

# On the Role of Magnesium in the Reaction of the Pyruvate Kinase from *Salmonella typhimurium*

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The kinetics of the two purified forms of pyruvate kinase from *Salmonella typhimurium* LT-2 were studied in assays at pH 6.8 where the relationships between the initial velocities of the catalysed reactions and  $Mg^{2+}$  are non-hyperbolic. The analysis show that  $Mg^{2+}$  display positive homotropic interactions in their binding behaviour with Hill coefficient values of 2.5 and 1.2 for the form I and II, respectively.

The binding sites of the cation to the pyruvate kinases seem to be independent to those for phosphoenolpyruvate and adenosine 5'-diphosphate; changes in the magnesium concentration might be of physiological significance in relation to a rapid regeneration of adenosine 5'-triphosphate by means of the pyruvate kinase reaction.

## Introduction

Pyruvate kinase (EC 2.7.1.40) plays a key role in cell metabolism since it catalyzes the substrate-linked phosphorylation reaction which produces adenosine 5'-triphosphate (ATP) and pyruvate from phosphoenolpyruvate (PEP) and adenosine 5'-diphosphate (ADP). The pyruvate thus produced can be used directly for the biosynthesis of the many cell metabolites that have their origins in pyruvate or acetyl-coenzyme A or it can be oxidized via the tricarboxylic acid cycle to yield more ATP. Multiple forms of the pyruvate kinase have been found in a variety of animals, plants and microorganisms and their properties and proposed metabolic functions have been reviewed [1–3].

In *Salmonella typhimurium* the presence of the two pyruvate kinases catalyzing the same reaction and the multiplicity of controls operative on these enzymes raise certain questions in relation to the physiological roles and their mechanisms of regulation. In this report we present a study of the effect of magnesium concentration on both enzymic activities, which can contribute to a better understanding of the function of the enzymes in the amphibolic Embden-Meyerhof pathway.

## Materials and Methods

### Bacteria and culture methods

The organism used was *Salmonella typhimurium* LT-2 obtained from the Department of Biochemistry, University of Leicester, U.K. Cells were cultured on glucose as described previously [4].

### Preparation of extracts

Extracts were prepared by ultrasonication of cells suspended in 50 mM Tris-HCl pH 7.5 containing 1 mM EDTA, 2 mM 2-mercaptoethanol and 50 mM KCl, as described previously [4].

### Protein estimation

Protein was measured either colorimetrically, with crystalline bovine serum albumin as standard, by the modified Folin method [5] or spectrophotometrically [6].

### Enzymatic preparations

Pyruvate kinase I and II were purified to homogeneity from the same culture by ammonium sulphate and gel filtration; anion exchange and affinity chromatographies as described by García-Olalla and Garrido-Pertierra [4].

### Enzyme assays

Pyruvate kinase activity was assayed by measuring the decrease in absorbance at 340 nm and 30 °C in a Beckman Model 35 recording spectrophotometer ac-

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cording to the method described previously [4]. The reaction mixture for pyruvate kinase I contained in 1 ml: Hepes buffer, pH 6.8 (30  $\mu$ mol),  $MgCl_2$  (5  $\mu$ mol), ADP (2  $\mu$ mol),  $NADH_2$  (0.15  $\mu$ mol), crystalline lactate dehydrogenase (5  $\mu$ g), PEP (10  $\mu$ mol) and purified pyruvate kinase I. For pyruvate kinase II assay the PEP amount was 1  $\mu$ mol and the purified pyruvate kinase II amount 4  $\mu$ g. The ratio of  $NADH$  oxidized to pyruvate formed was assumed to be unity. One enzyme unit is defined as the amount which catalyses the formation of 1  $\mu$ mol of pyruvate per min, and specific activity is enzyme units per milligram of protein. Assays under other conditions were as described in the text.

#### Calculation of kinetic parameters

The datum points illustrated in the Figures and the results show in the Table are mean values obtained from two independent sets of measurements, each conducted in duplicate. The Hill coefficients ( $h$ ) were calculated from Hill plots [7]. When sigmoidal kinetics were obtained from velocity *versus* substrate concentration plots, the apparent  $V_{max}$  values were estimated from Scatchard or double reciprocal plots.

#### Chemicals

The sodium salt of adenosine 5'-diphosphate, phosphoenolpyruvate, reduced nicotinamide adenine dinucleotide ( $NADH_2$ ), fructose 1,6-bis-

phosphate (FBP), EDTA, Hepes and rabbit muscle lactic dehydrogenase type II were purchased from SIGMA Chemical Co., St. Louis, Mo, USA. Magnesium chloride "Analar" grade and the other chemicals were from Merck, Darmstadt, West Germany.

#### Results and Discussion

Three main roles for the  $Mg^{2+}$  in the reaction mechanism of kinases seem possible: First, the  $MgADP^{2-}$  complex might be the true substrate of the reaction; second, free cation might bind to the enzyme, and then act as a link between the active site and ADP; third, the binding site for  $Mg^{2+}$  might be independent of the sites for the other substrates. When  $Mg^{2+}$  was the variable substrate, the purified pyruvate kinase I showed sigmoidal kinetics, indicating positive cooperative interaction between the bivalent cation and the enzyme. This behaviour was obtained both for a saturating PEP concentration and three fixed ADP concentrations as well as for an ADP concentration and two fixed PEP concentrations (Table I). Hill plots were linear with maximal slopes that indicate the existence of more than two binding sites for  $Mg^{2+}$  in the enzyme with some degree of interaction between them [8]. Likewise, pyruvate kinase II showed sigmoidal kinetics when  $Mg^{2+}$  was the variable substrate at a saturating PEP concentration and two fixed ADP concentrations as well as at an ADP concentration and two fixed PEP

Table I. Kinetic parameter of the homotropic interactions of pyruvate kinases from *S. typhimurium* with  $Mg^{2+}$ .\*

Enzyme	Substrate constant concentration [mm]	Substrate fixed concentration [mm]	$(S)_{0.5}$ [mm]	$h$ (Hill coefficient)	$V_{max}$ [nmol pyruvate formed/min]
Isozyme I	PEP 10.0	ADP 0.25	1.53	2.54	20.0
	PEP 10.0	ADP 0.50	1.50	2.48	30.3
	PEP 10.0	ADP 1.00	1.48	2.51	53.2
	ADP 0.25	PEP 3.50	1.54	2.52	18.0
Isozyme II	ADP 0.25	PEP 10.0	1.48	2.50	36.3
	PEP 3.0	ADP 0.1	0.93	1.23	13.6
	PEP 3.0	ADP 1.0	0.88	1.20	23.8
	ADP 0.25	PEP 0.25	0.94	1.21	20.7
	ADP 0.25	PEP 1.25	0.90	1.19	24.0

\* The experiments were carried out at variable  $Mg^{2+}$  concentration (0.01–5.0 mm) and under standard conditions. The enzymatic concentration used in each assay was 3  $\mu$ g/ml for the form I and 4  $\mu$ g/ml for the form II.

concentrations (Table I). Hill plots were linear with maximal slopes that indicate the existence of more than one binding site for  $Mg^{2+}$ . Since the kinetic parameters for pyruvate kinase I and II remain practically unchanged when ADP and PEP concentrations were varied, it is reasonable to think that the binding sites in both isozymes for ADP, PEP and  $Mg^{2+}$  should be independent between themselves. Moreover, the fact that the  $Mg^{2+}$  concentration necessary for maximum activities is independent of the ADP and PEP concentrations allows to interpret that the PEP, ADP and  $Mg^{2+}$  are the true substrates of the enzymes. These results are in agreement with those observed in homologous enzymes from different biological sources [9–16]. On the other hand when  $Mg^{2+}$  concentration was 5 mM optimum activities for both isozymes were found; according to Macfarlane and Ainsworth [17] and taking into account the different binding species of PEP, ADP with  $Mg^{2+}$  and hydrogen ions at pH 6.8, that  $Mg^{2+}$  concentration corresponds to a free  $Mg^{2+}$  concentration of 1.98 mM. When the purified pyruvate kinases were incubated at 70 °C with 2 mM  $MgCl_2$  and in absence of the other ligands we have found partial protection of the enzymes against thermal inactivation (unpublished experiments), which is an indicative fact that can confirm that  $Mg^{2+}$  is able to bind at the enzymes independently of the presence of the other substrates.

Pyruvate kinase I showed sigmoidal kinetics when PEP was the variable substrate at high ADP concentration and different fixed levels of  $Mg^{2+}$  (Fig. 1A). These data, when replotted in Hill plots, gave lines with maximal slopes ( $h$ ) close to 3.0, which support the existence of more than three PEP-binding sites in the enzyme with some degree of interaction between them [8]. However, when the pyruvate kinase II is treated in the same way as the I, the Hill coefficient decreased from 1.9 in the presence of 0.4 mM  $Mg^{2+}$  to 1.0 in the presence of 5 mM  $Mg^{2+}$  (Fig. 1B), indicating that the  $Mg^{2+}$  interacts with the enzyme eliciting marked conformation changes which can conduce to abolish the sigmoidal response and increase the affinity in the binding of PEP to the enzyme.

Pyruvate kinase I showed hyperbolic kinetics when ADP was the variable substrate at a high PEP concentration and four fixed  $Mg^{2+}$  concentrations (Fig. 2A). The double-reciprocal plots of the saturation data intersected on the ordinate axis, having estimated a  $V_{max}$  value of 72.7 nmol pyruvate formed/

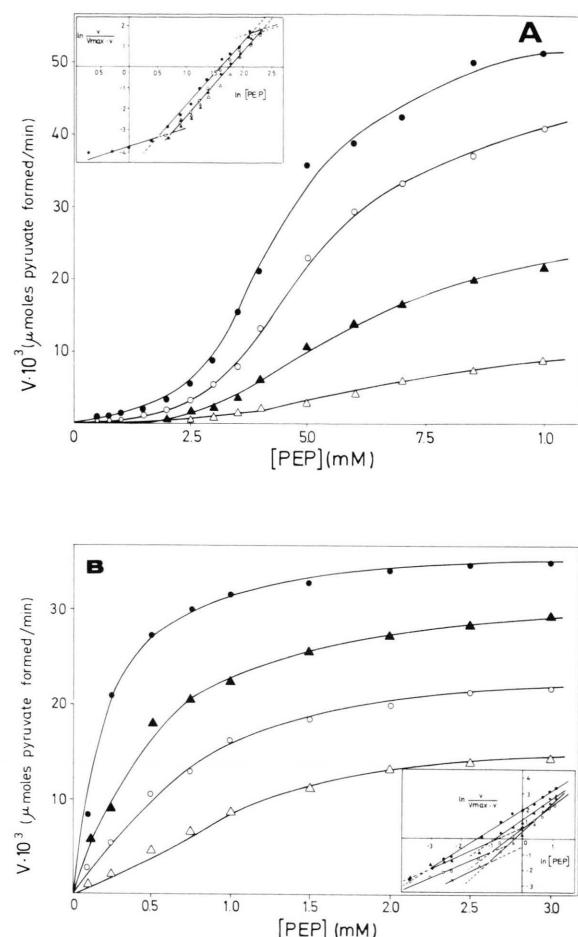


Fig. 1. Effect of the PEP concentration on the reaction catalyzed by pyruvate kinases in the presence of variable  $Mg^{2+}$  concentration.

(A) Assay mixtures contained in 1 ml: 30 mM Hepes buffer pH 6.8, 0.15 mM  $NADH_2$ , 5  $\mu$ g crystalline lactate dehydrogenase, 10.0 mM ADP, 3  $\mu$ g of purified pyruvate kinase I and four fixed  $Mg^{2+}$  concentrations, (Δ) 1.0 mM, (▲) 1.5 mM, (○) 2.5 mM and (●) 5.0 mM. (B) Assay mixtures contained in 1 ml: 30 mM Hepes buffer pH 6.8, 0.15 mM  $NADH_2$ , 5  $\mu$ g crystalline lactate dehydrogenase, 1.0 mM ADP, 4  $\mu$ g of purified pyruvate kinase II and four fixed  $Mg^{2+}$  concentrations, (Δ) 0.04 mM, (○) 0.75 mM, (▲) 1.5 mM and (●) 5.0 mM. Hill plots of the data saturation curves for PEP are shown in the insets.

min. The saturation effect with ADP appears to diminish the requirements for the bivalent cation, since the same  $V_{max}$  value was obtained irrespective of the  $MgCl_2$  concentration. Likewise, pyruvate kinase II showed hyperbolic saturation curves with respect to ADP concentration (Fig. 2B). The double-recipro-

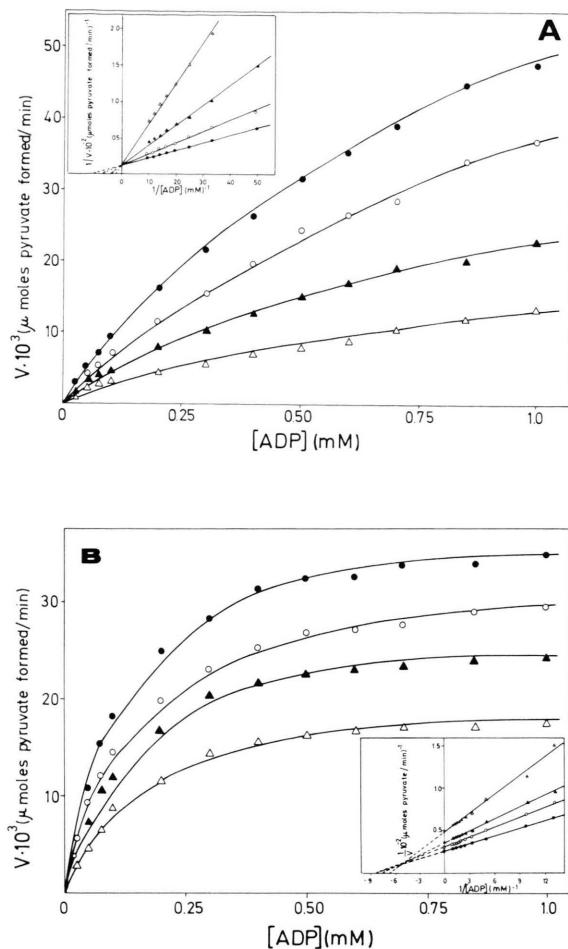


Fig. 2. Effect of ADP concentration on the reaction catalyzed by pyruvate kinases in the presence of variable  $Mg^{2+}$  concentration.

(A) Assay mixtures contained in 1 ml: 30 mM Hepes buffer pH 6.8, 0.15 mM NADH<sub>2</sub>, 5 μg crystalline lactate dehydrogenase, 10 mM PEP, 3 μg of purified pyruvate kinase I and four fixed  $Mg^{2+}$  concentrations, (Δ) 1.0 mM (▲) 1.5 mM, (○) 2.5 mM and (●) 5.0 mM. (B) Assay mixtures contained in 1 ml: 30 mM Hepes buffer pH 6.8, 0.15 mM NADH<sub>2</sub>, 5 μg crystalline lactate dehydrogenase, 3.0 mM PEP, 4 μg of purified pyruvate kinase II and four fixed  $Mg^{2+}$  concentrations, (Δ) 0.40 mM, (▲) 0.75 mM, (○) 1.5 mM and (●) 5.0 mM. Lineweaver-Burk plots are shown in the insets.

cal plots of the saturation data for a high PEP concentration and four fixed  $Mg^{2+}$  concentrations intersected to the left of the ordinate axis, thus indicating that the addition of  $Mg^{2+}$  and ADP to the enzyme did not follow an obligatory order.

In *S. typhimurium* pyruvate kinase activities are modified by some phosphorylated compounds where fructose 1,6-bisphosphate (FBP) and AMP were the best activators found for the form I and II, respectively [4]. The activator processes seem synergistic between PEP and FBP for pyruvate kinase I and PEP and AMP for pyruvate kinase II (Fig. 3), since at high concentrations of PEP no heterotropic activa-

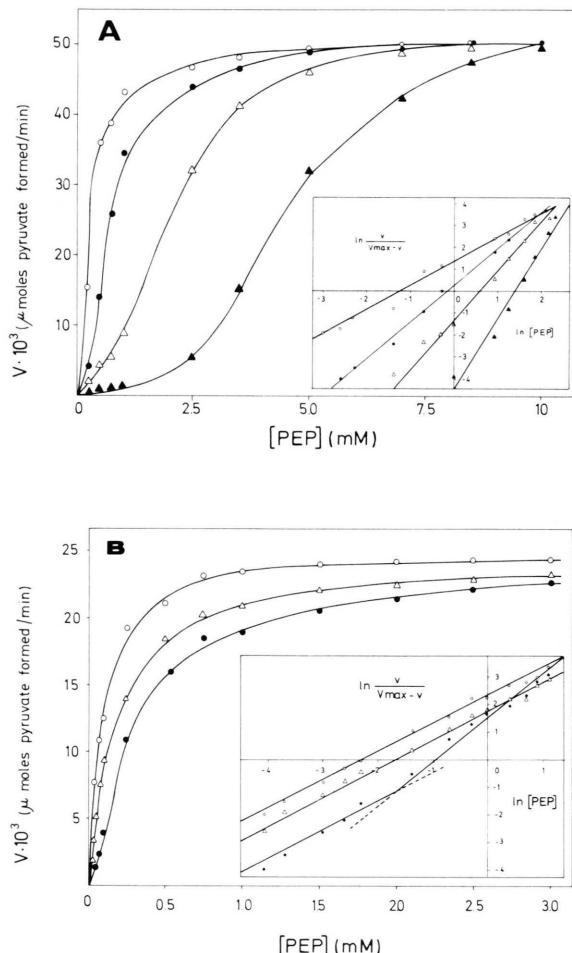


Fig. 3. Effects of the FBP and AMP on the kinetic responses of pyruvate kinases I and II with respect to PEP concentration.

(A) Assays were carried out in 30 mM Hepes pH 6.8 at 30 °C and 3 μg of purified pyruvate kinase I. FBP was added as indicated: (▲) without FBP; (Δ) 0.01 mM; (●) 0.05 mM; (○) 0.1 mM. (B) Assays were carried out in 30 mM Hepes buffer pH 6.8 and 4 μg of purified pyruvate kinase II. AMP was added as indicated: (●) without AMP; (Δ) 0.1 mM; (○) 1.0 mM. Hill plots are shown in the insets.

tions were observed and at high concentrations of effectors no homotropic interaction PEP sites were found. When the bacteria grow on glycolytic substrates the concentration of FBP is relatively high [18] and a concentration of free-Mg<sup>2+</sup> as low as 1 mM (Fig. 3A) should be enough to obtain the maximum activity of the form I. However, when *S. typhimurium* grows on gluconeogenic compounds the level of FBP is much lower than in glycolytic conditions. If a value of 0.24–0.30 mM for the intracellular PEP concentration during growth on gluconeogenic compounds can be assumed to be correct [18, 19] the form I is almost inactive whereas the form II can catalyse the reaction to almost half the

maximum reaction rate at a saturating Mg<sup>2+</sup> concentration. On the other hand, the activation of AMP on the pyruvate kinase II (Fig. 3B) might be important because an increasing of its concentration is a chemical signal of a decrease in the energy charge, and this activation is higher when the concentration of available Mg<sup>2+</sup> for the enzyme increases. Taking into account that an increasing in the Mg<sup>2+</sup> concentration changes the kinetic and the affinity of the enzyme by PEP, the intracellular concentration changes between free-Mg<sup>2+</sup> and bound-Mg<sup>2+</sup> might be of great significance in the regulation of the pyruvate kinase II when cells require a rapid transformation of PEP available into ATP.

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