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COMPARISON OF TYPE I HEXOKINASES FROM PIG HEART AND KINETIC EVALUATION OF THE EFFECTS OF INHIBITORS

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Summary

Type I hexokinase (ATP:D-hexose 6-phospotransferase, EC 2.7.1.1) of porcine heart exists in two chromatographically distinct forms. These do not differ significantly in size, electrophoretic mobility at pH 8.6 or kinetic properties. Both forms obey a sequential mechanism and are potently inhibited by glucose 6-phosphate. In contrast to observations of type I hexokinase from brain, inhibition by glucose 6-phosphate is not relieved by inorganic phosphate. Under most conditions, low concentrations of phosphate (<10 mM) have little effect on the kinetic behaviour of the enzyme but at higher concentrations this ligand is an inhibitor. Mannose 6-phosphate inhibits in a manner analogous to glucose 6-phosphate but the K_i is much greater. In view of the similarity of the kinetic parameters governing phosphorylation of mannose and glucose, this difference in affinity for the inhibitor site is seen as consistent with the existence of a separate regulatory site on the enzyme. MgADP inhibits hexokinase but behaves as a normal product inhibitor and inhibition is competitive with respect to MgATP and non-competitive with respect to glucose.

Introduction

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) occurs in animal tissues in four isoenzyme forms [1]. The most abundant of these, type I, has been purified to homogeneity from brain and heart and its molecular properties have been studied [2,3]. These studies indicate broadly similar physical properties for the enzymes from the two tissues. Easterby and O'Brien [3] reported that the heart type I enzyme could be fractionated on phosphocellulose into two forms. The minor of these reproducibly accounted for 8—13% of the total type I hexokinase extracted from the tissue and bound less

strongly to phosphocellulose at pH 6.5. Subsequently, Heumann et al. [4] reported the separation of two forms of type I hexokinase from human kidney but in this instance the chromatographic behaviour of one of the forms was quite different from that of the heart enzymes. More recently, Felgner and Wilson [5] have shown that rat brain type I hexokinase may be subdivided into at least two forms, one only of which will rebind to mitochondria. The present report compares the minor (type I_A) and major (type I_B) forms extracted from pig heart. The steady-state kinetic behaviour of the enzymes and their responses to the product inhibitors glucose 6-phosphate and MgADP have been studied together with the effects of P_i. The results obtained are contrasted with those obtained from similar studies of the brain type I [6,7] and skeletal muscle type II [8] enzymes.

Materials and Methods

Reagents. Analytical grade reagents were purchased from British Drug Houses (Poole, Dorset). Hepes was a product of Hopkins and Williams (Chadwell Heath, England). ATP was purchased from P-L Biochemicals (Milwaukee, U.S.A.) and all other nucleotides, coupling enzymes and sugar derivatives were products of C.F. Boehringer and Soehne (Mannheim, Germany). DEAE-cellulose and phosphocellulose P11 were products of Whatman (Maidstone, England) and Sephadex G-200 was obtained from Pharmacia (Uppsala, Sweden).

Protein concentration. During the purification of the enzyme the protein concentration was determined by the method of Warburg and Christian [9]. The concentration of the purified enzymes was determined by absorption measurements at 278 nm. A value of 6.0 was used for $A^{1\%}_{1cm}$ of freshly prepared enzyme [3].

Enzyme assays and kinetic measurements. The enzymes were assayed essentially as described by Easterby and O'Brien [3] by coupling to glucose-6-phosphate dehydrogenase (EC 1.1.1.49). The buffer used was 0.1 ionic strength (0.12 M) Tris-HCl (pH 7.5). The total Mg^{2+} concentration was maintained at 10 mM except when MgADP inhibition was studied. The Mg^{2+} concentration was then 20 mM. When mannose was used as hexose substrate or when glucose 6-phosphate-dependent inhibition was studied, hexokinase was coupled to pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) [10]. In all coupled assays the concentration of coupling enzymes was sufficient to keep the transition time below 10 s, in order to allow rapid maintenance of a steady state and accurate initial velocity measurements [11]. Prior to kinetic measurements, 0.5 mg/ml enzyme solution containing 1—2 mg/ml bovine serum albumin was dialysed against the assay buffer. During studies of the effects of P_i on the enzyme, the ionic strength was maintained at 0.18 by KCl addition.

Data were analysed using the simple equations of Alberty [12] and plotted according to Hanes [13]. Inhibitor constants were obtained from secondary plots of slopes and intercepts. All data were subjected to linear regression analysis. During the acquisition of data, aberrant observations were detected by

plotting in parameter space according to the method of Eisenthal and Cornish-Bowden [14].

Analytical ultracentrifugation. This was performed as described previously [3].

Polyacrylamide gel electrophoresis. This was performed as described by Davis [15]. The buffer was Tris/glycine (pH 8.6) and samples were layered through the electrode buffer in glycerol onto the gels. Protein concentrations in the range 50—500 μg/ml were used to detect heterogeneity. Gels were scanned on a Gilford spectrophotometer equipped with linear transport. Enzyme was stained as described by Katzen et al. [16].

Enzyme purification. This was conducted essentially as described by Easterby and O'Brien [3] except that stage 3 of the procedure was modified to include batchwise adsorption of the enzymes onto DEAE-cellulose (pH 8). The adsorbant was poured into a column (40 × 5.5 cm), washed with pH 8 phosphate buffer (I = 0.1, 0.05 M) and eluted in the normal way. Careful pooling of peak 1 activity from phosphocellulose (stage 4, [3]) allowed the minor enzyme (type I_A) to be obtained in homogeneous form after gel filtration on Sephadex G-200 (stage 5, [3]). The specific activities of the purified enzymes were: type I_A 80 units/mg and type I_B 100 units/mg, where the units have the normal μmol/min definition at 30°C. The yield from the purification procedure was 60—75%.

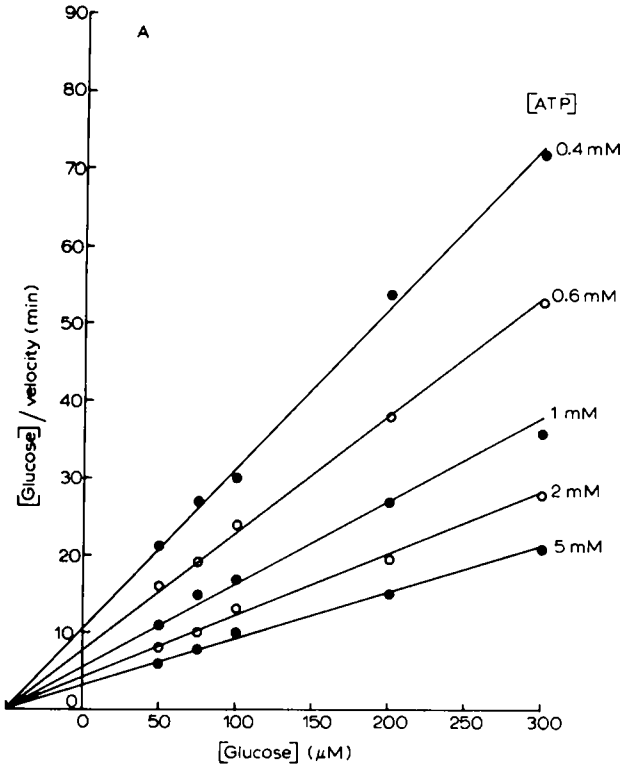
Results

Molecular properties of type I_A hexokinase

Heart hexokinase may be resolved on phosphocellulose at pH 6.5 into two distinct forms. These have previously been referred to as peak 1 and peak 2 activity but are now identified as type I_A and type I_B enzymes. Type I_A elutes first from phosphocellulose and accounts for 8—13% of the total, particulate hexokinase activity extracted from the tissue. In the present study it was obtained in homogeneous form and at pH 8.6 apparently had the same electrophoretic mobility as the major component, type I_B. Mixtures of the two enzymes gave a single protein and enzyme band on electrophoresis. It was also homogeneous as judged by sedimentation velocity analysis and $s_{20,w}$ was 5.4 S at a protein concentration of 2.5 mg/ml. The molecular weight determined by equilibrium ultracentrifugation was 98 600 and was also similar to that of the type I_B enzyme [3]. On SDS-polyacrylamide electrophoresis it behaved as a single polypeptide of 97 000 molecular weight. Like the type I_B enzyme, it dimerised in the presence of glucose 6-phosphate [17].

Substrate kinetic studies

Both the type I_A and type I_B enzymes exhibited a sequential mechanism with glucose or mannose as hexose substrate and MgATP as nucleotide (sample data are shown in Fig. 1). The K_m values were similar and the K_m for MgATP was independent of the hexose substrate (Table I). There was thus no distinction in behaviour between the two enzyme forms. Moreover, the hexose substrates were similar in behaviour suggesting that there is little discrimination between them at either enzyme's active site.



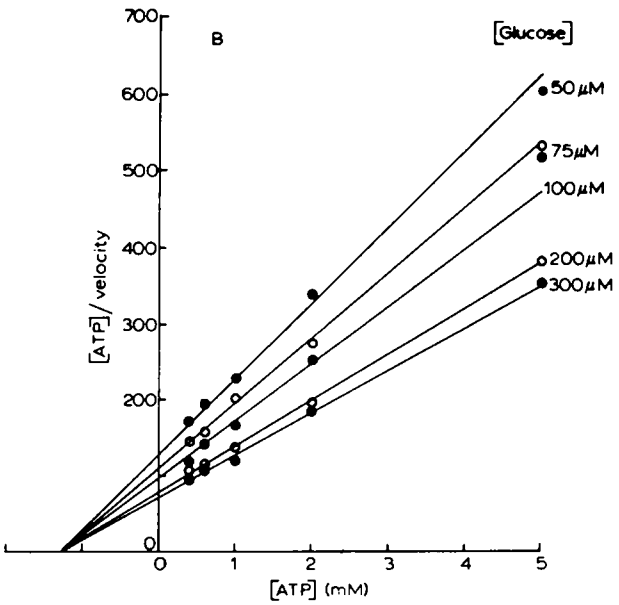


Fig. 1. The dependence of hexokinase type I_A rates on substrate concentration. The responses to (A) glucose and (B) MgATP are shown.

TABLE I

KINETIC PARAMETERS OF HEART HEXOKINASES

Where appropriate the range of values of kinetic coefficients obtained in different experiments is given.

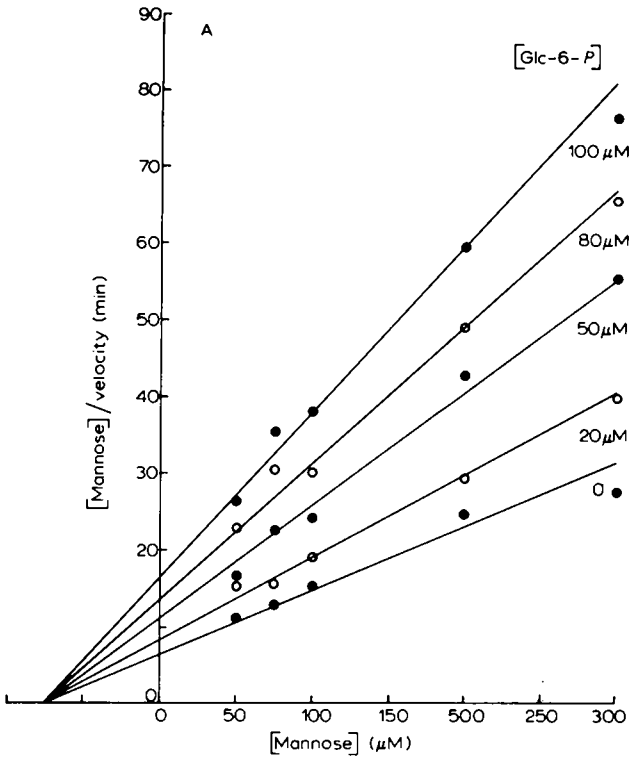
Enzyme	Varied substrate	Inhibitor	K_m (μM)	K_i (μM)
Type 1A				
	glucose		58	
	mannose		55	
	MgATP (vs. glucose)		900—1200	
	MgATP (vs. mannose)		1200	
	glucose	MgADP		3800—4600
	MgATP	MgADP		4800
	mannose	glucose 6-phosphate		53—84
	MgATP	glucose 6-phosphate		20
	glucose	mannose 6-phosphate		2500—2800
	MgATP	mannose 6-phosphate		3000—5100
Type 1B				
	glucose		57	
	mannose		55	
	MgATP (vs. glucose)		700—1100	
	MgATP (vs. mannose)		1100	
	glucose	MgADP		4400—4600
	MgATP	MgADP		3300
	mannose	glucose 6-phosphate		42
	MgATP	glucose 6-phosphate		44
	glucose	mannose 6-phosphate		2900
	MgATP	mannose 6-phosphate		3500—6400

Inhibition by glucose 6-phosphate and the effects of P_i

Inhibition of the type I_B enzyme by glucose 6-phosphate was reported previously to be competitive with respect to MgATP and non-competitive with respect to hexose [3]. This is confirmed by the present study for both type I_A and type I_B hexokinase (Fig. 2, Table I). Studies on the type I enzyme of brain have indicated that glucose 6-phosphate-dependent inhibition is relieved by P_i [6,7]. In the present study no relief of inhibition of type I_A or type I_B was seen, but the effects of P_i were complex and dependent on the buffer system employed. In Hepes, P_i at concentrations up to millimolar produced about 10% activation of the type I_B enzyme but at greater concentrations caused inhibition of both I_A and I_B (Fig. 3). In Tris-HCl buffers the activation was not seen but P_i again inhibited at high concentration. In neither buffer system did P_i relieve glucose 6-phosphate-dependent inhibition but in both buffers, glucose 6-phosphate largely abolished the component of inhibition due to P_i and the effects of the two ligands were not additive. The inhibitory effects of P_i were slightly more pronounced for the type I_A enzyme than for type I_B. In all cases the inhibition due to P_i could be reversed at high ATP concentration and in this respect was similar to that reported by Ellison et al. [7].

Inhibition by mannose 6-phosphate

Glucose and mannose display similarities as substrates for both hexokinases (Table I). However, mannose 6-phosphate differs from glucose 6-phosphate as an inhibitor. Like glucose 6-phosphate, mannose 6-phosphate is a competitive



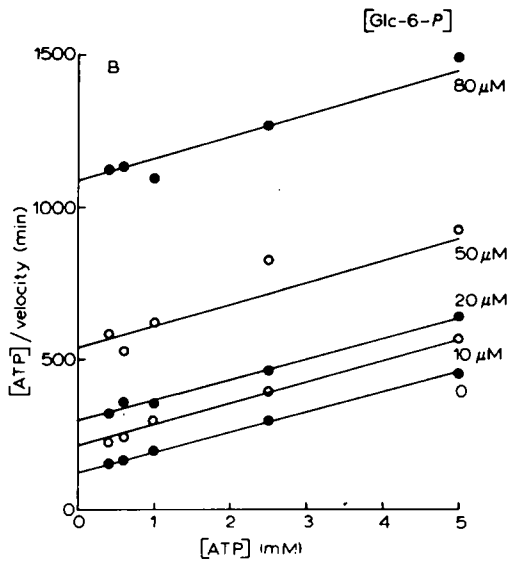


Fig. 2. Inhibition of hexokinase type I_A by glucose 6-phosphate. (A) Mannose concentration was varied with MgATP fixed at millimolar. (B) MgATP concentration was varried with mannose fixed at 100 μM.

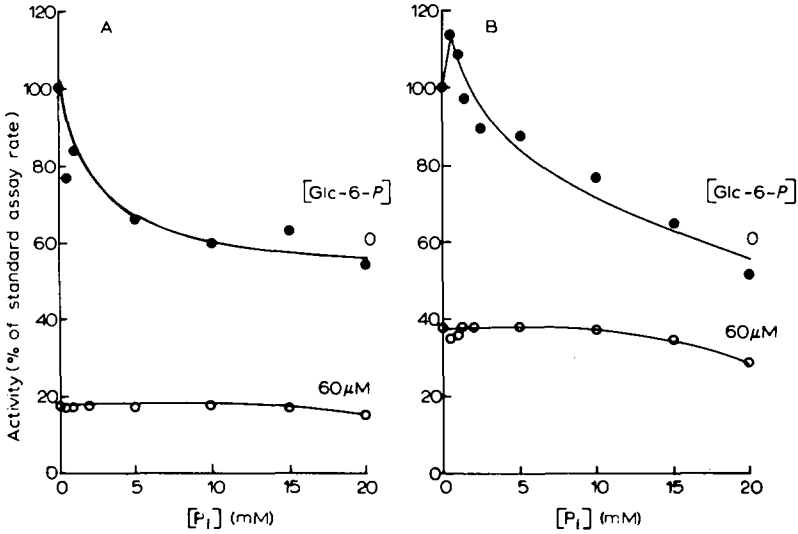


Fig. 3. The effect of P_i on hexokinases. (A) Type I_A; (B) type I_B. Data in the presence (○) and absence (●) of glucose 6-phosphate are shown. ATP concentration was 0.5 mM and Mg^{2+} 2 mM. The buffer was Hepes.

inhibitor relative to MgATP and non-competitive with respect to glucose but the inhibition is considerably weaker (Fig. 4, Table I).

Inhibition by MgADP

The pattern of $MgADP^-$ inhibition of both enzymes was a simple one. Linear inhibition was observed relative to $MgATP^{2-}$ and was competitive. Inhibition was non-competitive with respect to glucose (Fig. 5). The kinetic constants obtained are given in Table I. During experimentation the total Mg^{2+} concentration was 20 mM. This was at all times in excess of the total nucleotide concentration and both nucleotides would be expected to be present almost exclusively as their magnesium complexes. Although the free Mg^{2+} concentration varied during the experiment, the changes were found to have little effect on enzyme behaviour at fixed total nucleotide concentration (Fig. 6). Some inhibition of the type I_A enzyme at Mg^{2+} concentration greater than 10 mM in excess of the nucleotide concentration was noted and the data of Fig. 5 were corrected for this. The type I_B enzyme was unaffected by excess Mg^{2+} to 20 mM.

Although it would have been better to conduct these studies at constant free Mg^{2+} concentration, this was not practicable owing to the wide range of values reported for the stability constants of the $MgADP^-$ and $MgATP^{2-}$ complexes [18,19]. These vary between 1800 M^{-1} and 10 000 M^{-1} for $MgADP^-$ and 23 000 M^{-1} and 100 000 M^{-1} for $MgATP^{2-}$ under conditions similar to those used here. Using the most adverse values it is possible to calculate that at least 99% of ATP and 96% of ADP was present as Mg^{2+} complex at fixed total Mg^{2+} concentration of 20 mM. Therefore to correct for the effect of free Mg^{2+} on the enzyme it was assumed that Mg^{2+} in excess of total nucleotide represented the free level. In practice, however, such corrections were negligible (Fig. 6).

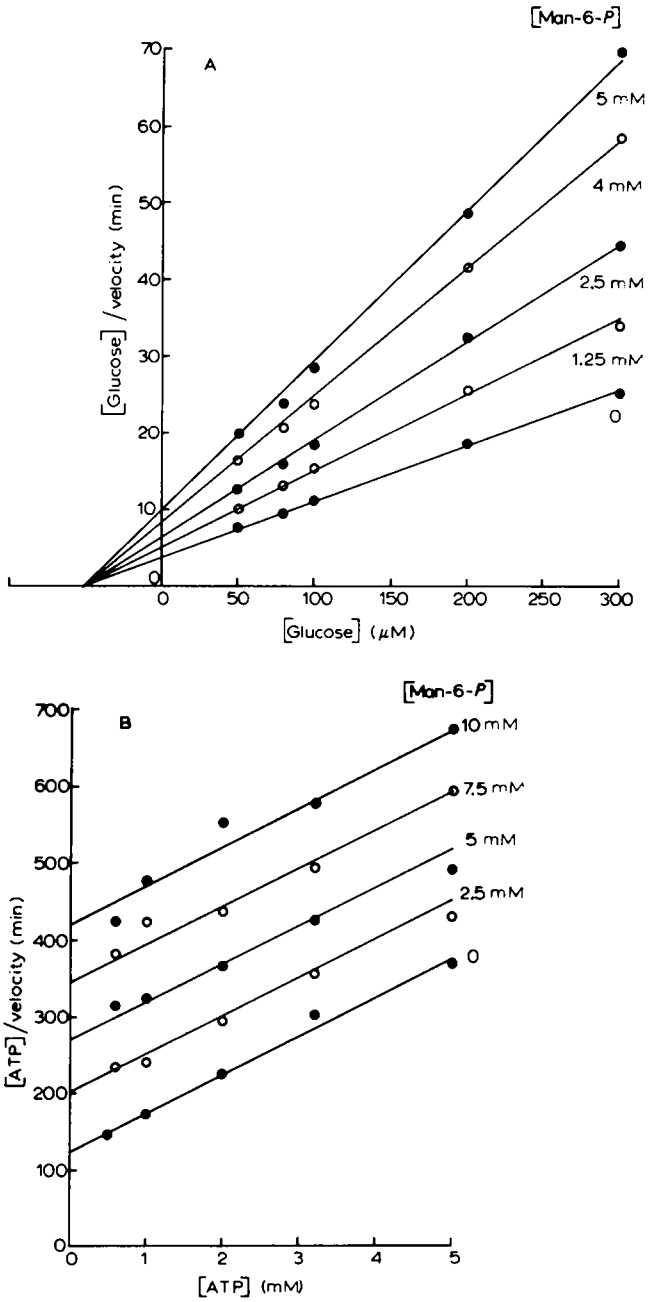
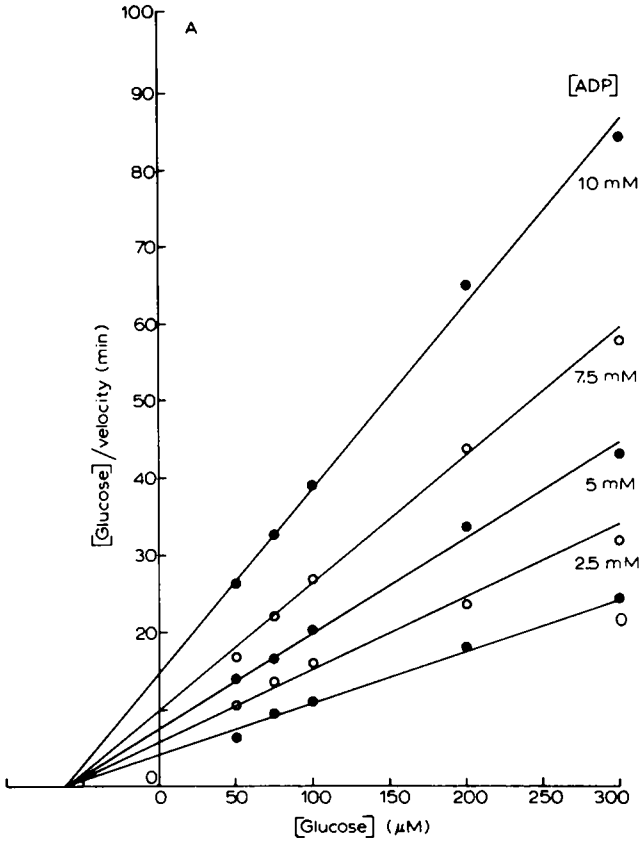


Fig. 4. Inhibition of hexokinase type I_B by mannose 6-phosphate. (A) Glucose concentration was varied with MgATP concentration fixed at millimolar. (B) MgATP concentration was varied with glucose fixed at 100 μM. Similar data were obtained with type I_A enzyme.

Fig. 5. Inhibition of hexokinase type I_B by MgADP. (A) Glucose concentration was varied with MgATP fixed at millimolar. (B) MgATP concentration was varied with glucose fixed at 100 μM. The total Mg^{2+} concentration was 20 mM. Similar data were obtained with type I_A enzyme. ⟶


A
[ADP]
10 mM
7.5 mM
5 mM
2.5 mM
0
[Glucose]/velocity (min)
[Glucose] (μM)
0
50
100
150
200
250
300
10
20
30
40
50
60
70
80
90
100

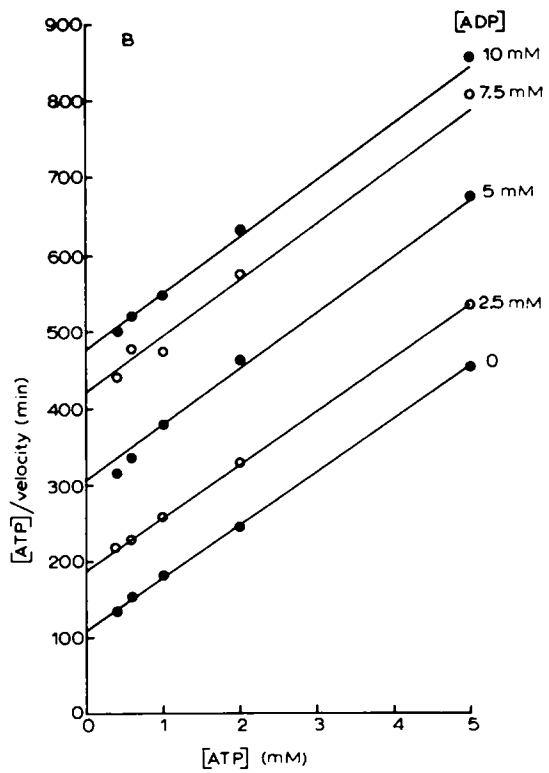

B
[ADP]
10 mM
7.5 mM
5 mM
2.5 mM
0
[ATP]/velocity (min)
[ATP] (mM)
0
1
2
3
4
5
100
200
300
400
500
600
700
800
900

Fig. 5

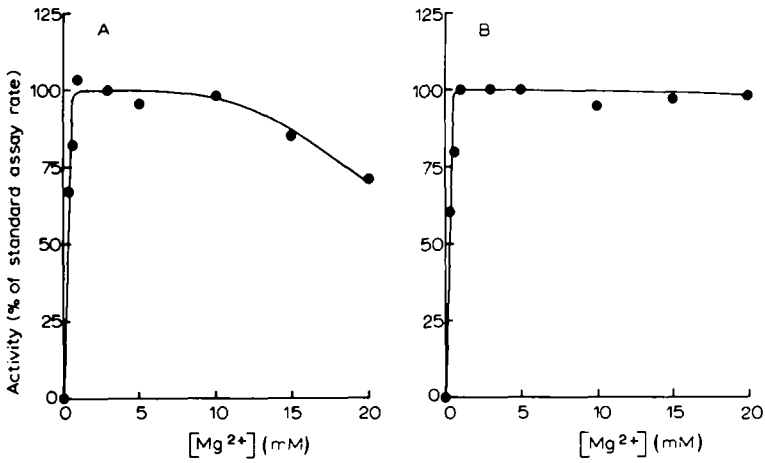


Fig. 6. The effect of Mg^{2+} on hexokinase activity. (A) Type I_A; (B) type I_B. The total ATP concentration was fixed at 0.4 mM.

Discussion

The minor component of heart hexokinase (type I_A) is broadly similar to the major component (type I_B) and at present only the chromatographic behaviour of the enzymes on phosphocellulose at pH 6.5 distinguishes them. The enzymes must differ in their titration curves between pH 6.5 and 8.6, the pH of electrophoresis, and it may be that type I_A is generated from I_B by proteolysis. However, such modification cannot be random as the proportions of the enzymes are the same in all preparations. Felgner and Wilson [5] have recently resolved brain hexokinase into several species which differ in their ability to rebind to mitochondria and speculate that they may differ either through proteolysis or phosphorylation. Preliminary investigations have shown that neither type I_A or type I_B hexokinase will rebind to mitochondria following purification and no evidence has been found for the phosphorylation of type I_B hexokinase by cyclic AMP-dependent protein kinase (EC 2.7.1.37) (Bannister, A. and Easterby, J.S., unpublished data). The possibility that the type I_A enzyme is a non-covalent dimer of I_B [17] remains and type I_A eluted from phosphocellulose in the same position as the dimer of I_B promoted by glucose 6-phosphate.

There has been considerable argument over the possibility of the existence on hexokinase of a regulatory site, distinct from a normal product binding site. The lack of competition between glucose and glucose 6-phosphate in kinetic studies [20] or during glucose 6-phosphate-dependent dimerisation of the brain and heart enzymes [17,21], have been cited as evidence for this. An alternative explanation is that a shift in the position of the glucose moiety follows or accompanies phosphorylation but the phosphate moiety overlaps the ATP binding site to produce direct competition with the nucleotide. Recently, Cassaza and Fromm [22] have shown that mannose 6-phosphate inhibition of brain hexokinase is qualitatively similar to glucose 6-phosphate-dependent inhibition. They see this observation, together with the inability to detect a

second product binding site, as evidence for the site overlap hypothesis. Similar results are obtained with the heart enzymes but are equally consistent with the presence of a separate regulatory site. While mannose and glucose are equally good substrates, mannose 6-phosphate is a poor inhibitor, suggesting a distinction between the active site and inhibitor site. The failure to detect a second, product binding site could well be attributed to the extreme weakness of binding as suggested by kinetic studies of the yeast and wheat germ enzymes [10,23]. Ellison et al. [7] have addressed themselves to this problem in studies of the brain enzyme and conclude that the presence of such a weak binding site is inconsistent with the Haldane relationship for the enzyme. However, this argument assumes a prior knowledge of the kinetic mechanism and equivalence between the dissociation constants of binary complexes and K_m for $MgADP^-$ and glucose 6-phosphate in the reverse reaction. The failure of P_i to promote the reverse reaction is seen as inconsistent with the presence of a distinct allosteric site for glucose 6-phosphate, but no account is taken of possible difference between the properties of the free enzyme and the enzyme-phosphate complex. The argument over the existence of an allosteric site therefore, hinges on whether glucose 6-phosphate exerts all of its effects by direct competition with substrate $MgATP^{2-}$ or through a conformational change in the enzyme. While the kinetic effects of the ligand on the brain enzyme can probably be adequately explained on the basis of direct competition [6] it seems clear from the studies of Wilson [24] that glucose 6-phosphate does produce conformational changes in the enzyme which might promote its release from the mitochondria. The studies of Chakrabarti and Kenkare [21] and Easterby [17] also demonstrate extensive conformational changes in the protein on binding glucose 6-phosphate. The ligand competition involved in these phenomena is similar to that seen in kinetic experiments and implies the existence of a single site responsible for all actions of the ligand. It has been proposed by Easterby [3,25] and by Colowick [26] that the low K_m mammalian hexokinases may have arisen from an ancestral form similar to the yeast and wheat germ hexokinase subunits or glucokinase by a process of gene duplication and fusion. A second substrate binding site introduced in this way could have become adapted to bind glucose 6-phosphate. The present observations are consistent with this idea, as are the recent studies by Holroyde et al. on rat liver glucokinase [27] and skeletal muscle hexokinase [28].

The competitive inhibition of hexokinase by $MgADP^-$ observed here contrasts with the non-competitive or mixed inhibitory behaviour reported for other mammalian hexokinases [29]. $MgADP^-$ appears to behave as a simple product inhibitor even when allowance is made for possible effects of variation in the level of free magnesium and the ligand does not seem to be involved at a regulatory site.

The effects of P_i on the enzymes are of considerable interest. In contrast to the brain type I enzyme [6,7] no relief of inhibition by glucose 6-phosphate was observed and the enzymes are similar to the type II enzyme of tumour cells in this respect [30]. Moreover, phosphate was itself inhibitory and this has previously been reported for the type II enzyme of skeletal muscle but not for the type I enzyme. Inhibition in the present case was less marked than in the skeletal muscle enzyme. Lueck and Fromm [8] have rationalised the differ-

ences in responses of the brain and skeletal muscle enzymes to P_i in terms of the patterns of energy usage by these tissues. The heart enzyme appears to fit into this scheme and heart represents an intermediate energy requirement. Glucose 6-phosphate accumulation can indicate the presence of alternative energy sources in this tissue and might therefore be expected to result in potent inhibition of hexokinase. Reversal of this inhibition by P_i would be inappropriate and inhibition due to P_i itself would be expected to be less pronounced than in skeletal muscle as its presence is not usually a signal for anaerobic glycogenolysis. Recently, Rijksen and Staal [31] have reported anomalous behaviour of erythrocyte hexokinase during studies of inhibition by glucose 1,6-bisphosphate. Their data suggest that the purified hexokinase may contain two forms, only one of which is subject to P_i release of inhibition. Erythrocytes have previously been reported to contain a 'complex' of type I hexokinases [32,33] and this behaviour is therefore consistent with that seen in heart.

The foregoing considerations suggest that heart hexokinase must contain a phosphate binding site distinct from the glucose 6-phosphate site and it is notable that while phosphate cannot reverse inhibition by glucose 6-phosphate, it can abolish the glucose 6-phosphate-dependent dimerisation of the enzyme [17]. This implies that the dimerisation is not a prerequisite of inhibition during kinetic experiments. However, at the higher enzyme concentrations prevailing in vivo (the minimum hexokinase concentration in heart is 0.1 mg/ml and will probably be higher owing to intracellular compartmentation) dimerisation might affect both activity and mitochondrial binding.

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