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AMINO ACID SEQUENCE AT THE PHOSPHORYLATED SITE OF RAT LIVER FRUCTOSE-1,6-DIPHOSPHATASE AND PHOSPHORYLATION OF A CORRESPONDING SYNTHETIC PEPTIDE

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SUMMARY

Rat liver fructose-1,6-diphosphatase was phosphorylated with (^{32}P)ATP and the catalytic subunit of cyclic AMP-dependent protein kinase from pig muscle. After digestion with pepsin, α-chymotrypsin and subtilisin a peptide with the amino-terminal sequence Ser-Arg-Tyr-(^{32}P)SerP-Leu-Pro-Leu-Pro was isolated. A synthetic unphosphorylated heptapeptide with the same amino acid sequence, ending with leucine, was phosphorylated with an apparent K_m of 400 μM, while the apparent K_m value for fructose-1,6-diphosphatase was 30 μM (subunit concentration). The V_{max} value was 20 times higher for the peptide than for the enzyme.

INTRODUCTION

The activity of rat liver fructose-1,6-diphosphatase (D-fructose-1,6-diphosphatase 1-phosphohydrolase, EC 3.1.3.11) is increased on intravenous injection of glucagon (which is associated with an elevation of the concentration of cyclic AMP) (1). The enzyme is phosphorylated and activated in vitro by the catalytic subunit of cyclic AMP-dependent protein kinase (2). Furthermore, ^{32}P-labelled enzyme can be isolated from rats injected with (^{32}P)phosphate (2). Thus,the activity of rat liver fructose-1,6-diphosphatase may be at least partially controlled by a phosphorylation-dephosphorylation mechanism.

The amino acid sequence around the phosphorylated site has been elucidated for a number of phosphorylatable proteins and some common features have been found. So far, identified protein substrates for cyclic AMP-dependent protein kinases are in general phosphorylated on a seryl residue separated on the amino-terminal side by one (or

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two) amino acid(s) from (one or) two basic residues. The structural requirements for phosphorylation have been studied with small peptides as model substrates. K_m values in the micromolar range and phosphorylation rates comparable to those for the native protein have been obtained with peptides representing part of the phosphorylatable site of rat liver pyruvate kinase, including two arginyl residues (3,4), while peptides containing only one arginine in the above-mentioned position are usually poorer substrates (3-8).

In order to study rat liver fructose-1,6-diphosphatase in this respect ^{32}P-labelled enzyme was digested with proteolytic enzymes and a peptide containing the (^{32}P)phosphorylated serine was isolated and its amino acid sequence determined. Phosphorylation experiments were performed with purified native fructose-1,6-diphosphatase and with a synthetic unphosphorylated peptide representing the amino acid sequence around the phosphorylation site.

EXPERIMENTAL

Materials: (^{32}P)ATP was a product of New England Nuclear, Boston. Pepsin (type EPBK) was purchased from Boehringer Mannheim. α-chymotrypsin (type II) and subtilisin Carlsberg (type VIII) were from Sigma. Sephadex gels were obtained from Pharmacia, Uppsala.

Methods: Fructose-1,6-diphosphatase was prepared by the method of Riou et al. (2), with slight modifications, from the livers of male Sprague-Dawley rats fed ad libitum on ordinary laboratory chow. Calculations were based upon the assumptions that $A^{0.1\%}_{280}$ = 1.0 and upon a subunit molecular weight of 36,000 (estimated from polyacrylamide gel electrophoresis in sodium dodecyl sulphate (9)).

The catalytic subunit of cyclic AMP-stimulated protein kinase from pig muscle was purified essentially according to Bechtel et al. (10) from the DEAE-cellulose peak I through the CM-Sephadex step (although potassium phosphate buffer, pH 7.4, was used instead of the 2-(N-morpholino)ethanesulfonic acid). One unit of protein kinase catalyzed the incorporation of 1 pmol of (^{32}P)phosphate per min into mixed histones (7.5 mg/ml) from 0.1 mM (^{32}P)ATP at pH 6.9 and 30°C.

10 mg of fructose-1,6-diphosphatase (with a purity of about 80% as judged from polyacrylamide gel electrophoresis) were incubated with 500,000 units of the catalytic subunit of protein kinase and 0.5 mM (^{32}P)ATP in the presence of 11 mM magnesium acetate at pH 6.5 and 30°C. Excess (^{32}P)ATP was separated from the enzyme after 45 min by chromatography at 4°C on a Sephadex G-50 column equilibrated and eluted with 5 mM potassium phosphate buffer, pH 7.0. Radioactivity was measured as Cerenkov radiation (11). The radioactive fractions

corresponding to the phosphorylated fructose-1,6-diphosphatase were pooled (30 ml), acidified with HCl (50 mM final concentration) and digested for 2 h at 25°C with pepsin (0.5 mg/ml). The digestion was stopped by raising the pH to 7.0 with 1 M NaOH. α-chymotrypsin (0.5 mg/ml) was added, and after 2 h subtilisin (0.5 mg/ml) was also admixed. The digestion was continued for another 2 h, whereafter the incubation mixture (44 ml) was chromatographed at room temperature on a 5.4 x 19 cm Sephadex G-25 column, equilibrated and eluted with 10 mM pyridine-acetic acid buffer, pH 3.1. The emerging peptide material was further purified by chromatography on SP-Sephadex C-25 and QAE-Sephadex A-25 at room temperature as described in the legend of Fig.1. Sephadex G-25 chromatography on a 2.2 x 47 cm column in the same buffer as above completed the purification procedure.

For amino acid analysis the purified peptides (peaks I and II in Fig.1) were hydrolyzed for 24 h at 110°C in sealed ampoules containing 6 M HCl and 1% phenol. A single-column Durrum amino acid analyzer was used. The values for serine and threonine were corrected for losses during hydrolysis.

Amino acid sequence analysis was performed by a dansyl-Edman method as described by Hartley (12). In order to determine which serine residue was phosphorylated, peptide at different stages of Edman-degradation was submitted to high-voltage electrophoresis in 50 mM sodium citrate buffer, pH 4.75, on Whatman 3 MM paper at 30 V/cm for 4 h and the radioactive ^{32}P-label was localized by autoradiography. A peptide with an amino acid sequence corresponding to the phosphorylated site of fructose-1,6-diphosphatase was synthesized and its composition checked as described earlier (3).

Phosphorylation of the synthetic peptide was performed for 5 min at 30°C and pH 6.9. The incubation mixture contained 0.1 mM (^{32}P)ATP, 0.5 mM magnesium acetate, 0.25 mM EGTA, 0.25 mM dithiothreitol, 5 mM 2-(N-morpholino)ethanesulfonic acid buffer, 0.13 mg bovine serum albumin per ml, 400 units of the catalytic subunit of protein kinase and peptide at the concentrations indicated. The total reaction volume was 40 µl. The reaction was interrupted by the addition of 10 µl of 250 mM HCl. 25 µl of each mixture was subjected to high voltage electrophoresis as described above. The incorporation of phosphate was measured as in ref. 3. In some experiments rat liver fructose-1,6-diphosphatase was added instead of peptide. A 90% pure (judged from polyacrylamide gel electrophoresis in sodium dodecyl sulphate) enzyme preparation that could be phosphorylated to 0.9 mol of (^{32}P)phosphate per mol of subunit was used. The incubation mixture was the same as for the peptide, except for the additional presence of 1 mM sodium malonate, which lowered the pH value to 6.8. The reaction was interrupted by the addition of 2 ml 10% (w/v) trichloroacetic acid containing 50 mM H_3PO_4 and 1 mg of bovine serum albumin was added. The precipitate was collected by centrifugation and dissolved in 0.2 ml 0.5 M NaOH. It was washed four times by repeated precipitation and dissolution. After the last addition of NaOH the radioactivity was measured (11) and the phosphate incorporation calculated. K_m and V_{max} values were estimated from Lineweaver-Burk plots.

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate of subtilisin treated, ^{32}P-labelled fructose-1,6-diphosphatase was performed. 30 µg of the enzyme were mixed with 1.2 µg of subtilisin in a total volume of 50 µl. After 45 min at 25°C and pH 7.4 the incubation was interrupted by the addition of 10 µl 7.5 mM phenylmethylsulfonyl fluoride, followed by denaturation (60 min at 60°C) and application on the gels (9). Controls were run in parallel. After staining for protein (13), the gels were sliced and the radioactivity was measured. The positions of radioactivity and protein bands were compared.

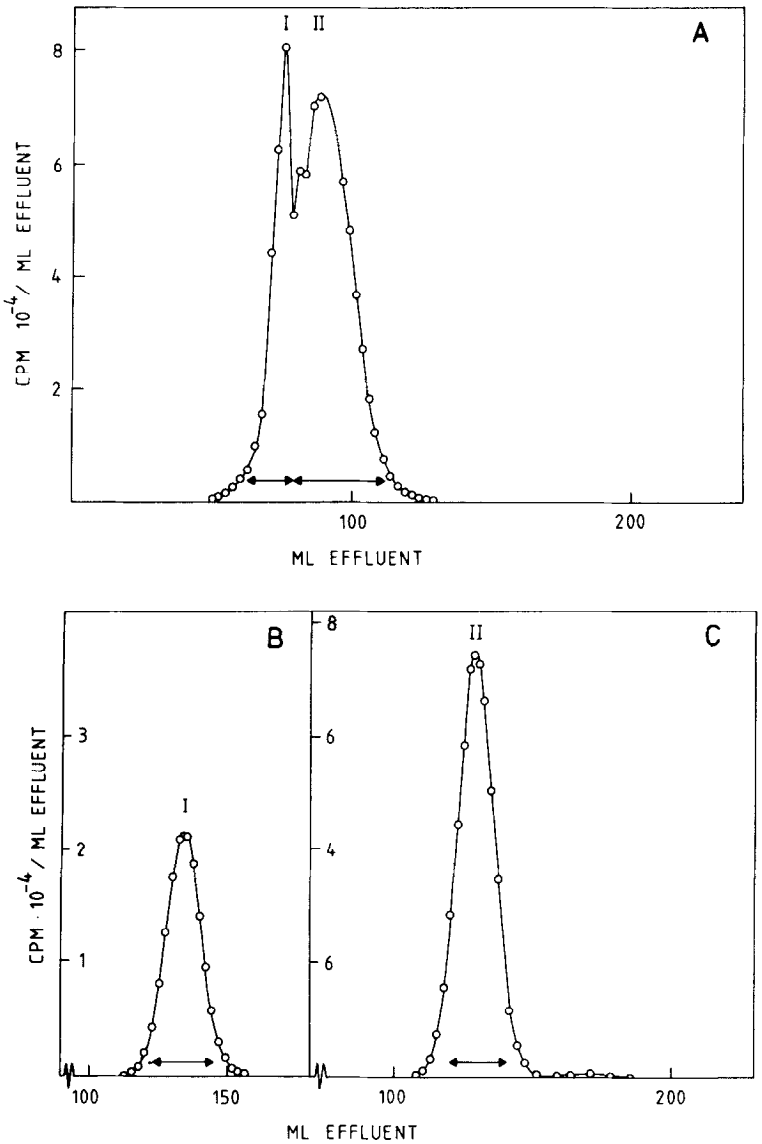

Fig. 1. Two successive chromatographies of ^{32}P-labelled peptide material from rat liver fructose-1,6-diphosphatase. The elution volumes indicated in the figure represent the effluent collected from the start of the gradients. The arrows indicate which material was pooled. o —— o = radioactivity.

A. Chromatography on a 0.9 x 30 cm SP-Sephadex C-25 column equilibrated with 10 mM pyridine-acetic acid buffer, pH 3.1. Elution was performed with one column volume of the same buffer, followed by a linear gradient (total volume 400 ml) formed from 10 mM and 150 mM pyridine-acetic acid buffer, pH 3.1.

B and C. Chromatography of peaks I and II, respectively, on a 0.9 x 30 cm QAE-Sephadex A-25 column equilibrated with 25 mM ammonium carbonate buffer, pH 8.8. Elution was performed with one column volume of the same buffer, followed by a linear gradient (total volume 400 ml) formed from 25 mM and 500 mM ammonium carbonate buffer, pH 8.8.

RESULTS AND DISCUSSION

The (^{32}P)phosphate incorporation into the fructose-1,6-diphosphatase was 0.36 mol per mol of subunit. The (^{32}P)phosphopeptide material emerged from the first (and second) Sephadex G-25 column as one symmetrical peak after 0.6 of the column volume. The pooled fractions corresponded to 98% of the radioactivity applied. After purification 5% of the initial (^{32}P)phosphopeptide material was recovered in peak I and 31% in peak II (Fig.1).

Table I shows the amino acid composition of these peptides, which we presume are derived from the same phosphorylation site. The peptide amount calculated from amino acid analysis data was about 50% greater (Table I) than that calculated from the (^{32}P)phosphate content, indicating that the enzyme contained some cold phosphate. This might have been derived from *in vivo* phosphorylation.

The total of peak I was consumed in the amino acid analysis. The remaining peak II material was sufficient in amount and purity for partial amino acid sequence analysis, where the amino-terminal

TABLE I

Amino acid composition of the purified peptides. 5.9 and 7.2 nmol of (^{32}P)phosphopeptide from peaks I and II, respectively, were submitted for amino acid analysis as described in Experimental.

	Peak I	Peak II		
Amino acid	Nmol analyzed	Nmol analyzed	Nmol analyzed / 10.5	Nearest integer
Aspartic acid	7.30	5.79	0.6	(1)
Threonine	15.09	19.43	1.9	2
Serine	42.21	31.36	3.0	3
Glutamic acid	13.89	12.61	1.2	1
Proline	45.41	41.89	4.0	4
Glycine	12.67	3.69	0.4	
Alanine	10.40	6.56	0.6	(1)
Valine	9.17	3.04	0.3	
Methionine	1.43	0.36	0.0	
Isoleucine	3.40	1.21	0.1	
Leucine	30.97	30.44	2.9	3
Tyrosine	8.38	10.29	1.0	1
Phenylalanine	1.06	-	-	
Histidine	0.77	-	-	
Lysine	0.58	-	-	
Arginine	11.06	11.45	1.1	1

part proved to be Ser-Arg-Tyr-Ser-Leu-Pro-Leu-Pro. This sequence was confirmed by analysis of a peptide isolated by the same procedure from another preparation of fructose-1,6-diphosphatase, phosphorylated to 0.65 mol of (^{32}P)phosphate per mol of subunit.The second seryl residue was identified by high voltage electrophoresis as the phosphate acceptor, since the ^{32}P-label left the peptide and migrated with orthophosphate when this residue was cleaved off.

When proteolytically modified, rat liver fructose-1,6-diphosphatase is converted to a more alkaline form with a lower sensitivity to inhibition by AMP (14). A corresponding conversion of rabbit liver fructose-1,6-diphosphatase is associated with splitting off of the amino-terminal 60 amino acids (as a peptide) (15). The amino acid sequence around the phosphorylated site of rat liver fructose-1,6-diphosphatase cannot be fitted into this rabbit liver enzyme peptide (16). Furthermore, subtilisin treatment of the rat liver enzyme reduced the subunit molecular weight by 25%, but the radioactive ^{32}P-labelling still coincided with the protein band on polyacrylamide gels. Thus, this enzyme is probably not phosphorylated in the region that is most readily attacked by proteolytic enzymes, which raises the possibility of two regulatory mechanisms - proteolytic modification and phosphorylation - for the enzyme in vivo.

The heptapeptide, Ser-Arg-Tyr-Ser-Leu-Pro-Leu, was synthesized for phosphorylation experiments. The peptide content of the preparation was 70% and the relative amounts of the amino acid residues were: Ser = 2.0, Arg = 1.0, Tyr = 0.8, Leu = 2.0 and Pro = 1.2.

Representative Lineweaver-Burk plots of data from phosphorylation experiments with the synthetic peptide and fructose-1,6-diphosphatase are shown in Fig.2A and B, respectively. The K_m and V_{max} values obtained with 400 units of protein kinase per test were 400 μM

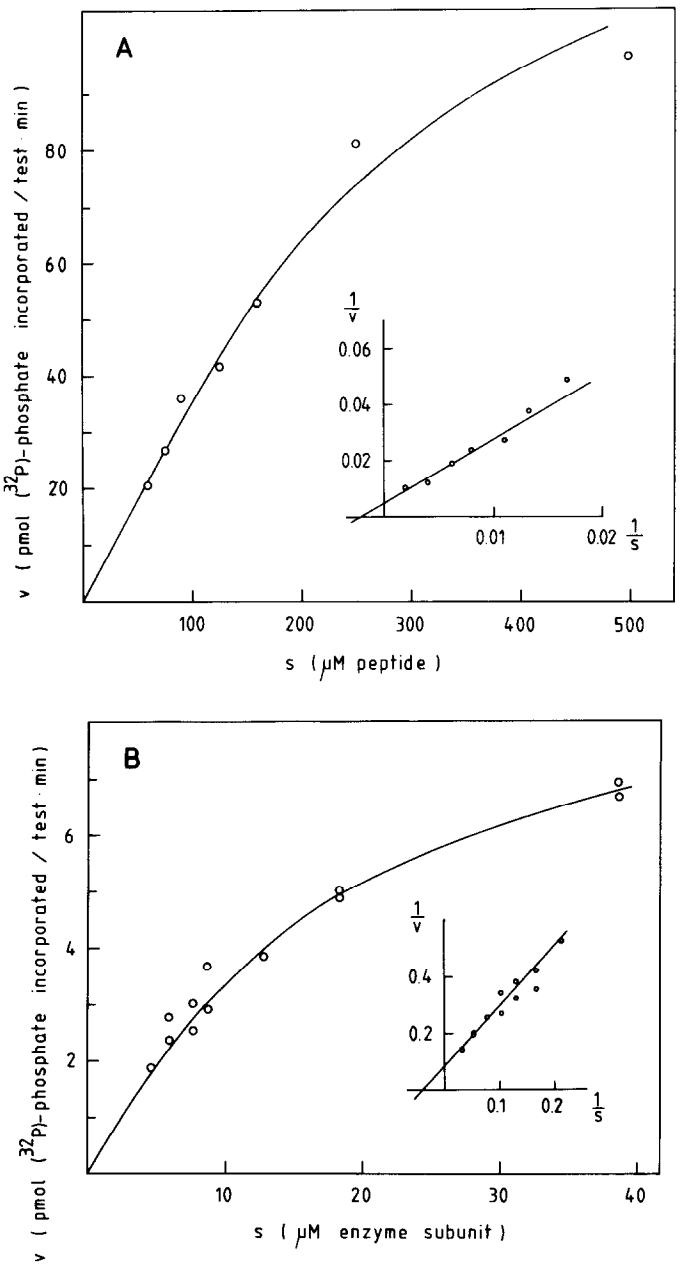

Fig. 2. Phosphorylation experiments as described in the text with A) synthetic peptide and B) fructose-1,6-diphosphatase. The ordinate represents the phosphate incorporation into the incubation mixture (total volume 40 μl). Lineweaver-Burk plots are inserted.

and 200 pmol (^{32}P)phosphate incorporated per min for the peptide and 30 μM and 10 pmol (^{32}P)phosphate incorporated per min for fructose-1,6-diphosphatase.

CONCLUSIONS

Rat liver fructose-1,6-diphosphatase was phosphorylated *in vitro* by the catalytic subunit of cyclic AMP-dependent protein kinase to a maximum of approximately one mol of phosphate per mol of subunit. In some preparations the phosphate incorporation was lower (0.3 - 0.4 mol of phosphate per mol of subunit), probably illustrating the need for rapid handling through the purification steps.

The amino acid sequence at the phosphorylated site of the enzyme contained one arginyl residue separated by one amino acid from the phosphoserine. A synthetic heptapeptide with this amino acid sequence was phosphorylated with a K_m value for the peptide of about 400 μM, in analogy with the relatively high K_m values obtained in phosphorylation experiments with other peptides containing only one arginyl residue in position Arg-X-Ser(P) or Arg-X-X-Ser(P) (4-8). Fructose-1,6-diphosphatase was, however, phosphorylated with a 10 times lower K_m value, indicating that the enzyme molecule contains further information for the protein kinase.

During the completion of this work Pilkis *et al.* (17) reported the partial amino acid sequence of a tryptic peptide obtained from ^{32}P-labelled rat liver fructose-1,6-diphosphatase. Their sequence was Ser-Arg-Pro-Ser(P)-Leu-Pro-Leu-Pro, thus differing from ours in the third position from the amino-terminal end. The reason for this discrepancy is not known.

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