ORIGIN OF THE DELAYED FEEDBACK CONTROL OF GLUCOSE UTILIZATION IN ASCITES TUMOR CELLS

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SUMMARY: Ascites cell hexokinase exhibits a biphasic response to its inhibitor glucose-6-P. Approximately half of the inhibition appears to be immediate. However a significant catalytic activity remains that falls to the steady rate very slowly. The half time of this latter response is 12 sec for the soluble enzyme and 130 sec for the enzyme bound to mitochondria. These results help to explain the biphasic character of the glucose utilization rate in tumor cells that follows the addition of glucose. The metabolic role of the delayed response of hexokinase to a feedback effector is discussed.

INTRODUCTION: Several laboratories have reported that glucose utilization in ascites cells begins with a rapid phase that last 20-60 sec, during which the hexokinase rate immediately following the addition of glucose is about 8-15 times greater than in the steady state (1-6). A number of hypotheses have been advanced to explain the difference in control of the hexokinase step during these two phases. Chance and Hess in 1956 noted that the glucose utilization rate changed in parallel to the O_2 uptake rate (5,6). Since the high initial O_2 uptake rate was attributed to the rapid generation of ADP by hexokinase, the much slower subsequent rate of glucose utilization was attributed to the non-availability of ATP in the cytosol, due to a presumed mitochondrial compartmentation of ATP.

In these early studies the role of glucose-6-P as a regulator of tumor hexokinase was not generally appreciated (7). More recent kinetic studies of the isolated enzyme showed this product to be a potent inhibitor with $K_i \cong 10^{-5}$ M (2,4,8). Although this fact, together with the measured levels of ATP and glucose in the cell, can satisfactorily explain the steady state rate of hexokinase, the transient high rate poses a problem. This is because careful measurements of the levels of intermediates during the transient phase indicate that the glucose-6-P level reaches a very high concentration of 0.5-1 mM in the cell prior to the onset of the inhibited state (9-11). The failure to correlate the time course of hexokinase inhibition with the changes in glucose-6-P concentration was attributed by Gumaa and McLean (10,11) to a slow release of hexokinase from the mitochondrial-bound state, in which it is less sensitive to inhibition by glucose-6-P (12,13). However, we have found that the

distribution of hexokinase between the mitochondrial and soluble phases is unaffected by the presence of glucose (14).

The present paper reports properties of hexokinase in vitro that indicate that the delayed feedback control seen in the whole cell is an intrinsic property of the enzyme.

METHODS: Soluble hexokinase, isoenzyme II, was isolated from Ehrlich-Lettre hyperdiploid ascites tumor cells as previously described (12). The enzyme used was purified through the DEAE-cellulose step. Isolated mitochondria from these cells were used as the source of bound hexokinse. Hexokinase was assayed by measuring the conversion of [2-^{3}H]glucose to [2-^{3}H]glucose-6-P by the following method. The reaction with radioactive glucose was terminated effectively by the addition of 100 μmoles of unlabeled glucose (providing greater than 1000-fold dilution) and 10 units phosphoglucoisomerase. Incubation with the isomerase was continued for 7 min to exchange all of the tritium of the [2-^{3}H]glucose-6-P with water. HCl was then added to a final concentration of 0.1 N and the water was sublimed in vacuo in a Y-tube such as described by Sprinson and Rittenberg (15) or by Englard and Hanson (16). The radioactivity of the water was measured by liquid scintillation spectrometry. Under the conditions used, this assay was linear with enzyme concentration and, in the absence of glucose-6-P, with time. Assays of [2-^{3}H]glucose-6-P by this method agree within 10% with values obtained spectrophotometrically (12). The isotopic method gives duplicates that agree to within about 3.5%. No detritiation is observed in the absence of either hexokinase or phosphoglucoisomerase and 97% of the tritium can be released by isomerase in the presence of excess hexokinase. Thus the hexokinase preparations used do not contain interferring enzymes which utilize either glucose-6-P or fructose-6-P and the tritium is confined to the 2 position of the glucose.

RESULTS: In considering possible explanations for the biphasic behavior of the glucose phosphorylation rate, one is compelled to explain the apparent insensitivity of the initial rate of the cellular hexokinase to the high glucose-6-P concentration that develops within 5 sec after the addition of glucose. It has previously been shown that yeast hexokinase incubated with substrates at low pH was in a highly inhibited state and responded slowly to activators such as citrate and P_i (17). Frieden has called those enzymes that respond slowly to a change in a ligand's concentration, hysteretic enzymes and has given a number of examples for which the ligand is either an activator or inhibitor of the catalytic process (18). It was, therefore, of considerable interest to determine the time course of inhibition that occurs when tumor hexokinase is suddenly exposed to high concentrations of glucose-6-P. The

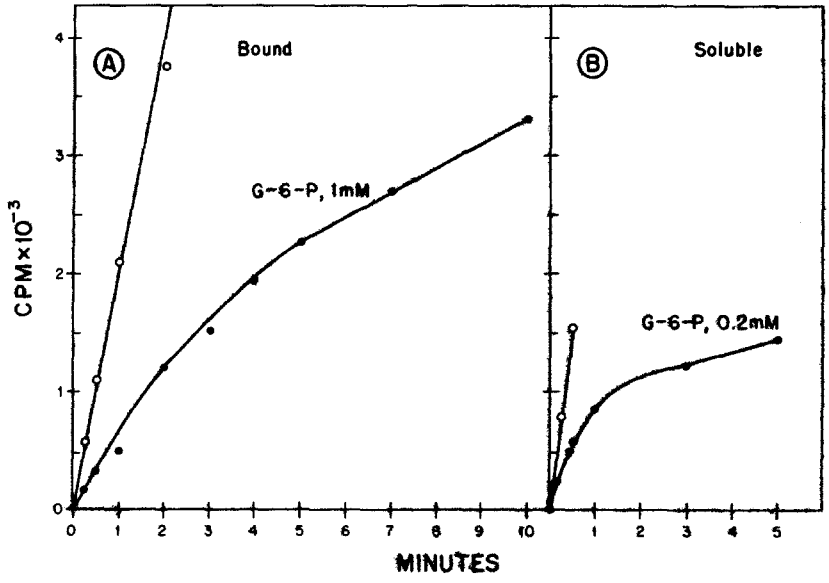

Figure 1: Time-dependence of glucose-6-P inhibition of hexokinase. Each tube contained: triethanolammonium-chloride, pH 8.0, 50 mM; ATP, 1 mM; $MgCl_2$, 2.5 mM; [2-^{3}H]glucose, 0.4 mM, 8 x 10^5 cpm/μmole. (A) contained 0.025 unit bound hexokinase per ml and glucose-6-P, 2 mM (●) or 0 mM (○). (B) contained 0.045 unit soluble hexokinase/ml and glucose-6-P, 0.2 mM (●) or 0 mM (○). Reactions were initiated by the addition of glucose and glucose-6-P where indicated. At the indicated times 0.15 ml samples were removed for assay as described in the Methods section. The ordinate represents the counting rate of water in the samples treated with isomerase. Under conditions of the experiment in A, 90% of the hexokinase remained bound.

results of such studies with hexokinase that is either bound to mitochondria or in solution are seen in Figures 1A and 1B. In both experiments, glucose, with or without glucose-6-P, was added to the otherwise complete reaction mixture. The amount of enzyme present was sufficiently low so that the glucose-6-P formed during the incubation did not bring about inhibition. When the bound enzyme is assayed in the presence of 1 mM glucose-6-P and 1 mM ATP the hexokinase has an initial rate of about 30% the uninhibited rate and after 5 min achieves a final steady state rate 12% of the uninhibited rate (Figure 1A). Similarly when the soluble hexokinase is assayed in the presence of 0.2 mM glucose-6-P and 1 mM ATP the initial rate is about 45% of the uninhibited rate and after 2 min achieves a final steady state rate 6% of the uninhibited rate (Figure 1B). The half time of the response to glucose-6-P was determined by replotting the data of Figure 1 as a semilogarithmic function of $v_t - v_f$ where v_t is the velocity at time t and v_f is the

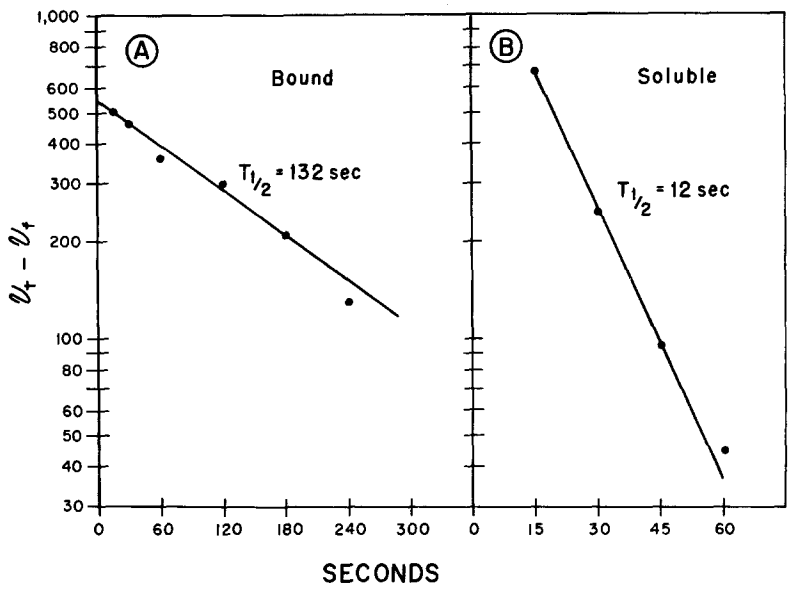

Figure 2: Half-time of response to glucose-6-P. The data from Figure 1A and 1B are replotted according to Frieden (18) as log (v_t-v_f) vs time. v_t-v_f is the velocity at time t minus the final velocity.

final steady state velocity (18) as shown in Figure 2. The $t_{\frac{1}{2}}$ is 132 sec for the bound and 12 sec for the soluble enzyme. That the slow fall in rate is not due to removal of substrates or increase in glucose-6-P level is shown by the control. That the observed slow decrease in reaction rate is not caused by inactivation in the presence of glucose-6-P was established by determining the hexokinase activity in the assay with glucose-6-P dehydrogenase. No losses of activity could be found after 10 min of incubation in the presence of high amounts of glucose-6-P under the experimental conditions used in Figure 1.

If the transformation of hexokinase to an inhibited state with glucose-6-P is, in part, a slow one, what is the time course of the activation of the enzyme after glucose-6-P is suddenly removed? To investigate this question, the experiment illustrated in Figure 3 was carried out. Hexokinase bound to mitochondria was incubated for 5 min with or without glucose-6-P and in the presence of unlabeled substrates. The mitochondrial hexokinase thereby placed in the inhibited or non-inhibited state, was pelleted by centrifugation and rapidly resuspended in an assay medium containing [2-^{3}H]glucose, with or without glucose-6-P, and assayed for glucose phosphorylation at successive intervals. The results show that when enzyme has been previously incubated with glucose-6-P and then transferred to a medium lacking glucose-6-P, the

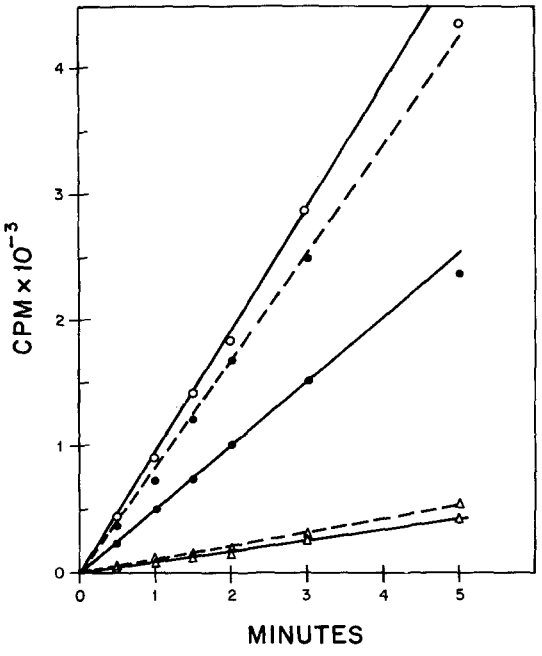

Figure 3: Time dependence for the reversal of glucose-6-P inhibition. Mitochondria containing 0.05 unit of bound hexokinase/ml were incubated with triethanolammonium-chloride, pH 8.0, 50 mM; $MgCl_2$ 2.5 mM; ATP, 1 mM; glucose 0.2 mM and glucose-6-P, 0 mM (○) or 1 mM (●,△). After 5 min at 25° the enzyme was pelleted by centrifugation at 15,000 x g for 2 min and resuspended in triethanolamine buffer, pH 8.0, 50 mM; ATP, 1 mM; $MgCl_2$, 2.5 mM; and [2-^{3}H]glucose 0.2 mM, 8 x 10^5 cpm/μmole and glucose-6-P 0 mM (○,●) or 1 mM (△). At the indicated times 0.2 ml samples were removed for assay of the tritium in glucose-6-P as described in the Methods section. The solid lines are drawn through the data points and dashed lines represent these points corrected for enzyme lost by incomplete centrigugation as judged by spectrophotometric assay (12) of an aliquot of the final incubation mix and normalized to the no glucose-6-P control.

activated rate is achieved without an observable time lag.

DISCUSSION: The initial transient phase of rapid glucose phosphorylation that follows the addition of glucose to ascites cells has been observed by various workers to endure for periods of time usually ranging from 15 sec (10) to 60 sec (5) and in some studies for as long as 2 min (19). These values for the delay in onset of the inhibited state *in vivo* are readily explained by the slow response of both the soluble and bound forms of hexokinase to glucose-6-P shown in Figure 1. A second feature of the whole cell kinetic studies that correlates with the *in vitro* studies relates to the initial rate of hexokinase in the presence of high levels of glucose-6-P. As is noted clearly in Figure

1A, the bound hexokinase seems to contain a form that responds immediately to the inhibitor since the initial transient rate is only about 30% of the fully uninhibited rate. Likewise in whole cell experiments it has been observed that the initial burst rate of glucose utilization corresponds to only about 30-45% of the V_{max} of hexokinse (2,11) in spite of the fact that the concentrations of glucose and ATP present in the cell initially should support rates close to V_{max} in the uninhibited state. The in vitro results cannot be readily attributed to the presence of different isozyme forms of hexokinase since an examination of the eluted and soluble hexokinase of the Ehrlich-Lettre cells by gel electrophoresis show it to be of the isozyme II form only. Yushok (20) has found initial rates of 2-deoxyglucose utilization in ascites cells that approach the V_{max} of hexokinase very closely. 2-deoxyglucose-6-P which accumulates in such cells is not inhibitory. Thus the lower-than-V_{max} initial rate seen with glucose is due to the immediate effect of the specific inhibitor, glucose-6-P on a portion of the hexokinase rate, as seen in vitro.

A simple tentative model that encompasses these characteristics is the following:

$$E_1 \underset{\text{slow}}{\rightleftharpoons} E_2 \overset{\text{G6P}}{\rightleftharpoons} E_2 \cdot \text{G6P}$$

The enzyme is proposed to exist in two conformers E_1 and E_2 that are slowly interconvertible. Both forms are equally active catalytically but only one, E_2, interacts with glucose-6-P to form the inactive complex. Upon removal of glucose-6-P from the fully inhibited enzyme by rapid dilution, Figure 3, there is an instantaneous return to the active form, E_2. Further experiments to test this scheme are in progress.

The present case may be the first in vivo example of a delayed response of an enzyme to the changed intensity of a regulatory signal. Glucose-6-P, the product of the hexokinase step, acts as an allosteric effector of the hexokinase activity of higher organisms, as was first clearly conceived by Crane and Sols in 1953 (21,22). Thus, any of the several pathways that utilize glucose-6-P can become mobilized through their influence on the size of the glucose-6-P pool. The action is allosteric as indicated both by specificity (22) and kinetic studies (12,23). One must ask, what is the advantage of a hysteretic response in the inhibition of hexokinase. First, it seems clear that the rise in glucose-6-P and ADP are important signals to the cell that glucose is available for metabolism. As already mentioned the ADP signal manifests itself in an immediate burst in aerobic metabolism (5), but further sensitive effects may be anticipated from actions of ADP as allosteric effectors of other processes (24,25). The point to make is that the signals of glucose availability are made almost instantaneously upon the addition of glucose to

the ascites cells. If hexokinase were sensitive to glucose-6-P inhibition during this initial period, the rate of build up of glucose-6-P and ADP would be greatly diminished, and the signals would be less intense until the steady state was reached. Using the data of Gumaa and McLean (11) as an indication of the amount of hexokinase, the steady state rate of glucose utilization and the apparent inhibition constant of glucose-6-P, one calculates that the time course of increase in glucose-6-P (and ADP) during the approach to steady state would be hyperbolic with a $t_{\frac{1}{2}}$ of about 12 sec, rather than linear with a rise time of the order of 3 sec as observed in vivo. A second effect of the burst in glucose-6-P concentrations is that the response of phosphofructokinase will likewise be rapid and maximal. It is important to its regulation, apparently, that phosphofructokinase responds in a sigmoidal manner to changes in fructose-6-P concentration (26,27). If the phosphofructokinase is to respond rapidly to the availability of glucose, the fructose-6-P should exceed the threshold for control. Therefore, by virtue of the hysteretic behavior of hexokinase towards inhibition by glucose-6-P allowing the rapid rise in both ADP and glucose-6-P (or fructose-6-P) phosphofructokinase is rapidly mobilized from an off to an on-state.

As noted in Figure 3 the response of hexokinase to a sharp decrease in the concentration of glucose-6-P is immediate. Thus the activation of any pathway that removes glucose-6-P or fructose-6-P will lead to an immediate stimulation of hexokinase. This again, has the virtue of producing a sharp parallel response in the ADP pool that can reinforce catabolic processes.

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