ACTA CHEMICA SCANDINAVICA 17 (1963) 2718–2723

Diserylphosphates and Serylpyrophosphates

Some Properties of O-Pyrophosphoryl-Serine Peptides

SVETLANA AVAEVA*, GEORG FÖLSCH, LARS STRID and OLOF MELLANDER

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

Several peptides of O-pyrophosphoryl-serine were prepared. They were hydrolyzed to orthophosphate and O-phosphorylserine peptides by crystalline inorganic yeast pyrophosphatase in the presence of Zn^{2+}. The stability of L-leucyl-(O-pyrophosphoryl)-L-serine at different pH was studied.

The increasing interest in the biological function of phosphoproteins attracts considerable attention to the phosphoprotein structure and particularly to the question of the bonding of the phosphoric acid residues to the protein.[1-4]

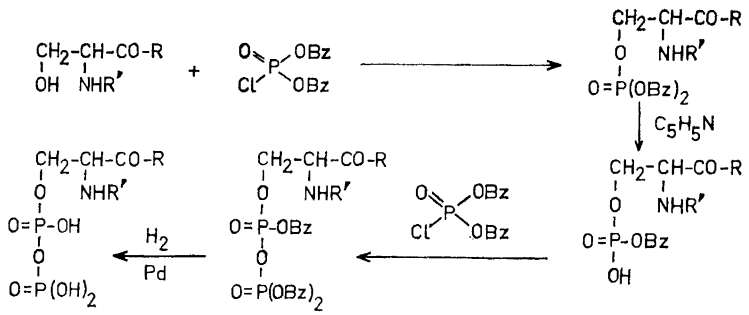
The monophosphate ester bond that is taken into account, to most investigators, does not alone explain the reactivity character of at least a part of the phosphate groups in phosphoproteins. The high rate of phosphate exchange, the transfer of phosphate groups connected with the activity of some enzymes being phosphoproteins and the relatively high thermodynamic potential [5] of at least some of the phosphate groups in phosphoproteins permits one to discuss the existence of other types of bonds between phosphate and protein, and, particularly, pyrophosphate bonds. At the present time, however, the question of the phosphate bonds in phosphoprotein has been decided primarily on the grounds of the action of different phosphatases, the specificity of which had been determined with very simple substrates having little in common with natural products.[1,2]

One of the possible means of studying the structure of phosphoproteins is to synthesize model compounds and to investigate their chemical and enzymatic reactions with the purpose of discovering specific reactions, which then may be used on phosphoproteins. In connection with studies of phosphoproteins and phosphopeptides in this laboratory,[6] a number of monophosphate derivatives of serine have been synthesized [7] and studied.[6-8] The investigations of

* Address: Chemistry Department, Moscow University, Moscow, U.S.S.R.

pyrophosphate derivatives of serine were started by Avaeva in the institute of Organic Chemistry, State University, Moscow.[9]

The isolation and investigation of a few peptides of O-pyrophosphoryl-serine is described in the present article. These pyrophosphates were observed in this laboratory to be byproducts in the preparation of corresponding monophosphate esters, when O-dibenzylphosphoryl-serine derivatives in pyridine solution were left for prolonged interaction with excess dibenzylphosphoryl chloride. Apparently, pyridine provokes mono-debenzylation giving O-monobenzylphosphoryl-serine derivative, which then reacts with a new molecule of dibenzylphosphoryl chloride:



Bz = $-CH_2C_6H_5$

The side reaction does not seem to stop at this stage, as formation of a small amount of the corresponding triphosphate esters was observed. The compounds were isolated by washing the reaction mixture with aqueous acid and base, hydrogenolysis of the neutral fraction and column chromatography on anion exchange resin (Dowex 1-X2) of the hydrogenolysate. Pyridinium formate was used as the eluent, and the acidic fractions were freed from pyridine by means of cation exchange resin (Dowex 50, H^+ form). After freeze-drying the pyrophosphates were obtained as white, glittering and very hygroscopic

Table 1. Peptides of O-pyrophosphorylserine isolated as byproducts in the preparation of monophosphate esters.

Compound	Ratio N/P	R_{Ser}
O-Pyrophosphoryl-L-seryl-L-leucine *	1.2	0.9 **
L-Leucyl-(O-pyrophosphoryl)-L-serine	1.0	0.9
α-L-Aspartyl-(O-pyrophosphoryl)-L-serine	1.3	0
γ-L-Glutamyl-(O-pyrophosphoryl)-L-serine	1.1	0

* Found: C 28.4, H 5.5, N 7.3, P 15.9. Calc. for $C_9H_{20}N_2O_{10}P_2$ (378.2): C 28.6, H 5.3, N 7.4, P 16.4.

** The monophosphate analogue [7] has R_{Ser} = 1.8. The R_{Ser} values refer to descending chromatography in the system butanol-acetic acid-water (4:1:1; v/v) and Whatman No. 1 paper.

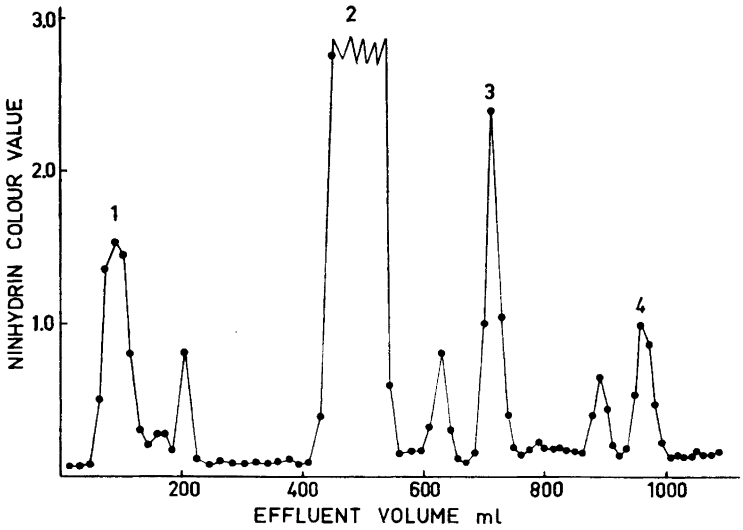


Fig. 1. Separation of the hydrogenated reaction products obtained from N-carbobenzoxy peptide benzyl esters, dibenzylphosphoryl chloride and pyridine after 60 h reaction time.

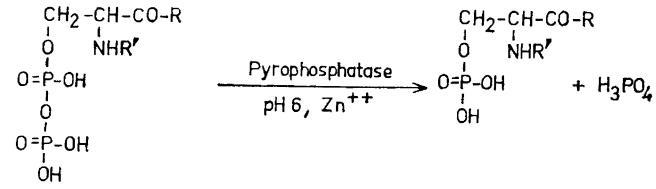
substances. They are represented in Table 1. The yields were always less than 5 % of the amount of material applied to the column (monophosphate peptide yield about 85 %).

Complete hydrolysis of the compounds (6 M HCl, 140°, 1 h) and paper chromatography in butanol-acetic acid-water (4:1:1) gave serine, the second amino acid, some O-phosphorylserine, and orthophosphate. The ratio N/P in the peptides was approximately 1. One of the peptides was subjected to elementary analysis with acceptable result (Table 1).

We have found, that crystalline inorganic pyrophosphatase from yeast [10] splits the pyrophosphate bond in these peptides of O-pyrophosphorylserine.

This enzyme has been used for the hydrolysis of inorganic pyrophosphate in the presence of Mg^{2+}. In 1960, Schlesinger and Coon [11] showed, the enzyme was capable of catalyzing the hydrolysis of nucleoside triphosphates as ATP, GTP and UTP, when Zn^{2+} was used in the place of Mg^{2+}. Diphosphates such as ADP, GDP and UDP were hydrolyzed considerably slower.

The pyrophosphatase was tested in the presence of Zn^{2+} on four of the new dipeptides having O-pyrophosphorylserine in C-terminal or N-terminal position. The enzyme splits off one phosphate group in each peptide:



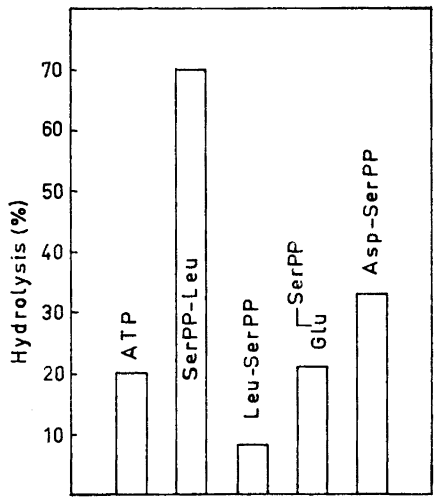


Fig. 2. Per cent hydrolysis of the pyrophosphate bond in O-pyrophosphorylserine (SerPP) peptides and of adenosine triphosphate (calc. as % terminal phosphate released). Crystalline inorganic pyrophosphatase, Zn^{2+}, pH 6.0, 37° and 30 min.

The rates of hydrolysis are recorded in Fig. 2 as per cent splitting of the pyrophosphate bond. Three of the peptides, including O-pyrophosphoryl-L-seryl-L-leucine, were hydrolyzed at a higher rate than ATP, whereas L-leucyl-(O-pyrophosphoryl)-L-serine was hydrolyzed at a considerably lower rate. It may be mentioned, that monophosphate dipeptides having O-phosphorylserine in C-terminal position are hydrolyzed by intestinal, bone, or kidney alkaline phosphatase at a lower rate than the reversed sequences.[8] The monophosphate peptides were completely resistent to the action of the yeast pyrophosphatase.

The stability of one of the peptides, L-leucyl-(O-pyrophosphoryl)-L-serine was studied at different pH. This compound was very stable in the pH interval from 2 to 10 (Fig. 3).

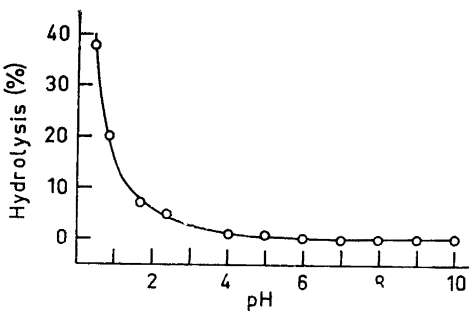


Fig. 3. Per cent hydrolysis (50°, 4.5 h) of L-leucyl(O-pyrophosphoryl)-L-serine (1 μmole) in buffer solutions (1 ml) of different pH.

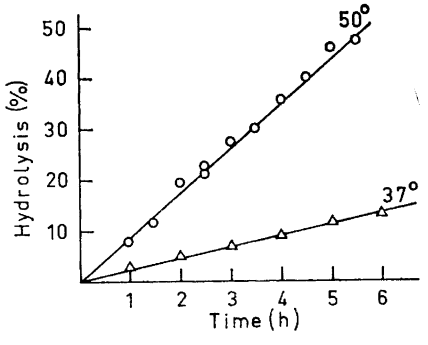


Fig. 4. Per cent hydrolysis of L-leucyl-(O-pyrophosphoryl)-L-serine (1 μmole) in 1 M HCl (1 ml).)

Below pH 2, however, a rapid hydrolysis of the pyrophosphate bond and formation of the corresponding monophosphate peptide was observed. The kinetics of hydrolysis was studied in 1 M HCl at 37° and 50° (Fig. 4). At both temperatures, the reaction is of first order with the rate constants $k = 4.0 \times 10^{-4}$ min^{-1} (37.0° ± 0.2) and $k = 17.5 \times 10^{-4}$ min^{-1} (50.0° ± 0.2). This instability of serine pyrophosphates in acid medium certainly excludes the discovering of such bonds in tissue phosphoproteins, isolated by methods commonly used[4] (*i.e.* trichloroacetic acid precipitation, *etc*).

EXPERIMENTAL

Isolation of pyrophosphopeptides. N-Carbobenzoxy dipeptide benzyl esters (with the sequences L-seryl-L-leucine, L-leucyl-L-serine, L-seryl-L-alanine, α-L-aspartyl-L-serine and γ-L-glutamyl-L-serine) were dissolved in anhydrous pyridine and treated with 50 % excess dibenzylphosphoryl chloride as described.[7] The reaction mixture was, however, left for a longer period (60 h) at + 4°. A neutral fraction was then isolated and hydrogenolyzed as usual in *t*-butanol-water solution and 10 % Pd/C catalyst. After filtration and evaporation to dryness, 3.0 g of the solid residue was dissolved in 20 ml of 0.1 M formic acid and chromatographed by the method described earlier.[12] A column of 2.0 cm diameter was packed to a height of 40 cm with Dowex 1−X2 (200−400 mesh) anion exchange resin in formate form, and the solution was applied. The column was eluted with a gradient from 0.1 M formic acid to 2 M pyridinium formate (mixing chamber volume was 1 l), and fractions of 16 ml (rate 1 ml/min) were collected. The separation was finished within 20 h. The fractions (100 μl of each) were tested[13] with ninhydrin (see Fig. 1). Peak 1 corresponds to free amino acids, peak 2 represents the main reaction product, *i.e.* the monophosphate ester of the peptide and peaks 3 and 4 contain more than one phosphate group per molecule of peptide. The ninhydrin-positive peaks were freeze-dried. The residue was dissolved in a small amount of distilled water and the pyridine removed by shaking with Dowex 50-resin in H^+ form, filtering and renewed freeze-drying.

Determination of N/P atomatic ratio was made as described.[12]

Enzymatic hydrolysis. The *inorganic pyrophosphatase* used was a twice crystallized yeast enzyme kindly provided by Dr. M. Kunitz, Rockefeller Inst., New York. It was used as a water solution containing 0.18 mg protein per ml, as determined spectrophotometrically.[14] The buffer used was 0.1 M tris-maleate, pH 6.0. Zinc acetate was used in 0.1 M solution. Adenosine-5-triphosphate used was obtained from Mann. Res. Lab. Inc., New York (C.P. quality 85 %, heavy metal free).

To the solution incubated at 37° and containing 1 μmole substrate, 2 μmoles zinc acetate and 120 μmoles of buffer solution (final volume 1.5 ml) was added 4.5 μg enzyme. The amount of orthophosphoric acid liberated after 30 min was determined by the modified Beerenblum and Chain method.[15]

Stability of pyrophosphate bond at different pH. Buffers for hydrolysis at pH 0.88, 1.68, 2.4, 9 and 10 were made from 0.1 M glycine and 0.1 M hydrochloric acid or 0.1 M sodium hydroxide; those of pH 3.5, and 6 from 0.1 M potassium hydrogen phthalate and 0.1 M hydrochloric acid or sodium hydroxide, and the buffer of pH 4 was 0.1 M potassium hydrogen phthalate. The buffers of pH 7 and 8 were finally made from 0.1 M barbital sodium and 0.1 M hydrochloric acid.

One μmole of L-leucyl-(O-pyrophosphoryl)-L-serine was incubated in 1 ml of buffer solution at 50° for 4.5 h. The amount of orthophosphoric acid liberated was determined as above.

Stability of pyrophosphate bond in 1 M HCl. One μmole of the above pyrophosphopeptide was incubated in 1 ml of 1 M hydrochloric acid at 37° or 50°. The amount of orthophosphoric acid liberated was determined as above after definite times of reaction.

Acknowledgment. We are indebted to Miss Monica Aldén for valuable technical assistance. The investigation was aided by a grant from the *Swedish Medical Research Council.*

REFERENCES

1. Perlmann, G. E. *Advan. Protein Chem.* **10** (1955) 1.
2. Sampath Kumar, K. S. V., Sundararajan, T. A. and Sarma, P. S. *Enzymologia* **18** (1957) 228; Sundararajan, T. A. and Sarma, P. S. *Enzymologia* **18** (1957) 234; Hofman, T. *Biochem. J.* **69** (1958) 139; Kalan, E. B. and Telka, M. *Arch. Biochem. Biophys.* **79** (1959) 275; **85** (1959) 273.
3. Anderson, L. and Kelley, J. J. *Federation Proc.* **18** (1959) 182; *J. Am. Chem. Soc.* **81** (1959) 2275; Lisovskaja, N. and Livanova, N. *Phosphoproteins*, A.N. USSR, Moscow 1960; Heald, P. J. *Biochem. J.* **80** (1961) 510; *Nature* **193** (1962) 451; Österberg, R. *Biochim. Biophys. Acta* **54** (1961) 424; Belec. J. and Jenness, R. *Biochim. Biophys. Acta* **63** (1962) 512; Taborsky, G. and Connelly Allende, C. *Biochemistry* **1** (1962) 406.
4. Ågren, G. *On the Nature of Phosphorus Linkages and Biological Functions of Intracellular Phosphoproteins*, Uppsala Universitets Årsskrift **5** (1958), Almqvist & Wiksell, Uppsala 1958.
5. Rabinowitz, M. and Lipmann, F. *J. Biol. Chem.* **235** (1960) 1043.
6. Mellander, O. *Phosphopeptides, Chemical Properties and Possible Role in the Intestinal Absorption of Metals*, in *The Transfer of Calcium and Strontium Across Biological Membranes*, (Ed. R.H. Wasserman), Academic Press, New York 1963.
7. Fölsch, G. *Acta Chem. Scand.* **9** (1955) 1039; **12** (1958) 561; **13** (1959) 1407, 1422; Fölsch, G. and Mellander, O. *Acta Chem. Scand.* **11** (1957) 1232; Fölsch, G. and Österberg, R. *J. Biol. Chem.* **234** (1959) 2298; *Acta Chem. Scand.* **15** (1961) 1963; Fölsch, G. *To be published.*
8. Mellander, O., Bennich, H. and Fölsch, G. *Acta Soc. Med. Upsaliensis* **64** (1959) 303; Fölsch, G., Mellander, O. and Strid, L. *Acta Chem. Scand.* **14** (1960) 625; Österberg, R. *Acta Chem. Scand.* **16** (1962) 2434; Fölsch, G., Mellander, O. and Strid, L. *To be published.*
9. Avaeva, S., Botvinik, M. and Siromajtnikova, I. *J. Gen. Chem. USSR* **33** (1963) 709; Avaeva, S., Botvinik, M. and Vaphina, M.; Avaeva, S., Siromajtnikova, I. and Botvinik, M. *J. Gen. Chem. In press.*
10. Kunitz, M. *J. Am. Chem. Soc.* **73** (1951) 1387; *J. Gen. Physiol.* **35** (1952) 423.
11. Schlesinger, M. J. and Coon, M. J. *Biochim. Biophys. Acta* **41** (1960) 30.
12. Strid, L. *Acta Chem. Scand.* **13** (1959) 1787.
13. Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907.
14. Kunitz, M. and Robbins, P. W. in *The Enzymes* **5** (1961) 169. Ed. by Boyer, P. D., Lardy, H. and Myrbäck, K. Academic Press, New York and London 1961.
15. Martin, J. B. and Doty, D. M. *Anal. Chem.* **21** (1949) 965.

Received July 31, 1963.