NON-DEPENDENCE ON NATIVE STRUCTURE OF PIG LIVER PYRUVATE KINASE WHEN USED AS A SUBSTRATE FOR CYCLIC 3',5'-AMP-STIMULATED PROTEIN KINASE.

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SUMMARY

Alkali-inactivated pig liver pyruvate kinase, type L, and a cyanogen bromide fragment from the same enzyme were shown to be phosphorylated by (^{32}P)ATP and cyclic 3',5'-AMP-stimulated protein kinase. In both cases the rate of phosphorylation was higher than with the native enzyme. Pyruvate kinases types A and M were not phosphorylated under the same conditions. From the ^{32}P-labelled cyanogen bromide fragment (^{32}P)phosphorylserine was isolated. The electrophoretic pattern of (^{32}P)phosphopeptides obtained on partial acid hydrolysis of the fragment indicated that the phosphorylated site of the fragment was identical with that of the native pyruvate kinase.

INTRODUCTION

Liver pyruvate kinase (ATP:pyruvate phosphotransferase; EC 2.7.1.40), type L, from the rat and pig has recently been reported to become phosphorylated on incubation with cAMP-stimulated protein kinase and (^{32}P)ATP (1,2). The purpose of our present work was to investigate some of the conditions under which the enzyme can be a substrate for this protein kinase. It was considered, for example, that one prerequisite for the reaction might be that the pyruvate kinase had to have a native conformation. Another possibility was that the amino acid sequence surrounding the serine residue that is phosphorylated had to be of a particular type.

Histones are phosphorylated by cAMP-dependent protein kinases and ATP (3,4). In aqueous solutions of low ionic strength histones appear to have

Abbreviations: cAMP, cyclic 3',5'-adenosine monophosphate.

low levels of ordered structure (5,6). NMR studies of F 1 histone, as well as a helix prediction method, suggest that the segment with the amino acids 1-47 constitutes a random coil even at high ionic strength (7). This segment contains the serine residue that is phosphorylated by protein kinase (5,8). Thus, a highly ordered structure does not seem to be a prerequisite for a protein to be a substrate for cAMP-dependent protein kinase. This view gets further support from the fact that myelin basic protein, which probably lacks tertiary structure, is also such a substrate (9,10). That ribosomal proteins are more easily phosphorylated after isolation using acid extraction than they are in intact ribosomes (11) also seems to favour the view, though it cannot be excluded that this effect results from reduction of steric hindrance of the protein kinase reaction.

This report describes experiments showing that inactivated pig liver pyruvate kinase (type L) as well as a cyanogen bromide fragment from the same enzyme are phosphorylated on incubation with cAMP-dependent protein kinase and (^{32}P)ATP.

MATERIALS AND METHODS

Pig liver pyruvate kinase (type L) was purified as described previously (2) and the enzyme activity was determined according to Kimberg and Yielding (12) with some modifications (1). Pyruvate kinase type A was prepared from pig kidney cortex (13) and type M from pig muscle (14). Pig liver protein kinase was purified and assayed essentially according to Kumon et al. (15). One unit of protein kinase activity is defined as that amount of the enzyme which catalyzes the transfer of one picomole of ^{32}P per minute from (^{32}P)ATP into histones under the conditions used. (γ-^{32}P)ATP was prepared according to Engström (16) and isolated as described by Mårdh (17). Radioactivity was measured as described earlier (1). Rabbit muscle 3-phosphoglyceraldehyde dehydrogenase (EC 1.2.1.12) was purchased from Boehringer and bovine serum albumin from Sigma. Guanidinium chloride was obtained from BDH Chemicals and purified according to Nosaki (18). Cyanogen bromide was a product of Pierce Chemical Company.

The amount of pyruvate kinase used in each experiment was calculated from the activity of the enzyme solution before treatment, assuming a specific activity of 175 units/mg for the pure pig liver L-type enzyme (2). The corresponding value for the A-type and M-type isoenzymes used was 500 units/mg. For calculations on a molar basis a subunit molecular weight (estimated from polyacrylamide gel electrophoresis in detergent) of 62,000 was used for pyruvate kinases types L (2,19) and M (unpublished result), and 60,000 for type A (13).

All gel chromatographies were performed at room temperature (22-23°C).

Phosphorylation of alkali-inactivated pyruvate kinase. Highly purified pig

liver pyruvate kinase was dialyzed against 50 mM potassium phosphate buffer, pH 7.0, containing 30% (v/v) glycerol and 0.1 mM dithiothreitol. 0.25 volume of cold 0.5 M NaOH was then added to an enzyme batch in an ice-water bath. 10 min later the pH of the incubation mixture was adjusted to 7.0 with 0.5 M HCl. No pyruvate kinase activity remained after this alkali treatment or reappeared during the phosphorylation experiments. 0.3 mg of inactivated enzyme was incubated at 30°C and pH 7.0 with 30 units of protein kinase and 0.4 mM (^{32}P)ATP (24,000 cpm/nmole) in the presence of 4 mM magnesium acetate and 0.04 mM cAMP. The final incubation volume was 1.6 ml. After different time intervals 0.2 ml samples were removed and their protein phosphorylation was determined (1). An equal amount of native pyruvate kinase with the addition of NaCl to correct the ionic environment was incubated with protein kinase and (^{32}P)ATP in the same way in parallel.

Native and alkali-treated type M and type A pyruvate kinases, 3-phosphoglyceraldehyde dehydrogenase and bovine serum albumin were also incubated with protein kinase and (^{32}P)ATP in the same way.

Cyanogen bromide cleavage of pig liver pyruvate kinase. To 1.5 mg of pyruvate kinase in 0.5 ml of 10 mM potassium phosphate buffer, pH 7.0, containing 30% (v/v) glycerol and 0.1 mM dithiothreitol, 8 M guanidinium chloride and 0.1 M dithiothreitol were added to final concentrations of 6 M and 10 mM, respectively. The pH was adjusted to 8.0 with 0.5 M KOH. After 22 hours at 23°C, iodoacetamide was added to a final concentration of 24 mM, and the solution was kept in the dark at 4°C for 3 hours (20). The material was then chromatographed on a 1.3 x 15 cm Sephadex G-50 column equilibrated and eluted with 70% (v/v) formic acid. The enzyme was detected by its absorbance at 280 nm.

In order to get a ^{32}P-labelled marker peptide a trace amount of pyruvate kinase (0.1 mg) was incubated with protein kinase and (^{32}P)ATP (23,000 cpm/nmole) and treated in the same way as the unlabelled enzyme. The phosphorylated enzyme was detected by its ^{32}P-labelling and added to the unlabelled pyruvate kinase.

8 mg of solid cyanogen bromide were then added, and the solution was stirred in the dark at room temperature for 45 hours (21). It was then dried over KOH and P_2O_5 in a vacuum desiccator. The material was dissolved in 1 ml of 50% (v/v) acetic acid and chromatographed on a 1.3 x 36 cm Sephadex G-50 column, equilibrated and eluted with 50% acetic acid. Most of the radioactivity was eluted as a sharp, symmetrical peak after 0.60-0.65 column volume. The volume of this material was reduced in the desiccator to about 1 ml prior to chromatography on a 1.3 x 40 cm Sephadex G-25 column in equilibrium with 10 mM HCl performed to reduce the ionic strength. The labelled material was eluted after 0.45 column volume. The yield, as calculated from the radioactivity of the ^{32}P-labelled enzyme eluted from the first Sephadex G-50 column, was 65%.

Phosphorylation of fragments. 0.1 ml aliquots of the fractions from the Sephadex G-25 chromatography were incubated for 40 min at 30°C with 33 units of protein kinase and 0.25 mM (^{32}P)ATP (20,000 cpm/nmole) in the presence of 12 mM each of imidazole-HCl and Tris-HCl buffers, pH 7.5, containing 10% (v/v) glycerol, 5 mM magnesium acetate, 0.02 mM cAMP and 0.05 mM dithiothreitol. The incubations were interrupted by the addition of 10% (w/v) sodium dodecyl sulphate and concentrated acetic acid to final concentrations of 0.2% (w/v) and 50% (v/v), respectively. The incubation mixtures were then chromatographed on 1.3 x 40 cm Sephadex G-25 columns equilibrated and eluted with 0.1% sodium dodecyl sulphate-50% acetic acid in order to separate excess (^{32}P)ATP from ^{32}P-labelled fragments. However, when material was prepared for acid hydrolysis, no sodium dodecyl sulphate was used. The extent of phosphorylation of the fragments was estimated by measuring the total radioactivity incorporated and correcting for the trace of radioactivity

present before the incubation. For comparison of rates of phosphorylation of fragments and enzyme an equivalent amount of native pyruvate kinase (0.02 mg) was phosphorylated with protein kinase and (^{32}P)ATP in the same way, except that the incubations were interrupted by adding trichloroacetic acid, and the phosphorylation of the enzyme was determined.

Isolation of (^{32}P)phosphorylserine. Unlabelled phosphorylserine and phosphorylthreonine were added to a sample of ^{32}P-labelled cyanogen bromide fragments (corresponding to 20,000 cpm) from the phosphorylation experiments. After acid hydrolysis (2 M HCl, 100°C, 23 h), chromatography on Dowex 50 and Dowex 1 was performed (22).

Electrophoretic (^{32}P)phosphopeptide pattern of partial acid hydrolysates of ^{32}P-labelled cyanogen bromide fragment material and native enzyme. Partial acid hydrolysates (6 M HCl, 100°C, 30 min) of ^{32}P-labelled cyanogen bromide fragments and ^{32}P-labelled native pig liver pyruvate kinase were subjected to high voltage electrophoresis in 0.05 M pyridine-acetic acid buffer, pH 3.5, on Whatman No. 3 MM paper at 40 V/cm and 90 mA for one hour, followed by radioautography (16).

RESULTS

Fig. 1 demonstrates that on incubation with protein kinase and (^{32}P)ATP the alkali-inactivated liver pyruvate kinase was more rapidly phosphorylated than the native enzyme. In addition, the maximal extent of the reaction (about 0.7 mole of ^{32}P/mole of enzyme subunit) seemed to be of the same order in the two cases.

When native and alkali-treated pyruvate kinase type A and type M, bovine serum albumin and 3-phosphoglyceraldehyde dehydrogenase were each incubated with protein kinase and (^{32}P)ATP, there was no significant phosphorylation in any case (less than 0.01 mole of ^{32}P/mole of enzyme subunit or protein molecule).

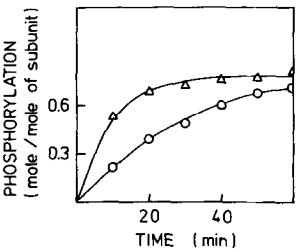

Figure 1. Phosphorylation of alkali-inactivated pig liver pyruvate kinase. Inactivated and native enzyme incubated with protein kinase and (^{32}P)ATP as described in the text. O——O, native enzyme; Δ——Δ, alkali-treated enzyme.

On incubation with (^{32}P)ATP and protein kinase of the cyanogen bromide fragments eluted from the Sephadex G-25 column, it was found that some material was phosphorylated. This material was eluted after 0.45 column volume in parallel with the ^{32}P-labelled marker fragment derived from the radioactive native enzyme. Fig. 2 shows that the cyanogen bromide fragment material from the peak fraction was much more rapidly phosphorylated than the native enzyme. However, the maximal phosphate incorporation (0.5 and 0.6 mole of phosphate per mole of enzyme subunit, respectively) did not seem to differ significantly.

All phosphate incorporated during the phosphorylation experiments was found to be, within experimental errors, serine-bound, since only (^{32}P)phosphorylserine could be isolated from an acid hydrolysate by chromatography on Dowex 50 and Dowex 1 and the recovery of ^{32}P-labelled phosphorylserine from the total radioactivity of the hydrolysate was found to be the same as that of added unlabelled phosphorylserine (as determined by its reaction with ninhydrin (23).

On radioautography of the electropherograms of partial acid hydrolysates the same pattern of radioactive bands was obtained from ^{32}P-labelled fragment material as from the native enzyme (Fig. 3).

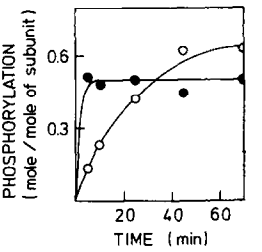

Figure 2. Phosphorylation of a cyanogen bromide fragment from pig liver pyruvate kinase. Incubation of fragment and native enzyme as described in the text. O——O, native enzyme; ●——●, cyanogen bromide fragment.

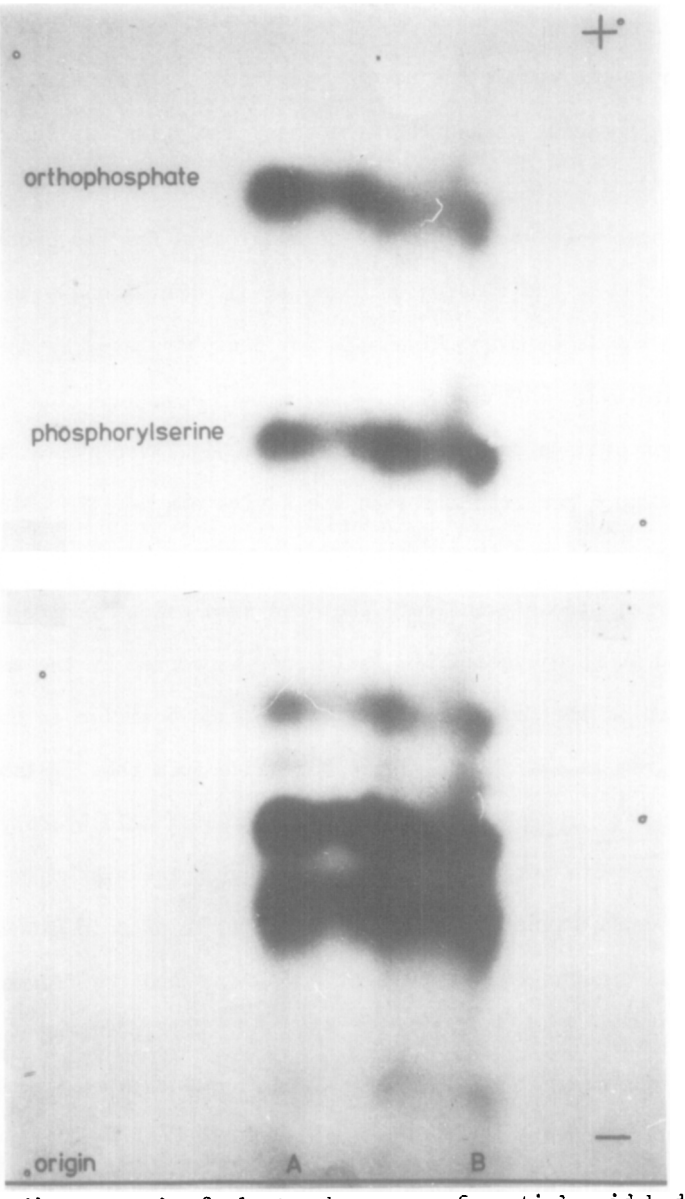

Figure 3. Radioautograph of electropherogram of partial acid hydrolysates of cyanogen bromide fragment and native enzyme. Fragment and enzyme were incubated with cAMP-dependent protein kinase and (^{32}P)ATP as described in the text. A, pig liver pyruvate kinase phosphorylated in the native state; B, phosphorylated cyanogen bromide fragment.

DISCUSSION

The results clearly show that pig liver pyruvate kinase does not need to be in its active state to be a substrate for cAMP-stimulated protein kinase. In fact, alkali-inactivated enzyme was more rapidly phosphorylated than the

native one under the conditions used. Experiments with other native and alkali-treated proteins showed that the phosphorylation was in any case a very specific reaction, as is evident by the fact that not even the other pig pyruvate kinase isoenzymes were effective as substrates for the protein kinase. This is supported by the finding of Cohen et al. that among other proteins tested rabbit muscle pyruvate kinase is not phosphorylated by cAMP-dependent protein kinase (24).

The cyanogen bromide fragment material from pig liver pyruvate kinase was phosphorylated much more rapidly than the active enzyme. The identical phosphopeptide patterns from ^{32}P-labelled native enzyme and cyanogen bromide fragment material strongly indicate that the same unique serine residue is phosphorylated in both cases. This is further supported by the extent of the phosphorylation of the material eluted at the same position in the Sephadex G-25 chromatogram as was the (^{32}P)phosphopeptide from the ^{32}P-labelled enzyme. Moreover, only (^{32}P)phosphorylserine was obtained on acid hydrolysis of the fragment. The results obtained are compatible with the view that only one cyanogen bromide fragment was phosphorylated in the protein kinase reaction.

The molecular weight of the fragment was low, since the fragment was clearly retarded on chromatography on Sephadex G-50. It therefore seems reasonable to assume that a small part of the pyruvate kinase polypeptide chains contains information sufficient to establish the structure required for phosphorylation. This may be a more general mechanism. The fact that similar amino acid sequences are found in several substrates of cAMP-dependent protein kinases supports this view. In the enzymes that are phosphorylated this serine is preceded by at least one basic amino acid residue at a distance of two to four amino acid residues, and is surrounded by neutral ones (24,25,26). This is also the case for histone F 1 (3) and troponin I (27), although serine residues in somewhat different sequences have been found to be phosphorylated in other histones (28) and in basic myelin protein (6).

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