Studies on the Interaction of Metal Ions with Pyruvate Kinase from Ehrlich Ascites-Tumour Cells and from Rabbit Muscle

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1. Pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40) from Ehrlich ascites-tumour cells was purified approximately fivefold by chromatography on DEAE-cellulose. The enzyme was shown to have an absolute requirement for one univalent and for one bivalent metal ion. 2. The univalent metal ion requirements were satisfied by K^+ , Rb^+ or NH_4^+ ; Na^+ and Cs^+ were weak activators but Li^+ was inactive. 3. Ca^{2+} exhibited 'non-competitive' and 'apparent competitive' effects in relation to the K^+ activation. 4. The bivalent metal ion requirements were satisfied by Mg^{2+} , Mn^{2+} or Co^{2+} ; Ba^{2+} , Sr^{2+} , Ca^{2+} , Ni^{2+} , Be^{2+} and Cu^{2+} were inactive. Mn^{2+} and Co^{2+} were better activators than Mg^{2+} . 5. The bivalent metal ion requirements of purified pyruvate kinase from rabbit muscle were satisfied by Mg^{2+} , Mn^{2+} , Co^{2+} and to a smaller extent by Ni^{2+} . Mn^{2+} and Co^{2+} were better activators than Mg^{2+} . 6. Ca^{2+} competitively inhibited the activation by Mg^{2+} , Mn^{2+} and Co^{2+} for both the tumour and rabbit enzymes. 7. It is concluded that there are no significant differences in metal ion specificity between the tumour and rabbit enzymes. 8. The possible role of metal ions in regulating enzymic and metabolic activities is considered further.

In the preceding paper (Bygrave, 1966) it was shown that inhibition of the pyruvate kinase (ATP-pyruvate phosphotransferase, EC 2.7.1.40) reaction by added Ca^{2+} largely accounted for the overall inhibition of glycolysis by these ions in extracts of Ehrlich ascites-tumour cells. The present studies on the metal ion activation of pyruvate kinase were carried out to gain a clearer insight into the mechanism of that inhibition.

Where under physiological conditions an enzyme is activated by one cation and inhibited by a second, an accurate control of the activity of that particular enzyme is possible by small alterations in the ionic environment. Particularly sensitive control would be expected where more than one cation was required for enzyme activation. Pyruvate kinase appears to be an ideal subject for further study of this phenomenon since some evidence is available for its activation by Mg^{2+} and K^+ and its inhibition by Ca^{2+} and Na^+ (Boyer, 1962), all of which are commonly occurring cell constituents. Because this evidence is incomplete and because the system as it occurs in tumour cells could lend itself to the type of

delicate control mentioned above, the relation of the Ca^{2+} inhibition to the Mg^{2+} and K^+ activation in particular was investigated in ascites-tumour cells.

The studies entailed an investigation into the metal ion activation of pyruvate kinase of Ehrlich ascites-tumour cells and into the effect of Ca^{2+} on these activations. Such studies have not previously been carried out with a pyruvate kinase from this source.

Studies on the bivalent metal ion requirements of the purified pyruvate kinase from rabbit muscle were also undertaken. It was hoped that these studies would determine whether significant differences in metal ion specificity existed between the pyruvate kinase obtained from rabbit muscle and that obtained from Ehrlich ascites-tumour cells.

MATERIALS AND METHODS

Ascites-tumour cells. The Ehrlich ascites-tumour cells were propagated and harvested as described previously (Bygrave, 1966). Since Na^+ inhibits pyruvate-kinase activity it was advantageous to prepare the tumour-cell suspension in 5 mM-tris-HCl buffer, pH 7.4, instead of in 0.9% NaCl.

Chemicals. Substrates, coenzymes, metal salts, tris and

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EDTA were obtained from the sources described previously (Bygrave, 1966).

Enzymes. Lactate dehydrogenase (L-lactate-NAD oxidoreductase, EC 1.1.1.27), type II, prepared from rabbit muscle, was a product of the Sigma Chemical Co., St Louis, Mo., U.S.A., and was found to be free from pyruvate kinase when assayed under the conditions described in Fig. 1. Crystalline pyruvate kinase from rabbit muscle was prepared according to the procedure of Bücher & Pfeleiderer (1955). Before use, a portion of the stock enzyme (stored at 4°) was dialysed against frequent changes of 0.05 M-tris-HCl buffer, pH 7.4, for 48 hr. at 4°. For enzyme assays, suitable dilutions of the stock enzyme were made in 0.05 M-tris-HCl buffer, pH 7.4.

Analysis of kinetic data. Values for the parameters of the primary double-reciprocal plots were obtained according to the method of Wilkinson (1961). All calculations were made by using an IBM 1620 computer.

Oxidation of NADH. Oxidation of NADH was followed by measuring the change in extinction at 340 m μ in a Beckman DU or in a Unicam SP.500 spectrophotometer. Reaction mixtures were contained in silica cuvettes having a 1 cm. light-path. The temperature was 25° in all experiments.

Assay of pyruvate-kinase activity. The assay adopted for measurement of pyruvate-kinase activity was a modification of the spectrophotometric procedure used by Bücher & Pfeleiderer (1955). Because the assay is a compound one involving two enzymes, the curve linking pyruvate-kinase activity, as measured by the decrease in extinction at 340 m μ , against time, became linear only after 30 sec. To obtain the initial reaction velocity, the decrease in extinction between 30 and 90 sec. was therefore measured. Initial reaction velocity determined in this way was proportional to enzyme concentration over a 20-fold range for both the 'tumour' and 'rabbit' enzymes.

Purification of pyruvate kinase from ascites-tumour cells. Although the limited availability of the ascites cells prevented a large-scale purification of pyruvate kinase, it was possible to purify the enzyme five- to seven-fold by using DEAE-cellulose.

The supernatant fraction obtained from the tumour-cell homogenate (prepared by ultrasonic disintegration) was dialysed for 48 hr. against frequent changes of 5 mM-tris-HCl buffer, pH 7.4, before chromatography. A sample of the dialysed supernatant (about 10 ml. containing 67 mg. of protein) was applied to a DEAE-cellulose column (7.5 cm. $\times$ 0.64 cm.²) under gravity. The adsorbed protein was eluted with 5 mM-tris-HCl buffer, pH 7.4, and 3.0 ml. fractions of the eluate were assayed for enzymic activity. Pyruvate-kinase activity was found in fractions 1-8 with a peak at fraction 4. Fractions 3, 4 and 5, containing 70% of the activity, were pooled for kinetic studies and this is referred to below as 'tumour enzyme'. Such a pattern of pyruvate-kinase activity in the eluate fraction as described above is completely reproducible. Purifications of pyruvate-kinase activity in the range five- to seven-fold were usually obtained with the peak fraction. The yield of tumour enzyme was generally only about 50%. The enzyme was unstable after chromatography and lost most of its activity after about 4 days when stored at 2-4°. It was therefore freshly prepared for each kinetic experiment. The tumour and rabbit enzymes were assayed at the concentrations of 30 μ g. and 3 μ g. respectively per cuvette.

RESULTS

Univalent metal ion specificity of tumour pyruvate kinase

In preliminary studies it was observed that, if either Mg²⁺ or K⁺ was omitted from the reaction mixture, enzymic activity was never more than 2% of the maximum attainable. Addition of EDTA to give a final concentration of 1 mM decreased this residual enzymic activity to less than 1% of the maximum attainable. These preliminary studies (Bygrave, 1964) also showed that those metal ions which influenced pyruvate-kinase activity (see below) did not influence lactate-dehydrogenase activity.

With the total added Mg²⁺ concentration held constant at 8 mM, the ability of various metal ions to replace K⁺ as univalent metal ion activator was studied. Of the ions tested at 75 mM only Rb⁺ and NH₄⁺ could replace K⁺ to any appreciable extent. Na⁺ and Cs⁺ showed a weak activating effect, but Li⁺ was without effect. Fig. 1 shows the effect of univalent metal ion concentration on the reaction velocity of the tumour enzyme at a fixed Mg²⁺ concentration. Maximum reaction velocities were observed at metal ion concentrations of 0.035 M, 0.06 M and 0.15 M for NH₄⁺, Rb⁺ and K⁺ respectively.

Fig. 1. Effect of K⁺, Rb⁺ and NH₄⁺ concentrations on the reaction velocity of the tumour enzyme. Each cuvette contained: tris-HCl buffer, pH 7.4, 40 μ moles; MgCl₂, 8 μ moles; ADP, 0.5 μ mole; NADH, 0.5 μ mole; phosphoenolpyruvate (added last), 0.25 μ mole; diluted lactate dehydrogenase, 0.02 ml.; tumour enzyme, 0.05 ml. The univalent metal ion concentrations were varied as indicated. The final volume was 1.0 ml. Incubation was at 25°. O, K⁺; ●, Rb⁺; Δ, NH₄⁺.

Fig. 2. Effect of Mg^{2+} , Mn^{2+} and Co^{2+} concentrations on the reaction velocity of tumour and rabbit pyruvate kinase. Assay conditions were the same as those described in Fig. 1, except that the metal ion concentrations were varied as indicated. The final volume was 1.0 ml. Incubation was at 25° . (a) 0.05 ml. of tumour enzyme; (b) 0.05 ml. of diluted rabbit enzyme. $\bullet$, Mn^{2+} ; Δ , Co^{2+} ; $\circ$, Mg^{2+} .

The reaction velocity fell off rapidly as the concentration of activating ions was increased beyond that required for maximum reaction velocity.

Bivalent metal ion specificity of pyruvate kinase

The following studies were carried out with the total K^+ concentration maintained at 75 mM. Of the bivalent metal ions tested (at a concentration of 2 mM) only Co^{2+} and Mn^{2+} could replace Mg^{2+} . Ni^{2+} showed a slight activating effect with the rabbit enzyme only. The following ions were tested but produced no activation: Ca^{2+} , Sr^{2+} , Ba^{2+} , Cu^{2+} , Y^{3+} , La^{3+} and Be^{2+} . Fe^{2+} , Zn^{2+} , Hg^{2+} , Cd^{2+} and Pb^{2+} interfered in the assay of pyruvate kinase.

The effects of Mg^{2+} , Mn^{2+} and Co^{2+} concentration on the reaction velocity of the tumour and rabbit enzymes are shown in Fig. 2. In each case typical Michaelis curves were produced. Maximum reaction velocities were observed at Co^{2+} , Mn^{2+} and Mg^{2+} concentrations of 1.5 mM, 2 mM and 8–10 mM respectively for the tumour enzyme and at 1–2 mM, 4–5 mM and 7–10 mM respectively for the rabbit enzyme. The velocity of the reaction fell off after the optimum concentration had been reached with both enzymes, rapidly with Co^{2+} and more gradually with Mn^{2+} or Mg^{2+} as bivalent metal ion activator.

Kinetic studies on the inhibition of pyruvate-kinase activity by added Ca^{2+}

Ca^{2+} inhibition of Mg^{2+} activation. The effect of variation of the total Mg^{2+} concentration ($[\text{Mg}_T^{2+}]$) at various concentrations of Ca^{2+} was studied.

Fig. 3. Influence of Ca^{2+} on the Mg^{2+} activation of rabbit pyruvate kinase. Assay conditions were the same as those described in Fig. 1, except that the total Mg^{2+} concentration was varied in the range of 0.1–0.25 mM and Ca^{2+} was added as indicated; 0.05 ml. of rabbit enzyme was used. The final volume was 1.0 ml. Incubation was at 25° . $\circ$, No added Ca^{2+} ; $\bullet$, 0.5 mM- Ca^{2+} ; Δ , 1 mM- Ca^{2+} .

Plots of the reciprocal of the reaction velocity ($1/v$) against the reciprocal of the total Mg^{2+} concentration ($1/[\text{Mg}_T^{2+}]$) were made with both the rabbit and tumour enzymes. As shown in Figs. 3 and 4, such plots had a common intercept. Further, in each case the ratios of slope to intercept increased with increasing concentrations of Ca^{2+} .

Since the data in Figs. 3 and 4 show that the relation of Ca^{2+} to Mg^{2+} was essentially the same for

Fig. 4. Influence of Ca^{2+} on the Mg^{2+} activation of tumour pyruvate kinase. Assay conditions were the same as those described in Fig. 1, except that the total Mg^{2+} concentration was varied in the range 0.12–0.48 mM and Ca^{2+} was added as indicated; 0.05 ml. of tumour enzyme was used. The final volume was 1.0 ml. Incubation was at 25°. $\circ$, No added Ca^{2+} ; $\bullet$, 0.3 mM- Ca^{2+} ; $\triangle$, 0.6 mM- Ca^{2+} .

Fig. 5. Influence of Ca^{2+} on the K^{+} activation of tumour pyruvate kinase. Assay conditions were the same as those described in Fig. 1, except that the total K^{+} concentration was varied in the range 20–60 mM and Ca^{2+} was added as indicated; 0.05 ml. of tumour enzyme was used. The final volume was 1.0 ml. Incubation was at 25°. $\circ$, No added Ca^{2+} ; $\bullet$, 1 mM- Ca^{2+} ; $\triangle$, 3 mM- Ca^{2+} .

Table 1. *Effect of K^{+} on the Ca^{2+} inhibition of tumour pyruvate kinase*

Assay conditions were the same as those described in Fig. 1, except that the K^{+} concentration was varied and that Ca^{2+} was added as indicated. The final volume was 1.0 ml. Incubation was at 25°.

Concn. of K^{+} (M)	Concn. of Ca^{2+} (M)	Reaction velocity ($\Delta E_{340}/\text{min.}$)	Inhibition by Ca^{2+} (%)
0.05	0	0.132	—
0.05	0.002	0.084	36
0.10	0	0.160	—
0.10	0.002	0.104	35
0.15	0	0.152	—
0.15	0.002	0.108	29

Ca^{2+} inhibition of K^{+} activation. Plots of the reciprocal of the reaction velocity ($1/v$) against the reciprocal of the total K^{+} concentration ($1/[\text{K}_T^{+}]$) were made with the tumour enzyme at different Ca^{2+} concentrations. As shown in Fig. 5 such plots did not have a common intercept and the ratios of slope to intercept decreased with increasing concentrations of Ca^{2+} . Additional studies showed that the Ca^{2+} inhibition of pyruvate kinase could be partially reversed by addition of further K^{+} (Table 1).

Effect of Ca^{2+} on the Mn^{2+} and Co^{2+} activation of tumour and rabbit pyruvate kinase. In view of the apparent antagonism between Ca^{2+} and Mg^{2+} towards pyruvate-kinase activity and since Mn^{2+} and Co^{2+} can replace Mg^{2+} as bivalent metal ion activator for this enzyme, it was decided to determine whether antagonistic relations also existed between Ca^{2+} and Mn^{2+} and between Ca^{2+} and Co^{2+} .

Fig. 6 shows the relation between Ca^{2+} inhibition and Co^{2+} activation of the tumour and rabbit enzymes. Plots of $1/v$ versus $1/[\text{Co}_T^{2+}]$ at different Ca^{2+} concentrations were made with each enzyme. Such plots had a common intercept and the ratios of slope to intercept increased with increasing Ca^{2+} concentration.

The relationship between Ca^{2+} inhibition and Mn^{2+} activation was similarly studied for each enzyme. Plots of $1/v$ versus $1/[\text{Mn}_T^{2+}]$ at different Ca^{2+} concentrations had a common intercept and the ratios of slope to intercept increased with increasing Ca^{2+} concentration (Fig. 7).

DISCUSSION

the rabbit and tumour enzymes, it was decided to determine whether the relation between Ca^{2+} inhibition and K^{+} activation of the tumour enzyme was similar to that of the rabbit enzyme as described by Kachmar & Boyer (1953).

The absolute requirement of pyruvate kinase for K^{+} was questioned by other workers (Happold & Beechey, 1958) on the grounds that high concentrations of K^{+} were required for activation. The

Fig. 6. Influence of Ca^{2+} on the Co^{2+} activation of pyruvate kinase. Assay conditions were the same as those described in Fig. 1, except that Co^{2+} (varied in the range 0.1–0.24 mM) replaced Mg^{2+} . The final volume was 1.0 ml. Incubation was at 25°. (a) 0.05 ml. of tumour enzyme; (b) 0.05 ml. of rabbit enzyme. ○, No added Ca^{2+} ; ●, 0.05 mM- Ca^{2+} ; △, 1.0 mM- Ca^{2+} .

Fig. 7. Influence of Ca^{2+} on the Mn^{2+} activation of pyruvate kinase. Assay conditions were the same as those described in Fig. 1, except that Mn^{2+} (varied in the range 1.0–0.24 mM) replaced Mg^{2+} . The final volume was 1.0 ml. Incubation was at 25°. (a) 0.05 ml. of tumour enzyme; (b) 0.05 ml. of rabbit enzyme. ○, No added Ca^{2+} ; ●, 0.6 mM- Ca^{2+} ; △, 1.5 mM- Ca^{2+} .

present studies have shown that the partially purified tumour enzyme has an absolute requirement for a univalent as well as for a bivalent metal ion and therefore confirm the findings of Kachmar & Boyer (1953), who used the purified rabbit enzyme.

Since K^+ , Rb^+ and NH_4^+ activate the tumour enzyme, and since Na^+ , Li^+ and Cs^+ were either very

weak activators or inactive, it is concluded that the univalent metal ion specificity of the tumour enzyme is the same as that found for the rabbit (Kachmar & Boyer, 1953), plant (Miller & Evans, 1957) and mammalian erythrocyte (Solovonuk & Collier, 1955) enzymes. The role of K^+ in phosphoryl group transfer is still uncertain. On the whole it does not appear to play any specific part in the phosphate

transfer (Boyer & Harrison, 1954) but is most probably concerned in combination effects with the enzyme independently of any combination with phosphoenolpyruvate (Kachmar & Boyer, 1953).

The bivalent metal ion specificity of the tumour enzyme is, like the univalent metal ion specificity, similar to that of the enzyme from rabbit (Mildvan & Cohn, 1965; Fig. 2b), plants (Miller & Evans, 1957) and mammalian erythrocytes (Solovuk & Collier, 1955). Mn^{2+} and Co^{2+} were better activators than Mg^{2+} with both the rabbit and tumour enzymes. That Ni^{2+} showed a weak activating effect with the rabbit and not with the tumour enzyme is most probably a reflection of the difference in the state of purity of the two enzymes. This difference in purity would also account for the slightly different patterns in activation curves (Fig. 2).

The inhibition of Mg^{2+} , Mn^{2+} and Co^{2+} activation by Ca^{2+} is on the whole competitive, although with the Co^{2+} -activated tumour enzyme (Fig. 6a) and the Mn^{2+} -activated rabbit enzyme (Fig. 7b) it is difficult to be certain of this. Since a metal ion-nucleotide complex is thought to be the functional substrate for phosphoryl group transfer enzymes (Nordlie & Lardy, 1962), a competitive inhibition could then be due to the fact that added Ca^{2+} decreases the concentration of the 'active' metal ion-adenine nucleotide complex by forming an 'inactive' Ca^{2+} -adenine nucleotide complex. The values for bivalent metal ions in the presence of the added Ca^{2+} would then be apparent rather than real. Relative values for the apparent stability constants for metal ion-nucleotide complexes are generally in the following decreasing order (Bock, 1960): Mn^{2+} ; Co^{2+} ; Mg^{2+} ; Ca^{2+} ; Sr^{2+} . The relative effectiveness of activation by Mn^{2+} , Co^{2+} and Mg^{2+} of pyruvate kinase is consistent with this order.

In the light of recent work on metal ion binding to pyruvate kinase (Mildvan & Cohn, 1965), however, the conclusion that the metal ion-nucleotide is the substrate in the pyruvate-kinase reaction must remain tentative. Mildvan & Cohn (1965) carried out magnetic-resonance studies on the bivalent metal ion complexes in the pyruvate-kinase reaction and concluded that the activating ion forms a metal ion-enzyme and not a metal ion-adenine nucleotide complex. The inhibitory effects of Ca^{2+} were considered to be due to competition of these ions with the activating ions to form the metal ion-enzyme complex.

Studies on the mechanism of inhibition by Ca^{2+} of the metal ion activation of pyruvate kinase are complicated by the strict requirements of the enzyme for both a univalent and a bivalent metal ion. Similar complications have been encountered elsewhere (see Passonneau & Lowry, 1963). Kachmar & Boyer (1953) considered, from their kinetic

studies on the rabbit enzyme, that Ca^{2+} has both 'apparent competitive' and 'non-competitive' effects in relation to K^{+} . Such a relation would appear to hold also for the tumour enzyme, as well as for the plant enzyme (Miller & Evans, 1957). It does seem possible that, in studying the inhibition by Ca^{2+} of either K^{+} or Mg^{2+} activation, interference with the other metal ion activator occurs.

Further considerations of the possible role of metal ions in regulating enzymic and metabolic activities. It has been established (Bygrave, 1966) that the inhibition of glycolysis by added Ca^{2+} in extracts of Ehrlich ascites-tumour cells is largely accounted for by inhibition of pyruvate-kinase activity. These studies have been extended in the present paper and have established that Ca^{2+} inhibits the pyruvate kinase from these cells by competing with Mg^{2+} and with K^{+} , metal ions on which this enzyme depends for activity. These observations permit an explanation for the mechanism of the Ca^{2+} inhibition of glycolysis by extracts of ascites-tumour cells. On the basis of the above observations, it is concluded that added Ca^{2+} inhibits glycolysis in extracts of Ehrlich ascites-tumour cells by antagonizing the stimulatory effects of Mg^{2+} and of K^{+} , the obligatory metal ion activators of the pyruvate-kinase reaction.

At a fixed Ca^{2+} concentration, the extent of glycolytic inhibition will also be governed by the concentration of Mg^{2+} and K^{+} present. Mg^{2+} stimulates pyruvate kinase and also glycolysis (F. L. Bygrave, unpublished work) at much lower concentrations than K^{+} . It is therefore likely that these reactions would be more sensitive to fluctuations in the concentration of Mg^{2+} than those of K^{+} . The emphasis in the proposed control mechanism of glycolysis (Bygrave, 1966) would then be placed on the competition between Ca^{2+} and Mg^{2+} to form the 'inactive' and 'active' metal ion-nucleotide complex, or, if the studies of Mildvan & Cohn (1965) hold, the 'inactive' and 'active' metal ion-enzyme complex respectively, in the pyruvate-kinase reaction.

After this work was completed, a brief report (Wyatt, 1964) appeared that substantiates the ideas put forward in the present hypothesis. Wyatt (1964) described a model system in which a number of metal ion-activated and metal ion-inhibited enzymes interacted so that, as the metal ion environment was altered, metabolism was switched to an alternative pathway. Such control becomes sensitive where an enzyme, like pyruvate kinase, has multiple metal activators (Mg^{2+} , K^{+}) and inhibitors (Ca^{2+} , Na^{+}).

The significance of the proposed relationship between the concentration of Ca^{2+} and the rate of glycolysis (Bygrave, 1966) is at present speculative. It will not be realized until the Ca^{2+} concentrations of a large number of tissues are correlated with their

glycolytic rates. Similarly, though the evidence adduced in the present work does not allow one to draw any conclusions as to the exact metabolic control exercised *in vivo* by small variations in the Ca^{2+} , Mg^{2+} , Na^{+} and K^{+} concentrations, it is believed that it has been a step in this direction.

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