# ACTIVITY OF RAT-LIVER PHOSPHOPROTEIN PHOSPHATASE ON PHOSPHOPEPTIDES FORMED IN THE CYCLIC AMP-DEPENDENT PROTEIN KINASE REACTION

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## 1. Introduction

The modification of protein function by phosphorylation and dephosphorylation reactions is now recognized as an important regulatory mechanism of intracellular processes [1]. In this laboratory attention has been paid to the phosphorylation and concomitant inactivation of liver pyruvate kinase (EC 2.7.1.40) by cyclic AMP-dependent protein kinase (EC 2.7.1.37) and MgATP [2], which is reversed by the action of a phosphoprotein phosphatase (EC 3.1.3.16) [3]. Apparently the native structure of pyruvate kinase is not required for its phosphorylation in vitro [4]. A pentapeptide, corresponding to the phosphorylated site of pyruvate kinase [5] has been shown to be phosphorylated [6].

Graves et al. showed that skeletal muscle phosphorylase *a* phosphatase (EC 3.1.3.17) could dephosphorylate specific phosphopeptides derived from phosphorylase *a* (EC 2.4.1.1) [7]. However, liver phosphoprotein phosphatase, as prepared by Meisler and Langan [8], failed to dephosphorylate tryptic phosphopeptides isolated from phosphoprotamine and phosphohistones. In this report the phosphopeptide Gly–Val–Leu–Arg–Arg–Ala–Ser(P)–Val–Ala, which includes the site phosphorylated on pyruvate kinase, type L, was found to be a substrate of rat-liver phosphoprotein phosphatase. Omitting the first four amino acids at the N-terminus of the phosphopeptide did not alter the rate of dephosphorylation significantly, whereas the elimination of the C-terminal alanine virtually abolished susceptibility to the phosphatase. The results suggest a requirement by the phosphatase of at least two amino acid residues C-terminal to the phosphorylserine of the phosphopeptide substrate.

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## 2. Experimental

Carboxymethylcellulose (CM-52) was purchased from Whatman. Dowex-50 ($H^+$) was from Sigma. Other analytical grade reagents were purchased from Merck and Sigma. [$\gamma$-$^{32}$P]ATP was prepared according to Mårdh's modification of the method of Engström [9,10]. Rat-liver phosphoprotein phosphatase and phosphorylated pyruvate kinase were prepared according to methods described previously [3,11]. The catalytic subunit of the cyclic AMP-dependent protein kinase of rabbit muscle was prepared from peak I through the CM–Sephadex step of method B, as described by Beavo et al. [12].

### 2.1. *Preparation of [$^{32}$P]phosphopeptides*

Peptides were synthesized and purified as described by Zetterqvist et al. [6]. The phosphorylation mixture (4.1 ml) usually contained 1 $\mu$mol peptide, 0.9 $\mu$mol [$\gamma$-$^{32}$P]ATP (100 cpm · pmol$^{-1}$), 40 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.7 (NaOH), 15 mM $\beta$-mercaptoethanol and 0.05 mg of the cata-



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lytic subunit of rabbit muscle cAMP-dependent protein kinase. The mixture was incubated at 30°C for 1 h during which 70–90% of the peptide became phosphorylated. The reaction was terminated by boiling the incubation mixture for 3 min. The mixture was then diluted 3-fold with ice-cold ammonium acetate buffer, pH 5.5, 0.025 M with respect to added acetic acid, and applied to a CM-52 column (1.4 × 18 cm), equilibrated with the dilution buffer. The [$^{32}$P]phosphopeptide was eluted at 4°C at a flow rate of 15 ml/h using a linear gradient, (100 ml + 100 ml) of 0.025 M–0.25 M ammonium acetate buffer, pH 5.5. Fractions 5.0–5.5 ml were collected. The pooled [$^{32}$P]phosphopeptide was freeze dried and the residue dissolved in 25 ml water and lyophilized again. This process was repeated twice. The final residue was dissolved in 0.5 ml water; the concentration of the phosphopeptide was determined by comparison with the specific activity of the [$\gamma$-$^{32}$P]ATP used during synthesis of the phosphopeptide. Analysis of the purified phosphopeptide by high-voltage paper electrophoresis followed by autoradiography [6] revealed one main spot containing not less than 98% of the radioactivity. The [$^{32}$P] phosphopeptides were eluted from the CM-52 column, well separated from residual [$\gamma$-$^{32}$P]ATP, [$^{32}$P]orthophosphate and dephosphopeptides. The [$^{32}$P]phosphopeptides are enumerated in table 1 and will be referred to by Roman numerals assigned to them.

Phosphopeptides, corresponding to the phosphorylation site of pyruvate kinase, but lacking either arginine could not be synthesized by protein kinase [6]. Therefore, tryptic phosphopeptides were prepared as follows: after terminating the phosphorylation of 1 $\mu$mol [$^{32}$P]phosphopeptide III, the reaction medium was adjusted to pH 7.8 with 1 M $NH_4HCO_3$. 40 $\mu$l of trypsin (16 $\mu$g) in 1 mM HCl were then added and the mixture incubated at 30°C for 15 min. The incubation was interrupted by boiling the mixture for 3 min. The tryptic digest was then chromatographed on a CM-52 column as described above, except that ammonium acetate buffer, pH 3.5, was used. A main [$^{32}$P] phosphopeptide was eluted at a buffer concentration, 0.05 M with respect to added acetic acid. Amino acid analysis of 23.5 nmol freeze dried [$^{32}$P]phosphopeptide gave the following results: arginine 23.5 nmol, serine 25.5 nmol, alanine 58.7 nmol and valine 31.4 nmol. From these data and the sequence of phosphopeptide III (table 1) the structure of the tryptic [$^{32}$P]phosphopeptide was concluded to be Arg–Ala–Ser(P)–Val–Ala.

Amino acid analysis of the tryptic [$^{32}$P]phosphopeptide was kindly performed by Dr Sjödahl accord-

Table 1
[$^{32}$P]Phosphopeptide substrates of rat-liver phosphoprotein phosphatase

| No. | Sequence | Apparent $K_m$ (mM) | Relative-$V_{max}$ |
|---|---|---|---|
| I | Gly–Val–Leu–Arg–Arg–Ala–Ser(P)–Val–Ala | 0.24 (3) | 1.0 |
| II | Val–Leu–Arg–Arg–Ala–Ser(P)–Val–Ala | 0.31 (2) | 1.0 |
| III | Leu–Arg–Arg–Ala–Ser(P)–Val–Ala | 0.57 (3) | 1.0 |
| IV | Arg–Arg–Ala–Ser(P)–Val–Alg | 0.28 (3) | 1.0 |
| V | Leu–Arg–Arg–Ala–Ser(P)–Val–Arg | 0.23 (3) | 1.0 |
| VI | Arg–Arg–Ala–Ser(P)–Val | – | – |
| VII | Leu–Arg–Arg–Ala–Ser(P)–Val | – | – |
| VIII | Ala–Arg–Thr–Lys–Arg–Ser–Gly–Ser(P)–Val | – | – |
| IX | Ala–Arg–Thr–Lys–Arg–Ser–Gly–Ser(P)–Val–Tyr | 0.40 (2) | 2.5 |
| X | Arg–Ala–Ser(P)–Val–Ala | 0.41 (2) | 1.0 |

The [$^{32}$P]phosphopeptides were prepared as described under Experimental. Phosphopeptides I–IV, VI–VII, and X correspond to the site phosphorylated on rat-liver pyruvate kinase [5]. Phosphopeptide V is based on the same sequence but has a carboxyl-terminal arginine. Phosphopeptides VIII and IX are based on the structure on the site phosphorylated on the $\beta$-subunit of phosphorylase *b* kinase [15]. The reactions were run in duplicate and the mean rates were used to calculate the slopes and intercepts of the Lineweaver-Burk diagrams by the method of least squares. Mean $K_m$-values are given; the figures in brackets indicate the number of determinations. The rates of dephosphorylation of these phosphopeptides were negligible, i.e., less than 2% of that of phosphopeptide I.

ing to the method of Spackman et al. [13] on a Beckman 121 M analyzer. The sample was hydrolyzed in 6 M HCl (1% phenol, w/v) at 110°C for 24 h. High-voltage electrophoresis was carried out as described earlier [6].

2.2. *Enzyme assay*

The dephosphorylation of the [$^{32}$P]phosphopeptides was followed in a reaction mixture (20 μl or 40 μl) containing 50 mM Tris/HCl, pH 7.5, 2.5 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mg bovine serum albumin/ml, 0.04–0.06 unit of protein phosphatase [11] and phosphopeptide at the concentrations specified in the legends of the figures. The incubation was initiated by addition of the phosphoprotein phosphatase, continued at 30°C for 5 min and terminated by the addition of 40 μl 0.2 M pyridine-acetate containing 10 mM $KH_2PO_4$, pH 3.1. The method of Nolan et al. [14] was used to determine the [$^{32}$P]orthophosphate released. Aliquots of the reaction mixture containing not more than 5 nmol phosphopeptide were applied to the Dowex-50 column. The [$^{32}$P]orthophosphate released was eluted with 4 × 1 ml of the buffer into polyethylene vials and the Cerenkov radiation determined in an Intertechnique Liquid Scintillation Spectrometer. A comparable amount of [$^{32}$P]orthophosphate could be extracted as [$^{32}$P]phosphomolybdate [16]. Furthermore, [$^{32}$P]orthophosphate could be demonstrated by high-voltage paper electrophoresis and autoradiography, indicating that the reaction products were orthophosphate and dephosphopeptides.

Blank incubations in the absence of phosphoprotein phosphatase were performed in parallel at all substrate concentrations tested. The blank values obtained were less than 1% of the total radioactivity present in the incubation mixture. The dephosphorylation of $^{32}$P-labelled proteins was carried out as described previously [3,11]. One unit of phosphoprotein phosphatase was the amount of enzyme which released 1 nmol [$^{32}$P]orthophosphate/min when tested under the conditions of the standard assay in the presence of 60 μM [$^{32}$P]phosphoprotamine.

## 3. Results and discussion

3.1. *Dephosphorylation of [$^{32}$P]phosphopeptides*

Experiments were performed at 20 μM [$^{32}$P]phosphopeptide which is close to the concentration of pyruvate kinase in the livers of rats given food rich in carbohydrate [3]. Upon incubation with phosphoprotein phosphatase, [$^{32}$P]orthophosphate was released in a time-dependent manner from [$^{32}$P]phosphopeptides I–V (fig.1) and [$^{32}$P]phosphopeptide X (results not illustrated). The rate of dephosphorylation of [$^{32}$P]phosphopeptides VI–VII was less than 2% of the rate of dephosphorylation of [$^{32}$P]phosphopeptide I. Thus, omitting four amino acid residues from the amino-terminus of [$^{32}$P]phosphopeptide I did not appreciably reduce the rate of dephosphorylation. However, the elimination of C-terminal alanine decreased the rate of dephosphorylation drastically. Substitution of arginine for alanine did not change the rate of dephosphorylation appreciably (fig.2B).

The role of C-terminal amino acid was further studied by using [$^{32}$P]phosphopeptides, corresponding

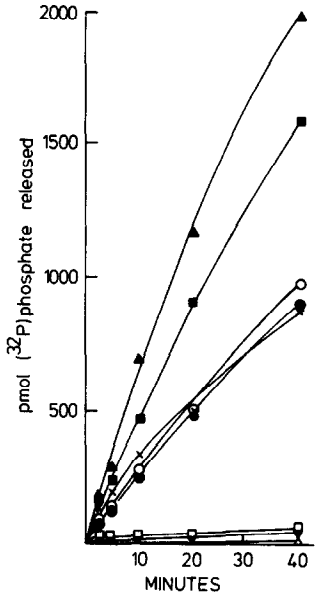


Fig.1. Dephosphorylation of [$^{32}$P]phosphopeptides by a rat liver phosphoprotein phosphatase. The reaction medium contained 20 μM [$^{32}$P]phosphopeptide and 0.23 unit protein phosphatase in final vol. 200 μl. At the times indicated 25 μl of the mixture were added to 50 μl 0.2 M pyridine-acetate buffer, containing $KH_2PO_4$, pH 3.1, and the [$^{32}$P]orthophosphate was determined. Other additions were described under Experimental. (▲-▲) I, (■-■) II, (○-○) III, (●-●) IV, (×-×) V, (△-△) VI, (▼-▼) VII, (□-□) VIII. The Roman numerals refer to the [$^{32}$P]phosphopeptides listed on table 1.

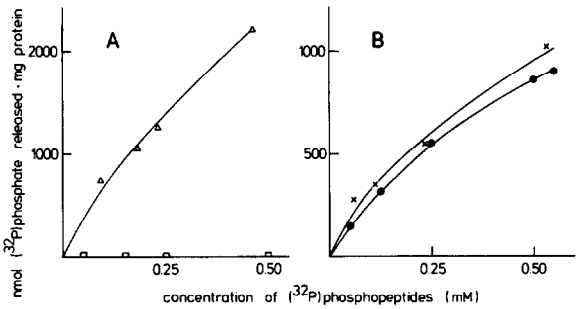


Fig.2. Effect of carboxyl-terminal amino acids of [$^{32}$P]phosphopeptides on the phosphatase reaction. The standard incubation medium was used. (A) (□-□) VIII, (△-△) IX. (B) (●-●) IV, (X-X) V. The Roman numerals are explained in table 1.

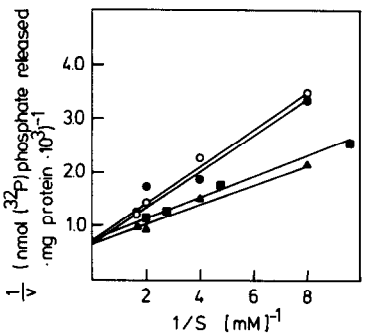


Fig.3. Dependence of the phosphatase reaction on the concentration of [$^{32}$P]phosphopeptides. (▲-▲) I, (■-■) II, (○-○) III, (●-●) IV. The Roman numerals refer to the [$^{32}$P]phosphopeptides listed in table 1.

to the site phosphorylated on the β-subunit of phosphorylase *b* kinase (EC 2.7.1.38) [15]. Phosphopeptide IX was susceptible to phosphoprotein phosphatase, but became resistant to the enzyme in the absence of the C-terminal tyrosine (fig.1 and fig.2A). Assuming that the phosphorylserine of phosphopeptides VIII and IX is identical to that phosphorylated in vivo [15], then these data and those above indicate a requirement by the phosphatase for at least two amino acid residues C-terminal to the phosphorylserine.

### 3.2. *Dependence of dephosphorylation rate on phosphopeptide concentration and ligands*

The dependence of the phosphoprotein phosphatase reaction on the concentrations of [$^{32}$P]phosphopeptides, representing the phosphorylation site of rat liver pyruvate kinase, is shown in fig.3. The apparent $K_m$ of [$^{32}$P]phosphopeptides I–IV and the tryptic [$^{32}$P]phosphopeptide ranged from 0.24–0.57 mM (table 1), i.e., about 9–20-fold greater than the $K_m$ of 0.027 mM found for the dephosphorylation of [$^{32}$P]phosphopyruvate kinase [11]. [$^{32}$P]Phosphopeptides VI and VII were essentially inactive when tested at concentrations up to 0.07 mM and 0.6 mM, respectively. The $V_{max}$ for the dephosphorylation of [$^{32}$P]phosphopeptides, as shown in fig.3, extrapolated to about 1.2 μmol [$^{32}$P]orthophosphate released/mg protein/min, which was close to the rate of dephosphorylation of [$^{32}$P]phosphoprotamine at a saturating concentration (60 μM).

The high $K_m$-values indicate that the phosphopeptides have low affinities for the phosphoprotein phosphatase and furthermore suggest that additional specificity determinants reside on the pyruvate kinase molecule. These views are supported by the observation that the rate of dephosphorylation of 5 μM [$^{32}$P]phosphopyruvate kinase, i.e., 20 μM with respect to [$^{32}$P]-phosphate moiety in the enzyme [3], was about 5-times higher than the rate of dephosphorylation of 20 μM [$^{32}$P]phosphopeptide I.

In further experiments the effects of selected ligands on the dephosphorylation were studied. Under the conditions of the standard assay, but in the absence of divalent cations, 1 mM ATP, ADP or phosphoenolpyruvate inhibited the dephosphorylation of 30 μM [$^{32}$P]phosphopeptide III by more than 60%. However, 2.5 mM $MgCl_2$ or $MnCl_2$ prevented this inhibition, as was the case with the dephosphorylation of [$^{32}$P]phosphopyruvate kinase [11]. Furthermore, $MnCl_2$ (2.5 mM) stimulated the rate of dephosphorylation of phosphopeptides I–V and IX two-fold while $MgCl_2$ (2.5 mM), added alone, did not affect the reaction with the same phosphopeptides. Neither in the presence nor absence of divalent cations were phosphopeptides VI–VIII dephosphorylated by the phosphoprotein phosphatase.

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