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INFLUENCE OF Mg^{2+}, ITP^{4-} AND ATP^{4-} ON HUMAN PLATELET PHOSPHOFRUCTOKINASE

JAN WILLEM N. AKKERMAN[a], GERTIE GORTER[a], JAN J. SIXMA[a] and GERARD E. J. STAAL[b]

[a]*Division of Haemostasis and Thrombosis and* [b]*Division of Haematology, State University Hospital, Utrecht (The Netherlands)*

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SUMMARY

In the reaction catalyzed by human platelet phosphofructokinase, Mg^{2+} is required for optimal activity. Maximal Mg^{2+} activation was obtained at $[Mg^{2+}] = [MgITP^{2-}]$ or higher. At high $MgATP^{2-}$ concentrations there is an increase in the allosteric inhibition by ATP^{4-}.

INTRODUCTION

6-Phosphofructokinase (EC 2.7.1.11) catalyzes the conversion of Fru-6-*P* to Fru-1,6-P_2, coupled with the dephosphorylation of ATP to ADP. This catalysis depends on Mg^{2+}. It is generally accepted that Mg^{2+} is important as a constituent of the $MgATP^{2-}$ complex and that $MgATP^{2-}$ is the substrate for the enzyme [1, 2]. Since in a solution the $MgATP^{2-}$ complex is in equilibrium with the free ions, interference of Mg^{2+}_{free} and ATP^{4-}_{free} in kinetic studies might be present.

Such interference has indeed been observed. Lardy and Parks [19] found inhibition by free ATP in several enzymes for which MgATP is the substrate. Paetkau and Lardy [15] showed that free ATP^{4-} acted as a powerful inhibitor and that Mg^{2+} was required for the reaction of rabbit muscle phosphofructokinase.

In view of the important function of phosphofructokinase in regulating human platelet glycolysis, we characterized the partially purified enzyme [3–5]. Phosphofructokinase exhibits normal Michaelis–Menten kinetics towards $MgATP^{2-}$. At increased $MgATP^{2-}$ the enzyme activity is suppressed. This allosteric inhibition is absent when $MgITP^{2-}$ is involved in the reaction.

The present paper describes the kinetics of purified platelet phosphofructokinase towards $MgATP^{2-}$ and $MgITP^{2-}$ with special attention to the role of the free ions. It will be shown that the enzyme kinetics towards both phosphate donors are highly dependent on the levels of Mg^{2+} and ATP^{4-} in the assay medium.

MATERIALS AND METHODS

The nucleotide phosphates, added as sodium salts, glycolytic intermediates,

cofactors and enzymes used for measurement of the phosphofructokinase activity were purchased from Boehringer Mannheim. $MgCl_2$ was prepared from 99.998% MgO (Koch–Light Laboratories, Colnbrook, England) and constant boiling HCl and its concentration was determined by complexometric titration. All other chemicals used were of analytical grade.

Human platelet phosphofructokinase was partially purified as described previously [4]. The enzyme activity was measured by coupling the formation of Fru-1,6-P_2 to the α-glycerophosphate dehydrogenase reaction and following the oxidation of NADH at 340 nm in a Perkin–Elmer-124 spectrophotometer at 30 °C. The assay medium contained in a final volume of 3 ml: 50 mM Tris–HCl (pH 8.0), 6 mM KCl, 0.05 ml dialyzed auxiliary enzymes (fructose diphosphate aldolase, 10 mg/ml; triosephosphate isomerase, 2 mg/ml; glycerophosphate dehydrogenase, 2 mg/ml), 0.2 mM disodium NADH, 1.0 mM Fru-6-*P* and $MgCl_2$ and Na_2ATP or Na_3ITP at the various concentrations required for the levels of free and complexed ions indicated in Results. These levels were calculated with the aid of a stability constant for $MgATP^{2-}$ of 20 000 M^{-1}, which has been determined for a medium that was closely similar to the one used by us [6, 7]. The $MgITP^{2-}$ complex was assumed to have an identical stability constant [1, 8]. Complex formation between Mg^{2+} and Fru-6-*P* and between K^+ and ATP^{4-} was considered negligible because of the low stability constants of these complexes [1, 7, 9]. The formation of $HATP^{3-}$ and $MgHATP^-$ was neglected since these complexes represent less than 1% of the total nucleotide content at pH 8.0 [10]. A unit of enzyme activity is defined as the amount of enzyme activity catalyzing the formation of 1 μmole of Fru-1,6-P_2 per min at 30 °C. The various purified phosphofructokinase preparations ($n = 12$) had a specific activity of about 7 units/mg protein, as tested in the assay medium described above at 4 mM Fru-6-*P*, 0.4 mM ATP_{total}, 5 mM $MgSO_4$ and an additional 5 mM disodium EDTA. The protein content was based on a protein determination according to Lowry et al. [11], using crystalline bovine serum albumin (Sigma, St. Louis, U.S.A.) as a standard. The influences of ATP^{4-}, ITP^{4-} and Mg^{2+} reported here, were not contaminated by effects of ionic strength or Na^+ concentrations and corrections were unnecessary.

RESULTS

Human platelet phosphofructokinase shows normal Michaelis–Menten kinetics with respect to substrate $MgITP^{2-}$. The enzyme activity at various $MgITP^{2-}$ concentrations is illustrated in Fig. 1. It is shown that different activities were measured when the Mg^{2+} concentration was fixed, and therefore the concentration of ITP^{4-} varied, and when the concentration of ITP^{4-} was kept constant thus changing the Mg^{2+} concentrations. At 0.2 mM $MgITP^{2-}$ the enzyme activity increased about 75% when the Mg^{2+} concentration was raised from 20 μM to 0.5 mM and the concentration of ITP^{4-} was decreased from 0.5 mM to 20 μM. Similar results were obtained at other $MgITP^{2-}$ levels.

The data indicate that Mg^{2+} may activate or ITP^{4-} may inhibit the phosphofructokinase activity. Further information about these effects was obtained by studying the possible activation by Mg^{2+} and possible inhibition by ITP^{4-} separately. Constant $MgITP^{2-}$ complex concentrations were used to eliminate any

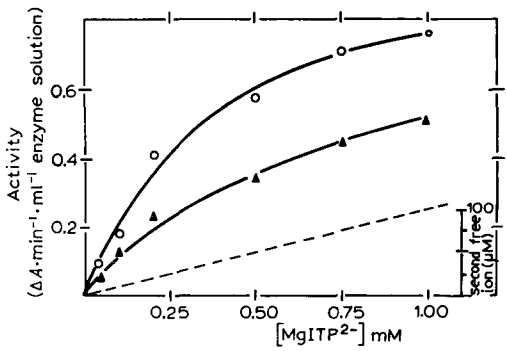

Fig. 1. Phosphofructokinase activity, expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution at various $MgITP^{2-}$ concentrations, both at a fixed ITP^{4-} of 0.5 mM (▲—▲) and at a fixed Mg^{2+} concentration of 0.5 mM (○—○). The corresponding concentrations of Mg^{2+} and ITP^{4-}, respectively, are indicated by the dotted line.

influence of the substrate level on the enzyme activity. Such measurements are difficult, since at a fixed $MgITP^{2-}$ concentration any change in the Mg^{2+} concentration is accompanied with an opposite alteration of the ITP^{4-} concentration.

The influence of ITP^{4-} is shown in Fig. 2, which demonstrates that a slight increase of the ITP^{4-} concentration resulted in a pronounced inhibition both at saturating and non-saturating $MgITP^{2-}$ levels. The inhibition was maximal at 4–5 mM ITP^{4-}, independent of the $MgITP^{2-}$ concentration.

The influence of Mg^{2+} is shown in Fig. 3. The Mg^{2+} concentration which caused maximal stimulation was equal to the concentration of the substrate $MgITP^{2-}$ used. This relation was consistent from levels as low as 0.05 mM $MgITP^{2-}$ up to concentrations of 0.6 mM, indicating a dependence of Mg^{2+} stimulation from the

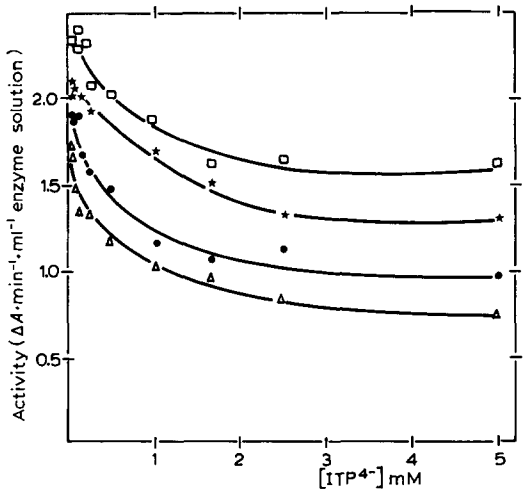

Fig. 2. Phosphofructokinase activity, expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution at various ITP^{4-} and different fixed $MgITP^{2-}$ concentrations of 2.0 mM (□—□), 0.6 mM (★—★), 0.3 mM (●—●) and 0.15 mM (△—△).

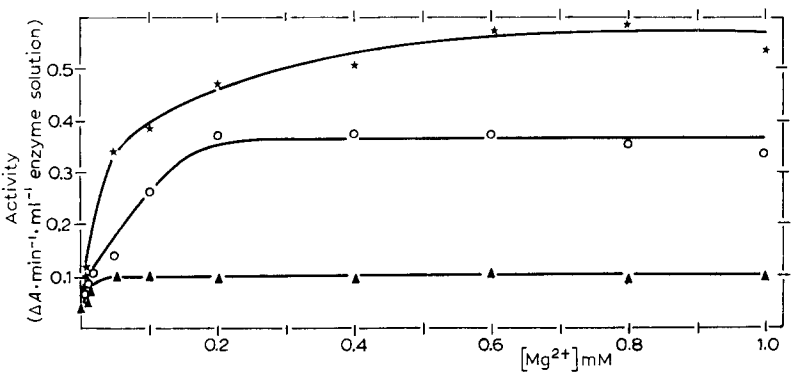

Fig. 3. Phosphofructokinase activity, expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution at various Mg^{2+} concentration and different fixed $MgITP^{2-}$ concentration of 0.6 mM (*—*), 0.2 mM (○—○) and 0.05 mM (▲—▲).

concentration of the $MgITP^{2-}$ complex. This point was further clarified by relating the phosphofructokinase activity to the $[Mg^{2+}]/[MgITP^{2-}]$ ratio. The $MgITP^{2-}$ concentration was kept fixed to exclude any direct influence of the substrate. Fig. 4 shows that this method of recording the results illustrates a pronounced inhibition at $[Mg^{2+}]/[MgITP^{2-}]$ ratios less than 1.0. No effect was obtained at ratios of 1.0 or above.

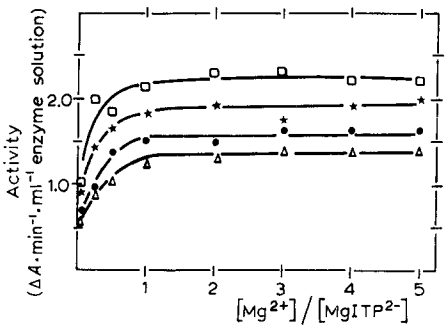

Fig. 4. Phosphofructokinase activity, expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution at various $[Mg^{2+}]/[MgITP^{2-}]$ ratios and different fixed $MgITP^{2-}$ concentration of 2.0 mM (□—□), 0.6 mM (*—*), 0.3 mM (●—●) and 0.15 mM (△—△).

These data might be thought to indicate that free Mg^{2+} is required in concentrations equal to or higher than the substrate $MgITP^{2-}$ concentrations to give optimal phosphofructokinase activity. We may however not yet exclude the possibility that these effects are produced by inhibition by ITP^{4-}. We therefore tried to relate the enzyme activity at a fixed $MgITP^{2-}$ concentration to the $[ITP^{4-}]/[MgITP^{2-}]$ ratio. No clear relation between changes in enzyme activity and this ratio could be demonstrated (Fig. 5). At a fixed $[ITP^{4-}]/[MgITP^{2-}]$ ratio an increase of the $MgITP^{2-}$ concentration is not clearly reflected by an increasing enzyme activity. Especially at a $[ITP^{4-}]/[MgITP^{2-}]$ ratio of 1.0 the activity is independent of the substrate concen-

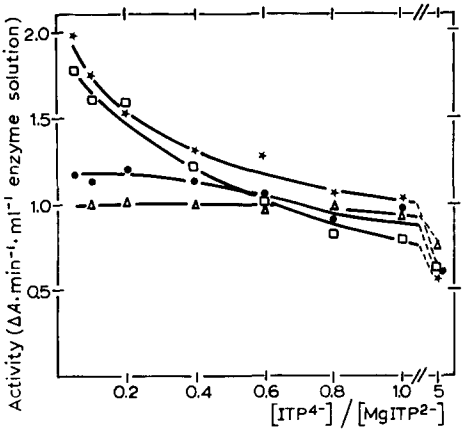

Fig. 5. Phosphofructokinase activity, expressed as $\Delta A \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ enzyme solution at various $(ITP^{4-}]/[MgITP^{2-}]$ ratios and different fixed $MgITP^{2-}$ concentration of 2.0 mM (□—□), 0.6 mM (*—*), 0.3 mM (●—●) and 0.15 mM (△—△).

tration, which is in sharp contrast with Fig. 4. No absolute conclusion about the involvement of ITP^{4-} may be drawn from these data, but they are highly suggestive for a crucial role of free Mg^{2+} in phosphofructokinase activity.

The role of free Mg^{2+} is demonstrated in more detail in Fig. 6, illustrating the strong inhibition especially at low $[Mg^{2+}]/[MgITP^{2-}]$ ratios. Replotting these activity data in a reciprocal plot demonstrates that there still remains a "basic" phosphofructokinase activity at very low $[Mg^{2+}]/[MgITP^{2-}]$ ratios, indicating the absence of an absolute requirement for free Mg^{2+}.

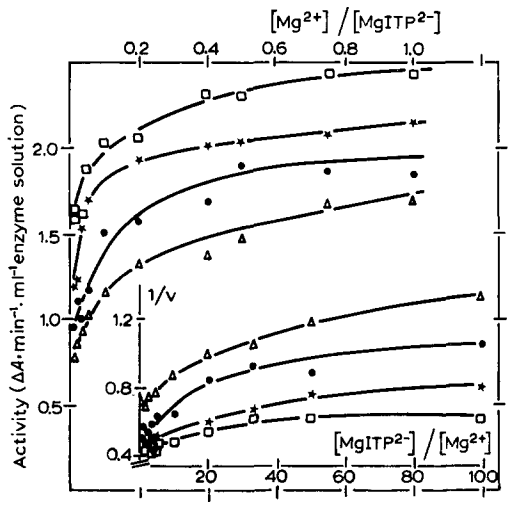

Fig. 6. Phosphofructokinase activity, expressed as $\Delta A \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ enzyme solution at various $[Mg^{2+}]/[MgITP^{2-}]$ ratios less than 1. and different fixed $MgITP^{2-}$. Symbols as in Fig. 4. The insert shows the Lineweaver–Burk plot of the same data.

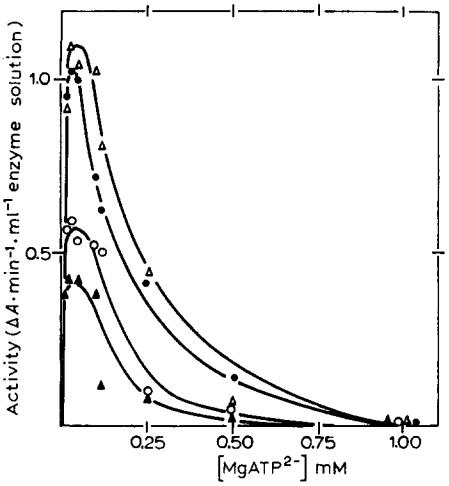

Fig. 7. Phosphofructokinase activity, expressed as $\Delta A \cdot min^{-1} \cdot ml^{-1}$ enzyme solution at various $MgATP^{2-}$ concentration and different fixed ATP^{4-} concentration of 50 μM (▲—▲), 25 μM (○—○), 12,5 μM (●—●) and 10 μM (△—△). The ratio $[Mg^{2+}]/[MgATP^{2-}]$ was $\geqslant$ 1.0.

The results obtained with MgITP may probably be extrapolated to MgATP. The influence of free Mg^{2+} would thus be negligible at $[Mg^{2+}]/[MgATP^{2-}]$ ratios higher than 1.0. Such a situation is depicted in Fig. 7 which shows the well known allosteric inhibition at high substrate concentrations, but also points out how strongly this inhibition was increased by slight raises of the ATP^{4-} concentrations.

DISCUSSION

The study of metal–adenine nucleotide complexes as parts of enzyme-catalyzed reactions is hampered by the fact that only one of the three constituents, the two free ions and the complex itself, can be fixed, whereas the changes in concentrations of the second component automatically varies the third. The variation of the levels of free ions at fixed complex concentrations and the use of MgITP, which shows no allosteric inhibition, in stead of MgATP alleviates studies of these interactions. The use of high levels of one of the free ions and therefore very low concentrations of the other has been applied to eliminate one of the two variables [12]. In our experiments significant influences of very low Mg^{2+} concentrations could be detected (Fig. 6), thus making this approach useless. The use of other buffer systems in which higher stability constants have been determined, showed no real improvements.

The influence of free ions appeared indeed to be of substantial importance for phosphofructokinase activity. Mg^{2+} activated or ITP^{4-} inhibited. The Mg^{2+} effect was directly related to the substrate concentration, whereas the influence of ITP^{4-} was not dependent on the substrate level. The activation by Mg^{2+} was only present at $[Mg^{2+}]/[MgITP^{2-}]$ ratios lower than 1.0. Double reciprocal plots of velocity vs $[Mg^{2+}]/[MgITP^{2-}]$ ratio suggested that still a basic phosphofructokinase activity was present at very low $[Mg^{2+}]/[MgITP^{2-}]$ ratios.

Assuming a similar dependence on the Mg^{2+} concentration when $MgATP^{2-}$

is involved in the phosphofructokinase activity, a lack of free Mg^{2+} could be excluded by keeping the Mg^{2+} concentrations equal or above the $MgATP^{2-}$ concentrations. Under these conditions ATP^{4-} increased the allosteric inhibition by $MgATP^{2-}$. The requirement for free Mg^{2+} in amounts equal to the substrate $MgITP^{2-}$ concentration suggests that Mg^{2+} has at least two roles in the reaction mechanism: (a) it forms a complex with ITP^{4-} to form $MgITP^{2-}$ which is the substrate for the enzyme, and (b) it forms a complex with the enzyme to activate the enzymic reaction [12–16].

In contrast to the enzyme from yeast [1], platelet phosphofructokinase is inhibited by free ATP^{4-}, since these ions increase the allosteric inhibition by the $MgATP^{2-}$ complex.

Platelets contain about 2 μmoles/ATP/10^{11} cells, which is involved in metabolic processes [17]. The Mg^{2+} content of the platelet has not yet been determined precisely, but probably varies between 2 and 3 μmoles/10^{11} cells (Holmsen, H., personal communication). Since magnesium is bound to various other components of the cell, it seems feasible to assume that the Mg^{2+} concentration is inadequate for binding all the metabolic ATP, thus leaving free ATP^{4-} in the cytoplasma. The level of free Mg^{2+} will then be very low. In rat tissue only 10% of the total Mg^{2+} content is present as free ions [18]. If this holds true also for the human platelet, the requirement for free Mg^{2+} and the inhibitory action of free ATP^{4-} may provide important mechanisms for the regulation of phosphofructokinase activity in the circulating platelet.

REFERENCES

1 Mavis, R. D. and Stelwagen, E. (1970) J. Biol. Chem. 245, 674–680
2 Blangy, D., Buc, H. and Monod. J. (1968) J. Mol. Biol. 31, 13–35
3 Akkerman, J. W. N., Gorter, G., Corbey, H. M. A., Staal, G. E. J. and Sixma, J. J. (1973) in Erythrocytes, Thrombocytes and Leukocytes (Gerlach, E., Moser, K., Deutsch, E. and Wilmanns, W., eds), pp. 135–138, Georg Thieme Publishers, Stuttgart
4 Akkerman, J. W. N., Gorter, G. Sixma, J. J. and Staal, G. E. J. (1974) Biochim. Biophys. Acta 370, 120–129
5 Akkerman, J. W. N., Gorter, G., Staal, G. E. J. and Sixma, J. J. (1974) Eur. J. Clin. Invest., in the press
6 TaquiKhan, M. M. and Martell, A. E. (1962) J. Phys. Chem. 66, 10–15
7 O'Sullivan, W. J. and Perrin, D. D. (1964) Biochemistry 3, 18–26
8 Purich, D. L. and Fromm, H. J. (1972) Biochem. J. 130, 63–69
9 McGilvery, R. W. (1965) Biochemistry 4, 1924–1930
10 Morrison, J. F. and Heyde, E. (1972) Annu. Rev. Biochem. 41, 29–54
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
12 Keech, B. and Barrit, G. J. (1967) J. Biol. Chem. 242, 1983–1987
13 Bais, R. and Keech, B. (1972) J. Biol. Chem. 247, 3255–3261
14 Dugal, B. S. (1973) Biochem. Biophys. Res. Commun. 54, 1603–1610
15 Paetkau, V. and Lardy, H. A. (1967) J. Biol. Chem. 242, 2035–2042
16 Wimhurst, J. M. and Manchester, K. L. (1972) FEBS Lett. 27, 321–326
17 Kotelba-Witkowska, B., Holmsen, H. and Mürer, E. M. (1972) Br. J. Haematol. 22, 429–435
18 Veloso, D., Guynn, R. W., Oskarsson, M. and Veech, R. L. (1973) J. Biol. Chem. 248, 4811–4819
19 Lardy, H. A. and Parks, Jr, R. E. (1956) in Enzymes: Units of Biological Structure and Function, p. 584, Academic Press, New York