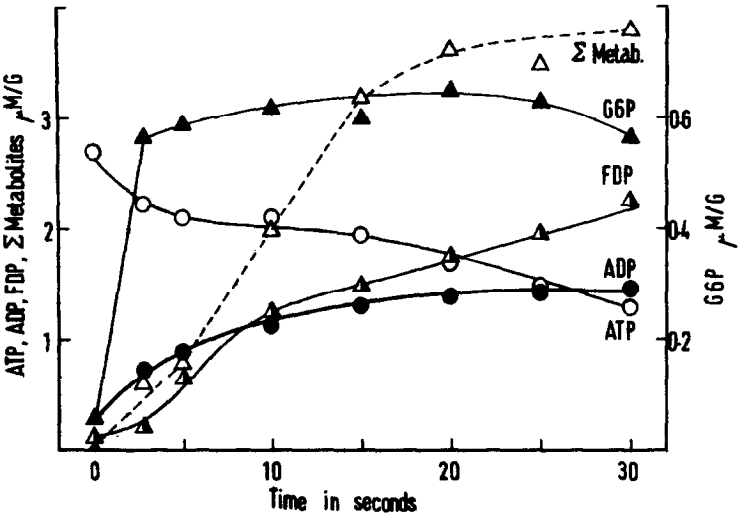

Figure 1. Interrelationship between G6P, FDP, ATP, ADP and the total rate of glucose phosphorylation ($\sum$ metabolites) during the 30 sec. after addition of glucose to Krebs ascites cells.

The cells were incubated aerobically with glucose, 10 mM initial concentration. The results are given as μmoles/g packed cells. The $\sum$ metabolites were summated from measurements of all the intermediates of the glycolytic pathway between glucose and lactate (with the exception of 1:3 diphosphoglycerate) together with intermediates of the pentose phosphate pathway.

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30 sec. after addition of glucose are shown in Fig. 1. The total rate of glucose phosphorylation in the intact cell was estimated from the sum of the metabolites ($\sum$ metabolites) accumulating between glucose and lactic acid, corrected for the endogenous concentration at zero time. This method is in principle the same as that used by Rose (3). The total rate of glucose phosphorylation was linear for the first 15 sec. at a rate of 12 μmoles of glucose phosphorylated/min. at 37°, i.e. approximately 50% of V_{max} of hexokinase, after which the rate declined rapidly to less than 10% of the initial value. In contrast to this, G6P increased sharply over the first 3 sec. to the maximum value, a rate of accumulation equivalent to the phosphorylation of 11 μmoles of glucose/min. at 37°, and thereafter remained constant. The interrelationship between the decrease in ATP, the increase in ADP and the accumulation of FDP, the latter following a sigmoid curve, is also shown.

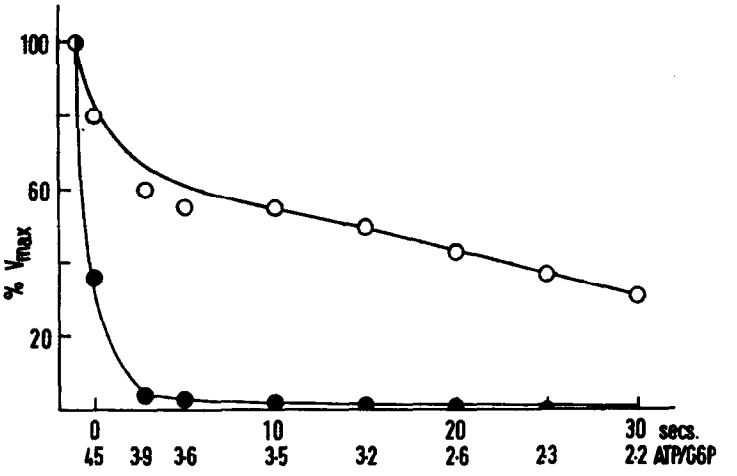

Figure 2. Activity of partially purified hexokinase in simulated systems. The hexokinase activity in the presence of ATP concentration found in the cell at time intervals from 0-30 sec. after addition of glucose are shown by O - O; the activity in the presence of ATP plus G6P concentrations at the same time intervals by ●-●. The time scale and the ATP and G6P concentrations are similar to those shown in Fig. 1. The ATP/G6P quotient is given for the appropriate time intervals. The results are expressed as a percentage of the maximum velocity measured in the presence of optimum substrate concentrations in the absence of G6P (●).

The activity of partially purified soluble hexokinase in simulated systems is shown in Fig. 2. The effect of the decreasing ATP content is shown by the upper curve and the combined effect of changes in the ATP/G6P quotient by the lower curve. The latter shows a fall in the rate of hexokinase to less than 5% of V_{max} in the presence of concentrations of ATP and G6P similar to those found in the cell 3 sec. after addition of glucose. This is in sharp contrast to the linear rate of glucose phosphorylation found at this time in intact cells as shown by the $\sum$ metabolites (Fig. 1).

FDP accumulates in high concentration in ascites cells and the effect of this metabolite on hexokinase, again in simulated systems, was studied. In the presence of 3.7 mM ATP, 7.4 mM Mg^{++} and 2 mM FDP a 90% inhibition of soluble hexokinase was obtained; the Mg^{++} content of washed ascites cells was determined as 7.0 mM. Raising the Mg^{++} content of the assay system to

15 mM decreased the FDP inhibition to only 23%, suggesting the involvement of Mg^{++}, ATP and FDP interactions in this inhibition.

DISCUSSION

The present results may be examined from the viewpoint of the sequence of control mechanisms operating at hexokinase in the intact cell. The very rapid initial accumulation of G6P might be expected to cause an immediate feedback inhibition of hexokinase activity. The K_i of hexokinase for G6P is well below the concentration found in ascites cells within 3 sec. after addition of glucose {K_i G6P v ATP, 0.02 mM; K_i G6P v glucose, 0.16 mM (15)}; further, an ATP/G6P quotient of 3 has been cited as causing a 90-95% inhibition of hexokinase in the presence of 1 mM ATP and 3 mM Pi (1). Nevertheless, it is clear that a rapid and linear rate of glucose phosphorylation continues for 15 sec. after glucose addition, after which the feedback inhibition appears to be operative. The simulated systems show that the soluble hexokinase of ascites cells is indeed susceptible to inhibition by G6P and the ATP/G6P content existing in the cell. Thus other factors must be operative to counteract the inhibition of hexokinase activity during this initial period, the most obvious of which is the binding of hexokinase to mitochondria which has been shown to cause a modification in the properties of the enzyme towards G6P inhibition (7,11,12). Rose & Warms (16) have shown that 0.2 mM G6P causes a release of 60% of bound hexokinase and further that the halftime for release of hexokinase was 18 sec. at 35° with 0.1 mM G6P. The figures correlate well with the present changes observed in the intact cell, where the concentration of G6P is 0.4-0.5 mM and the time of onset of inhibition 15-20 sec. after addition of glucose. Thus the second phase may be characterised by a release of bound hexokinase and onset of feedback inhibition by G6P.

In the present experiments only 28% of hexokinase was in the bound form but it is possible that the osmotic shock used to disrupt the cells may have altered the distribution. In Ehrlich ascites cells 60% of hexokinase was

recovered in the mitochondrial fraction in a method using 0.25 M mannitol - 0.3 mM EDTA and disruption with glass beads in a Nossal shaker (17); in Krebs ascites cells 53% of bound form was recovered in cells homogenised with 0.25 M sucrose without osmotic shock (18).

A second possibility for the onset of inhibition at 15 sec. might be the accumulation of metabolites other than G6P which could reinforce the inhibition; FDP appeared the most likely since it is present at a concentration of 1.5 mM. Studies with a simulated system showed that with the cellular concentration of Mg^{++} (7 mM) up to 90% inhibition of hexokinase was produced by 2 mM FDP, this effect was markedly dependent on the Mg^{++} concentration. This effect may be considered in relation to the operation of oscillating systems. Higgins (19) has defined the basic requirement for an oscillating system, shown in Fig. 3, and the hexokinase : phosphofructokinase coupled system appears to fulfil these conditions, the inhibitory effect of FDP on hexokinase and activation of phosphofructokinase by F6P and ADP providing the crossover coupling of opposite character. While oscillating systems have been widely studied in yeast and heart extracts (see Hess & Boiteux, ref. 20), only preliminary evidence appears to exist for continuous oscillations in the glycolytic pathway in ascites cells (21).

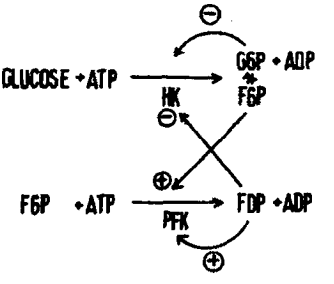

Figure 3. Interrelationships between G6P, F6P and FDP as modifiers of hexokinase (HK) and phosphofructokinase (PFK) based on the Higgins model for general oscillatory mechanisms (19). This requires that one product must tend to activate its own production (FDP on PFK), that the other product must tend to inactivate its own production (G6P on HK) and that there must be cross coupling of opposite character (FDP inhibition of HK and F6P activation of PFK).

The present results appear to give some *in vivo* evidence for the importance of binding in the control of glucose phosphorylation and in the modification of the feedback inhibition by G6P.

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