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PURIFICATION OF A CA^{2+}-ACTIVATED PROTEASE FROM RAT ERYTHROCYTES AND ITS POSSIBLE EFFECT ON PYRUVATE KINASE IN VIVO

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A Ca^{2+}-activated protease with [^{32}P]phosphopyruvate kinase as substrate was purified to about 50% from rat erythrocytes. The purification involved chromatography on Sepharose/Sephadex gels, DEAE-cellulose and $(NH_4)_2SO_4$ precipitation. The protease required 3.3 mM Ca^{2+} for full activity. When pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) was purified from erythrocytes incubated with [^{32}P]phosphate it contained 0.5 mol [^{32}P]phosphate/mol enzyme subunit. When 3.3 mM Ca^{2+} were added at hemolysis this incorporation decreased. The possible importance of this Ca^{2+}-activated protease for the regulation of pyruvate kinase in erythrocytes is discussed.

Introduction

Pyruvate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) from human erythrocytes has similar immunological determinants to the liver enzyme (type L) and has similar sigmoidal kinetics towards its substrate, phospho*enol*pyruvate [1]. It is easily modified in vivo and in vitro to a still active form with approx. the same molecular weight as the L-type pyruvate kinase [2]. Kahn et al. [3], therefore, proposed that the erythrocyte enzyme is a precursor to the L-type pyruvate kinase. They gave the designation L′ to the subunits of the unmodified erythrocyte enzyme.

Marie et al. [4] and Kiener et al. [5] demonstrated that this L′ enzyme form could be phosphorylated in vitro under the influence of an endogenous cyclic AMP-dependent protein kinase and [^{32}P]ATP. This phosphorylation was accompanied by a decrease in the activity of the pyruvate kinase at unsaturating concentrations of phospho*enol*pyruvate [5], as previously observed for the liver enzyme [6]. The phosphorylation of pyruvate kinase from erythrocytes has also been demonstrated in vivo [4], but the influence of this phosphorylation on the activity of the enzyme was not studied.

Proteolytic attack on L′ pyruvate kinase yields different modified enzyme forms, depending on which protease is used. Some of these enzymes modified in vitro can still be phosphorylated, but not the pyruvate kinase modified in vivo [2].

Dahlqvist-Edberg [7] found a pyruvate kinase from rat erythrocytes which showed immunological behaviour similar to that of the L-type pyruvate kinase from rat liver, but had kinetics towards phospho*enol*pyruvate similar to that of a partially degraded L-type pyruvate kinase [8]. The enzyme could not be phosphorylated. Dahlqvist-Edberg, therefore, suggested that the erythrocyte pyruvate kinase was an L-type that had been proteolytically modified in the cell or during the purification procedure [7]. This enzyme form could correspond to the pyruvate kinase derived from human erythrocytes and modified in vivo, since they cannot be phos-

Abbreviation: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

phorylated and since they have the same $K_{0.5}$ for phospho*enol*pyruvate [3,5,7].

It was of interest to look for the step at which the proteolysis of the erythrocyte pyruvate kinase occurred (if it did occur) and to attempt to find an endogenous protease that could account for this proteolytic modification. Reports have been published on a group of proteolytic enzymes from different tissues called Ca^{2+}-activated proteases which uses as substrates enzymes involved in phosphorylation/dephosphorylation reactions (for review see Ref. 9). Ca^{2+}-activated protease of rat liver has as one of its substrates, L-type pyruvate kinase in its phosphorylated form [9].

We have looked for a similar enzyme activity in erythrocytes.

Materials and Methods

Bovine serum albumin, ATP, ADP, cyclic AMP, dithiothreitol, NADH, phospho*enol*pyruvate and fructose 1,6-diphosphate were obtained from Sigma. Rabbit lactate dehydrogenase was purchased from Boehringer-Mannheim. Sephadex and Sepharose gels were obtained from Pharmacia, Uppsala, Sweden. [^{32}P]ATP was bought from New England Nuclear, Boston. An $A^{0.1\%}_{280nm}$ value of 1.0 was used for protein estimations.

Pyruvate kinase from rat liver was purified as described by Titanji et al. [10] and phosphorylated as described by Bergström et al. [8]. The activity of pyruvate kinase was estimated by coupling this enzyme reaction to the lactate dehydrogenase reaction and measuring the reduction of NADH as described previously [6], or by the modified colorimetric method of Kimberg and Yielding [11]. For calculation of phosphate incorporation, a specific activity of 290 units/mg [12] was used for the pyruvate kinase from rat erythrocytes.

The assay of Ca^{2+}-activated protease was performed as described by Ekman and Eriksson [9]. In order to investigate the size of the [^{32}P]phosphopeptide released from phosphopyruvate kinase, the volume was doubled. This incubation was interrupted by the addition of 1 mg bovine serum albumin and trichloroacetic acid to a final concentration of 10% (w/v). After centrifugation the supernatant was run on a Sephadex G-25 column (1.75 × 20 cm) equilibrated and eluted with 50% (v/v) acetic acid.

In order to determine which peptide bond is attacked by Ca^{2+}-activated protease, [^{32}P]phosphopyruvate kinase was incubated with this enzyme. The incubation was interrupted and the [^{32}P]phosphopeptide material separated from digested and undigested [^{32}P]phosphopyruvate kinase by chromatography on a Sephadex G-200 column (100 ml) equilibrated and eluted with 40 mM potassium phosphate buffer (pH 7.0)/30% glycerol/0.1 mM dithiothreitol. The pooled peptide material was run on a Sephadex G-25 column (5.6 × 20 cm) equilibrated and eluted with 50% acetic acid (v/v). The [^{32}P]phosphopeptides were slightly retarded and appeared as one peak. The fractions were pooled and the material was applied to a SP-Sephadex C-25 column (0.9 × 30 cm) equilibrated with 10 mM pyridine in acetic acid (pH 3.1). The column was washed with 1 vol. buffer and eluted with a linear 400 ml gradient (10–300 mM) pyridine in acetic acid (pH 3.1). The material of the distinct [^{32}P]phosphopeptide peak eluted at a pyridine/acetic acid buffer concentration of about 75 mM was pooled. This material, corresponding to about 5 nmol (based on the radioactivity), was analyzed for N-terminal amino acids and total amino acid composition as described by Humble et al. [13]. [^{32}P]Orthophosphate was determined by the method of Martin and Doty [14].

Erythrocytes from rat were prepared as described by Dahlqvist-Edberg [7]. After the last centrifugation 10 ml packed red cells were halved, and each was incubated with 20 ml 35 mM Tris-HCl buffer (pH 7.4)/130 mM NaCl/5 mM KCl/1.7 mM $MgCl_2$/2.5 mM sodium phosphate/20 mM glucose/3 ml [^{32}P]orthophosphate (37 MBq) in 0.25 M sucrose for 30 min, at 30°C, in a shaking water-bath under 96.5% O_2/3.5% CO_2. Cyclic AMP was then added to give a final concentration of 0.1 mM and the incubation was continued for 4 h. The erythrocytes were washed three times with 2 vol. 20 mM potassium phosphate buffer (pH 7.0)/100 mM NaCl/50 mM NaF. The red cells in one sample were then lysed with 2 vol. 1 mM EDTA/10 mM 2-mercaptoethanol. The cells of the other sample were lysed with 2 vol. 10 mM 2-mercaptoethanol/25 µl 1 M $CaCl_2$. Both samples were immediately frozen in liquid nitrogen and stored at –80°C overnight. The samples were then rapidly thawed by immersion in a water-bath at 8–10°C. Potassium

phosphate buffer (pH 7.0) was added to give a final concentration of 20 mM phosphate buffer. The samples were centrifuged at 17 500 $\times g$ for 45 min at 4°C. Under stirring, 23 g solid $(NH_4)_2SO_4$ were added/100 ml superantant. After 30 min at 4°C the precipitates were collected by centrifugation at 48 000 $\times g$ for 20 min at 4°C, dissolved in 50 mM Tris-HCl buffer, (pH 8.0), reprecipitated with $(NH_4)_2SO_4$ and collected as above. These precipitates were washed with 10 mM potassium phosphate buffer (pH 7.0)/23 g solid $(NH_4)_2SO_4$/100 ml, centrifuged and dissolved in and dialysed against 50 mM potassium phosphate buffer (pH 7.0)/30% glycerol/0.1 mM dithiothreitol. To isolate ^{32}P-labelled pyruvate kinase, antiserum against pyruvate kinase (prepared by the method of Ljungström and Ekman [15]) in 5-fold excess was added to the dialysed samples. After incubation on an ice-water bath for 3 h, the samples were applied to 0.5 ml columns of protein A-Sepharose. Non-adsorbed protein was washed from the column with 0.1 M potassium phosphate buffer (pH 7.0)/1 M NaCl. The pyruvate kinase-antibody complex was eluted with 50 mM citric acid (pH 3), 4 M urea, mixed with 1 mg bovine serum albumin and precipitated by the addition of 25% (w/v) trichloroacetic acid to a final concentration of 10%. The pellets were collected by centrifugation, dissolved in 0.5 ml 0.5 M NaOH and analyzed for ^{32}P-radioactivity.

The specific radioactivity of [^{32}P]ATP was determined in 500 μl samples withdrawn before the hemolysis and immediately added to 2 ml ice-cold 10% (w/v) trichloroacetic acid. From this step, the procedure was done as described by Ljungström and Ekman [15].

For purification of Ca^{2+}-activated protease erythrocytes were isolated as described by Dahlqvist-Edberg [7]. Data will be given for a preparation from 50 g packed blood cells. 100 ml 0.5 mM dithiothreitol/0.5 mM EDTA were added to the packed cells and the cells were lysed for at least 2 h under stirring. The hemolysate was centrifuged at 17 300 $\times g$ for 45 min and saturated $(NH_4)_2SO_4$ (pH 7) was added to give 50% saturation. This mixture was stirred for 90 min and then centrifuged for 60 min at 100 000 $\times g$. The pellet was suspended in 5 ml 50 mM Tris-acetic acid buffer (pH 7.0)/2 mM EDTA/1 mM EGTA/5 mM 2-mercaptoethanol. The suspension was dialyzed against 5 mM $KHCO_3$/5 mM EDTA/5 mM 2-mercaptoethanol until it was dissolved, which was generally achieved after 5 h.

The dialysate was run on a Sepharose 6B column (3 X 33 cm) equilibrated and eluted with buffer 1 (20 mM Tris-acetic acid buffer (pH 7.0)/1 mM EDTA/1 mM EGTA/2 mM 2-mercaptoethanol). Ca^{2+}-activated protease was eluted as a rather broad peak (Fig. 1) and the fractions were pooled.

This material was run on a DEAE-cellulose column (2 X 6 cm) equilibrated with the same buffer and the column washed with 1 vol. buffer 1. The column was

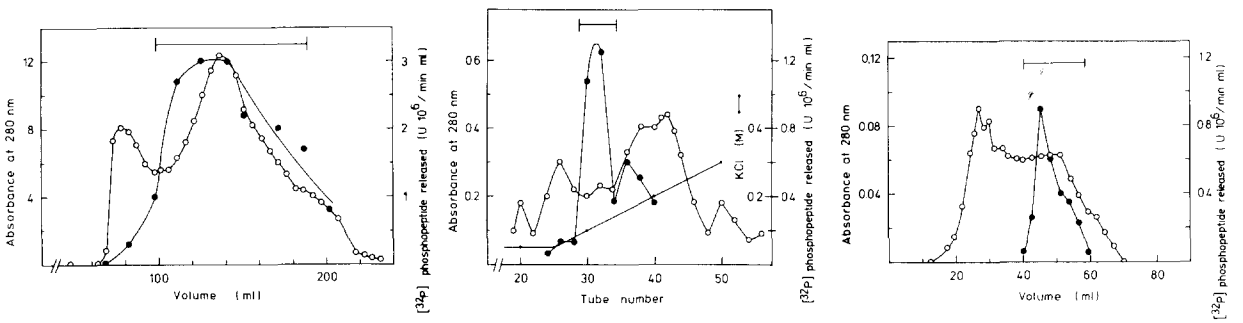

Fig. 1. Chromatography of Ca^{2+}-activated protease on Sepharose 6B (3 X 33 cm). 4-ml fractions were collected. o———o, absorbance; •———•, [^{32}P]phosphopeptide released. (Left-hand figure.)

Fig. 2. Chromatography of Ca^{2+}-activated protease on DEAE-cellulose. o———o, absorbance; •———•, [^{32}P]phosphopeptide released. (Centre figure.)

Fig. 3. Chromatography of Ca^{2+}-activated protease on Sephadex G-200 (2.2 X 30 cm). 3-ml fractions were collected. o———o, absorbance; •———•, [^{32}P]phosphopeptide released. (Right-hand figure.)

eluted with a linear 0–0.4 M KCl gradient in buffer 1 (Fig. 2).

The fractions with Ca^{2+}-activated protease activity (at 150 mM KCl) were pooled and precipitated at 50% satn. $(NH_4)_2SO_4$.

The pellet was dissolved in 2 ml 50 mM Tris-acetic acid buffer (pH 7.0)/2 mM EDTA/1 mM EGTA/2 mM 2-mercaptoethanol and was then chromatographed on a Sephadex G-200 column (2.2 × 30 cm). The column was equilibrated and eluted with buffer 1 containing 30% (v/v) glycerol (Fig. 3). The enzyme was eluted at a column volume corresponding to a molecular weight of about 100 000. The enzyme samples were pooled and stored at −18°C.

Results

Purification of Ca^{2+}-activated protease. The results from a typical purification are given in Table I. The yield was low and the losses were greatest during DEAE-cellulose chromatography. However, the degree of purification in this step was high. Phosphatase activity could only be found in the hemolysate and not at later stages of purification.

The specific activity after Sephadex G-200 chromatography was about 50% of that achieved for Ca^{2+}-activated protease from rat liver [9]. This was in agreement with the results from SDS-polyacrylamide gel electrophoresis under reducing conditions [16], which gave an apparently 50% pure enzyme. The main component had a molecular weight of 80 000, which was the same as that of the liver enzyme.

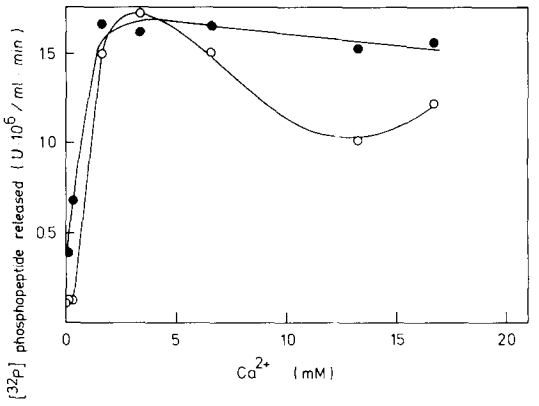

Fig. 4. The [^{32}P]phosphopeptide release from ^{32}P-labelled pyruvate kinase as a function of the Ca^{2+} concentration. The incubation time was 30 min. o——o, the proteolytic activity of Ca^{2+}-activated protease and •——•, the proteolytic activity of the hemolysate.

Kinetic properties of Ca^{2+}-activated protease. Under the conditions used, the activity of Ca^{2+}-activated protease was linear with time and the amount of enzyme protein up to 10 min. When the substrate, [^{32}P]phosphopyruvate kinase, was increased, the activity of Ca^{2+}-activated protease, increased proportionally up to the highest concentration tested i.e., $18 \cdot 10^{-9}$ M. The dependence on pH was the same as for Ca^{2+}-activated protease from rat liver [9]. The effects of Ca^{2+} on the activity of the erythrocyte enzyme (Fig. 4) showed a pattern similar to that obtained for the liver enzyme [9]. Maximal

TABLE I

THE PURIFICATION OF Ca^{2+}-ACTIVATED PROTEASE FROM 50 g PACKED RED BLOOD CELLS FROM THE RAT

The amount of [^{32}P]phosphopyruvate kinase was 8.7 pmol in each test.

Purification step	Total activity (U · 10^6)	Total protein (mg)	Specific activity (U · 10^6/mg)	Purification factor	Recovery (%)
Hemolysate	1430	4756	0.30	–	100
50% $(NH_4)_2SO_4$ precipitate	1170	1364	0.86	2.9	82
Sepharose 6B	686	542	1.27	4.2	48
DEAE-cellulose	44.9	7.48	6.0	20	3
50% $(NH_4)_2SO_4$ precipitate	47.5	3.9	12.2	41	3
Sephadex G-200	15	0.51	29.4	98	1

stimulation of the [^{32}P]phosphopeptide release from phosphorylated pyruvate kinase was observed at about 3.3 mM Ca^{2+} for both the purified Ca^{2+}-activated protease and the hemolysate.

Effect of Ca^{2+}-activated protease on ^{32}P-labelled pyruvate kinase. The [^{32}P]phosphopeptides from the digestion of rat liver pyruvate kinase with Ca^{2+}-activated protease or hemolysate in the presence of Ca^{2+} were eluted from a Sephadex G-25 column after a column volume of 0.47. The material obtained from the hemolysate-treated [^{32}P]phosphopyruvate kinase in the absence of Ca^{2+} and in the presence of 6.25 mM EGTA was also analyzed by chromatography. In this case most of the released radioactivity was eluted at the same volume at which phosphate was eluted (Fig. 5). Thus, the release of [^{32}P]phosphopeptides seems to require the presence of Ca^{2+}. The activity of the pyruvate kinase partially digested by Ca^{2+}-activated protease was tested as a function of the concentration of phospho*enol*pyruvate (Fig. 6). It was shown that the $K_{0.5}$ increased for this substrate when the enzyme was proteolytically modified, from 1.2 mM for the phosphorylated enzyme to 2.0 mM for the modified enzyme (Fig. 6).

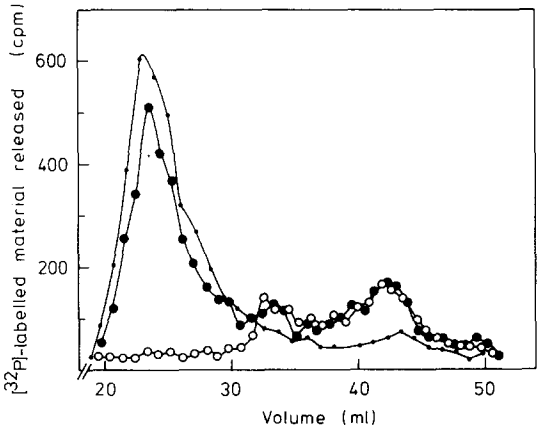

Fig. 5. Chromatography of [^{32}P]phosphorylated material, obtained by digestion of ^{32}P-labelled pyruvate kinase with Ca^{2+}-activated protease or hemolysate on a Sephadex G-25 column (1.75 × 20 cm^2), •———•, material after Ca^{2+}-activated protease digestion in the presence of Ca^{2+}; •———•, material after digestion with hemolysate in the presence of Ca^{2+}; o———o, material after digestion with hemolysate in the absence of Ca^{2+} but in the presence of 6.25 mM EGTA. 1-ml fractions were collected.

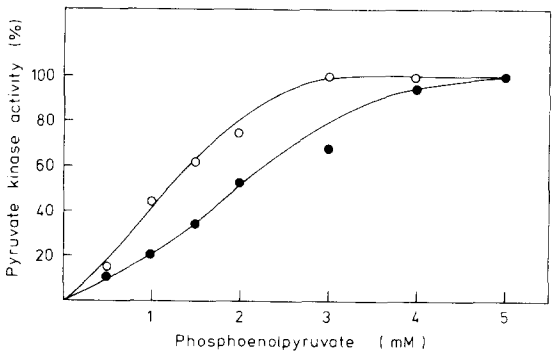

Fig. 6. The activity of pyruvate kinase as a function of phospho*enol*pyruvate. o———o, phosphorylated pyruvate kinase and •———•, phosphorylated pyruvate kinase treated with Ca^{2+}-activated protease.

Site of cleavage by Ca^{2+}-activated protease. Glycine became the N-terminal amino acid in the peptide isolated from pyruvate kinase after treatment with Ca^{2+}-activated protease. This, in addition to the amino acids presents in the peptide, indicated that Ca^{2+}-activated protease had cleaved the phosphorylated site of pyruvate kinase (Pro-Ala-Gly-Tyr-Leu-Arg-Arg-Ala-SerP-Val-Ala-Gln-Leu-Thr-Gln-Glu-Leu) [17], between Ala and Gly at the N-terminal side and between Gln and Glu at the C-terminal side.

Pyruvate kinase prepared from erythrocytes incubated with [^{32}P]orthophosphate. Erythrocytes were incubated in the presence of 37 MBq [^{32}P]-orthophosphate (1.5 mM), then hemolysed, partially purified, and incubated with antiserum against liver pyruvate kinase. The ^{32}P-labelled antigen-antibody complex was then isolated on protein A-Sepharose. The incorporation of [^{32}P]phosphate was about 0.5 mol/mol subunit into the pyruvate kinase. When Ca^{2+} was added to the medium at the same time as the hemolysis was induced, the isolated pyruvate kinase only contained about 0.05 mol [^{32}P]phosphate/mol enzyme subunit.

Discussion

The phosphorylated site of pyruvate kinase is more easily removed by subtilisin [8] and Ca^{2+}-activated protease from rat liver [9] than the unphosphorylated phosphorylatable site. Both these proteases remove the phosphorylated sites without any appreciable change in the maximal activity and

molecular weight of pyruvate kinase [8,9]. No analysis of rat liver cell cytosol for Ca^{2+}-activated proteolytic activity could be made, probably because of high phosphatase activity, but in hemolysate this activity seemed to account for the major proteolytic modification of phosphopyruvate kinase (Fig. 5).

A hemolysate and the Ca^{2+}-activated protease purified from erythrocytes showed the same demand for Ca^{2+} when the proteolytic activity was assayed against phosphopyruvate kinase. The peptide material obtained was retarded to the same extent on a Sephadex G-25 column in both preparations. The fact that the amount of released peptide material was similar in both cases, suggests that Ca^{2+}-activated protease of erythrocytes contributes to the modification of pyruvate kinase, as it has been very difficult to purify an unmodified phosphorylatable enzyme from these cells [2,7]. In the experiments in which erythrocytes were incubated with [^{32}P]phosphate, the pyruvate kinase, after isolation, was phosphorylated to about 50%, but when Ca^{2+} was added during hemolysis the degree of phosphorylation decreased to about 5%, which could indicate activation of the Ca^{2+}-dependent protease.

The concentration of Ca^{2+} found in vivo [18] is, however, not consistent with the levels needed to activate this proteolytic activity. One explanation for this discrepancy may be compartmentation of Ca^{2+} in the cell. The importance of the existence of the L-isozyme in mature erythrocytes is difficult to understand, since these cells have no gluconeogenetic activity. Perhaps the capacity for regulatory phosphorylation is of importance to the immature erythrocyte. The accumulation of the proteolytically-modified pyruvate kinase in mature erythrocytes may be due to a low but significant activity of Ca^{2+}-activated protease, whereas further degradation of pyruvate kinase is low compared with the high turnover of the enzyme in liver cells, for example. Whether the modification of the erythrocyte pyruvate kinase with Ca^{2+}-activated protease is of special advantage to the metabolism of the erythrocyte is not known.

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