Specific Spectrophotometric Assay for the M Isoenzyme of Pyruvate Kinase in Plasma Samples Containing Mixtures of the Muscle (M) and Liver (L) Isoenzymes

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Plasma pyruvate kinase (EC 2.7.1.40) activity contains two major isoenzymes. The "L" type originates from liver (erythrocytes contain a similar isoenzyme), the "M" type predominantly from skeletal muscle. The diagnostic use of pyruvate kinase as an index of muscle leakage requires an assay of only the M type. An M-type-selective assay for mixtures of the two isoenzymes was devised, based on the differences that the L form has a higher $K_m$ for phosphoenolpyruvate and is inhibited by about 95% by a high concentration of ADP. All relevant conditions of the assay have been examined and the reaction system has been optimized at 30°C. The assay yields the expected $K_m$ values for M type pyruvate kinase. An apparent increase in activity in hemolysed plasma samples was found to be derived from leukocytes.

Additional Keyphrases: Duchenne dystrophy · carrier detection · leakage of enzyme from muscle

Pyruvate kinase (EC 2.7.1.40) in normal plasma consists of two isoenzymes; the L form (L-PK) is derived from erythrocytes and liver, and the M form (M-PK) is derived mainly from skeletal muscle and heart, and to a small extent from some other tissues (1, 2). Plasma PK determination has been used to aid in the detection of carriers of Duchenne’s muscular dystrophy; boys affected with the disease have grossly above-normal values (1) while female carriers of the disease usually do not suffer from muscle disease, but many have above-normal PK values (3, 4). It is still to be determined whether or not PK assay is superior to assay of creatine kinase (EC 2.7.3.2) for carrier detection, but it has been suggested that the carrier detection rate is improved by assaying for both enzymes (5). Assay for PK activity in fetal plasma samples may also be of value for prenatal diagnosis of the disease.

To achieve these objectives requires an assay for PK that measures exclusively the muscle form of the enzyme under optimum conditions of catalytic activity in a small volume of plasma. Previously reported assay systems for PK are similar in principle and measure both muscle and liver isoenzyme activities (1, 3). This paper reports an investigation leading to an optimized assay that is essentially specific for M-PK in small plasma samples containing a mixture of the two isoenzymes.

Materials and Methods

Imidazole, NADH, ADP, LDH, and DTT were all obtained from Sigma London Chemical Company. PEP (monocyclohexylamine salt) and all other reagents were obtained from British Drug Houses, Poole, Dorset, U.K.; these were of analytical purity.

All blood samples were collected into heparin, centrifuged immediately, and the plasma either stored on ice and used without undue delay or, for longer-term storage, kept under liquid nitrogen.

For the assay of muscle type pyruvate kinase the final assay system established was as follows. (Departures from this system used in the investigation are indicated, as appropriate, in the Results section.) The final concentrations in the reaction mixture were imidazole · HCl, 100 mmol/L, pH 7.4; KCl 150 mmol/L; MgCl$_2$ 5 mmol/L; NADH 0.17 mmol/L; ADP 5 mmol/L; DTT 5 mmol/L; PEP 0.25 mmol/L, and lactate dehydrogenase, 10 kU/L.

In practice, three stock solutions were prepared containing:

(a) Imidazole · HCl 110 mmol/L, pH 7.4; KCl 165 mmol/L; MgCl$_2$ 5.5 mmol/L; NADH 0.19 mmol/L; ADP 5.5 mmol/L; and DTT 5.5 mmol/L. This solution can be stored frozen for at least one week.

(b) 500 U of lactate dehydrogenase dissolved in 1 mL of doubly distilled water. This solution can be stored frozen in small aliquots for at least three weeks.

(c) PEP, 12.5 mmol/L, in doubly-distilled water. This solution is prepared freshly each day.

The assay procedure: add 455 µL of solution a, 10 µL of solution b, and 25 µL of plasma to a cuvette, mix well, and incubate for 5 min at 30°C. Then add 10 µL of solution c to initiate the reaction.

We followed the decrease in absorbance of NADH at 340 nm with a Cary 16S spectrophotometer thermostatted at 30°C. A short lag phase of about 1.5 min was followed by a linear rate of absorbance change corresponding to the M-PK activity. The reaction rate was measured for about 5 min. Correction for blanks was checked, but found to be ordinarily unnecessary.

One unit (U) of M-PK activity is the amount that will consume 1 µmol of NADH per minute under the assay conditions described above. The results are expressed in terms of units of activity per litre of plasma. The molar absorbivity of NADH was taken as 6.22 cm$^2$/µmol.

Results

Analytical Variables

Buffer and pH. The optimum pH for M-PK is pH 7.4 (Figure 1). Imidazole · HCl buffer, 100 mmol/L, was chosen because the pK of imidazole (7.1) is near the optimum pH of the assay. This buffer concentration was chosen to ensure adequate buffering of the relatively high ADP concentration we used as compared with previous workers.

ADP and PEP. In previous standard enzyme assays, 2 mmol of ADP per liter with 2 mmol of PEP per liter was used to estimate total PK activity, or 0.2 mmol of PEP per liter to measure approximately the M-type PK activity. Figure 2 compares the reaction rate of plasma PK as a function of PEP concentration at fixed 2 mmol/L and 5 mmol/L ADP concentrations. Both curves show a plateau at about 0.2 mmol of
PEP per liter. With 5 mmol of ADP per liter no further increases in the reaction rate occur as the PEP concentration is further increased, but a secondary increase is seen with 2 mmol of ADP per liter. The first plateau is ascribable to the maximum activity of the M-PK, which has a low $K_m$ for PEP (6). The secondary increase is not seen with 5 mmol of ADP per liter, because at this concentration of ADP all or most of the L-PK is inhibited (6). However, 2 mmol ADP per liter is insufficient to inhibit L-PK and a secondary rise in PK activity is observed owing to this isoenzyme, which has a higher $K_m$ for PEP. A PEP concentration of 0.25 mmol/L was therefore chosen for routine assay to give maximum activity of the muscle isoenzyme. We did not use higher concentrations, to avoid the possibility of interference by activating L-PK.

In addition to the inhibitory effect of ADP on L-PK, the concentration is critical for its action as a substrate. In Figure 3 one can see that maximum activity requires at least 3 mmol of ADP per liter. Therefore 5 mmol of ADP per liter is adequate to give maximum activity of M-PK, and inhibition of L-PK.

**Fig. 1.** Effect of pH on plasma M-PK activity

By assaying a plasma sample with 5 mmol of ADP per liter and various concentrations of PEP, we calculated the $K_m$ for PEP to be 0.03 mmol/L. The $K_m$ for ADP was derived in a similar manner and found to be 0.8 mmol/L. Both of these values agree reasonably with $K_m$'s calculated from use of pure skeletal muscle pyruvate kinase (6).

**LDH.** A lag phase occurring after addition of PEP was found to depend on the activity of the linking enzyme, LDH. Figure 4 shows that the lag phase can be shortened to 1.5 min by increasing the amount to 10 kU/L. A further increase in LDH did not further shorten lag time.

**Enzyme concentration.** By assaying a series of dilutions of a muscle homogenate, we found the relationship between enzyme concentration and measured enzyme activity to remain linear up to 350 U/L (Figure 5). The amount of plasma in the assay mixture had no interfering effects up to 50 μL of plasma in a total reaction volume of 0.5 mL (Table 1).

**Storage of plasma.** Storage of plasma at 4 or −20 °C results in a progressive loss of enzyme activity. For a normal blood sample that had been cooled and the plasma promptly separated after withdrawal, typical activity losses are shown in Table 2. This lost activity could be almost completely regained by including 5 mmol of DTT per liter in the assay mixture. A similar result was obtained after simulated aging by bubbling oxygen through a fresh plasma sample on ice for 20 min (Table 2).

**Fig. 2.** Effect of phosphoenolpyruvate concentration on plasma pyruvate kinase activity
The reaction was carried out with 2 mmol/L ADP (O) and 5 mmol/L ADP (△)

**Fig. 3.** Effect of ADP concentration on the activity of plasma M-PK

**Fig. 4.** Dependence of the lag phase of the assay for plasma M-PK on the amount of lactate dehydrogenase in the reaction mixture.
Lactate dehydrogenase activity (Sigma Type XI) was 935 kU/g of protein at 37 °C.
No loss of activity is observed with plasma samples stored in liquid nitrogen, although for routine assays DTT was always included in the reaction mixture.

Hemolysis. Mechanical damage resulting in hemolysis was simulated by whirlly-mixing 1-mL aliquots of whole blood for different lengths of time between 0 and 3 min. The plasma was separated and its M-PK activity measured. Hemoglobin was used as an index of hemolysis and was measured by converting it to cyanmethemoglobin (7) and comparing the absorbance at 419 nm with that of standard solutions.

Slight hemolysis is shown to have little effect on the assay system (Figure 6). This was expected because erythrocytes contain the L-form of PK only. However, with greater hemolysis the apparent activity of plasma M-PK is progressively increased (Figure 6). This could be due to the contribution of L-PK from erythrocytes, M-PK from leukocytes, or other unknown interfering substances. We re-examined the effect of hemolysis, using whole blood from which the leukocytes had been removed by centrifugation (1500 × g for 10 min) and removal of the plasma and the buffy coat. The erythrocytes were resuspended in the plasma and the process was repeated three times. Finally, a portion of erythrocytes was resuspended in fresh plasma so that the hematocrit of the blood was restored to the original value; the buffy coat was added to another portion of erythrocytes and the appropriate volume of fresh plasma added. This gave two blood samples, one depleted of leukocytes, the other enriched in them. Mechanical damage to the blood was carried out as before. Figure 6 shows clearly that the increase in M-PK activity is due entirely to release from damaged leukocytes. Although much L-PK would be released from the erythrocytes, this isoenzyme does not interfere with the assay.

Discussion

In the past, plasma PK activity has been measured by a variety of adaptations of the assay system. In most cases total plasma PK activity has been measured. However, some assessment of the relative proportions of the two major isoenzymes was made by Harano et al. (7), by assaying plasma samples with two concentrations of PEP, 0.2 and 2 mmol/L, both with 2 mmol of ADP per liter. By taking the ratio of the two results, the proportion of M-type PK was determined. Smith and Thomson (8) used two assay systems. In the first, a low PEP concentration (0.2 mmol/L) was used to select for M-PK; in the second, the PEP concentration was increased to 1 mmol/L and L-PK was inhibited by including 2 mmol of DTT.

Table 1. Measured M-PK Activity in a Sample of Normal Plasma

<table>
<thead>
<tr>
<th>Plasma vol., µL</th>
<th>M-PK activity, U/L</th>
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<tbody>
<tr>
<td>10</td>
<td>34.5</td>
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<tr>
<td>20</td>
<td>37.2</td>
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<tr>
<td>25</td>
<td>37.5</td>
</tr>
<tr>
<td>30</td>
<td>38.7</td>
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<tr>
<td>40</td>
<td>37.9</td>
</tr>
<tr>
<td>50</td>
<td>38.0</td>
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</table>

* The results are the mean of triplicate assays. Up to 50 µL of plasma in a total reaction volume of 500 µL does not have any adverse effects on the measured activity.

Table 2. Effect of Dithiothreitol on the M-PK Activity of Fresh and Stored Normal Plasma Samples and on Samples Artificially Aged by Bubbling Oxygen through Fresh Plasma

<table>
<thead>
<tr>
<th></th>
<th>Relative M-PK activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>without DTT</td>
</tr>
<tr>
<td>Fresh plasma</td>
<td>99</td>
</tr>
<tr>
<td>Plasma stored 4 °C for 1 day</td>
<td>72</td>
</tr>
<tr>
<td>Plasma stored frozen for a week</td>
<td>68</td>
</tr>
<tr>
<td>Oxygenated plasma</td>
<td>66</td>
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* Each percentage figure represents the mean of five determinations.

Fig. 5. Linearity of the assay for M-PK as a function of the amount of enzyme present
A 100 g/L homogenate of human muscle was used as the source of M-PK.

Fig. 6. Effect of hemolysis on plasma M-PK activity
This was measured after mechanical hemolysis of whole blood (O), whole blood enriched with leukocytes (L), and whole blood depleted of leukocytes (A).
alanine, a known allosteric inhibitor of the liver isoenzyme, per liter. Neither of these two methods is ideal for a selective M-PK assay. In the first case, conditions are not optimal for M-PK and the L-PK is not inhibited, so if the plasma contains above-normal activity of the L-type isoenzyme, this will interfere. In the second case, with a PEP concentration of 1 mmol/L an alanine concentration of 2 mmol/L will not be a very effective inhibitor. In fact, it can be predicted (9) that only about 55% of the L-PK would be inhibited. Further, a 2-h preincubation at room temperature was required to overcome the effect of cold-desensitization during storage. Percy et al. (10) measured M-PK only in serum. In their assay they used, per liter, 0.14 mmol of PEP and 0.4 mmol of ADP. With such a low PEP concentration, L-PK is unlikely to have much activity, but both substrate concentrations are well below the optimum for M-PK. Under these conditions the reaction rate will depend on substrate concentrations, so that a small variation in either will change the reaction rate and result in an inaccurate estimate of the activity.

In our procedure, M-PK is assayed at optimal substrate concentrations at 30 °C whilst L-PK is totally inhibited. The advantages of using this assay for PK is that by measuring M-PK alone a more specific assessment of muscle leakage is produced. Improved accuracy is also obtained by using the substrates at saturating conditions so that small errors arising from pipetting and weighing inaccuracies will not affect the reaction rate. Any variations in the plasma L-PK activity unrelated to muscle disease will not be recorded. Complete inhibition of the L-PK is particularly advantageous for reliable results to be obtained, because it is this isoenzyme that causes problems in assay because of the temperature-sensitive allosterism phenomenon characteristic of the L-form (9). For the assay system described here no prolonged thermal equilibration is required to ensure that L-PK is in its allosterically active form.

This work was supported by the Muscular Dystrophy Group of Great Britain.

References