ROLE OF BIVALENT CATIONS IN THE CONTROL OF ENZYMES INVOLVED IN GLUCONEOGENESIS

Janet M. WIMHURST and K.L. MANCHESTER
Department of Biochemistry, University College London, Gower Street, London, WC1 E 6BT, England

Received 10 July 1970

1. Introduction

Krebs et al. [1] found that gluconeogenesis by kidney slices was dependent on the availability of Ca^{2+} in the medium. The function of Ca^{2+} is uncertain but may be to prevent the breakdown of phosphoenolpyruvate by pyruvate kinase. Since the three key enzymes required for the reverse of the glycolytic sequence – pyruvate carboxylase, phosphoenolpyruvate carboxykinase and fructose-1,6-diphosphatase – are all activated by bivalent cations such as Mg^{2+}, it is likely that their action is also inhibited by Ca^{2+} [2,3]. Thus the control of the relative rates of glycolysis and gluconeogenesis could result from translocation of Ca^{2+} between mitochondrion and cytoplasm, differentially altering the extent of inhibition of pyruvate kinase and pyruvate carboxylase [4]. This would assume that phosphoenolpyruvate carboxykinase, which is cytosolic in species like the rat and fructose-1, 6-diphosphatase are not seriously inhibited by Ca^{2+}. Its effect on the activity of these enzymes in rat liver has not been systematically investigated, though inhibition of fructose-1,6-diphosphatase from trout liver and from muscle has recently been reported [5,6].

Mn^{2+} can offset the inhibitory effect of Ca^{2+} on mitochondrial carboxylation of pyruvate [3]; this may result partly because both Mn^{2+} and Mg^{2+} can activate carboxylase [3], which when activated by Mn^{2+} is much less sensitive to inhibition by Ca^{2+}. In the present study, a comparison of the ability of Mn^{2+} and Mg^{2+}, separately or together, to activate the three key gluconeogenic enzymes has been made and the effect of these ions on Ca^{2+} inhibition has been investigated.

2. Methods

All assays were performed at 30° and at optimal substrate concentrations as determined in this laboratory. Rats were killed by decapitation, exsanguinated, the livers were excised and frozen at -80°.

Pyruvate carboxylase was assayed spectrophotometrically in extracts from mitochondria prepared as previously described [3] and was resuspended at room temperature in the minimum volume of 50% sucrose [7]. Protein concentration was measured by the method of Lowry et al. [8]. The assay medium used was that described by Utter and Keech [9] and contained in final concentration 112 mM tris-HCl, ATP 2 mM, magnesium sulphate 8 mM, sodium bicarbonate 20 mM, sodium pyruvate 2mM, NADH 0.2 mM, acetyl-CoA 0.24 mM (prepared *in situ* from CoA, acetylphosphate and phosphotransacetylase [10]) and 2.2 units malate dehydrogenase; the final pH was 7.2 at 30°. The reaction was initiated by the addition of 0.1 ml of the enzyme extract to 0.9 ml of assay medium and the rate of disappearance of NADH was followed for about 10 min. The rate of removal of NADH in the absence of acetyl-CoA was substracted.

Phosphoenolpyruvate carboxykinase was measured by an assay based on that of Foster et al. [11]. The incubation medium contained in final concentration 62 mM tris-HCl, 1 mM glutathione, 6 mM ITP, 6 mM magnesium sulphate, 13 mM sodium fluoride, 4.5 mM oxaloacetate and when stated 0.1 mM manganese chloride; pH 7.0 at 30°. Liver was homogenised in 9 or 14 volumes cold 0.25 M sucrose and the reaction started by the addition of 0.2 ml of the 100,000 *g*

supernate to 0.55 ml of the incubation medium. Both enzyme extract and incubation medium were pre-incubated for 5 min at 30° separately before mixing. The reaction was stopped after 0, 3, 6 and 9 min by the addition of 3 ml cold 10% TCA. P_i was then measured before and after the hydrolysis of the phosphoenolpyruvate formed by the enzyme by addition after neutralisation of 0.5 ml of 1% mercuric chloride [13].

Fructose-1,6-diphosphatase was assayed in extracts prepared by the method of Underwood and Newsholme [14]. Liver was homogenised in 19 volumes of 0.15 M KCl containing 5 mM cysteine-HCl and 2 mg per ml bovine plasma albumin. The pH of the homogenate was adjusted to 5.2 with HCl, and the extract incubated at 37° for 10 min. After cooling on ice to 0°, the supernatant after centrifugation at 35,000 *g* for 45 min, was collected and neutralised with KOH. The activity of the enzyme was followed spectrophotometrically in 3 ml of medium containing 50 mM tris-HCl, pH 7.5; 20 mM mercaptoethanol; 5 mM $MgSO_4$; 0.05 mM fructose-1,6-diphosphate; 0.2 mM NADP; 0.05 mM EDTA and 0.7 and 2 units per ml of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase respectively. The reaction was started by the addition of 0.05 ml of the enzyme extract, and the reduction of NADP followed.

3. Results

3.1. *Pyruvate carboxylase*

Activity of pyruvate carboxylase rises with increasing $[Mg^{2+}]$ (fig. 1). The sigmoid shape of the curve is consistent with activation by free Mg^{2+} as well as a requirement for $MgATP^{2-}$ formation. Calculation of likely concentrations of free Mg^{2+}, which take into account complexing of the metal by other anions present, gives an estimated K_a for Mg^{2+} of 0.16 – 0.28 mM, with random order binding and noncompetitive inhibition by ATP^{4-} providing the best fit for the data [15]. Activation is optimal at about 8 mM (corresponding to a free ion concentration of 0.82 mM). With Mn^{2+}, activation is similar but is optimal at far lower concentrations (2 mM when [ATP] = 2 mM). Higher concentrations lead to a sharp fall in activity [15]. The calculated concentration of the free ion under optimal conditions is about 0.04 mM. A mixture of both ions at optimal concentrations – Mg^{2+} at 8 mM and Mn^{2+} at 1 or 2 mM – produced no greater activity than either alone. At optimal concentrations of either ion, Ca^{2+} was inhibitory – 1 mM caused 60% inhibition with Mg^{2+} but only 25% with Mn^{2+} [3]. The reciprocal plots (fig. 2) indicate a K_i at low $[Ca^{2+}]$ of 0.25 mM.

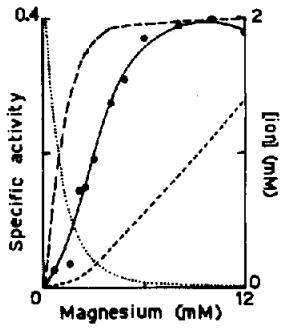

Fig. 1. Effect of magnesium on the activity of pyruvate carboxylase. Concentration of ATP is 2 mM. From the appropriate stability constants the concentration of the ion Mg^{2+} - . - . - ., $MgATP^{2-}$ - - - - - and ATP^{4-} . . . . . have been calculated [15]. •——•, activity is expressed as μmole NADH oxidised per mg protein per min at 30°.

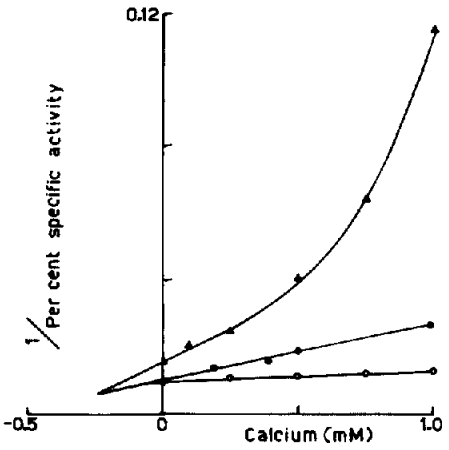

Fig. 2. Dixon plot showing the complexity of the inhibition of pyruvate carboxylase by calcium in the presence of magnesium or manganese as the activating ion. o——o: $MnCl_2$ 2 mM, ATP 2 mM; •——•: $MgSO_4$ 8 mM, ATP 2 mM; Δ——Δ: $MgSO_4$ 3.2 mM, ATP 2 mM. Activity is μmole NADH oxidised per mg protein per min at 30°.

3.2. *Phosphoenolpyruvate carboxykinase*

Phosphoenolpyruvate carboxykinase is activated by either Mg^{2+} or Mn^{2+} separately, the optimal concen-

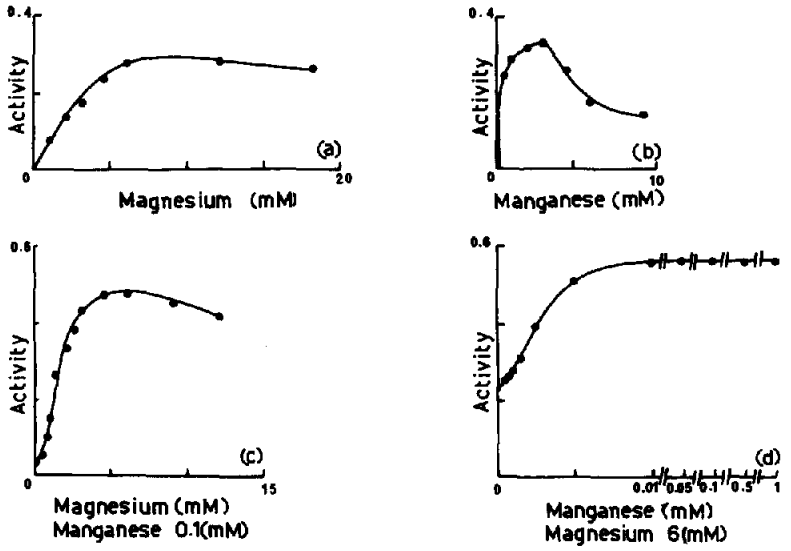

Fig. 3. Activation of phosphoenolpyruvate carboxykinase by metal ions; (a) $MgSO_4$ alone; (b) $MnCl_2$ alone; (c) increasing concentrations of $MgSO_4$ in the presence of 0.1 mM $MnCl_2$; (d) increasing concentrations of $MnCl_2$ in the presence of 6 mM $MgSO_4$. ITP was 6 mM in all cases. Activity is μmole phosphoenolpyruvate produced per g liver per min at 30°.

tration for $[Mg^{2+}]$ being in excess of [ITP], whereas a lower $[Mn^{2+}]$ produces similar activation (fig. 3). In contrast with pyruvate carboxylase however, addition of Mn^{2+} in the presence of Mg^{2+} produced a marked increase in the activity over that in the presence of either ion alone ([11] and fig. 3). Ca^{2+} inhibition was observed, but again was less pronounced with Mn^{2+} than with Mg^{2+} (fig. 4). When both ions were present – Mg^{2+} 6 mM, Mn^{2+} 0.1 mM – the K_i observed (1.5 mM) was intermediate between those for the Mn^{2+} activated system (3 mM) and the Mg^{2+} activated system (0.6 mM).

3.3 *Fructose-1,6-diphosphatase*

Fructose-1,6-diphosphatase is activated by Mg^{2+} (fig. 5a) but the optimal concentration depends in part on the fructose-1,6-diphosphate concentration which above 0.2 mM is inhibitory [14]. Mn^{2+} also activates the rat liver enzyme. The optimal ion concentration appears to be independent of the concentration of the substrate and excess Mn^{2+} rapidly leads to inhibition. Unlike for either pyruvate carboxylase or phosphoenolpyruvate carboxykinase, Mn^{2+} is inhibitory in the presence of Mg^{2+} at all concentrations greater than that of EDTA in the assay (fig. 5c); higher concentrations of Mg^{2+} possibly gave some protection.

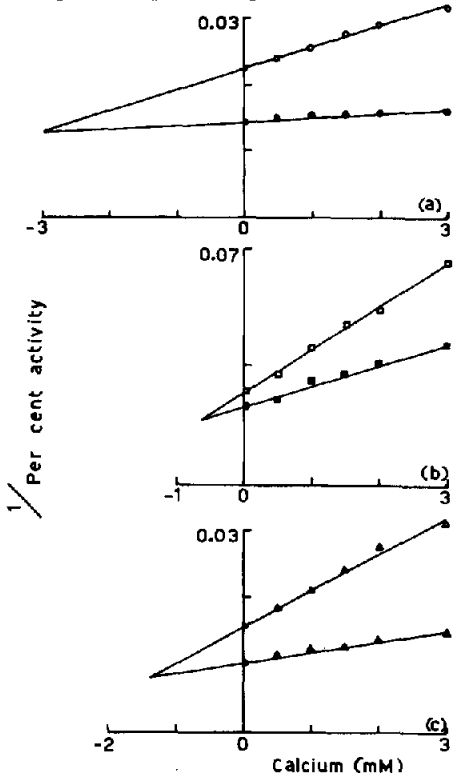

Fig. 4. Dixon plots showing the inhibition of phosphoenolpyruvate carboxykinase by calcium in the presence of (a) magnesium, (b) manganese and (c) a mixture of magnesium and manganese. ●—●: $MgSO_4$ 6 mM; ○—○: $MgSO_4$ 2 mM; ■—■: $MnCl_2$ 3 mM; □—□ $MnCl_2$ 0.5 mM; ▲—▲: $MgSO_4$ 6 mM, $MnCl_2$ 0.1 mM; △—△: $MgSO_4$ 2 mM, $MnCl_2$ 0.1 mM. ITP was 6 mM in all cases. Activity is μmole phosphoenolpyruvate produced per g liver per min at 30°.

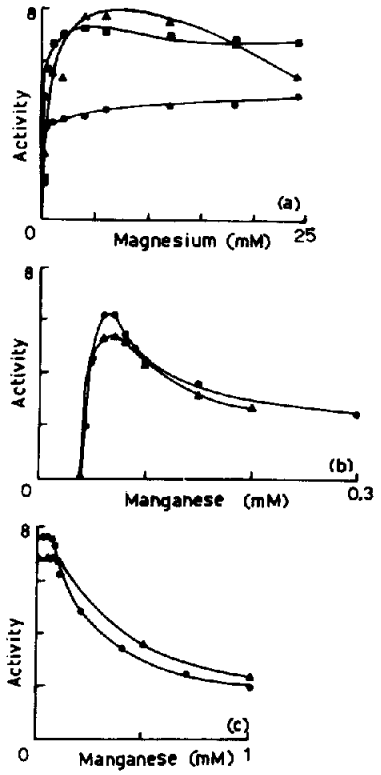

Fig. 5. Effect of bivalent cations on the activity of fructose 1:6-diphosphatase. (a) Mg^{2+} with fructose-1,6-diphosphatase; ▲—▲: 0.05 mM; ■—■: 0.1 mM; ●—●: 0.5 mM; (b) Mn^{2+} with fructose-1,6-diphosphate; ●—●: 0.05 mM; ▲—▲: 0.3 mM; (c) Mn^{2+} in the presence of Mg^{2+}; ●—●: 5 mM; ▲—▲: 12 mM, fructose-1,6-diphosphate 0.05 mM. All assays included 0.05 mM EDTA. Activity is μmole NADP reduced per g liver per min at 30°.

Ca^{2+} strongly inhibits rat liver fructose-1,6-diphosphatase (fig. 6) in the presence of either activating ion. Both Mn^{2+} and Mg^{2+} protect against Ca^{2+}, optimal concentrations of either being equally effective, although the actual $[Mn^{2+}]$ is far lower than that of Mg^{2+}. 1 mM Ca^{2+} inhibits fructose-1,6-diphosphatase 80% in the presence of 5 mM $MgSO_4$ compared with 60% for pyruvate carboxylase at 8 mM $MgSO_4$ and 25% for phosphoenolpyruvate carboxykinase at 6 mM $MgSO_4$. Under optimal Mg^{2+} or Mn^{2+} activation (1–5 mM and 0.07 mM respectively) fructose-1,6-diphosphatase is the most susceptible of the three enzymes to Ca^{2+}, with 50% inhibition at approximately 0.1 mM Ca^{2+}.

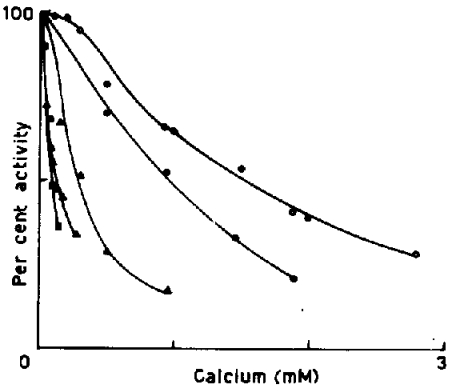

Fig. 6. Inhibition of fructose-1,6-diphosphatase by Ca^{2+} in the presence of different concentrations of Mg^{2+} or Mn^{2+} as the activating ion. △—△: $MnCl_2$ 0.07 mM; ○—○: $MnCl_2$ 0.15 mM; ■—■: $MgSO_4$ 1 mM; ▲—▲: $MgSO_4$ 5 mM; ●—●: $MgSO_4$ 10 mM; fructose-1,6-diphosphate 0.05 mM. Activity is μmole NADP reduced per g liver per min at 30°.

4. Discussion

The three enzymes respond differently to Mn^{2+} in the presence of Mg^{2+}; they are all inhibited by Ca^{2+}, but, with the exception of fructose-1,6-diphosphatase, to a less extent when activated by Mn^{2+}. The Ca^{2+} content of rat liver is equal or greater than 1 mM [16], which is sufficient to affect the enzymes studied. The hepatic content of Mn^{2+} (0.1 – 0.15 mM [ref. 17 and unpublished measurements]), is also sufficient to influence activities, but for both Ca^{2+} and Mn^{2+} lack of knowledge of their location and extent of sequestration precludes judgement of the role *in vivo*. Uptake of Ca^{2+} by mitochondria is reported to facilitate Mn^{2+} accumulation also [18]. Such an effect would minimise any inhibition of pyruvate carboxylase by Ca^{2+}.

Acknowledgement

We are grateful to the Medical Research Council for financial support for this work.

References

[1] H.A. Krebs, D.A.H. Bennett, P. De Gasquet, T. Gascoyne and T. Yoshida, Biochem. J. 86 (1963) 22.

[2] G.A. Kimmich and H. Rasmussen, J. Biol. Chem. 244 (1969) 190.
[3] E.J. Harris, C. Berent and J. Wimhurst, FEBS Letters 6 (1970) 93.
[4] W. Gevers and H.A. Krebs, Biochem. J. 98 (1966) 720.
[5] H.W. Behrisch and P.W. Hochachka, Biochem. J. 111 (1969) 287.
[6] H. Vaugham and E.A. Newsholme, Biochem. J. 114 (1970) 81 P.
[7] M.C. Scrutton and M.F. Utter, J. Biol. Chem. 240 (1965) 1.
[8] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[9] M.F. Utter and D.B. Keech, J. Biol. Chem. 238 (1963) 2603.
[10] G. Michal and H.U. Bergmeyer, in: Methoden der Enzymatischen Analyse, ed. H.U. Bergmeyer (Verlag Chemie, Weinhouse/Berstrasse, 1963) p. 512.
[11] D.O. Foster, H.A. Lardy, P.D. Ray and J.B. Johnston, Biochemistry 6 (1967) 2120.
[12] O.H. Lowry and J.A. Lopez, in: Methods in Enzymology, Vol. 3., eds. S.P. Colowick and N.O. Kaplan (Academic Press, New York, 1957) p. 845.
[13] H. Lohman and O. Meyerhof, Biochem. Z. 273 (1934) 60.
[14] A.H. Underwood and E.A. Newsholme, Biochem. J. 95 (1965) 767.
[15] J.M. Wimhurst and K.L. Manchester, Biochem. J., in press.
[16] C. Long, Biochemists Handbook (Spon, London, 1967).
[17] E.J. Lorenzen and S.E. Smith, J. Nutrition 33 (1947) 143.
[18] L. Mela and B. Chance, Biochemistry 7 (1968) 4059.