Kinetic studies on the reaction catalysed by phosphofructokinase from *Trypanosoma brucei*

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The steady-state kinetics of the reaction catalysed by the bloodstream form of *Trypanosoma brucei* were studied at pH 6.7. In the presence of 50 mM-potassium phosphate buffer, the apparent co-operativity with respect to fructose 6-phosphate and the non-linear relationship between initial velocity and enzyme concentration, which were found when the enzyme was assayed in 50 mM-imidazole buffer (Cronin & Tipton, 1985) Biochem. J. 227, 113–124, are not evident. Studies on the variations of the initial rate with changing concentrations of MgATP and fructose 6-phosphate, the product inhibition by fructose 1,6-bisphosphate and the effects of the alternative substrate ITP were consistent with an ordered reaction pathway, in which MgATP binds to the enzyme before fructose 6-phosphate, and fructose 1,6-bisphosphate is the first product to dissociate from the ternary complex.

INTRODUCTION

Phosphofructokinase (ATP: d-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) from bloodstream forms of *Trypanosoma brucei* has been shown to differ from that enzyme in other species in several of its allosteric properties (Cronin & Tipton, 1985). Such differences may reflect the specialized metabolism of this organism, which includes the compartmentation of many of the enzymes of glycolysis within the glycosome (see Opperdoes & Borst, 1977; Oduro et al., 1980a,b) and the absence of a functional tricarboxylic acid cycle (Flynn & Bowman, 1973) or cytochrome chain (see Bowman & Flynn, 1976). The pathways of glucose metabolism under aerobic (Grant & Sargent, 1960; Bowman & Flynn, 1976; Opperdoes et al., 1977) and anaerobic (Opperdoes et al., 1976; Opperdoes & Borst, 1977; Brohn & Clarkson, 1980; Hammond & Bowman, 1980; Visser et al., 1981) conditions, although not yet fully elucidated, appear to differ in several respects from those known in other animal, plant and bacterial species.

Although the kinetic behaviour of phosphofructokinase from mammalian species has been studied by several groups [for reviews see Bloxham & Lardy (1973) and Uyeda (1979)] there is no clear agreement on the mechanism followed. The possibility that the metabolic requirements of specific cell types may have led to the retention or evolution of advantageous kinetic behaviour (see MacFarlane & Ainsworth, 1974; Tipton, 1980) and the differences in regulatory behaviour previously reported (Cronin & Tipton, 1985) suggest that there is no reason a priori to assume that the kinetic behaviour of the enzyme from *T. brucei* would resemble that of the mammalian enzyme. This paper reports the results of a study on the steady-state kinetics of phosphofructokinase from *T. brucei* which was carried out under conditions similar to those used in earlier studies of its allosteric behaviour (Cronin & Tipton, 1985).

MATERIALS AND METHODS

Stabilate inocula of *T. brucei* (427–12/ICI-060) were kindly given by Dr. H. P. Voorheis, who has described the history of this monomorphic laboratory strain (Voorheis et al., 1979). Trypanosomes were grown and phosphofructokinase was purified from them as described previously (Cronin & Tipton, 1985). The sources of the other materials used in this work are also described in that paper. Protein concentration was determined by the method of Bradford (1976), with hen’s-egg-white lysozyme as standard.

Phosphofructokinase activity was assayed spectrophotometrically at 37 °C and pH 6.7 in a total volume of 2.0 ml by coupling the product formation to the oxidation of NADH. The assay mixture contained 50 mM-potassium phosphate/KOH buffer, pH 6.7, 10 mM-MgSO₄, 100 mM-KCl, 5 mM-2-mercaptoethanol and 0.2 mM-NADH. When ADP formation was being monitored, 20 μg of pyruvate kinase, 20 μg of lactate dehydrogenase and 0.2 mM-phosphoenolpyruvate were included, and, to monitor the production of fructose 1,6-bisphosphate, 200 μg of aldolase, 20 μg of triosephosphate isomerase and 20 μg of glyceraldehyde dehydrogenase were present. All coupling enzymes were desalted by gel-filtration through Sephadex G-25 equilibrated with 55.56 mM-potassium phosphate/KOH buffer, pH 6.7.

The reaction, which was followed at 340 nm, was started by the addition of 0.1 μg, unless otherwise stated, of purified phosphofructokinase. A unit of enzyme activity is defined as the amount catalysing the formation of 1 μmol of product/min.

In common with many other kinases (see Cohn, 1963), the true substrate for phosphofructokinase from *T. brucei* has been found to be the magnesium complex of ATP (Cronin & Tipton, 1987). Furthermore, it appears that free Mg²⁺ ions are also required, and a Kₘ value of 294 ± 18 μM has been calculated for them (Cronin & Tipton, 1987). The procedure of Storer & Cornish-Bowden (1976) was used to determine the concentration of the MgATP complex. The greatest concentration of ATP used in the present studies was 1 mM, although ITP at a maximum concentration of 1 mM was also present in some studies. By using the values of the stability constants previously taken (Cronin & Tipton, 1987) together with the value of 7.6 x 10⁴ M⁻¹ for the
combination of Mg$^{2+}$ with HPO$_4^{2-}$ (Bock, 1960) and $7.76 \times 10^6$ and $4.53 \times 10^{18}$ M$^{-1}$ for the association constants for the combination of H$^+$ with HPO$_4^{2-}$ and PO$_4^{3-}$ respectively (Pettigrew & Frieden, 1979a; Weast, 1974), it could be calculated that under these conditions the ATP used in these studies will be predominantly in its complexed form, and there would also be sufficient free Mg$^{2+}$ ions to saturate the enzyme. In the studies of product inhibition by fructose 1,6-bisphosphate with respect to ATP, the total concentration of MgSO$_4$ was increased to 25 mm to prevent a decrease of the free Mg$^{2+}$ concentration below that required for maximum activity.

Calculation of the species present in solution by the procedure of Storer & Cornish-Bowden (1976) indicated that only $83 \pm 1\%$ would be in the MgATP$^{2-}$ form, which is the true substrate for the enzyme, throughout the experimental conditions employed. Since the other ionic species of this complex do not inhibit the enzyme (Cronin & Tipton, 1987), the Michaelis constants determined for ATP were corrected to express them in terms of the MgATP$^{2-}$ species alone.

Kinetic data were analysed by the non-linear regression method of Wilkinson (1961). Secondary (slope and intercept) plots were fitted by linear regression. Data are presented as double-reciprocal plots for illustrative purposes.

**RESULTS**

The activity of phosphofructokinase from *T. brucei*, like that enzyme from several other eukaryotic sources (Underwood & Newsholme, 1965; Hulme & Tipton, 1971b; Leaver & Burt, 1981), has been shown to be non-linearly dependent on enzyme concentration when assayed in imidazole buffer, pH 6.7 (Cronin & Tipton, 1985). However, in the presence of the same concentration of phosphate buffer, a strictly linear relationship between initial rate and enzyme concentration was observed (Fig. 1). The dependence of initial velocity on the concentration of fructose 6-phosphate was sigmoid in imidazole buffer but hyperbolic in the phosphate buffer (Cronin & Tipton, 1985). In either buffer double-reciprocal plots of initial velocity against ATP concentration were linear.

**Initial-rate studies in the absence of products**

Double-reciprocal plots of the variation of initial velocity as a function of the concentrations of ATP and fructose 6-phosphate are shown in Figs. 2(a) and 2(b) respectively. The families of intersecting lines obtained clearly rule out a double-displacement (two-step transfer, or Ping Pong) mechanism. The linearity of these plots also excludes any significant co-operative effects, and

![Fig. 1. Dependence of the initial velocity of the reaction catalysed by phosphofructokinase from *T. brucei* on the enzyme concentration](image)

Activities were determined by measuring the formation of fructose 1,6-bisphosphate as described in the text. The concentrations of the substrates ATP and fructose 6-phosphate were both 1.0 mm.

![Fig. 2. Effects of substrate concentrations on the initial rates of the reaction catalysed by phosphofructokinase from *T. brucei*](image)

Activities were determined by measuring the formation of fructose 1,6-bisphosphate as described in the text. (a) The effects of varying the ATP concentrations at the following fixed concentrations of fructose 6-phosphate: 0.25 mm (▲), 0.4 mm (□), 0.75 mm (■), 2.0 mm (○) and 5 mm (●). (b) The effects of varying the fructose 6-phosphate (F6P) concentrations at the following fixed concentrations of ATP: 25 μM (▲), 31.25 μM (△), 41.7 μM (□), 62.5 μM (■), 125 μM (○) and 1.0 mm (●).
Table 1. Kinetic constants for the reaction catalysed by phosphofructokinase from *T. brucei*

The constants were determined from initial-rate determinations as described in the text. The value of $k_{cat}$ was calculated by using a value of 49000 for the $M_r$ of one subunit (Cronin & Tipton, 1985), assuming each subunit in the tetrameric enzyme to have the same activity.

<table>
<thead>
<tr>
<th>Michaelis constant</th>
<th>Value</th>
<th>Specificity constant</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_M^{\text{ATP}}$</td>
<td>39.3 $\mu M$</td>
<td>$k_{cat}/K_M^{\text{ATP}}$</td>
<td>$6.2 \times 10^4$ s$^{-1}$M$^{-1}$</td>
</tr>
<tr>
<td>$K_M^{\text{ADP}}$</td>
<td>64.8 $\mu M$</td>
<td>$k_{cat}/K_M^{\text{ATP}}$</td>
<td>$3.7 \times 10^4$ s$^{-1}$M$^{-1}$</td>
</tr>
<tr>
<td>$K_M^{\text{Pi}}$</td>
<td>999 $\mu M$</td>
<td>$k_{cat}/K_M^{\text{Pi}}$</td>
<td>$2.4 \times 10^8$ s$^{-1}$M$^{-1}$</td>
</tr>
<tr>
<td>$V_m$</td>
<td>296 units/mg</td>
<td>$k_{cat}$</td>
<td>242 s$^{-1}$</td>
</tr>
</tbody>
</table>

Table 2. Kinetic constants for nucleoside triphosphates as substrates for phosphofructokinase from *T. brucei*

Assays were carried out by monitoring fructose 1,6-bisphosphate formation as described in the text and at a fixed fructose 6-phosphate concentration of 0.5 mM.

<table>
<thead>
<tr>
<th>Nucleoside triphosphate</th>
<th>Apparent $K_m$ ($\mu M$)</th>
<th>Apparent $V$ (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>46.9±2.0</td>
<td>100.6±1.4</td>
</tr>
<tr>
<td>GTP</td>
<td>92.7±1.4</td>
<td>115.0±0.6</td>
</tr>
<tr>
<td>ITP</td>
<td>113±2.3</td>
<td>101.2±0.6</td>
</tr>
</tbody>
</table>

suggests that a random-order mechanism operating under steady-state conditions is unlikely, although, as pointed out by Pettersson (1970), departure from Michaelis–Menten behaviour may not always be apparent with such a mechanism. Graphs of the slopes and intercepts of the lines shown in Fig. 2 against the reciprocal concentrations of the fixed substrates (Florini & Vestling, 1957), which were linear in all cases, were used to determine the kinetic constants for the reaction (Table 1).

Studies with alternative substrates

The enzyme was shown to be active with either ITP or GTP in place of ATP. The apparent $K_m$ and maximum-velocity values for these substrates, determined at a fixed fructose 6-phosphate concentration of 0.5 mM, are shown in Table 2.

The effects of ITP as a competing substrate with ATP were studied. Fig. 3(a) shows the double-reciprocal pattern obtained when the formation of fructose 1,6-bisphosphate was monitored in the presence of 0.5 mM-fructose 6-phosphate, different concentrations of ATP and a range of fixed ITP concentrations. Fig. 3(b) shows the results obtained in the presence of 50 μM-ATP, different concentrations of fructose 6-phosphate and a series of fixed concentrations of ITP. The non-linear, apparently competitive, pattern shown in Fig. 3(a) and the mixed-type pattern in Fig. 3(b) would be consistent with a sequential mechanism with ATP binding to the free enzyme (Fromm, 1964; Huang, 1979). The apparent linearity of the plots shown in Fig. 3(b) might indicate a random order of substrate addition, but, as pointed out by Ricard et al. (1972), a number of conditions may exist where curvature may not be apparent.

Several substrates were tested as alternative phosphate acceptor substrates, but none was found to be suitable.

**Fig. 3. Effects of the alternative substrate ITP on the activity of phosphofructokinase from *T. brucei***

Activities were determined by measuring the formation of fructose 1,6-bisphosphate as described in the text. (a) Initial rates were determined in the presence of 0.5 mM-fructose 6-phosphate, different concentrations of ATP and the following fixed total ITP concentrations: 0 (●), 25 μM (○), 62.5 μM (■), 125 μM (□), 250 μM (▲) and 1.0 mM (△). (b) Initial rates were measured in the presence of 50 μM-ATP, different concentrations of fructose 6-phosphate (F6P) and the following fixed total concentrations of ITP: 0 (●), 62.5 μM (■), 125 μM (□) and 1.0 mM (△).
Fig. 4. Inhibition of phosphofructokinase from *T. brucei* by
fructose 1,6-bisphosphate

Activities were assayed by monitoring the formation of
ADP as described in the text. (a) Initial rates were
determined in the presence of 1 mM-fructose 6-phosphate,
25 mM-MgSO₄ and the following concentrations of
fructose 1,6-bisphosphate: 0 (●), 5 mM (○), 10 mM (■)
and 20 mM (□). (b) Initial rates were determined in the
presence of 50 µM-ATP, 10 mM-MgSO₄, various
concentrations of fructose 6-phosphate (F6P) and the
following concentrations of fructose 1,6-bisphosphate: 0
(●), 1.25 mM (○), 5 mM (■) and 7.5 mM (□).

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D-Sedoheptulose 7-phosphate, which has also been reported
to be a substrate for phosphofructokinase from rabbit
muscle and liver (Karadsheh *et al.*, 1973), showed no
activity with the enzyme from *T. brucei* at a concentration
of 1 mM and in the presence of 1 mM-ATP. D-Glucose
1-phosphate, which has also been reported to be a
substrate for the rabbit muscle enzyme (Eyer *et al.*, 1971),
was also ineffective at 1 mM in the presence of
0.1 mM-ATP, and under similar conditions d-fructose
1-phosphate, also a substrate for the rabbit muscle
enzyme (Uyeda, 1972), was not effective at concentrations
up to 20 mM.

**Product-inhibition studies**

It was not possible to use ADP as a product inhibitor,
since it behaves as an activator of this enzyme (Cronin
& Tipton, 1985). The inhibition by fructose 1,6-
bisphosphate with respect to both ATP and fructose
6-phosphate is shown in Fig. 4. Replots of the slopes and
intercepts of the lines against the inhibitor concentration
were linear in all cases. The *Kᵢ* values for this product
acting as an inhibitor with respect to fructose 6-phosphate
were calculated to be 10.7 mM for *Kᵢ₁* (slope replot) and
15.8 mM for *Kᵢ₂* (intercept replot).

When the inhibition towards ATP was determined at
50 mM-fructose 6-phosphate, a concentration about 50
times the *Kᵢᵢ* value for this substrate (see Table 1),
the inhibition was still mixed, as shown in Fig. 5, although
the lines intersected at a point below the abscissa (cf. Fig.
4), indicating the slope effect to be less important at the
higher fructose 6-phosphate concentration. The inhibition
by fructose 1,6-bisphosphate with ATP as the
varied substrate was determined at a range of fructose
6-phosphate concentrations, and the *Kᵢ* values were
determined in each case. Fig. 6 shows a graph of these
values against the fructose 6-phosphate concentration.
The curves indicate that the value of *Kᵢᵢ* increases
linearly with substrate concentration, whereas *Kᵢᵠ* tends
Phosphofructokinase kinetics

6. Phosphofructokinase to a mechanism of behaviour uncompetitive, mechanism can be

\[ V = \frac{K_{ATP}}{K_{m}^{ATP}} \]

where \( V \) is the maximum velocity, \( K_{m} \) values are the substrate concentrations that give half-maximal velocity at saturating concentrations of the other substrate, and \( K_{ATP}^{m} \) represents the apparent value of \( K_{ATP}^{m} \) when the fructose 6-phosphate concentration tends to zero. The kinetic constants determined from the initial-rate data are shown in Table 1.

It has been suggested (Dalziel, 1957; Cleland, 1970) that a mechanism of this type may show high substrate inhibition, owing to the formation of an abortive ternary complex by the binding of the second substrate to the enzyme–product binary complex. The abortive complex that might be formed in the mechanism shown in eqn. (1) would thus be E·F6P·ADP. Attempts to demonstrate significant high-substrate inhibition of the enzyme by fructose 6-phosphate were, however, unsuccessful. At an ATP concentration of 0.1 mM the activity at 90 mM-fructose 6-phosphate, determined by monitoring ADP formation, was found to be about 95% of that at 15 mM-fructose 6-phosphate.

**DISCUSSION**

There have been conflicting reports on the kinetic mechanism followed by phosphofructokinase preparations from animal sources. Under a variety of conditions, apparently parallel double-reciprocal plots have been observed, which were interpreted as being consistent with a double-displacement mechanism [for reviews see Bloxham & Lardy (1973) and Uyeda (1979)]. Subsequent work, however, showed that the mechanism was indeed sequential (Uyeda, 1972; Bar-Tana & Cleland, 1974a; Pettigrew & Frieden, 1979b), and this was consistent with evidence from isotope-exchange studies which failed to demonstrate a significant role for a free modified-enzyme intermediate (Uyeda, 1970; Hulme & Tipton, 1971a; Hanson et al., 1973). For the enzyme from *T. brucei* the primary plots (Fig. 2) are clearly intersecting, allowing a double-displacement mechanism to be excluded. This derives from the relatively high value of \( K_{ATP}/K_{m}^{ATP} \) of 0.61, whereas for the enzyme from rabbit muscle the corresponding ratio is 0.03 (Bar-Tana & Cleland, 1974a). It has been pointed out that this behaviour of the mammalian enzyme will make its activity relatively insensitive to fluctuations of the concentrations of one substrate at low concentrations of the other (Tipton, 1980; see also Tipton et al., 1987). In contrast, the higher value of this ratio will result in the activity of the *T. brucei* enzyme being more responsive to substrate concentration under such conditions.

Uyeda (1972) proposed that the enzyme from rabbit muscle followed a compulsory-ordered mechanism similar to that shown in eqn. (1). However, other workers have suggested the order of substrate addition
Michaelis–Menten behaviour. In the present work advantage has been taken of the effects of 50 mM-phosphate in abolishing co-operative behaviour to study the kinetics at the same pH as was previously used in studies of the regulatory behaviour of the enzyme. Recent studies (D. Nolan & H. P. Voorheis, unpublished work) have shown the intracellular pH value of bloodstream T. brucei to be at, or just below, 7.0. It is to be hoped that these studies will prove to be of value in assessing the behaviour of the enzyme within the trypanosome and its role in the regulation of glycolysis. However, such analysis will have to await a more detailed knowledge of the concentrations of metabolites and ions within the glycosome.

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