The roles of magnesium ions in the reaction catalysed by phosphofructokinase from *Trypanosoma brucei*

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The involvement of Mg^{2+} ions in the reaction catalysed by phosphofructokinase from *Trypanosoma brucei* was studied. The true substrate for the enzyme was shown to be the $MgATP^{2-}$ complex, and free Mg^{2+} ions are also required for enzyme activity. At concentrations of $MgATP^{2-}$ of 2.92 mM and greater, and a fructose 6-phosphate concentration of 1 mM and in the presence of EDTA as a Mg^{2+} buffer, the K_m value for Mg^{2+} was determined to be $294 \pm 18 \mu M$. Neither $MgATP$ nor free ATP is an inhibitor of the enzyme, although apparent inhibition by the latter can be observed as a consequence of the decrease in free Mg^{2+} by chelation.

INTRODUCTION

The enzyme phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) from the bloodstream form of *Trypanosoma* (*Trypanozoon*) *brucei brucei* (*T. brucei*) has been shown to differ from that enzyme from several other species in its allosteric and kinetic behaviour (Cronin & Tipton, 1985, 1987). These differences may reflect the specialized metabolism of this parasitic protozoan (see Brohn & Clarkson, 1978, 1980; Bowman & Flynn, 1976; Oppenheimer *et al.*, 1976) and the compartmentation of many of the enzymes of glycolysis within a subcellular organelle, which has been called the glycosome (Oppenheimer & Borst, 1977; Oduro *et al.*, 1980a,b).

As with many kinases (see Cohn, 1963), the true substrate for phosphofructokinase preparations from a variety of sources appears to be the complex between ATP and Mg^{2+} ion rather than free ATP (see, e.g., Cottam & Uyeda, 1973). A common feature of phosphofructokinase preparations from many sources is the inhibition by high concentrations of ATP (see Uyeda, 1979), and this phenomenon, together with the activation or reversal of this inhibition by AMP, ADP and P_i , have been interpreted in terms of the regulation of the activity of this enzyme by the cellular energy charge (see Atkinson, 1970).

In the present paper, the role of Mg^{2+} ions in the activity of phosphofructokinase has been studied, with particular reference to the possible inhibition by high concentrations of ATP.

MATERIALS AND METHODS

Stabulate inocula of *T. brucei* (427-12/ICI-060) were kindly given by Dr. H. P. Voorheis, who has described the history of this strain (Voorheis *et al.*, 1979). Procedures for the growth of the trypanosomes, in the bloodstream of albino Wistar rats, and the subsequent isolation of phosphofructokinase were as previously described (Cronin & Tipton, 1985). The sources of the other materials used in this work are also described in that paper.

The activity of phosphofructokinase was assayed spectrophotometrically at 37 °C by monitoring the

production of fructose 1,6-bisphosphate by a coupled assay containing, in a total volume of 2.0 ml, 50 mM-imidazole/HCl buffer, pH 6.7, 100 mM-KCl, 5 mM-2-mercaptoethanol, 0.2 mM-NADH, 200 μg of aldolase, 20 μg of triosephosphate isomerase and 20 μg of glycerol-3-phosphate dehydrogenase. The substrate concentrations and other assay components are described in the text. Coupling enzymes were desalted before use by gel filtration through Sephadex G-25 equilibrated with 55.56 mM-imidazole/HCl buffer, pH 6.7. Reactions were initiated by the addition of 0.1 μg of purified phosphofructokinase, and the time course of the decrease in A_{340} was followed. One unit of enzyme activity is defined as the amount catalysing the formation of 1 μmol of product in 1 min under these conditions.

Protein concentration was determined by the method of Bradford (1976), with hen's egg-white lysozyme as the standard.

The stability constant for the formation of the $MgATP^{2-}$ complex ($MgATP$) was determined in 50 mM-imidazole/HCl buffer, pH 6.7, containing 100 mM-KCl, at 37 °C by the 8-hydroxyquinoline method of Burton (1959) to be $7042 M^{-1}$. This value was used to determine the concentrations of free ATP, free Mg^{2+} and $MgATP$. In this calculation 'free ATP' represents all ATP species (ATP^{4-} , $KATP^{3-}$, $NaATP^{3-}$, $HATP^{3-}$ etc.) that are not complexed with Mg^{2+} . A fuller description of the concentrations of free and complexed species, used for obtaining the data shown in Figs. 5 and 6, was obtained by the procedure of Storer & Cornish-Bowden (1976). The computer program COMPLX, described by those authors, was translated into BASIC by Dr. B. A. Orsi of this Department. Use of this program allows the concentration of one species to be maintained constant, which in the present case was chosen to be the H^+ concentration, corresponding to pH 6.7. The equilibria considered in these calculations and their stability constants are shown in Table 1. Na_2EDTA was added, where indicated, as a Mg^{2+} buffer.

Kinetic data which followed Michaelis–Menten kinetics were fitted by non-linear regression by use of a computer program based on the procedure of Wilkinson (1961), and the values obtained are given as mean values $\pm$ S.E.M. Data which showed a sigmoid dependence of initial rate on substrate concentration were plotted in

Table 1. Reactions considered in calculations of the concentrations of different Mg and ATP species

The concentrations of the various complexes and free species were calculated as described in the text.

Reaction	$K_{\text{assoc.}} \text{ (M}^{-1}\text{)}$	Reference
1. $\text{H}^+ + \text{ATP}^{4-} \rightleftharpoons \text{HATP}^{3-}$	9.08×10^6	Phillips <i>et al.</i> (1966)
2. $\text{Mg}^{2+} + \text{HATP}^{3-} \rightleftharpoons \text{MgHATP}^-$	5.37×10^2	
3. $\text{Mg}^{2+} + \text{ATP}^{4-} \rightleftharpoons \text{MgATP}^{2-}$	3.48×10^4	
4. $\text{H}^+ + \text{HATP}^{3-} \rightleftharpoons \text{H}_2\text{ATP}^{2-}$	8.5×10^3	O'Sullivan & Perrin (1964)
5. $\text{K}^+ + \text{ATP}^{4-} \rightleftharpoons \text{KATP}^{3-}$	1.4×10^1	
6. $\text{Na}^+ + \text{ATP}^{4-} \rightleftharpoons \text{NaATP}^{3-}$	1.4×10^1	
7. $\text{Mg}^{2+} + \text{H}_2\text{ATP}^{2-} \rightleftharpoons \text{MgH}_2\text{ATP}$	2.0×10^1	Noat <i>et al.</i> (1970)
8. $\text{Mg}^{2+} + \text{MgATP}^{2-} \rightleftharpoons \text{Mg}_2\text{ATP}$	4.0×10^1	
9. $\text{Mg}^{2+} + \text{Cl}^- \rightleftharpoons \text{MgCl}^+$	3.4×10^0	Blair (1970)
10. $\text{Mg}^{2+} + \text{SO}_4^{2-} \rightleftharpoons \text{MgSO}_4$	2.51×10^2	O'Sullivan (1969)
11. $\text{Mg}^{2+} + \text{F6P} \rightleftharpoons \text{MgF6P}$	3.9×10^1	Schwarzenbach & Anderegg (1957)
12. $\text{Mg}^{2+} + \text{EDTA}^{4-} \rightleftharpoons \text{MgEDTA}^{2-}$	5.01×10^8	O'Sullivan (1969)
13. $\text{H}^+ + \text{EDTA}^{4-} \rightleftharpoons \text{HEDTA}^{3-}$	7.94×10^{10}	Pettigrew & Frieden (1979)
14. $\text{H}^+ + \text{HEDTA}^{3-} \rightleftharpoons \text{H}_2\text{EDTA}^{2-}$	1.45×10^6	
15. $\text{H}^+ + \text{H}_2\text{EDTA}^{2-} \rightleftharpoons \text{H}_3\text{EDTA}^-$	4.88×10^2	

linear form by a rearrangement of the Hill (1910) equation. The maximum-velocity values used to construct these plots were obtained by interpolation of the data, when plotted in double-reciprocal form, to the reciprocal-velocity axis.

RESULTS

Fig. 1 shows the dependence of the initial velocity on the total concentration of ATP determined in the presence of 10 mM-MgSO₄. Apparent inhibition occurred at ATP concentrations above 2 mM.

The effects of varying the concentration of MgATP at two different concentrations of MgSO₄ are shown in Fig. 2. The dependence of the initial velocity on the concentration of MgATP at a total concentration of 5 mM-MgSO₄ was similar to that shown in Fig. 1, but the behaviour at a total MgSO₄ concentration of 0.5 mM revealed much more powerful inhibition. Although these data might be interpreted as indicating potent inhibition by MgATP, the dependence of the velocity on the concentrations of free ATP and free Mg²⁺, shown in the inset to Fig. 2, show the activity to increase with increasing concentrations of the metal ion, but to decrease with increasing free ATP concentrations. These results might suggest that free ATP is not the true substrate for the enzyme.

The possibility that the MgATP species is inhibitory at 0.5 mM total MgSO₄ may be excluded, since one might expect this inhibition to be also manifest at a total MgSO₄ concentration of 5 mM. The inhibition may thus be due to a decrease in the concentration of free Mg²⁺ ions or an increase in the concentration of free ATP. In an attempt to resolve these possibilities, the concentration of free ATP was kept constant in one experiment, whereas in another the concentration of MgATP was maintained. The total concentrations of Mg²⁺ ions and ATP required to obtain these conditions were determined using the equations described by Ainsworth (1977).

Fig. 3 shows the variation of initial velocity with respect to the MgATP concentration at a fixed concentration of 50 μM free ATP. The inhibition observed could result from high substrate inhibition by

MgATP or from the large concentrations of MgSO₄, added to maintain the concentration of free ATP constant at 50 μM, resulting in inhibition either directly or because of the large changes in ionic strength, which is also shown in Fig. 3. Evidence that the latter of these represents the explanation of the observed inhibition under these conditions is given by data obtained when the total concentration of ATP was varied at a large fixed concentration of MgSO₄ (100 mM). The results obtained (not shown) gave a maximum velocity of 38 units/mg,

Fig. 1. Variation of the activity of phosphofructokinase from *T. brucei* with the total ATP concentration, [ATP]_T

Enzyme activity was assayed in the presence of 10 mM-MgSO₄ and the following concentrations of fructose 6-phosphate: ●, 0.5 mM; ○, 2.0 mM, ■, 10.0 mM.

Fig. 2. Effects of free ATP, free Mg²⁺ and MgATP²⁻ on the activity of phosphofructokinase from *T. brucei*

Enzyme activity was assayed in the presence of 1 mM-fructose 6-phosphate, various concentrations of ATP and either 0.5 mM- (●) or 5.0 mM- (○) MgSO₄. The concentration of the MgATP²⁻ complex was calculated by using a stability constant of 7042 M⁻¹. Inset: variation of initial velocity with the concentrations of free ATP ('ATP_F') (□) and free magnesium ions (Mg_F²⁺) (■) when the total concentration of MgSO₄ was 0.5 mM. 'ATP_F' represents all ATP species that are not complexed with Mg²⁺.

some 70% lower than that obtained in Fig. 3. However, MgATP concentrations up to 15 mM caused no significant high-substrate inhibition of phosphofructokinase activity. In this experiment the maximum concentration of free ATP, at 15 mM-MgATP, was only 25 μM, and the total change in ionic strength was only 0.028. These data do not distinguish between inhibition resulting from the increase in ionic strength and that due to the high concentrations of MgSO₄ present. The possibility that the inhibition might result, at least in part, from the high concentrations of free sulphate ions, which increased from 0 to 30 mM under the conditions of Fig. 3, was tested by comparing the effects of KCl, NaCl and Na₂SO₄ on the activity of the enzyme assayed in the presence of 10 mM-MgSO₄, 100 μM-ATP and 5 mM-fructose 6-phosphate. Under these conditions 100 mM-NaCl or -KCl behaved similarly in increasing the activity of the enzyme by approx. 40%, whereas 50 mM-Na₂SO₄ caused 30% inhibition.

Fig. 4 shows the variation of initial velocity with the concentrations of free ATP or free Mg²⁺ ions at a fixed MgATP concentration of 1 mM. As discussed in connection with the inhibition data shown in Fig. 2, it is not possible to decide whether the variations observed result from a requirement for free Mg²⁺ ions or from inhibition of the enzyme by free ATP. This is because the free Mg²⁺ concentration is inversely proportional to the free ATP concentration under these conditions, as shown in the inset to Fig 4.

In order to resolve this, the computer program of Storer & Cornish-Bowden (1976) was used to calculate

Fig. 3. Dependence of the activity of phosphofructokinase from *T. brucei* on the concentration of MgATP²⁻ at a fixed concentration of free ATP

Activity was determined in the presence of 1 mM-fructose 6-phosphate and various concentrations of MgSO₄ in order to maintain the free ATP concentration constant at 50 μM. The change in ionic strength (μ) is also shown.

Fig. 4. Dependence of the activity of phosphofructokinase from *T. brucei* on the concentrations of free Mg²⁺ or free ATP at a fixed concentration of MgATP²⁻

Activity was determined in the presence of 1 mM-fructose 6-phosphate, 1 mM-MgATP²⁻ and various concentrations of either free ATP ('ATP_F') (●) or free Mg²⁺ (Mg_F²⁺) (○). 'ATP_F' represents all ATP species not complexed with Mg²⁺. Inset shows the inverse relationship between free Mg²⁺ and free ATP concentrations under these experimental conditions.

Fig. 5. Dependence of the activity of phosphofructokinase from *T. brucei* on the concentrations of free ATP and ATP⁴⁻

Activity was determined in the presence of 1 mM-fructose 6-phosphate, saturating concentrations of MgATP²⁻ (≥ 2.92 mM), a fixed total concentration of 10 mM-MgSO₄, 5–20 mM total ATP (○), 5 mM total ATP and Na₂EDTA concentrations ranging between 2 and 7.5 mM (△), and 10 mM total ATP and Na₂EDTA concentrations ranging between 1 and 7 mM (■). The concentrations of the different species were calculated from the data in Table 1 as described in the text. (a) Dependence of activity on the concentration of free ATP ('ATP_F'), which represents all ATP species not present as MgATP²⁻. (b) Dependence of activity on the concentration of ATP⁴⁻. In these experiments the ionic strength varied from 0.16 to 0.25 and the free sulphate ion concentration ranged from 7.21 to 9.71 mM.

the concentrations of the free and complexed species by using the data shown in Table 1 and in the presence of EDTA as a Mg²⁺ buffer. The activity of the enzyme was measured at a fixed total concentration of 10 mM-MgSO₄ and various amounts of total ATP, ranging from 5 to 20 mM in the absence of Na₂EDTA and in the presence of two fixed total ATP concentrations, 5 and 10 mM, with Na₂EDTA concentrations in the ranges 2–7.5 mM and 1–7 mM respectively. The minimum concentration of MgATP obtained in this study was 2.92 mM, which, from the data shown above, should have been sufficient to saturate the enzyme.

When the variation of initial velocity was expressed as a function of the concentration of free ATP or ATP⁴⁻, the data obtained from the three experimental procedures gave rise to separate dependencies, as shown in Fig. 5. In contrast, a single curve was obtained when the three sets of data were used to plot the variation of initial velocity as a function of the free Mg²⁺ concentration. This is shown in Fig. 6. These data gave an apparent K_m value for free Mg²⁺ of $294 \pm 18 \mu\text{M}$. The close fit of the data to a single curve also suggests that free ATP, which varies between 0.73 and 10.8 mM in these experiments, does not significantly inhibit the activity of the phosphofructokinase under these conditions. The obedience of the data in Fig. 6 to simple Michaelis–Menten behaviour would suggest that the data are not significantly affected by the changes in ionic strength, and would be consistent with the changes in the free SO₄²⁻ concentrations being the dominant factor in the inhibition shown in Fig. 3.

The above calculations do not include any effects arising from complex-formation between Mg²⁺ ions and the imidazole component of the buffer. No data were available on the stability constants for such complex-formation. However, values of 44.7 and 17.8 M⁻¹ have been reported for the stability constants of the Mn-imidazole and Mn₂-imidazole complexes res-

pectively (Martin & Edsall, 1958). These values are similar to those for the formation of the Mg²⁺-fructose 6-phosphate complex, which the analysis showed to have no significant effects on the overall behaviour of the system. Incorporation of these data and a value of $1.32 \times 10^7 \text{ M}^{-1}$ for the association constant for the formation of the H-imidazole species (Brooks & Davidson, 1960), but omitting the association with fructose 6-phosphate (Table 1, eqn. 11), since the COMPLX program allows only nine reactants, had no significant effect on the K_m value determined for free Mg²⁺, a value of $305 \pm 18 \mu\text{M}$ being obtained.

Phosphofructokinase from *T. brucei* was also active

Fig. 6. Dependence of the activity of phosphofructokinase from *T. brucei* on the concentration of free Mg²⁺ (Mg_F²⁺)

Experimental details and the designations of the symbols are as described in Fig. 5 legend.

Table 2. Apparent Michaelis constants for the activity of phosphofructokinase from *T. brucei* towards ATP in the presence of Mg²⁺ or Mn²⁺

The enzyme was assayed in the presence of 0.5 mM-fructose 6-phosphate, 10 mM-MgSO₄ or 10 mM-MnCl₂ and various concentrations of ATP.

Cation	<i>V</i> (units/mg)	<i>K_m</i> (μM)
Mg ²⁺	92.8 ± 1.4	67.4 ± 3.9
Mn ²⁺	63.2 ± 0.8	19.2 ± 1.6

when MgSO₄ was replaced by MnCl₂. Double-reciprocal plots of the dependence of the initial velocity on the total ATP concentration, in the range 0.04–1 mM and in the presence of either 10 mM-MgSO₄ or 10 mM-MnCl₂, were linear, and allowed the apparent *K_m* and maximum-velocity values shown in Table 2 to be determined. Since the stability constants for the MgATP²⁻ and MnATP²⁻ complexes are similar (see O'Sullivan, 1969), the decrease in maximum velocity when Mg²⁺ was replaced by Mn²⁺ may result from differences in the catalytic efficiency with the latter complex as a substrate, or from differences in the activity of the Mn²⁺–enzyme form.

The dependence of initial velocity on the concentration of fructose 6-phosphate in the presence of either Mg²⁺ or Mn²⁺ ions is shown in the form of Hill plots, in Fig. 7. Although a linear dependence was obtained in the

Fig. 7. Dependence of the activity of phosphofructokinase from *T. brucei* on the concentration of fructose 6-phosphate

Activities were determined in the presence of 1 mM-ATP, various concentrations of fructose 6-phosphate (F6P) and 10 mM-MgSO₄ (●), 10 mM-MnCl₂ (○) or 10 mM-MnCl₂ plus 1 mM-AMP (□). Data are expressed as Hill (1910) plots.

presence of Mg²⁺, the data obtained with Mn²⁺ appeared to show some deviation from linearity. The apparent maximum velocities were 252 and 204 units/mg with Mg²⁺ and Mn²⁺ respectively, and for Mg²⁺ the Hill coefficient, *h*, was 1.4 and the concentration of fructose 6-phosphate that gave half-maximum velocity was 830 μM. In the presence of 1 mM-AMP and 10 mM-MnCl₂, *h* and the half-saturating fructose 6-phosphate concentration were 0.9 and 210 μM respectively, and the maximum velocity was 145 units/mg (Fig. 7). An *h* value of 1.0 and a decrease in both the half-saturating substrate concentration and the maximum velocity by this concentration of AMP have previously been reported in the presence of MgSO₄ (Cronin & Tipton, 1985).

The activity of the enzyme assayed in the presence of 50 μM-ATP, 0.4 mM-fructose 6-phosphate and 5 mM-MgSO₄ was unaffected by the addition of CaSO₄, CuSO₄ or FeSO₄ at concentrations of 200 μM. ZnSO₄, however, was inhibitory, causing 50% inhibition at a concentration of about 120 μM.

DISCUSSION

The data presented above indicate that, in common with the enzyme from several other sources (see, e.g., Cottam & Uyeda, 1973), the true substrate for phosphofructokinase from *T. brucei* is the MgATP complex. There have been some reports that a free bivalent cation may also be essential for the activity of phosphofructokinase. This was first considered by Paetkau & Lardy (1967), who found the enzyme from rabbit muscle to be inactive unless the concentration of Mg²⁺ exceeded that of ATP. A high-affinity binding site for Mn²⁺, with a dissociation constant of 20 μM, was also identified in the enzyme from this source (Cottam & Uyeda, 1973). Mavis & Stellwagen (1970), using the alternative substrate ITP, showed that the activity of yeast phosphofructokinase was dependent on the concentrations of both MgITP and free Mg²⁺.

The investigation of whether a free bivalent cation is required for activity is complicated by the fact that of the three variables, free metal ion, free nucleotide and metal–nucleotide complex, only one can be maintained constant under experimental conditions unless an additional metal-buffering system is also added. Because of this, claims suggesting free Mg²⁺ to be essential for the activities of the enzymes from human platelets (Akerman *et al.*, 1974) and erythrocytes (Etiemble *et al.*, 1981), and that free ATP is an inhibitor of the monkey liver enzyme (Mattheyse *et al.*, 1979), are not conclusive.

The results presented here indicate that ATP and its magnesium complex are not inhibitors of phosphofructokinase from *T. brucei*, although the former species may cause inhibition indirectly by removing, through chelation, essential Mg²⁺ ions. This contrasts with the suggestion by Nwagwu & Oppendoes (1982) that ATP is an inhibitor of the enzyme from this source.

Phosphofructokinase preparations from a number of sources have been reported to be inhibited by ATP (for reviews see Bloxham & Lardy, 1973; Uyeda, 1979), but the enzyme from some other sources, including *Lactobacillus casei* and *Lactobacillus plantarum* (Doelle, 1972), *Flavobacterium thermophilum* (Yoshida, 1972) and *Dictyostelium discoideum* (Baumann & Wright, 1968), appears to be insensitive to inhibition by this nucleotide.

In some cases, including phosphofructokinase from mouse liver (Hosey *et al.*, 1980), yeast (Mavis & Stellwagen, 1970), pig spleen (Hickman & Weidemann, 1975) and the human platelet isoenzyme F4 (Meienhofer *et al.*, 1980), MgATP rather than the free nucleotide has, however, been reported to be inhibitory.

With phosphofructokinase from *T. brucei*, neither ATP nor its complexes with Mg^{2+} are inhibitory, although indirect inhibition by removal of these ions, which are essential for activity, might be a factor regulating the activity of the enzyme. It is, however, not possible to assess the likely importance of such an effect, since, although the total cellular concentrations of ATP in *T. brucei* have been estimated under both aerobic and anaerobic conditions (Oppendoes *et al.*, 1976; Visser & Oppendoes, 1980), no data are available on the free Mg^{2+} ion concentrations.

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