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PHOSPHORYLATION AND INHIBITION OF HUMAN ERYTHROCYTE PYRUVATE KINASE BY ERYTHROCYTE MEMBRANES

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Summary: The enzymatic activity of human erythrocyte pyruvate kinase was found to decrease on incubation of the purified enzyme with red blood cell ghosts, ATP and cAMP. If [^{32}P]γATP was used radioactivity was found associated with the protein after gel electrophoresis. Various effectors protected the enzyme against phosphorylation. Treatment of the modified enzyme with a protein phosphatase restored enzymatic activity and also caused the loss of the radioactive label. Modification of the pyruvate kinase in this way altered the affinity of the enzyme for one of its substrates (phosphoenolpyruvate), but the binding of the other substrate (ADP) was unaffected.

Pyruvate kinase, isolated from liver, can be phosphorylated on incubation with ATP, cAMP and a cAMP-dependent protein kinase from liver cells (1), or in whole cells phosphorylation can be induced by glucagon (2) or by catecholamines (3). The phosphorylation results in a decrease in affinity of the enzyme for P-enolpyruvate (4) and it is thought that this may play a significant role in the balance between gluconeogenesis and glycolysis: if pyruvate kinase was fully active during gluconeogenesis there would be unwanted cycling between P-enolpyruvate and pyruvate.

Phosphorylation of the erythrocyte pyruvate kinase has been looked for previously, but not found (5). During the course of this present study Marie and co-workers have independently found that the pyruvate kinase can be phosphorylated *in vivo* by an endogenous cAMP-dependent protein kinase (6). However the kinetic properties of the phosphorylated enzyme and the effect of various ligands on the rate of phosphorylation have not previously been reported. Since the red blood cell does not carry out gluconeogenesis it is not clear why the pyruvate kinase should be phosphorylated.

Abbreviations: Fru-1,6-P_2, fructose-1,6-bisphosphate; P-enolpyruvate, phospho-enolpyruvate; 2,3-DPG, 2,3-diphosphoglycerate; DTE, dithioerythritol; Hepes, N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid; SDS, sodium dodecyl sufate.

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Materials and methods: Human blood (unfilled units) was obtained from the Red Cross, Springfield, MA. Matrex Gel Red "A" was purchased from Amicon Ltd. The cyclic nucleotides, fructose bisphosphatase, histone and protein kinase inhibitor (from rabbit muscle) were purchased from Sigma Chemical Co., and caffeine was purchased from J. T. Baker. Protein phosphatase was a gift from Dr. Donald Graves, Iowa State University. The sources of all other materials were as described previously (7).

Pyruvate kinase was assayed essentially as previously described (7); the final concentrations of assay reagents were: 90mM Hepes, pH 7.4; 5.7mM $MgCl_2$; 100mM KCl; 8mM P-enolpyruvate; 4.1mM ADP; 0.38mM NADH and 1mM EDTA. Any variations from this are described in the text. To minimize the lag period (7) all reagents were incubated at 30°C for 10 minutes prior to initiation of the assay with ADP.

Protein kinase activity was assayed by the method described by Boivin and Galand (8); one unit is described as the amount of enzyme required to transfer 1 pmole of [^{32}P]γATP to 1mg of histone in ten minutes.

Protein concentration was estimated with coomassie blue dye (9) with bovine serum albumin as a standard. SDS gel electrophoresis in 8% acrylamide was done according to the method of Laemmli (10).

Purification of the pyruvate kinase was essentially as described by Marie and co-workers (11) except that Matrex Gel Red "A" was used in the final affinity chromatography step. The enzyme was stored as a 50% ammonium sulfate suspension in 2mM DTE, and desalted on a Sephadex G-25 column prior to use. Specific activities between 120 and 240 units/mg were routinely obtained. In the preparations where proteolytic cleavage was to be kept to a minimum, 1mM phenylmethylsulfonylfluoride was added to the buffer used to lyse the cells.

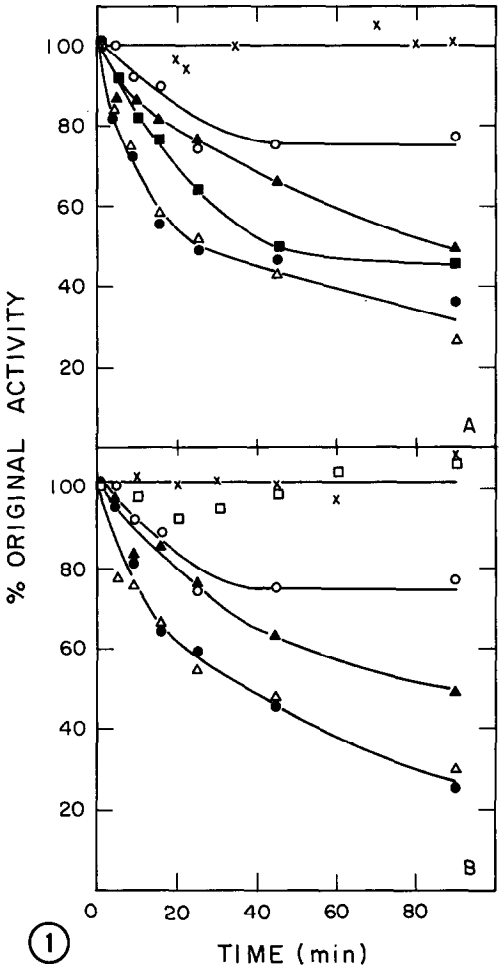
The erythrocyte ghosts were prepared by the method of Hanahan and Ekholm (12). The membranes were stored at 4°C and prepared fresh each week.

It was found that Fru-1,6-P_2 could not be completely or reliably removed from the pyruvate kinase in a short time by gel filtration or dialysis. However treatment of the enzyme with an ammonium sulfate suspension of fructose bisphosphatase to give a final concentration of 0.1 - 0.3 units/ml, consistently removed the effector quite rapidly without the loss of enzymatic activity.

The phosphorylation of pyruvate kinase (1 - 2 units) was carried out at pH 7.5 in potassium phosphate buffer (20mM) containing 10mM NaF, 10mM caffeine, 10mM DTE, 20 - 50 units protein kinase, 1.25mM ATP and various concentrations of effectors. Any variations from this are described in the text. Fru-1,6-P_2 was hydrolysed by fructose bisphosphatase before the enzyme was phosphorylated.

Results and discussion: If pyruvate kinase, in the absence of Fru-1,6-P_2, was incubated with red blood cell membranes, ATP and either cAMP or cGMP, there was a progressive loss in activity; this loss was much slower in the absence of cAMP or cGMP (Figure 1). If the ghosts were stored for more than two weeks this cyclic nucleotide-independent inactivation of pyruvate kinase increased.

The rate of the inactivation of the enzyme was dependent on the concentration of protein kinase activators present. At concentrations of cAMP above 10^{-6}M the rate of inactivation was constant, below 10^{-6}M the inactivation slowed. Higher concentrations of cGMP (>10^{-4}M) were needed to bring about the same rate of inactivation (Figures 1A and 1B). Protein kinase inhibitor, at a final



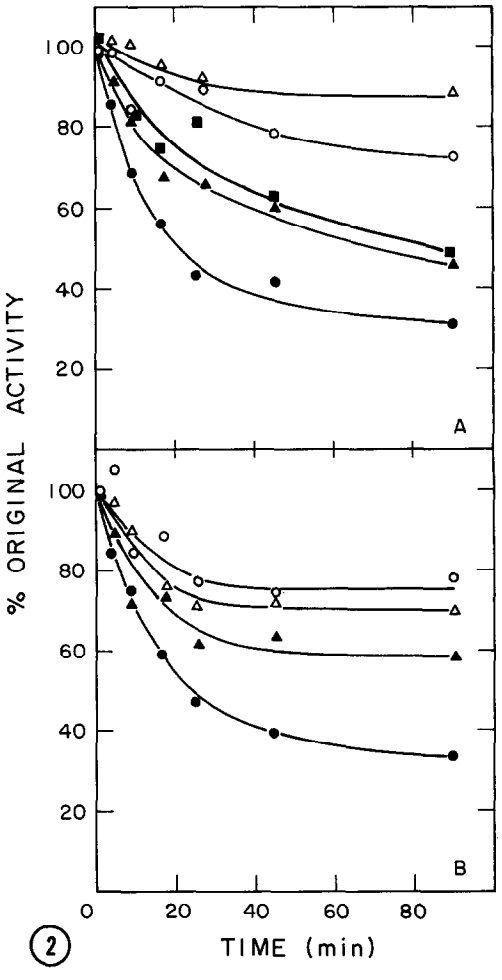


Figure 1A. cAMP stimulated inactivation of pyruvate kinase by erythrocyte membranes pH 7.5 25°C. The inactivation conditions were as described under "Methods" with the addition of various concentrations of cAMP: X , 7.57 μM cAMP but no membranes; O , no cAMP; ▲ , 0.077 μM cAMP; ■ , 0.76 μM cAMP; Δ , 7.57 mM cAMP, and ● , 757 μM cAMP. Samples were withdrawn and pyruvate kinase activity determined at 0.4mM P-enolpyruvate, in the absence of Fru-1,6-P_2 at 30°C, pH 7.4.

Figure 1B. cGMP stimulated inactivation of pyruvate kinase, pH 7.5, 25°C: X , 36 μM cAMP, 0.5mg/ml protein kinase inhibitor; □ , 89 μM cGMP, 0.5 mg/ml protein kinase inhibitor; O , no cGMP; ▲ , 6.35 μM cGMP; Δ , 64.2 μM cGMP; ● , 635 μM cGMP. Assays were done as in Figure 1A.

Figure 2A. Protection of pyruvate kinase by 2,3-DPG against inactivation by erythrocyte membranes: O , no cAMP; ● , 75 μM cAMP; ▲ , 75 μM cAMP, 2mM 2,3-DPG; Δ , 5.32 μM cAMP, 14mM 2,3-DPG; and ■ , 9.2 μM cAMP, 4.8 mM 2,3-DPG; Pyruvate kinase activity was determined as described in the legend to Figure 1A.

Figure 2B. Protection of pyruvate kinase by Fru-1,6-P_2 against inactivation by erythrocyte membranes: O , no cAMP; ● , 62 μM cAMP; ▲ , 62 μM cAMP, 5 μM Fru-1,6-P_2 and Δ , 62 μM cAMP, 50 μM Fru-1,6-P_2. Samples were withdrawn and incubated with 50 x 10^{-3} units fructose bisphosphatase in the assay reagents for 10 minutes prior to initiation of the assay with ADP: the P-enolpyruvate concentration was 0.4mM.

concentration of 0.3 to 0.5 mg/ml completely protected the pyruvate kinase from cAMP or cGMP stimulated inactivation.

The rate of inactivation was also reduced by both 2,3-DPG and Fru-1,6-P_2. 14mM 2,3-DPG almost completely protected the enzyme, whereas lower concentrations of 2,3-DPG and 5 or 50μM Fru-1,6-P_2 only gave partial protection (Figures 2A and 2B).

Treatment of the modified enzyme with protein phosphatase brought about a complete regain of enzymatic activity of the pyruvate kinase (Figure 3): the optimum conditions for the dephosphorylation reaction were not determined so it is likely that the reaction in vivo occurs more rapidly.

SDS gel electrophoresis of the modified enzyme labeled with [^{32}P]γATP revealed three protein bands in the region corresponding to about 60,000 daltons (Figure 4A). The most intense band, with the highest molecular weight corresponds to the L' form of pyruvate kinase: the form that has not been cleaved by proteolysis (13). The other two bands correspond to the L form (proteolytically

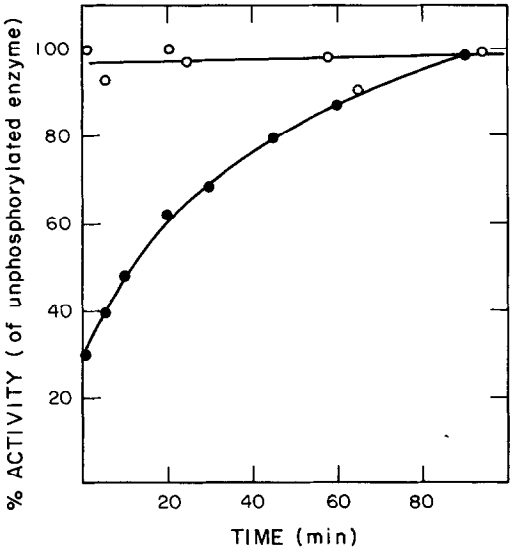


Figure 3. Regain of enzymatic activity on treatment of phosphorylated pyruvate kinase with protein phosphatase: ● , phosphorylated enzyme; O , unphosphorylated enzyme. The phosphorylated enzyme (1 to 2 units) was treated with 3.2 units of the phosphatase in pH 7.4 Hepes buffer, containing 100 mM KCl; 5.7 mM $MgCl_2$; 1 mM EDTA; 10 mM caffeine, and 10 mM DTE, at 30°C. Samples were withdrawn and assayed as described in the legend to Figure 1.

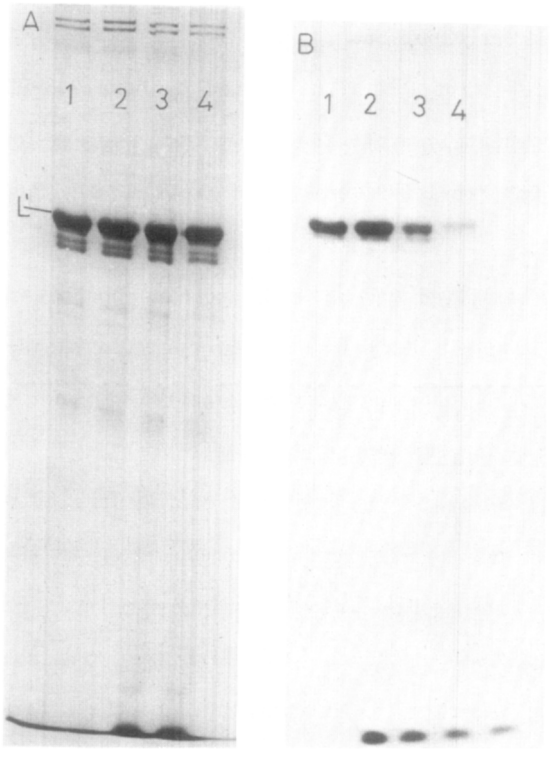


Figure 4A. SDS gel electrophoresis of pyruvate kinase, stained with coomassie blue: 1) pyruvate kinase (5 units) phosphorylated, as described in the methods section, in 90 mM Hepes buffer, pH 7.4, containing 100 mM KCl; 5.7 mM $MgCl_2$; 1 mM EDTA; 10 mM caffeine, and 10 mM DTE: 2) as for 1): 3) phosphorylated pyruvate kinase subsequently treated with protein kinase inhibitor (final concentration 0.15 mg/ml) and 1.6 units protein phosphatase for 3 hr at 30°C, pH 7.4: 4) as for 3) but treated with 3.2 units protein phosphatase. The proteins were precipitated with 10% trichloroacetic acid, spun down and washed with acetone:ether (50:50), and then dissolved in 2% SDS (w/v) and 1% mercaptoethanol. Equal amounts of protein were loaded for each sample.

Figure 4B. Autoradiogram of gel (4A).

cleaved) and either a third form of the enzyme or an impurity. These three bands were seen in all the enzyme preparations.

Two of these three bands were phosphorylated (Figure 4B). The L form is not phosphorylated (Axel Kahn, personal communication) so these bands correspond to the L' form and the third form or impurity. On incubation of the enzyme with protein phosphatase the radioactive label was gradually lost from both bands (Figure 4B). In the absence of phosphatase the label was stable.

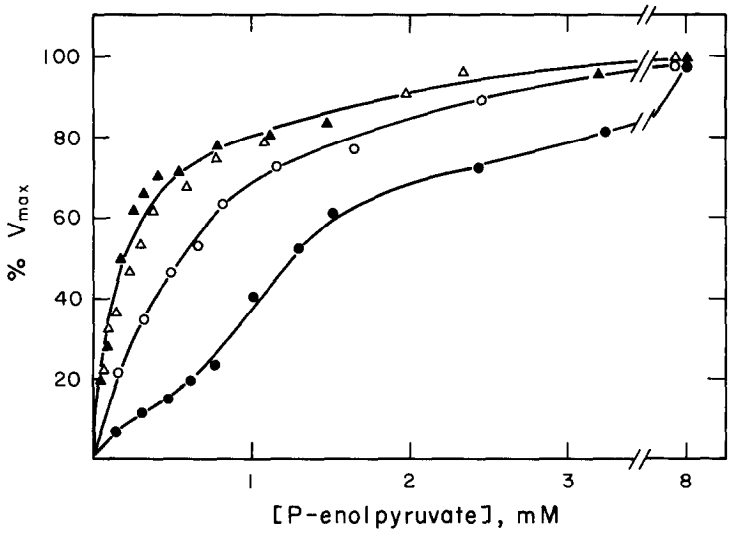


Figure 5. P-enolpyruvate saturation curves for pyruvate kinase in the presence (▲△) or absence (●○) of Fru-1,6-P_2 (100 μM): ▲●, phosphorylated enzyme; △○, unphosphorylated enzyme. The modification of the enzyme and the kinetic assays were done as previously described.

In all of these experiments no marked differences were seen between enzyme prepared from cells lysed in the presence of phenylmethylsulfonylfluoride, and enzyme prepared in its absence.

Figure 5 shows the saturation curves for the binding of P-enolpyruvate to phosphorylated and unphosphorylated enzyme. With Fru-1,6-P_2 present in the assays no differences could be detected between the two forms of the enzyme. Values of 0.18 ± 0.015 and 0.22 ± 0.05mM were obtained for the $K_{0.5}$ (substrate concentration at half the maximum velocity) of the phosphorylated and unphosphorylated enzymes respectively, with Hill coefficients of 1.04 ± 0.15 and 1.12 ± 0.04 respectively. In the absence of the effector the phosphorylated enzyme had a higher $K_{0.5}$ (1.38 ± 0.13mM) and showed a higher degree of co-operativity (Hill coefficient 1.86 ± 0.26) than did the unphosphorylated enzyme ($k_{0.5}$ 0.8 ± 0.18mM, Hill coefficient 1.45 ± 0.2).

At saturating P-enolpyruvate concentrations, the two forms of the enzyme showed no differences in affinity for ADP (data not shown). $K_{0.5}$ values of 0.26 ± 0.07 and 0.27 ± 0.08mM and Hill coefficients of 1.21 ± 0.15 and 1.24 ± 0.09 were obtained for the phosphorylated and unphosphorylated forms respectively.

Accurate values for the $K_{0.5}$ for the binding of Fru-1,6-P_2 could not be readily obtained since fructose bisphosphatase had to be added first to completely remove the effector. However both forms had $K_{0.5}$ values below 2×10^{-7}M.

The effect of phosphorylation of pyruvate kinase is marked at low P-enolpyruvate concentrations, the enzymatic activity is reduced by at least two or three fold. The concentration of P-enolpyruvate in the red blood cell is about 20μM (14) and pyruvate kinase is not saturated, thus at the physiological concentration of P-enolpyruvate the phosphorylated enzyme will have lower activity. Since the enzyme has been found to be phosphorylated both *in vivo* and *in vitro* it is probably subject to regulation by this mechanism *in vivo*. However the reason for such control is not clear. Unlike the liver, the red blood cell does not carry out gluconeogenesis. One possibility is that reducing the activity of pyruvate kinase would promote a build-up of metabolites preceeding the enzyme in the glycolytic pathway; this would include 2,3-DPG (15). In red blood cells incubated with catecholamines or cAMP together with phosphodiesterase inhibitors an increase in the levels of 2,3-DPG has been observed (15). 2,3-DPG binds preferentially to deoxyhemoglobin so an increase in the level of 2,3-DPG could bring about a change in the state of oxygenation of the cell, aiding in the release of oxygen to the tissues.

The rate of phosphorylation, and the activity of the phosphorylated enzyme can be further controlled by 2,3-DPG and Fru-1,6-P_2. Both metabolites inhibit phosphorylation, and at saturating concentrations, Fru-1,6-P_2 overcomes the effects of phosphorylation. Thus Fru-1,6-P_2 may play a very important role in the regulation of pyruvate kinase activity, both in the scheme outlined here, and as an allosteric activator. Brewer has reported that the concentration of this effector in the erythrocyte is about 5μM (14); this would be high enough to mask the effects of phosphorylation of the pyruvate kinase. This suggests that the concentration of Fru-1,6-P_2 available to pyruvate kinase in the cell may vary.

The level of adenylate cyclase in the human erythrocyte is thought to be rather low and the enzyme is only slightly activated by catecholamines (16). It

therefore seems unlikely that rapid changes in the level of cAMP and hence the phosphorylation state of pyruvate kinase (via a cAMP-dependent protein kinase) would occur. The mechanism for the control of the enzyme by phosphorylation is not clear; perhaps phosphorylation is stimulated by alternative pathways.

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