Phosphofructokinase from *Plasmodium berghei*

Influence of Mg^{2+} , ATP and Mg^{2+} -complexed ATP*

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The control enzyme phosphofructokinase is of regulatory significance in the metabolism of glucose by the malarial parasite *Plasmodium berghei*. (1) The enzyme was partially purified from erythrocytic stages of *P. berghei* by precipitation with poly(ethylene glycol) and chromatography on 2',5'-bisphosphoadenosine–Sephadex 4B. (2) Similarly to various other phosphofructokinases, the enzyme from *P. berghei* shows an allosteric behaviour. It is activated by fructose 6-phosphate and inhibited by ATP. (3) The effects of Mg^{2+} -complexed ATP, free ATP and Mg^{2+} were studied by keeping constant the concentration of one of these and varying the concentrations of the other two. (4) The enzyme is shown to be allosterically inhibited by free ATP and by higher concentrations of Mg^{2+} . Compared with phosphofructokinase of erythrocytes, inhibition by ATP is weaker by two orders of magnitude. Mg^{2+} -complexed ATP has no effect on allosteric regulation. (5) The proposed kinetic model provides an adequate description of the data.

INTRODUCTION

The enzyme phosphofructokinase (PFK, EC 2.7.1.11) is one of the most important control enzymes in glucose metabolism [1–3]. The enzyme of *Plasmodium berghei* has been shown to differ in its kinetic behaviour from that of many other species [4,5]. These differences reflect the adaptation of *P. berghei* to an exclusively anaerobic utilization of glucose, which results in a 10–30-fold increased glycolytic flux of infected erythrocytes [6].

Malarial PFK shows allosteric properties, although it differs from the mammalian enzyme with respect to a number of allosteric effectors. *P. berghei* PFK is activated by fructose 6-phosphate and inhibited by higher concentrations of ATP, but this inhibition is weaker by two orders of magnitude compared with PFK of erythrocytes. The shift of the pH-dependence to the acidic range renders a high glycolytic flux possible at the low pH values occurring in parasitaemia [7], where glycolysis by the erythrocytes is strongly inhibited [8].

In order to elucidate the interaction between ATP and the enzyme it is necessary to investigate the influence of Mg^{2+} -complexed ATP on PFK activity [9], which has not been done hitherto. In the present paper results are presented concerning the effect of Mg^{2+} , of Mg^{2+} -complexed ATP ($MgATP$) and of free non- Mg^{2+} -complexed ATP (ATP_{free}) on partially purified PFK from *P. berghei*.

MATERIALS AND METHODS

Materials

Poly(ethylene glycol) 6000 was purchased from Serva (Heidelberg, Federal Republic of Germany), 2',5'-bisphosphoadenosine–Sephadex 4B was from Pharmacia (Uppsala, Sweden) and the auxiliary enzymes and all other fine chemicals were from Boehringer (Mannheim, Federal Republic of Germany). The ATP used was the disodium salt, also from Boehringer, containing less than 0.5% ADP and AMP.

Preparation of the enzyme

PFK was partially purified from the stroma-free lysate of parasite cells that had been obtained as described in ref. [5].

In the first step the enzyme was fractionally precipitated by poly(ethylene glycol). The stroma-free lysate was adjusted to pH 6.0 by addition of the same volume of 50 mM-potassium phosphate buffer. PFK was precipitated by addition of solid poly(ethylene glycol) at a total concentration of 5% (w/w). After 30 min the precipitated protein was separated by centrifugation at 15000 g for 10 min and resuspended in 50 mM-potassium phosphate buffer, pH 6.0. To remove non-soluble material the suspension was centrifuged once more (15000 g for 10 min).

In the second step the enzyme was purified by chromatography on 2',5'-bisphosphoadenosine–Sephadex 4B. After binding of the PFK on a column (1.2 cm × 10 cm) equilibrated with 0.1 mM-potassium phosphate buffer, pH 6.0, and successive washing with 20 ml of 0.1 M-potassium phosphate buffer, pH 6.0, 20 ml of 0.1 M-potassium phosphate buffer, pH 8.0, and 10 ml of 0.1 M-Tris/HCl buffer, pH 8.0, the enzyme was eluted by increasing the ionic strength (flow rate 20 ml/h, linear gradient of 0–0.15 M-KCl in 0.1 M-Tris/HCl buffer, pH 8.0). All buffers also contained 1.0 mM-EDTA, 1.0 mM-6-aminohexanoic acid and 0.3 mM-2-mercaptoethanol. All preparations were carried out at 4 °C.

Protein concentration was determined by the method of Lowry *et al.* [10].

Assay of the enzyme activities

The activity of PFK was measured with an Eppendorf photometer at 366 nm with an assay mixture containing 0.2 mM-NADH, 1.0 mM- $MgCl_2$, 90 mM-KCl, 1.0 mM- K_2HPO_4 , 8.0 µg of fructose-bisphosphate aldolase, 3.3 µg of triose-phosphate isomerase and 3.3 µg of glycerol-3-phosphate dehydrogenase in 80 mM-Tris/HCl buffer, pH 6.8, in a total volume of 0.5 ml at 37 °C. The concentrations of the substrates varied in the ranges 0.03–3.0 mM-fructose 6-phosphate and 0.04–4.8 mM-ATP.

Abbreviation used: PFK, phosphofructokinase.

* Dedicated to Professor Dr. sc. nat. E. Hofmann on his 60th birthday.

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Within the ranges of PFK activity used the rate of reaction was directly proportional to the concentration of the enzyme.

The activity of pyruvate kinase was recorded with an assay mixture containing 1.9 mM-ADP, 1.0 mM-phosphoenolpyruvate, 0.5 mM-NADH, 19 mM-MgSO₄, 50 mM-KCl and 6.2 µg of lactate dehydrogenase in 50 mM-triethylamine/HCl buffer, pH 7.6, in a total volume of 0.5 ml at 37 °C.

Calculation of the dissociation equilibria

To calculate the concentrations of Mg²⁺, Mg²⁺-complexed ATP and free ATP the following dissociation equilibria were considered:

For simplicity the following notation is used:

MgATP is the sum of all Mg²⁺-containing complexes of ATP, i.e.:

$$[\text{MgATP}] = [\text{MgATP}^{2-}] + [\text{MgHATP}^-] + [\text{MgH}_2\text{ATP}] + [\text{Mg}_2\text{ATP}]$$

ATP_{free} is the sum of all species not complexed with Mg²⁺, i.e.:

$$[\text{ATP}_{\text{free}}] = [\text{ATP}^{4-}] + [\text{HATP}^{3-}] + [\text{H}_2\text{ATP}^{2-}] + [\text{NaATP}^{3-}] + [\text{KATP}^{3-}]$$

The concentrations of all species were calculated from the total concentrations of ATP (ATP_T), Mg²⁺ (Mg_T), Na⁺ and K⁺ and the pH value by using a Newton procedure [14] for the solution of implicit equations. The program, which was written in BASIC, also allows the calculation of the total concentrations of ATP and Mg²⁺ required to obtain given values of [Mg²⁺], [MgATP] or [ATP_{free}]. It allows the concentration of one species to be kept constant while varying the concentrations of the others at a given pH.

Determination of the kinetic parameters

For the determination of the kinetic parameters a non-linear-regression procedure based on a Gauss-Newton algorithm was used [15].

The program NLREG [16] allows one subsequently to fit several possible models to the data and to estimate the parameters with the standard errors (S.E.M.). To obtain maximum-likelihood estimates the data were weighted with the reciprocals of the squares of the standard deviation. It includes visual inspection of the residuals as proposed by Atkins [17] as well as statistical methods for the discrimination between models and testing them for adequacy [18]. We applied non-parametric tests (runs test, signs test and Wilcoxon test), parametric tests (χ² test and *F* test for nested models) and two asymptotic procedures proposed recently [19,20], the first for assessment of the accuracy of a model (*U_k*) and the second for discrimination between alternative models (*T_{kl}*):

$$U_k = \frac{N}{2} \left(\frac{SQ_k}{N} - 1 \right) \quad (1)$$

$$T_{kl} = \frac{N}{4} \left(\frac{SQ_k}{N} - \frac{SQ_l}{N} \right) \left(\frac{1}{N} \sum_i s_i^{-2} (v_{ik} - v_{il})^2 \right)^{-\frac{1}{2}} \quad (2)$$

where *N* is the number of data, *SQ_k* is the sum of weighted least squares of model *k*, *s_i* is the standard deviation of the experimental error of the *i*th measurement and *v_{ik}* is the rate law according to model *k*, *i*th measurement.

A model is accepted to be adequate whenever *U_k* < *u_{1-α}* (1-α quantile of the standard normal distribution). Two models *k* and *l* are regarded to differ significantly if |*T_{kl}*| > *t_{1-α/2}* (1-α/2 quantile of Student's *t* distribution).

The experimental data used for modelling are derived from at least two repetitions. From these and additional investigations we have estimated an experimental error of constant standard deviation of 0.035 *V_{max}*.

RESULTS

The results obtained during the partial purification of *P. berghei* PFK are shown in Table 1. Precipitation of the enzyme by poly(ethylene glycol) is an attractive alternative to (NH₄)₂SO₄ precipitation in many cases in view of its denaturation-preventing effect and the high rate of recovery of enzyme that can be achieved [21,22]. Here it was used in order to remove most of the protein, especially haemoglobin and its degradation products as well as pyruvate kinase and other enzymes.

Since gels commonly used for the purification of PFK (e.g. Cibacron Blue-Sepharose and ATP-Sepharose) failed in the case of PFK from *P. berghei* or were not efficient enough (e.g. DEAE-Sephadex and CM-Sephadex), other chromatographic media were tested (e.g. AMP-Sepharose, phenyl-Sepharose, Procion Yellow HE-3G-Sepharose). The best results were obtained with 2',5'-bisphosphoadenosine-Sepharose 4B.

Table 1. Purification of PFK from *P. berghei* by precipitation with poly(ethylene glycol) and chromatography on 2',5'-bisphosphoadenosine-Sepharose 4B

For experimental details see the Materials and methods section.

Step	Volume (ml)	Activity (nkat)	Specific activity (nkat/mg)	Yield (%)	Purification (fold)	PFK/pyruvate kinase activity ratio
Stroma-free lysate	6.0	278	0.512	100	1	0.33
Poly(ethylene glycol) precipitation						
Supernatant	12.0	1.9	0.004			0.03
Precipitate	5.0	238	3.35	86	6.6	1.25
2',5'-Bisphosphoadenosine-Sepharose 4B chromatography						
Washing, pH 6.0	10.0	2.2	0.145			0.02
Washing, pH 8.0	10.0	34.0	0.82	12.2		1.0
Elution, pH 8.0, with KCl gradient	2.0	109	428	39	837	100

Fig. 1. Dependence of the activity of *P. berghei* PFK on $[ATP_T]$ at several fixed values of [fructose 6-phosphate]

[Fructose 6-phosphate] was 0.1 mM (■), 0.5 mM (▲) or 1.0 mM (●). $[Mg_T]$ was 1.0 mM. The lines correspond to the fit of eqn. (3) to the data.

A preparation with a specific activity of 428 nkat/mg of protein was obtained that was free of activities affecting the kinetic measurements and that was sufficient for the kinetic experiments.

Fig. 1 shows the dependence of PFK activity on the concentration of total ATP. Although the activity is increased by higher concentrations of fructose 6-phosphate (Fru6P), the biphasic effect of ATP remains unchanged.

The data can adequately be described by the following rate law:

$$v = \frac{V_{\max} [MgATP] [Fru6P]}{([MgATP] + K_{ma}) ([Fru6P] + K_i)} \cdot \frac{1}{1 + L_0 \left(\frac{1 + \frac{[ATP_T]}{K_a}}{1 + \frac{[Fru6P]}{K_{i1}}} \right)^n} \quad (3)$$

where $V_{\max}$ is the maximum activity, K_{ma} and K_i are the Michaelis constants for MgATP and fructose 6-phosphate, L_0 is the equilibrium constant for the transition between the R state and the T state, n is the number of subunits (this value had not been fixed but was estimated together with the other parameters), K_a is the inhibition constant for ATP and K_{i1} is the activation constant for fructose 6-phosphate. (Kinetic nomenclature is in accordance with Cleland [23] and Monod *et al.* [24].)

The goodness-of-fit was assessed by the tests described above.

Fig. 2. Dependence of the activity of *P. berghei* PFK on $[ATP_T]$ at several fixed values of $[Mg_T]$

$[Mg_T]$ was 0.1 mM (■), 1.0 mM (●) or 10 mM (▲). [Fructose 6-phosphate] was 1.0 mM. The lines correspond to the fit of eqn. (4) to the data.

Fig. 3. Dependence of the activity of *P. berghei* PFK on $[MgATP]$ (a) and on $[ATP_{free}]$ (b) at several fixed values of $[Mg^{2+}]$

$[Mg^{2+}]$ was 0.03 mM (▼), 0.1 mM (▲), 0.3 mM (●), 0.7 mM (○), 2.0 mM (△), 8.0 mM (▽) or 16 mM (□). [Fructose 6-phosphate] was 1.0 mM. The values of $[Mg_T]$ and $[ATP_T]$ were chosen appropriately to fulfil these conditions. The lines correspond to the fit of eqn. (4) to the data.

The residuals were normally distributed without a trend, either with respect to the concentrations or with the measured activity. None of the applied tests gave indication to reject the model, and the value of $U_k = 1.42 < u_{0.95}$ directly supports the proposed rate law.

The ATP-dependence of malarial PFK for $[Mg_T]$ values 0.1 mM, 1.0 mM and 10 mM is shown in Fig. 2. Obviously, enzyme activity decreases at low as well as at high total concentrations of Mg^{2+} . These findings might be due to different mechanisms, and a decision in favour of a certain mode of allosteric regulation is impossible.

In an attempt to resolve these possibilities the concentration of free Mg^{2+} was kept constant at several values, whereas the concentrations of ATP_{free} and MgATP were varied. In other experiments the concentrations of MgATP and ATP_{free} respectively were maintained. The total concentrations of ATP and Mg^{2+} required to obtain these conditions were determined as described above. Fig. 3(a) shows the dependence of the activity of *P. berghei* PFK on $[MgATP]$ at fixed concentrations of free Mg^{2+} . At $[Mg^{2+}] \leq 2$ mM no inhibition occurs at lower concentrations (≤ 0.3 mM) of MgATP. At higher values of $[MgATP]$ inhibition is due to increasing concentrations of free ATP. In the presence of high concentrations of free Mg^{2+} (8 mM and 16 mM) the enzyme is inhibited although the concentration of ATP_{free} is negligible. Thus PFK from *P. berghei* is inhibited by both free ATP and Mg^{2+} . At this stage no indication was found for an inhibitory effect of MgATP. These effects are also seen in Fig. 3(b), which shows the dependence of the enzyme on $[ATP_{free}]$. As $[Mg^{2+}]$ is increased the enzyme is increasingly inhibited at fixed concentrations of free ATP. Since MgATP does not inhibit the enzyme, the decrease in activity is due to Mg^{2+} itself.

Although the proposed actions of Mg^{2+} , ATP_{free} and MgATP

Table 2. Kinetic parameters of PFK from *P. berghei* according to the proposed model (eqn. 4)Values of the parameters are given $\pm$ S.E.M.

Parameter	Value
K_t	0.032 ± 0.004 mM
K_{t1}	0.59 ± 0.08 mM
K_{ma}	0.020 ± 0.003 mM
K_a	0.72 ± 0.04 mM
K_m	0.78 ± 0.06 mM
L_0	4.10 ± 0.37
n	4 ± 0.12

seem to be probably correct, this model has to be shown to provide an adequate description of the data.

Therefore this and a number of other possible Monod–Wyman–Changeux models were fitted to the data and involved in model discrimination. The best representation of the experimental data was provided by a model assuming MgATP to be the substrate without any allosteric effect and free ATP and Mg^{2+} being allosteric inhibitors with separate binding sites. Fructose 6-phosphate (Fru6P) is a substrate and a positive effector. The rate equation reads as:

$$v = \frac{V_{\max} [MgATP] [Fru6P]}{([MgATP] + K_{ma}) ([Fru6P] + K_t) (1 + L)} \quad (4)$$

where

$$L = L_0 \left(\frac{\left(1 + \frac{[ATP_{free}]}{K_a}\right) \left(1 + \frac{[Mg^{2+}]}{K_m}\right)}{1 + \frac{[Fru6P]}{K_{t1}}} \right)^n$$

The estimated values of the kinetic parameters are given in Table 2. The model provides an adequate description of all the data (126 points). The following models could be rejected.

Model 1: both inhibitors are bound to the same site. Free ATP and Mg^{2+} would have a competitive effect.

Model 2: Mg^{2+} ions are bound to the R state and the T state (according to the Monod–Wyman–Changeux model) with different affinities. Free ATP would only have an apparent inhibitory effect.

Model 3: competitive inhibition by free ATP and MgATP but not by Mg^{2+} . In this case Mg^{2+} would only have an apparent effect by shifting the complexing of the nucleotide.

Model 4: the same, but non-competitive inhibition.

If more complex models were fitted to the data, including the favoured one as a submodel (nested models), no significant increase in the goodness of fit was achieved. Such models were as follows.

Model 5: in addition to the favoured model, MgATP is bound to the T state (competitively towards free ATP).

Model 6: the same, but non-competitively towards free ATP.

Model 7: effects of Mg^{2+} and MgATP on the R state and the T state of the enzyme.

The results of discrimination and model testing are shown in Table 3.

DISCUSSION

The existence of a parasite-specific PFK in *Plasmodium* sp. was first demonstrated by Kruckeberg and co-workers [4] and it has been partially characterized with respect to its kinetic properties in a previous paper [5]. The effects of free and Mg^{2+} -complexed ATP could not be differentiated, and the measurements were carried out with a crude preparation of PFK.

These results (not shown) have been confirmed for a purified enzyme. The agreement between the kinetic properties of the purified and those of the non-purified enzyme suggests that no kinetically relevant proteolytic modifications occurred during purification.

The binding of the substrate MgATP to PFKs from different origins shows a hyperbolic dependence with half-saturation values in the range 10–100 μ M in most of the cases [25–30]. The same holds true for the enzyme from *P. berghei*.

In contrast with this the inhibition by ATP, which has been found for all allosteric PFKs, varies by orders of magnitude. Inhibition of the *P. berghei* enzyme is weaker by two orders of magnitude compared with the erythrocyte enzyme under identical conditions [25]. This inhibitory effect is also much less pronounced than that on muscle PFK [31,32]. A discrimination between the effects of free ATP and MgATP was investigated in only some cases, for instance for PFKs from yeast [33,34], erythrocytes [25] and *Trypanosoma brucei* [35,36].

For yeast PFK both forms were shown to inhibit the enzyme whereas PFK from erythrocytes is only inhibited by free ATP. In contrast with these examples no inhibition by either free ATP or

Table 3. Results of testing the goodness-of-fit of different models using the U_k statistics and discrimination between them by the T_{kl} test

Model 0 is the favoured model according to eqn. (4); models 1–7 are described in the text.

	U_k	T_{kl}					
		Model 0	Model 1	Model 2	Model 3	Model 4	Model 5
Model 0	0.86*						
Model 1	2.14	3.4					
Model 2	5.17	7.4	6.3				
Model 3	8.81	12.4	9.1	8.4			
Model 4	9.27	15.6	12.9	9.2	5.4		
Model 5	0.81†	0.88‡	−3.9	−7.7	−11.0	−15.9	
Model 6	0.84†	0.54‡	−4.4	−7.1	−13.2	−16.1	0.37‡
Model 7	4.13	6.2	5.6	0.63‡	−7.5	−9.6	7.4
							7.2

* Not significant at $\alpha = 5\%$ (adequate model).

† Not significant at $\alpha = 5\%$ (trivial since models 5 and 6 include the adequate model 0 also as a submodel).

‡ No significant differences between the two models ($\alpha = 5\%$).

MgATP is reported for the enzyme from *T. brucei*. The observed inhibitory effect in the presence of high concentrations of ATP was shown to be due to the decrease of $[Mg^{2+}]$ caused by complexing of ATP. In general the effect of Mg^{2+} on PFKs of different origins varies. For most of the enzymes an activation is reported [27–30]. For the yeast enzyme [34] and PFKs from rabbit muscle [32] and *T. brucei* [36] the presence of free Mg^{2+} is essential in addition to the complexing of ATP. PFK from erythrocytes is inhibited by Mg^{2+} .

With respect to the kinetics of PFK from *P. berghei* the effect of Mg^{2+} cannot be reduced to the complexing of ATP. In addition to this, higher concentrations of free Mg^{2+} inhibit the enzyme. It was not possible to describe the observed inhibition by ATP by the decrease of Mg^{2+} , as was proposed for PFK from *T. brucei*. A model was favoured assuming an allosteric binding site for Mg^{2+} that is different from that for the nucleotides, as has also been proposed for yeast PFK [34].

The inhibition of the enzyme by higher concentrations of Mg^{2+} is not caused by the increase of ionic strength, as was demonstrated by additional experiments not shown here. The effects of increased concentrations of KCl and Tris in the relevant ranges on PFK activity are negligible.

A possible effect of Mg^{2+} on the association–dissociation behaviour of the enzyme has not been taken into consideration. The role of Mg_2ATP as a positive or negative effector of PFK has not yet been examined. Because its concentration *in vivo* will not exceed 3% of $[ATP_t]$ (independent of pH for $[Mg^{2+}] \leq 10$ mM) the influence of this species is of little interest.

Although the pH-dependence of the Mg^{2+} complexing of ATP was taken into account, the kinetic effects were only related to the sums of free ATP and MgATP. In the range pH 6.5–7.2, which is of especial physiological importance for the infection of erythrocytes by *P. berghei*, shifts of pH have only little effect on $[ATP_{free}]$, $[MgATP]$ and $[Mg^{2+}]$, despite changes in the pH-dependent dissociation equilibria. Thus the earlier observed pH-dependence of the inhibition by ATP [5] is indeed related to protonation steps of the enzyme, probably of imidazole residues, and not of the substrates.

The relative constancy of $[MgATP]$ seems to be one prerequisite for the pH-independence of the observed substrate affinity for the nucleotide (K_{ma}) [5].

PFKs from mammalian sources and from malarial parasites show large differences with respect to allosteric regulation, whereas the binding of the substrates is similar. This indicates that the catalytic site of the enzyme is much more highly conserved than are the allosteric sites.

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