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PRODUCT INHIBITION STUDIES OF YEAST PHOSPHOGLYCERATE KINASE EVALUATING PROPERTIES OF MULTIPLE SUBSTRATE BINDING SITES

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Summary

Product inhibition studies on yeast phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) have been performed with 1,3-P_2-glycerate. The results indicate that:

1. The catalytic reaction can be affected via four substrate binding sites, two for $MgATP^{2-}$ and two for 3-*P*-glycerate.
2. There is one catalytic centre per enzyme molecule.
3. The catalytic reaction primarily occurs at the 'first' or 'high affinity' $MgATP^{2-}$ and 3-*P*-glycerate binding sites. The 'second' set of sub-sites for these substrates are located in a region for regulation of the catalytic reaction.
4. The products of the reaction, 1,3-P_2-glycerate and ADP, are preferentially bound to the regulatory region.
5. $MgATP^{2-}$ and 1,3-P_2-glycerate are able to bind simultaneously to this region. When liganded with $MgATP^{2-}$ the apparent K_i value for 1,3-P_2-glycerate increases from 3 μM to 20 μM.

Introduction

The phosphoglycerate kinase (ATP:3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) reaction proceeds via a ternary complex between the enzyme and its two substrates [1—6]. It has been possible to describe the mechanism as being rapid equilibrium random [2,7], when studied in the so

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called backward reaction, i.e. in the direction of gluconeogenesis, with $MgATP^{2-}$ and 3-phospho-D-glycerate (3-*P*-glycerate) as substrates. At high concentrations of Mg^{2+}, substrate activation appears [1]. This phenomenon was suggested as being due to multiple substrate binding sites [1]. Later on equilibrium studies have shown that the enzyme is capable of binding two molecules of 3-*P*-glycerate [8] and more than one molecule of the nucleotide [8,9]. Multiplicity of the latter is also evident from the fact that ATP^{4-} causes activation of the enzyme in the backward reaction [10] and ADP^{3-} is a strictly non-competitive inhibitor of $MgATP^{2-}$ [2]. Also $MgADP^{-}$ is non-competitive inhibitor of $MgATP^{2-}$ but a competitive contribution appears also especially in the concentration range for substrate activation. At this stage it appeared important to get the inhibition patterns with 1,3-diphospho-D-glycerate (1,3-P_2-glycerate), especially the behaviour relative 3-*P*-glycerate. As phosphoglycerate kinase is activated by anions [11,12] and evidence for a specific anion binding site has been presented [13] two questions became obvious: (1) Is the second binding site for $MgATP^{2-}$ identical with the second binding site for 3-*P*-glycerate, and if not, (2) do these additional sites form a second catalytic centre?

To make the product inhibition studies possible a new method for assaying the enzymatic activity was worked out (presented in the section 'Activity measurements').

Materials and Methods

Enzymes. Commercially available yeast phosphoglycerate kinase, which was further purified [14], or enzyme prepared from baker's yeast [15] were used in parallel experiments. The main electrophoretic component (IIB [14], also called 2 [15]) was utilized. Pyruvate kinase and glyceraldehydephosphate dehydrogenase from rabbit muscle, lactate dehydrogenase from bovine heart and alcohol dehydrogenase from yeast were products from Sigma Chemical Co.

Reagents. Most of the reagents were from Sigma Chemical Co. The potassium salt of phospho*enol*pyruvate was obtained from Boehringer, Mannheim. Only analytical grade reagents and glass-distilled water were used. Contaminating metal ions were removed as described earlier [17].

Preparation of 1,3-P_2-glycerate. 1,3-P_2-glycerate was synthesized according to Negelein and Brömel [18] in an enzyme system utilizing the glyceraldehydephosphate dehydrogenase and alcohol dehydrogenase reactions. After 15 min at 25°C, the reaction was stopped by cooling in an ice-bath. The reaction mixture was applied to a Dowex 1-formate column as described by Krimsky [19]. After elution with 0.6 mM ammonium formate, the 1,3-P_2-glycerate was concentrated by precipitation in ethanol. The precipitate was then dissolved in 50 mM Tris-HCl buffer (pH 7.8 at 25°C), 0.25 M KCl, to a final concentration of about 5 mM. The concentration of 1,3-P_2-glycerate was determined as described earlier [18]. No decomposition appeared during storage in an ice-bath for 5 h, the time required for the performance of an experiment, or at −20°C for three days.

Activity measurements. The activity of phosphoglycerate kinase was mea-

sured spectrophotometrically at 366 nm. The activity was expressed as $v = (dA_{366}/dt)_{t=0}$, in min^{-1} [20,17].

Inhibition by 1,3-P_2-glycerate of the backward reaction of phosphoglycerate kinase was studied in an assay system using the combined pyruvate kinase and lactate dehydrogenase reactions as ADP trap. The measurements were performed in 50 mM Tris-HCl buffer (pH 7.8 at 25°C), 0.25 M KCl. As a standard, 10 mM phospho*enol*pyruvate and 0.5 mM NADH were used in the assay medium. The reaction was followed at 366 nm and the rate expressed as described above. It was found suitable to use about 32 U pyruvate kinase and about 67 U lactate dehydrogenase per ml substrate. The phosphoglycerate kinase reaction appeared to be the rate determining step if the initial velocity did not exceed 0.3 min^{-1}. About 0.2 U of this enzyme was utilized in the present work. $MgATP^{2-}$, 3-*P*-glycerate and 1,3-P_2-glycerate concentrations up to 5, 10 and 2.5 mM, respectively, were shown not to affect the reaction velocity of the pyruvate kinase-lactate dehydrogenase system. Phospho*enol*-pyruvate appeared to be a weak inhibitor of phosphoglycerate kinase competitive with 3-*P*-glycerate, and causing the above assay media to show about 90% of its enzymatic activity. Following the phosphoglycerate kinase reaction via the creatine kinase system did not seem to be very successful.

Estimation of the free concentrations of Mg^{2+} and the substrates. Computer programs were used to calculate the concentrations of the divalent metal ion and the substrates occurring in free form or complexed to the metal ion. The following dissociation constants were used: $MgATP^{2-}$, 0.08 mM (cf. [2]); $MgADP^{-}$, 0.6 mM [21]; 3-*P*-glycerate-Mg^{2+}, 10 mM [22]; and Mg^{2+}-phospho*enol*pyruvate, 5 mM [23]. The possible binding of Mg^{2+} to 1,3-P_2-glycerate was neglected.

Results

Influence of high $MgATP^{2-}$ concentrations on the kinetics of 3-P-glycerate. In the experiment presented in Fig. 1 the $MgATP^{2-}$ concentration was kept at such high levels that the second nucleotide binding site was becoming saturated. As the results show each of the Lineweaver-Burk plots for 3-*P*-glycerate can be represented by two straight lines. In both 3-*P*-glycerate concentration ranges the apparent K_m value is independent of the $MgATP^{2-}$ concentration.

Overall inhibition by 1,3-P_2-glycerate. Inhibition of the backward phosphoglycerate kinase reaction with increasing concentration of the product 1,3-P_2-glycerate is illustrated in Fig. 2. Up to about 0.4 mM the inverted rate of the reaction appears linearity related to the concentration of the inhibitor. Above this value deviation from linearity occurs. The phenomenon indicates that multiple 1,3-P_2-glycerate binding sites exist.

Inhibition by 1,3-P_2-glycerate at variable concentrations of 3-P-glycerate or $MgATP^{2-}$. The 1,3-P_2-glycerate concentration was kept constant at various levels well within the linear low concentration range presented in Fig. 1. As is shown in Fig. 3A, 1,3-P_2-glycerate is a non-competitive inhibitor of 3-*P*-glycerate at substrate concentrations below about 0.6 mM. The lines intersect just above the horizontal axis. At higher concentrations of the variable

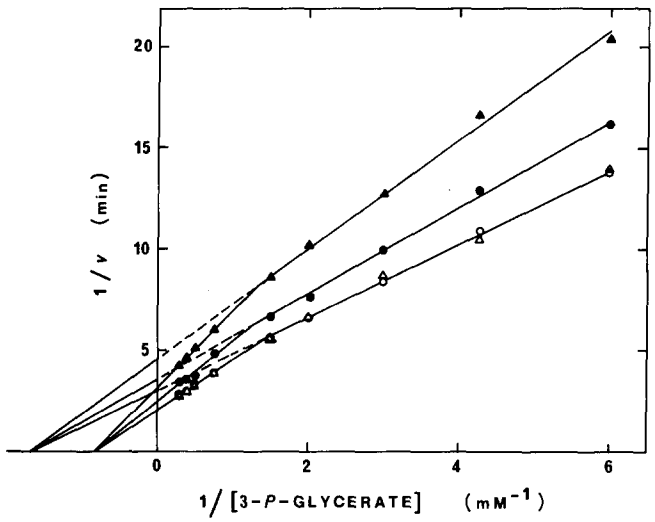

Fig. 1. The effect of high $MgATP^{2-}$ concentrations on the 3-*P*-glycerate kinetics. The concentrations of $MgATP^{2-}$ were ▲, 2 mM; ●, 4 mM; ○, 6 mM; and △, 10 mM. The assay mixture contained 10 mM free Mg^{2+}.

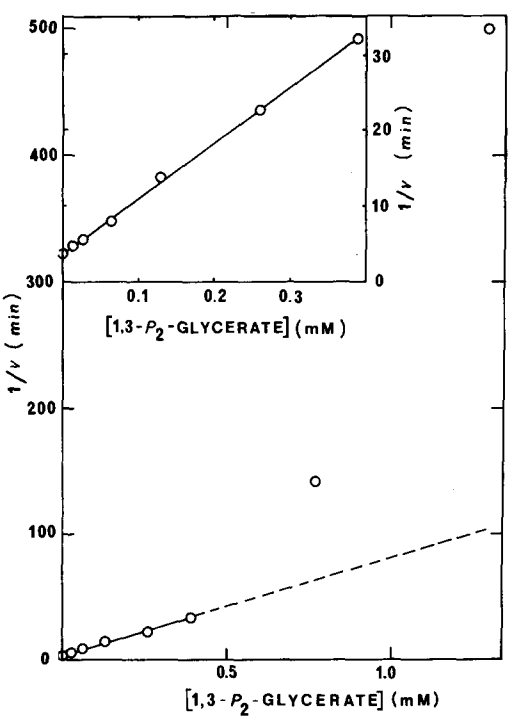

Fig. 2. The inhibition of phosphoglycerate kinase by 1,3-P_2-glycerate. The assay mixture contained 5 mM ATP, 10 mM $MgCl_2$ and 2 mM 3-*P*-glycerate.

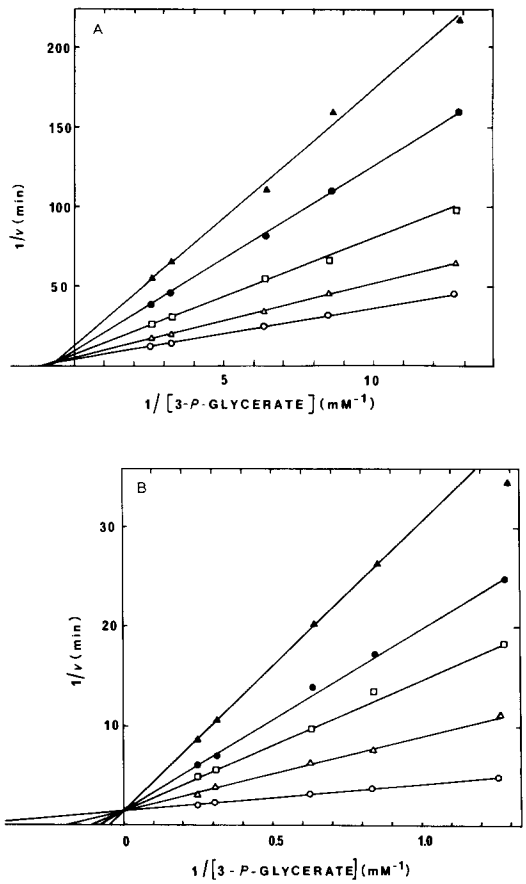

Fig. 3. The effect of 1,3-P_2-glycerate on the 3-*P*-glycerate kinetics. A, The 3-*P*-glycerate concentration was varied between 0.1 mM and 0.5 mM. The 1,3-P_2-glycerate concentrations were ○, none; △, 6.3 μM; □, 16 μM; ●, 31 μM; and ▲, 47 μM. B, The 3-*P*-glycerate concentration was varied between 1 mM and 5 mM. The 1,3-P_2-glycerate concentrations were ○, none; △, 16 μM; □, 33 μM; ●, 49 μM; and ▲, 81 μM. The assay mixture contained 5 mM ATP and 10 mM $MgCl_2$. The experiments A and B were performed separately.

substrate the type of inhibition shifts. Within this concentration interval 1,3-P_2-glycerate is competitive with 3-*P*-glycerate (Fig. 3B).

Also, when the $MgATP^{2-}$ concentration is varied the type of inhibition changes at a substrate concentration of about 0.6 mM. Below this value the product competes with $MgATP^{2-}$ (Fig. 4A). When $MgATP^{2-}$ is varied within a higher concentration interval the lines intersect at a point above the abscissa axis (Fig. 4B). Thus, non-competitive inhibition with a competitive contribution occurs. The inhibition patterns in Fig. 4 are the same if the total Mg^{2+} concentration is 10 mM or the free concentration is 5 mM.

Estimation of the apparent inhibitor constants. The inhibition has been treated separately in the two concentration ranges of the substrates, without correction for possible overlap of the inhibition patterns observed in the two intervals. In the experiments presented in Figs. 3B and 4B both the substrates

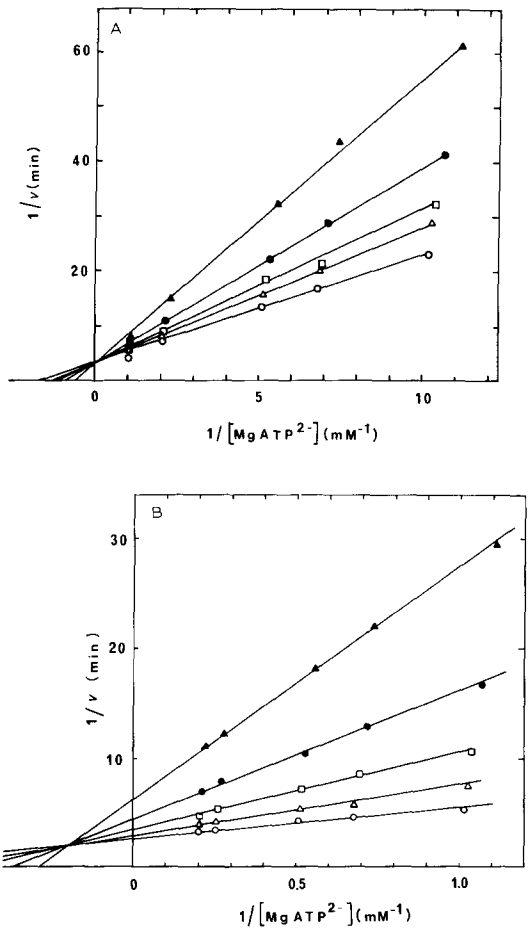

Fig. 4. The effect of 1,3-P_2-glycerate on the $MgATP^{2-}$ kinetics. A, The $MgATP^{2-}$ concentration was varied between 0.1 mM and 1 mM. The 1,3-P_2-glycerate concentrations were ○, none; △, 1.2 μM; □, 3.0 μM; ●, 5.8 μM; and ▲, 11 μM. B, The $MgATP^{2-}$ concentration was varied between 1 mM and 5 mM. The 1,3-P_2-glycerate concentrations were ○, none; △, 5.6 μM; □, 14 μM; ●, 27 μM; and ▲, 51 μM. The assay mixture contained 5 mM free Mg^{2+} and 2 mM 3-*P*-glycerate. The experiments A and B have been performed separately.

occurred in the concentration ranges for activation. The experimental data of these figures appear to fit the following rate equation:

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_A}{[A]} + \left(1 + \frac{[I]}{K_i'}\right)\frac{K_B}{[B]} + \left(1 + \frac{[I]}{K_i}\right)\frac{K_A K_B}{[A][B]}\right] \quad (1)$$

The symbols used represent: A, $MgATP^{2-}$; B, 3-*P*-glycerate; K_A and K_B, the respective apparent Michealis constants obtained from experimental data in the figure valid for the concentration interval used; and I, 1,3-P_2-glycerate. The inhibitor constants K_i and K_i' represent inhibitor binding to the enzyme when the second $MgATP^{2-}$ binding site is assumed to be free or occupied, respec-

tively. K_i as estimated from data in Fig. 3B was 3.0 μM, and from Fig. 4B, 3.4 μM. In both cases a K_i' of 20 μM was obtained.

In the experiment presented in Fig. 3A 3-*P*-glycerate was varied in the 'low' concentration range. $MgATP^{2-}$ was within the concentration interval where activation appears. Fitting the experimental data to the rate equation:

$$\frac{1}{v} = \frac{1}{V}\left[1 + \left(1 + \frac{[I]}{K_i}\right)\frac{K_A}{[A]} + \left(1 + \frac{[I]}{K_i'}\right)\frac{K_B}{[B]} + \left(1 + \frac{[I]}{K_i}\right)\frac{K_A K_B}{[A][B]}\right] \quad (2)$$

gave K_i equal to 3.0 μM and K_i' equal to 21 μM. The symbols are the same as in Eqn. 1.

The results in Fig. 4A present kinetic patterns for $MgATP^{2-}$ in the 'low' concentration range. 3-*P*-glycerate was within the concentration interval where activation appears. Fitting the experimental data to the rate equation:

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \left(1 + \frac{[I]}{K_i}\right)\frac{K_A K_B}{[A][B]}\right] \quad (3)$$

gave K_i equal to 3.4 μM. The same symbols as in Eqn. 1 were used.

Discussion

The catalytic mechanism of phosphoglycerate kinase seems to be somewhat complex, so that a simple mechanism, which fits the available experimental data is hard to find. An increased understanding can, however, be offered by the present results, which give further evidence for the reality of a non-Michaelian type of kinetics, i.e., substrate activation (cf. also [1,12]). As the enzyme is monomeric and has an asymmetric structure [24,25] the possibility of negative interaction between identical sub-sites can be excluded. So can also a possible alternative pathway of the reaction via a ping-pong mechanism [5]. Earlier results (Figs. 2 and 4 in [1]), which were very similar to the results presented in Fig. 1, showed amazingly that the double-reciprocal plot for either substrate could be represented by two straight lines intersecting at the horizontal axis when the concentration of the second substrate was varied. These results gave the first evidence for the fact that the two substrates bind independently to the catalytic centre and that substrate activation occurs. As the second substrate in both the earlier experiments almost exclusively was in the lower concentration range it was interesting to study whether higher concentrations of the second substrate affects the substrate activation caused by the variable substrate.

The results presented in Fig. 1, with 3-*P*-glycerate as the variable substrate and $MgATP^{2-}$ at high enough concentrations for saturation of both the supposed nucleotide binding sites, show that the biphased double-reciprocal plot for the variable substrate is independent of the concentration of the second substrate. Thus, it appears that there are two binding sites for either substrate, which can influence the rate of the catalytic reaction. That the enzyme contains multiple binding sites for the two substrates has been shown earlier [8,9].

However, only one active centre per enzyme molecule seems to exist. The additional sub-sites may have some regulatory function.

Some knowledge about the properties and possible function of the additional substrate binding sites can be obtained by inspection of available product inhibition patterns. For this discussion we assume that the sites of phosphoglycerate kinase for $MgATP^{2-}$ and 3-*P*-glycerate can be represented by Fig. 5. A and A′ are the sites for $MgATP^{2-}$ and B and B′ the sub-sites for 3-*P*-glycerate. A and B are the primary binding sites also named catalytic. 1,3-P_2-Glycerate is non-competitive with 3-*P*-glycerate (Fig. 3A) and competitive with $MgATP^{2-}$, (Fig. 4A) when these substrates are assumed to bind at the catalytic centre. Such an inhibition pattern agrees with for example an ordered two-substrate mechanism accepting $MgATP^{2-}$ as the first candidate at the active centre and keeping 1,3-P_2-glycerate as the last product to dissociate from the enzyme [26]. An analogous inhibition pattern was obtained, however, with $MgADP^{-}$, which appeared non-competitive with $MgATP^{2-}$ and competitive with 3-*P*-glycerate [2]. These results rule out the previous suggested sequence of substrate binding to and product dissociation from the enzyme. That the two products behave analogously to their corresponding substrates is further evidence for a random two-substrate mechanism with the products and their respective substrates binding preferentially to different forms of the enzyme, probably at different sites. With 3-*P*-glycerate and $MgATP^{2-}$ varied in the concentration range for the suggested activation, thus binding to B′ and A′, 1,3-P_2-glycerate is competitive (Fig. 3B) and non-competitive (Fig. 4B), respectively. Hence 1,3-P_2-glycerate appears to bind to the same form, or probably to the same site (B′), as the second 3-*P*-glycerate molecule. The inhibition patterns also suggest that the A′ and B′ sub-sites simultaneously can be occupied by $MgATP^{2-}$ and 1,3-P_2-glycerate, respectively. This conclusion supports the earlier results showing that yeast phosphoglycerate kinase is able to catalyse phosphorylation of adenosine 5′-triphosphate to adenosine 5′-tetraphosphate [27].

The fact that $MgADP^{-}$ is competitive with 3-*P*-glycerate and 1,3-P_2-glycerate is competitive with $MgATP^{2-}$ might appear to contradict the results of others [28,29] who report that 3-*P*-glycerate and $MgADP^{-}$ bind to the enzyme simultaneously. The discrepancy might be only superficial, however. In our product inhibition studies mainly the sites causing activation of the backward reaction are occupied by the inhibiting products. Binding of $MgADP^{-}$ to the substrate site at elevated concentrations would account for the formation of the observed enzyme-$MgADP^{-}$-(3-*P*-glycerate) complex. Complementary kinetic

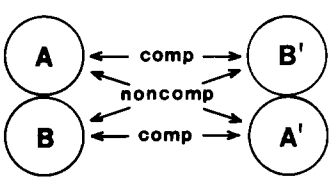

Fig. 5. Schematic illustration of the interactions between the four substrate-product binding sites in the enzyme molecule. A represents $MgATP^{2-}$, B 3-*P*-glycerate, A′ ADP^{3-} or $MgADP^{-}$ (also $MgATP^{2-}$ when bound as activator), and B′ 1,3-P_2-glycerate (also 3-*P*-glycerate when bound as activator).

investigations on yeast phosphoglycerate kinase in the forward reaction are missing. When 1,3-P_2-glycerate as substrate is varied substrate activation of the erythrocyte enzyme occurs [30], however.

In the crystalline state phosphoglycerate kinase, both from yeast [24] and horse muscle [25], appears to bind one mol of adenine nucleotide per mol of enzyme. There is structural evidence for one more nucleotide binding domain, however [24,25]. Phosphoglycerate kinase appears to be a monomer, but evidence that the enzyme tends to form dimers has been presented [14,31]. As early as 1947 Bücher [20] suggested that one binding site for the substrate ATP and another for the corresponding product ADP are involved in the catalytic reaction. Our present data indicate that this suggestion should be extended to include one site for 3-*P*-glycerate and another for 1,3-P_2-glycerate also. As the extent of substrate activation is relatively low it is reasonable to assume that this phenomenon appears only in vitro. The product inhibition patterns indicate, however, that the properties of the enzyme offer possibilities for the substrates, even at 'low' concentrations, to regulate very effectively the direction of the reversible reaction at in vivo conditions.

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