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THE INFLUENCE OF DIVALENT CATIONS ON ALLOSTERIC BEHAVIOUR OF MUSCLE PYRUVATE KINASE FROM THE SEA MUSSEL *MYTILUS EDULIS L.*

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Abstract—1. Pyruvate kinase of the adductor muscle of the sea mussel displays an absolute requirement for Mg^{2+} or Mn^{2+}. By the use of Ca^{2+} or Zn^{2+} no enzyme activity is obtained.

2. In the presence of Mn^{2+}, in contrast to Mg^{2+}, always hyperbolic substrate saturation curves are obtained.

3. There is evidence that Mn^{2+} not only acts by forming an ADP–Mn^{2+} complex, but also as an allosteric activator.

4. Ca^{2+} is a strong inhibitor but the enzyme becomes less sensitive to this inhibition when Mg^{2+} is replaced for Mn^{2+}.

INTRODUCTION

Mytilus edulis, an intertidal bivalve, keeps its valves tightly closed upon emersion at low tide. During this period of anoxia the animals use a metabolic pathway for energy production which is identical to the Embden–Meyerhof–Parnas scheme up to the stage of phosphopyruvate (PYR-P). In contrast to vertebrate skeletal muscle, which converts PYR-P to pyruvate and accumulates lactate, the main end products are alanine and succinate (De Zwaan *et al.*, 1973). Alanine is the initial end product and its production is restricted to early stages of anoxia. Gradually succinate takes over the position of alanine. The conversion of PYR-P into alanine or succinate depends upon the competition between PYR-P carboxylase (EC 4.1.1.32) and pyruvate kinase (EC 2.7.1.40). For this reason it is clear that pyruvate kinase is a key enzyme in the regulation of anaerobic glycolysis.

Mytilus edulis lives in habitats with fluctuating salinities. The sea mussel behaves as a poikilosmotic organism. In the cell inorganic ions contribute to about 50% of the osmoconcentration. The rest is made up by taurine, betaine and amino acids (Schoffeniels & Gilles, 1972). It is likely that carbohydrates form the source for amino acid production (Hammen, 1969). This indicates that pyruvate kinase can play an active role as regulator of free amino acid levels in the cell.

In previous papers (De Zwaan *et al.*, 1972; Holwerda *et al.*, 1973) we studied the influence of organic compounds on pyruvate kinase activity. We found that adductor muscle pyruvate kinase (as well as other tissues as gill, mantle and hepatopancreas) possesses allosteric properties which are similar to L (liver)-type pyruvate kinase of vertebrate except in its behaviour to pH. We now report about the influence of inorganic ions on pyruvate kinase activity in relation to pH.

MATERIALS AND METHODS

All (bio)chemicals used were of analytical grade. Sea mussels were obtained from the Institute for Mussel Research (Texel, The Netherlands) and collected from beds in the Waddenzee. Pyruvate kinase was isolated from fresh posterior adductor muscle as described before (De Zwaan *et al.*, 1972). The enzyme preparation, which was preserved in 30 vol % glycerol, had a specific activity of about 45 μmole/min per mg protein at pH 7·6.

Pyruvate kinase activity was measured according to Bücher & Pfleiderer (1955). Oxidation of NADH was followed at 25°C and at 340 nm in a Zeiss PM *Q*II spectrophotometer. All tests were performed with 1–3 μg protein. The figures presented are calculated for the same protein concentration and are therefore direct comparable. The test medium contained 0·1 M imidazole–HCl buffer, 67 mM KCl, 0·067 mM NADH and 36 units LDH (Boehringer from hog muscle, in 50% glycerol). No interference of glycerol with pyruvate kinase was observed.

RESULTS

Figure 1 shows the substrate saturation curve at pH 7·6 and 6·2 with Mg^{2+} as divalent cation. At the higher pH the curve is slightly sigmoid (Hill coefficient (n_H): 1·4 and apparent K_m: 0·25 mM PYR-P). Lowering of the pH to 6·2 results in a sharp increase in sigmoidicity (n_H = 1·9) as well in apparent K_m (0·81 mM).

Figure 2 shows the same relation when a fixed amount of FDP (0·1 mM) is added to the reaction medium. At both pH values there is a decrease of apparent K_m (at pH 7·6 apparent K_m = 0·07 mM, at pH 6·2 = 0·10 mM) and of the Hill coefficients. Both curves give a n_H of 1·1, reflecting a hyperbolic saturation curve. As can be concluded from Figs. 1 and 2 stimulation of pyruvate kinase by FDP depends on pH and PYR-P concentration.

Figure 3 presents the same relation as Figs. 1 and 2, but with Mg^{2+} replaced by Mn^{2+}. Neither curve

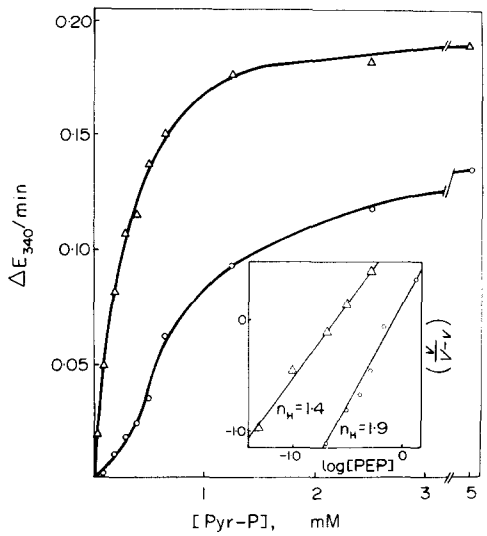

Fig. 1. Relation between pyruvate kinase activity and PYR-P concentration at pH 7·6 (△) and pH 6·2 (○), with Mg^{2+} as the divalent cation (10 mM $MgSO_4$). A Hill plot is inserted.

exhibits sigmoidicity: Hill coefficients have become 1·1. Addition of FDP has no influence on the activity, neither at pH 7·6 nor at 6·2. Nearly the same apparent K_m values are obtained as in the case with Mg^{2+} in the presence of FDP.

Figure 4 shows pH optimum curves. With Mg^{2+} maximal activity is found at pH 7·4. At lower pH values there is a strong decrease of activity. In the presence of FDP the activity changes only marginally between pH 7·5 and 6·2. With Mn^{2+} in the reaction medium instead of Mg^{2+} the result is completely different: without and with FDP added enzyme activity remains practically constant between pH 7·5 and 6·2. Also alanine, which is a strong inhibitor when Mg^{2+} is the divalent ion present (Holwerda *et al.*, 1973), has no influence at any pH value between 7·5 and 6·2.

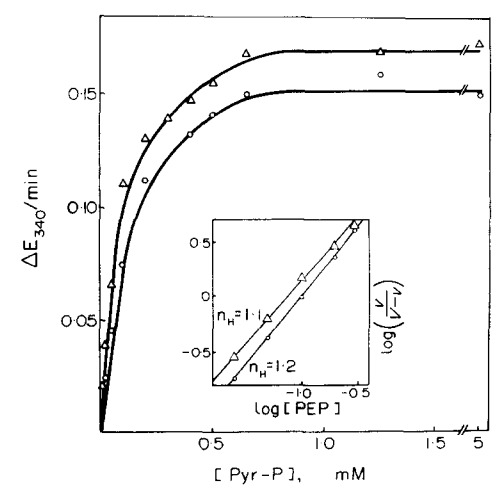

Fig. 2. Relation between pyruvate kinase activity and PYR-P concentration in the presence of 0·1 mM FDP at pH 7·6 (△) and pH 6·2 (○), with Mg^{2+} as in Fig. 1. A Hill plot is inserted.

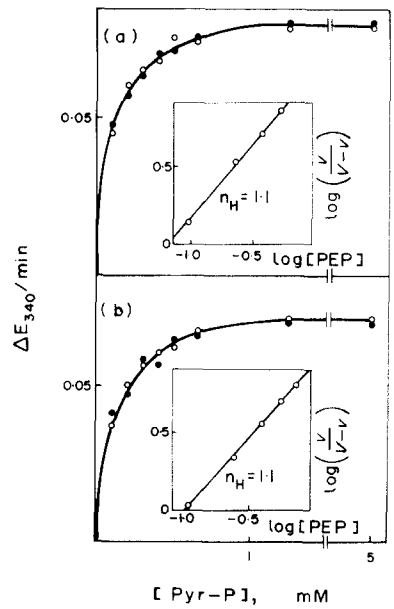

Fig. 3. Relation between pyruvate kinase activity and PYR-P concentration in the presence (○) or absence (●) of 0·1 mM FDP, at pH 7·6 (a) and pH 6·2 (b). In all cases Mn^{2+} was the divalent ion used (10 mM $MnSO_4$). Hill plots are inserted.

In Fig. 5 saturation curves for the metal ions at pH 7·6 and 6·2 are shown. Values of V_{max}, apparent K_m and n_H have been collected in Table 1. With Zn^{2+} or Ca^{2+} up to 10 mM no enzyme activity was obtained.

Figure 6 shows Ca^{2+} inhibition curves in the presence of 10 mM Mg^{2+} or Mn^{2+}. In the first case the enzyme is strongly inhibited by Ca^{2+} (K_i = 3·6 mM) but with Mn^{2+} the enzyme is almost insensitive to the calcium ion.

Figure 7 shows the activation of pyruvate kinase by Mn^{2+} in the presence and absence of 5 mM Mg^{2+} at pH 6·2. It can be seen from Fig. 5 that ΔE/min at 5 mM Mg^{2+}, without Mn^{2+} added, is 0·03 and

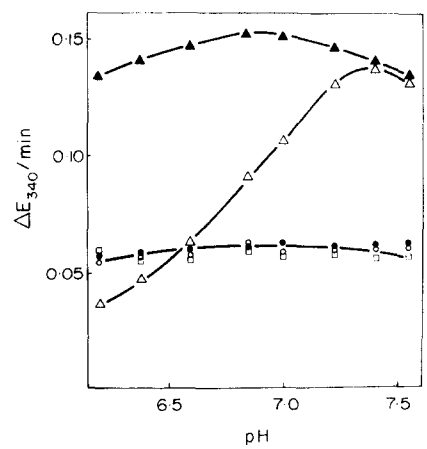

Fig. 4. Effect of the divalent metal ion on the pH optimum curve in the absence and presence of various effectors. [PYR-P] = 0·5 mM. —△— 10 mM $MgSO_4$; —▲— 10 mM $MgSO_4$ plus 0·1 mM FDP; —○— 10 mM $MnSO_4$; —●— 10 mM $MnSO_4$ plus 0·1 mM FDP; —□— 10 mM $MnSO_4$ plus 4 mM alanine.

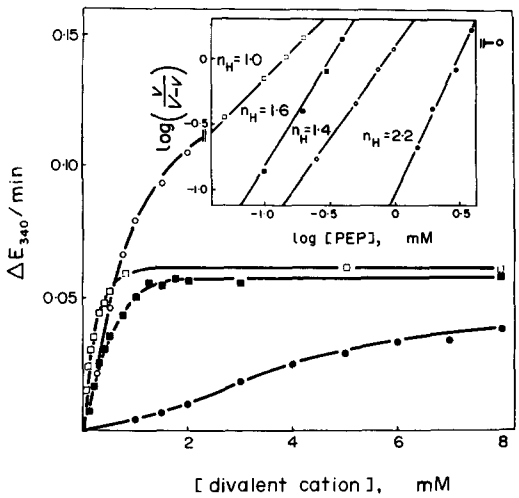

Fig. 5. Activity of pyruvate kinase at [PYR-P] = 0·5 mM as a function of Mg^{2+} concentration (—○— pH 7·6; —●— pH 6·2) and Mn^{2+} concentration (—□— pH 7·6; —■— pH 6·2). Hill plots are inserted.

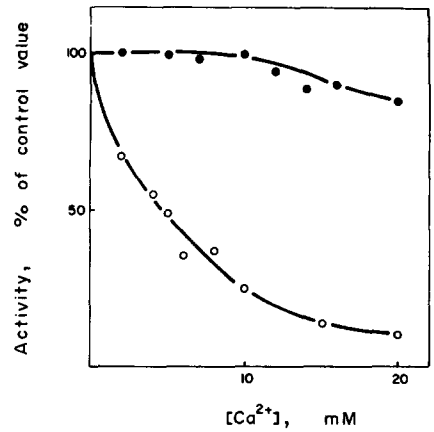

Fig. 6. Inhibition of pyruvate kinase activity by Ca^{2+} with 10 mM Mg^{2+} (—○—) or 10 mM Mn^{2+} (—●—) in the reaction medium. [PYR-P] = 0·5 mM; pH = 7·6.

at saturation concentration of Mn^{2+}, without Mg^{2+} added, 0·06 (Table 1). So, for the upper curve of Fig. 7 we expected that the reaction velocity would increase from 0·03 to 0·06 (reached when all Mg^{2+} is replaced by Mn^{2+}). But this curve reaches a maximum at a relatively small Mn^{2+} concentration. This maximum is substantially higher than the maximum for the Mn^{2+} activation curve.

DISCUSSION

Pyruvate kinase from the adductor muscle of the sea mussel can exist in two kinetically different forms with a hyperbolic and a sigmoidal saturation curve respectively. The sigmoidal saturation curve can be altered into the hyperbolic by increasing the pH (Fig. 1), adding FDP (Fig. 2) or replacing Mg^{2+} by Mn^{2+} (Fig. 3). The latter may explain the rapid increase of enzyme activity with increasing concentration of Mn^{2+} in the presence of 5 mM Mg^{2+} (Fig. 7, upper curve). From Fig. 5 and Table 1 it is seen that at pH 6·2 both maximal activity and apparent K_m of the enzyme for Mn^{2+} is higher than for Mg^{2+}. So, it was expected that in the presence of 5 mM Mg^{2+} there would be gradually increase of activity with increasing concentration of Mn^{2+}, since the ADP-Mg^{2+} complex becomes replaced by the ADP-Mn^{2+} complex. However, the rapid rise of the upper curve in Fig. 7 over the middle curve indicates that not only the complex ADP-Mn^{2+} but also free Mn^{2+} is responsible for the activation of the enzyme. Free Mn^{2+} could enhance the affinity of the enzyme towards Mg^{2+} as is the case with increasing the pH (Table 1). Replacing Mg^{2+} by Mn^{2+} and increasing the pH had also the same effect on the PYR-P saturation curve.

Table 1. Values of V_{max}, apparent K_m and n_H of the stimulation curves for Mg^{2+} and Mn^{2+} at two pH values

pH	Cation	V_{max}	App. K_m	n_H
7·6	Mg^{2+}	0·15	0·87	1·4
7·6	Mn^{2+}	0·06	0·15	1·0
6·2	Mg^{2+}	0·04	3·16	2·2
6·2	Mn^{2+}	0·06	0·46	1·6

V_{max} expressed in ΔE/min; apparent K_m in mM.

For pyruvate kinase of *Mucor rouxii* too the manganous ion has been found to be an allosteric activator (Passeron *et al.*, 1969). Recently it has been shown (Leonard, 1972) that for erythrocyte pyruvate kinase Mn^{2+} changes the sigmoidal substrate saturation curve into a hyperbole and causes desensitization for FDP.

The results presented indicate that changes of pyruvate kinase activity may be the result not only of variations in the concentration of organic substances such as PYR-P, ATP, alanine and FDP, but also in the intracellular concentrations of metal ions. Potts (1958) reported concentrations of 7·3 and 34 mg ion for Ca^{2+} and Mg^{2+} respectively in *Mytilus edulis*. In our laboratory we found the Mn^{2+} concentration to vary between 0·05 and 0·15 mg ion. This concentration range of the manganous ion is below the apparent K_m values presented in Table 1. This is important as a high concentration of Mn^{2+} would keep the enzyme in the hyperbolic form with loss of its regulatory properties.

During anoxia in bivalves the Ca^{2+} concentration increases (Crenshaw *et al.*, 1969). This may play a role in inhibiting pyruvate kinase (Fig. 6), thus leaving PYR-P available for production of succinate.

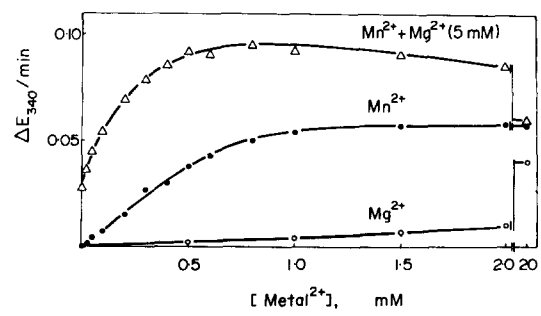

Fig. 7. Effect of variation of the concentration of Mg^{2+} (—○—), Mn^{2+} (—●—) and Mn^{2+} plus 5 mM Mg^{2+} (—△—) on the enzyme activity. [PYR-P] = 0·5 mM pH = 6·2.

REFERENCES

BÜCHER TH. & PFLEIDERER G. (1955) Pyruvate kinase. In *Methods in Enzymology* (Edited by COLOWICK S. P. & KAPLAN N. D.), Vol. 1, pp. 435–436, Academic Press, New York.

CRENSHAW M. A. & NEFF J. M. (1969) Decalcification at the mantle shell interface in molluscs. *Am. Zoologist* **9**, 881–885.

HAMMEN C. S. (1969) Metabolism of the oyster, *Crassostrea virginica*. *Am. Zoologist* **9**, 309–318.

HOLWERDA D. A. & ZWAAN A. DE (1973) Kinetic and molecular characteristics of allosteric pyruvate kinase from muscle tissue of the sea mussel *Mytilus edulis* L. *Biochim. biophys. Acta* **309**, 296–306.

LEONARD H. A. (1972) Human pyruvate kinase. Role of the divalent cation in the catalytic mechanism of the red cell enzyme. *Biochemistry* **11**, 4407–4414.

PASSERON S. & TERENZI H. (1969) Activation of pyruvate kinase of *Mucor rouxii* by manganese ions. *Febs Lett.* **6**, 213–216.

POTTS W. T. W. (1958) The inorganic and amino acids composition of some lamellibranch mussels. *J. exp. Biol.* **35**, 749–764.

SCHOFFENIELS E. & GILLES R. (1972) Ionregulation and osmoregulation in Mollusca. In *Chemical Zoology* (Edited by FLORKIN M. & SCHEER B. T.), Vol. **6**, pp. 393–420, Academic Press, New York.

ZWAAN A. DE & HOLWERDA D. A. (1972) The effect of phosphoenol-pyruvate, fructose-1,6-diphosphate and pH on allosteric pyruvate kinase in muscle tissue of the bivalve *Mytilus edulis* L. *Biochim. biophys. Acta* **276**, 430–433.

ZWAAN A. DE & MARREWIJK W. J. A. VAN (1973) Anaerobic glucose degradation in the sea mussel *Mytilus edulis* L. *Comp. Biochem. Physiol.* **44B**, 429–439.

Key Word Index—*Mytilus edulis*: muscle pyruvate kinase; divalent cations; anaerobic metabolism; anoxia.