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KINETICS OF HUMAN ERYTHROCYTE HEXOKINASE

INFLUENCE OF TEMPERATURE, ATP^{4-} AND MAGNESIUM IONS

GERT RIJKSEN and GERARD E.J. STAAL

Haematological Department, Unit of Medical Enzymology, State University Hospital, Utrecht (The Netherlands)

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Summary

Human erythrocyte hexokinase (EC 2.7.1.1) is inhibited competitively with respect to $Mg \cdot ATP^{2-}$ by uncomplexed Mg^{2+} (K_i = 16—18 mM) and ATP^{4-} (K_i = 1.6 mM). No real activation by low concentrations of Mg^{2+} could be detected and no allosteric behaviour was observed under the conditions tested.

The temperature dependence of the enzyme was studied in relationship to the presence of Mg^{2+} or ATP^{4-}. At equal concentrations of Mg^{2+} and ATP^{4-} a break in the Arrhenius plot was observed at 27.5° C, the higher temperature form of the enzyme having the lower activation energy.

This break point in the Arrhenius plot was shifted to 36° C in the presence of 5 mM Mg^{2+}. A straight-line relationship was observed in the presence of 2.5 mM ATP^{4-}.

The K_m for $Mg \cdot ATP^{2-}$ showed a linear increase at temperatures over about 36° C independent of the presence of Mg^{2+} or ATP^{4-}. The nature of these phenomena is discussed.

Introduction

The reaction catalysed by hexokinase (EC 2.7.1.1), glucose + ATP ⇌ glucose-6-phosphate + ADP has an absolute requirement for a divalent cation. There is a general agreement that the $Mg \cdot ATP^{2-}$-complex is the true substrate. Since the $Mg \cdot ATP^{2-}$ complex is in equilibrium with its free constituents, possible interference of free Mg^{2+} and ATP^{4-} with the phosphorylation of glucose must be taken into account.

Although hexokinase type I from different tissues has well been characterized by a number of investigators, discrepancies exist with respect to the action of Mg^{2+} and ATP^{4-} on the enzyme. The allosteric activation of hexokinase type I from guinea-pig brain by uncomplexed magnesium was described by Bachelard

[1]. Meanwhile, this was shown to be incorrect by Pürich and Fromm [2], who converted the kinetic data of Bachelard to strictly hyperbolic kinetics by using an appropriate association constant for the Mg · ATP-complex. In a study on human erythrocyte hexokinase Gerber et al. [3] reported that uncomplexed magnesium up to 4 mM is capable of activating human erythrocyte hexokinase twofold with a K_{Mg} = 1.0 mM. At higher concentrations an inhibition was observed. Two binding sites were suggested for magnesium, one for its activating and one for its inhibitory effect. Furthermore no inhibition by uncomplexed ATP was detected, whereas hexokinase from other tissues is reported to be strongly inhibited by ATP^{4-} [4,5].

Preliminary investigations in our laboratory on purified human erythrocyte hexokinase did not confirm the results of Gerber et al. [3] in every respect and a more extensive investigation of this problem has been carried out. For the reason that the exhibition of allosteric behaviour can be dependent on the reaction temperature [6], this investigation was extended to the temperature dependence of the hexokinase reaction in relation to the influence of uncomplexed Mg^{2+} and ATP^{4-}.

Materials and Methods

Reagents. ATP (disodium salt), $NADP^+$ (disodium salt) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were obtained from Boehringer Mannheim. D(+)-glucose and $MgCl_2 \cdot H_2O$ were purchased from Merck. All other reagents were of analytical grade of purity. Fresh human erythrocytes were supplied by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam (The Netherlands).

Preparation of enzyme. Purified human erythrocyte hexokinase was prepared as described earlier [7], with a final specific activity of about 25 I.U. mg protein at 37°C.

All experiments were carried out immediately after the last step of the purification procedure.

Determination of enzyme activity. Hexokinase was measured spectrophotometrically in a system coupled with glucose 6-phosphate dehydrogenase. The assay medium contained in a final volume of 3 ml: 33 mM Tris · HCl (pH 8.0), 10 mM glucose, 0.33 mM $NADP^+$, 0.15 I.U. glucose 6-phosphate dehydrogenase and ATP and $MgCl_2$ at the concentrations as indicated in the text. The reaction was started by adding 0.020 I.U. purified human erythrocyte hexokinase. In the experiments concerning the temperature dependency the Tris · HCl buffer was replaced by a Tris/maleate buffer with the same molarity and pH.

Initial rate measurements were performed by following the reduction of $NADP^+$ at 340 nm with a Perkin Elmer spectrophotometer Model 124. One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 1 μmol of glucose-6-phosphate per min.

Calculations of Mg · ATP^{2-}, ATP^{4-} and Mg^{2+}. The levels of free and complexed ions were calculated using a stability constant for Mg · ATP^{2-} of 20 000 mol^{-1}, which had been determined for a medium very similar to the one we used [8,9]. Although the stability constants for Mg · ATP^{2-} vary considerably with temperature [10], these differences were neglected in the experiments concerning the

temperature dependency, since the results are hardly influenced by these changes in the stability constant in the case of equal amounts of the free ions, and not at all in the case that excess of one of the free ions is used.

The formation of $HATP^{3-}$ and $Mg \cdot HATP^{-}$ was neglected, since these complexes represent less than 1% of the total nucleotide content at pH 8.0 [11].

Statistics. For the statistical treatment of the kinetic data in the double reciprocal plots the method of the least squares is used with weighting factors proportional to $(velocity)^4$, which is assumed to be valid, if the velocity measurements have been made using a constant amount of enzyme [12]. In the temperature dependency experiments an Algol-computer program is used derived for the statistical treatment and drawing of Arrhenius plots.

Results

Influence of free ions

To examine the influence of a little excess of one of the free ions is rather impossible because the other free ion will be present in a significant concentration and therefore its action can not be excluded. Therefore a larger excess of either ion is used to eliminate the influence of the other. The kinetic data thus obtained are used in a rate equation describing the initial velocity in presence of both ions, in order to compare the calculated velocities with the experimental values at low concentrations of both ions.

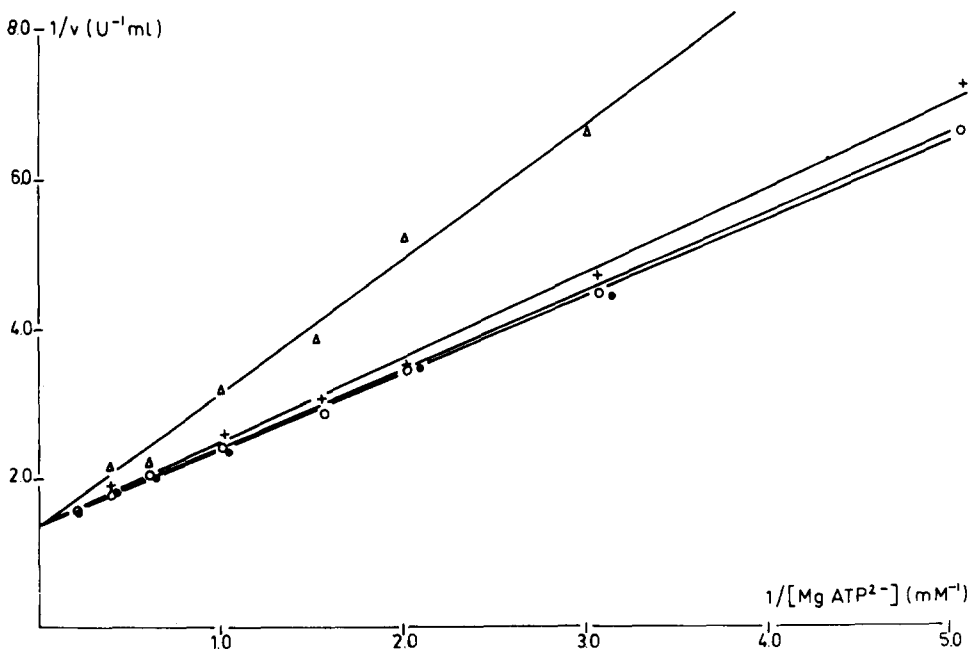

Fig. 1. Lineweaver-Burk plots of hexokinase activity vs. $[Mg \cdot ATP^{2-}]$ at excess of Mg^{2+} over ATP^{4-} of 2.0 mM, (●); 7.0 mM, (○); 10.0 mM, (+); 20.0 mM, (△) at 37°C. $[ATP^{4-}]$ in this experiment varies between 120—20 μM depending on the concentration of Mg^{2+} and $Mg \cdot ATP^{2+}$; In the calculation of K_i Mg^{2+} the influence of these concentrations of ATP^{4-} is neglected. Further conditions are as described in Materials and Methods.

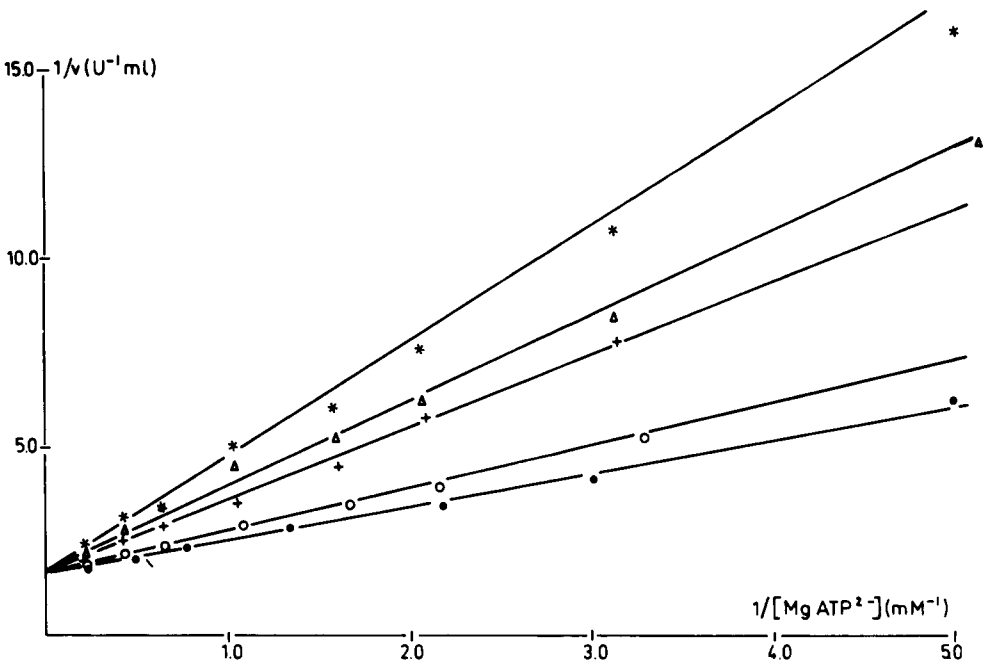

Fig. 2. Lineweaver-Burk plots of hexokinase activity vs. [Mg · ATP^{2-}] at excess of ATP^{4-} over Mg^{2+} of 0.0 mM, (●); 1.0 mM (○); 2.0 mM, (+); 3.0 mM, (△); 4.0 mM (∗) at 37°C. [Mg^{2+}] in this experiment varies between 300—20 μM dependent on the concentrations of ATP^{4-} and Mg · ATP^{2-}. Further conditions are as described in Materials and Methods.

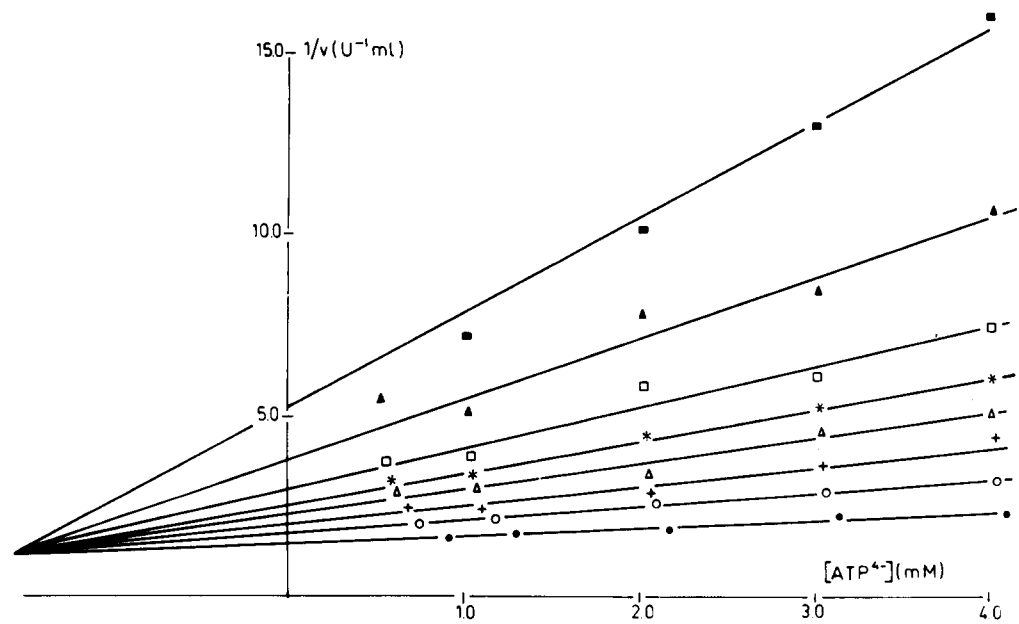

Fig. 3. Dixon plots for the inhibition by [ATP^{4-}] at concentrations of Mg · ATP^{2-} of 5.0, (●); 2.5 mM, (○); 1.65 mM, (+), 1.0 mM, (△); 0.65 mM, (*); 0.5 mM, (□); 0.33 mM, (▲); 0.2 mM, (■) at 37°C. [Mg^{2+}] in this experiment varies between 300—20 μM. In the calculation of K_i ATP^{4-} the influence of these concentrations of Mg^{2+} is neglected. Further conditions are as described in Materials and Methods.

Fig. 1 shows the double reciprocal plot of the inhibition by uncomplexed Mg^{2+} at various concentrations of $Mg \cdot ATP^{2-}$. The inhibition appears to be competitive with respect to $Mg \cdot ATP^{2-}$. An inhibition constant of K_i Mg^{2+} = 16—18 mM can be calculated from lines obtained with the highest concentrations of magnesium.

The inhibition by ATP^{4-} is shown in Fig. 2. A competitive inhibition to $MgATP^{2-}$ is found. A Dixon plot, in which the reciprocal of the velocity is plotted versus the concentration of ATP^{4-} at various concentrations of $Mg \cdot ATP^{2-}$ (Fig. 3), was used to calculate an inhibition constant of K_i ATP^{4-} = 1.60 mM. The extrapolated values of $1/v$ for $[ATP^{4-}] = 0$ from Fig. 3 are used in a double reciprocal plot versus $[Mg \cdot ATP^{2-}]$ to obtain the theoretical K_m for $Mg \cdot ATP^{2-}$ at zero concentration of ATP^{4-} and Mg^{2+}. The affinity constant of K_m $Mg \cdot ATP^{2-}$ = 0.50 mM could be calculated. The inhibition of Mg^{2+} and ATP^{4-} appeared to be noncompetitive with respect to glucose (results not shown). A sequential mechanism is now generally accepted for the hexokinase type I reaction, probably a rapid equilibrium random one, as was pointed out for hexokinase type I from brain [4,13] and from human erythrocytes [3]. Thus the corresponding rate equation describing the initial velocity of the hexokinase reaction in presence of the competitive inhibitors Mg^{2+} and ATP^{4-} can be written as follows:

$$v = \frac{V}{1 + \frac{K_m\text{glucose}}{[\text{glucose}]} + \frac{K_m\text{MgATP}^{2-}}{[Mg \cdot ATP^{2-}]}\left(1 + \frac{[Mg^{2+}]}{K_iMg^{2+}} + \frac{[ATP^{4-}]}{K_iATP^{4-}}\right) + \frac{K_i\text{glucose}\ K_m\text{MgATP}^{2-}}{[\text{glucose}][Mg \cdot ATP^{2-}]}\left(1 + \frac{[Mg^{2+}]}{K_iMg^{2+}} + \frac{[ATP}{K_iAT}\right.}$$

in which K_i glucose stands for the dissociation constant of the enzyme-glucose complex. This constant can be derived from Lineweaver-Burke plots given earlier [7].

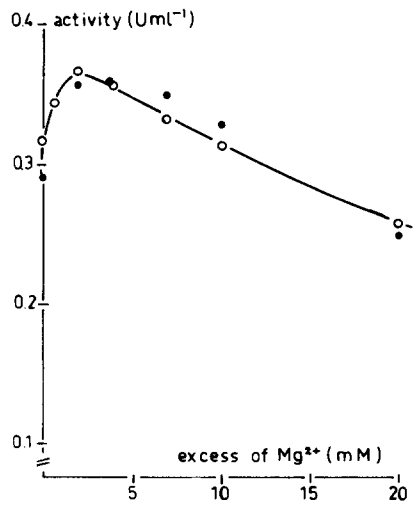

Fig. 4. Plots of initial velocity of the hexokinase reaction at 37°C versus excess of free Mg^{2+} at fixed $[ATP]_{total}$ of 0.65 mM. •, values measured as described in Materials and Methods; ○, values calculated according to the rate equation as described in the text.

TABLE I

KINETIC CONSTANTS DESCRIBING THE HEXOKINASE REACTION

Parameter	Value	Derived from
V	0.70 units/ml^{-1}	Figs. 1, 2
K_m Mg · ATP^{2-}	0.50 mM	Fig. 4
K_m glucose	0.064 mM	ref. 7
K_i glucose	0.095 mM	ref. 7
K_i Mg^{2+}	16-18 mM	Fig. 1
K_i ATP^{4-}	1.60 mM	Fig. 3

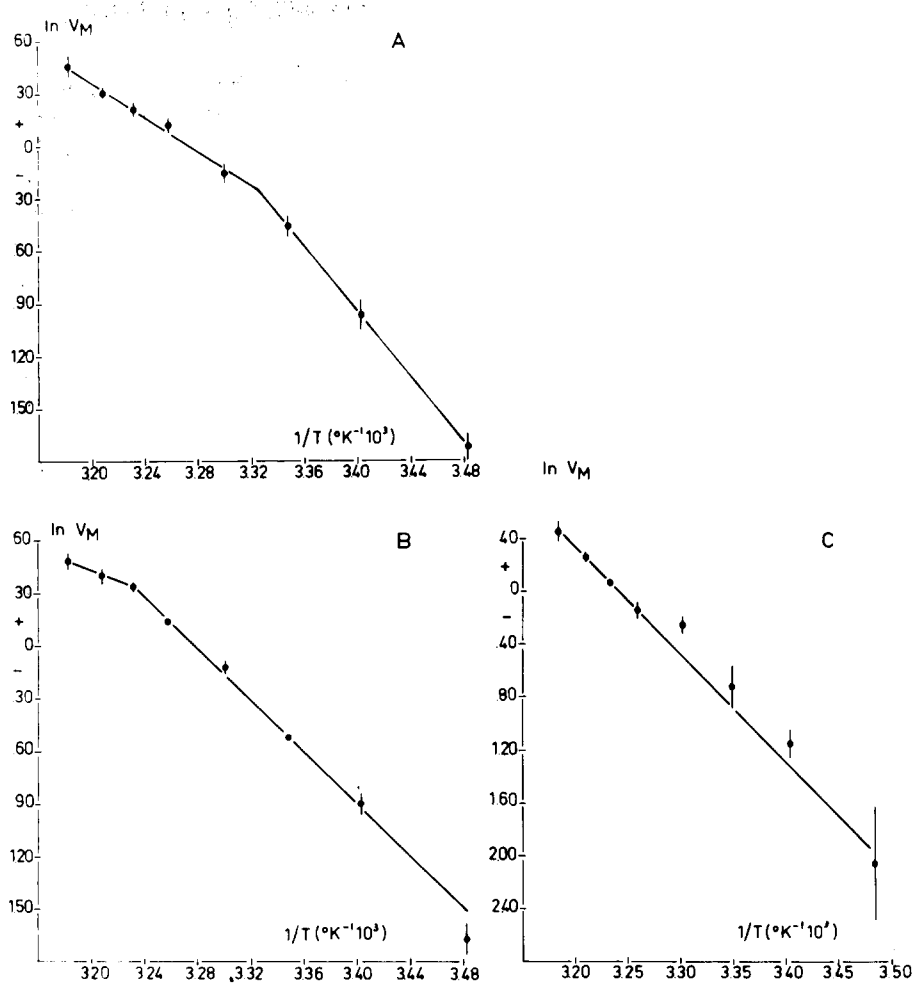

Fig. 5. Arrhenius plots of hexokinase activity versus the temperature in presence of equivalent amounts of the free ions (A), Mg^{2+} and ATP^{4-} (B), excess of 5 mM Mg^{2+} and (C), excess of 2.5 mM ATP^{4-}. The lines are drawn by the computer using the statistical program as described in Materials and Methods, using 1/variance of the velocity (brackets) as weighting factors.

The values of the kinetic constants in this rate equation are summarized in Table I.

The velocity of the hexokinase reaction versus the concentration of Mg^{2+} can now be calculated. Comparison with the experimental values shows that this equation gives a rather good fit (Fig. 4). In all conditions Hill constants near unity were observed (Hill plots not shown). So an allosteric behaviour of hexokinase could be ruled out under all conditions tested.

Influence of temperature

The influence of temperature on the hexokinase activity is studied in the presence of a fixed amount of 10 mM glucose. Although purified human erythrocyte hexokinase is very unstable, this concentration of glucose is stabilizing enough to produce linear increasing absorbance at 340 nm during the first 5 min of measurement at all temperatures tested. Values for V and K_m $Mg \cdot ATP^{2-}$ and their standard errors are calculated from double reciprocal plots using the statistics as described in Materials and Methods.

The results, dependent on the presence of either of the free ions, are plotted in Arrhenius plots (Fig. 5). The parameters describing these Arrhenius plots are summarized in Table II. In the presence of equal amounts of Mg^{2+} and ATP^{4-} a break point in the Arrhenius plot is observed at 27.5° C (Fig. 5A). This break point is shifted to 36° C in the presence of excess 5 mM Mg^{2+} (Fig. 5B). However, a fairly good straight-line relationship is observed in the presence of excess 2.5 mm ATP^{4-} (Fig. 5C). The activation energy (E_a) of the enzyme in the presence of ATP^{4-} is the same as for the lower temperature part of the other curves.

The possibility that the breaks in the Arrhenius plots reflect a conformational transition between two different forms of the enzyme or a structural alteration in the protein involving the catalytic site was investigated by studying the dependency of the K_m on temperature. A constant value for the affinity constant for $Mg \cdot ATP^{2-}$ was found up to about 36°C (Fig. 6). At higher temperatures a gradual increase of K_m was found. This phenomenon was independent of the presence of Mg^{2+} or ATP^{4-}, so the influence of these ions on the Arrhenius plots was not reflected on the dependency of K_m on the temperature.

Again in all conditions and at all temperatures tested Hill constants near unity were observed (Hill plots not shown).

TABLE II

PARAMETERS DESCRIBING THE ARRHENIUS PLOTS

In V estimated in presence of	T_{break} (°C)	E_α(kcal · mol^{-1})		
		$T < T_{br}$	$T > T_{br}$	
$[Mg^{2+}]_{total} = [ATP^{4-}]_{total}$	27.5 ± 0.7	18.7 ± 0.1	9.7 ± 0.8	
Excess of $[Mg^{2+}] = 5.0$ mM	36.0 ± 0.4	14.7 ± 0.6	5.9 ± 0.3	
Excess of $[ATP^{4-}] = 2.5$ mM	—			17.9 ± 0.8

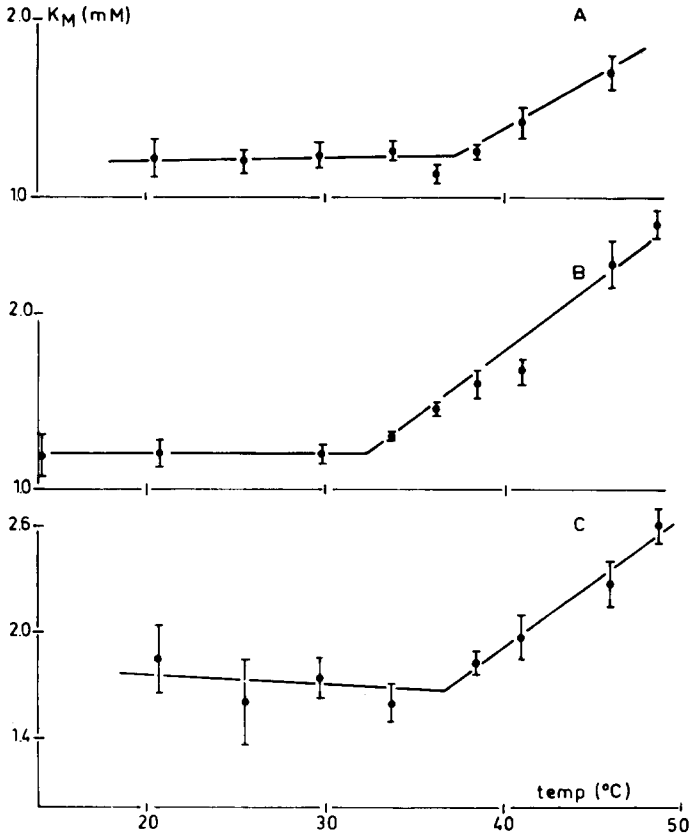

Fig. 6. Plot of K_m for Mg · ATP^{2-} versus temperature in the presence of (A), equal amounts of Mg^{2+} and ATP^{4-} (B), excess of 5 mM Mg^{2+} and (C), excess of 2.5 mM ATP^{4-}. The values for K_m and the standard error of K_m (brackets) are calculated using the statistics as described in Materials and Methods.

Discussion

The results show a competitive inhibition of Mg^{2+} and ATP^{4-} with respect to Mg · ATP^{2-} with inhibition constants of K_i Mg^{2+} = 16—18 mM and Ki ATP^{4-} = 1.60 mM. When the velocity of the hexokinase reaction is plotted versus the concentration of Mg^{2+} at a fixed concentration of ATP_{total} (Fig. 4) and apparent activation is observed in the low concentration range. However, this activation can be fully explained by the raised concentration of substrate and simultaneously lowered concentration of ATP^{4-} when introducing a small excess of free magnesium. It is possible that Gerber et al. [3] came to the conclusion that magnesium at low concentrations is an activator of the human erythrocyte enzyme and that there are two possible binding sites for the ion, because these authors did not detect an inhibition by free ATP^{4-}, which is present in considerable amounts at low concentrations of Mg^{2+}.

Our data on human erythrocyte hexokinase are consistent with reports on the inhibition of free magnesium and ATP^{4-} of hexokinase type I from other tissues [4,5] and confirm the conclusion of Pürich and Fromm [2] about the

nonallosteric behaviour of the enzyme. Although Gerber et al. [3] found a straight-line relationship between log v and 1/temperature (°K) for human erythrocyte hexokinase in the range of 4—37°C, we observed a break in the Arrhenius plot dependent on the presence of Mg^{2+} or ATP^{4-}. At equivalent amounts of Mg^{2+} and ATP^{4-} a break-point is observed at 27.5°C, which is shifted to 36°C in the presence of an excess of 5 mM Mg^{2+}. No breakpoint is observed in the presence of an excess of ATP^{4-}.

This influence of the free ions is not reflected when the Michaelis-Menten constant for $Mg \cdot ATP^{2-}$ is plotted versus the temperature. A break point at about 36°C is observed regardless of the presence of ATP^{4-} or Mg^{2+}. So we may conclude that this phenomenon is not likely to be related to the effects shown in the Arrhenius plots, but rather reflects either a gradual conformational change at temperatures over about 36°C to a different form of the enzyme or a denaturation effect at the catalytic site which reduces the affinity for the substrate, but does not express itself in the maximal velocity.

The presence of a considerable amount of lipid in our human erythrocyte hexokinase preparation was reported before [7]. This lipid appeared to be a mixture of phospholipids and cholesterol upon analysis by thin-layer chromatography. The origin of this lipid might be the mitochondrial membrane to which the hexokinase is possibly bound in the reticulocyte like in other tissues. Untill now detailed analysis was not possible because of the small amount of material available. Since it is known that Mg^{2+} influences the temperature of the phase transition of phospholipids [14,15], it is possible that the breaks in the Arrhenius plots are the result of phase transitions of the phospholipid as is described for (Na^+, K^+)-ATPase [14] rather than a conformational change between two forms of the protein part. If this is true, it is very important to study more closely possible lipid effects on the enzyme activity and its regulation, especially regarding the influence of the temperature and the regulation by ATP^{4-} and Mg^{2+}.

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