Purification and regulatory properties of phosphofructokinase from *Trypanosoma (Trypanozoon) brucei brucei*

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Phosphofructokinase (EC 2.7.1.11) from *Trypanosoma (Trypanozoon) brucei brucei* was purified to homogeneity by using a three-step procedure that may be performed within 1 day. Proteolysis, which removes a fragment of M_r approx. 2000, may occur during the purification, but this can be prevented by including antipain, an inhibitor of cysteine proteinases, in the buffers during the purification. The subunits of the enzyme appear to be identical in size, with an M_r of 49000. The M_r of the native enzyme was estimated to be approx. 220000, suggesting a tetrameric structure. Kinetic studies showed the activity to depend hyperbolically on the concentration of ATP but sigmoidally on the concentration of fructose 6-phosphate. Although cyclic AMP, AMP and ADP stimulated the enzyme activity at low concentrations of fructose 6-phosphate, the last two nucleotides were inhibitory at high concentrations of this substrate. Phosphoenolpyruvate behaved as an allosteric inhibitor of the phosphofructokinase. Citrate, fructose 1,6-bisphosphate, fructose 2,6-bisphosphate and P_i did not influence significantly the activity of the enzyme.

The salivarian trypanosomes *Trypanosoma (Trypanozoon) brucei gambiense* and *T. (T.) brucei rhodesiense* are the agents responsible for African sleeping sickness. Together with the morphologically indistinct animal parasite *T. (T.) brucei brucei* these organisms are known collectively as the *T. brucei* complex. Because the trypanosomes are capable of avoiding the host immune response by antigenic variation (Vickerman, 1976; Turner, 1982), it may not prove possible to prevent this disease by vaccination. In attempts to devise a more effective treatment for mammalian trypanosomal infections, the bloodstream form of *T. (T.) brucei brucei* (referred to below simply as *T. brucei*) has been studied extensively.

Although trypanosomal glycolysis is functionally similar to that found in other cell types, a number of major differences in its organization have been described. Opperdoes & Borst (1977) reported the discovery in *T. brucei* of a new type of subcellular organelle that, because it contained many of the enzymes of glycolysis, they termed the glycosome. Those glycolytic enzymes that appear not to be present within this particle include phosphoglycerate mutase, enolase and pyruvate kinase

(Opperdoes & Borst, 1977; Oduro *et al.*, 1980a,b). The relatively constant concentration of glucose in the host cardiovascular system allows trypanosomes to derive all their energy from glycolysis, excreting pyruvate as the sole waste product of this metabolism under aerobic conditions (Brohn & Clarkson, 1978). Thus each glucose molecule metabolized yields only two molecules of ATP. Because bloodstream-form trypanosomes do not possess a functional tricarboxylic acid cycle (Flynn & Bowman, 1973) or respiratory chain (see Bowman & Flynn, 1976), the NADH formed during glycolysis is believed to be reoxidized by the coupled action of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate oxidase (Grant & Sargent, 1960; Bowman & Flynn, 1976). The latter enzyme, which is unique to trypanosomes, is located within the kinetoplast mitochondrion (Opperdoes *et al.*, 1977b).

Under anaerobic conditions, or similarly when the terminal oxidase is inhibited by salicylhydroxamic acid (Opperdoes *et al.*, 1976), the end products of metabolism are approximately equal amounts of pyruvate and glycerol (Opperdoes *et al.*, 1976; Brohn & Clarkson, 1980). The survival of these organisms under such conditions is believed to result from the formation of ATP through a reversal of the glycerol-3-phosphate kinase reac-

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tion (Opperdoes & Borst, 1977; Hammond & Bowman, 1980; Visser *et al.*, 1981), an enzyme whose glycosomal location has been described (Opperdoes & Borst, 1977; Oduro *et al.*, 1980 *a,b*).

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is of primary importance in the regulation of glycolytic flux (Mansour, 1972; Ramaiah, 1974; Hofmann, 1976). The cumulative list (Sols, 1981) of those compounds that have been found to influence the activity of this enzyme is very large. In view of the compartmentation and other unusual aspects of trypanosomal glycolysis, it was considered that a study of phosphofructokinase from *T. brucei* would be of intrinsic biochemical interest, and also of possible therapeutic relevance. The present paper describes the purification and some regulatory properties of this enzyme. Some of the results of this study, particularly those with respect to the effects of AMP on the activity of phosphofructokinase, differ markedly from those reported by Nwagwu & Opperdoes (1982), who employed crude enzyme preparations for their study.

Materials

ATP (disodium salt), AMP (disodium salt), cyclic AMP (sodium salt), phosphoenolpyruvate (monopotassium salt), fructose 2,6-bisphosphate (tetrasodium salt), antipain (dihydrochloride), aprotinin, leupeptin, chymostatin, pepstatin A, bovine serum albumin (fraction V), ovalbumin, dithiothreitol and phenylmethanesulphonyl fluoride were obtained from Sigma Chemical Co., Poole, Dorset, U.K. ADP (disodium salt), fructose 6-phosphate (disodium salt), fructose 1,6-bisphosphate (trisodium salt) and NADH (disodium salt) were purchased from C. F. Boehringer and Soehne, Mannheim, West Germany. One sample of the fructose 2,6-bisphosphate used in this work was kindly given by Dr. E. Van Schaftingen, International Institute of Cellular and Molecular Pathology, Brussels, Belgium.

All other chemicals were of the highest available purity and were obtained from either BDH Chemicals, Poole, Dorset, U.K. or Hopkin and Williams, Chadwell Heath, Essex, U.K. DEAE-cellulose (DE-52) was from Whatman, Maidstone, Kent, U.K., and ATP-agarose (type 2) was purchased from P-L Biochemicals, Milwaukee, WI, U.S.A.

Fructose 1,6-bisphosphate aldolase (EC 4.1.2.13), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), triosephosphate isomerase (EC 5.3.1.1), pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27) and malate dehydrogenase (EC 1.1.1.37) were obtained from Boehringer. Glyceraldehyde-3-phosphate dehydro-

genase (EC 1.2.1.12) and β -galactosidase (EC 3.2.1.23) were supplied by Sigma. Hen's-egg-white lysozyme (EC 3.2.1.17) was purchased from Armour Laboratories, Chicago, IL, U.S.A.

Methods

Growth and isolation of trypanosomes

Stabulate inocula of *T. brucei* (427-12/ICI-060) were used as the source of trypanosomes during the entire work. Initial stocks of this organism were a gift from Dr. H. P. Voorheis, who has described the history of this monomorphic trypanosomal strain (Voorheis *et al.*, 1979).

Trypanosomes were grown in 200–600 g albino Wistar rats, which were maintained on food and water *ad libitum* until parasitaemia levels were high enough for cell harvesting (about 3 days). Infected rat blood was withdrawn, under ether anaesthesia, from the aorta into a syringe containing 1 ml of 100 mM-EGTA, pH 7.5. Trypanosomes were largely separated from blood components by centrifugation at 600 g for 10 min before purification on DEAE-cellulose (Lanham, 1968) that had been equilibrated with 50 mM- Na_2HPO_4 /3 mM- NaH_2PO_4 /43.6 mM- NaCl /10 mM-glucose, pH 8.0.

Enzyme assays

Phosphofructokinase activity was measured spectrophotometrically at 37°C by monitoring the oxidation of NADH at 340 nm with coupled enzyme reactions. During the purification this activity was determined in 50 mM-Tris/HCl buffer, pH 8.0 containing 10 mM- MgSO_4 , 5 mM-2-mercaptoethanol, 0.2 mM-NADH, 1 mM-fructose 6-phosphate, 200 μg of aldolase, 20 μg of triosephosphate isomerase and 20 μg of glycerol-3-phosphate dehydrogenase in a total volume of 2 ml. Reactions were initiated by the addition of fructose 6-phosphate after recording the blank rate, if any, which was subtracted from the measured rate. In this standard assay the coupling enzymes were not desalted before use. Under these conditions the initial rate was found to be directly proportional to the concentration of enzyme.

The regulatory kinetic properties were studied by coupling the formation of either fructose 1,6-bisphosphate (Fru-1,6- P_2 -coupled assay) or ADP (ADP-coupled assay) to the oxidation of NADH. In either system activities were measured in 50 mM-imidazole/HCl buffer, pH 6.7, containing, unless otherwise indicated, 10 mM- MgSO_4 , 100 mM-KCl, 5 mM-2-mercaptoethanol, 0.2 mM-NADH and substrate and effector concentrations as indicated in a final volume of 2 ml. When the Fru-1,6- P_2 -coupled assay was used the reaction mixtures included 200 μg of aldolase, 20 μg of triosephosphate isomer-

ase and 20 μ g of glycerol-3-phosphate dehydrogenase. When the ADP-coupled assay was used the reaction mixtures included 20 μ g of lactate dehydrogenase, 20 μ g of pyruvate kinase and 0.2 mM-phosphoenolpyruvate. All coupling enzymes were desalted before use by passage through a column (16.5 cm $\times$ 2 cm) of Sephadex G-25 (fine grade) equilibrated with 50 mM-imidazole/HCl buffer, pH 6.7.

Unless otherwise stated, all reactions were initiated by the addition of 0.1 μ g of purified phosphofructokinase in a volume of 20 μ l. Stock solutions of the enzyme were diluted to 5 μ g/ml in plastic Minifuge tubes with 50 mM-Tris/HCl buffer, pH 7.8, containing 1 mg of bovine serum albumin/ml, 5 mM-dithiothreitol and 10% (w/v) glycerol, and stored on ice. All substrate and effector solutions were prepared freshly each day, adjusted to pH 6.7 with NaOH or HCl when necessary, and stored on ice. After an initial lag phase the reaction progress curves were linear and the steady-state velocities attained when either ATP, fructose 6-phosphate or phosphofructokinase was used to initiate the reaction were similar.

In all cases 1 unit of enzyme activity is defined as the amount of enzyme required to catalyse the formation of 1 μ mol of product, either ADP or fructose 1,6-bisphosphate, in 1 min.

Malate dehydrogenase (Mehler *et al.*, 1948), lactate dehydrogenase (Kornberg, 1955), pyruvate kinase (Valentine & Tanaka, 1966) and β -galactosidase (Craven *et al.*, 1965) were assayed essentially as described by these authors.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed on 12.5% slab gels with the buffer system described by Laemmli (1970). Bovine serum albumin (M_r 68000), hen's-egg ovalbumin (M_r 43000) and the subunits of the rabbit muscle enzymes pyruvate kinase (M_r 57200) and glyceraldehyde-3-phosphate dehydrogenase (M_r 36600) served as M_r standards. Electrophoresis was performed at 4°C and at a constant 80 V. Protein bands were stained as described by Taucher *et al.* (1975).

Non-denaturing polyacrylamide-gel electrophoresis was performed on 2.5%, 5% and 7% discs gels as described by Davis (1964). The 2.5% polyacrylamide-disc-gel electrophoresis was also performed with the continuous phosphate system described by Varon *et al.* (1967) except that the solution C described in that work was replaced with a solution containing 10% (w/v) acrylamide and 1.324% (w/v) bisacrylamide in water. With either system, electrophoresis was performed at 4°C and with a constant current of 2.5 mA per gel.

After electrophoresis the gels were fixed and stained for 30 min in acetic acid/methanol/water (1:5:4, by vol.) containing 1.25% Coomassie Blue R250. Excess stain was removed by washing with acetic acid/methanol/water (7:10:83, by vol.).

Protein samples that were too dilute for electrophoresis were precipitated with an equal volume of 20% (w/v) trichloroacetic acid at 4°C for 1 h. After centrifugation for 5 min in an Ole Dich Minifuge, the pellets were washed twice with acetone before resuspension in the appropriate buffer.

Ultracentrifugation

The M_r of native phosphofructokinase was estimated by subjecting 10 μ g of the purified enzyme to centrifugation on linear 10–25% (v/v) glycerol gradients (5 ml total vol.) in 100 mM-imidazole/HCl buffer, pH 6.7, containing 5 mM-2-mercaptoethanol, 1 mg of bovine serum albumin/ml and substrate and effector concentrations as indicated. Samples of the enzyme (40 μ g/ml) were prepared in the appropriate buffers by dilution of a 0.45 mg/ml stock enzyme solution, which had been obtained by concentrating the purified enzyme in a collodion bag (Sartorius). Gradients were prepared and fractionated by using a peristaltic pump operated at a flow rate of 40 ml/h. Centrifugation was performed at 45500g for 6–7 h at 4°C in a Sorvall AH650 swing-out rotor in a Sorvall OTD-50B ultracentrifuge. Pig heart mitochondrial malate dehydrogenase (M_r 68000), pig muscle lactate dehydrogenase (M_r 142000), rabbit muscle pyruvate kinase (M_r 237000) and *Escherichia coli* β -galactosidase (M_r 520000) (50 μ g of each) were used as M_r standards. The standard marker enzymes were centrifuged under conditions identical with those for phosphofructokinase except that effectors of that enzyme were not present. The location of phosphofructokinase, which was run separately, was determined by assay at pH 8.0 as described above.

Treatment of kinetic data

Initial-rate data for the influence of substrates or effectors on the activity of phosphofructokinase were linearized by plotting the data in terms of the Hill (1910) equation. The values of V_{max} used to construct these plots were obtained by extrapolating the data, when plotted as double reciprocals or by the method of Dixon (1953), through the $1/v$ axis. Kinetic data for the variation of velocity with ATP concentration, which yielded linear double-reciprocal plots, were fitted to rectangular hyperbolae by using the computer program of Cleland (1967), which was translated into BASIC for use with a WANG 2200 computer by Dr. B. A. Orsi of this department.

Protein determinations

The concentration of purified enzyme protein was determined by using the Bio-Rad microassay procedure (Bio-Rad Technical Bulletin 78-0791), which is based on the method of Bradford (1976). All other protein determinations were performed by using the Lowry method. With either method lysozyme served as standard.

Purification of phosphofructokinase

All steps in the procedure were carried out at 4°C and could be performed within 1 day.

Step 1: subcellular fractionation. A subcellular fraction enriched in glycosomes was isolated from purified trypanosomes (2×10^{10} – 5×10^{10} cells) essentially as described by Oppendoes *et al.* (1977a). The purified cell suspension, obtained as described above, was centrifuged at 600g for 10 min. The supernatant was discarded, and the pellet was washed twice, by suspension and centrifugation, with 20 ml of 20 mM-Tris/HCl buffer, pH 7.6, containing 320 mM-sucrose, 1 mM-K₂EDTA and 20 µg of antipain/ml (29.5 µM) (homogenization buffer). An approximately equal volume of packed glass beads (type 1-W; Sigma), which had been washed twice with the same buffer, was mixed with the trypanosome pellet in a chilled mortar and ground for 3–4 min until a smooth paste was obtained.

The mixture was then suspended in 25 ml of homogenization buffer and centrifuged at 1000g for 10 min to remove the abrasive, whole cells, nuclei and cell debris. The supernatant was kept and the pellet was resuspended in 15 ml of homogenization buffer and centrifuged as before. The supernatants were combined and centrifuged at 14500g for 10 min. The glycosome-enriched pellet thus obtained was resuspended in 25 ml of 20 mM-Tris/HCl buffer, pH 8.0, containing 35% saturation (NH₄)₂SO₄ (5.225 g), 1 mM-K₂EDTA, 5 mM-MgCl₂, 5 mM-2-mercaptoethanol, 1% (w/v) Triton X-100 and 20 µg of antipain/ml.

Step 2: ammonium sulphate fractionation. The suspension from the previous step was homogenized in a tight-fitting Dounce homogenizer (20–30 strokes) and kept on ice for 15 min. The suspension was then centrifuged at 15000g for 30 min and the supernatant transferred to a plastic container.

Step 3: chromatography on ATP-agarose. The supernatant from step 2 was mixed with an equal volume of 20 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-MgCl₂, 5 mM-2-mercaptoethanol and 1 mM-K₂EDTA (equilibration buffer) and passed through an ATP-agarose column (2.8 cm × 2.0 cm diam.) that had been equilibrated with the same buffer. After sample application the column was washed successively with the following solutions: 50 ml of equilibration buffer, 5 ml of

equilibration buffer containing 5 mM-NADH and 5 mM-ATP, 40 ml of equilibration buffer and 50 ml of equilibration buffer containing 0.5 M-KCl, 0.15 mM-ADP and 0.15 mM-fructose 6-phosphate. Phosphofructokinase was then eluted with a linear gradient of KCl (0.5–3.5 M) prepared in equilibration buffer containing 0.15 mM-ADP and 0.15 mM-fructose 6-phosphate. Although the last two compounds were not essential for the elution of the enzyme, their presence resulted in a sharper peak of enzyme activity. All fractions (5 ml each) were collected in plastic vials.

The active peak fractions were pooled and concentrated in Visking tubing, surrounded by solid sucrose, to less than 10 ml. After this the enzyme was dialysed against two changes (500 ml each) of 50 mM-Tris/HCl buffer, pH 7.8, containing 60% (w/v) glycerol, 1 mM-K₂EDTA and 10 mM-dithiothreitol (storage buffer). The enzyme was concentrated further by this procedure, and when stored at –20°C lost about 13% of its activity per month. Concentration of phosphofructokinase by ultrafiltration on an Amicon PM-10 membrane [240 kPa (35 lbf/in²) above atmospheric pressure] resulted in appreciable losses of enzyme activity. Towards the end of the work it was found that concentration in collodion bags (Sartorius) allowed higher protein concentrations to be obtained without detectable losses of enzyme activity.

Results and discussion

Purification of phosphofructokinase

Table 1 summarizes a typical purification of phosphofructokinase from *T. brucei*. The enzyme eluted from ATP-agarose by the KCl gradient yielded a single protein-staining band when subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, as shown in Fig. 1. When fresh, the ATP-agarose could adsorb as much as 70 units of phosphofructokinase/ml. On continued use the capacity of this material decreased steadily, resulting in some of the applied activity being unretarded and, in some cases, a small peak of activity being eluted before the KCl gradient was applied. The regeneration procedure described by Ramadoss *et al.* (1976) did not result in a significant re-gain in capacity, and thus fresh batches of this affinity resin were used whenever the capacity declined below practicable levels (about 5 units/ml).

The specific activity of the enzyme purified by this procedure was in the range 140–180 units/mg, which was comparable with those of homogeneous phosphofructokinase preparations isolated from other sources (see Uyeda, 1979). The entire purification, starting with purified trypanosomes, could be performed in about 6 h.

Table 1. Purification of phosphofructokinase from *T. brucei*

The purification procedure and assay of phosphofructokinase at pH 8.0 were carried out as described in the text. One unit of enzyme activity is defined as the amount catalysing the formation of 1 μ mol of product in 1 min.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Homogenate	25	105.9	142.2	1.34	(—)	(100)
Glycosome fraction	25	10.2	51.6	5.06	3.8	36.3
35%-satn.-(NH ₄) ₂ SO ₄ supernatant	24	4.48	47.6	10.6	7.9	33.5
Post-ATP-agarose, concentrated and dialysed	7.0	0.095	15.56	164	122	10.9

Fig. 1. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of phosphofructokinase purified from *T. brucei*

Electrophoresis and staining were carried out as described in the text. Track 1 contained 10 μ g of the protein purified at pH 6.7 in the absence of antipain by the procedure described in the text. Track 2 contained 10 μ g of the protein purified at pH 8.0 in the presence of antipain. Track 3 contained 10 μ g each of bovine serum albumin, pyruvate kinase, glyceraldehyde-3-phosphate dehydrogenase and ovalbumin.

The purification of approximately 100-fold suggests that as much as 1% of *T. brucei* protein is present as phosphofructokinase. A similar value has been calculated for pyruvate kinase from this source (Flynn & Bowman, 1980). These values emphasize the dependence of the bloodstream form of *T. brucei* on glycolysis for its energy requirements.

The rather low yields of enzyme activity in the preparation of the glycosome fraction are similar to values obtained by Opperdooes & Borst (1977). Examination of the activities of other fractions indicated the remaining activity to be evenly distributed between the 1000g pellets and the 14500g supernatant, suggesting that it might represent unbroken cells and ruptured glycosomes respectively. If this were the case changes in the extent of grinding would be unlikely to increase the yield of glycosomes. Oduro *et al.* (1980a) compared a number of different methods for producing a glycosome-enriched fraction and concluded that the grinding procedure, similar to that used in the present work, was the most satisfactory. It is unlikely that the material lost in this step represents a phosphofructokinase with very different properties, since double-reciprocal plots of initial-velocity data with ATP (20–1000 μ M) or fructose 6-phosphate (67–4000 μ M) obtained at pH 8.0 with the crude homogenate showed no evidence of non-linearity and gave K_m values similar to those obtained with the purified enzyme.

Endogenous proteolysis

Initially, phosphofructokinase was purified in a similar manner to that described above with the exception that the Tris buffer used during steps 2 and 3 was replaced with a similar concentration of imidazole/HCl buffer, pH 6.7, and without the presence of antipain (an inhibitor of the cysteine group of proteinases; see Aoyagi, 1978). The enzyme purified in this fashion yielded two protein-staining bands on electrophoresis in the presence of sodium dodecyl sulphate, as shown in Fig. 1. The M_r values of these bands, estimated from a standard curve of $\log M_r$ versus R_f for the marker proteins shown in Fig. 1 were 49000 ± 180 (mean $\pm$ S.E.M., $n = 5$) and 47000 ± 150 (mean $\pm$ S.E.M., $n = 5$). After one particular preparation made by using this procedure, only the faster-migrating band was detected after electrophoresis. Since the specific activity of this single-band preparation was similar to that found for the 'double-

band' enzyme (and similar also to that found subsequently for material purified in the presence of proteolytic inhibitors at pH 8.0), it was evident that some form of interference with the enzyme was occurring during isolation that did not significantly affect its activity under these conditions.

All attempts at electrophoresis of phosphofructokinase in the absence of a detergent, as described in the Methods section, failed. After electrophoresis and staining, protein could be observed on the top surface of each gel. The failure of phosphofructokinase to enter polyacrylamide gels under non-denaturing conditions has been reported previously for the rabbit liver enzyme (Kemp, 1971).

Although it was found that the modification could be largely prevented by including AMP (2mM), ADP (3mM) and fructose 6-phosphate (3mM) in the glycosome buffer, subsequent work showed that the interference could be completely avoided by including the proteolytic inhibitors leupeptin, chymostatin, pepstatin A, aprotinin and antipain (each at 20 µg/ml) together with 0.2mM-phenylmethanesulphonyl fluoride in the buffers of steps 1 and 2. Eventually it was found that antipain alone could prevent the modification, whereas pepstatin A or phenylmethanesulphonyl fluoride could not. The effectiveness of the remaining inhibitors, individually, to prevent the proteolysis was not tested.

When phosphofructokinase was purified at pH 8.0 in the absence of antipain, it was found that the proteolysis was retarded, as might be expected from the pH optimum of the cysteine class of proteinases (see Kay, 1982). Nevertheless, as an additional precaution, antipain was included routinely during the purification procedure at pH 8.0. The single protein-staining band from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of phosphofructokinase obtained by the pH 8.0 procedure was unchanged during storage,

indicating that the proteinase activity responsible for the alteration was absent from the purified enzyme preparation. Cysteine-proteinase activities have been identified in *T. congolese* (Rautenberg *et al.*, 1982) and *T. cruzi* (Rangel *et al.*, 1981).

Relative molecular mass

The M_r of purified phosphofructokinase was estimated by subjecting the enzyme (10 µg) to ultracentrifugation on linear glycerol gradients under the conditions listed in Table 2. After each run, the enzyme activity was detected as a single peak and the M_r of the enzyme was estimated from a plot of $\log M_r$ versus elution volume obtained for a number of marker proteins. Under all conditions the M_r was similar (Table 2), and the average value obtained from the data of 222000 ± 4000 (mean $\pm$ S.E.M., $n = 9$) suggested the enzyme to be tetrameric, in agreement with the oligomeric structure found for the majority of preparations of this enzyme from other sources (see Uyeda, 1979). A quite similar value for the M_r of the enzyme from *T. (T.) brucei rhodesiense* has been reported by Jaffe *et al.* (1971). The M_r value of the subunit determined in the present work was, however, smaller than that of about 80000 reported for phosphofructokinase from eukaryotes and higher than the value of about 35000 reported for the enzyme from prokaryotes (see Uyeda, 1979).

Effect of enzyme concentration

The effect of enzyme concentration on the activity of purified phosphofructokinase (with a specific activity of 152 units/mg in the pH 8.0 assay) at pH 6.7 and at a low concentration of fructose 6-phosphate, in the presence or in the absence of AMP (activator) or phosphoenolpyruvate (inhibitor), is shown in Fig. 2. Although, in the absence of added effectors, the initial velocity was proportional to the enzyme concentration over most of the studied range, this relationship was not maintained at low enzyme concentrations. The

Table 2. Determination of the M_r of phosphofructokinase purified from *T. brucei*
Samples (10 µg) of the enzyme were subjected to glycerol-gradient centrifugation at 45 500g for 6–7 h as described in the text in the presence of the substrate and effectors shown below.

Conditions	M_r	Recovery (%)
Control	210 000	48
100 µM-ATP, 10 mM-fructose 6-phosphate	220 000	48
10 mM-Phosphoenolpyruvate	225 000	66
1 mM-AMP	205 000	52
5 mM-MgSO ₄ , 50 µM-ATP, 0.5 mM-fructose 6-phosphate	240 000	71
Control	240 000	19
5 mM-MgSO ₄ , 100 µM-ATP, 1 mM-fructose 6-phosphate	225 000	28
10 mM-MgSO ₄	220 000	46
0.1 mM-MgSO ₄	215 000	18

Fig. 2. Effects of enzyme concentration on the activity of *T. brucei* phosphofructokinase assayed in imidazole buffer, pH 6.7. Activity was determined by the coupled assay for the formation of fructose 1,6-bisphosphate, as described in the text, in the presence of 0.25 mM-fructose 6-phosphate and 0.2 mM-ATP (●) plus 2 mM-AMP (○) or 2 mM-phosphoenolpyruvate (Δ). The data shown within the dashed lines of (a) are shown in enlarged form in (b).

linearity at higher enzyme activities used and in the presence of AMP together with the excess of coupling enzyme activities used, in relation to their kinetic parameters, are not consistent with this behaviour being due to failure to estimate the true steady-state velocities of the reaction (for discussion see Tipton, 1978). This type of behaviour, which has been observed previously with a number of phosphofructokinase preparations from eukaryotes (Underwood & Newsholme, 1965; Hulme & Tipton, 1971; Leaver & Burt, 1981), is consistent with a dissociation of the enzyme as its concentration falls.

In the presence of AMP or phosphoenolpyruvate the dependence of velocity on enzyme concentration was essentially linear, although, in the latter case, the curve did not pass through the origin, suggesting some degree of dissociation at very low protein concentrations. The behaviour of bovine heart and rat liver phosphofructokinase (Hulme & Tipton, 1971; Reinhart & Lardy, 1980) was consistent with allosteric activators and inhibitors showing a preference for different polymeric states of the enzyme. However, such a model might not account adequately for the behaviour of the *T. brucei* enzyme, since there was no indication that, in the presence of phosphoenolpyruvate, the curve of the plot of velocity versus enzyme concentration would rise to parallel the control curve at high enzyme concentrations.

In the absence of added effectors a sigmoidal dependence of velocity on fructose 6-phosphate concentration was obtained at all enzyme concentrations studied (0.002–0.1 μg/ml). The Hill co-

Table 3. Effects of enzyme concentration on the kinetics of fructose 6-phosphate phosphorylation by phosphofructokinase from *T. brucei*

Enzyme activity was determined by using the coupled assay for fructose 1,6-bisphosphate formation. $[\text{Fru-6-P}]_{0.5}$ is the concentration of fructose 6-phosphate required to give half-maximum velocity, and h is the Hill constant.

Concn. of enzyme (μg/ml)	$V_{\max.}$ (units/mg)	$[\text{Fru-6-P}]_{0.5}$ (mM)	h
0.100	267	0.82	1.2
0.010	267	1.00	1.3
0.005	267	1.26	1.3
0.002	267	1.91	1.4

efficient (h) obtained from the fructose 6-phosphate saturation curves decreased with increasing enzyme concentrations (Table 3), an observation reported also for the enzyme from pig spleen (Hickman & Weidemann, 1975). At each concentration of the *T. brucei* enzyme, the maximum velocity, when expressed as specific activity, was similar (Table 3), indicating the velocity to be linearly dependent on enzyme concentration at saturating concentrations of fructose 6-phosphate. To avoid the complications of apparent enzyme dissociation at low enzyme concentrations, subsequent kinetic studies were performed with an enzyme concentration of 0.05 μg/ml, which was well within the linear portion of the control curve shown in Fig. 2.

Effect of substrate concentration

The effects of varying the fructose 6-phosphate concentration at different fixed concentrations of ATP are shown as Hill plots in Fig. 3. It can be seen that, although the concentration required to give half-maximum velocity, $[Fru-6-P]_{0.5}$, was dependent on the concentration of ATP, the degree of apparent co-operativity was not. When ATP was expressed as the variable substrate the data, from the same experiment, yielded linear double-reciprocal plots (see inset to Fig. 3), indicating Michaelis-Menten kinetics to be obeyed by the nucleotide substrate. The apparent K_m for ATP was dependent on the concentration of fructose 6-phosphate, and values of 13.0, 16.5, 22.5 and 26.2 μM respectively were obtained at fructose 6-

Fig. 3. Effects of substrate concentrations on the rate of the reaction catalysed by phosphofructokinase from *T. brucei*. Enzyme activity was determined at pH 6.7 by the coupled assay for ADP formation as described in the text. The concentrations of total ATP ($[ATP]_T$) were 25 μM ($\square$), 50 μM ($\blacksquare$), 100 μM ($\circ$) and 500 μM ($\bullet$) and the data are presented as a Hill (1910) plot. The value of the Hill constant was found to be 1.2 in each case. Inset: double-reciprocal plot of the data with ATP as the variable substrate. The fructose 6-phosphate concentrations ($[Fru-6-P]$) were 0.25 mM ($\square$), 0.4 mM ($\blacksquare$), 0.75 mM ($\circ$) and 2.0 mM ($\bullet$).

phosphate concentrations of 0.25, 0.4, 0.75 and 2.0 mM.

Inhibition of phosphofructokinase

Phosphoenolpyruvate behaved as an inhibitor of *T. brucei* phosphofructokinase, as shown in Fig. 4. These data show the effects of phosphoenolpyruvate and fructose 6-phosphate to be complementary. Raising the concentration of either one of these ligands caused an increase in the co-operativity and a decrease in the apparent affinity of the enzyme towards the other. The data indicate also that, when the concentration of fructose 6-phosphate was varied, phosphoenolpyruvate did not alter the extrapolated value of the maximum velocity.

This behaviour, together with that observed in the presence of substrates alone, would appear to be consistent, qualitatively, with the theory of a two-state concerted K-system as postulated by Monod *et al.* (1965), which has been proposed to account for the behaviour of this enzyme from other sources (see, e.g., Blangy *et al.*, 1968; Goldhammer & Hammes, 1978; Pettigrew & Frieden, 1979). In the present case, inhibitors, such as phosphoenolpyruvate, would preferentially bind to an inactive or less active T-state, whereas the substrate, fructose 6-phosphate, would show a preference for a more active R-state. ATP would exhibit equal affinity towards either form. It is implied here that the observed behaviour results from the manifestations of a number of different tetrameric enzyme species and that the interrelationships found are not complicated by enzyme dissociation (see above). Although the ultracentrifugation studies described above would appear to support this contention, these results may not necessarily validate such an interpretation, because of the much lower enzyme concentrations used for the kinetic studies.

Citrate, an allosteric inhibitor of phosphofructokinase from many sources (see Uyeda, 1979), had only a weak effect on the activity of the *T. brucei* enzyme. In the presence of 5 mM-citrate and 1 mM-ATP, the value of $[Fru-6-P]_{0.5}$ was increased from 0.93 to 1.41 mM, with no significant effect on the maximum velocity or the degree of co-operativity. This finding may reflect the fact that the bloodstream-form trypanosomes do not possess a functional tricarboxylic acid cycle (see Bowman & Flynn, 1976; Flynn & Bowman, 1973).

Activation of phosphofructokinase

AMP, cyclic AMP and ADP were found to increase the activity of *T. brucei* phosphofructokinase when the concentration of fructose 6-phos-

Fig. 4. Inhibition of phosphofructokinase from *T. brucei* by phosphoenolpyruvate

Activity was determined at pH 6.7 by the coupled assay for fructose 1,6-bisphosphate formation (a) or ADP formation (b) in the presence of 1 mM-ATP. The results are presented as Hill (1910) plots. (a) The concentration of fructose 6-phosphate ([Fru-6-P]) was as indicated, the concentrations of phosphoenolpyruvate were 0 (●), 2 mM (○), 5 mM (■) and 10 mM (□), and the concentration of MgSO_4 was 15 mM. $V_{\max}$, the maximum velocity in the presence of a saturating concentration of fructose 6-phosphate, was 295 units/mg. (b) The concentration of phosphoenolpyruvate ([Pyr-P]) was as indicated, the concentrations of fructose 6-phosphate were 0.71 mM (□), 2.0 mM (■), 5.0 mM (○) and 10.0 mM (●), and the concentration of MgSO_4 was 20 mM. $V_{\max}$ for each line was the velocity determined in the absence of phosphoenolpyruvate. The value of the Hill constant for each set of data is shown.

phate was low (< 0.5 mM and with 1 mM-ATP). The results obtained, with the Fru-1,6- P_2 -coupled assay, when each activator concentration was varied separately in the presence of 0.25 mM-fructose 6-phosphate and 1 mM-ATP (results not shown) indicated that all three nucleotides bound co-operatively, when plots of $\log[v - v_0/V_{\max} - (v - v_0)]$ versus $\log[\text{nucleotide}]$ were constructed [v_0 being the velocity in the absence of activator and $V_{\max}$ was the maximum value of $v - v_0$ obtained from a graph of $1/(v - v_0)$ versus the reciprocal of the nucleotide concentration]. Under these conditions the nucleotide concentrations required to give half-maximal activation were 0.40, 0.64 and 1.80 mM for cyclic AMP, AMP and ADP respectively. The rather high value of the constant obtained for cyclic AMP, relative to the concentration of this nucleotide *in vivo* (see Mancini & Patton, 1981), lends support to the arguments advanced by Sols (1981) that this compound may be acting as an analogue of AMP in this case. At high concentrations of AMP or ADP, Hill plots, when the concentration of fructose 6-phosphate was

varied, had slopes of unity and the value of $[\text{Fru-6-P}]_{0.5}$ was decreased 2–3-fold (results not shown). In terms of the concerted model discussed above, the simplest interpretation of these results would be to suggest that activators bound to the R-enzyme state. However, when the concentration of fructose 6-phosphate was raised above 0.5 mM, ADP and AMP were inhibitory. The results obtained for AMP are shown in Fig. 5. The decrease of $V_{\max}$ of *E. coli* phosphofructokinase by ADP was proposed to result from a competitive interaction between the activator and ATP at the substrate-binding site (Blangy *et al.*, 1968). In the present study, after an initial activatory effect was overcome, a competitive-type of inhibition pattern was obtained when the concentration of ATP was varied at different fixed concentrations of AMP, and at a constant (0.5 mM) concentration of fructose 6-phosphate (results not shown; Fru-1,6- P_2 -coupled assay). However, the extent of inhibition of $V_{\max}$ with respect to fructose 6-phosphate by different concentrations of AMP did not appear to result from inhibition at the ATP substrate-

Fig. 5. Effects of AMP on the activity of phosphofructokinase from *T. brucei*

Activity was determined at pH 6.7 by the coupled assay for the formation of fructose 1,6-bisphosphate in the presence of 1 mM-ATP, various concentrations of fructose 6-phosphate ([Fru-6-P]) and the following fixed concentrations of AMP: 0 (●), 0.25 mM (○), 1.0 mM (■), 2.0 mM (□) and 3.0 mM (△). For clarity not all the experimental points are shown. Inset: dependence of the apparent maximum velocity (V_m) on the concentration of AMP.

binding site alone, since the former inhibition was clearly partial in nature (see inset to Fig. 5), whereas a slope replot of the AMP versus ATP data was linear (not shown). Nwagwu & Oppenheimer (1982) did not observe inhibition of this enzyme by AMP at high concentrations of fructose 6-phosphate. The reason for this discrepancy, which might be related to differences in the assay conditions or to the crude enzyme preparation used by these workers, is unclear.

To investigate further this behaviour, the effect of a series of fixed concentrations of ADP on the maximum velocity was examined. The results of this study are shown in Fig. 6. The value of $V_m^{\max}$ (see the legend to Fig. 6) corresponded to 32% inhibition of the maximum enzyme activity in the absence of ADP. This value was in good agreement with the value of 31% obtained from Fig. 5 for the AMP inhibition data, suggesting that both nucleotides influenced the enzyme activity in a similar manner.

For the same reasons discussed above in connection with AMP, the partial nature of the inhibition

produced by ADP was not consistent with a purely competitive displacement of ATP from the substrate-binding site. Furthermore, if ADP were considered to activate by promoting the predominance of the R-form of the enzyme, i.e. the form preferred by fructose 6-phosphate, it would not be expected that ADP should bind co-operatively at saturating concentrations of this substrate, although, as shown in Fig. 6, this appeared to be the case. The activation by nucleotides might be better understood if these compounds were considered to induce a conformational change in the enzyme structure, resulting in an enzyme form that, although having a lower maximum velocity with respect to the R-form, showed a greater affinity towards fructose 6-phosphate. The existence of such a third form has been suggested to account for the behaviour of this enzyme from rabbit muscle (Kemp & Foe, 1983) and yeast (Laurent *et al.*, 1984). However, the behaviour of the *T. brucei* enzyme is complicated further by the observation that, when $MgSO_4$ was replaced by $MnCl_2$ (10 mM), the Hill coefficient for fructose 6-phosphate in the presence of 1 mM-AMP and 1 mM-ATP was 0.9 (C. N. Cronin & K. F. Tipton, unpublished work), suggesting that, under these conditions, fructose 6-phosphate was bound with negative cooperativity. Although additional evidence would be required to show that this behaviour reflected substrate binding, a concerted model of cooperativity could not account for these observations, and a more elaborate model of allostery, such as that proposed by Koshland *et al.* (1966), would be necessary.

Fructose 2,6-bisphosphate has been found to be a potent stimulator of phosphofructokinase from a number of sources (Van Schaftingen *et al.*, 1980, 1982; Hers & Van Schaftingen, 1982; Pilkis *et al.*, 1982). However, as with phosphofructokinase from *E. coli* and plant cytosol (see Hers *et al.*, 1982), fructose 2,6-bisphosphate was without effect on the *T. brucei* enzyme. When present at concentrations up to 200 μ M, this compound had no significant effect, irrespective of the concentration of fructose 6-phosphate or in the presence of inhibitory concentrations (5 mM) of phosphoenolpyruvate, on the enzyme activity determined by the ADP-coupled assay. In addition, fructose 1,6-bisphosphate, which has been found to activate the enzyme from many sources (see Uyeda, 1979), had no effect on the activity of the *T. brucei* enzyme, when present at a concentration of 2 mM, under similar conditions to those described above for fructose 2,6-bisphosphate. Inorganic phosphate had little influence on the values of $[Fru-6-P]_{0.5}$, $V_m^{\max}$, or the Hill coefficient when present at low concentrations (<5 mM; activity determined by the Fru-1,6- P_2 -coupled assay), but it completely

Fig. 6. Effects of ADP on the maximum velocity of the reaction catalysed by phosphofructokinase from *T. brucei*. Activity was determined at pH 6.7 by the coupled assay for fructose 1,6-bisphosphate formation, in the presence of 1 mM-ATP and various concentrations of fructose 6-phosphate and ADP. (a) Maximum velocities plotted against the ADP concentration. (b) Data presented as a Hill (1910) plots, where ΔV_m is the change in the maximum velocity caused by a fixed concentration of ADP and $\Delta V_m^{\max}$ is the maximum change in this value, obtained from a plot of $1/\Delta V_m$ versus $1/[ADP]$. This value was found to be 91 units/mg, which corresponded to 32% inhibition.

abolished the co-operativity towards fructose 6-phosphate when present at a concentration of 50 mM (phosphate/KOH buffer, pH 6.7, replacing imidazole HCl buffer), again without greatly influencing the values of $[Fru-6-P]_{0.5}$ or $V_{\max}$.

These studies show that the phosphofructokinase from the bloodstream form of *T. brucei* is insensitive to many compounds that are effectors of this enzyme from other sources (Sols, 1981). This may reflect the specialized metabolism of this organism as well as the glycosomal localization of the enzyme. In view of the differences in energy metabolism during different stages of the life-cycle of the trypanosome, it would be particularly interesting to investigate whether these are reflected in changes in the regulatory properties of phosphofructokinase.

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