ACTIVATION OF HUMAN ERYTHROCYTE PYRUVATE KINASE BY HEXOSE-BISPHOSPHATES

John A. Badwey and Edward W. Westhead
Department of Biochemistry
University of Massachusetts, Amherst, MA 01003

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Summary: Human erythrocyte pyruvate kinase (E.C. 2.7.1.40, ATP-pyruvate phosphotransferase) is activated by hexose-1,6-bisphosphates in the order: fructose- > mannose- > glucose-bisphosphate. These activators promote substantial reductions (7 to 10 fold) in the K_M for the substrate phosphoenolpyruvate but do not effect the maximum velocity. The ability of mannose- and glucose-1,6-bisphosphate to function as activators of both human erythrocyte phosphofructokinase (Rose, I.A. and Warms, J.V.B. (1974), Biochem. Biophys. Acta 156, 231-239) and pyruvate kinase may account for the presence of these metabolites in red cells at relatively high levels. Fructose-1,6-bisphosphate also induces slight increases in the K_M values for the active substrate, Mg·ADP, at subsaturating levels of phosphoenolpyruvate but is without effect at saturating levels of phosphoenolpyruvate. A possible role for these activators in maintaining a balance in the flux through the pyruvate kinase reaction is proposed.

Activation of human erythrocyte pyruvate kinase (E.C. 2.7.1.40, ATP-pyruvate phosphotransferase) by fructose-1,6-P_2 has been reported by a number of laboratories (1-8). In vitro experiments on whole cells, however, have shown that pyruvate kinase does not become a more efficient catalyst under conditions in which fructose-1,6-P_2 accumulates (9,10). A possible reason for this discrepancy could be the existence in red cells of additional activators capable of mimicking the effects of fructose-1,6-P_2. Human red cells are known to contain relatively high concentrations (0.1 to 0.20 mM) of glucose-1,6-P_2 and mannose-1,6-P_2 (11). Since both of these metabolites have recently been shown to be important physiological activators of human erythrocyte phosphofructokinase (12), we have investigated the possibility that these metabolites might also function as activators of pyruvate kinase.

Materials and Methods

Mannose-1,6-P_2 (lithium salt) was a generous gift from Dr. I. A. Rose. α-D-glucose-1,6-P_2 (potassium salt) was obtained from Calbiochem, Los Angeles, Calif. The sources of all other materials and the procedures used to prepare the enzyme are described in detail elsewhere (13).

Abbreviations: fructose-1,6-P_2, fructose-1,6-bisphosphate; mannose-1,6-P_2, mannose-1,6-bisphosphate; glucose-1,6-P_2, glucose-1,6-bisphosphate; P-enolpyruvate, phosphoenolpyruvate; glycerate-1,3-P_2, glycerate-1,3-bisphosphate.

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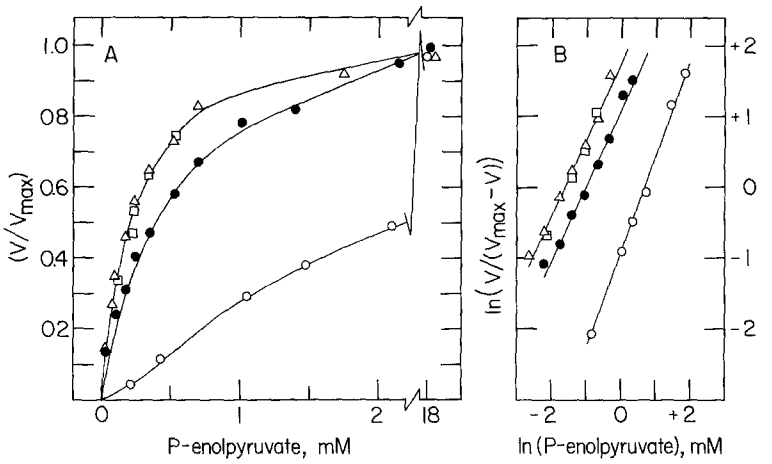

Figure 1. Effects of hexose bisphosphates on the P-enolpyruvate saturation kinetics. All assay conditions are described under Methods and the symbols correspond to the following additions: (o) no hexose-bisphosphate added, control; (•) 0.3 mM fructose-1,6-P_2; (Δ) 0.3 mM mannose-1,6-P_2; and (□) 0.3 mM mannose-1,6-P_2 plus 1.0 mM glucose-1,6-P_2. The data of part A were used to construct the Hill plots shown in part B.

Assay Procedure--Pyruvate kinase activities were measured by a modification of the coupled assay procedure proposed by Bücher and Pfleiderer (14). The reaction mixture of 0.7 ml contained 8.0 mM P-enolpyruvate, 4.1 mM ADP, 0.38 mM NADH, 5.7 mM $MgCl_2$, 100 mM KCl, 90 mM Hepes buffer (pH - 7.4), and 50 units of lactate dehydrogenase. The reactions were initiated by the addition of enzyme. Variations from this are detailed in the corresponding figure legends. The disappearance of NADH was followed at 340 nm on a Gilford 240 spectrophotometer. Temperature was maintained at 30°C. The velocity was obtained from the slope of the linear, fast phase of the reaction progress curve (13).

All of the Hill coefficients and K_a values reported were obtained from a linear regression fit of the data by the method of least squares analysis using a programmable Hewlett-Packard HP-25 calculator. The program was supplied by the manufacturer.

Results

P-enolpyruvate Saturation Kinetics--In the absence of activators, pyruvate kinase displays a slightly sigmoidal saturation curve (n_H = 1.2 to 1.4) for P-enolpyruvate with a $K_{0.5}$ of approximately 2.1 mM (Fig. 1). Addition of either fructose-1,6-P_2 or mannose-1,6-P_2 converted this curve to a normal rectangular hyperbola (n_H = 1.0) with a greatly reduced K_M (Fig. 1). A similar "K-type" (15) activation by glucose-1,6-P_2 has previously been described (16). Enzyme activated by saturating mannose-1,6-P_2 could not be stimulated further by the addition of glucose-1,6-P_2 (Fig. 1). Mannose-1,6-P_2 was added to the

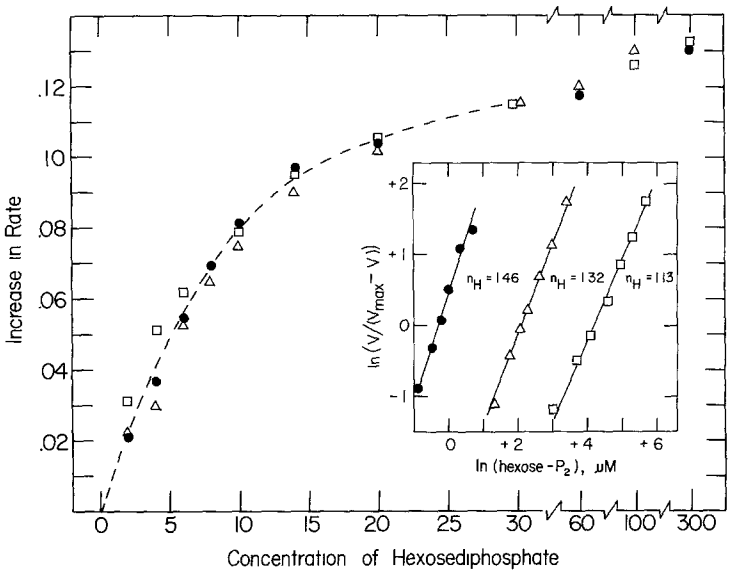

Figure 2. Activation of human erythrocyte pyruvate kinase by fructose-1,6-P_2 (●), M x 10^{-7}; mannose-1,6-P_2 (△), M x 10^{-6}; glucose-1,6-P_2 (□), M x 10^{-3}. Note the different orders of magnitude used in the abscissa for each of the hexose bisphosphates. The concentration of P-enolpyruvate utilized was 1.0 mM and all other assay conditions are those of the standard assay mixture described under Methods. Numerical values in the ordinate represent the differences in $A_{340/min}$ of assays with and without activator. The insert shows the Hill plots of the data.

reaction mixtures as the lithium salt, but at the concentrations employed in these studies, lithium had no effect on the enzyme.

Hexose-P_2 Activation Profiles--The activation profiles of human erythrocyte pyruvate kinase by hexose-bisphosphates are shown in Figure 2. The concentrations of hexose-P_2 required to produce half-maximal activation (K_a) are 0.73 μM, 8.4 μM and 6.4 μM for fructose-1,6-P_2, mannose-1,6-P_2, and glucose-1,6-P_2 respectively. The dashed line is drawn to emphasize the similar shapes of the dependence of rate upon activator concentrations.

Mg·ADP-Saturation Kinetics--Regardless of the concentration of P-enolpyruvate present, only hyperbolic saturation curves (n_H = 1.0) were observed (Fig. 3) for the active substrate complex, Mg·ADP (for a review of the role of the divalent cation in forming the catalytically active species, see

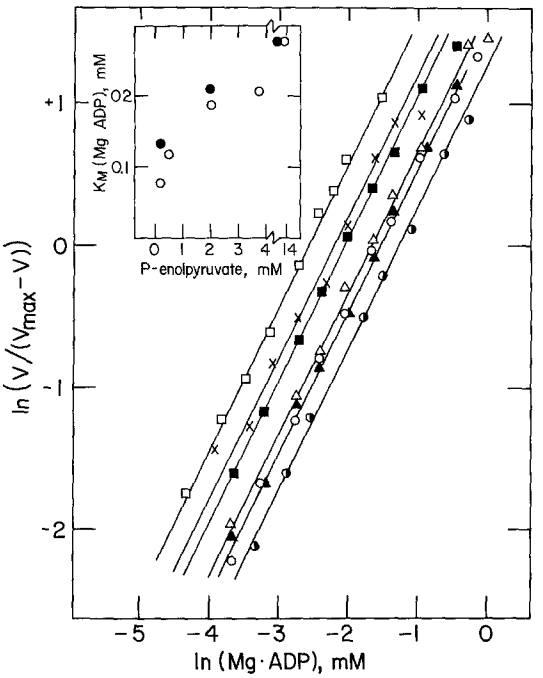

Figure 3. Hill plots of the saturation kinetics of human erythrocyte pyruvate kinase with the Mg·ADP complex as the variable substrate. The following symbols correspond to the concentrations of the fixed substrate, P-enolpyruvate: (□) 0.2 mM; (X) 0.5 mM; (△) 2.0 mM; (○) 4.0 mM and (◑) 14.0 mM. The corresponding closed symbols (■ and ▲) are for kinetic studies performed under the same conditions described above except for the presence of fructose-1,6-P_2 (0.1 mM). All other assay conditions are described under Methods. The insert shows the relationship between the K_M for the Mg·ADP complex and the assay concentration of P-enolpyruvate in the absence (o) and in the presence (●) of 0.1 mM fructose-1,6-P_2. Decreasing the assay concentration of NADH four-fold did not affect these values (data not shown).

The concentrations of Mg·ADP were calculated from the cubic equation which results from the solution of the simultaneous equations arising from the two equilibria of Mg^{++} with ADP and with P-enolpyruvate. The values utilized for the dissociation constants of the metal substrate complexes were 6.0×10^{-4}M for Mg·ADP (21) and 5.5×10^{-3}M for Mg·P-enolpyruvate (22).

References 17 to 20). The K_M values for this substrate increase as the degree of saturation of the enzyme with P-enolpyruvate increases (Insert, Figure 3). Fructose-1,6-P_2 (0.1 mM) promotes a slight increase in the K_M values for this substrate at subsaturating P-enolpyruvate levels (Insert, Figure 3) but has no effect at saturating PEP levels nor has it any effect on the Hill coefficients. Since the K_M values for the Mg·ADP complex increase as the degree of saturation of the enzyme with P-enolpyruvate increases (Insert, Figure 3), these

increased K_M values for (Mg·ADP) in the presence of fructose-1,6-P_2 are consistent with the fact that fructose-1,6-P_2 increases the affinity of the enzyme for P-enolpyruvate (Fig. 1) and probably does not reflect a change in the intrinsic affinity of the enzyme for Mg·ADP.

Discussion

Mannose-1,6-P_2 is an extremely effective activator of red cell pyruvate kinase *in vitro*, about 8 x more effective than glucose-1,6-P_2 (Fig. 2). Considering its high concentration in erythrocytes (100 to 200 μM (11,12) *vs* 2 to 4 μM for fructose-1,6-P_2 (12,23)), it may well function as an important metabolic activator.

Rose (24) has recently reported that red cells contain an enzyme, glucose-1,6-P_2 synthase, capable of synthesizing glucose-1,6-P_2 and mannose-1,6-P_2 according to the following equation:

glycerate-1,3-P_2 + glucose-1-P (or mannose-1-P) → glycerate-3-P + glucose-1,6-P_2 (or mannose-1,6-P_2)

Since the concentration of glycerate-1,3-P_2 in red cells is inversely related to the activity of pyruvate kinase (10), glycosyl-bisphosphate synthesis would be expected to become stimulated when pyruvate kinase is inhibited. Inhibition of pyruvate kinase should occur when the level of free glycerate-2,3-P_2 becomes elevated (7,25), such as during oxygenation of red cells (26,27). Increased synthesis of glycosyl-bisphosphates might then be important in modulating this inhibition to maintain a balance in the flux of metabolites through the pyruvate kinase reaction. Evidence for such a scheme is provided by the increased levels of both glucose-1,6-P_2 and glycerate-2,3-P_2 in pyruvate kinase deficient cells (12).

This scheme may also account for the failure of pyruvate kinase to become a more efficient catalyst under conditions in which whole cells accumulate fructose-1,6-P_2 (9,10) since under those conditions the levels of glycerate-1,3-P_2 and glycerate-2,3-P_2 would also be expected to be elevated (9,10). The parallel increases in the levels of the inhibitor, glycerate-2,3-P_2 (7,25) and

the hexose bisphosphate may balance each other. An alternative explanation would be that pyruvate kinase is always saturated with respect to its activators Although this latter possibility appears reasonable in view of the low *in vitro* activation constants shown in Figure 2, it should be remembered that under *in vivo* conditions (low P-enolpyruvate, high ATP levels), the activation constants would be expected to be considerably higher. Unfortunately, the hysteretic behavior of this enzyme (13) prevents detailed kinetic studies under simulated *in vivo* conditions by conventional methods.

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