REGULATION OF HUMAN ERYTHROCYTE HEXOKINASE BY GLUCOSE-1, 6-DIPHOSPHATE AND INORGANIC PHOSPHATE

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1. Introduction

Considerable amounts of glucose 1, 6-diphosphate (Glc-1,6-P_2) exist in mammalian erythrocytes [1–3], mainly as a result of the phosphoglucomutase reaction [2,3]. It is involved in glycolysis by its positive effects on phosphofructokinase [4] and pyruvate kinase [5] and by its inhibitory action on hexokinase [6,7]. The inhibition constants reported for hexokinase type I from human erythrocytes [6,7] are well in or below the range of the intracellular concentrations [1–3], suggesting a role for Glc-1,6-P_2 in regulation of hexokinase activity in vivo. In contrast Beitner et al. [8] reported that Glc-1,6-P_2 exerted a selective inhibitory effect on hexokinase type II and had no effect on type I hexokinase.

A striking pH-dependency of the inhibition by Glc-1,6-P_2 was reported by Rose and Warms [9] for hexokinase type II from Ehrlich-Lettré hyperdiploid cells and for hexokinase type I from Sarcoma 37 cells.

Recently we reported [10] that the inhibition of purified hexokinase from human erythrocytes by glucose 6-phosphate and fructose 6-phosphate is strongly regulated by inorganic phosphate. In this paper we show that the inhibition of human erythrocyte hexokinase by Glc-1,6-P_2 is similarly regulated by inorganic phosphate and that the same pH-dependency exists as reported by Rose and Warms [9] for hexokinase from Ehrlich-Lettré and Sarcoma 37 cells.

2. Materials and methods

Glucose 1,6-diphosphate (Glc-1,6-P_2, tetracyclohexylammonium salt), ATP (disodium salt), $NADP^+$ (disodium salt) and glucose-6-phosphate dehydrogenase were obtained from Boehringer, Mannheim. NaH_2PO_4 (P_i) and all other chemicals were of analytical grade of purity.

Human erythrocyte hexokinase was purified as reported earlier [11], with a specific activity of 35 U/mg of protein. The enzyme solution contained 0.05 M Tris–HCl (pH = 8.0), 0.5 M NaCl and 0.003 M β-mercaptoethanol.

Hexokinase activity was determined at 37°C in a system coupled with glucose-6-phosphate dehydrogenase as described before [10]. $MgCl_2$ was added in the concentrations necessary to maintain an excess of 5 mM Mg^{2+} over ATP. The concentration of glucose was 10 mM. The pH of the assay buffer ranged from 6.8–8.3. All pH values were determined at 37°C in the complete reaction mixture. The reaction was started by adding 0.03 units of hexokinase. One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of glucose 6-phosphate per min at 37°C.

Inhibition constants were calculated from double reciprocal plots of enzyme activity vs. [$MgATP^{2-}$] in the presence and absence of Glc-1,6-P_2 at a concentration near the K_i. These plots have been statistically treated as described before [12].

3. Results

Human erythrocyte hexokinase is inhibited by Glc-1,6-P_2 competitively with respect to $MgATP^{2-}$ (fig.1). The secondary plot of slope of the Lineweaver-Burke plots vs. inhibitor concentration is linear up to inhibitor concentrations of about twice the

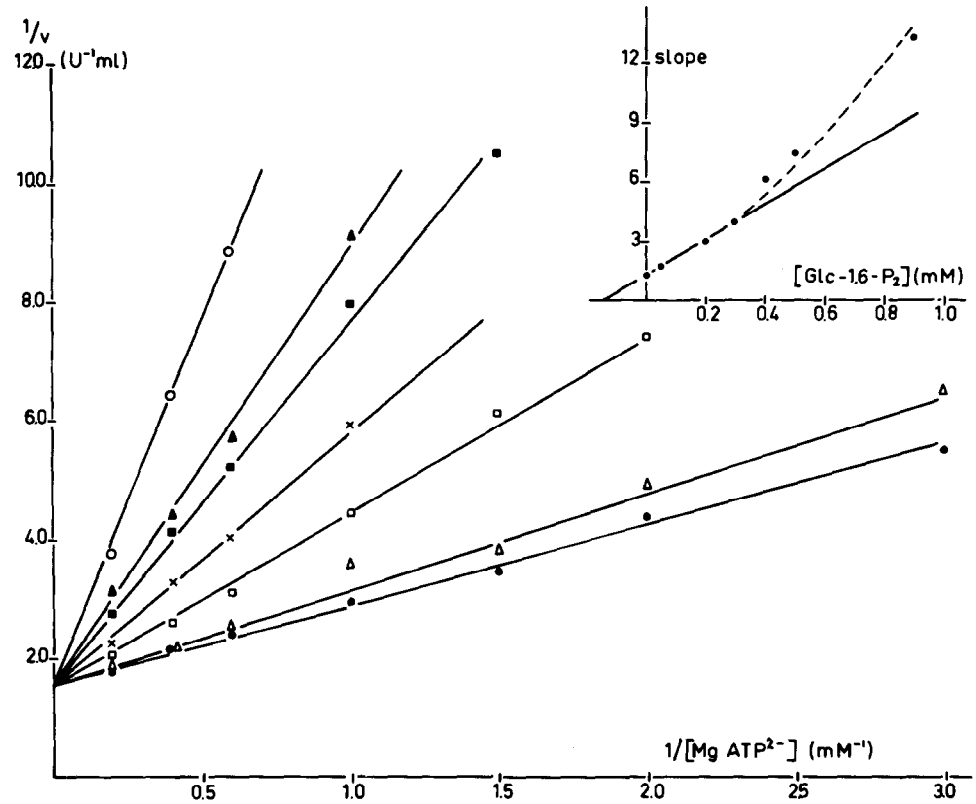


Fig.1. Lineweaver-Burk plots of hexokinase activity vs. [$MgATP^{2-}$] at concentrations of Glc-1, 6-P_2 of 0.0 mM (●), 0.05 mM (△), 0.2 mM (□), 0.3 mM (×), 0.4 mM (■), 0.5 mM (▲) and 0.9 mM (○). pH = 7.7. *Inset:* Secondary plot of slope vs. added Glc-1, 6-P_2.

K_i (inset fig.1). At higher concentrations the curve bends upwards. Inorganic phosphate was able to overcome, at least partly, the inhibition by Glc-1, 6-P_2 (fig.2). This influence of P_i is competitive with respect to Glc-1,6-P_2. A similar pattern of inhibition and a similar influence of P_i was found either at pH 7.7 (figs.1 and 2) or at pH 7.15 (results not shown), although K_i Glc-1,6-P_2 was much lower at pH 7.15 (see below).

Using modified Michaelis-Menten equations the influence of P_i on the inhibition constant of an inhibitor could be described in terms of enzyme activity in presence and absence of inhibitor as a function of P_i [10]. If P_i competes completely with the inhibitor, the function equals $f(P_i) = 1 + P_i/K_{P_i}$. However the funtion describing the influence of P_i on the inhibitor by Glc-1,6-P_2 is not linear (fig.3), reflecting the inability of P_i to overcome the inhibition completely. K_i Glc-1,6-P_2 is maximally increased by a factor of about 3 at pH 7.7 and by a factor of about 4 at pH 7.15 at higher concentrations of P_i. From the linear part of the function the activation constant of P_i, KP_i, can be calculated. Up to concentrations of about 1 mM P_i the activation constant of P_i is approx. 0.8 mM at both pH values.

Figure 4 shows that the inhibition by Glc-1,6-P_2 is pH dependent, the inhibition constant increases exponentially above pH 7.0. The inhibition was competitive towards $MgATP^{2-}$ at all pH values tested. The inhibion by glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-diphosphate, observed at pH 7.7 [10] was not pH-dependent.

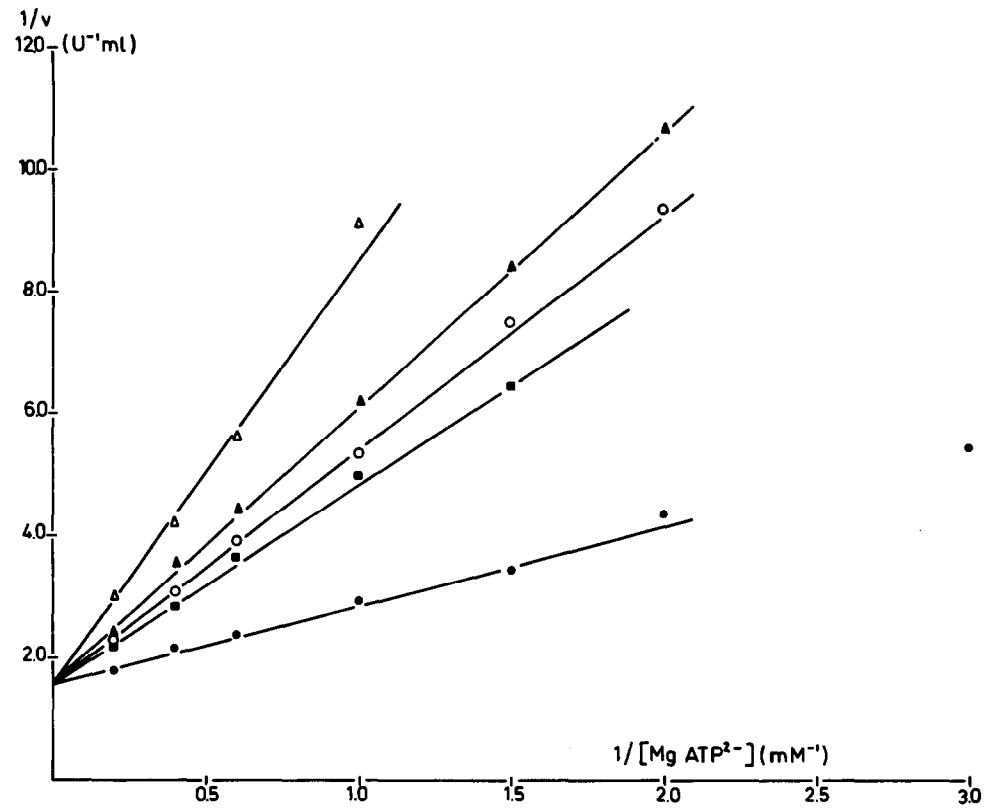
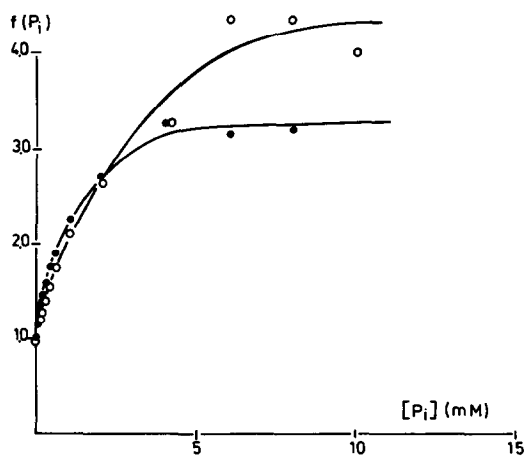


Fig.2. Influence of P_i on the inhibition by Glc-1, 6-P_2 at pH 7.7. The reciprocal of hexokinase activity is plotted vs. the reciprocal of [$MgATP^{2-}$] in the presence (△,▲,○,■) and absence (●) of 0.5 mM Glc-1, 6-P_2. The concentrations of added phosphate are 0.0 mM (●,△), 0.2 mM (▲), 0.8 mM (○) and 5.0 mM (■).



4. Discussion

The results show that the competitive inhibition of human erythrocyte hexokinase by Glc-1,6-P_2 with respect to $MgATP^{2-}$ is similarly regulated by phosphate as the inhibition by glucose-6-P and fructose-6-P described previously [10]. To explain the effects of P_i a model, proposed by Ellison et al. [13] for brain hexokinase, was modified: hexokinase exists in

Fig.3. Influence of P_i on the inhibition by Glc-1, 6-P_2 by 0.5 mM Glc-1, 6-P_2 at pH 7.7 (●) and by 0.1 mM Glc-1, 6-P_2 at pH 7.15 (○). f(P_i) is determined as defined in the text. [$MgATP^{2-}$] was kept constant at 0.5 mM.

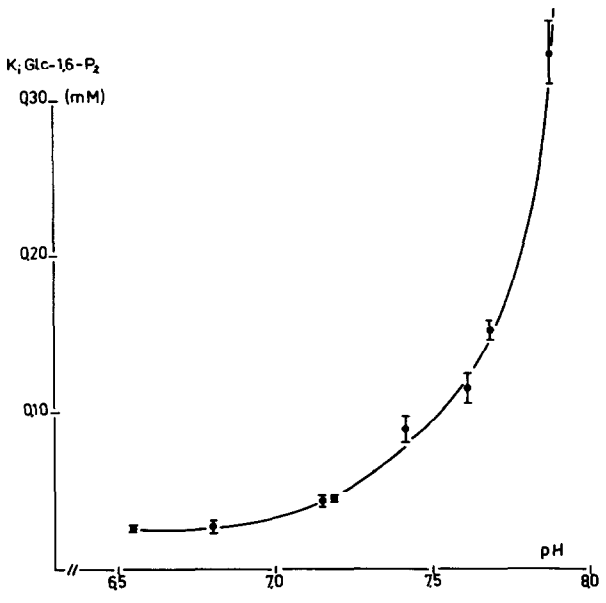


Fig.4. Influence of pH on the inhibition constant of Glc-1, 6-P_2. The values for K_i and the standard error of K_i (brackets) are calculated as described in Materials and methods.

equilibrium between a free and a phosphate associated form with the same kinetic properties except in their ability to bind hexose-phosphate. The phosphate associated form has a reduced but still substantial affinity for the inhibitor. An alternative explanation of the results is the presence of two different enzymes, one with a high affinity for hexose-phosphate, being sensitive to regulation by P_i, one with a lower affinity for hexosephosphate being insensitive to P_i; the K_m $MgATP^{2-}$ being the same for both enzymes. The secondary plot of fig.1, which is deviating from linearity at higher concentrations of Glc-1,6-P_2 is in favour of the latter model.

The pH-dependency of the inhibition of human erythrocyte hexokinase by Glc-1,6-P_2 is completely in agreement with the results of Rose and Warms [9] on hexokinase from Ehrlich-Lettré and Sarcoma 37 cells. They argued that the loss of inhibition at higher pH is attributed to the appearance of an additional negative charge. The phosphate-group at the C_1-atom is bound to the enzyme as a mono-anion but is excluded as a dianion.

At pH 7.2, the intracellular pH of the red blood cell, the inhibition constant of Glc-1, 6-P_2 is about 50 μM. This value is measured at a concentration of 5 mM of free Mg^{2+}. Under in vivo conditions this inhibition constant may even be lower, because intracellular concentrations of free Mg^{2+} (approx. 0.7 mM, ref. [14]) minimize the formation of Mg-Glc-1, 6-P_2 (K_{diss}= 2.5 mM), which is not inhibitory [9]. The value of K_i is well below the range of intracellular concentrations of Glc-1,6-P_2 (80–150) μM, ref. [1–3], suggesting an important role for Glc-1,6-P_2 in regulating hexokinase activity in vivo. From this data it is probable that Glc-1,6-P_2 is at least as important in regulating hexokinase activity in vivo as is glucose-6-P. Because P_i exerts its most pronounced effects in the range of its physiological concentration, the importance of P_i in regulating hexokinase activity is once more demonstrated.

The strong pH-dependency of the inhibition of human erythrocyte hexokinase by Glc-1,6-P_2 suggests that red cell glycolysis is influenced by pH not only at the phosphofructokinase step but also at the hexokinase step.

In glucosephosphate isomerase (GPI) deficiency increased levels of glucose 6-phosphate and an increased formation of glycogen in erythrocytes and liver are reported [15]. The latter suggests that in addition to the increased level of glucose-6-P the amount of Glc-1,6-P_2 might be increased too. Defective GPI thus may affect strongly the regulation of hexokinase, which may account for its strong impact on erythrocyte mechanism reflected in severe haemolysis. On the contrary the formation of P_i during glycogen formation will have a positive effect on hexokinase activity. Unfortunately there is little information available about the red cell contents of Glc-1,6-P_2 and P_i in GPI-deficiency.

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References

[1] Bartlett, G. R. (1968) Biochim. Biophys. Acta.156, 231–239.
[2] Passonneau, J. V., Lowry, O. H., Schulz, D. W. and Brown, J. G. (1969) J. Biol. Chem. 244, 902–909.
[3] Gerber, G., Winczuk, E. and Rapoport, S. (1973) Acta Biol. Med. Germ. 30, 759–771.
[4] Rose, I. A. and Warms, J. V. B. (1974) Biochem. Biophys. Res. Commun. 59, 1333–1340.
[5] Koster, J. F., Slee, R. G., Staal, G. E. J. and Van Berkel, Th. J. C. (1972) Biochim. Biophys. Acta 258, 763–768.
[6] Gerber, G., Preissler, H., Heinrich, R. and Rapoport, S. M. (1974) Eur. J. Biochem. 45, 39–52.
[7] Rose, I. A., Warms, J. V. B. and Kosow, D. P. (1974) Arch. Biochem. Biophys. 164, 729–735.
[8] Beitner, R., Haberman, S. and Livni, L. (1975) Biochim. Biophys. Acta 397, 355–369.
[9] Rose, I. A. and Warms, J. V. B. (1975) Arch. Biochem. Biophys. 171, 678–681.
[10] Rijksen, G. and Staal, G. E. J. (1977) Biochim. Biophys. Acta (in press).
[11] Rijksen, G. and Staal, G. E. J. (1976) Biochim. Biophys. Acta 445, 330–341.
[12] Rijksen, G. and Staal, G. E. J. (1976) Biochim. Biophys. Acta 452, 382–391.
[13] Ellison, W. R., Lueck, J. D. and Fromm, H. J. (1975) J. Biol. Chem. 250, 1864–1871.
[14] Gerber, G., Berger, H., Jänig, G. R. and Rapoport, S. M. (1973) Eur. J. Biochem. 38, 563–571.
[15] Van Biervliet, J. P. G. and Staal, G. E. J. (1977) Acta. Ped. Scan. 66, 311–315.