Kinetic Properties of Cerebral Pyruvate Kinase

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Partly purified guinea-pig brain pyruvate kinase is not activated by fructose 1,6-diphosphate and gives hyperbolic substrate-saturation curves with phosphoenolpyruvate. It is therefore different from the L-type pyruvate kinase of mammalian liver. Inhibition by MgATP^{2-} was competitive for MgADP^- but not for phosphoenolpyruvate, and the enzyme is therefore different from the M-type pyruvate kinase, which is said to be competitively inhibited by MgATP^{2-} with respect to both substrates. The K_i (MgATP^{2-}) value of approx. 8mm for the brain enzyme is higher than the values (about 2mm) reported for the muscle enzyme. Stimulation of enzymic activity was observed at low (1–2mm) concentrations of MgATP^{2-} . Substrate kinetic constants were K_m (MgADP^-) = 0.47mm, K_m (phosphoenolpyruvate) = 0.08mm. Free Mg^{2+} at very high concentrations (over 10mm) was inhibitory (K_i = 20–32mm). Neither ADP^{3-} nor $5'\text{-AMP}^{2-}$ inhibited the activity. The brain enzyme was concluded to be different from both the M-type and the L-type of other mammalian organs such as muscle and liver.

Two types of pyruvate kinase (ATP pyruvate phosphotransferase; EC 2.7.1.40) are known to occur in mammalian organs: 'L'-pyruvate kinase in the liver and 'M'-pyruvate kinase in muscle. The M-type also occurs in liver. The L-type is distinguished from the M-type by many criteria, which include its possessing insulin-inducibility, activation by fructose 1,6-diphosphate and sigmoidal dependence of its rate on phosphoenolpyruvate concentration (Tanaka *et al.*, 1967; Haeckel *et al.*, 1968; Llorente *et al.*, 1970; Middleton & Walker, 1972). The yeast enzyme is also activated by fructose diphosphate (Hess *et al.*, 1966; Haeckel *et al.*, 1968; Macfarlane & Ainsworth, 1972).

Although relatively little is known of the enzyme in the mammalian brain, it behaves on electrophoresis like the M-type rather than the L-type (Tanaka *et al.*, 1967). The cerebral enzyme is considered to be one of the control stages of glycolysis: it catalyses a non-equilibrium reaction (Rolleston & Newsholme, 1967) and it is activated in controlled hypoxia *in vivo* (Bachelard *et al.*, 1974). The present paper reports the results of a study of its substrate and product-inhibition kinetic properties which indicate that the enzyme present in the brain under normal conditions is different from the L- or M-type.

Materials and Methods

Preparation of enzyme

Guinea pigs were killed by stunning and exsanguination and their brains were rapidly removed. Each whole brain, except for cerebellum, was homogenized in a Teflon-pestle homogenizer (type C; clearance 0.15–0.23mm; A. H. Thomas and Co., Philadelphia,

Pa., U.S.A.) for 2min at 2000rev./min at 0°C in 9vol. of 0.1M-triethanolamine-hydrochloride buffer (adjusted to pH7.5 with 1M-NaOH). After centrifugation at 150000g for 1h, the supernatant was brought to 50% saturation by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ at 0°C (Nicholas & Bachelard, 1969). The precipitate was removed by centrifugation at 20000g for 15min and the supernatant was brought to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$. The precipitate, obtained after centrifugation at 20000g for 15min, was taken up into the original buffer (2ml/g of fresh tissue) and dialysed against that buffer until free from SO_4^{2-} . It contained 85% of the pyruvate kinase activity of the original homogenate (125 $\mu\text{mol/min per g}$) and had a specific activity of 65 $\mu\text{mol/min per mg}$ of protein. The other cytoplasmic enzymes that might compete for Mg^{2+} and adenine nucleotides were present in negligible amounts; hexokinase activity was less than 1 $\mu\text{mol/min per mg}$ of protein and phosphofructokinase activity was not detected in the sample volumes used in these studies.

Determination of pyruvate kinase activity

The partially purified brain extract was diluted before use in 0.1M-triethanolamine hydrochloride buffer (adjusted to pH6.8 with triethanolamine base) and pyruvate kinase activity was measured, in the direction of ATP formation, at 340nm in a Unicam SP.800 spectrophotometer equipped with constant-temperature cell housing at 37°C. The reaction mixture contained (final concns.): 0.1M-triethanolamine hydrochloride-triethanolamine (pH6.8); lactate dehydrogenase [6 units/ml; dialysed free from $(\text{NH}_4)_2\text{SO}_4$ against the triethanolamine buffer

before use]; 0.16mM-NADH; brain extract (0.1–0.2 μ g of protein/ml). Other constituents (KCl, phosphoenolpyruvate, MgSO_4 and ADP) were present in the concentrations indicated in the text. The ADP (free acid) was adjusted to pH 7.5 with triethanolamine base. In some experiments 2mM-glucose and hexokinase (5 units/ml) were included to ensure regeneration of ADP from ATP, but this had no effect on the observed reaction rates.

Reagents

ADP (free acid), ATP (sodium salt), NADH (sodium salt), phosphoenolpyruvate (potassium salt), lactate dehydrogenase (EC 1.1.1.27; from ox muscle; 300 units/mg), hexokinase (EC 2.7.1.1; from yeast; 140 units/mg) and triethanolamine hydrochloride were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. The ATP was freed from sodium by passage at 0°C through a column of Dowex 50 (H^+ form) and adjusted to pH 7.5 with triethanolamine base. Triethanolamine (free base) was obtained from Hopkin and Williams Ltd., Romford, Essex, U.K. All other reagents were from British Drug Houses Ltd., Poole, Dorset, U.K.

Calculations of MgADP^- and MgATP^{2-}

The amounts of MgSO_4 to be added to the reaction mixtures were calculated from the stability constants of 4000M^{-1} for MgADP^- and 80000M^{-1} for MgATP^{2-} (O'Sullivan & Perrin, 1964). A computer program was set so that for all concentrations of total ADP^{3-} added, at least 97% was in the form MgADP^- unless stated otherwise. When ATP^{4-} was also present, further Mg^{2+} was added in amounts sufficient to keep the same proportion of ADP^{3-} as MgADP^- (97%) and at least 99% of the ATP^{4-} as MgATP^{2-} .

The ATP^{4-} was found to contain 1.1% ADP^{3-} (from enzymic measurement with a crystalline suspension of rabbit muscle pyruvate kinase, and also indirectly from its P_i content). This was corrected for in the calculations when ATP^{4-} was present in the reaction mixture.

Computer programs

For calculation of slopes and intercepts of linear double-reciprocal plots, a program designed by Miss B. Kinsley (Biometrics Unit, Institute of Psychiatry) was applied. For calculation of secondary plots, program BMD 05 R (copyright: Regents of the University of California, Calif., U.S.A.; made available on Library disc, University of London Computing Centre) was used. Calculation of the equations for curved kinetic plots was made with program 'POLI'

(programmed by J. H. Zar, Zoology Department, University of Illinois, Ill., U.S.A.).

Results

Effect of Mg^{2+}

To have at least 97% of the ADP^{3-} as MgADP^- , a considerable excess of Mg^{2+} (up to 8mM excess) must be present, because of the relatively low stability constant of MgADP^- . It seemed important to test the possibility that free excess of Mg^{2+} might activate or inhibit the pyruvate kinase activity. Mg^{2+} and ADP^{3-} were added to the reaction mixture in proportions calculated to give a series of set concentrations of MgADP^- with various concentrations of free excess of Mg^{2+} . Initial rate versus $[\text{Mg}^{2+}]$ plots showed that increasing the $[\text{free Mg}^{2+}]$ up to 10mM (Fig. 1) had no effect on activity at MgADP^- concentrations from 0.25 to 1.3mM, but at higher concentrations Mg^{2+} was clearly inhibitory. At concentrations of free Mg^{2+} above 10mM, the inhibition appeared to be competitive with respect to MgADP^- , from the double-reciprocal plots of Fig. 2. Secondary plots (Cleland, 1963a) of the slopes from Fig. 2 versus $[\text{Mg}^{2+}]$ were linear (Fig. 3); extrapolation of the lines to the abscissae gave K_i values of 20mM- Mg^{2+} at 75mM- K^+ (Fig. 3a) and 32mM- Mg^{2+} at 100mM- K^+ (Fig. 3b). Calculated concentrations of free excess of

Fig. 1. Initial velocity of pyruvate kinase plotted against excess of $[\text{free Mg}^{2+}]$ at fixed MgADP^- concentrations of ○, 0.25mM; ●, 1.3mM

In these experiments, depending on the concentration of Mg^{2+} added, the proportion of total ADP as MgADP^- varied between 89 and 97.5% (see the Materials and Methods section and Fig. 4).

Fig. 2. Plots of $1/v$ versus $1/[\text{MgADP}^{3-}]$ at excess of $[\text{Mg}^{2+}]$ of (mm): ●, 10; ○, 15; ■, 20; □, 25. The concentration of phosphoenolpyruvate was 2.0mm, and of K⁺, 75mm (a) and 100mm (b).

Fig. 3. Secondary plots of slopes versus excess of $[\text{Mg}^{2+}]$ from Fig. 2. (a) 75mm-K⁺; K_i (Mg²⁺) = 20mm. (b) 100mm-K⁺; K_i (Mg²⁺) = 32mm. For further details see Fig. 2.

Fig. 4. Initial velocity of pyruvate kinase plotted against excess of $[\text{free ADP}^{3-}]$ at fixed MgADP^{3-} concentrations of (mm): ○, 0.25; ●, 0.50; □, 0.75; ■, 1.0; ▼, 1.3.

In these experiments the concentration of free excess of Mg^{2+} varied between 2 and 10mm (see Fig. 1).

Mg^{2+} never exceeded 10mm in the following experiments, so the free Mg^{2+} present is concluded not to affect the results.

Effects of adenine nucleotides

Free ADP³⁻ and AMP²⁻. Neither free ADP³⁻ nor 5'-AMP²⁻ was inhibitory to cerebral pyruvate kinase when tested in reaction mixtures containing 2mm-phosphoenolpyruvate, 0.25–1.3mm-MgADP³⁻ and 100mm-K⁺. AMP at 10mm gave less than 5% inhibition. Fig. 4 shows the lack of effect of free ADP³⁻ over a range of MgADP³⁻ concentrations from 0.25 to 1.3mm. The rate of enzymic activity varied with the concentration of MgADP³⁻ but not of ADP³⁻.

MgADP⁻ as substrate. MgADP⁻ is clearly the substrate form of ADP as shown by the hyperbolic rate versus [MgADP⁻] curve of Fig. 5, in contrast with the lack of dependence on [free Mg²⁺] (Fig. 1) or [free ADP³⁻] (Fig. 4). The curve of Fig. 5 is that predicted by insertion of the experimental values for v (18 at each value for MgADP⁻) into the Michaelis-Menten equation. The fit of the line to the experimental points confirms that MgADP⁻ is the substrate. The experiments shown were performed in the presence of 75mM-K⁺ and the K_m value from the calculation of the line was 0.58mM, and 0.48mM at 100mM-K⁺, only slightly higher than the value of 0.47mM obtained

Fig. 5. Initial velocity of pyruvate kinase plotted against [MgADP⁻] at various concentrations of excess of free ADP⁻ of (mM): ○, 0.01–0.03; ●, 0.015–0.06; □, 0.02–0.095; ■, 0.025–0.125; ▼, 0.03–0.16

The vertical bars give the spread of 18 observed values at each calculated concentration of MgADP⁻.

directly from double-reciprocal plots at 100mM-K⁺ (see below). Double-reciprocal plots of $1/v$ versus $1/[MgADP^-]$ (Fig. 6a) at various fixed concentrations of phosphoenolpyruvate from 0.25 to 2.0mM were linear, but plots of $1/v$ versus $1/[phosphoenolpyruvate]$ at various fixed concentrations of MgADP⁻ (not shown) were curved, concave up. The K_m value for MgADP⁻ (0.47mM) was taken from the line at saturating concentrations of phosphoenolpyruvate (2mM) at 100mM-K⁺. Secondary plots (Fig. 6b) of the slopes from Fig. 6(a) versus $1/[phosphoenolpyruvate]$ gave a value for K_m (phosphoenolpyruvate) of 0.08mM.

MgATP²⁻. Cerebral pyruvate kinase activity was increased when MgATP²⁻ was present in low concentrations (up to 1 or 2mM, depending on the concentrations of other reactants), and decreased at higher concentrations of MgATP²⁻. This pattern of stimulation and inhibition was observed under all concentrations tested of MgADP⁻ (see below) and of phosphoenolpyruvate (0.25–2.0mM). Fig. 7 shows the initial rate versus [MgATP²⁻] plots at MgADP⁻ concentrations from 0.25 to 1.25mM, at phosphoenolpyruvate concentrations of 0.25mM (Fig. 7a) and 1.0mM (Fig. 7b). The inhibitory parts of the curves, from 4 or 5mM to 9mM-MgATP²⁻, are presented as double-reciprocal plots of $1/v$ versus $1/[MgADP^-]$ in Fig. 8. The inhibition appears to be competitive with respect to MgADP⁻. Secondary plots of slopes versus [MgATP²⁻] were curved (insets on Fig. 8); Dixon plots of $1/v$ versus [MgATP²⁻] (not shown) were also curved, concave up. Double-reciprocal plots of $1/v$ versus $1/[phosphoenolpyruvate]$ at fixed concentrations of MgADP⁻ and a range of concentrations of

Fig. 6 (a) Plots of $1/v$ versus $1/[MgADP^-]$ at phosphoenolpyruvate concentrations of (mM): ●, 0.25; ○, 0.4; ■, 0.8; □, 2.0; and (b) secondary plot of slopes versus $1/[phosphoenolpyruvate]$ from (a)

The concentration of K⁺ was 100mM. From (a) K_m (MgADP⁻) = 0.47mM; from (b) K_m (phosphoenolpyruvate) = 0.08mM. For further details see the text.

Fig. 7. Plots of rate versus $[MgATP^{2-}]$ at $MgADP^-$ concentrations of (mM): $\bullet$, 1.25; $\circ$, 1.0; $\blacksquare$, 0.75; $\square$, 0.5; $\blacktriangledown$, 0.25. The concentration of K^+ was 100 mM, and of phosphoenolpyruvate, 0.25 mM (a) and 1.0 mM (b). The rates between 0 and 1 mM- $MgATP^{2-}$ (c) were determined separately under the same conditions as in (b), i.e. $[phosphoenolpyruvate] = 1.0$ mM.

Fig. 8. Inhibition by $MgATP^{2-}$: plots of $1/v$ versus $1/[MgADP^-]$ at the inhibitory concentrations of $MgATP^{2-}$ from Fig. 7. Secondary plots of slopes versus $[MgATP^{2-}]$ are inset. (a) and (b) as for Fig. 7(a) and 7(b) respectively. Concentrations of $MgATP^{2-}$ (mM): $\bullet$, 0; $\circ$, 4; $\blacktriangledown$, 5; $\blacksquare$, 7; $\square$, 9.

$MgATP^{2-}$ were all curved, concave up, and showed no tendency to converge, although, owing to the curvature, they could not be said to be parallel. The pattern was clearly not competitive, which indicates that inhibition by $MgATP^{2-}$ is competitive with respect to $MgADP^-$ but not with respect to phosphoenolpyruvate.

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Lack of co-operative interactions

The stimulation of L-type pyruvate kinase activity by fructose diphosphate was not observed with the brain enzyme and the rate versus $[phosphoenolpyruvate]$ plots were hyperbolic rather than the sigmoid curves produced by the L-enzyme, referred to in the introduction.

Discussion

Substrate kinetic properties

The results of the present work confirm previous suggestions (Tanaka *et al.*, 1967) that cerebral pyruvate kinase is different from the inducible L-enzyme of the mammalian liver: the brain enzyme does not appear to be activated by fructose diphosphate and gives hyperbolic rate versus [phosphoenolpyruvate] curves. Also the Michaelis constants for the brain enzyme differ from those of the L-enzyme of liver. Our value for K_m (phosphoenolpyruvate) of 0.08 mM agrees well with the values reported for the enzyme from guinea-pig brain (Takagaki, 1968) and rat brain (Schwark *et al.*, 1971), in contrast with values of 0.2–0.84 mM reported for the liver enzyme (Tanaka *et al.*, 1967; Susor & Rutter, 1968; Llorente *et al.*, 1970). The muscle enzyme gives K_m values for phosphoenolpyruvate of the same order as those of the brain enzyme (0.03–0.086 mM; Kachmar & Boyer, 1953; Reynard *et al.*, 1961; Tanaka *et al.*, 1967). Recorded values of K_m (MgADP⁻) for the liver enzyme (0.1 mM; Tanaka *et al.*, 1967) and for the muscle enzyme (0.2–0.3 mM; McQuate & Utter, 1959; Reynard *et al.*, 1961; Tanaka *et al.*, 1967) are lower than the value of 0.47 mM found here for the brain enzyme. The brain enzyme appears also to be different from the muscle enzyme in the kinetic properties of MgATP²⁻ inhibition, discussed below.

The substrate kinetic constants show that the enzyme is unlikely to be sensitive to changes in the cerebral intracellular concentration of phosphoenolpyruvate which at over 1 mM (McIlwain & Bachelard, 1971) is considerably higher than the K_m value of 0.08 mM. On the other hand, the enzyme should be very sensitive to changes in concentrations of adenine nucleotides. The K_m value for MgADP⁻ of 0.47 mM is of the same order as the intracellular concentration of total ADP³⁻ (0.5 mM) and higher than the concentration of MgADP⁻ there. A concentration of free Mg²⁺ of 1 or 1.5 mM (Bachelard & Goldfarb, 1969) would give a maximum concentration of MgADP⁻ of 0.35 or 0.40 mM, even if all of the free Mg²⁺ were available for complexing with ADP³⁻. The presence of higher concentrations of ATP⁴⁻, totalling some 4 mM or more, would severely limit the Mg²⁺ available for MgADP⁻ formation. The extent to which Mg²⁺ would be limited in this way cannot be assessed with any accuracy because of the lack of quantitative knowledge of the intracellular distribution of ATP⁴⁻.

Activation and inhibition

The present results confirm that MgADP⁻ is the substrate for the enzyme, rather than ADP³⁻, which had no effect on the activity. Free Mg²⁺ also had no effect at concentrations calculated to occur in the brain *in situ* (Bachelard & Goldfarb, 1969). The inhibi-

tion by excess of free Mg²⁺, competitive with respect to MgADP⁻ with K_i of 32 mM at normal concentrations of K⁺ (100 mM), only occurred at concentrations of above 10 mM, far higher than are ever likely to be encountered *in vivo*.

5'-AMP, a known inhibitor of the allosteric enzyme of yeast (Haeckel *et al.*, 1968), did not inhibit the brain enzyme and the pattern of inhibition by MgATP²⁻ was different from that reported for the L- or M-type enzyme. The liver enzyme does not appear to be inhibited by MgATP²⁻ (Irving & Williams, 1973), whereas the enzymes from yeast and muscle are. The inhibition of the muscle enzyme has been reported to be competitive with respect to both substrates, MgADP⁻ and phosphoenolpyruvate (Ainsworth & Macfarlane, 1973), with a K_i value for MgATP²⁻ of 2 mM. This is similar to the K_i value for the yeast enzyme (1.22–1.84 mM) which, like the brain enzyme, is activated by low concentrations of the inhibitor (Haeckel *et al.*, 1968). Nevertheless, the brain enzyme seems different from all of these: at 1–2 mM-MgATP²⁻ it is slightly stimulated and the K_i value for MgATP²⁻ is considerably higher. Owing to the curvatures of the Dixon plot (not shown) and the secondary plots of Fig. 8, the K_i value had to be calculated indirectly (Cleland, 1963b). The experimental values of Fig. 8 were fitted according to a computer program both for second- and third-order equations. The second-order equation of the form (Cleland, 1963b) $y = a + bx + cx^2$ gave the best fit. The values for K_i (MgATP²⁻) with respect to varied concentrations of MgADP⁻ (Fig. 8) were 8 mM for fixed phosphoenolpyruvate concentrations of 2, 1 and 0.8 mM, and 7.5 mM for a phosphoenolpyruvate concentration of 0.4 mM, giving a mean K_i value of 7.9 mM.

It seems reasonable to conclude therefore that cerebral pyruvate kinase under normal conditions is not only clearly different from the inducible liver enzyme, but also shows inhibition kinetic properties which are qualitatively and quantitatively different from those exhibited by the enzymes from muscle or from yeast.

Controlled hypoxia causes activation of cerebral pyruvate kinase under conditions of increased rates of glycolysis but no change in rate of respiration and no detectable change in concentration of any of the adenine nucleotides (Bachelard *et al.*, 1974). The activation of the enzyme under these conditions can hardly be associated with its sensitivity, noted above, to changes in ADP or ATP concentrations and an alternative interaction must therefore be sought. Under hypoxic conditions there is increased entry of glucose to the brain and it remains to be seen if the raised carbohydrate concentrations result in induced activity as happens in the liver. In the present study, the partially purified enzyme represented 83% of that present in the original homogenate. It is possible that

a minor proportion of the normal activity is different and could be increased in proportion in a changed physiological state. Although Tanaka *et al.* (1967) found only one form of the enzyme on electrophoresis, Susor & Rutter (1968) noted two.

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References

- Ainsworth, S. & Macfarlane, N. (1973) *Biochem. J.* **131**, 223–236
- Bachelard, H. S. & Goldfarb, P. S. G. (1969) *Biochem. J.* **112**, 579–586
- Bachelard, H. S., Lewis, L. D., Pontén, U. & Siesjö, B. K. (1974) *J. Neurochem.* **22**, 395–401
- Cleland, W. W. (1963a) *Biochim. Biophys. Acta* **67**, 173–187
- Cleland, W. W. (1963b) *Nature (London)* **198**, 463–465
- Haeckel, R., Hess, B., Lauterborn, W. & Wuster, K. H. (1968) *Hoppe-Seyler's Z. Physiol. Chem.* **349**, 699–714
- Hess, B., Haeckel, R. & Brand, K. (1966) *Biochem. Biophys. Res. Commun.* **24**, 824–831
- Irving, M. G. & Williams, J. F. (1973) *Biochem. J.* **131**, 287–301
- Kachmar, J. F. & Boyer, P. D. (1953) *J. Biol. Chem.* **200**, 669–682
- Llorente, P., Marco, R. & Sols, A. (1970) *Eur. J. Biochem.* **13**, 45–54
- Macfarlane, N. & Ainsworth, S. (1972) *Biochem. J.* **129**, 1035–1047
- McIlwain, H. & Bachelard, H. S. (1971) *Biochemistry and the Central Nervous System*, 4th edn., pp. 47, 104, Churchill, London
- McQuate, J. T. & Utter, M. F. (1959) *J. Biol. Chem.* **234**, 2151–2157
- Middleton, M. C. & Walker, D. G. (1972) *Biochem. J.* **127**, 721–731
- Nicholas, P. C. & Bachelard, H. S. (1969) *Biochem. J.* **112**, 587–594
- O'Sullivan, W. J. & Perrin, D. D. (1964) *Biochemistry* **3**, 18–26
- Reynard, A. M., Hass, L. F., Jacobsen, D. D. & Boyer, P. D. (1961) *J. Biol. Chem.* **236**, 2277–2283
- Rolleston, F. S. & Newsholme, E. A. (1967) *Biochem. J.* **104**, 524–533
- Schwark, W. S., Singhal, R. L. & Ling, G. M. (1971) *J. Neurochem.* **18**, 123–134
- Susor, W. A. & Rutter, W. J. (1968) *Biochem. Biophys. Res. Commun.* **30**, 14–20
- Takagaki, G. (1968) *J. Neurochem.* **15**, 903–916
- Tanaka, T., Harano, T., Sue, F. & Morimura, H. (1967) *J. Biochem. (Tokyo)* **62**, 71–91