PYRUVATE KINASE PHOSPHATASE

Mary-Frances JETT, Louis HUE and Henri-Géry HERS
Laboratoire de Chimie Physiologique, Université de Louvain and *International Institute of Cellular and Molecular Pathology, Brussels, Belgium*

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1. Introduction

Liver pyruvate kinase can be phosphorylated and simultaneously inactivated by cAMP-dependent protein kinase; the inactivated enzyme can be reactivated by a multifunctional protein phosphatase (review [1]). The inactivation of pyruvate kinase has been observed in isolated hepatocytes incubated in the presence of glucagon and in this case the decrease in pyruvate kinase activity was correlated with the stimulation of gluconeogenesis [2]. The glucagon induced inactivation of pyruvate kinase is however transient and reactivation occurred within 20 min when sub-optimal doses of glucagon were added. This reactivation was more rapid in the presence of insulin [2,3].

The purpose of this work was to investigate the nature of the enzyme that reactivates pyruvate kinase and its relationship with the well-known protein phosphatases that act on glycogen phosphorylase *a*.

2. Methods

The inactive form of pyruvate kinase was partially purified from livers of anesthetized rats given glucagon (i.v., 1 mg/kg) 5 min prior to sacrifice. Livers were homogenized at 0°C in 4 vol. 0.1 M KF, 15 mM EGTA and 50 mM glycyl glycine, at pH 7.4. Following centrifugation at 18 800 × *g* for 10 min, pyruvate kinase was purified essentially as in [4] by treatment at pH 5.2, ammonium sulfate precipitation and a single chromatography on DEAE-cellulose. The enzyme was eluted from the DEAE-column (1.5 cm × 10 cm) with 100 mM potassium phosphate (pH 7.2) at a rate of 5–6 ml/min. The peak fraction was dialyzed overnight against a buffer containing 30% glycerol, 20 mM mercaptoethanol, and 100 mM Tris at pH 7.2 and stored at 5°C. When the final pyruvate kinase preparation was assayed as described below at 0.15 mM PEP ($v_{0.15}$), the activity was consistently <10% of the maximal velocity measured at 5 mM PEP (V_5). This value of the $v_{0.15}/V_5$ ratio is characteristic of the inactive form of pyruvate kinase [2,5]. When purified pyruvate kinase was incubated in the presence of 10 mM $MgCl_2$, no reactivation occurred indicating that no magnesium-dependent phosphatase activity was present in this preparation. The addition of exoggenous pyruvate kinase phosphatase caused a complete reactivation of pyruvate kinase as evidenced by an increase in the $v_{0.15}/V_5$ ratio from 0.1–0.45.

The liver extract, used as a source of pyruvate kinase phosphatase was prepared as follows. Livers were homogenized in 2 vol. 50 mM imidazole (pH 7.4), 250 mM sucrose and 0.5 mM dithiothreitol and the homogenate was centrifuged at 18 800 × *g* for 10 min at 0°C. The high-speed supernatant was prepared by centrifuging the extract at 106 500 × *g* for 30 min. Additional treatment is noted in the legend of table 1.

The reactivation of pyruvate kinase was measured at 25°C in the presence of 50 mM Tris (pH 7.2), 5% glycerol, bovine serum albumin (1 mg/ml), 25 mM KCl, 10 mM $MgCl_2$, 20 mM mercaptoethanol and non-saturating levels of pyruvate kinase (8 units/ml), unless noted otherwise. The reaction was initiated by the addition of the pyruvate kinase phosphatase preparation. The amount of pyruvate kinase present in the pyruvate kinase phosphatase preparation represented ≤15–20% of the total pyruvate kinase present in the assay. The reaction was stopped by freezing an aliquot (20 μl) in tubes placed in a dry ice/acetone bath. The frozen samples were stored at −20°C for ≤2 days. They were then thawed in 0.78 ml buffer containing 0.1 M KF, 15 mM EGTA, 5 mM EDTA, bovine serum albumin (2 mg/ml), 5 mM potassium phosphate and

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50 mM glycyl glycine (pH 7.4). Samples of 25 μl of this solution were then used for the determination of pyruvate kinase activity ($v_{0.15}$) as described below. The initial rate of the reactivation reaction was linear with respect to the amount of pyruvate kinase phosphatase added and the time of incubation. One unit of pyruvate kinase phosphatase is that amount of enzyme that catalyzes an increase in pyruvate kinase activity ($v_{0.15}$) of 1 unit [5]/min under the described conditions.

Pyruvate kinase activity was measured at 0.15 mM phosphoenolpyruvate as in [5]. Skeletal muscle phosphorylase *a* was prepared and liver phosphorylase phosphatase was assayed and its unit defined as in [6]. All phosphoprotein phosphatase preparations were filtered on Sephadex G-25 equilibrated with 50 mM Tris (pH 7.2) and 0.5 mM dithiothreitol prior to being assayed. Treatment of the high-speed supernatant or of the pH 5.2 fractions with trypsin was performed as in [6].

3. Results

3.1. *Separation of pyruvate kinase phosphatase from phosphorylase phosphatase*

It had been shown that when a liver extract prepared from liver of fed rats was subjected to high-speed centrifugation, 90% of the spontaneously-active phosphorylase phosphatase is recovered in the glycogen pellet [6,7]. This is confirmed by the data in table 1 which also shows that 80% of the enzyme which catalyzes the reactivation of pyruvate kinase was found in the high-speed supernatant. It has also been reported that when a crude liver extract is centrifuged at pH 5.2, the majority of the spontaneously active phosphorylase phosphatase ('native' phosphatase) and also the activity which can be revealed by trypsin ('latent' phosphatase) is sedimented [6]. This observation is confirmed by the data shown in table 1 which also indicates that under the same conditions the majority of the enzyme which reactivates pyruvate kinase is present in the supernatant.

3.2. *Effect of magnesium and trypsin treatment*

The reactivation of pyruvate kinase is highly dependent on the presence of magnesium chloride. When the reactivation was studied in an extract or in a high-speed supernatant a 10-fold stimulation of the reaction was observed at 10 mM magnesium (fig.1).

When a high-speed supernatant is treated with trypsin, the phosphorylase phosphatase activity is increased many-fold [6–8]. In an experiment in which a 10-fold increase was observed, there was little change in the pyruvate kinase phosphatase activity when measured in the presence of 10 mM magnesium; but there was an 8-fold increase in the activity measured in the absence of the divalent cation (not shown). Magnesium had, therefore, little effect after trypsin treatment.

3.3. *Effect of the heat-stable inhibitory protein*

It is known that the activity of phosphorylase phosphatase which has been revealed by trypsin, is

Table 1
Separation of pyruvate kinase phosphatase from 'native' and 'latent' phosphorylase phosphatase (units/g liver)

	High-speed centrifugation		Centrifugation at pH 5.2		
	Phosphorylase phosphatase	Pyruvate kinase phosphatase	Phosphorylase phosphatase		Pyruvate kinase phosphatase
			– Trypsin	+ Trypsin	
Fraction					
Extract	22.7 ± 4.8	6.2 ± 1.0	22.3 ± 0.2	252 ± 1.7	6.8 ± 0.8
Supernatant	2.4 ± 2.1	5.8 ± 0.4	0.6 ± 0.2	33.8 ± 5.4	4.8 ± 0.6
Pellet	18.4 ± 3.9	0.7[a]	29.5 ± 2.9	194 ± 37	2.4 ± 0.6

[a] Average of 2 expts

Liver extract was either centrifuged at 100 000 × *g* for 30 min or was brought to pH 5.2 with 1 M acetic acid and then centrifuged at 10 000 × *g* for 20 min. The pellets were resuspended in homogenization buffer to the initial volume and the pH 5.2 supernatant was neutralized with 2 M Tris base. Trypsin treatment was applied to the extract and following the pH 5.2 fractionation, as indicated. The values shown are means ± SEM of 3 separate expts

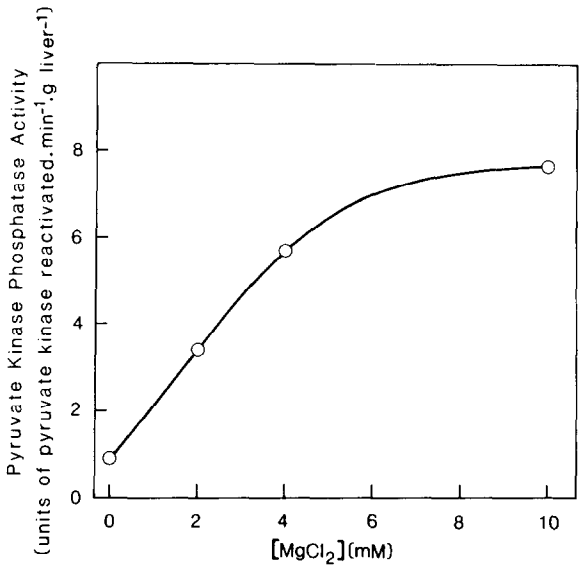

Fig.1. Effect of magnesium on pyruvate kinase phosphatase activity. A liver high-speed supernatant was used as a source of pyruvate kinase phosphatase.

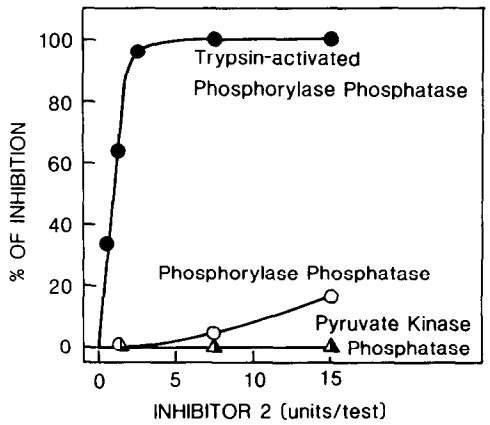

Fig.2. Effect of skeletal muscle inhibitor 2 on phosphorylase phosphatase and on pyruvate kinase phosphatase before and after trypsin treatment. A high speed supernatant, treated (closed symbols) or not (open symbols) with trypsin, was the source of phosphatases. The inhibitor 2 was purified from rat skeletal muscle according to [10]. One unit of inhibitor is the amount of inhibitor that causes 50% inhibition of the trypsin activated phosphorylase phosphatase in the conditions of the assay (total vol. 100 μl). The non-inhibited activities measured before and after trypsin treatment and expressed in units/g liver were 0.8 and 27.5 for phosphorylase phosphatase, 9.5 and 13 for pyruvate kinase phosphatase measured with magnesium and 0.8 and 8.5 for the same enzyme measured without magnesium. The same lack of inhibition was observed when pyruvate kinase phosphatase was measured before or after trypsin treatment with or without magnesium.

greatly decreased by an inhibitory protein which can be obtained by heating various tissue extracts [6–9]. In contrast the phosphorylase phosphatase activity which can be directly measured in a liver preparation is negligibly affected by this inhibitor [6,7]. As seen in fig.2 the activity of pyruvate kinase phosphatase was not affected by concentrations of the inhibitor which decreased the activity of the trypsin-treated phosphorylase phosphatase by ~90%. After trypsin treatment, pyruvate kinase phosphatase, assayed in the presence or absence of 10 mM magnesium, remained insensitive to the heat-stable inhibitory protein.

4. Discussion

The main conclusion that we can draw from this work is that the enzyme which reactivates pyruvate kinase in a liver extract is different from both the native phosphorylase phosphatase [6] and the multifunctional protein phosphatase which can be revealed by treatment of the high-speed supernatant with trypsin [6–8]. Indeed, 'native' phosphorylase phosphatase is glycogen-bound and can be separated from the enzyme which reactivates pyruvate kinase by high-speed centrifugation. It can also be separated from the non-specific 'latent' phosphorylase phosphatase, although in a less complete fashion, by centrifugation at pH 5.2. Under these conditions, the majority of pyruvate kinase phosphatase remains soluble.

Another characteristic of pyruvate kinase phosphatase is its magnesium dependency. Very high, and presumably non-physiological concentration of magnesium are required for a maximum activity. The physiological implication of this observation is not clear.

Finally, pyruvate kinase phosphatase, treated or not with trypsin, is completely insensitive to the heat-stable inhibitory protein of protein phosphatases and shares this property with the native phosphorylase phosphatase.

The general impression that one can draw from this work is that the protein phosphatases that play a role in metabolism of glycogen and in glycolysis are specific enzymes which are not inhibited by the heat-stable inhibitory protein.

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