THE ACTION OF THE ACID PHOSPHOMONOESTERASE OF WHEAT BRAN ON THE METHYLAMIDE OF N-BENSOYL-O-PYROPHOSPHOSERINE

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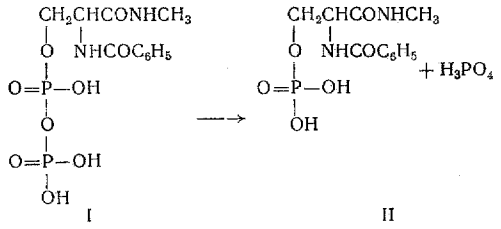
One possible approach to solving questions concerning the chemical nature of the phosphate bonds of phosphoproteins and their conversions in metabolism is the synthesis of model compounds and the investigation of their properties. Therefore, we have developed methods for the synthesis and have studied some properties of diseryl pyrophosphates [1] and seryl pyrophosphates [2].

The pyrophosphate bonds of the seryl pyrophosphates are readily hydrolyzed by such enzymes as the alkaline phosphatase of E. coli [3] and the inorganic pyrophosphatase of yeast [4], while O-pyrophosphoserine is hydrolyzed by the acid phosphatase of wheat bran [5]. When serine or ethanolamine is present in the reaction mixture, in addition to the hydrolysis of the methylamide of N-benzoyl-O-pyrophosphoserine (I) by the alkaline phosphatase of E. coli, a phosphotransferase reaction takes place, i. e., there is an enzymatic synthesis of phosphoserine or phosphoethanolamine [6].

This paper gives the results of the hydrolysis of the methylamide of N-benzoyl-O-pyrophosphoserine (I) by the acid phosphatase of wheat bran in order to elucidate the possibility of this enzyme's catalyzing the phosphotransferase reaction with the participation of seryl pyrophosphate (I) and p-nitrophenyl phosphate. It was established that under the action of the acid phosphatase the seryl pyrophosphate (I) is rapidly hydrolyzed, forming, in the first place, the methylamide of N-benzoyl-O-phosphoserine (II) and phosphoric acid.



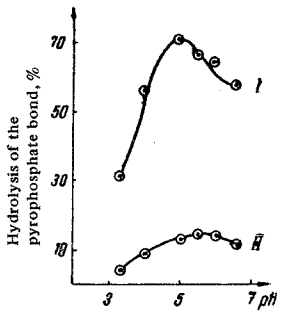
I II

We have investigated the dependence of the percentage of enzymatic cleavage of seryl pyrophosphate (I) on the pH of the reaction medium (figure). It follows from the results obtained that seryl pyrophosphate (I) is hydrolyzed by wheat bran phosphatase at a high rate: the enzyme exhibits its greatest activity in the pH range from 5.0–5.5. To compare the rates of cleavage of pyrophosphate and phosphomonoester bonds, the hydrolysis of seryl monophosphate (II) was also performed (see figure). It must be emphasized that the rate of cleavage of the pyrophosphate bond of seryl pyrophosphate (I) is considerably higher than that of the phosphomonoester bond of seryl monophosphate (II). Thus, at pH 5.0 (37° C) in an acetate buffer, after 15 min seryl pyrophosphate (I) is cleaved to the extent of 71% and seryl monophosphate (II) to the extent of 12.8%.

The composition of the phosphate buffer has little influence on the percentage hydrolysis of I and II (at pH 5.0 in a citrate buffer, I is 65.4% hydrolyzed and in an acetate buffer 71%). It has been reported previously that the composition of the buffer has a substantial influence on the rate of hydrolysis of O-pyrophosphoserine (at pH 5.5 in a citrate buffer O-pyrophosphoserine was hydrolyzed to the extent of 53%, in an acetate buffer to the extent of 16% [5]).

It is known that many alkaline [7] and acid [8] phosphatases possess a phosphotransferase, besides a phosphatase, activity. It was necessary to elucidate the capacity of the acid phosphatase for catalyzing the synthesis of phosphate esters. As the donor of the phosphoric acid residue we used the seryl pyrophosphate I and as the acceptor ethanolamine. An analysis of the reaction mixture showed that the hydrolysis of the seryl pyrophosphate I by the acid phosphatase from wheat bran in the presence of an acceptor is not accompanied by the phosphotransferase reaction. It remained unclear with what the absence of synthetic activity was connected—with features of the donor or of the

enzyme. Consequently, investigations were carried out with one of the most widely used donors in phosphotransferase reactions of phosphomonoesterases—p-nitrophenyl phosphate. The course of the reaction was monitored by determining the amounts of p-nitrophenyl and phosphoric acid. Glucose, ethanolamine, methanol, propanol, glycerol, and butane-1, 4-diol were used as the acceptors. The ratio of the concentration of p-nitrophenyl phosphate to that of the acceptor was varied in the following way for glucose: 1 : 12.5; 1 : 25; 1 : 50; 1 : 100; 1 : 200; 1 : 300; 1 : 1000; and for ethanolamine: 1 : 100; 1 : 200; 1 : 300; 1 : 500. For the other acceptors this ratio was 1 : 500. The time of incubation with glucose and ethanolamine was from 5 to 30 min and with propanol from 15 min to 3 hr. For the other acceptors the time of incubation was 15 min, the reaction being carried out at 25 and 37° C and at pH 5.5—and also pH 5.2 in the case of propanol. However, in all cases equimolar amounts of p-nitrophenol and inorganic phosphate were detected. All these results show the absence of phosphotransferase activity in the acid phosphatase. The results obtained permit the assumption that the structure of the active center and the mechanism of the action of the acid phosphatase of wheat bran differ from those for the nonspecific alkaline and acid phosphatases with a phosphotransferase activity.



Hydrolysis of the pyrophosphate bond of seryl pyrophosphate (I) and the phosphomonoester bond of seryl monophosphate (II) by the acid phosphatase from wheat bran as a function of the pH.

It is reported in the literature that a preparation hydrolyzing monophosphates and phytin, but possessing no phosphotransferase activity, has been isolated from the seeds of rye [9].

EXPERIMENTAL

The work was carried out with a freeze-dried preparation of the acid phosphatase of wheat bran of the film of "Reanal", Hungary; an aqueous solution containing 1.5 mg in 1 ml was prepared. The methylamide of N-benzoyl-O-pyrophosphoserine (I) and the methylamide of N-benzoyl-O-phosphoserine (II) were synthesized as described previously [10–11]. The amount of inorganic phosphate was determined by a modification of the Fiske-Subbarow method [12] and that of p-nitrophenol spectrophotometrically by measuring the density of its coloration in an alkaline medium at 400 mμ and comparing it with the density of a standard solution of p-nitrophenol.

The concentrations of the solutions of seryl pyrophosphate (I), seryl monophosphate (II), and p-nitrophenyl phosphate were found by mineralizing the substances to inorganic phosphate (with $HClO_4$ at 140–150° C for 3 hr).

Analysis of the reaction mixtures. The analysis was carried out on an Evans Electroselenium Limited (England) "LGD" amino acid analyzer after the halting of the reaction by the addition of an equal volume of 10% TCA, centrifuging at 3000 rpm for 10 min, and the bringing of the solution to pH 2.2. The mixture was deposited on a column (0.9 × 150 cm) of Amberlite CG-120 (Cl) resin; elution was carried out with 0.2 M citrate buffer, pH 3.25.

The reaction mixtures were analyzed by column chromatography on the anion-exchange resin Dowex 1 × 2 (200–400 mesh, 47 × 1.8 cm). The substances were eluted with a constantly increasing gradient passing from 0.1 M formic acid to 0.1 M pyridine formate (volume of the mixing vessel 650 ml). Under these conditions, phosphoethanolamine issued in the first 100 ml of eluate. The eluate was evaporated in vacuum to small volume (0.3–0.5 ml) and analyzed by paper chromatography in the propanol–2 N ammonia (70 : 30) system, by electrophoresis (1000 V, 2 hr, pH 5.6) and by mineralization with $HClO_4$ at 140–150° C for 3 hr.

Enzymatic hydrolysis. The process was studied using a reaction mixture containing 1 μM of the substrate, 2 μM

of $MgSO_4$, 50 μM of a citrate or acetate buffer, and 150 μg of enzyme in 1 ml; the mixture was incubated at 37° C for 15 min. The reaction was stopped by the addition of an equal volume of 10% TCA, and the amount of inorganic phosphate split off was determined.

Phosphotransferase activity of the acid phosphatase. The investigation was carried out in a reaction mixture containing 5 μM of the seryl pyrophosphate I or p-nitrophenyl phosphate, 500 μM of ethanolamine previously brought to pH 5.5 by the addition of HCl, and 250 μg of enzyme in 0.5 ml. The mixture was incubated at 37° C for 30 min and was analyzed on the amino acid analyzer.

A reaction mixture (pH 5.5) containing 5 μM of seryl pyrophosphate (I), 1000 μM of ethanolamine, and 135 μg of enzyme in 0.5 ml was kept at 37° C for 30 min and was analyzed by column chromatography.

A mixture containing 1 μM of p-nitrophenyl phosphate, 500 μM of methanol, propanol, glycerol, glucose, or butane-1, 4-diol, 50 μM of acetate buffer with pH 5.5, and 115 μM of enzyme in 0.5 ml was incubated at 25° C for 15 min. The reaction was stopped by the addition of 10% TCA. The amounts of p-nitrophenol and phosphoric acid were measured in an aliquot part of the resulting solution. In all cases the formation of equimolar amounts of the two hydrolysis products was observed.

How the concentration of the acceptor affects the phosphotransferase activity of the enzyme was determined by several methods: A) in a reaction mixture containing 5 μM of p-nitrophenyl phosphate, 500, 1000, or 2000 μM of glucose, 50 μM of acetate buffer (pH 5.5), and 150 μg of enzyme in 0.5 ml; the mixture was incubated at 25° C for 10 min and the amounts of p-nitrophenol and phosphoric acid liberated were determined in an aliquot part of the reaction mixture, equimolar amounts of the substances being found; B) a reaction mixture (pH 5.5) containing 5 μM of p-nitrophenyl phosphate, 500, 1000, or 1500 μM of ethanolamine, and 150 μg or 600 μg of enzyme in 0.5 ml was incubated at 37° C for 5 min. The subsequent procedure was as in (A).

An investigation of the influence of the time of incubation on the phosphotransferase activity of the enzyme was carried out: in a reaction mixture (pH 5.5) containing 5 μM of p-nitrophenyl phosphate, 500 μM of ethanolamine, and 150 μg of enzyme in 0.5 ml; the mixture was incubated at 37° C for 5, 20, and 40 min; b) a reaction mixture containing 1.0 μM of p-nitrophenyl phosphate, 500 μM of propanol, 50 μM of acetate buffer (pH 5.5 or 5.2) and 30 μg of enzyme in 0.5 ml was incubated for 1 or 2 hr at 25° C. In all cases the formation of equimolar amounts of p-nitrophenol and inorganic phosphate took place.

The seryl-containing compounds used in the work were derivatives of D, L-serine.

CONCLUSIONS

The pyrophosphate bond of the methylamide of N-benzoyl-O-pyrophosphoserine is cleaved by the acid phosphatase from wheat bran. The rate of cleavage of the pyrophosphate bond is considerably higher than that of the phosphomonoester bond. The acid phosphatase preparation from wheat bran does not catalyze the phosphotransferase reaction, which shows a difference in the structures of the active centers and in the mechanisms of the action of the acid phosphatase of wheat bran and the alkaline phosphatase of E. coli.

REFERENCES

1. S. M. Avaeva, M. M. Botvinik, and I. F. Syromyatnikova, ZhOKh, collection: The Chemistry of Natural Compounds, Their Analogs and Fragments [in Russian], 154, 1965.

2. S. M. Avaeva, S. N. Kara-Murza, and M. M. Botvinik, ZhOKh, 36, 1509, 1965.

3. S. M. Avaeva, S. N. Kara-Murza, N. V. Ras'kova, and M. M. Botvinik, KhPS [Chemistry of Natural Compounds], 3, 328, 1967; S. M. Avaeva, S. N. Kara-Murza, G. L. Kogan, N. V. Ras'kova, and M. M. Botvinik, DAN SSSR, **172**, 1436, 1967.

4. S. M. Avaeva, S. N. Kara-Murza, and M. M. Botvinik, Biokhim., **32**, 5, 1967.

5. S. M. Avaeva, G. L. Kogan, S. N. Kara-Murza, and M. M. Botvinik, Vestnik MGU, ser. khim., no. 4, 87, 1967.

6. S. M. Avaeva, N. V. Ras'kova, and M. M. Botvinik, Biokhim., **33**, 847, 1968.

7. O. Meyerhof and H. Green, Science, **110**, 503, 1949; R. Morton, Biochem. J. **70**, 139, 1957; J. Wilson, J. Dayan, and K. Cyr, J. Biol. Chem., **239**, 4182, 1964.

8. B. Axelrod, J. Biol. Chem., **172**, 1, 1948; O. Meyerhof and H. Green, J. Biol. Chem., **178**, 655, 1949;

R. Morton, Biochem. J. **70**, 150, 1958; J. Ullrich and M. Calvin, Biochem. Biophys. Acta, **63**, 1, 1962.
9. C. Anagnostopoulos and A. Lino, Bull. Soc. Chim. Biol., **39**, 781, 1957.
10. S. M. Avaeva, N. V. Ras'kova, and A. T. Mevkh, Vestnik MGU, no. 4, 121, 1968.
11. S. M. Avaeva, M. M. Botvinik, and I. F. Syromyatnikova, Acta Chim. Hung, **44**, 223, 1965.
12. H. Weil-Malherbe and R. Grenn. Biochm. J., **49**, 286, 1951.

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