Biochimica et Biophysica Acta, 370 (1974) 102–112

BBA 67327

HUMAN PLATELET 6-PHOSPHOFRUCTOKINASE

PURIFICATION, KINETIC PARAMETERS AND THE INFLUENCE OF SULPHATE IONS ON ENZYME ACTIVITY

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(Received April 24th, 1974)

SUMMARY

1. Human platelet 6-phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) was partially purified. The final preparation had a specific activity of 7.1 μmoles Fru-1,6-P_2 formed per min per mg protein at 25 °C.

2. SO_4^{2-} activated the enzyme activity especially at low Fru-6-*P* concentrations. This effect is due to diminished cooperativity with respect to Fru-6-*P*.

3. SO_4^{2-} diminished the allosteric inhibition by ATP. Other nucleotide phosphates caused no inhibition when tested in the presence of 3 mM SO_4^{2-}. In the absence of SO_4^{2-} CTP also inhibited enzyme activity.

INTRODUCTION

In recent years increasing awareness of the importance of metabolic regulation has reflected itself in a number of review articles about control of glycolysis. The important key enzyme 6-phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) has received much attention. Phosphofructokinase catalyzes the conversion of Fru-6-*P* to Fru-1,6-P_2, coupled with the dephosphorylation of MgATP to MgADP. The enzyme has been purified from a great number of tissues and its main characteristics have been described [1–5]. The enzyme from human blood platelets has not been characterized before, mainly due to poor availability of the material and instability of the enzyme.

The most important function of the platelet is to aggregate. On the presence of aggregation-inducing agents the cells react by an increase of glycolytic flux which may deliver part of the energy for shape change, aggregation and release reaction [6]. The transmitting mechanism between extracellular stimuli and intracellular glycolysis is still unknown, but may perhaps involve phosphofructokinase [7–9]. Furthermore, this enzyme may function in balancing glycolysis and the citric acid cycle by its sensitivity for citrate [6, 9].

In view of the proposed importance of phosphofructokinase in control of

glycolysis, we started an investigation on the enzyme from human platelets. The purified enzyme could be activated and stabilized by SO_4^{2-}. Although stabilization by SO_4^{2-} has been reported for the enzyme from rat jejunal mucosa [10], no special attention has been given to the influence of SO_4^{2-} on the kinetics of phosphofructokinase. Furthermore, kinetic studies on the effect of NH_4^+ [3] and Mg^{2+} [11] have been carried out with variable amounts of $MgSO_4$ and $(NH_4)_2SO_4$, without considering a possible participation of SO_4^{2-}.

We characterized the purified enzyme, giving special attention to the role of SO_4^{2-}. It will be shown that the properties of the enzyme towards Fru-6-*P*, ATP and CTP are markedly influenced by SO_4^{2-}.

MATERIALS AND METHODS

Chemicals

All nucleotide phosphates, glycolytic intermediates and enzymes used for measurement of phosphofructokinase activity and molecular-weight determinations were obtained from Boehringer Mannheim. β-Mercaptoethanol was from Fluka A.G., Buchs and ε-aminocapronic acid from Mogepha, Alkmaar, The Netherlands. Whatman DE-11 cellulose, Sepharose 6B and Sephadex G-25 and G-200 were purchased from Pharmacia, Uppsala. Affinity chromatography was carried out on Cibacron-blue F3G, purchased from Ciba A.G., Basel. All other chemicals used were of analytical grade.

Preparation of platelet suspensions

6–8 l freshly drawn blood, obtained from 12–16 donors, was collected in plastic 500-ml bags (Blood Transfusion Centre, Amsterdam), containing 100 ml 2.7% disodium citrate anhydride and 2.3% glucose. The bags were centrifuged (4 °C, 300 × *g*, 15 min) to remove red blood cells and leucocytes and the platelets were washed 6 times in modified Ringer solution according to Karpatkin [12]. The final preparation contained less than 0.01% red blood cells and no detectable leucocytes. After addition of 5 mM β-mercaptoethanol lysis was obtained by freezing the sample in liquid nitrogen. The frozen preparation was kept at −90 °C overnight.

Assay of phosphofructokinase activity

Phosphofructokinase activity was determined by coupling the formation of Fru-1,6-P_2 to the α-glycerophosphate dehydrogenase reaction. The oxidation of NADH was followed at 340 nm in a Perkin–Elmer-124 spectrophotometer at 25 °C. The reaction mixture contained in 3 ml: 0.2 M Tris–HCl (pH 8.1 or pH 7.1); 6 mM KCl; 0.05 ml dialyzed auxiliary enzymes (fructose bis-phosphate aldolase 10 mg/ml; triose phosphate isomerase 2 mg/ml, glycerophosphate dehydrogenase 2 mg/ml); 0.2 mM NADH (disodium salt); $MgCl_2$ in concentrations of $[Mg^{2+}_{total}] = 3 \times [ATP^{4-}_{total}]$ and the concentrations of substrates of the reaction catalyzed by phosphofructokinase as indicated in Results. The reaction was started by adding 0.02 ml purified human platelet phosphofructokinase. Before assaying the enzyme activity, the preparation was dialyzed for 3 h against 2 × 10 000 times its volume of 0.1 M Tris–HCl (pH 8.1) containing 1 mM β-mercaptoethanol, 0.2 mM EDTA (disodium salt) and 30% (v/v) glycerol. A unit of activity is defined as the amount of enzyme

catalyzing the formation of 1 μmole of Fru-1,6-P_2 per min at 25 °C. The specific activity is defined as units per mg protein. Since the enzyme was purified in a medium containing 10 mM SO_4^{2-} (see Results), the specific activity at the various purification steps was measured in an assay medium (pH 8.1) containing 4 mM Fru-6-*P*, 0.4 mM ATP and an additional 10 mM SO_4^{2-}. The protein was determined according to Lowry et al. [13] using crystalline bovine serum albumin as a standard. Enzyme solutions were concentrated in an Amicon ultrafiltration apparatus using Diaflo XM-50 filters.

Molecular-weight determinations and electrophoresis

The molecular weight of phosphofructokinase was estimated on Sepharose 6B (column size 6 cm × 100 cm), according to Andrews [14]. Glutathione reductase (118 000) [15], lactate dehydrogenase (135 000) [16], pyruvate kinase (237 000) [17] and urease (500 000) [18] were used as reference proteins. Electrophoresis was carried out on starch gel, polyacrylamide and cellulose acetate strips (Cellogel, Chemetron, Milano) at 4 °C according to Layzer et al. [3].

RESULTS

Purification of human platelet phosphofructokinase

The whole procedure was carried out at 4 °C. All the buffers used contained 0.3 mM EDTA (disodium salt), 1 mM β-mercaptoethanol and 1 mM ε-aminocapronic acid, except when stated otherwise. Stabilizing agents were added to the buffers in the indicated concentrations.

The purification method contained the following steps: (1) Centrifugation of platelet lysate (3500 × *g*, 10 min) to remove insoluble material. (2) Dialysis against 5 mM potassium phosphate buffer (pH 7.0) for 3 h. (3) Adsorbtion on DEAE-cellulose column (column size 3.5 cm × 10 cm), which was previously equilibrated with 5 mM potassium phosphate buffer (pH 7.0). After adsorbtion, the column was washed with the same buffer. The enzyme was eluted with 0.3 M potassium phosphate buffer (pH 8.1), containing 10 mM $(NH_4)_2SO_4$ and 0.2 mM Fru-1,6-P_2. (4) Precipitation of the protein fraction by 25% (w/v) $(NH_4)_2SO_4$, adjusting the pH to pH 8.1 with 1 M KOH. Further stabilization during this step was obtained by adding 1 mM ATP. After 3 h the suspension was centrifuged (15 000 × *g*, 15 min) and the precipitate was dissolved in 0.1 M potassium phosphate buffer (pH 8.1), containing 10 mM $(NH_4)_2SO_4$, 0.2 mM Fru-1,6-P_2 and an increased β-mercaptoethanol concentration of 20 mM. (5) Heat treatment, keeping the sample at 60 °C for 30 s. After this step the preparation was quickly cooled and centrifuged (15 000 × *g*, 15 min). (6) Column chromatography on Sepharose 6B (column size 2.5 cm × 45 cm), which was equilibrated with 0.1 M potassium phosphate buffer (pH 8.1) containing $(NH_4)_2SO_4$ and Fru-1,6-P_2 in concentrations mentioned above. The effluent was concentrated by ultrafiltration. (7) Column chromatography on Sephadex G-200 (column size 2.5 cm × 60 cm) under the conditions mentioned in 6. After concentration by ultrafiltration the enzyme solution was placed on a Sephadex G-25 column (column size 1.5 cm × 30 cm), equilibrated with 50 mM potassium phosphate buffer (pH 7.1), containing 0.5 mM EDTA (disodium salt) and 5 mM β-mercaptoethanol. (8) Affinity chromatography according to Böhme et al. [19]. The enzyme preparation was applied on

a Cibacron-blue F3G column (column size 1.5 cm × 3 cm), which was equilibrated with 50 mM potassium phosphate buffer (pH 7.1), containing 0.5 mM EDTA (di-sodium salt) and 5 mM β-mercaptoethanol. The column was washed with the same buffer and the enzyme preparation was eluted with 0.2 M potassium phosphate buffer (pH 8.5) containing 5 mM ATP and 1.5 M $(NH_4)_2SO_4$. When 1 mM cyclic 3',5'-AMP was added to the elution buffer the yield of this purification step increased. After elution, $(NH_4)_2SO_4$ and nucleotides were removed on a Sephadex G-25 column as described earlier and the solution was concentrated by ultrafiltration, as described under Methods.

Table I shows a typical example of 12 enzyme purifications. Attempts to remove other platelet proteins such as actomyosin [20] from the lysate before starting purification were unsatisfactory. The final preparation had a specific activity of 7.1 units/mg protein, as measured in the assay medium containing an additional 10 mM SO_4^{2-}. This specific activity corresponds to a purification rate of 240 times.

TABLE I

PURIFICATION OF HUMAN PLATELET PHOSPHOFRUCTOKINASE

The specific activity has been determined in an assay medium containing an additional 10 mM SO_4^{2-}.

Step of purification	Volume (ml)	Concentration (units/ml)	Total activity (units)	Protein concentration (mg/ml)	Specific activity (units/mg)	Yield (percent)
1. Lysate	38	0.79	29.2	25.00	0.031	(100)
2. Dialysis	40	0.84	33.6	25.30	0.036	115
3. DEAE-batch	300	0.18	53.3	1.83	0.098	183
4. 30% $(NH_4)_2SO_4$	24	2.21	53.0	15.00	0.148	182
5. Heat treatment	20	2.38	48.2	5.93	0.403	165
6. Sepharose 6B	106	0.24	25.4	0.38	0.655	89
7. Sephadex G-200	52	0.43	22.5	0.31	1.380	77
8. Cibacron-blue	21	0.85	18.5	0.34	7.100	64

Electrophoresis

Electrophoresis with partially purified enzyme was carried out on different gels as described under Methods. After detecting enzyme activity according to Layzer et al. [3] only one band containing phosphofructokinase activity was observed. This disagrees with the findings of Layzer and Conway [21] who detected two iso-enzymes by DEAE-cellulose chromatography of the crude lysate, but our purification procedure may account for this apparent discrepancy.

Molecular weight and influence of pH

After chromatography on a Sepharose 6B column human platelet phosphofructokinase showed a molecular weight of $(3.6 \pm 0.05) \cdot 10^5$ (mean and range of three determinations), which corresponds well with the values described for the enzyme from rabbit muscle [1, 22] and human erythrocytes [3].

Optimal enzyme activity was found between pH 8.2 and pH 8.4 at a Fru-6-*P* concentration of 2 mM. The physiological pH inside the platelet is probably pH 7.1 [23] and at this pH the enzyme showed about 50% of its optimal activity in 0.2 M

Tris–HCl, 7% in 0.2 M Tris–maleate and 60% in 0.2 M tri–ethanolamine hydrochloride–NaOH buffer.

Kinetics

Human platelet phosphofructokinase is activated and stabilized by SO_4^{2-}. Fig. 1 shows the influence of increasing SO_4^{2-} concentration on the enzyme activity, as measured at various Fru-6-*P* concentrations at pH 8.1. In the presence of SO_4^{2-} the curve is less sigmoidal. This resulted in activation of enzyme activity, especially at low Fru-6-*P* concentrations. Optimal activation was reached at 3 mM SO_4^{2-}. A further increase of the SO_4^{2-} concentration only produced further activation at low [Fru-6-*P*], but markedly reduced the apparent *V* values.

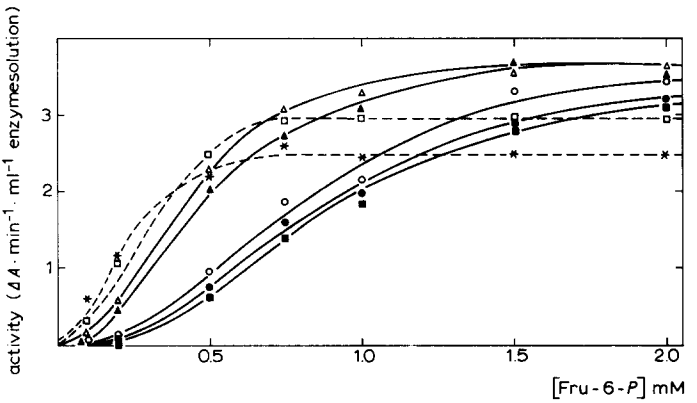

Fig. 1. Phosphofructokinase activity (expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution) vs [Fru-6-*P*] at various [SO_4^{2-}]: 0 mM (■—■); 0.5 mM (●—●); 1.0 mM (○—○); 2.0 mM (▲—▲); 3.0 mM (△—△); 5.0 mM (□—□) and about 58 mM (★—★) as tested at pH 8.1 and [ATP] = 0.5 mM.

A similar activation was measured at pH 7.1. The increased activity in the presence of SO_4^{2-} may be due to a slight rise of ionic strength. When this increase of ionic strength was compensated by decreasing the concentration of the buffer similar results were obtained.

The influence of SO_4^{2-} on the kinetic properties of platelet phosphofructokinase was investigated by measuring the enzyme activity at various substrate concentrations in the presence or absence of 3 mM SO_4^{2-}.

Fig. 2 shows the $1/v$ vs 1/[Fru-6-*P*] plots at various [ATP] at pH 8.1. SO_4^{2-} markedly reduced the cooperativity with respect to Fru-6-*P* (Fig. 2A). The lowest dotted line in Fig. 2A represents the $1/v$ vs 1/[Fru-6-*P*] data at infinite ATP concentration (the values were derived from the $1/v$ vs 1/[ATP] plot at various [Fru-6-*P*], not given in a figure). The relationship between $1/v$ vs 1/[Fru-6-*P*] at [ATP] = ∞ was almost linear, indicating minimal cooperativity towards Fru-6-*P*. This was further supported by a Hill coefficient of 1.2 (see insert). When the same experiments were carried out in the absence of SO_4^{2-}, Fig. 2B was obtained. Again the lowest dotted line represents the $1/v$ vs 1/[Fru-6-*P*] data at [ATP] = ∞. The cooperativity as expressed by the Hill coefficient was 1.7 in the absence of SO_4^{2-}.

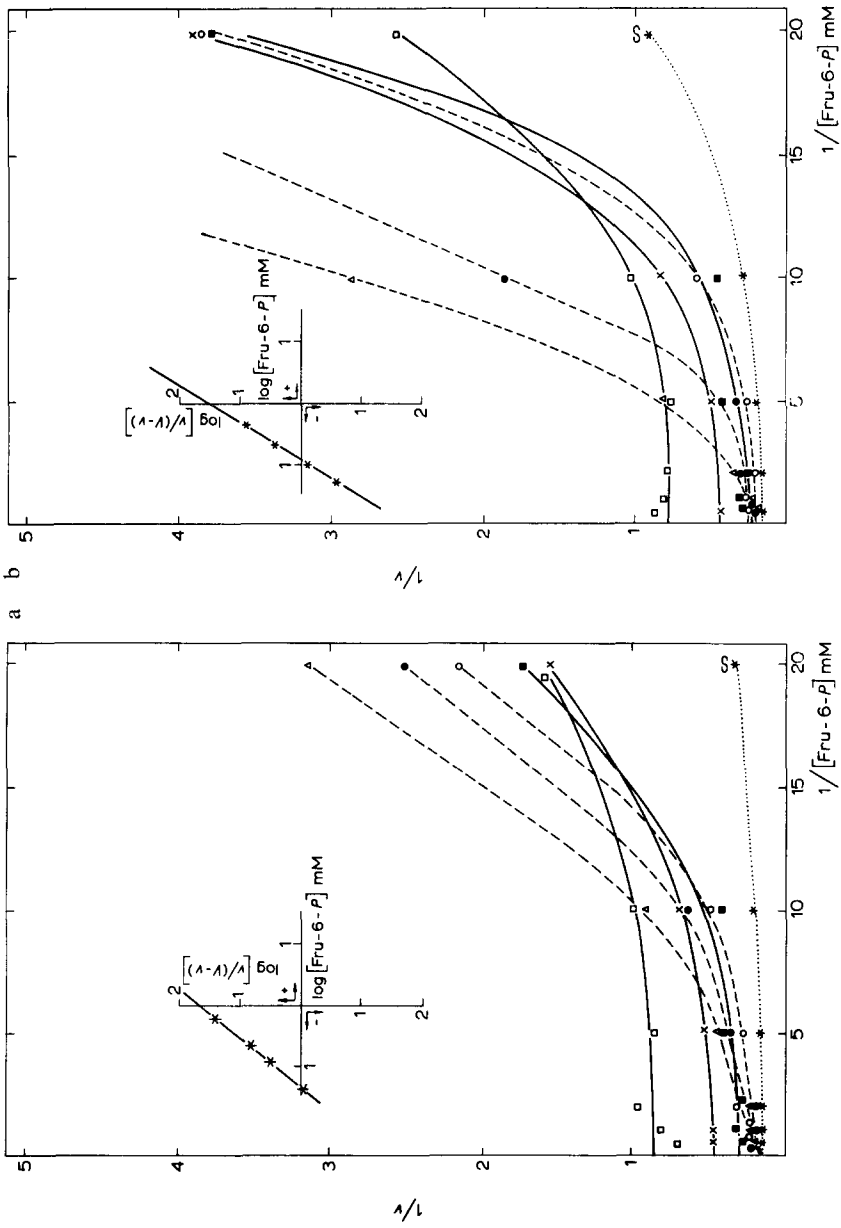

Fig. 2. Lineweaver–Burk plots of phosphofructokinase activity (expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution) versus [Fru-6-*P*] at various [ATP]: 1.0 mM (△—△); 0.5 mM (●—●); 0.2 mM (○—○); 0.1 mM (■—■); 0.05 mM (×—×) and 0.025 mM (□—□). Values at [ATP] = ∞ (*—*) were calculated from $1/v$ vs 1/[ATP] plots by extrapolation to 1/[ATP] = 0. Data from one enzyme preparation as tested at pH 8.1 in the presence (Fig. 2A) and absence (Fig. 2B) of 3 mM SO_4^{2-}. Data represent one of seven similar experiments. Reciprocal values >5.0 were not shown in the figures. The inserts show Hill plots at [ATP] = ∞.

It is known that the pH has a considerable influence on the kinetic behaviour of phosphofructokinase from various sources. Fig. 3 shows the $1/v$ vs 1/[Fru-6-*P*] plot at various [ATP] at pH 7.1. In the presence of 3 mM SO_4^{2-} (Fig. 3A) a Hill coefficient of 2.1 was calculated (see insert), while this value increased to 2.7 when SO_4^{2-} was omitted from the assay medium (Fig. 3B).

The different kinetic data as can be calculated from Figs 2 and 3 are summarized in Table II. From this table it can be concluded that at lower pH the *n* value is

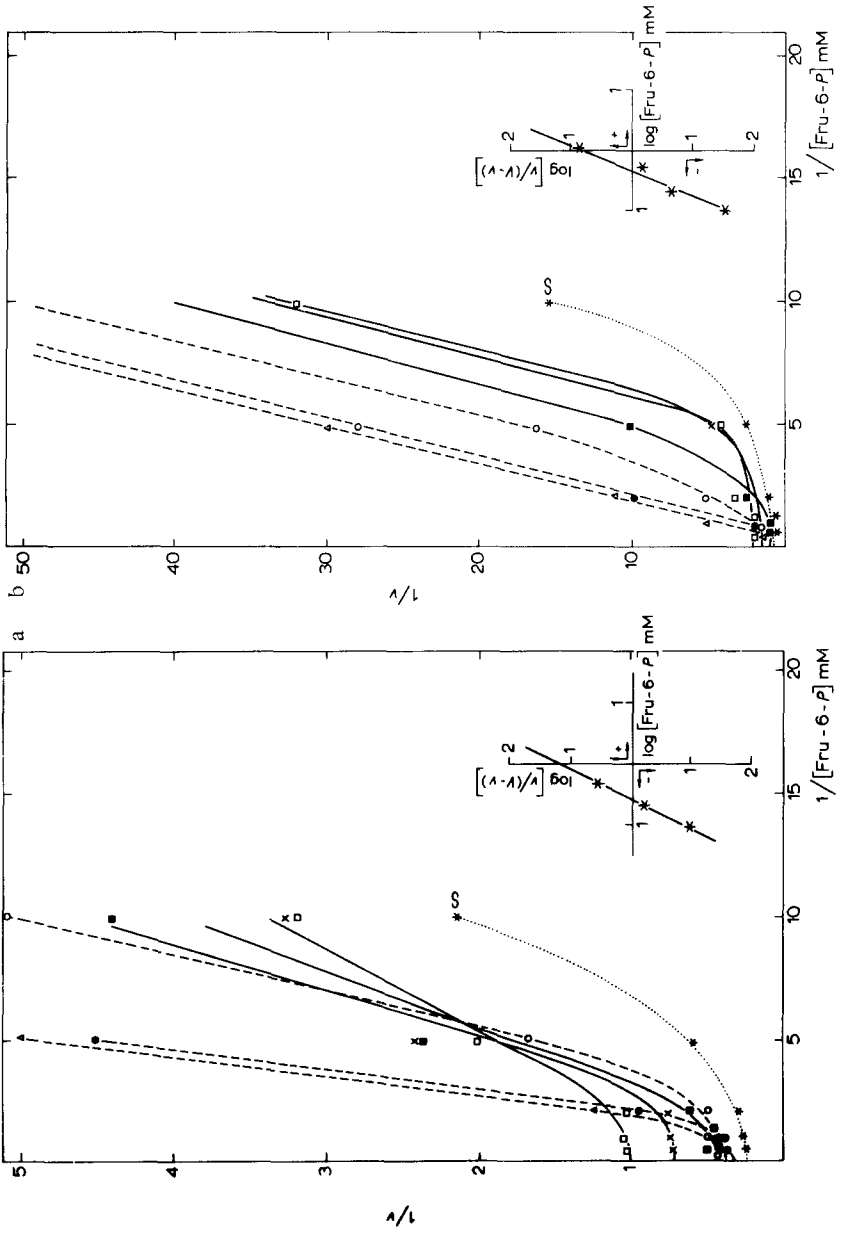

Fig. 3. Lineweaver–Burk plots of phosphofructokinase activity (expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution) versus [Fru-6-*P*] at various [ATP] as tested at the enzyme preparation used in Fig. 2 at pH 7.1 in the presence (Fig. 3A) and absence (Fig. 3B) of 3 mM SO_4^{2-}. The symbols are defined in Fig. 2. Data represent one of seven similar experiments. Reciprocal values > 50 were not shown in the figures. The inserts show Hill plots at [ATP] = ∞.

increased. In the presence of SO_4^{2-} the Hill coefficient is lower. The same is valid for the K_m for Fru-6-*P*, while the K_m for ATP is not changed significantly.

Figs 2 and 3 also show that ATP strongly inhibits phosphofructokinase activity, as has been reported for the enzyme from other sources [1–5, 24]. SO_4^{2-} diminished this allosteric inhibition by ATP, both at pH 8.1 (Fig. 4) and pH 7.1 (not shown). This effect was optimal at 3 mM SO_4^{2-}. A further increase of the SO_4^{2-} concentration produced no further activation but increased again the inhibition by

TABLE II

MICHAELIS–MENTEN CONSTANTS, HILL COEFFICIENTS AND MAXIMAL ENZYME ACTIVITY OF PHOSPHOFRUCTOKINASE AT VARIOUS CONDITIONS

K_m and n values were calculated from Hill plots of data from Figs 2 and 3 and represent one of seven similar experiments.

Reaction medium	K_m for Fru-6-*P* (mM)	Hill coefficient	K_m for ATP (mM)	V ($\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution)
pH 8.1; + SO_4^{2-}	0.05	1.2	0.09	6.3
pH 8.1; − SO_4^{2-}	0.13	1.7	0.07	5.7
pH 7.1; + SO_4^{2-}	0.27	2.1	0.07	4.2
pH 7.1; − SO_4^{2-}	0.45	2.7	0.05	2.5

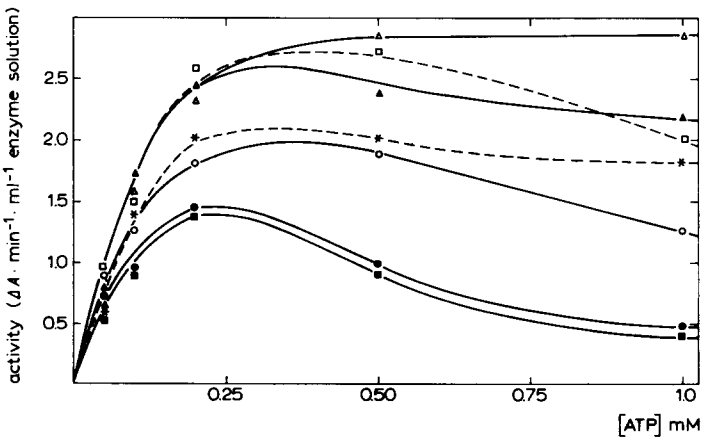

Fig. 4. Phosphofructokinase activity (expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution) vs [ATP] at various [SO_4^{2-}]: 0 mM (■—■); 0.5 mM (●—●); 1.0 mM (○—○); 2.0 mM (▲—▲); 3.0 mM (△—△); 5.0 mM (□—□) and about 58 mM (*—*) as tested at pH 8.1 and [Fru-6-*P*] = 0.5 mM.

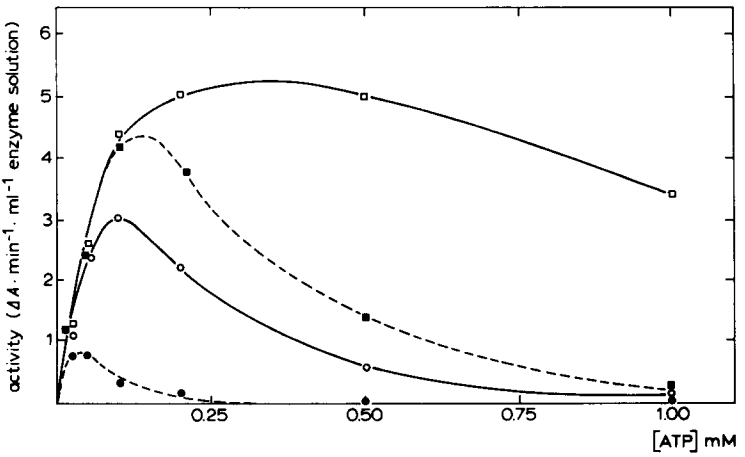

Fig. 5. Phosphofructokinase activity (expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution) vs [ATP] in the absence (dark symbols) and presence of 3 mM SO_4^{2-} (open symbols) and at [Fru-6-*P*] 2.0 mM (■—□) and 0.5 mM (●—○) as measured at pH 7.1.

ATP. The allosteric inhibition by ATP can be abolished by increasing [Fru-6-*P*]. Since in the absence of SO_4^{2-} the enzyme activity is much stronger inhibited the amount of Fru-6-*P* to abolish this effect is much higher than in the presence of 3 mM SO_4^{2-} (Fig. 5).

It is generally assumed that the inhibition of phosphofructokinase activity by high concentrations of ATP involves an inhibitory site which is highly specific for this nucleotide [5, 24]. We could support this by investigating the influence on the enzyme activity of other nucleotide phosphates such as ITP, CTP, GTP and UTP. For the enzyme from erythrocytes, yeast and other sources it was found that these nucleotide phosphates do not inhibit the enzyme activity [5, 24]. With the platelet enzyme this specificity is also observed when SO_4^{2-} are present in the assay medium. In the absence of SO_4^{2-} at pH 7.1, however, inhibition was also obtained with CTP (Fig. 6).

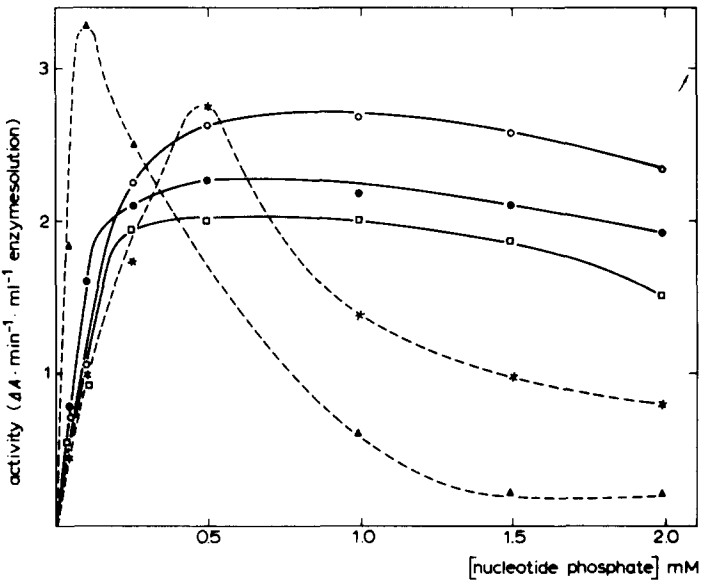

Fig. 6. Specificity of the inhibition of phosphofructokinase activity (expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution) at increased concentrations of nucleotide phosphates as tested at pH 7.1 in the absence of SO_4^{2-}. [Fru-6-*P*] = 0.5 mM; ATP (▲—▲); CTP (*—*); ITP (□—□); GTP (●—●); UTP (○—○).

DISCUSSION

Investigations on platelet glycolysis have recently indicated that phosphofructokinase plays a key role in glycolytic control [7, 8], although its properties were still unknown. Kinetic measurements on the platelet enzyme are highly handicaped by the limited availability of great amounts of fresh platelets and the lability of the enzyme.

The purification method described here results in a 240 times purification rate and a good yield of enzyme activity (about 60%). The partially purified enzyme is electrophoretically homogenous for its phosphofructokinase activity. This disagrees

with the results of Layzer and Conway who eluted two fractions from an ion-exchanging column [21]. The molecular weight of $3.6 \cdot 10^5$ for the human platelet enzyme corresponds very well with the value reported for the enzyme from other sources and agrees with the tetrameric nature of the enzyme from most tissues [1, 3, 22]. In agreement with other workers [2, 3, 25] we observed often small variations in kinetic parameters determined in different enzyme preparations. This may be due to slight differences of the final protein concentrations in the reaction mixture [1, 25]. Comparisons of kinetic data, therefore, can only be made on one single enzyme preparation. The results reported here, however, are representive for 12 purified human platelet phosphofructokinase preparations.

SO_4^{2-} acts as an allosteric activator on the reaction catalyzed by human platelet phosphofructokinase. In the absence of SO_4^{2-} and especially at pH 7.1 the enzyme exhibits strong cooperative interactions with respect to the substrate Fru-6-*P*. There is a pronounced allosteric inhibition at increased [ATP] and to a minor extent at increased [CTP]. The presence of SO_4^{2-} in the assay medium favours an enzyme conformation, which shows a) diminished cooperative interactions towards Fru-6-*P*, b) the inhibition by ATP is less pronounced and c) no inhibition by other nucleotide phosphates is observed. SO_4^{2-} alone are unable to suppress these effects completely. Increased [Fru-6-*P*] and alkaline pH both contribute to the action of SO_4^{2-} in favouring an enzyme conformation with almost normal Michaelis–Menten kinetics.

Influence of SO_4^{2-} on enzyme kinetics of phosphofructokinases have not been reported before or were denied [26]. If the sensitivity for SO_4^{2-} represents a common property of phosphofructokinases from most tissues, the findings reported here may influence kinetic measurements of the enzyme from jejunal mucosa [10], erythrocytes [3, 5], thymocytes [27] and other sources [11], which all have been carried out in the presence of SO_4^{2-}. The divergent reports about the influence of citrate on the enzyme from erythrocytes [3, 5] might also have been caused by the use of different amounts of SO_4^{2-} in the assay mixture.

The influences of both substrates on the enzyme kinetics support the concept of a regulatory function of phosphofructokinase in human platelet glycolysis. The addition of various aggregation inducers to platelets is followed by an increased glucose consumption and lactate production [6, 8], reflecting stimulation of glycolysis probably due to increased phosphofructokinase activity [7, 8]. Furthermore, shape change, aggregation and especially release reaction are energy-dependent processes in which, at least partly, glycolysis is involved. During performance of these functions utilization of metabolic ATP is completely compensated by rephosphorylation of ADP and only during release reaction a fall of metabolic ATP is observed [28]. Rather than one of the nucleotides alone, the ratio (ATP + $\frac{1}{2}$ ADP)/(ATP + ADP + AMP) might be important in regulation of platelet glycolysis [8, 28]. This makes the role of metabolic ADP of special interest, especially in connection with the activating properties on phosphofructokinase activity.

ACKNOWLEDGEMENTS

The platelet suspensions were supplied by Dr E. Borst-Eilers, head of the Blood Transfusion Laboratory, University Hospital, Utrecht, for whose cooperation we are most grateful.

The authors are indebted to Dr J. F. Koster, Department of Biochemistry, Erasmus University, Rotterdam, The Netherlands, for valuable help in preparing the manuscript.

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