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ENDOGENOUS, CYCLIC 3'-5' AMP-DEPENDENT PHOSPHORYLATION OF HUMAN RED CELL PYRUVATE KINASE

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Summary : ATP-depleted human red cells have been incubated in a glucose-containing medium with ortho [^{32}P] phosphate in the presence and in the absence of cyclic AMP and dibutyril cyclic AMP. Then pyruvate kinase has been purified to homogeneity. It was found that pyruvate kinase was intensively phosphorylated in the presence of cyclic 3'-5' AMP and 5 times less in its absence. The phosphorylated site was readily cleaved by subtilisin.

Introduction : Liver L-type pyruvate kinase can be phosphorylated by a cyclic 3'-5' AMP-dependent protein kinase isolated from the cytosolic fraction of liver cells (1). This phosphorylation is associated with decrease of the affinity for phosphoenolpyruvate and increase of the inhibition by ATP (2). In whole cells such a phosphorylation can be induced by glucagon (3-5). It is expected that the glucagon-induced phosphorylation of liver pyruvate kinase plays a major role in the balance between the glycolytic and gluconeogenic pathways : inhibition of pyruvate kinase would avoid the "futile cycle" between pyruvate and phosphoenolpyruvate, then would allow the gluconeogenic pathway to proceed (3-6).

We have recently established that red cell-type pyruvate kinase (L'_4) may be considered as a "precursor form" of liver L-type enzyme (L_4), the former being transformed into the latter by partial proteolysis (7,8). It was therefore important to determine whether red cell enzyme was phosphorylatable, and whether it could, indeed, be endogenously phosphorylated.

Methods : Blood, anticoagulated with citrate phosphate dextrose (CPD) and stored under sterile conditions for 6 days at 4°, was obtained from a blood bank. It was washed three times with a 35 mM Tris-HCl buffer (pH 7.4)/130 mM NaCl/5 mM KCl/ 1.7 mM $MgCl_2$ (buffer A) containing 0.1 mM Na_2HPO_4, the white cells being carefully removed. Two samples of 40 ml of red cells were mixed

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with 60 ml of buffer A containing 20 mM glucose, 1 mM adenosine, 2.5 mM Na_2HPO_4 and 5 mCi ortho [^{32}P] phosphate (carrier free ^{32}Pi), which corresponded to a ^{32}Pi specific radioactivity of 52 CPM/p mole. After 30 mn incubation at 37° with gentle agitation 0.1 mM cyclic 3'-5' AMP (cAMP) and 0.1 mM dibutyril cyclic 3'-5' AMP (Bt_2cAMP) were added to the first sample and incubation was continued for 4 hrs. Then the red cells were washed three times in a 20 mM phosphate buffer (pH 7)/ 100 mM NaCl/ 50 mM KF.

Hemolysis was provoked by adding to the packed red cells two volumes of distilled water containing 1 mM diisopropylfluorophosphate, 1 mM phenylmethylsulfonylfluoride, 1 mM EDTA and 10 mM β mercaptoethanol, then KF and phosphate buffer pH 7 were added to a final concentration of 50 mM and 20 mM respectively. The membranes were eliminated by centrifugation (30 mn at 105,000 g). Pyruvate kinase was totally purified by $(NH_4)_2SO_4$ fractionation and Blue Dextran Sepharose-4 B chromatography as previously reported (7-9) except that 40 mM KF instead of KCl were added to all the buffers used. After specific elution of pyruvate kinase by fructose 1,6 P_2 (7-9), a part of the preparation was lyophylised and dissociated by SDS ; the other part was mixed with 0.2 mg/ml bovine albumin , then precipitated by $(NH_4)_2SO_4$ (to 80% saturation). Radioactivity of the trichloroacetic acid-precipitated proteins was counted in liquid scintillation, using the filter method as described elsewhere (10).

After SDS-polyacrylamide gradient gel electrophoresis (using the discontinuous buffer system described by Laemli (11), the protein bands stained with Coomassie blue were detected with a Gelman gel scanner (model DCD-16). Each channel of the gel was sliced into 2 mm-thick slices whose radioactivity was counted separately.

Proteolytic attack of non-dissociated pyruvate kinase by trypsin (7) and subtilisin (12) was carried out as described previously.

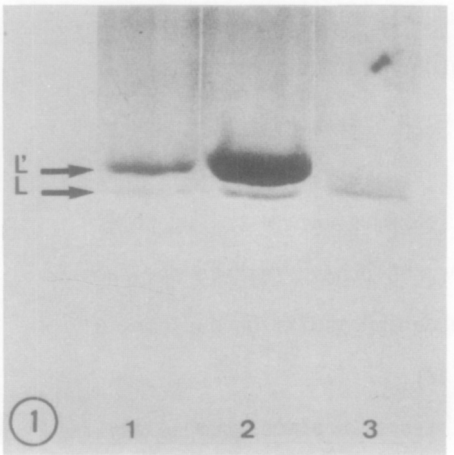
Results and discussion : Table I shows the results of a typical experiment. When the red cells were incubated with cAMP and Bt_2cAMP, pyruvate kinase was intensively labelled : its specific radioactivity was 7.9 CPM/p mole of enzyme subunit for an initial ^{32}Pi specific radioactivity of 52 CPM/p mole. In the absence of cAMP and Bt_2cAMP, labelling was only 1.6 CPM/p mole. By comparison, labelling of glucose-6 phosphate dehydrogenase purified from the same samples was less than 0.2 CPM/p mole (unpublished).

SDS-polyacrylamide gel (fig. 1 and 2) electrophoresis showed that all the radioactivity was found at the level of the L' protein band. In the experiment represented here the L' subunits accounted for 90% of the total subunits (which corresponds to a ratio about of 20% of $L_2L'_2$ and 80% of L'_4 tetramers in the undissociated preparation (7)); this small amount of L subunits made difficult the comparison of the labelling of the L' and L subunits.

TABLE I : Purification of pyruvate kinase from red cells incubated with ^{32}Pi, with (I) and without (II) 0.1 mM cAMP and Bt_2cAMP. Specific radioactivity of ^{32}Pi was 52 CPM/p mole.

	ENZYME ACTIVITY (IU)		SPECIFIC ACTIVITY IU/mg of proteins		CPM		CPM/mg of proteins		CPM/p mole[1] of subunit	
	I	II	I	II	I	II	I	II	I	II
CYTOSOL	96	91	0.009	0.009	1.85×10^7	1.44×10^7	2.4×10^3	1.8×10^3		
AMMONIUM SULPHATE PRECIPITATE	77	67	4.6	4.6	5.17×10^5	3.5×10^5	32×10^3	20×10^3		
ELUATE OF THE BLUE DEXTRAN SEPHAROSE -4B COLUMN BY FRUCTOSE 1,6 P_2	44.5	38	440	350	12.6×10^3	2.83×10^3	125×10^3	25.8×10^3	7.87	1.63

(1) The specific radioactivity of pure pyruvate kinase was calculated with molecular weight of the L' subunits : 63,000 (7,8).



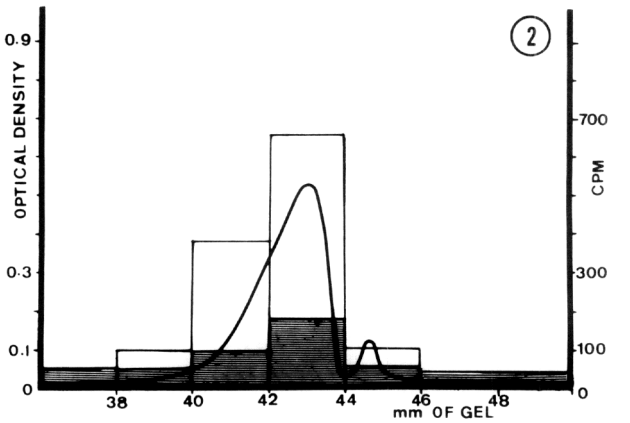


Fig. 1 : SDS-POLYACRYLAMIDE GRADIENT GEL OF ERYTHROCYTE PYRUVATE KINASE

Channel 1 : pyruvate kinase, 5 µg protein ; channel 2 : pyruvate kinase, 20 µg protein ; channel 3 : catalase, 5 µg protein.
Enzyme represented here corresponds to sample I. Exactly the same pattern was obtained with either preparation I or preparation II.
The arrows indicate the position of the L' and L subunits (7, 8).

Fig. 2 : SCANNING OF THE COOMASSIE BLUE-STAINED POLYACRYLAMIDE GEL (see fig. 1) AND COUNT OF ^{32}Pi RADIOACTIVITY.

After electrophoresis the gel was stained with Coomassie Blue, then the electrophoretic diagram was recorded with a gel scanner. The gel channels corresponding to a load of 20 µg of pyruvate kinase (sample I and II) were sliced each 2 mm, and ^{32}Pi radioactivity of the slices was counted in liquid scintillation.

——— Absorbance at 550 nm

Rectangles : radioactivity of the gel slices. White rectangles : sample I, shared rectangles : sample II.

In order to allow an easy comparison between the L' and L peaks, we used an extended scale. Only the two protein peaks showed here existed, and radioactivity was only detected at this level.

From another experiment we obtained some evidences that both the precursor L' subunits and the partially proteolysed L subunits were phosphorylated. This point, however, is to be confirmed by further studies.

Our results are just the opposite of those recently reported by Dahlqvist Edberg (13). This author claimed that erythrocyte pyruvate kinase was not phosphorylatable and could correspond to a proteolysed molecule of liver L-type enzyme ; she worked with impure red cell pyruvate kinase preparations

and did not use antiproteolytic agent for the enzyme purification. Although it is impossible to determine exactly the reasons of these discrepancies, it could be that Dahlqvist Edberg has been working with enzyme degraded by proteolytic enzymes from red cell stroma or contaminant leukocytes. We have demonstrated indeed, their richness in proteolytic enzymes active on red cell pyruvate kinase (7). These proteolytic substances could have removed the phosphorylatable site of erythrocyte pyruvate kinase during isolation and partial purification of the enzyme, as subtilisin does (12).

Subtilisin is able to remove the phosphorylated site almost completely as it does with liver L-type PK(12)(Table II). This seems to indicate that the phosphorylatable site is the same on the L' subunits of red cell enzyme and L

TABLE II : Lability of the bound radiophosphorous of erythrocyte pyruvate kinase to proteolytic treatment.

	I (+ cAMP)		II (- cAMP)	
	CPM	%	CPM	%
UNTREATED ENZYME	456	100	481	100
+ TRYPSIN	380	83		
+ SUBTILISIN	125	27	126	26
+ TRYPSIN + SUBTILISIN	77	17		

About 4 µg of pyruvate kinase sample I and 20 µg of pyruvate kinase sample II were incubated for 1 hr at 37°, in the presence of 1 mg/ml bovine albumin with trypsin (10 µg/ml) or subtilisin (0.25 µg/ml) at pH 7.3 (7).
Reaction was stopped with 10% (w/v) trichloroacetic acid, the precipitated proteins were collected on Millipore filters (type HA 0.4 µm), then washed with the same trichloroacetic acid solution. The filters were placed in polyethylene vials containing 10 ml of scintillator liquid and radioactivity was counted.

I : red cells incubated in the presence of 0.1 mM cAMP and Bt_2cAMP.
II : incubation without cyclic nucleotides.