TRANSIENT ISOLATION OF THE HEXOKINASE REACTION FROM THE GLYCOLYTIC SEQUENCE ON INITIATION OF GLYCOLYSIS IN ASCITES TUMOR CELLS

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The kinetics of accumulation of glycolytic intermediates during the first 10 sec after glucose addition to Ehrlich ascites tumor cells indicate that hexokinase operates alone for the first 3 sec, utilizing a pre-existing pool of ATP. Between 4 and 5 sec, hexokinase activity declines, while the remainder of the glycolytic sequence begins a coordinated acceleration; after 5 sec, hexokinase activity increases again, following the sharply rising phosphofructokinase activity. The 5 sec point coincides with the minimum ATP level in the cells and also marks the beginning of the coupling between the ATP-utilizing kinases in the head-end of glycolysis and the ATP-generating systems.

Recently Gumaa and McLean (1) reported evidence that the initial period of rapid glucose phosphorylation by Krebs ascites tumor cells was independent of feedback inhibition by G6P and suggested that the slowing of glucose phosphorylation observed after 15 sec was a consequence of a release of bound hexokinase, which release rendered it accessible to inhibition by the G6P. The kinetic studies of glycolysis in Ehrlich ascites tumor cells below support Gumaa and McLean's contention that G6P is not a major controlling factor in this period, but also indicate a shift in the source of the ATP used for glucose phosphorylation at about 5 sec and suggest a control by ADP.

METHODS

A hypotetraploid strain of Ehrlich ascites carcinoma cells was grown for 9 days in Swiss white mice and was prepared in 54 mM phosphate buffer solution (phosphate-Locke, pH 7.3-7.4) as described previously (2). To facilitate rapid sampling, the 25 ml incubation flasks were fitted with

stoppers equipped with inlet and outlet tubes; a vacuum line was attached to the outlet tube so that measured volumes could be quickly sucked into the cell suspension contained in the flask. In the experiment described in detail below, 6 ml of buffer containing 0.29 ml of packed cells was placed in the flask at 23°; 0.50 ml of 10 mM glucose in buffer was drawn into the cell suspension, with continual manual agitation, to give a final glucose concentration of 0.77 mM (17.2 μmoles/ml cells) and 2 to 10 sec later, 3 ml 14% perchloric acid was drawn into the suspension to quench the reactions. The acid supernatants were neutralized with KOH, and after removal of the $KClO_4$, were analyzed for nucleotides and glycolytic intermediates by specific enzymatic procedures (3). Lactic acid was determined by the method of Barker and Summerson (4).

RESULTS

The accumulation patterns of G6P, F6P, and FDP shown in Fig. 1 are typical. Both G6P and F6P pass through definite maxima near 5 sec, the F6P lagging slightly behind the G6P. FDP often exhibits a brief decline during the first second or two after glucose addition and then rises dramatically as

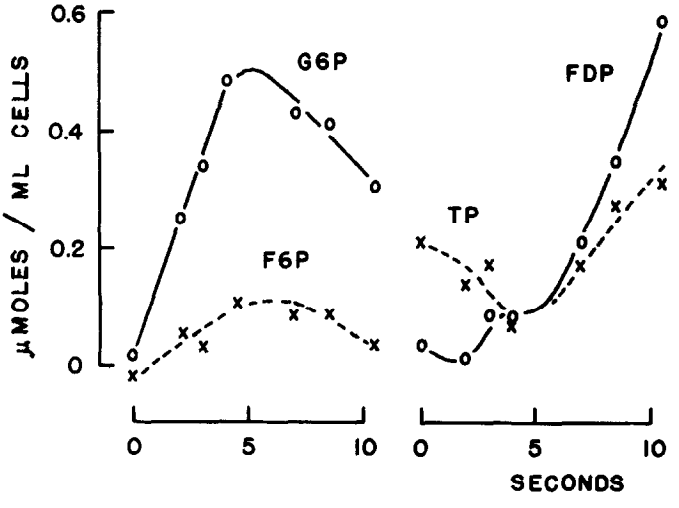

Fig. 1. Accumulation of glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-diphosphate (FDP), and triose-phosphate (TP). Values are given as μmoles per ml of packed cells; glucose is added to aerated cells at zero time.

G6P begins its decline. The early part of triose phosphate curve is variable; when the initial level is high, as in Fig. 1, it declines for the first few seconds, whereas when it is low, it tends to rise slowly. However, after 5 sec. triose phosphate consistently rises along with the FDP curve.

The cyclic change in ATP (Fig. 2) has been observed repeatedly (3,5,6). Although not measured in this particular experiment, the ADP curve invariably approximates a mirror image of the ATP curve during the cycle, rising from a baseline of from 0.5 to 1.0 μmole/ml cells (3,6).

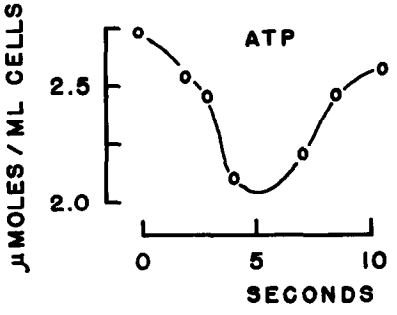

Fig. 2. Changes in ATP after glucose addition.

Accumulation of lactic acid represents a major component in the rate calculations after 5 sec, but its measurement is imprecise because of the high initial level of lactate (11-12 μmoles/ml cells in this experiment). To improve precision, values from three incubation series with the same cell preparation but different glucose concentrations were averaged (Fig. 3). The results with the highest concentration (0.77 mM) are those represented in Figs. 1 and 2. Lower glucose levels were used in the other two series (0.15 and 0.08 mM), but earlier studies (7) have demonstrated that the initial rate of lactate accumulation is insensitive to glucose concentration in this range. After a brief decline, lactate begins to accumulate at a rate approaching 10 μmoles/ml cells/min, in agreement with rates observed previously (3,7). The initial decrease has been observed in other experiments, but careful balance studies over longer intervals (7,8) indicate that its effect

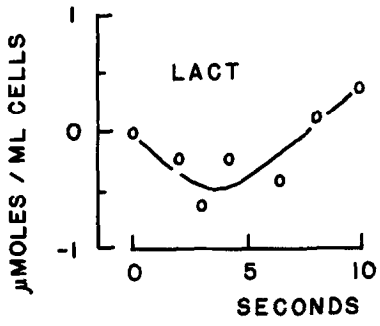

Fig. 3. Accumulation of lactate.

on the overall balance is negligible.

Estimates of phosphoglyceric acids, phosphoenol pyruvate, and pyruvate in comparable experiments indicate only minor accumulation of these intermediates over the first 10 sec, the total being less than the change in triose phosphate, and calculation of the total rate of accumulation consistently gives values less than 1.0 μmole/ml cells/min (7,8).

By summing the rates of accumulation of the measured intermediates, it is possible to obtain approximate velocities for individual enzymes (7). Two assumptions are made in these approximations: (a) lactate production via glycolysis is zero until 4 sec, after which it rises along the curve shown in Fig. 3; and (b) contribution of the phosphoglyceric acids and pyruvate to the overall balance is small. The underlying assumption is that glycolysis operates without complicating side reactions. The calculated velocities for hexokinase (V_H), phosphofructokinase (V_P) and lactate dehydrogenase (V_L) in glucose equivalents are shown in Fig. 4. Even allowing for the mentioned uncertainties, a general shift in glycolysis is apparent between 4 and 5 sec; previous to this time, hexokinase is operating alone; after the shift, the whole glycolytic sequence is operating, the "head end" being more rapid than the "tail end," as is usual in the early periods (6). Although the velocity of phosphoglucose isomerase begins at 1.8μ moles/min above V_P, it approaches V_P by 5 sec. The later decline in F6P indicates that the isomerase is not

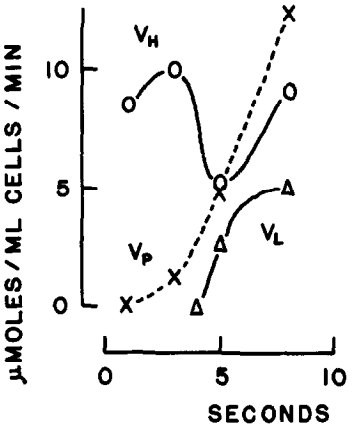

Fig. 4. Calculated velocities of hexokinase (V_H, O ——), phosphofructokinase (V_P, X ---), and lactate dehydrogenase (V_L, Δ ——) in glucose equivalents.

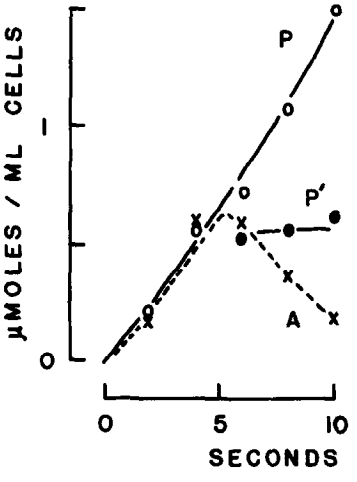

Fig. 5. Total and net ATP investment into phosphorylation of glycolytic intermediates compared to the change in ATP level. P, O ——, total phosphate esterified in accumulated sugar phosphates; P', O ——, phosphate esterified minus ATP generated by the tail end of glycolysis; A, X ---, decrease in ATP content of cells (-Δ ATP). Calculations described in text.

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regulating the phosphofructokinase velocity.

The ATP utilized to accumulate phosphorylated glycolytic intermediates is depicted by line P of Fig. 5: $P = \Delta G6P + \Delta F6P + 2\Delta FDP + \Delta TP$. Comparison of the actual decline in ATP level (-ΔATP), curve A, illustrates that consump-

tion of pre-existing ATP could completely provide for the accumulation of sugar phosphates over the first 5 sec. The divergence of the A and P curves after 5 sec indicates that sugar phosphorylation is coupled to the ATP-generating systems after this time and is no longer drawing upon some reserve pool of ATP. Curve P' was obtained by subtracting lactate accumulation from P ($P' = P - \Delta L$) and represents the net requirement of glycolysis for ATP, assuming one ATP generated per lactate produced. From P', it is apparent that glycolysis could be self-supporting with respect to phosphorylation after 5 sec.

DISCUSSION

The conclusions based on Figs. 4 and 5 suggest that hexokinase acts something like a "pump primer" for glycolysis, preloading the system with a supply of G6P by using a reserve pool of ATP. Once the priming has occurred, the whole glycolytic sequence begins to accelerate in a coordinated fashion and is thereafter coupled to the ATP-generating systems of the cell. This shift suggests possible changes in the nature of the hexokinase. It is conceivable, for example, that the secondary rise in hexokinase activity represents activation of a different population of hexokinase molecules.

The possible effects of potential controlling agents can be approximated from K_i values in the literature. Some of these values were obtained with rat brain and muscle hexokinase by Grossbard and Schimke (9), whereas others were measured with Ehrlich ascites carcinoma hexokinase by Uyeda and Racker (10) (see Table I). The latter workers observed that inorganic phosphate reversed the inhibitory effects of G6P but not ADP, and the values for G6P inhibition in 10 mM phosphate was estimated from one of their tables, since this would approximate the intracellular phosphate concentration (11,12). The K_m values for tumor hexokinase used were 0.43 mM for ATP (10) and 0.03 mM for glucose (E. Bacal and E. L. Coe; unpublished results). In the period from 0 to 5 sec, ATP declines from 2.7 to 2.0 mM, G6P increases from 0.02 to 0.50 mM, glucose remains near 0.77 mM, and ADP will probably increase from

TABLE I

CALCULATED INHIBITION OF HEXOKINASE BY

GLUCOSE-6-PHOSPHATE AND ADP

Substrate	Inhibitor	P_i (mM)	K_i (mM)	Ref.	Relative Activity at 0 sec	Relative Activity at 5 sec
Glucose	G6P	-	0.16	(9)	1.00	0.90
ATP	G6P	0	0.02	(9)	0.88	0.18
ATP	G6P	10	-	(10)	1.00	0.55
ATP	ADP	-	0.05*	(10)	0.42	0.22

P_i: inorganic orthophosphate. Concentrations of substrates and inhibitors are given in text. * K_i reported for ascites tumor hexokinase; values for rat tissue hexokinases were over 10 times higher (9).

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about 0.5 to 1.0 mM (3,6). The possible inhibitions calculated from these values are summarized in Table I. The minimum hexokinase velocity at 5 sec is about 50% of the initial velocity (Fig. 4) which could be consistent with either G6P or ADP inhibition; however the relatively high velocity at 3 and 8 sec would not be consistent with the elevated G6P level at these times (Fig. 1). In comparison, the shape of the velocity curve corresponds roughly to the ATP curve (Fig. 2), suggesting that ADP inhibition may play a role in the control of hexokinase.

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