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KINETIC STUDIES ON THE REACTION CATALYZED BY PHOSPHOGLYCERATE KINASE

II.* THE KINETIC RELATIONSHIPS BETWEEN 3-PHOSPHOGLYCERATE, $MgATP^{2-}$ AND ACTIVATING METAL ION

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SUMMARY

1. The Michaelis constants for the two substrates of phosphoglycerate kinase (ATP:D-3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3), $MgATP^{2-}$ and 3-phosphoglycerate, are each independent of the concentration of the second substrate. Three prevalent mechanisms can describe this situation.

2. The Mg^{2+} complex of 3-phosphoglycerate does not appear to be in an active substrate form.

3. Mg^{2+} at high concentrations inhibits the enzyme non-competitively with respect to 3-phosphoglycerate.

4. High concentrations of Mg^{2+} change the kinetic relationships and non-linear Lineweaver–Burk plots are obtained for the two substrates. The curves can be approximated to two straight lines. The two intersection points with the abciss axis are independent of the concentration of the second substrate.

5. The data are interpreted in terms of two different binding sites for each substrate, the second set of sites being involved only at high Mg^{2+} concentrations. Various mechanisms for this effect are considered and it is suggested that these could also be important for other enzymes involving reaction with ATP.

INTRODUCTION

Kinetic examinations of the enzyme phosphoglycerate kinase (ATP:D-3-phosphoglycerate 1-phosphotransferase, EC 2.7.2.3) have mostly been concerned with the relationships between the activating divalent metal ion (Mg^{2+} or Mn^{2+}) and the nucleotide substrate[1–4]. With regard to the substrates only the Michaelis constants at given conditions are known[1–6].

* For paper I in this series see ref. 4.
Abbreviation: PGA,3-phosphoglycerate.

To obtain further information about the reaction mechanism it seemed necessary to study the possible mutual dependence of the two substrates, $MgATP^{2-}$ and PGA, as well as the possible connection between the metal ion and PGA. An earlier kinetic study of this problem has been made on a pea-seed enzyme[5], but the results did not allow a clear distinction between the various possibilities.

MATERIAL AND METHODS

Enzymes

Crystalline phosphoglycerate kinase from yeast was obtained from Boehringer and Soehne, Mannheim, W. Germany.

Reagents

The same reagents and the same kind of purification methods and precautions to remove possible contaminating metal ions were used as described earlier[4]. Disodium NADH, however, was obtained this time from Sigma Chemical Co.

Activity measurement

The activity of phosphoglycerate kinase was determined by the spectrophotometric method of BÜCHER[2] and expressed as the initial velocity, $v = \left(\frac{dA_{366}}{dt}\right)$ (in min^{-1}, *cf.* ref. 4).

RESULTS

The influence of PGA on the $MgATP^{2-}$ kinetics

Fig. 1 shows that Lineweaver–Burk plots in terms of the concentration of $MgATP^{2-}$ at different PGA concentrations give a set of lines with a common inter-

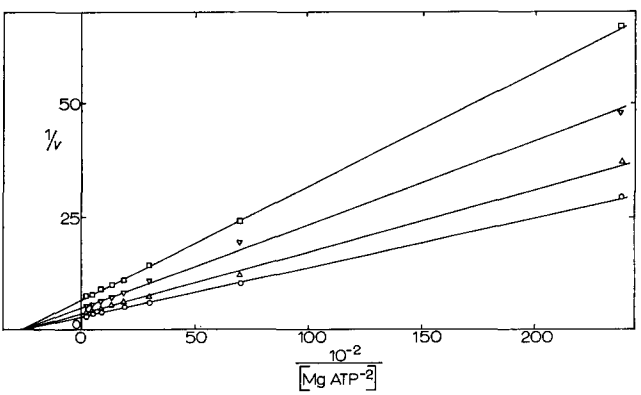

Fig. 1. Effect of the concentration of $MgATP^{2-}$ on the activity of phosphoglycerate kinase at different concentrations of PGA. ○—○, 3.0 mM; △—△, 1.5 mM; ▽—▽, 0.75 mM; □—□, 0.375 mM. The total concentrations of ATP and $MgCl_2$ are varied in the experiment and these are kept equal in each experimental point. 50 mM Tris–acetic acid buffer (pH 7.8, 25°) and about 0.2 μg phosphoglycerate kinase per ml are used in the experiment. A dissociation constant for $MgATP^{2-}$ of $8 \cdot 10^{-5}$ M is used as described earlier[4]. A K_m value equal to 0.4 mM is obtained for $MgATP^{2-}$.

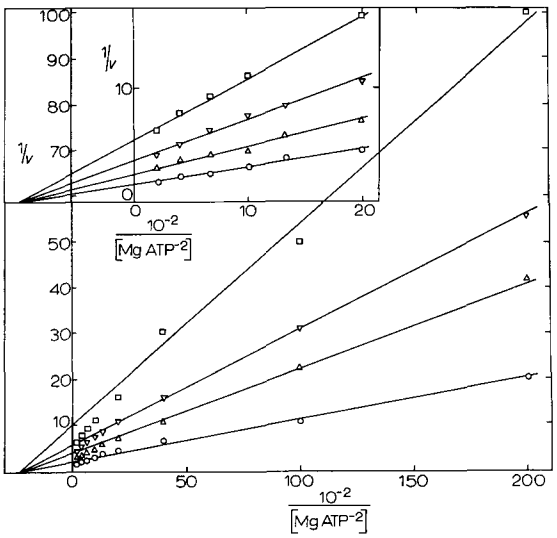

Fig. 2. Effect of the concentration of $MgATP^{2-}$ on the activity of phosphoglycerate kinase at different concentrations of PGA. ○—○, 5.85 mM; △—△, 1.17 mM; ▽—▽, 0.585 mM; □—□, 0.293 mM. The total ATP concentrations are varied in the experiment, while the total $MgSO_4$ concentration is kept constant at 10 mM. 50 mM Tris-HNO_3 buffer (pH 7.8, 25°) and about 0.2 μg phosphoglycerate kinase per ml are used in the experiment. A dissociation constant for $MgATP^{2-}$ of $8 \cdot 10^{-5}$ M is used as described earlier[4]. A separate enlargement is made for total ATP concentrations between 0.5 and 5 mM. K_m values for $MgATP^{2-}$ equal to 0.5 and 1.0 mM are obtained in the two concentration ranges.

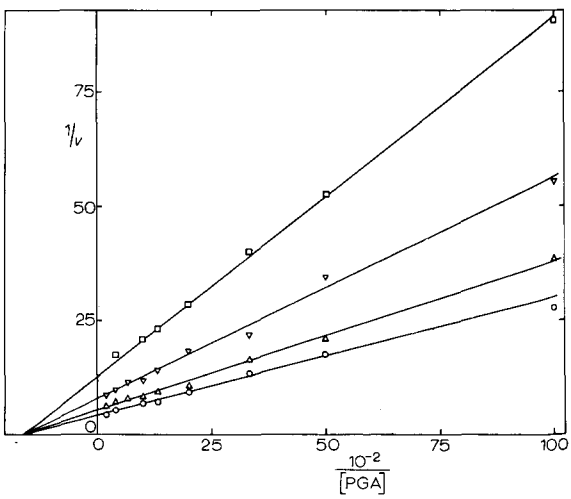

Fig. 3. Effect of the concentration of PGA on the activity at different and equal concentrations of ATP and $MgCl_2$. ○—○, 2.0 mM; △—△, 1.0 mM; ▽—▽, 0.5 mM; □—□, 0.25 mM. 50 mM Tris–acetic acid buffer (pH 7.8, 25°) and about 0.2 μg phosphoglycerate kinase per ml are used in the experiment. A K_m value equal to 0.62 mM is obtained for PGA.

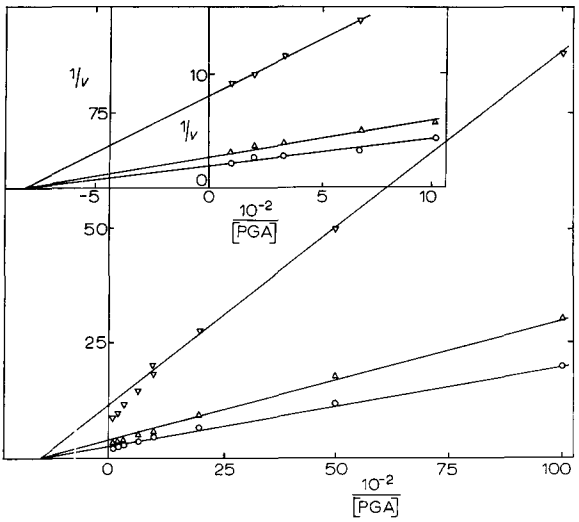

Fig. 4. Effect of concentration of PGA on the activity of phosphoglycerate kinase at different concentrations of total ATP. ○—○, 1.0 mM; △—△, 0.5 mM; ▽—▽, 0.1 mM. The total $MgSO_4$ concentration is kept fixed at 5.0 mM. 50 mM Tris–HNO_3 (pH 7.8, 25°) and about 0.2 μg phosphoglycerate kinase per ml are used in the experiment. A separate enlargement is made for PGA concentrations between 1 and 10 mM. K_m values for PGA equal to 0.63 and 1.23 mM are obtained in the two concentration ranges.

section on the $[MgATP^{2-}]^{-1}$ axis. This indicates that the Michaelis constant for $MgATP^{2-}$ is independent of the PGA concentration. If the total Mg^{2+} concentration* is kept at 10 mM (Fig. 2) the Lineweaver–Burk plots are non-linear. Curves slightly convex upwards are obtained, each of which can be approximated to two straight lines (Fig. 2). Two sets of lines are thus obtained with intersections on the $[MgATP^{2-}]^{-1}$ axis. Similar results are obtained at 5 mM Mg^{2+}.

The influence of $MgATP^{2-}$ on the PGA kinetics

Lineweaver–Burk plots in terms of the PGA concentration at different $MgATP^{2-}$ concentrations (Fig. 3) give linear relations. The intersection point on the $[PGA]^{-1}$ axis indicates that the Michaelis constant for PGA is independent of the $MgATP^{2-}$ concentration. At a high Mg^{2+} concentration* (Fig. 4) curved plots like those described in the previous section (Fig. 2) are obtained.

The influence of Mg^{2+} on the PGA kinetics

Non-linear Lineweaver–Burk plots in terms of PGA at different Mg^{2+} concentrations are shown in Fig. 5. The curved plots can be approximated to two straight lines (Fig. 5). The common intersection points on the $[PGA]^{-1}$ axis indicate that Mg^{2+} does not change the Michaelis constant for PGA. This is evidence that Mg^{2+} is a non-

* Different anions are used in the experiments at low (Figs. 1 and 3) and high (Figs. 2, 4 and 5) free Mg^{2+} concentrations. Unpublished results have shown that no bigger differences can be expected for the different anions in the concentration ranges used in this paper. A slight increase in ionic strength is obtained at high Mg^{2+} concentrations. This seems not to cause the non-linear Lineweaver–Burk plots, however.

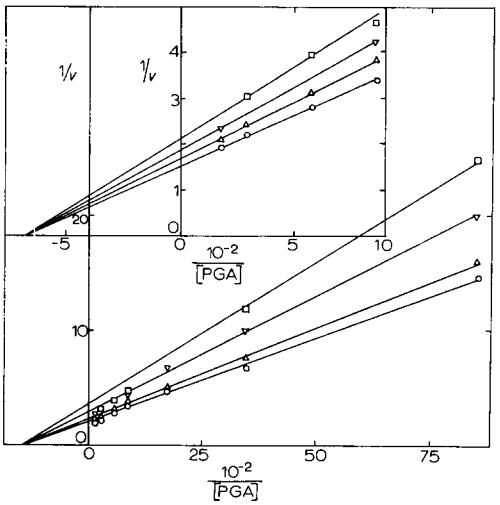

Fig. 5. Effect of the concentration of PGA on the activity of phosphoglycerate kinase at different high total concentrations of $MgSO_4$. ○—○, 10.0 mM; △—△, 7.5 mM; ▽—▽, 5.0 mM; □—□, 2.5 mM. The total ATP concentration is kept fixed at 1.0 mM. 50 mM Tris–HNO_3 buffer (pH 7.8, 25°) and about 0.2 μg phosphoglycerate kinase per ml are used in the experiment. A separate enlargement is made for total PGA concentrations between 1.0 and 5.0 mM PGA. K_m values for PGA equal to 0.63 and 1.27 mM are obtained in the two concentration ranges, respectively, after correction for the MgPGA concentration. A dissociation constant of 14 mM is used for this complex[5].

competitive inhibitor to PGA. If the Mg^{2+} concentration in Fig. 5 is varied in a concentration range lower than the total ATP concentration, an intersection point on the $[PGA]^{-1}$ axis is obtained as well, but in that case an activation is obtained. This is quite in accord with earlier results[4] showing that $MgATP^{2-}$ is the active substrate form of ATP.

DISCUSSION

Linear Lineweaver–Burk plots are obtained for the two substrates in the phosphoglycerate kinase catalyzed reaction (see Figs. 1 and 3; also Figs. 2 and 4 in limited concentration ranges). The Michaelis constant for each substrate is independent of the second substrate. The initial reaction velocity can then be expressed as

$$v = \frac{V}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_{AB}}{[A][B]}}$$ (*cf.* refs. 7–9) and $K_A \cdot K_B = K_{AB}$.

(A, B, K_A and K_B represent $MgATP^{2-}$, PGA and their respective Michaelis constants, V represents the maximal initial velocity.) Of the many prevalent two-substrate mechanisms there are three that fit these relations: (1) random order of reaction for the two substrates with the formation of a ternary complex, which is in rapid equilibrium with the enzyme and the substrates, (2) one of the substrates has to combine with the enzyme before the second one can react and thus form the ternary complex, the kinetics is of the steady-state type since equilibrium kinetics is not consistent with $K_A \cdot K_B = K_{AB}$ and (3) the catalytic mechanism involves two binary complexes[7].

Mechanism 1 is favoured, even if Mechanism 2 cannot be quite excluded. Mechanism 1 requires linear Lineweaver–Burk plots.

Arguments may be raised that the curvature in Figs. 1 and 3 is too small to be seen and that high Mg^{2+} concentrations (Figs. 2, 4 and 5) may in some way increase the curvature resulting from steady-state kinetics. REINER[9] has analyzed the random-order steady-state kinetic case for two substrates. The curves he gives for that mechanism are concave upwards. The curves here in Figs. 2 and 4 are instead, concave downwards. Other possible effects of high Mg^{2+} concentrations are discussed later.

No direct evidence against Mechanism 2 has been found, but preliminary equilibrium dialysis studies indicate independent binding of $MgATP^{2-}$ and PGA to phosphoglycerate kinase. Mechanism 3 is possible if the maximal initial velocity, V, in the forward and reverse (the reaction presented in this paper) reaction are equal. However, BÜCHER[2] has shown that V (forward) $= 8.8 \times V$ (reverse). Although his results were obtained at different conditions, it is doubtful if Mechanism 3 applies.

In order to check if MgPGA is an active substrate form, the results in Fig. 5 were used to determine v as a function of the MgPGA concentration at certain constant concentrations of free PGA. The results showed that MgPGA apparently inhibits the reaction. Since the dissociation constant for this complex is about 14 mM (ref. 5) a high free Mg^{2+} concentration is needed to obtain a significant concentration of the complex. The inhibition by MgPGA can be accounted for by the free Mg^{2+} present in the reaction mixture. If MgPGA should be responsible for the increase in activity at high Mg^{2+} and PGA concentrations in Figs. 4 and 5, this can scarcely cause the increase in activity in Fig. 2. The increase in activity at 1.17 mM PGA, with a concentration of $MgATP^{2-}$ between 1 and 5 mM (Fig. 2) is about twice as high as would be expected from the simultaneous lowering of the Mg^{2+} and MgPGA concentrations only.

The non-linear Lineweaver–Burk plots at high Mg^{2+} concentrations might be explained by assuming that there are two active and independent centres on the enzyme molecule and that these normally are catalytically equal (Figs. 1 and 3), but that the enzyme molecule is not symmetrical. High Mg^{2+} concentrations might then change the second centre in such a way that the kinetic parameters for the substrates will change (Figs. 2, 4 and 5). This case would be analogous to that of two enzymes acting on the same substrate (ref. 9, p. 98).

TABLE I

EFFECT OF HIGH METAL-ION CONCENTRATION ON THE MAXIMAL INITIAL VELOCITIES

The data in Figs. 1–4 are treated as if the set of lines at the particular concentration ranges were quite independent of the neighbouring concentration ranges. V' and V'' are the estimated maximal initial velocities when both substrates are at infinite concentration for the lower and higher concentration range, respectively. All data are normalized to about the same concentration of enzyme.

Data	$[Mg^{2+}]_{total}$	V'	V''	$\frac{V''}{V'}$
Fig. 1	$[ATP]_{total}$	0.434		
Fig. 2	10 mM	0.425	0.590	1.4
Fig. 3	$[ATP]_{total}$	0.434		
Fig. 4	5 mM	0.360	0.510	1.4

Table I shows that a second set of substrate-binding sites gives a considerable increase in the maximal initial velocity (V) while the corresponding V in the lower concentration range of the substrates remains at about the same value as is obtained when the Mg^{2+} concentration is equal to the total ATP concentration. An alternative explanation would be that there are two kinds of binding sites for the substrates. One of these is a direct part of the active center and the other is located at a "regulatory site". In this case either the binding at the "regulatory site" or the regulating function of the substrate is induced or modified by the metal ion (ref. 10, see also ref. 9, p. 66).

Consideration of the present data, together with published results for other enzymes involving reaction with ATP, suggest that different mechanisms for the metal-ion activation might operate at different experimental conditions.

Non-linear Lineweaver–Burk plots in terms of the concentration of ATP are obtained for mitochondrial adenosine triphosphatase when $[Mg^{2+}]_{total}/[ATP]_{total} > 1$ (see ref. 11).

In the pyruvate kinase case contradictory results concerning the metal-ion activation have been obtained[12–15]. Some data indicate that the divalent metal ion takes part in the catalytic mechanism by forming a complex with the enzyme[12,13]. Other data favour the mechanism where the metal-ion complex of the nucleotide is the active form of the nucleotide substrate[14,15]. It has recently[13] been shown that there seem to be two different kinds of substrate-binding sites in pyruvate kinase. No kinetic significance, however, was found for the second set of sites[13]. The two suggested mechanisms for metal-ion activation of pyruvate kinase are obtained at not quite overlapping concentration ranges[12–15]. Therefore, it would be interesting if substrate kinetics in the pyruvate kinase studies were made over wider concentration ranges of both the substrates and the divalent activating metal ion. The difference in the activation of pyruvate kinase and phosphoglycerate kinase might be due to the fact that pyruvate kinase interacts more strongly[12] with its divalent activating metal ion than does phosphoglycerate kinase[3]. In the phosphoglycerate kinase case, the weak metal-ion binding[3] suggests that the sites operating in a mechanism involving an enzyme–metal ion complex would only function at high metal-ion concentrations, a fact which is in agreement with the present results. In the pyruvate kinase case, however, at low concentrations of the divalent metal ion and the substrates, the predominant mechanism for metal-ion activation might involve an enzyme–metal ion complex, but at higher concentrations of these reacting species, activation over a nucleotide–metal ion complex might also operate in the catalytic mechanism.

In order to obtain more information about the effect of high Mg^{2+} concentration, as well as about the two-substrate mechanism of phosphoglycerate kinase, valuable results might be obtained from studies of the binding of the two substrates to the enzyme and also from studies of the structure of the enzyme itself. Such investigations have been initiated.

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