ACTA CHEMICA SCANDINAVICA 19 (1965) 1575–1582

Hydrolysis of Phosphopeptides

III. The Action of Alkaline Phosphatase Preparations from Kidney, Bone and Yeast on O-Phosphorylated Model Compounds

HEDVIG CSOPAK, GEORG FÖLSCH, LARS STRID and OLOF MELLANDER

Department of Medical Biochemistry, University of Göteborg, Göteborg, Sweden

Various peptides of O-phosphoryl-L-serine including Asp-SerP-Ala and similar sequences * have been studied as substrates for alkaline phosphatases from kidney, bone and yeast. Connections between the structure around the O-phosphoryl group and the rate of hydrolysis is discussed.

A review of earlier work on phosphoproteins and phosphopeptides in this laboratory can be found in Ref. 1. Special attention has been paid to the fact that O-phosphorylated peptides form rather strong and highly soluble metal complexes. The phosphopeptides of natural origin as well as those synthesized in this laboratory are rather resistant to proteolytic degradation, as compared with corresponding peptides missing the phosphate groups.[1,2] Having their phosphate groups intact, they may be expected to survive in physiological conditions and possibly have metal ion transport functions. The metal ion and/or phosphate group could finally be delivered to specific sites by help of tissue phosphatase and suitable peptidases. It is of interest therefore, to study the action of phosphatases of different origin on O-phosphorylated peptides. We have demonstrated that intestinal alkaline phosphatase acts on various phosphopeptides.[3] This paper will describe the preparation of alkaline phosphatases from swine kidney, calf bone and baker's yeast, and the action of these enzymes on various O-phosphorylated peptides.

* Abbreviations used as in Ref. 2.

EXPERIMENTAL

Substrates

The synthesis of the following substrates (*cf.* Table 1) and their analytical data, have been published:

O-phosphoryl-L-serine;[4] O-phosphoryl-DL-serine methyl ester and O-phosphoryletanolamine;[5] O-phosphoryl-DL-serylglycine, O-monophenylphosphoryl-DL-serylglycine, glycyl-(O-phosphoryl)-DL-serine, and glycyl-(O-phosphoryl)-DL-serylglycine;[6] O-phosphoryl-L-seryl-L-glutamic acid, O-phosphoryl-L-seryl-L-leucine, α-L-glutamyl-(O-phosphoryl)-L-serine, and L-leucyl-(O-phosphoryl)-L-serine;[7] O-phosphoryl-L-seryl-L-lysine.[8]

The synthesis and data for the following substrates are published separately:[9] O-phosphoryl-L-threonine, O-phosphoryl-L-seryl-L-alanine, L-isoleucyl-(O-phosphoryl)-L-serine, α-L-aspartyl-(O-phosphoryl)-L-serine, γ-L-glutamyl-(O-phosphoryl)-L-serine, L-lysyl-(O-phosphoryl)-L-serine, α-L-aspartyl-(O-phosphoryl)-L-serylglycine, α-L-glutamyl-(O-phosphoryl)-L-serylglycine, α-L-aspartyl-(O-phosphoryl)-L-seryl-L-alanine, α-L-glutamyl-(O-phosphoryl)-L-seryl-L-alanine, α-L-aspartyl-(O-phosphoryl)-L-seryl-L-glutamic acid, and L-leucylglycyl-(O-phosphoryl)-L-serine.

The following commercial substrates were used: phenyl phosphate sodium salt (KEBO AB, Stockholm, Sweden); *o*-carboxyphenyl phosphate (Lot c 2216, MANN Res.Labs., New York 6, N.Y., U.S.A); *p*-nitrophenyl phosphate di-sodium salt (Lot 101B—102—1, SIGMA Chem. Co., St. Louis, Missouri, U.S.A.); β-glycerophosphate sodium salt, 5 H_2O (B.D.H. Ltd., Poole, England); creatine phosphate di-sodium salt,6 H_2O (Lot E 1996, MANN Res. Labs.); adenosine-5′-triphosphate (85 %, Lot D 1897, MANN Res. Labs.). Substrate solutions were made immediately before use, since several of the compounds were markedly hydrolyzed when stored in water solution.

Preparation of alkaline phosphatases

From swine kidney. The method of Binkley [10] was used with the modification that gel filtration on Sephadex G-75 was used instead of chromatography on ECTEOLA, as the latter procedure repeatedly gave inactive preparations. A column (3.0 × 80.0 cm) was packed with Sephadex G-75 as described by Flodin [11] and equilibrated with 0.05 M Tris-HCl buffer pH 8.2. Binkley's barium fraction 0.6—0.9 was applied, washed into the column with two 6 ml portions of the above buffer, and 10 ml fractions were collected. The frac-

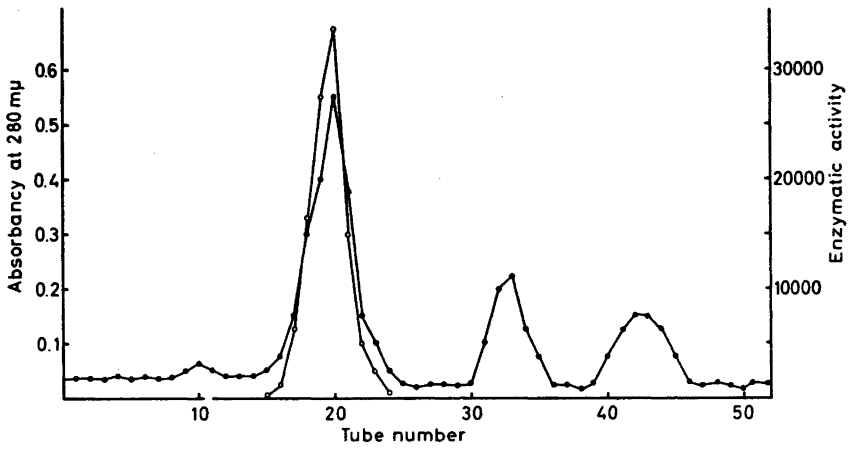

Fig. 1. Gel filtration of alkaline phosphatase from kidney on Sephadex G-75. ● Light absorbancy at 280 mμ; O Phosphatase activity.

tions having phosphatase activity (Fig. 1) were combined and precipitated with barium acetate and ethanol according to Binkley. The precipitate was collected by centrifuging (16 000 *g*; 20 min) and dissolved in 10 ml of distilled water. The activity of this enzyme solution, using β-glycerophosphate as the substrate, was 86 000 μg P/mg N/min.

From calf bones. The method described by Volkin [12] was used, but the enzyme obtained was further purified. A column (2.9 × 60.0 cm) was packed with Sephadex, having a water regain of 14 ± 1 g/g. After equilibration with 0.02 M Tris-acetate buffer pH 8.0 containing 0.05 M magnesium acetate, the enzyme solution (7 ml) was applied and fractions of 7 ml were collected. The active fractions (Fig. 2) were combined and chromato-

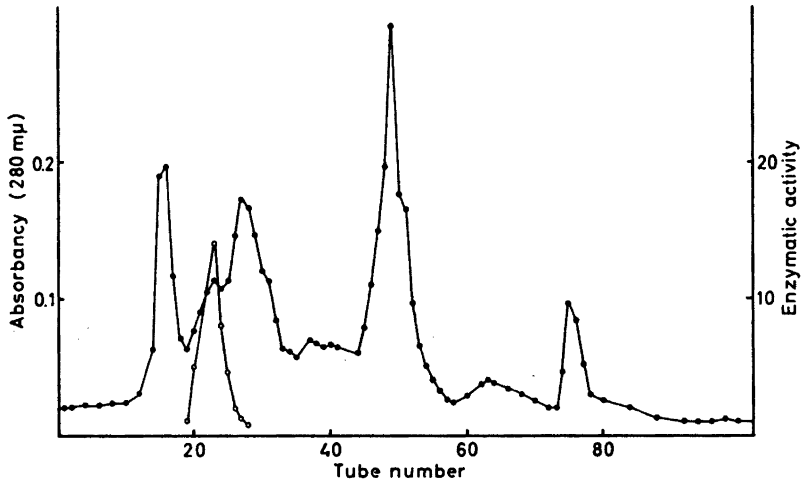

Fig. 2. Gel filtration of alkaline phosphatase from calf bones on Sephadex (water regain 14 ± 1 g/g) ● Absorption at 280 mμ; O Enzyme activity.

graphed on a column (3.0 × 55.0 cm) of DEAE-cellulose according to Peterson and Sober.[13] The column was equilibrated with 0.01 M Tris-acetate buffer containing 0.008 M magnesium acetate, and the gel filtered solution applied and washed into the column with two 5 ml portions of the same buffer. Elution was performed with stepwise increases in magnesium acetate concentration (Fig. 3), and 5 ml fractions were collected. The enzyme appeared with 0.04 M Mg^{2+}. The active fraction was rechromatographed in the same way, whereby a single protein peak coincided with a single activity peak. The specific activity, 670 μg *p*-nitrophenol/mg N/min, was the same as after the first DEAE run.

The activity was determined by measuring the increase in optical density at 400 mμ due to liberation of *p*-nitrophenol from *p*-nitrophenyl phosphate. The reaction was carried out at 37° using 3 ml of 1 mM *p*-nitrophenyl phosphate mixed with 1 ml of 1 mM magnesium chloride, and 1 ml of enzyme solution mixed with 5 ml of 0.1 M Tris-HCl buffer pH 8.8. The optical density was measured at 30 sec intervals.

From baker's yeast. The preparation and properties of this enzyme is described separately.[14] The enzyme solution used here had the specific activity 14 300 μg *p*-nitrophenol/mg N/min. The activity was determined as in the case of the bone enzyme, except that Tris-HCl buffer of pH 8.1 and a final concentration of 0.005 M magnesium acetate was used.

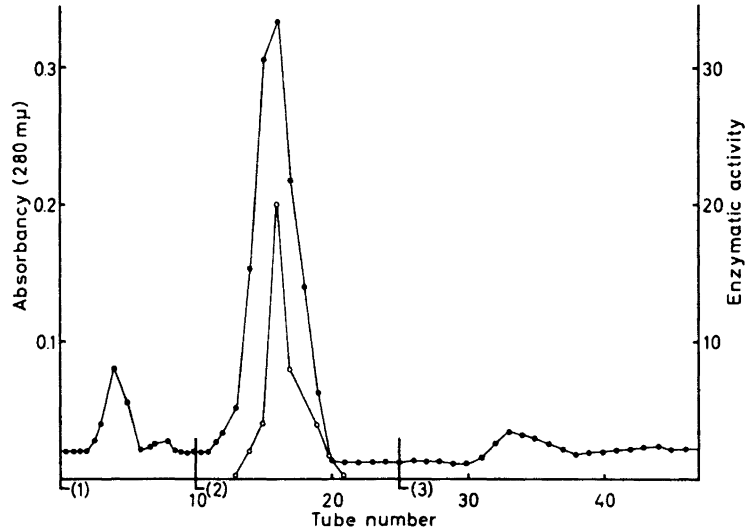

Fig. 3. Chromatography of alkaline phosphatase from calf bones on DEAE-cellulose. Eluted in the steps using 0.01 M Tris-acetate buffer pH 8.0 containing (1) 0.008 M; (2) 0.04 M; (3) 0.2 M magnesium acetate. O Light absorbancy at 280 mμ; ● Phosphatase activity.

Hydrolysis of the substrates

Kidney phosphatase hydrolysis. The enzyme (50 μl) was incubated at 38° with 25 μl 0.05 M magnesium chloride, 150 μl distilled water, and 25 μl 0.02 M ethanolamine-HCl buffer, pH 9.2. In the same way the substrate (800 μl 0.0156 M solution in water) was incubated with 100 μl 0.05 M magnesium chloride and 100 μl of the buffer. After incubating 20 min, the two solutions were mixed. Aliquots of 100 μl were taken after different times (1 – 60 min) for determination [15] of the inorganic phosphate liberated. Blank runs omitting the enzyme, were run in parallel. The rates of hydrolysis were calculated as % phosphate liberated in the first 20 min.

Bone phosphatase hydrolysis. The substrate (800 μl 0.0156 M) was incubated 15 min at 37° with 125 μl 0.05 M magnesium chloride and 125 μl 0.05 M Tris-HCl buffer pH 8.8. The enzyme (200 μl) was added and the rates of hydrolysis determined as in the case of kidney enzyme.

Yeast phosphatase hydrolysis. The substrate (800 μl 0.0156 M) was incubated for 15 min at 37° with 125 μl 0.05 M magnesium chloride and 225 μl 0.05 M Tris HCl buffer pH 8.1. The enzyme (100 μl) was added and the rate of hydrolysis determined as in the case of the kidney enzyme.

RESULTS AND DISCUSSION

The rates of phosphate liberation from different substrates catalyzed by three different enzyme preparations are given in Table 1 as per cent hydrolysis in 20 min.* The differences are small as compared with those obtained in the

* Owing to lack of enzyme, only a few substrates could be studied with the yeast enzyme.

Table 1. Rates of phosphate liberation from substrates using alkaline phosphatase preparations from swine kidney, calf bone and baker's yeast. Given as % hydrolysis in 20 min; conditions specified under Experimental.

Substrate	Per cent hydrolysis in 20 min by alkaline phosphatase from		
	kidney	bone	yeast
1 O-Phosphoryl-L-threonine	25.7	7.5	20.5
2 O-Phosphoryl-L-serine	26.0	10.3	19.0
3 O-Phosphoryl-DL-serine methyl ester	20.4	10.0	—
4 O-Phosphoryl-etanolamine	6.0	7.2	—
5 SerP-Gly	18.0	10.4	18.7
6 SerP-Ala	16.9	7.4	—
7 SerP-Leu	12.9	4.8	10.2
8 SerP-Glu	21.0	7.4	—
9 SerP-Lys	13.0	4.7	—
10 Gly-SerP	10.0	8.4	15.1
11 Leu-SerP	3.3	1.2	0.9
12 Ile-SerP	1.2	1.3	—
13 α-Asp-SerP	4.7	7.0	—
14 α-Glu-SerP	3.5	2.5	—
15 γ-Glu-SerP	4.8	2.7	—
16 Lys-SerP	7.7	1.2	—
17 Gly-SerP-Gly	23.6	11.5	—
18 α-Asp-SerP-Gly	10.0	4.8	—
19 α-Glu-SerP-Gly	9.9	5.3	—
20 α-Asp-SerP-Ala	6.8	4.4	—
21 α-Glu-SerP-Ala	3.8	5.1	—
22 α-Asp-SerP-Glu	5.5	5.7	—
23 Leu-Gly-SerP	0.74	3.5	—
24 Ser φ P-Gly	0.0	0.0	0.0
25 Phenyl phosphate	—	—	21.0
26 o-Carboxyphenyl phosphate	—	3.0	—
27 p-Nitrophenyl phosphate	23.6	11.6	24.1
28 β-Glycerophosphate	21.4	13.5	16.0
29 Creatine phosphate	13.3	14.8	—
30 Adenosine triphosphate	5.4	4.9	—

leucine aminopeptidase hydrolysis [2] of the peptides studied. There are, however, some obvious connections between structure and the rate of hydrolysis, especially with regard to the position of the phosphate group. As was the case of intestinal alkaline phosphatase,[3] the three present enzyme preparations dephosphorylated dipeptides having O-phosphorylserine in the N-terminal position (substrates 5—9) definitely faster than the reversed sequences (substrates 10 — 16; Table 1). It is to note, that this relationship applies also to *O-pyrophosphorylserine* peptides in their hydrolysis catalyzed by inorganic pyrophosphatase from yeast.[16] The hydrolysis rates of tripeptides having O-phosphorylserine in the middle (substrates 17 — 22) seem to lie between those of the dipeptide groups. An exception is the peptide Gly-SerP-Gly, which is one of the best substrates tested. The peptide Leu-Gly-SerP, having

its free α-amino group far from the phosphate group, is slowly dephosphorylated. The O-phosphoryl derivatives of L-threonine, L-serine, and DL-serine methyl ester (substrates 1—3) are some of the best substrates, whereas the O-phosphoryl derivative of 2-aminoethanol (substrate 4) is hydrolyzed notably slower.

The rate of hydrolysis seems, also, to be influenced by the nature of the amino acid bound to the O-phosphorylserine residue. Peptides containing glycine are hydrolysed faster than those having alanine, leucine, or isoleucine, *i.e.* amino acids with hydrophobic and, possibly, sterically hindering side chains.* Less clear are the effects on the rate given by the charged side chains of aspartic acid, glutamic acid, or lysine, since the enzyme from kidney and that from bone behaved differently. Whereas SerP-Lys was a poor substrate for both enzymes, Lys-SerP was poor only for the bone enzyme, but was hydrolyzed rather rapidly by the kidney enzyme. A similar, although less pronounced, irregularity was observed (Table 1) for the peptides SerP-Glu (fast hydrolysis by the kidney enzyme) and Asp-SerP-Glu (somewhat faster hydrolysis than of Asp-SerP-Gly by the bone enzyme).

The enzymes used were not purified enough to allow a decision about if they are different enzymes, possibly having different specificity, or if they are identical. The irregularities in rates mentioned above may indicate that some of the preparations may contain more than one enzyme. Although none of them showed diesterase activity against substrate (24), they hydrolyzed the N-phosphorylated derivative, creatine phosphate, a compound not hydrolyzed by pure *E.coli* phosphatase.[17] Also, the presence of enzymes other than phosphatases cannot be excluded. A presence of leucine aminopeptidase in the bone enzyme preparation would easily explain why this liberated phosphate from Leu-Gly-SerP faster than did the kidney enzyme, which was the more active enzyme in other cases. The dipeptide Gly-SerP formed by peptidase action would be dephosphorylated rather rapidly.

Regarding the mechanism of the dephosphorylation of the O-phosphorylated serine derivatives, it seems probable that the phosphoryl group is transferred from the serine hydroxyl of the substrate to a specific serine hydroxyl group of the enzyme forming a phosphoryl enzyme intermediate. The phosphoryl group is then transferred from the phosphoryl enzyme to a water molecule.** The formation of an intermediate phosphoryl enzyme involving a specially reactive serine residue has been demonstrated in the case of several phosphatases.[17] Milstein,[19] Lipmann and collaborators,[20] as well as Engström,[21] have further shown that the active serine residue in alkaline phosphatases of different origin is found in the sequence Asp-Ser-Ala. In the case of Asp-SerP-Ala (substrate 20 in Table 1) the phosphate group, therefore, is transferred between two identical amino acid sequences (Fig. 4). It is evident, that this tripeptide substrate shows no unusual reactivity in the rate of dephosphorylation.

* The glycine peptides used all contain DL-serine, but it seems, that the optical configuration at the serine α-carbon atom has little influence on hydrolysis rates.[3]

** In those experiments where Tris buffer was used, the hydroxyl groups of the buffer base may compete with water as the phosphate group acceptor[18]. Therefore, the absolute values for a given substrate obtained for the different enzymes cannot be directly compared.

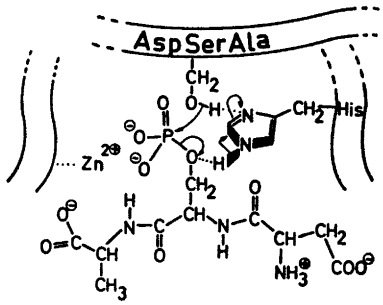

Fig. 4. Some functional groups possibly involved in a transition state of phosphate transfer from the substrate, α-L-aspartyl-(O-phosphoryl)-L-seryl-L-alanine, to the enzyme, alkaline phosphatase, forming a phosphoryl enzyme. The substrate may be activated and held in place by the enzyme-bound metal ion, and the transfer assisted by imidazole catalysis. (*cf.* Hummel, J. P. and Kalnitzky, G. *Ann. Rev. Biochem.* 33 (1964) 15).

If the "active center" of alkaline phosphatase has the structure shown in Fig. 4, *i.e.* consisting mainly of a serine hydroxyl group, a negatively charged aspartic acid side chain, a positively charged metal ion (Zn^{2+}), and a further basic group (imidazole), it would be hydrophilic in character. The attraction for substrates having the hydrophobic amino acids leucine or isoleucine near the phosphate group may then be expected to be small, and possibly result in a comparably small rate of hydrolysis. This was actually found. The high rates obtained with the substrates containing hydrophobic phenyl groups (substrates 25 and 27 in Table 1) might be expected, since *aryl*oxy-phosphate bonds are cleaved in these substrates. *o*-Carboxyphenyl phosphate (substrate 26 in Table 1) which is an aryloxy phosphate but has a carboxyl group *ortho* to the phosphoryloxy group, was found to be hydrolyzed slowly. This may be due to competition for the enzyme metal ion between the closely placed phosphate and carboxyl groups. This competition by phosphate and carboxyl groups may also explain the decrease in the rate of hydrolysis as the phosphate group is placed closer to the carboxyl group in a series of O-phosphorylserine peptides, where the relative rates are SerP-X > X-SerP-Y > X-SerP (Table 2). The same may apply to peptides [16] of O-pyrophosphorylserine. The high rates for O-phosphorylserine and O-phosphorylthreonine may be due to neutralization of the carboxyl group by the closely placed α-amino group.

Table 2. Mean values of rates of phosphate liberation from di- and tripeptides having O-phosphorylserine (SerP) in different positions, using alkaline phosphatases from kidney and bone. The amino acids (X, Y) used were glycine, alanine, leucine, isoleucine, aspartic acid, glutamic acid, and lysine. Substrate numbering and individual rates are given in Table 1.

Enzyme source	Mean values of % dephosphorylation in 20 min		
	SerP-X (substr. 5—9)	X-SerP (substr. 10—16)	X-SerP-Y (substr. (17—22)
Swine kidney	16.4	5.0	9.9
Calf bone	6.9	3.5	6.1

The results obtained in this work confirm our previous conclusions [3] that the structure around the O-phosphorylserine residue in peptides is of considerable importance for the sensitivity of the phosphate group to phosphatase action. The biological consequences of this finding and the possible differences in specificity of the phosphatases from different tissues cannot at present be evaluated, further work on these aspects being considered.

This work was aided by a grant from the *Swedish Medical Research Council* (Project No. U 422).

REFERENCES

1. Mellander, O. *Phosphopeptides, Chemical Properties and Possible Role in the Intestinal Absorption of Metals.* In Wasserman, R. H. *The Transfer of Calcium and Strontium Across Biological Membranes,* Academic Press, New York 1963.
2. Fölsch, G., Strid, L. and Mellander, O. *Acta Chem. Scand.* **19** (1965) 1566.
3. Mellander, O., Bennich, H. and Fölsch, G. *Acta Soc. Med. Upsalien.* **64** (1959) 303.
4. Fölsch, G. and Mellander, O. *Acta Chem. Scand.* **11** (1957) 1232.
5. Fölsch, G. and Österberg, R. *J. Biol. Chem.* **234** (1959) 2298.
6. Fölsch, G. *Acta Chem. Scand.* **12** (1958) 561.
7. Fölsch, G. *Acta Chem. Scand.* **13** (1959) 1407.
8. Fölsch, G. and Österberg, R. *Acta Chem. Scand.* **15** (1961) 1963.
9. Fölsch, G. *Acta Chem. Scand.* **20** (1966). *In press.*
10. Binkley, F. *J. Biol. Chem.* **236** (1961) 735.
11. Flodin, P. *J. Chromatog.* **5** (1961) 103.
12. Volkin, E. *Methods in Enzymology* **2** (1955) 539.
13. Peterson, E. A. and Sober, H. A. *Methods in Enzymology* **5** (1962) 3.
14. Csopak, H. *Acta Chem. Scand.* **18** (1964) 2414.
15. Berenblum, J. and Chain, E. *Biochem. J.* **32** (1938) 295.
16. Avaeva, S., Fölsch, G., Strid, L. and Mellander, O. *Acta Chem. Scand.* **17** (1963) 2718.
17. Hummel, J. P. and Kalnitsky, G. *Ann. Rev. Biochem.* **33** (1964) 15.
18. Dayan, J. and Wilson, I. B. *Biochim. Biophys Acta* **81** (1964) 620.
19. Milstein, C. *Biochim. Biophys. Acta* **67** (1963) 171; *Biochem. J.* **92** (1964) 410.
20. Schwartz, J. H., Crestfield, A. M. and Lipmann, F. *Proc. Natl. Acad. Sci.* U.S. **49** (1963) 722.
21. Engström, L. *Biochim. Biophys. Acta* **92** (1964) 79.

Received May 18, 1965.