The Role of Mitochondria in Modifying Calcium-Sensitive Cytoplasmic Metabolic Activities

MODIFICATION OF PYRUVATE KINASE ACTIVITY

By J. MELI and F. L. BYGRAVE

Department of Biochemistry, School of General Studies, Australian National University,
Canberra, A.C.T. 2600, Australia

(Received 15 February 1972)

1. The modification of pyruvate kinase activity *in vitro* was examined by altering the environmental $[Mg^{2+}]/[Ca^{2+}]$ ratio with EDTA on the one hand and isolated rat liver mitochondria on the other. 2. Controlled additions of Ca^{2+} and EDTA caused pyruvate kinase activity to be alternately and rapidly switched on and off. 3. By being able to accumulate Ca^{2+} in preference to Mg^{2+} rat liver mitochondria were able to alter the $[Mg^{2+}]/[Ca^{2+}]$ ratio in the vicinity of pyruvate kinase and thereby modify the activity of this enzyme. 4. The possible role of mitochondria in modifying pyruvate kinase and other ion-sensitive cytoplasmic enzyme activities is discussed.

Many of the metabolic processes that occur in the cell proceed at optimum rates *in vitro* only in the presence of an appropriate bivalent metal ion (see Dixon & Webb, 1964). This generalization reflects the fact that some of the enzymes themselves involved in the metabolic processes have an absolute requirement for a metal ion. The metal-ion requirement is usually satisfied by Mg^{2+} , which is the predominant bivalent ion in most cells. Often Ca^{2+} antagonizes the activation brought about by Mg^{2+} (Heilbrunn, 1952). These properties are seen, for example, in the glycolytic enzyme, pyruvate kinase, which has an obligatory requirement for Mg^{2+} (or Mn^{2+}) and is inhibited by Ca^{2+} (Kachmar & Boyer, 1953; Bygrave, 1966b; Mildvan & Cohn, 1966).

It is possible to envisage that under certain circumstances the $[Mg^{2+}]/[Ca^{2+}]$ ratio could be a physiological factor that influences the final rate of a single enzyme or metabolic pathway sensitive to this ratio. If cells were then able to alter this ratio in a specific environment they would have at their disposal a further means of controlling or, at the very least, modifying metabolism.

Mitochondria isolated from liver cells are known to readily accumulate Ca^{2+} in preference to Mg^{2+} (for review see Lehninger *et al.*, 1967). In principle then the liver cell should be able to alter the $[Mg^{2+}]/[Ca^{2+}]$ ratio in the cytosol by means of its mitochondria and thereby influence the activity of enzymes and pathways sensitive to this ratio.

The present work was undertaken to test the feasibility of these ideas. A system has been constructed *in vitro* that enables pyruvate kinase to be measured in the presence of rat liver mitochondria. The ion-accumulation properties of these mitochondria have been used to vary the $[Mg^{2+}]/[Ca^{2+}]$

ratio outside the mitochondria. This variation in turn can subsequently lead to rapid and reversible modification of pyruvate kinase activity.

Experimental

Assay of pyruvate kinase

Pyruvate kinase was assayed according to the method of Bücher & Pfeleiderer (1955). The oxidation of NADH was followed at 340 nm with a Unicam SP.800 spectrophotometer linked to a Beckman chart recorder. Unless indicated otherwise the final reaction mixture contained 50 mM-triethanolamine-HCl buffer (pH 7.5), 75 mM-KCl, 0.15 mM-NADH, 0.25 mM-ADP, 0.16 mM-phosphoenolpyruvate, 5 μ M-EDTA and 10 μ g of lactate dehydrogenase in the final volume of 3.0 ml. Pyruvate kinase and $MgSO_4$ were present at the concentrations indicated in the legends to the figures.

The complete reaction mixture excluding phosphoenolpyruvate was preincubated at 30°C for 3–6 min and the reaction, which was also carried out at 30°C, was started by the addition of phosphoenolpyruvate. The extinction at 340 nm was read against a blank, which contained the reaction mixture minus the substrate.

Preparation of rat liver mitochondria and supernatant

Mitochondria were prepared from the livers of Wistar-strain albino rats (200 g) by a slight modification of the method of Schneider (1948). The isolation medium was 0.25 M-sucrose containing 2 mM-triethanolamine-HCl (pH 7.5). The mitochondria were washed twice by centrifugation at 8000 g for 10 min

in a Sorvall refrigerated centrifuge and finally resuspended in the isolation medium at a concentration of 50–60 mg of mitochondrial protein/ml. The clear supernatant obtained after centrifuging the homogenate, at 105 000g for 60 min in a Spinco model L refrigerated centrifuge, was used where indicated as a source of pyruvate kinase.

Protein was assayed by the biuret method.

Measurement of Ca^{2+} accumulation and release by mitochondria

Movement of Ca^{2+} was followed by using $^{45}\text{CaCl}_2$. Samples (50 μl) of the reaction mixture housed in the spectrophotometer were rapidly removed and the mitochondria separated from the medium by means of Millipore filtration, with Gelman GN-6 filters of 0.45 μm pore size. Pre-filter and post-filter washings with a medium containing 50 mM-triethanolamine (pH 7.5) and 75 mM-KCl were employed. The filter was placed in a glass vial, dried and 5 ml of scintillation fluid containing 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene made up in 1 litre of toluene were added. Radioactivity was determined in a Beckman LS-100 liquid-scintillation counter by using the ^{14}C channel.

Materials

Pyruvate kinase (rabbit muscle; approx. specific activity 150 units/mg), lactate dehydrogenase (free from pyruvate kinase), ADP, NADH and phosphoenolpyruvate were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. All other reagents were of A.R. grade. $^{45}\text{CaCl}_2$ (specific radioactivity, 0.507 mCi/mmol) was a product of The Radiochemical Centre, Amersham, Bucks., U.K.

Results

It has been previously shown that inhibition by Ca^{2+} of the Mg^{2+} or Mn^{2+} -activated pyruvate kinase is competitive in nature (Bygrave, 1966a; Mildvan & Cohn, 1966). These findings were confirmed in preliminary experiments in the present study. However, the inhibition by Ca^{2+} , although still competitive, was found to be rather unusual when examined at low Mg^{2+} concentrations. Fig. 1 shows the effect of varying the Mg^{2+} concentration in the range 0.04–0.4 mM in the presence of three different concentrations of Ca^{2+} . The plot at zero Ca^{2+} is a straight line. However, as the Ca^{2+} concentration is increased the plots become increasingly non-linear, indicating deviation from Michaelis–Menten enzyme kinetics. This effect, which is observed only at low Mg^{2+} concentrations, was not further investigated in the present study.

Fig. 1. Influence of Ca^{2+} on the Mg^{2+} activation of pyruvate kinase

The reaction was carried out as described in the Experimental section. In addition to the basic reaction mixture (final vol. 3 ml) each cuvette contained 0.12 μg of pyruvate kinase and various concentrations of magnesium as indicated. ●, No added calcium; ○, 50 μM - Ca^{2+} ; ▲, 100 μM - Ca^{2+} ; △, 200 μM - Ca^{2+} .

Bailey *et al.* (1968) always included EDTA in the medium used for assay of pyruvate kinase. In the present work 2–4 μM -EDTA was found to stimulate pyruvate kinase activity about twofold and so was always used in the reaction mixture.

Influence of EDTA on Ca^{2+} inhibition of pyruvate kinase

Since the inhibition of pyruvate kinase by Ca^{2+} can be reversed by Mg^{2+} , it should also be possible to reverse the inhibition by removing Ca^{2+} with a suitable chelating agent. Because EDTA has a higher affinity for Ca^{2+} than for Mg^{2+} (Sillen & Martell, 1964) it will preferentially sequester Ca^{2+} in a solution containing both of these ions. Fig. 2 shows that the inhibition by Ca^{2+} is gradually overcome as the concentration of EDTA is increased. The concentrations of EDTA causing maximum reversal of the inhibition by 0.17 mM- and 0.33 mM- Ca^{2+} are 0.24

and 0.42 mM respectively. Further increasing the EDTA concentration decreases the pyruvate kinase activity because Mg^{2+} is now also being chelated.

As it is possible to relieve the Ca^{2+} inhibition of pyruvate kinase with EDTA it should also be possible alternately to inhibit or activate the enzyme by adding Ca^{2+} or EDTA respectively. This is shown in Fig. 3. The time required to switch from the inhibited to the activated state or vice versa is of the order of 30 s and may be even shorter than indicated by this experiment. The lags observed in Fig. 3 are not likely to be due to the time required to activate or inhibit the enzyme but rather to the employment of the coupled enzyme system in the assay (McClure, 1969). The concentration of the common intermediate pyruvate must reach a steady state before the assay becomes linear. This can take up to 30 s.

Fig. 2. Influence of EDTA on the activity of pyruvate kinase

The reaction was carried out as indicated in the Experimental section. In addition to the basic reaction mixture (final vol. 3 ml) each cuvette contained 0.4 mM- Mg^{2+} and 0.25 μg of pyruvate kinase. Ca^{2+} and EDTA were added as indicated. ○, 0.17 mM- Ca^{2+} ; ●, 0.33 mM- Ca^{2+} .

Hence the observed metal-ion effects may be almost instantaneous.

Alteration of pyruvate kinase activity mediated by mitochondria

The above experiments indicated that pyruvate kinase could be activated or inhibited by sequestering or adding Ca^{2+} . The present experiments were carried out with a view to determining whether mitochondria could similarly alter the pyruvate kinase activity by influencing the $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$ ratio in the incubation medium. Accumulation of Ca^{2+} by liver mitochondria is an energy-requiring process with little ability to accumulate Mg^{2+} (Lehninger *et al.*, 1967). Succinate may be used as a respiratory substrate to support Ca^{2+} accumulation and maintenance (Drahota *et al.*, 1965). Atractyloside and rotenone must be present in the reaction medium to prevent adenine nucleotide translocation and pyruvate oxidation respectively. Preliminary experiments showed that the addition of succinate, sucrose, atractyloside, rotenone and carbonyl cyanide *m*-chlorophenylhydrazone, either separately or together, had no effect on the pyruvate kinase activity (see below). These experiments also showed that rat liver mitochondria could accumulate $^{45}\text{Ca}^{2+}$ in the pyruvate kinase assay medium with similar efficiency to that in the medium of Drahota *et al.* (1965). Over 90% of the added Ca^{2+} was accumulated when Ca^{2+} was present at a concentration of 71 nmol/mg of mitochondrial protein.

Results in Fig. 4 show how the ion-accumulation

Fig. 3. Control of pyruvate kinase activity by alteration of the free Ca^{2+} concentration

The reaction was carried out as indicated in the Experimental section. In addition to the basic reaction mixture (final vol. 3 ml) the cuvette contained 0.4 mM- Mg^{2+} and 0.25 μg of pyruvate kinase. Ca^{2+} and EDTA were added in the amounts indicated. Values in brackets indicate the activity of pyruvate kinase expressed as a percentage of the initial activity, which is taken as 100. In this experiment 100% equals 18.5 nmol of pyruvate formed/min.

Fig. 4. Alteration of pyruvate kinase activity mediated by mitochondria

The reaction was carried out as described in the Experimental section. In addition to the basic reaction mixture (final vol. 3ml) each cuvette contained $50\mu\text{M-MgSO}_4$, $20\mu\text{M-atractyloside}$, $0.25\mu\text{M-rotenone}$, $2.5\mu\text{g}$ of pyruvate kinase and 2mg of mitochondrial protein. Phosphoenolpyruvate was added only to the test cuvette. Subsequent additions of Ca^{2+} and succinate were made to the test and blank cuvettes simultaneously. Values in brackets indicate the activity of pyruvate kinase expressed as a percentage of the initial rate: 100% is equivalent to 36.7nmol of pyruvate formed/min. The broken line indicates the control rate, in which both Ca^{2+} and succinate were omitted.

properties of liver mitochondria can be manipulated so as to alter the $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$ ratio outside the mitochondria and thus alter pyruvate kinase activity. Addition of $50\mu\text{M-Ca}^{2+}$ to the reaction medium causes about 70% inhibition of the enzyme activity. This agrees with the extent of inhibition observed under similar conditions but in the absence of mitochondria. Addition of succinate then allows Ca^{2+} to be removed from the environment of the enzyme and pyruvate kinase activity returns to the original value. The time taken for this to occur corresponds to the time required for the mitochondria to accumulate all of the Ca^{2+} .

Numerous control experiments were carried out to ascertain that only pyruvate kinase activity was being measured under these conditions. It should be emphasized that mitochondria were present in both the blank and test cuvettes. The reaction was started by addition of phosphoenolpyruvate to the test

Fig. 5. Effect of Ca^{2+} concentration on the reversal of calcium inhibition of pyruvate kinase by mitochondria

The experiment was carried out as described in Fig. 4, except that the amount of Ca^{2+} added was varied as indicated here. ●, Initial pyruvate kinase activity; ○, pyruvate kinase activity on addition of Ca^{2+} ; △, maximal pyruvate kinase activity after addition of succinate. Below an added concentration of 240nmol of Ca^{2+}/mg of protein, pyruvate kinase activity remained constant for 2–5 min after the addition of succinate. Above an added concentration of 240nmol of Ca^{2+}/mg of protein, maximal pyruvate kinase activity occurred about 2 min after addition of succinate (△) and the activity subsequently decreased to a constant rate (▲) between 4 and 6 min after addition of succinate.

cuvette. All subsequent additions were made simultaneously to both the cuvettes.

Reversal of Ca^{2+} inhibition by mitochondria as a function of Ca^{2+} concentration

Mitochondria have only a limited capacity for Ca^{2+} accumulation under 'limited-loading' conditions (Lehninger *et al.*, 1967). Hence if too high a concentration of Ca^{2+} is added to the system described in Fig. 4 not all of the Ca^{2+} will be accumulated by the mitochondria and thus pyruvate kinase activity will not be completely reversed.

Results in Fig. 5 show that at Ca^{2+} concentrations up to $100\text{nmol}/\text{mg}$ of protein, mitochondria can accumulate Ca^{2+} and allow pyruvate kinase activity to return to the initial uninhibited rate. Between 100 and 220nmol of Ca^{2+}/mg of protein the final activity

is consistently greater than the initial rate. It is possible that mitochondria may be releasing endogenous Mg^{2+} at the higher Ca^{2+} concentrations (see Lee *et al.*, 1971a). Irreversible damage to isolated mitochondria is also known to occur at these concentrations of Ca^{2+} (Lehninger *et al.*, 1967; Bygrave & Reed, 1970).

Between 160 and 230 nmol/mg of mitochondrial protein there is also a decrease in the final rate of pyruvate kinase activity. However, this rate is still considerably greater than that of the inhibited rate when only Ca^{2+} is present. This implies that, even at such high Ca^{2+} concentrations, the mitochondria are still capable of accumulating Ca^{2+} . Below 230 nmol/mg of protein, pyruvate kinase activity remains constant at a final rate for at least 4 min. Above this concentration another effect can be seen. The pyruvate kinase activity increases to a maximum approx. 2 min after the addition of succinate and decreases to a level which is the same as that in the presence of only Ca^{2+} . This is probably due to the Ca^{2+} being released from the mitochondria when too high a $[Ca^{2+}]/[\text{mitochondrial protein}]$ ratio exists (Drahota *et al.*, 1965).

This experiment also indicates that approx. 200 nmol/mg of protein is being accumulated by the mitochondria. This is a somewhat greater amount than expected (see above). It is possible, though, that pyruvate is being accumulated as a permeant anion along with the Ca^{2+} . Permeant anions are known to facilitate accumulation of Ca^{2+} (see Lehninger *et al.*, 1967). Pyruvate may be acting in a similar manner in this system.

Thus these experiments have shown that the interaction of pyruvate kinase, Ca^{2+} and mitochondria can be suitably investigated at Ca^{2+} concentrations in the range 0–100 nmol of Ca^{2+} /mg of mitochondrial protein.

Simultaneous measurement of pyruvate kinase activity and Ca^{2+} movements

Results in Fig. 6 show the change in pyruvate kinase activity in relation to the movement of Ca^{2+} in and out of the mitochondria. The reciprocal relation between enzyme activity and the Ca^{2+} concentration outside the mitochondria is clearly evident. Once all of the Ca^{2+} has been accumulated by the mitochondria, carbonyl cyanide *m*-chlorophenylhydrazone, a potent uncoupler of oxidative phosphorylation, can be added. This causes a rapid release of Ca^{2+} from the mitochondria and subsequent inhibition of pyruvate kinase activity. Thus it is possible to alter the activity of pyruvate kinase several times in succession by the removal or addition of Ca^{2+} mediated by mitochondria. The response of pyruvate kinase to the changes in Ca^{2+} concentration is again very rapid.

Fig. 6. *Simultaneous measurement of pyruvate kinase activity and Ca^{2+} movements*

The reaction mixture was as described for Fig. 5 except that $50 \mu M$ - Ca^{2+} (containing $0.5 \mu Ci$ of $^{45}Ca^{2+}$), $5 mM$ -succinate and $10 \mu M$ -carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were added as indicated. Samples ($50 \mu l$) were removed at the appropriate times to measure $^{45}Ca^{2+}$ taken up by the mitochondria. Pyruvate kinase ($2.5 \mu g$) from rabbit muscle was employed in (a) and pyruvate kinase from rat liver supernatant ($0.22 mg$ of protein) was employed in (b). In each case the mitochondria were obtained from liver. o, Percentage of maximum pyruvate kinase activity [(a) 100% = 21.0 nmol of pyruvate formed/min; (b) 100% = 14.5 nmol of pyruvate formed/min]. ●, Percentage of total added $^{45}Ca^{2+}$ present outside the mitochondria.

Discussion

Experiments in the present study have clearly illustrated the potential role of liver mitochondria in modifying the activity of cytoplasmic enzyme systems that are activated by Mg^{2+} and inhibited by Ca^{2+} . It was shown that by simply altering the $[Mg^{2+}]/[Ca^{2+}]$ ratio in the immediate environment of pyruvate kinase, mitochondria could dictate the final rate of the enzyme reaction. Furthermore it was established that by this means the rate of the reaction could be rapidly altered and the degree of inhibition (which could be substantial) depended solely on this ratio. The concentrations of Mg^{2+} and Ca^{2+} employed also were well within the range known to exist in the

cell cytoplasm (see Thiers & Vallee, 1957). Finally, the inhibition observed was readily reversible. Each of these points is important if this form of control is to be seriously considered as a regulating component in cell metabolism.

That the ionic environment does play a major role in regulating cell metabolism is a concept that has been developed only recently (Bygrave, 1964, 1966b, 1967; Kimmich & Rasmussen, 1969; Rasmussen, 1970; Rasmussen & Nagata, 1970). The present experiments provide appreciable support for this concept and bear out some earlier suggestions that Ca^{2+} accumulation by mitochondria may be intimately involved in altering and/or maintaining the composition of the ionic environment in the cell cytoplasm (Lehninger, 1964; Bygrave, 1964, 1967).

Apart from its obvious suitability in this work for illustrating the principle of the ionic control concept, pyruvate kinase is also a key enzyme of glycolysis and gluconeogenesis (see Scrutton & Utter, 1968). Previous work has shown that the high sensitivity of this enzyme to Ca^{2+} (Kachmar & Boyer, 1953; Bygrave, 1966b; Mildvan & Cohn, 1966) is mainly responsible for the inhibition of glycolysis by this ion in cell-free extracts (Geiger, 1940; Bygrave, 1966a). There is some evidence that concentrations of cytoplasmic Ca^{2+} may play a role in gluconeogenesis (Gevers & Krebs, 1966; Kimmich & Rasmussen, 1969; Wimhurst & Manchester, 1970), possibly through interactions at the pyruvate kinase step (Rasmussen & Nagata, 1970). Ionic control of pyruvate kinase is thus potentially important for the control of both glycolysis and gluconeogenesis.

Another metabolic process located in the cytoplasm and sensitive to the $[\text{Mg}^{2+}]/[\text{Ca}^{2+}]$ ratio is phospholipid synthesis (Kennedy & Weiss, 1956; Porcellati *et al.*, 1970a,b; J. B. Roberts & F. L. Bygrave, unpublished work). Similar sensitivity applies also to protein synthesis in *Escherichia coli* extracts (Roy, 1971) but has yet to be demonstrated in preparations from mammalian tissue. Thus we envisage that, apart from their other numerous cellular activities, mitochondria play a more significant role than previously imagined in modifying the activity of ion-sensitive cytoplasmic enzyme systems. We suggest that this form of control is a fine one superimposed upon the primary control mechanisms involving, for example, cellular metabolites (for review see Scrutton & Utter, 1968).

Little can be said at this time of the mechanisms that could induce liver mitochondria *in vivo* to accumulate and release Ca^{2+} in a manner co-ordinated with the overall cellular metabolic network. The recently

described cytoplasmic metabolic factor (Lee *et al.*, 1971a,b) may shed some light on this aspect of the problem.

Requests for reprints should be addressed to F.L.B.

References

- Bailey, E. E., Stirpe, F. & Taylor, C. B. (1968) *Biochem. J.* **108**, 427
- Bücher, Th. & Pfeleiderer, G. (1955) *Methods Enzymol.* **1**, 435
- Bygrave, F. L. (1964) Ph.D. Dissertation, University of Queensland
- Bygrave, F. L. (1966a) *Biochem. J.* **101**, 480
- Bygrave, F. L. (1966b) *Biochem. J.* **101**, 488
- Bygrave, F. L. (1967) *Nature (London)* **214**, 667
- Bygrave, F. L. & Reed, K. C. (1970) *FEBS Lett.* **7**, 339
- Dixon, M. & Webb, E. C. (1964) *Enzymes*, 2nd. edn., p. 420, Longmans, London
- Drahota, Z., Carafoli, E., Rossi, C. S., Gamble, R. L. & Lehninger, A. L. (1965) *J. Biol. Chem.* **240**, 2712
- Geiger, A. (1940) *Biochem. J.* **34**, 465
- Gevers, W. & Krebs, H. A. (1966) *Biochem. J.* **98**, 720
- Heilbrunn, L. V. (1952) *An Outline of General Physiology*, 3rd. edn., p. 536, W. B. Saunders, Philadelphia
- Kachmar, J. F. & Boyer, P. D. (1953) *J. Biol. Chem.* **200**, 669
- Kennedy, E. P. & Weiss, S. B. (1956) *J. Biol. Chem.* **222**, 193
- Kimmich, G. A. & Rasmussen, H. (1969) *J. Biol. Chem.* **244**, 190
- Lee, N. M., Weidemann, I. & Kun, E. (1971a) *Biochem. Biophys. Res. Commun.* **6**, 1030
- Lee, N. M., Weidemann, I. & Kun, E. (1971b) *FEBS Lett.* **18**, 81
- Lehninger, A. L. (1964) *The Mitochondrion*, p. 175, W. A. Benjamin, New York
- Lehninger, A. L., Carafoli, E. & Rossi, C. S. (1967) *Advan Enzymol. Relat. Areas Mol. Biol.* **29**, 259
- McClure, W. R. (1969) *Biochemistry* **8**, 2782
- Mildvan, A. S. & Cohn, M. (1966) *J. Biol. Chem.* **241**, 1178
- Porcellati, G., Biason, M. G. & Arienti, G. (1970a) *Lipids* **5**, 725
- Porcellati, G., Biason, M. G. & Pirotta, M. (1970b) *Lipids* **5**, 734
- Rasmussen, H. (1970) *Science* **170**, 404
- Rasmussen, H. & Nagata, N. (1970) in *Calcium and Cell Function* (Cuthbert, A. W., ed.), p. 209, Macmillan, London
- Roy, A. K. (1971) *Biochim. Biophys. Acta* **246**, 349
- Schneider, W. C. (1948) *J. Biol. Chem.* **176**, 259
- Scrutton, M. C. & Utter, M. F. (1968) *Annu. Rev. Biochem.* **37**, 249
- Sillen, L. G. & Martell, A. E. (1964) *Chem. Soc. Spec. Publ.* **17: Stability Constants**
- Thiers, R. E. & Vallee, B. L. (1957) *J. Biol. Chem.* **226**, 911
- Wimhurst, J. M. & Manchester, K. L. (1970) *FEBS Lett.* **10**, 33