AMINO ACID SEQUENCE OF A (^{32}P)PHOSPHOPEPTIDE FROM PIG LIVER PYRUVATE KINASE PHOSPHORYLATED BY CYCLIC 3′,5′-AMP-STIMULATED PROTEIN KINASE AND γ-(^{32}P)ATP

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SUMMARY

Pig liver pyruvate kinase (type L) was ^{32}P-labelled by incubation with (^{32}P)ATP and cyclic 3′,5′-AMP-stimulated protein kinase from the same source. One major (^{32}P)phosphopeptide was isolated from a peptic hydrolysate of the enzyme. Its amino acid sequence was Leu-Arg-Arg-Ala-(^{32}P)SerP-Leu.

INTRODUCTION

Recently we reported the presence of three proteins in rat liver cell sap which are phosphorylated by cyclic 3′,5′-AMP-stimulated protein kinase (1). One of the components has been shown to be derived from the main isoenzyme (L type) of liver pyruvate kinase (ATP:pyruvate phosphotransferase; EC 2.7.1.40) (2). A corresponding phosphorylation of the pig liver enzyme has been described (3). In both cases the activity of the enzyme is decreased by phosphorylation, especially at low substrate concentrations, and (^{32}P)SerP has been isolated from an acid hydrolysate of ^{32}P-labelled enzyme (2,3).

The aim of the present investigation was to find out whether a specific serine residue in pig liver pyruvate kinase is phosphorylated on incubation with (^{32}P)ATP and cyclic 3′,5′-AMP-stimulated protein kinase. This was done by isolating one major (^{32}P)phosphopeptide from a peptic digest of ^{32}P-labelled enzyme. The amino acid sequence of the peptide was determined.

MATERIALS AND METHODS

Pig liver pyruvate kinase and protein kinase were purified as described previously (3). 5 or 10 mg of enzyme in 3 mM potassium phosphate buffer, pH

7.0, were incubated with 0.1 mM γ-(^{32}P)ATP (29000 cpm/nmole), prepared as described before (2), and 0.9 units of protein kinase for 30 min at 30°C in the presence of 0.01 mM cyclic 3',5'-AMP, 10 mM magnesium acetate, 0.05 mM Fru-1,6-P_2, 0.05 mM dithiothreitol and 15% glycerol (v/v). The total volume was 20 ml. Excess γ-(^{32}P)ATP was removed by chromatography at 5°C on a 2.1 x 40 cm Sephadex G-25 column equilibrated and eluted with 2 mM potassium phosphate buffer, pH 7.0. The total radioactivity of the fraction was estimated by measuring its Cerenkov radiation (4). The molar incorporation of phosphate into the tetrameric enzyme (5) was calculated, assuming the specific activity of the pure enzyme to be 170 units/mg (3) and the subunit molecular weight to be 62000 (3,5).

1 M HCl was added to the ^{32}P-labelled enzyme to a final concentration of 0.05 M. 2.7 mg of pepsin (Boehringer, Mannheim GmbH, type 15445 EPBK) were then added and the solution was kept at 25°C for 2 hours. The digestion was interrupted by separating the pepsin from the main ^{32}P-labelled material by chromatography on a 5.4 x 44 cm Sephadex G-25 column equilibrated and eluted with 50 mM pyridine-acetic acid buffer, pH 3.2. The radioactive peak appeared after 0.7 column volume.

Amino acid sequence analysis was performed, using a dansyl-Edman method according to Hartley (6).

All preparations were carried out at room temperature (23°C - 25°C) unless otherwise stated.

RESULTS

In one experiment using 5 mg of enzyme, 2.9 moles of (^{32}P)phosphate/mole of enzyme were incorporated. The pooled (^{32}P)phosphopeptide material obtained after pepsin digestion and Sephadex G-25 chromatography - corresponding to 90% of the radioactivity of the ^{32}P-labelled enzyme - was lyophilized, dissolved in 13 ml of 50 mM pyridine-acetic acid buffer, pH 3.2, and chromatographed on an SP-Sephadex column, as described in Figure 1A. Only one major (^{32}P)phosphopeptide peak, corresponding to 60% of the radioactivity eluted

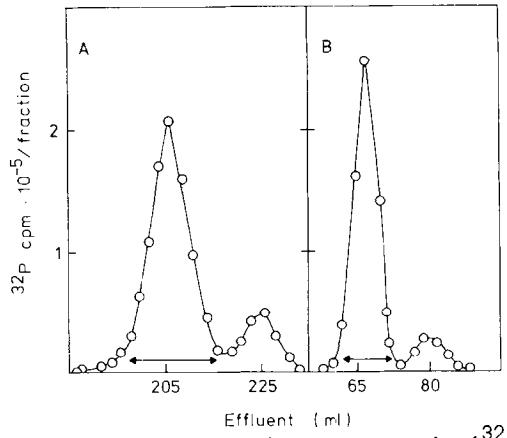

Figure 1. Two successive chromatographies of a peptic (^{32}P)phosphopeptide from pig liver pyruvate kinase. First a 0.9 x 15 cm SP-Sephadex C-25 column, equilibrated with 0.05 M pyridine-acetic acid buffer, pH 3.2, was used. Elution was performed with 10 ml of the same buffer, pH 3.2, and a linear gradient (total volume 200 ml) formed from 0.05 M and 0.2 M pyridine-acetic acid buffer, pH 3.2 (Figure 1A). For further chromatography a 0.9 x 15 cm QAE-Sephadex A-25 column, equilibrated with 0.05 ammonium carbonate buffer, pH 8.5, was used. The column was eluted with 10 ml of the same buffer, pH 8.5, and a linear gradient (total volume 200 ml) formed from 0.05 M and 0.5 M ammonium carbonate buffer, pH 8.5 (Figure 1B). 2.3 ml fractions were collected. The radioactivity of the fractions was measured and the material was pooled as indicated. For details see text. o—o = ^{32}P radioactivity.

from the preceding column, was obtained. The ^{32}P-labelled material was lyophilized, dissolved in 20 ml of 50 mM ammonium carbonate buffer, pH 8.5, and further chromatographed on a QAE-Sephadex column (Figure 1B). 70% of the radioactivity applied was eluted as one peak. This material (21 nmoles) was lyophilized and dissolved in 1.0 ml of water. On determination of the N-terminal amino acid (6) only leucin was obtained, showing that the (^{32}P) phosphopeptide was sufficiently pure for amino acid sequence analysis. The sequence was found to be Leu-Arg-Arg-Ala-(^{32}P)SerP-Leu.

The same amino acid sequence was obtained in another experiment using 10 mg of pyruvate kinase from a different preparation with a molar incorporation of 2.0 mole of (^{32}P)phosphate/mole of enzyme. The amino acid composition of the (^{32}P)phosphopeptide isolated (TABLE 1) was found to correspond to the result of the sequence analysis.

DISCUSSION

The fact that only one major peptic peptide, containing six amino acid

Table 1.

Amino acid composition of the peptic (^{32}P)phosphopeptide isolated from ^{32}P-labelled pig liver pyruvate kinase. 8 nmoles of peptide were hydrolyzed for 24 hours at 110° in a sealed ampoule containing 6 M HCl. A one-column Durrum amino acid analyser was used.
Numbers given are mole of amino acid per mole of alanine where the nearest integer is given within brackets.

Amino acid	
Serine[x]	1.0 (1)
Alanine	1.0 (1)
Leucine	1.6 (2)
Arginine	1.8 (2)

[x]Corrected to zero time of hydrolysis.

residues, was obtained from ^{32}P-labelled pig liver pyruvate kinase shows that a specific site in the enzyme was phosphorylated. This further supports the view (3) that the enzyme belongs to the group of enzymes whose activity is regulated by phosphorylation-dephosphorylation.

The amino acid sequence of the phosphorylated site in pig liver pyruvate kinase has not so far been found in other enzymes which are regulatorily phosphorylated. However, it has some similarities to those sequences found in different glycogen phosphorylase (EC 2.4.1.1) and glycogen synthetase (EC 2.4.1.11) preparations, which can be summarized as $\begin{matrix}\text{Arg}\\\text{Lys}\end{matrix}$-Gln-Ile-SerP-$\begin{matrix}\text{Ile}\\\text{Val}\end{matrix}$-Arg (7,8). In these cases the SerP residue is surrounded by hydrophobic residues and the third residue before SerP is a basic amino acid.

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