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DEPHOSPHORYLATION AND REACTIVATION OF PHOSPHORYLATED PYRUVATE KINASE BY A CYTOSOLIC PHOSPHOPROTEIN PHOSPHATASE FROM HUMAN ERYTHROCYTES

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Summary: A cytosolic phosphoprotein phosphatase activity which is capable of removing the phosphate group from phosphorylated human erythrocyte pyruvate kinase has been found in the red blood cell. Removal of the phosphate group results in the reactivation of the pyruvate kinase. The phosphatase is not markedly sensitive to fluoride or chelating agents; it is inhibited by ligands containing phosphate groups. Adenosine diphosphate was found to be the most effective inhibitor. Gel filtration of the preparation suggests that there is more than one form of phosphatase present

Despite an earlier report to the contrary (1), it has recently been found that human erythrocyte pyruvate kinase can be phosphorylated by erythrocyte membranes (2) or in the intact red blood cell (3). At low P-enolpyruvate concentrations the phosphorylated enzyme has reduced activity suggesting that phosphorylation of pyruvate kinase could be a mechanism for regulating its activity *in vivo*. Lagrange and co-workers have also found that erythrocyte phosphofructokinase is phosphorylated (4) but there was no apparent effect on its activity. However, in contrast to the liver, the regulatory role that phosphorylation of enzymes might play is not clear since the erythrocyte does not carry out gluconeogenesis.

If phosphorylation is an important regulatory mechanism in the red blood cell there must be a mechanism for dephosphorylating the phosphorylated proteins. An enzyme capable of dephosphorylating spectrin has been found in the lysate of red blood cells (5,6) but as yet no phosphatase activity capable of dephosphorylating other phosphorylated proteins has been reported.

Abbreviations: Fru-1,6-P_2, fructose-1,6-bisphosphate; P-enolpyruvate, phosphoenolpyruvate; 2,3-DPG, 2,3-diphosphoglycerate; DTE, dithioerythritol; HEPES, N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-N,N'tetraacetic acid.

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This paper describes the identification of a cytosolic phosphoprotein phosphatase that will dephosphorylate and reactivate pyruvate kinase. The lack of such an enzyme in the erythrocyte would have suggested that the phosphorylation of enzymes, previously reported, was an artefact of the non-physiological conditions used.

Materials and methods: Casein, protein kinase from bovine heart, and the proteolytic inhibitors were obtained from Sigma Chemical Co., [^{32}P] γATP (2500 Ci/mmole) was obtained from New England Nuclear, Scintiverse was obtained from Fisher Scientific Co. and Sephadex G-200 was obtained from Pharmacia. The sources of all other materials were as described previously (2).

Pyruvate kinase and the erythrocyte membranes were prepared as described previously (2). The protein phosphatase was partially purified from lysed red blood cells (in 1 mM phenylmethylsulfonylfluoride) by ammonium sulfate precipitation at pH 7.5 (25-45% fraction) followed by batchwise chromatography on DEAE Sephadex at pH 7.0 and passage through a Matrex Gel Red 'A' column (to remove the pyruvate kinase). The purification through these stages was at least 200-fold.

Pyruvate kinase, in the absence of Fru-1,6-P_2 (2), was phosphorylated by incubation with 0.2 mM ATP (50-100 μCi), 50 μM cyclic AMP and red blood cell membranes in pH 7.4 HEPES buffer (2) containing 10 mM caffeine and 10 mM DTE, for at least 3 hours at 25°C. Casein was dephosphorylated by the method described by Reimann et al. (7) and subsequently phosphorylated by incubation with 0.2 mM ATP (0.1 mCi), 50 μM cyclic AMP and protein kinase (0.1 mg/ml), for 3 hours at 25°C, in pH 7.4 HEPES containing 10 mM caffeine. Spectrin was phosphorylated by the incubation of the erythrocyte ghosts with 1 μM ATP (25 μCi) and 50 μM cyclic AMP for 3 hours at 25°C.

Pyruvate kinase was assayed as described previously (2), any deviations from this are described in the text.

Phosphatase activity towards casein or phosphorylated pyruvate kinase was determined by following the loss of radioactive label from the substrates. The reactions were stopped by precipitating the proteins with trichloroacetic acid, essentially as described by Boivin and Galand (8). The final precipitate was dissolved in 0.1 ml of 1 M NaOH and counted in 5 ml of Scintiverse. Phosphatase activity against p-nitrophenylphosphate was measured by following the increase in absorbance at 400 nm, at pH 7.4 and 30°C.

SDS gel electrophoresis was done according to the method of Laemmli (9), in 8% acrylamide. Gels and autoradiograms were scanned on a Gelman ACD 18 densitometer.

Molecular weights were determined on a Sephadex G200 superfine column (70 x 1.6 cm). The column was equilibrated with pH 7.4 HEPES buffer containing 1 mM DTE and run at 4°C; 1.2 ml fractions were collected.

Results and discussion: Pyruvate kinase that had been phosphorylated as described above was found to contain 2.63 ± 0.71 phosphate groups per tetramer (based on a subunit molecular weight of 66,000 and a specific activity of 150 units/mg). Densitometric scans of the gel autoradiograms showed at least 90% of the radioactivity corresponded to labeled pyruvate kinase.

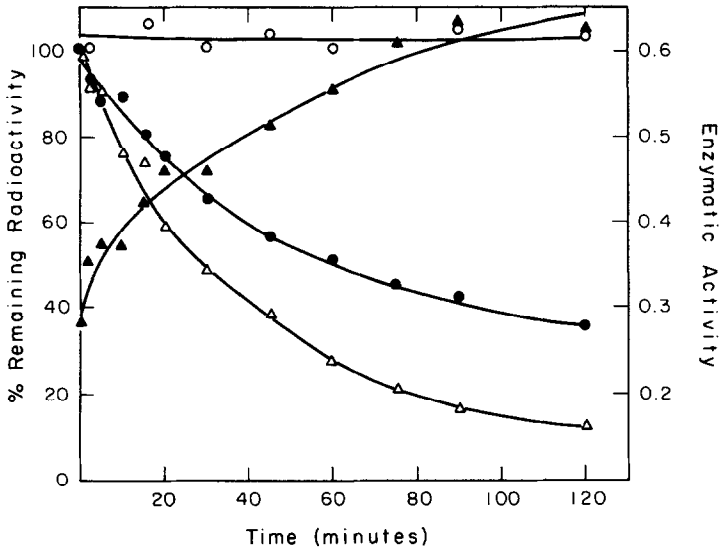

Figure 1. Dephosphorylation and reactivation of pyruvate kinase at 30 °C in HEPES buffer, pH 7.4, containing 5.7 mM Mg^{++}. o—o radioactivity of phosphorylated pyruvate kinase (21 units/ml) alone; ●—● radioactivity of phosphorylated pyruvate kinase (21 units/ml) + hemolysate (final concentration 20 mg/ml protein); Δ—Δ radioactivity of phosphorylated pyruvate kinase (21 units/ml) + partially purified phosphatase (final concentration 0.15 mg/ml protein); ▲—▲ enzymatic activity of pyruvate kinase (21 units/ml) + partially purified phosphatase (final concentration 0.15 mg/ml protein). Pyruvate kinase assays were carried out at 30 °C ; the final concentrations of assay reagents were: 90 mM HEPES, pH 7.4; 5.7 mM $MgCl_2$; 100 mM KCl; 0.4 mM P-enolpyruvate; 4.1 mM ADP; 0.38 mM NADH and 1 mM EDTA.

If this phosphorylated pyruvate kinase was incubated with the lysate of human erythrocytes (in 1 mM phenylmethylsulfonylfluoride and 0.1 mg/ml trypsin inhibitor) there was a progressive loss of the radioactive label (Figure 1). Washed red blood cell membranes showed less than 1% of the hydrolysis rate of the hemolysate.

Phosphorylated pyruvate kinase treated with the partially purified phosphatase also lost the phosphate group, with a concomitant increase in enzymatic activity. The regain of activity closely followed the loss of label with no detectable time lag between the two (Figure 1). Electrophoresis and subsequent autoradiography of the gel showed that the loss of label was not accompanied by any changes in the pyruvate kinase bands (Figures 2A, 2B). It is unlikely that the loss of radioactivity is due to proteolytic action since the limited pro-

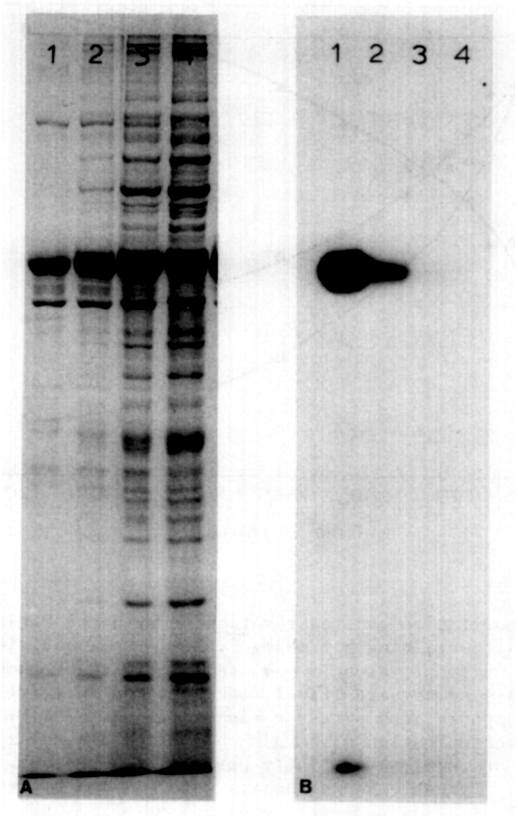

Figure 2A. SDS gel electrophoresis of pyruvate kinase, stained with Coomassie blue. 1) phosphorylated pyruvate kinase (5 units); 2) phosphorylated pyruvate kinase (5 units) + protein phosphatase preparation (10 µg); 3) phosphorylated pyruvate kinase (5 units) + phosphoprotein phosphatase preparation (100 µg); 4) phosphoprotein phosphatase preparation alone (100 µg). All samples were incubated in HEPES buffer, pH 7.4, at 30 °C for 3 hours.

Figure 2B. Autoradiogram of gel 2A.

teolysis of pyruvate kinase produces a change in the gel pattern (10), and also the radioactivity was still lost in a variety of different proteolytic inhibitors.

Pyruvate kinase from erythrocytes (unfilled units or freshly drawn blood) that had been lysed in the presence of KF and proteolytic inhibitors, was rapidly partially purified by passage of the lysate through a Matrex Gel column and elution of the enzyme with 1 M KCl. This enzyme showed no increase in activity upon incubation with the phosphatase preparation which suggests that it is unlikely that in unstimulated cells the pyruvate kinase is significantly phosphorylated so as to reduce its activity.

The phosphatase also removed the phosphate from phosphorylated spectrin, without any apparent change in the gel pattern. Phosphocasein was also hydrolysed as was p-nitrophenylphosphate. Hydrolysis of phosphocasein and pyruvate kinase, labeled to similar extents and at the same concentration occurred at very similar rates.

Preliminary results show that the hydrolysis of phosphocasein by this phosphatase preparation, at pH 7.4 in HEPES buffer (containing Mg^{2+}), occurs in the presence of EDTA (33 mM) or EGTA (13 mM). Similarly the hydrolysis of phosphorylated pyruvate kinase was only slightly inhibited by EDTA (20 mM) (unpublished experiments). Fluoride did not have any marked effect on the hydrolysis of phosphocasein.

The hydrolysis of phosphocasein by this phosphatase was inhibited to varying extents by ligands containing phosphate (unpublished experiments). The most effective inhibitor was ADP; phosphate, 2,3-DPG, ATP, Fru-1,6-P_2 and P-enolpyruvate all inhibited the enzyme to lesser extents. ADP was also the most effective inhibitor of the hydrolysis of phosphorylated pyruvate kinase.

Phosphocasein was used in most of these experiments so that it would be clear that any changes in the rate of dephosphorylation arose from ligand interactions with the phosphatase rather than with the pyruvate kinase. Several of these ligands bind to pyruvate kinase and the rate of phosphorylation of the enzyme is altered by such compounds (2).

After gel filtration of the phosphatase preparation, measurement of activity against p-nitrophenylphosphate revealed two phosphatase peaks. One eluted at the position corresponding to a molecular weight of about 80,000, the other was broader and eluted at a position corresponding to a molecular weight of about 175,000 (Figure 3). The broad peak at higher molecular weight showed the most activity against phosphocasein; the maximum activity eluted at a position corresponding to a molecular weight of about 135,000. This is similar to that reported for some phosphoprotein phosphatases (13,14) and this red blood cell enzyme would seem to correspond to the Class I isozymes of the divalent cation-

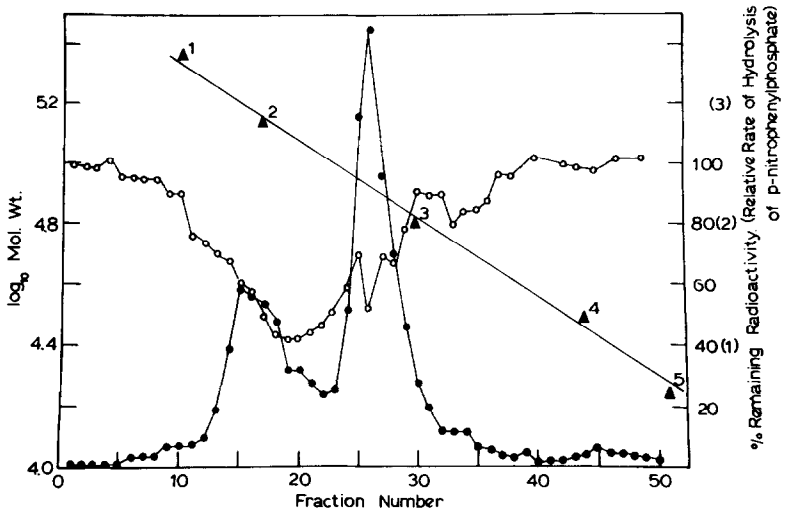

Figure 3. Gel filtration on Sephadex G-200 superfine of the phosphoprotein phosphatase preparation in HEPES buffer, pH 7.4, at 4 °C. ●—● activity against p-nitrophenylphosphate; o—o activity against phosphocasein; ▲—▲ standards, 1) catalase (bovine liver), 2) glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), 3) hemoglobin (human), 4) carbonic anhydrase (bovine erythrocytes), 5) myoglobin (equine muscle).

independent non-specific phosphatases from cardiac muscle (11). The protein that eluted at a molecular weight of 80,000 showed much more activity against p-nitrophenylphosphate than against phosphocasein. These results do suggest that there is more than one type of phosphatase present. In the experiments reported here we have not looked at the phosphatase under conditions which might dissociate a smaller catalytic subunit.

Since this phosphatase preparation dephosphorylates casein, spectrin and pyruvate kinase it may well be the same enzyme as that reported by Graham and co-workers (5,6), but there are both similarities and differences between the two preparations. Neither of them seem to show a requirement for a divalent metal, though there could be a very tightly bound divalent cation (11). Both preparations were inhibited by compounds containing phosphate; in this present study ADP was the most effective inhibitor, rather than ATP. We have also found that KF did not strongly inhibit the phosphatase whilst in the previous study KF was an effective inhibitor. Antoniw and Cohen have found that the in-

hibition of liver protein phosphatase is very dependent on the metal ions present, Mn^{2+} overcoming the effects of KF (12).

These differences may arise because the earlier study was done with the lysate from red blood cells whilst the present experiments have been carried out on a partially purified preparation, or because in the previous study only the effects on spectrin were studied.

This phosphatase may play a very important role in metabolic control within the red blood cell. In certain patients suffering from non-spherocytic hemolytic anemia the pyruvate kinase shows kinetic properties that resemble the low P-enolpyruvate affinity form of the enzyme produced by oxidation or phosphorylation. It would be likely that a patient lacking this phosphoprotein phosphatase would show lower pyruvate kinase activity with abnormal P-enolpyruvate saturation kinetics. Quite likely some other abnormalties would be found in other cells.

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