The in vitro Modification of Phosphorylated Pyruvate Kinase by a Ca$^{2+}$-activated Protease from Rat Liver

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A Ca$^{2+}$-activated protease from rat liver cell sap was prepared. It was shown to act on rat liver pyruvate kinase that had been phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase, the activity being optimum at neutral pH. The modified pyruvate kinase had the same $V_{max}$ as the phosphoenzyme but showed a lower affinity for the substrate phosphoenolpyruvate. The possibility that this proteolytic attack is the step that initiates further degradation in the cell is discussed.

Fairly little is known about the intracellular degradation of proteins. One theory is that initially some rate-limiting modification of the proteins takes place in the cytoplasm, followed by total rapid degradation by the lysosomal enzymes.\(^1\)

For rat liver pyruvate kinase type L (EC 2.7.1.40) it has been shown that the phosphorylated form is about ten times more sensitive to subtilisin with respect to removal of the phosphorylated site than is the unphosphorylated enzyme with regard to removal of the corresponding phosphate-accepting site.\(^2\)

As proteolytic sensitivity in vitro seems to be correlated to that in vivo,\(^3\) the phosphorylation of pyruvate kinase may not only regulate the activity of the enzyme but might also be of importance for the regulation of pyruvate kinase degradation in the cell. The aim of the present investigation was to find a protease in rat liver cell sap that acted similarly to subtilisin on pyruvate kinase. A Ca$^{2+}$-activated protease (\(=\) CAP) has been found, among other tissues, in rabbit muscle.\(^4\) Phosphorylase kinase, phosphorylase,\(^4\) glycogen synthetase,\(^5\) phosphorolase phosphatase\(^6\) and cyclic AMP-dependent and independent protein kinases\(^7\) can be modified by this type of enzyme in vitro. This CAP could be an endogenous enzyme acting also on other phosphorylatable proteins, e.g. liver pyruvate kinase type L. A similar protease was purified from rat liver and its action on liver pyruvate kinase was examined.

EXPERIMENTAL

Purification of CAP. CAP from rat liver was purified according to the method of Dayton et al.\(^9\) up to the Sephadex G-200 step. The homogenization was achieved by four strokes with a Potter-Elvehjem homogenizer. The enzyme obtained had a specific activity of about $45 - 60 \times 10^{-6}$ U/mg. One unit is defined as the amount of enzyme that releases 1 $\mu$mol of $[^{32}P]$phosphate per min from $[^{32}P]$phosphorylpyruvate kinase under the conditions used. The activity of the enzyme decreased by about 50% when stored for two weeks at $-18 \, ^\circ\text{C}$ in 20 mM Tris-acetate buffer, pH 7.0, containing 2 mM 2-mercaptoethanol and 1 mM EDTA. The purification was followed by polyacrylamide gel electrophoresis in detergent under reducing conditions\(^10\) and the enzyme was finally about 90% pure.

Preparation of $[^{32}P]$phosphorylated pyruvate kinase. Pyruvate kinase from rat liver was prepared as described previously\(^11\) and phosphorylated as described by Bergström et al.\(^12\) The phosphorylated enzyme (1 mg in a total volume of 2 ml) was chromatographed on a Sephadex G-50 column (2 x 15 cm), equilibrated and eluted with 10 mM potassium phosphate buffer, pH 7.0, containing 30% glycerol, 0.1 mM dithiothreitol, 25 mM NaF and 0.1 mM EDTA. It was stored at $-18 \, ^\circ\text{C}$ until used. The incorporation of $[^{32}P]$phosphate was 0.9 mol/mol of enzyme subunit.
Assays. The coupled assay of pyruvate kinase was performed as described by Ekman et al.\textsuperscript{13} The standard assay of CAP was carried out in 50 mM Tris-acetate buffer, pH 8.0, containing 50 mM KCl, 3.3 mM CaCl\textsubscript{2}, 5 mM 2-mercaptoethanol, 50 \mu M EDTA, 0.5 mM NaN\textsubscript{3} and the \textsuperscript{32}P-labelled pyruvate kinase, in a total volume of 75 \mu l. After 10 min at 20 °C the incubations were interrupted by 2 ml of 10 % (w/v) trichloroacetic acid, and 1 mg of bovine serum albumin was added. The \textsuperscript{32}P-labelled precipitable proteins were collected by centrifugation. The pellets were dissolved in 0.2 ml of 0.5 M NaOH and reprecipitated. After centrifugation the precipitates were dissolved in 0.5 ml of 0.5 M NaOH. The Cerenkov radiation was measured in the washed dissolved pellets and in the first supernatants. The [\textsuperscript{32}P]orthophosphate of the supernatants was determined as described by Martin and Doty.\textsuperscript{14}

Phosphorylation of pyruvate kinase after treatment with CAP. In some experiments the pyruvate kinase (15 pmol) was treated with CAP before phosphorylation. Incubation with the protease was carried out as described above and was interrupted by the addition of 10 \mu l of 0.1 M EGTA. The catalytic subunit of cyclic AMP-dependent protein kinase from rat liver capable of phosphorylating 65 pmol of histone/min,\textsuperscript{15} 10 \mu l of 0.1 M magnesium acetate and 10 \mu l of 1 mM [\textsuperscript{32}P]ATP were added. Phosphorylation was performed at 30 °C for 30 min and was interrupted with 2 ml of 10 % trichloroacetic acid. Precipitated protein was washed as described previously\textsuperscript{16} and the radioactivity was measured. The incorporation of [\textsuperscript{32}P]phosphate was 0.9 mol/mol of subunit in the control samples run in the absence of CAP.

RESULTS

Pyruvate kinase as substrate of CAP. The phosphorylated and unphosphorylated forms of pyruvate kinase were tested and found to be susceptible to CAP (Fig. 1).

The release of [\textsuperscript{32}P]phosphopeptides and phosphorylatable sites was studied as a function of time. The release of [\textsuperscript{32}P]phosphopeptides was rapid compared to that of the phosphorylatable sites. That is, phosphoryruvate kinase was a better substrate for CAP than the unphosphorylated enzyme. The rate of release of [\textsuperscript{32}P]phosphopeptides seemed to decrease with time. This was due to the contaminating phosphatase, which dephosphorylated the labelled peptide at a slower rate than the peptide was released.

The heptapeptide Leu-Arg-Arg-Ala-[\textsuperscript{32}P]SerP-Val-Ala, which has the same sequence as the phosphorylated site of pyruvate kinase, was tried as substrate, using the methods described earlier.\textsuperscript{17} The duration of incubation with CAP was 45 min. There was no release of amino acids or peptides by CAP from this [\textsuperscript{32}P]phosphopeptide. The specificity of CAP for peptide bonds was not tested further.

![Fig. 1](image_url) Treatment of pyruvate kinase with CAP. The graph shows the time course of the decrease of \textsuperscript{32}P-labeling and the release of [\textsuperscript{32}P]phosphate from phosphorylated pyruvate kinase (○) and the decrease of phosphate-accepting sites from unphosphorylated pyruvate kinase (●). The amount of pyruvate kinase was 15 pmol in each case and the amount of CAP protein used was 250 μg/ml.

![Fig. 2](image_url) Ca\textsuperscript{2+}-dependence of CAP with \textsuperscript{32}P-labelled pyruvate kinase as substrate. The amount of pyruvate kinase was 4 pmol and the incubation time was 10 min.
Fig. 3. The activity of CAP as a function of the concentration of CAP protein. The activity of the contaminating phosphatase is also included. The amount of pyruvate kinase used in the assay was 9.5 pmol and the incubation time was 10 min.

The effect of pH and Ca$^{2+}$ on the activity of CAP. The activity of CAP was analysed at different pH values, with phosphorylated pyruvate kinase as substrate. The concentration of Ca$^{2+}$ was 3.3 mM. The pH optimum was 8, which was used in all subsequent tests. The concentration of Ca$^{2+}$ needed for maximal activity of CAP was 2–8 mM (Fig. 2). A Ca$^{2+}$ concentration of 3.3 mM was chosen for all other tests.

The release of [$^{32}$P]phosphopeptides as a function of the concentration of CAP. The release of [$^{32}$P]phosphopeptides increased with increasing amounts of CAP (Fig. 3). The contaminating phosphatase had such low activity that it was not detected at an amount of CAP corresponding to less than 60 µg protein/ml. The phosphatase dephosphorylated the produced [$^{32}$P]phosphopeptide, which explains why the release of [$^{32}$P]phosphopeptides did not increase linearly for increasing amounts of CAP. For that reason the amount of CAP chosen in the experiments corresponded to 80 µg protein/ml, when the release of phosphopeptides was almost at a maximum and the activity of the phosphatase was low.

The activity of CAP treated pyruvate kinase as a function of the phosphoenolpyruvate concentration. When phosphorylated pyruvate kinase is treated with subtilisin in vitro the partially degraded enzyme has a lower affinity for phosphoenolpyruvate than the phosphoform of the enzyme. A similar decrease in the affinity for phosphoenolpyruvate with no change in $V_{\text{max}}$ was observed when the [$^{32}$P]phosphorylated pyruvate kinase was incubated with CAP so that it lost 85% of its radioactivity. The apparent $K_m$ increased from 0.7 to 1.8 mM after treatment with CAP (Fig. 4). The same increase has been obtained by digestion with subtilisin.

Inactivation of CAP. When CAP was incubated in the absence of a substrate it became inactivated. After 10 min 95% of its activity remained, and after 30 min 55%. It was therefore important to store the preparations in the frozen state and to use the thawed enzyme immediately.

DISCUSSION

The Ca$^{2+}$-activated protease was prepared by adopting the method which Dayton et al. used for porcine muscle. A similar protease has been found in rat brain, rabbit skeletal muscle, calf uterus, macrophages, rat liver and other tissues. The protease preparations show a broad specificity. Some of them seem to be involved in phosphorylation-dephosphorylation reactions, since they either have phosphorylatable proteins as substrates or activate protein kinases. Whether or not they are the same enzyme is yet to be determined.

In this study CAP was found to exhibit activity on phosphorylated pyruvate kinase similar to the activity of substilisin on the latter enzyme. CAP was also active on other phosphorylated and unphosphorylated substrates of cyclic AMP-dependent protein kinase, such as histone and protamine (data not given). This might indicate a specificity of CAP for proteins involved in phosphorylation-dephosphorylation reactions. For pyruvate kinase the modification by CAP may possibly initiate the degradation of the protein, thus regulating the amount and thereby the activity of pyruvate kinase in the cell. The biological significance of the activity of CAP on phosphorylated pyruvate kinase remains to be established, however.

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REFERENCES


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