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INTERACTION OF MAGNESIUM ION WITH PYRUVATE KINASE*

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ABSTRACT - Using the technique of gel filtration, the enzyme pyruvate kinase is shown to bind approximately 40 magnesium ions per molecule of enzyme at 25°C and pH 7.0. The intrinsic binding constant at low ionic strengths was estimated to be 4×10^4 liters/mol. No effect on the binding of Mg^{++} could be detected when Li^+ was substituted for K^+ at an ionic strength of 0.025M.

In addition to its two substrates, pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40) requires the presence of both Mg^{++} and K^+. (Boyer and Lardy, 1942). Up to the present time, no complete explanation of the role played by either of the two metal ions has been given. Kinetic studies in our laboratory (Melchior, 1965) have indicated that the monovalent ion exerts its effect through a direct interaction with the enzyme protein, while the divalent ion is involved in the formation of a complex ion with ADP. On the other hand, Mildvan and Cohn (1965) have been able to use the technique of nuclear magnetic resonance by substituting Mn^{++} for Mg^{++} and have shown that the divalent cation functions as a metal-enzyme complex. These workers further demonstrated that one divalent cation is bound per active site of the enzyme protein. More recent kinetic studies have tended to support this view and have suggested that free ADP rather than the Mg^{++} complex ion is the active species in the catalytic event. (Ainsworth and MacFarlane, 1975). It is apparent that two totally conflicting pieces of evidence about the role of the divalent ion have emerged.

It should be noted that Melchior and Melchior (1958) have shown the inherent difficulty of using kinetic data to determine the interactions in a system involving complex ions. Equilibrium measurements have been made using Mn^{++} as ligand, however several workers have reported that the properties of the enzyme in the presence of Mn^{++} differ significantly from those observed in the present of the physiological activator. (Suelter and Melander, 1963); Kayne and Price (1972) Leonard (1972).

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It is apparent that only equilibrium binding measurements can resolve these problems. Because of these considerations, we have employed gel filtration to measure the binding of Mg^{++} by pyruvate kinase.

METHODS

Rabbit muscle pyruvate kinase (PK) obtained from Calbiochem, was stored at 2°C as a suspension in 2 M ammonium sulfate. Before use, the crystals of enzyme were collected by centrifugation at 10,000 x g for 1 hour, followed by dialysis in the chamber described by Colowick and Wormack (1969) until free of ammonium ions. The enzyme concentration was determined by using the extinction coefficient of 0.52 at 278 mμ. The PK is a tetramer composed of four non-active monomers weighing approximately 60,000 daltons. For comparison, measurements were made of bovine serum albumin (BSA, 65,000 daltons) and β-lactoglobulin (βLG, 35,000 daltons). Both reference proteins were crystalline products, and the concentrations were calculated directly from the dry weights of protein.

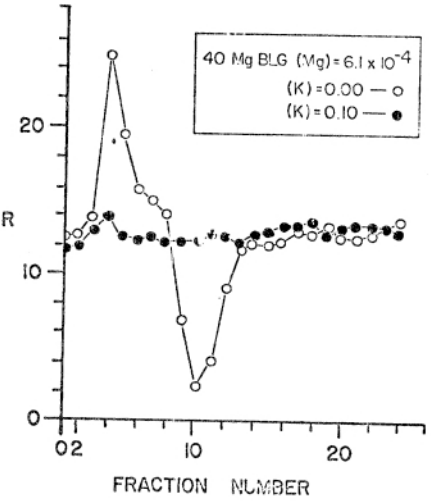
The columns used to measure the binding were filled with Sephadex (G-25-300) obtained from Sigma. The gel particles act as small spherical semipermeable membranes which allow the entrance of small molecules but exclude large ones; thus the protein-Mg^{++} complex moves rapidly through the column and is separated from its binding region. One ml sample of protein dissolved in a salt-buffer solvent was placed on top of the column which was equilibrated with the same solvent. Fractions were collected in preweighed disposable plastic tubes at a rate of 2.5 ml every 10 minutes. The tubes were recapped and reweighed to determine the exact volume of each fraction. Mg^{++} was determined on an aliquot of each fraction either by titration with ehtylenediamine tetraacetic acid using erichrome black-T as indicator or by use of a Perkin Elmer model 303 atomic absorption spectrophotometer.

The columns were 0.9 x 60 cm. and were water-jacketed. Temperature was maintained at 25°C. All solutions were kept at pH 7.0 by using a solution containing 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 2.5 mM tetraethylammonium or tetramethylammonium hydroxide.

Figure 1 illustrates a measurement of the binding of Mg^{++} by βLG; the protein-Mg^{++} complex moves through the column ahead of the "hole" which presents the amount of Mg^{++} removed by the protein.

FIGURE 1

BINDING OF MAGNESIUM BY β-LACTOGLOBULIN AS DETERMINED SEPHADEX GEL CHROMATOGRAPHY



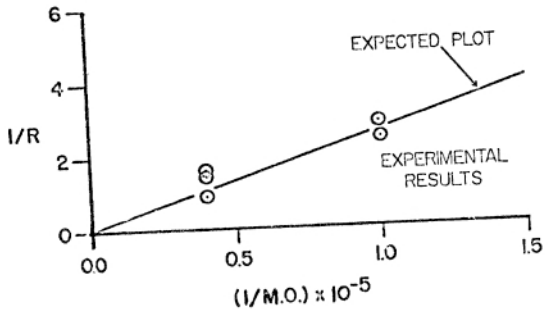
$$R = \frac{\text{Volume of Titrant}}{\text{Volume of Fraction}}$$

The binding data is presented in the form developed by Scatchard (1949). The reciprocal of R, which is defined as molecules of ligand bound per molecule of protein, is plotted against the reciprocal of the concentration of ligand. It has been shown that the slope of these graphs can be used to calculate the intrinsic binding constant, while the intercept is the reciprocal of the number of binding sites.

To verify the method, experiments were made on the binding of methyl orange by BSA at 7.5°C and compared to the classical equilibrium dialysis experiments of Klotz, et.al. (1946). Figure 2 shows the excellent agreement of the two methods.

FIGURE 2

BINDING OF METHYL ORANGE BY BSA



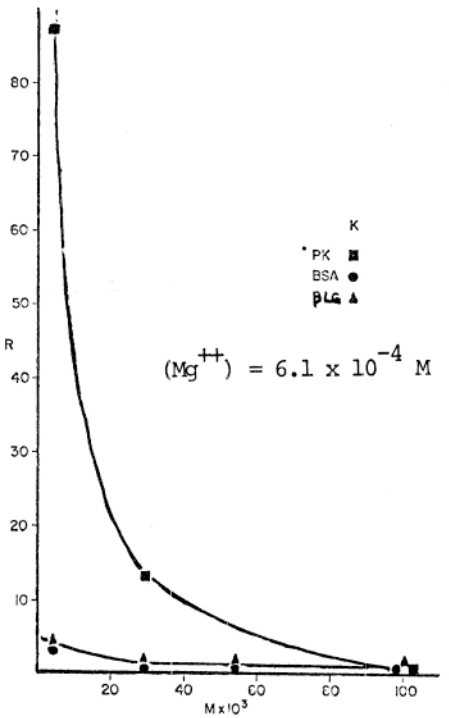
RESULTS

There are two major experimental problems in making equilibrium measurements of interactions of ions with pyruvate kinase. One is related to the stability of concentrated solutions; we have never achieved a solution of more than 3 x 10^{-5}M, which is equilivalent to 1.2 x 10^{-4}M active sites. This has necessitated making the studies of binding in extremely dilute solutions of ligand. The second problem is related to the fact that 0.1 M KCl is required for optimal activity of the enzyme; thus measurements of the completely active enzyme require high ionic strength.

The effect of ionic strength on the binding of Mg^{++} is illustrated in Figure 3. It is apparent that pyruvate kinase is particularly sensitive to changes in ionic strength: βLG and BSA show much smaller changes in binding as the salt concentration is increased.

FIGURE 3

BINDING OF Mg^{++} VERSUS IONIC STRENGTH



The sensitivity of binding to ionic strength coupled with the difficulty in achieving high concentrations of binding sites make estimation of binding of Mg^{++} by PK at its optimal catalytic activity impossible by any of the existing equilibrium methods. Measurements were made on the two reference proteins at low ionic strength and at 0.025 M; the results shown in Figures 4 and 5. It is apparent that the major effect of electrolytes is on the intrinsic binding constant rather than on the number of binding sites.

FIGURE 4

BINDING OF Mg^{++} BY BSA

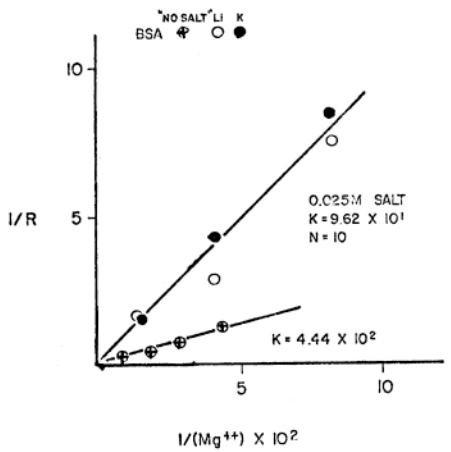
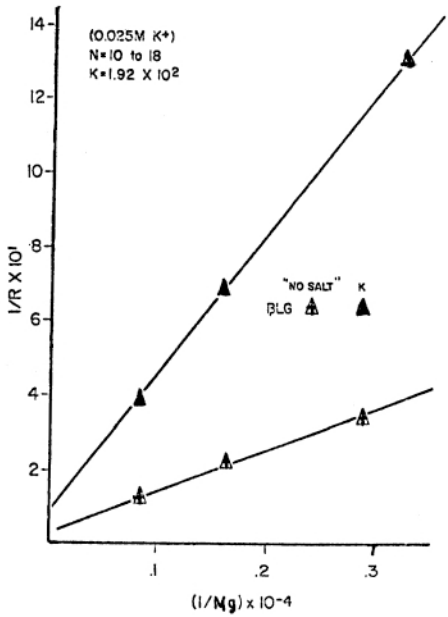


FIGURE 5

BINDING OF Mg^{++} BY βLG IN PRESENCE OF 0.025M K^{+}



The problems inherent in making measurements on PK are emphasized in Figure 6. In the absence of background electrolyte, the inhibition of binding at high ionic strength produces an apparent maximum in the binding curve. Attempts to gather data at 0.025 M salt solution produced a wide scatter of results which made calculation of the usual constants meaningless. However, it was noted that no difference in the effects of Li^+ and K^+ could be observed.

FIGURE 6

BINDING OF Mg^{++} BY PYRUVATE KINASE

R VERSUS (Mg^{++})

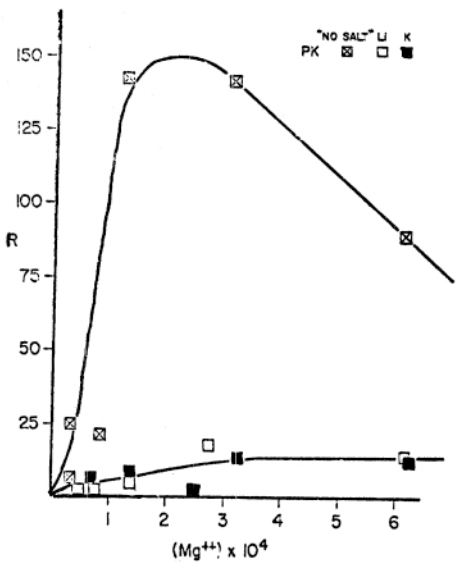
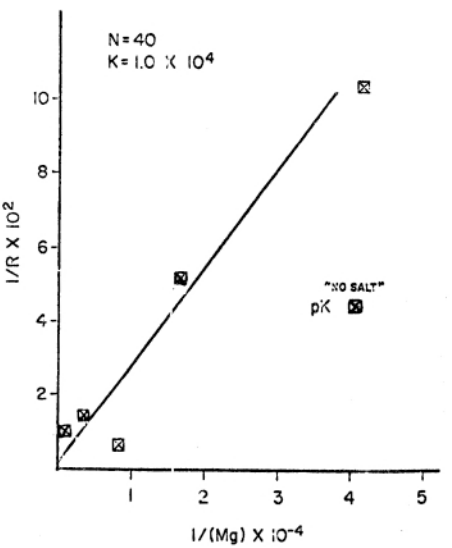


FIGURE 7

BINDING OF Mg^{++} BY PYRUVATE KINASE

NO K^+ OR Li^+ PRESENT



A Scatchard plot of the data collected the lowest ionic strength is shown in Figure 7. PK appears to bind approximately 40 Mg^{++} with an intrinsic binding constant of about 1 x 10^4 liters per mole.

DISCUSSION

It is apparent that equilibrium binding measurements are required to establish the exact role of the metal ion activators in the pyruvate kinase reaction. However, the high ionic strength required for optimal activity of the enzyme coupled with the instability of concentrated solutions of the enzyme preclude obtaining valid results by any of the known techniques. At low ionic strengths, PK binds Mg^{++} strongly, showing an intrinsic binding constant that is approximately 20 fold greater than that observed with BSA or βLG.

The data presented on the binding of Mg^{++} by BSA and βLG indicated that the effect of ionic strength is to alter the apparent intrinsic binding constant, not the number of binding sites. The effects of ionic strength on the activity of a protein are complex and difficult to predict (Tanford, 1967). From the results presented here, it appears that increasing the salt concentration lowers the activity coefficients of the free ligand or the protein more than that of the Mg^{++} protein complex. Assuming the same situations holds for PK, we can conclude that even when fully activated, PK can bind up to 40 molecules of Mg^{++} per mole of enzyme, or about 10 per active site. This is quite different from the conclusion of Mildvan and Cohn (1965) who employed Mn^{++} to demonstrate that one divalent cation is bound per active site when the enzyme is fully activated by the monovalent cation.

These results emphasize the great caution that must be used in translating kinetic studies into conclusions about equilibrium binding. Because pyruvate kinase is specifically affected by both monovalent and divalent cations, it is impossible to study the effects of ionic strength per se on its catalytic potency.

A number of previous attempts to find an effect of K^+ on the enzyme that is not duplicated by the non-activating ion Li^+ have met with failure. Techniques that have been used include circular dichromism (Wildes, et. al., 1971), binding of ADP and Phosphoenolpyruvate (Betts and Evans, 1968), sovent perterbation techniques (Melchior, et. al., 1969, 1970). To this list we can now add the apparent lack of any difference in the binding of Mg^{++} when 0.025 M Li^+ is substituted for 0.025 M K^+.

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