THE MINIMUM SUBSTRATE OF CYCLIC AMP-STIMULATED PROTEIN KINASE, AS STUDIED BY SYNTHETIC PEPTIDES REPRESENTING THE PHOSPHORYLATABLE SITE OF PYRUVATE KINASE (TYPE L) OF RAT LIVER

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SUMMARY

Synthetic peptides, representing part of the phosphorylatable site of rat liver pyruvate kinase, were phosphorylated by (^{32}P)ATP and the catalytic subunit of cyclic AMP-stimulated protein kinase. The shortest peptide which could be significantly phosphorylated was Arg-Arg-Ala-Ser-Val, with an apparent K_m of 0.08 mM. The apparent K_m for Arg-Arg-Ala-Ser-Val-Ala was 0.02 mM and that for Leu-Arg-Arg-Ala-Ser-Val-Ala was less than 0.01 mM. Peptides in which threonine was substituted for serine, or leucine for the one or the other arginine of the pentapeptide were not detectably phosphorylated. Substitution of phenylalanine for valine increased, and substitution of lysine or glycine for valine considerably decreased the rate of phosphorylation.

INTRODUCTION

Cyclic AMP-stimulated protein kinase phosphorylates a number of intracellular proteins (1), including liver pyruvate kinases (type L) of rat and pig (2-3). The functional and structural diversity of the proteins which can be phosphorylated raises the question of a possible common denominator, such as an area of shared sequence, which makes them all substrates of cyclic AMP-stimulated protein kinase. Recently, Humble et al. showed that both alkali-inactivated pig liver pyruvate kinase (type L) and a cyanogen bromide fragment from the same enzyme could be phosphorylated by (^{32}P)ATP and cyclic AMP-stimulated protein kinase. In both cases the rate of phosphorylation was higher than with the native enzyme. Thus a small part of the pyruvate kinase polypeptide chains fulfills the structural requirements

for phosphorylation (4). A similar conclusion was drawn by Daile and Carnegie, who showed that peptides from enzymatic digests of the basic protein of human myelin were substrates of cyclic AMP-stimulated protein kinase (5). By studying genetic variants of β casein, Kemp et al. obtained further evidence supporting the importance of the primary structure in the region of the phosphorylatable seryl residue in this reaction (6). In the latter report, reference was also given to an unpublished observation that a synthetic hexapeptide, corresponding to the region around Ser-24 in chicken lysozyme, is a substrate of the protein kinase. Daile et al. have recently shown that a synthetic octapeptide, corresponding to amino acid residues 106-113 in the basic protein of human myelin, is phosphorylated by cyclic AMP-stimulated protein kinase (7).

In this laboratory, a peptic phosphopeptide containing 20 residues was recently isolated from rat liver pyruvate kinase phosphorylated by cyclic AMP-stimulated protein kinase. The amino acid sequence of the peptide was determined (8) and was the basis for the present work, the aim of which was to investigate the minimum structural requirements for the phosphorylation of the pyruvate kinase.

MATERIALS AND METHODS

(γ-^{32}P)ATP was prepared according to the method of Engström (9), and purified as described by Mårdh (10). It was mixed with unlabelled ATP to give a specific radioactivity of 30-50 cpm/pmol. In some experiments the specific radioactivity was 300 cpm/pmol, as indicated in Table 1.

Enzyme preparations. Cyclic AMP-stimulated protein kinase of rat liver was purified as described before through the hydroxyapatite step (11). The catalytic subunit was then isolated essentially as outlined by Kumon et al. (12), by adding cyclic AMP to the final concentration of 4 μM, and chromatographing the sample on a similar column of hydroxyapatite as used in the previous step (11), this time, however, equilibrated and eluted with buffers containing 4 μM cyclic AMP. The protein kinase activity now appeared at a higher concentration of potassium phosphate (i.e. about 150 mM). The active fractions were pooled and stored at -18°C. The activity was assayed essentially as described by Corbin and Reimann (13). One unit of protein kinase catalyzed the incorporation of 1 pmol of (^{32}P)phosphate per min into mixed histones at 5 mg/ml and 0.10 mM (^{32}P)ATP.

The catalytic subunit of the cyclic AMP-stimulated protein kinase of rabbit skeletal muscle was prepared from peak I through the CM-Sephadex step of method B, as described by Beavo et al. (14). Pyruvate kinase of rat liver was purified as described by Titanji et al. (11).

Synthesis and purification of peptides. Peptides were synthesized by the solid phase method as described by Merrifield (15-16) with the use of a Beckman Model 990 Peptide Synthesizer. All coupling steps were performed twice. Products were stripped from the resin using liquid HF, evaporated, extracted with 10% acetic acid and carefully lyophilized.

102 mg of the raw heptapeptide Leu-Arg-Arg-Ala-Ser-Val-Ala were subjected to chromatography on a carboxymethyl cellulose (Whatman CM 32) column (1.4 cm x 16 cm), equilibrated with ammonium acetate, pH 5.5, 0.025 M in ammonium ions. Elution was performed with a 600 ml, linear gradient of ammonium acetate pH 5.5, 0.025 M to 0.25 M, at a flow rate of 21 ml/h. The peptide, which appeared between 285 and 324 ml, was traced by the absorbance at 235 nm, lyophilized, dissolved in water and lyophilized once more to give 47 mg of a crystalline material. This preparation, labelled M 57, contained insignificant amounts of ammonium ions as seen from the amino acid analysis.

Amino acid analysis. The raw peptide preparations as well as the purified heptapeptide were analysed with respect to their amino acid content, after hydrolysis in sealed tubes for 24 h at 110°C in 6 M HCl, containing 1% phenol. The analyses were performed on an updated Beckman Spinco Model MS Amino Acid Analyzer. The values for serine and threonine were corrected for a loss of 11% and 5%, respectively. The experimental data for each amino acid were within 3% of the theoretical values, unless otherwise indicated. The peptide content of the different preparations was about 70%.

Phosphorylation experiments. The phosphorylation was performed at 30°C and pH 7.3 in a mixture containing synthetic peptide and (^{32}P)ATP at the concentrations indicated, 1 µM cyclic AMP, 5 mM magnesium acetate, 40 mM potassium phosphate, 7.5% (w/v) glycerol, 0.1 mM dithiothreitol and 4 units of protein kinase. Total reaction volume was 0.1 ml. The reaction was interrupted by the addition of 0.025 ml of 250 mM HCl. 0.025 ml of each mixture was subjected to high voltage electrophoresis in sodium citrate - citric acid buffer, pH 3.4 and 0.05 M with respect to total citrate. The electrophoresis was performed on Whatman 3 MM paper at 20 V/cm. (^{32}P)ATP and (^{32}P)phosphopeptides were localized by autoradiography on Kodak X-ray film for about 24 h, and the radioactive spots were cut out and counted in a liquid scintillation counter. In some experiments, rat liver pyruvate kinase was added instead of peptide, and the incorporation of (^{32}P)phosphate was measured by the method of Corbin and Reimann (13).

RESULTS AND DISCUSSION

Phosphorylatable peptides and effects of substitutions. Fig. 1 shows that Arg-Arg-Ala-Ser-Val (labelled "active pentapeptide") was the shortest peptide that permitted an appreciable rate of phosphorylation by the catalytic subunit of a cyclic AMP-stimulated protein kinase from rat liver. An identical pattern (data not shown) was obtained with the catalytic subunit of a cyclic AMP-stimulated protein kinase prepared from rabbit skeletal muscle. The rate with the active pentapeptide was about 40-50% of that for the hexapeptide Arg-Arg-Ala-Ser-Val-Ala and the heptapeptide Leu-Arg-Arg-Ala-Ser-Val--Ala (Table 1). Under the conditions used, the peptide Arg-Ala-Ser-Val-Ala

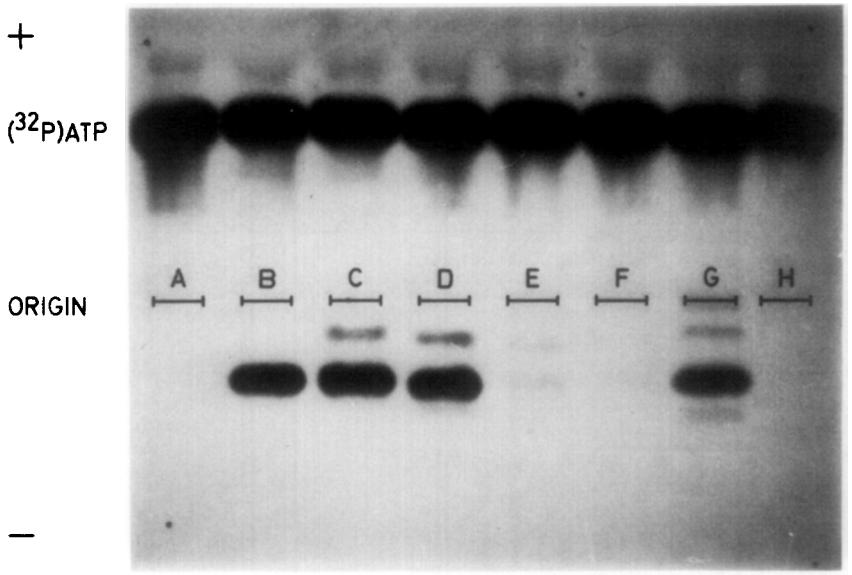

Figure 1. Autoradiography of an electropherogram of synthetic peptides phosphorylated by (^{32}P)ATP and the catalytic subunit of a rat liver cyclic AMP-stimulated protein kinase. The synthetic peptides represented the amino acid sequence around the phosphorylatable seryl residue of rat liver pyruvate kinase. The concentration of peptide and (^{32}P)ATP was 0.10 mM and the incubations were performed for 5 min, as described in the text. The structure of the peptides and the peptide number are shown in Table 1. A, no peptides. B, peptide M 57. C, peptide M 57 R. D, peptide M 66 R. E, peptide M 67 R. F, peptide M 68 R. G, peptide M 69 R. H, peptide M 70 R.

was phosphorylated at a rate 50 times lower than that of the active pentapeptide. No phosphorylation of Ala-Ser-Val-Ala was detected. Peptides having a leucyl residue substituted for either arginyl residue of the active pentapeptide were not detectably phosphorylated. This may suggest that the presence of both arginyl residues is important for high rate phosphorylation. However, additional analogues are required to investigate this possibility. The presence of an amino acid residue linked to the carboxyl end of the phosphorylatable seryl residue was essential, since Arg-Arg-Ala-Ser was not significantly phosphorylated. In addition, the results suggest that the preferred side chain of this residue is hydrophobic, since substitution of phenylalanine for valine increased the rate of phosphorylation by 38%, while substitution of glycine or lysine for valine decreased the rate by 93% and

Table 1. Sequence of synthetic peptides and relative rate of their phosphorylation by (^{32}P)ATP plus the catalytic subunit of a cyclic AMP-stimulated protein kinase from rat liver.

Peptide number	Amino acid sequence	Rate of phosphorylation (% of rate with M 69 R)
M 57	Leu-Arg-Arg-Ala-Ser-Val-Ala	251
M 57 R	Leu-Arg-Arg-Ala-Ser-Val-Ala	207
M 66 R	Arg-Arg-Ala-Ser-Val-Ala	193
M 67 R	Arg-Ala-Ser-Val-Ala	2
M 68 R	Ala-Ser-Val-Ala	< 1
M 69 R	Arg-Arg-Ala-Ser-Val	100
M 70 R	Arg-Arg-Ala-Ser	< 1
M 72 R	Leu-Arg-Ala-Ser-Val	< 1
M 73 R	Arg-Leu-Ala-Ser-Val	< 1
M 75 R	Arg-Arg-Ala-Ser-Gly	7
M 76 R	Arg-Arg-Ala-Ser-Phe	138
M 77 R	Arg-Arg-Ala-Ser-Lys	17
M 79 R	Arg-Arg-Ala-Thr-Val	< 1

The incubations were performed for 5 min as described in the text, at 0.10 mM peptide and 0.10 mM (^{32}P)ATP. Peptides M 67 R through M 73 R, and M 79 R, were also incubated at a specific radioactivity of (^{32}P)ATP of 300 cpm/pmol. ("R" stands for raw peptide). The rates of phosphorylation were estimated after isolation of the (^{32}P)phosphopeptides by electrophoresis and autoradiography. Only the main (^{32}P)phosphopeptide of each experiment was measured. The value for peptide M 67 R refers to the least positively charged of two equally ^{32}P-labelled compounds (c.f. Fig. 1, sample E). Serine content of peptide M 57 was 0.96 mol per mol of peptide. Arginine and glycine contents of M 75 R were 1.92 and 1.05 mol per mol of peptide, respectively.

83%, respectively. It is also shown that the threonyl residue was not significantly phosphorylated when inserted into the pentapeptide instead of the seryl residue.

The influence of contaminating material of the raw peptide preparations. Each peptide was investigated by use of the unpurified preparation. In order to study the influence of contaminating material, the raw heptapeptide was

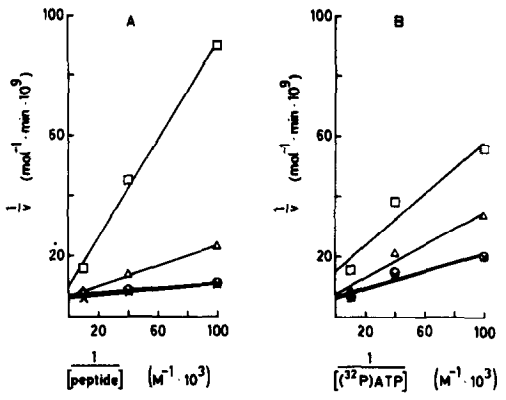

Figure 2. Lineweaver-Burk plots of data from phosphorylation experiments with synthetic peptides. Incubations were performed for 5 min, and the rate of phosphorylation was estimated after isolation of the (^{32}P)phosphopeptides by electrophoresis and autoradiography, as described in the text. Only the main (^{32}P)phosphopeptide of each experiment was measured. The ordinate refers to the phosphorylation in the incubation mixture (total volume 0.1 ml). Slopes and intercepts of the lines were calculated by the least squares method, while the points represent experimental data. x—x, peptide M 57. o—o, peptide M 57 R. Δ—Δ, peptide M 66 R. □—□, peptide M 69 R. A, variation of concentration of peptide at 0.10 mM (^{32}P)ATP. B, variation of concentration of (^{32}P)ATP at 0.10 mM peptide.

purified on carboxymethyl cellulose. As seen from Table 1 and Fig. 2A, the removal of contaminants did not appreciably increase the rate of formation of the main (^{32}P)phosphopeptide. It was concluded from this that the comparison of the different peptides could be performed using the raw products. The nature of the minor ^{32}P-labelled components of the phosphorylated raw peptides (Fig. 1) was not investigated. However, the radioactivity of this material represented only trace amounts. Each of the peptides having a rate of phosphorylation lower than that of peptide M 69 R were incubated together with the purified heptapeptide at the same concentration, i.e. 0.10 mM. In all cases the phosphorylation was similar to that obtained with the heptapeptide alone, indicating that the low rate of phosphorylation, as listed in Table 1, was a property of the peptide and not due to inhibition of the protein kinase.

Conditions of phosphorylation. It was found that the rate of phosphorylation of the peptides, as studied in the experiments of Fig. 2, was essentially linear with time up to 10 min of incubation (data not shown). Therefore, 5 min was chosen as the standard incubation time. From the data of Fig. 2A it is concluded that the rate of phosphorylation at 0.10 mM peptide was almost equal to the apparent V_{max} for the heptapeptide and hexapeptide, but about 60% of V_{max} for the pentapeptide. The V_{max} of the heptapeptide is one order of magnitude higher than the rate of phosphorylation of mixed histones at similar conditions and a concentration of 5 mg/ml. The data of Fig. 2B are interpreted as showing that 0.10 mM (^{32}P)ATP represents almost saturating conditions at 0.10 mM peptide. It is concluded that 0.10 mM peptide and 0.10 mM (^{32}P)ATP represent suitable substrate concentrations to allow a meaningful comparison between the different peptides.

Apparent K_m-values for the phosphorylatable peptides. As can be estimated from Fig. 2A, the apparent K_m for the pentapeptide was 0.08 mM, that for the hexapeptide 0.02 mM, and that for the heptapeptide less than 0.01 mM. It is interesting to notice that the K_m-value of the heptapeptide approaches the concentration of pyruvate kinase subunits in rat liver, which is about 0.001 mM in normally fed rats and about 0.004 mM in rats given carbohydrate-rich food (11). When native pyruvate kinase was phosphorylated at a calculated subunit concentration of 0.01 mM, the rate was 0.01 nmol/min, i.e. similar to the rate for the active pentapeptide at the same concentration, but lower than that for the hexa- and heptapeptides. This is comparable to a previous report (4).

Conclusions. Apparently, the minimum structural requirements for a significant rate of phosphorylation of rat liver pyruvate kinase are fulfilled by the pentapeptide Arg-Arg-Ala-Ser-Val. Together with previous evidence from this and other laboratories (4-7) the present results also suggest a method to investigate in detail the rules for the substrate specificity of the cyclic AMP-stimulated protein kinase.

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