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HYDROLYSIS OF THE PYROPHOSPHATE BOND OF SERYL PYROPHOSPHATES BY THE ALKALINE PHOSPHATASE OF ESCHERICHIA COLI

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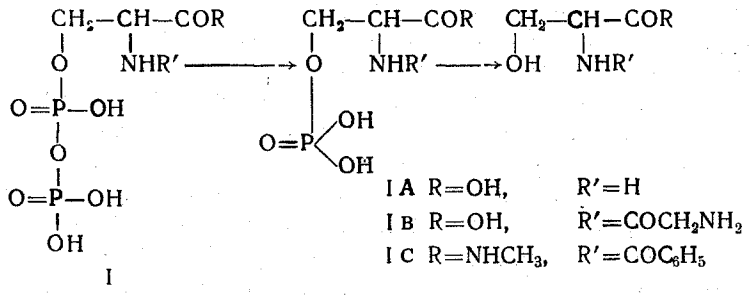
The high lability, reactivity, and energy of the phosphate bonds of the phosphoproteins that participate in the metabolic processes shows the possibility of the existence in these proteins of not only seryl monophosphate but also seryl pyrophosphate groupings. In view of this, some years ago an investigation was begun of compounds with different types of seryl pyrophosphate bonds. Symmetrical diseryl pyrophosphates were synthesized and some of their properties were studied [1-4]. At the present time, methods have been developed for the synthesis of the first representatives of another class of serine pyrophosphates, seryl pyrophosphates of type I. The stability of these compounds at various pH values, the kinetics of their acid hydrolysis, and their enzymatic hydrolysis by the inorganic pyrophosphatase of yeasts have been studied [5-7]. In the preceding communication, information was given on the cleavage of type I seryl pyrophosphates by the alkaline phosphatase of E. coli which takes place by the mechanism



It was found that the incubation (pH 8.0, 37° C, 30 min) of 1 μmole of I with 2.5 γ of the alkaline phosphatase of E. coli led to the liberation of 23-29% of the total phosphorus of I. Chromatographic and electrophoretic analyses showed that under the conditions given the main reaction products were phosphoserine and its derivatives, while serine and its derivatives were formed in only very small amounts, i.e., hydrolysis took place primarily at the pyrophosphate

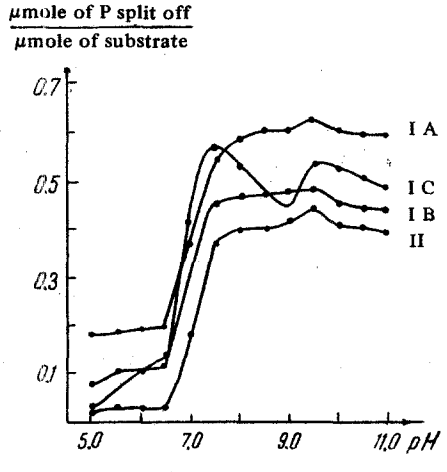


Fig. 1. Influence of pH on the hydrolysis of seryl pyrophosphate and phosphoserine by the alkaline phosphatase of E. coli.

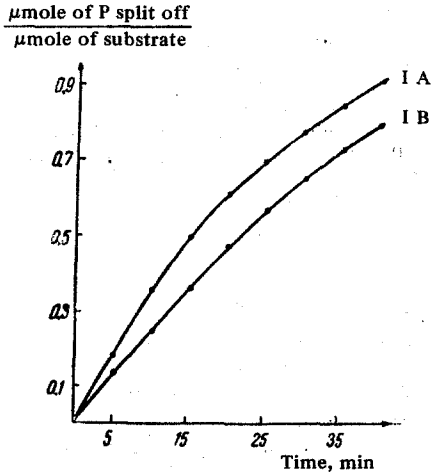


Fig. 2. Dependence of the enzymatic hydrolysis of seryl pyrophosphates on the incubation period.

bond of the seryl pyrophosphate. In a study of the influence of the pH on the hydrolysis of seryl pyrophosphate it was found that the enzyme possesses considerable activity over a wide pH range, from 7.5 to 11.0 (Fig. 1). The rate of enzymatic hydrolysis of IA and IB at pH 8.0 is directly proportional to the incubation period (Fig. 2).

Table 1

Influence of Inhibitors on the Monophosphatase and Pyrophosphatase Activity of the Enzymes

Inhibitor	Percentage of the initial activity	
	phospho-serine	pyrophos-phoserine
Acetone	69.4	69.1
Urea	87.7	88.3
Co^{++}	57.3	57.0
Cd^{++}	60.4	59.5

Table 2

Thermal Inactivation of the Enzyme and Subsequent Restoration of its Activity

Substrate	Percentage of the original activity after thermal inactivation for			
	0 hr	1 hr	4 hr	24 hr
Phosphoserine	22.5	30.4	40.0	48.9
Pyrophospho-serine	22.0	30.2	39.2	47.9

Until recently it was considered that the alkaline phosphatase of E. coli is a phosphomonoesterase, so it appeared necessary to obtain some information in favor of the view that the hydrolysis of the pyrophosphate bond and the hydrolysis of the monophosphate bond were effected by the same enzyme. A comparative study of the enzymatic hydrolysis of two substrates, O-pyrophospho-D, L-serine (IA) and O-phospho-D, L-serine (II), was carried out, and it was shown that the rate of cleavage of the phosphomonoester bond of II and the pyrophosphate bond of IA were similar and the enzyme was active with respect to these substrates over the same pH range (cf. Fig. 1). The influence of partial inactivation of the enzyme on its monophosphatase and pyrophosphatase activity was also considered. It was found that the partial inactivation of the enzyme produced by the incubation of equal volumes of solutions of the enzyme with acetone, with 10 N urea, and with 0.4 M solutions of $CoCl_2$ and $CdSO_4$ led in each case to completely identical changes in the phosphatase and pyrophosphatase activity (Table 1). Thermal inactivation of the enzyme (90° C, 1 hr) also caused identical losses of activity in relation to both substrates. Complete parallelism was also found in the reduction of the activity of the enzyme after thermal treatment (Table 2). It is also an important fact that the action of the enzyme on an equimolar mixture of saturated concentrations of both substrates did not lead to an amount of phosphorus equal to the sum of those liberated by each substrate separately (Table 3).

The combination of the results obtained showed that the capacity for cleaving seryl monophosphate and seryl pyrophosphate is a property of a single enzyme, i.e., the alkaline phosphatase of E. coli, and that the hydrolysis of these substrates apparently takes place at a single active center of this enzyme. This is also confirmed by the literature, where the capacity of the alkaline phosphatase of E. coli for hydrolyzing pyrophosphate bonds of inorganic polyphosphates and of nucleoside polyphosphates has been reported previously [8]. All these facts indicate a less well-defined specificity of the alkaline phosphatase of E. coli than was previously thought.

Table 3

Action of the Enzyme on an Equimolar Mixture of Substrates

Substrate	Phosphorus liberated, γ
Phosphoserine (1 μmole)	14.4
Pyrophosphoserine (1 μmole)	18.0
Phosphoserine + pyrophosphoserine (1 μmole + + 1 μmole)	18.4

Experimental

The work was carried out with a sample of the alkaline phosphatase of E. coli BAP-C 61-31 of the Worthington Biochemical Corporation with a concentration of protein in the solution of 5 ml/mg. The experiments were carried out with a solution of the enzyme prepared by mixing 10 μl of the original solution with 1 ml of a 0.02 M buffer solution of tris-HCl (pH 8.0).

The substrates O-pyrophospho-D, L-serine, glycyl-O-pyrophospho-D, L-werine, and the methylamide of N-benzoyl-O-pyrophospho-D, L-serine were synthesized by the carbodiimide and acid chloride methods [5]. The experiments were carried out with approximately 0.01 M aqueous solutions of seryl pyrophosphates and phosphoserine. The exact concentration of the solutions was found from their total phosphorus content. Phosphorus was determined by the molybdate method, the intensity of the coloration being measured by a SF-4 spectrophotometer at 660 mμ [9].

In a typical experiment on the study of enzymatic hydrolysis, 100 μl of a ~ 0.01 M aqueous solution of the substrate, 500 μl of a 0.05 M buffer solution of tris-HCl with pH 8.0, and 50 μl of a solution of the enzyme (2.5 γ of protein) was incubated at 37° C for 30 min. The enzyme was inactivated by the addition of 500 μl of 35% trichloroacetic acid, and the amount of inorganic phosphate split off was determined. Blank experiments without the enzyme

were carried out in parallel.

The experiments on the influence of the pH on the hydrolysis of I and II were carried out in the presence of 100 μl of a 0.01 M solution of $Mg(CH_3COO)_2$; in the work with IC, 50 μl of substrate solution was taken. 0.05 M tris maleate buffer solutions with pH 5.0 and 5.5, buffer solutions of tris-HCl with pH 6.0–9.0, and borate buffer solutions with pH 0.5–11.0 were used.

In the study of the dependence of the hydrolysis on the time, compounds IA and IB were incubated for from 10 to 50 min. In the experiments on the inactivation of the enzyme by inhibitors, mixtures of equal volumes of an enzyme solution with the following substances were incubated at 20° C: acetone (4 hr), 10 M urea (3 hr), and 0.1 M solutions of $CoCl_2$ or $CdSO_4$ (1 hr). Experiments with the inactivated enzyme (50 μl of enzyme plus 50 μl of water) and experiments with 100 μl of the enzyme–inhibitor (1:1) mixture were carried out in parallel.

In the study of thermal inactivation, a solution of the enzyme was heated at 90° C for 1 hr and was rapidly cooled to 20° C, and the activity of 100 ml of the enzyme solution was determined immediately after it had been cooled and also after incubation at 20° C for 1, 4, and 24 hr; experiments with 100 μl of inactivated enzyme were carried out in parallel. The action of the enzyme on an equimolar mixture of substrates was studied under the conditions of a typical experiment.

Paper chromatography was carried out by the descending method in the propanol–2N ammonia (70:3) system, and paper electrophoresis in pyridine–acetic acid–water buffer, pH 4.3, at 900 V. The chromatograms and electrophoregrams were revealed with ninhydrin and a reagent for phosphorus [10].

The sample of the alkaline phosphatase of E. coli was kindly provided by Prof. F. Lipman (Rockefeller Institute, New York).

Summary

It has been shown that the pyrophosphate bond of seryl pyrophosphates is cleaved by the alkaline phosphatase of E. coli.

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