TRYPTIC HYDROLYSIS OF INORGANIC PYROPHOSPHATE MODIFIED BY PHOSPHATE AND O-PHOSPHOETHANOLAMINE

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UDC 577.15.04

By the peptide map method, a phosphorylated peptide has been isolated from a tryptic hydrolysate of phosphorylated yeast inorganic pyrophosphatase (I), and this is a direct proof of the formation of a covalent bond between (I) and phosphate in the course of this reaction. The isolation and analysis of the peptide from the tryptic hydrolysate shows that the phosphate acceptor is probably the aspartic acid residue 240 or 248. Analysis of a tryptic hydrolysate of (I) modified with O-phosphoethanolamine has shown that O-phosphoethanolamine forms an amide bond with the carboxy group of the same aspartic acid residue. In an alkaline medium, the phosphate residue migrates to the imidazole ring of a histidine residue, apparently that present in position 222.

Yeast inorganic pyrophosphatase is an enzyme that catalyzes the hydrolysis of pyrophosphoric bonds in pyrophosphate and ester of phosphoric acid. It is a metal-dependent enzyme exhibiting activity only in the presence of activator metals such as Mg^{2+}, Zn^{2+}, and Mn^{2+}.

Recently, it has become known that the reaction with the product of enzymatic hydrolysis, inorganic phosphate, takes place at two centers of the enzyme: at a low concentration of p_i (1.0 mM). a specific noncatalytic center is phosphorylated, and with a further increase in the concentrate of phosphate the synthesis of pyrophosphate in the active center of the enzyme also begins [1]. The radioactively phosphorylated enzyme has previously been isolated after deep denaturation of the protein and has been partially hydrolyzed with pepsin, reduced with sodium tetrahydroborate, and subjected to complete acid hydrolysis with the subsequent isolation of labeled homoserine [2]. The most direct method of demonstrating the formation of a covalent bond between a protein and phosphate is the tryptic hydrolysis of the phosphorylated protein and the isolation of phosphorylated peptides. This type of investigation opens up the possibility of the subsequent localization of the phosphate acceptor in the amino acid sequence. However, the extremely high lability of the derivative with an acyl phosphate bond that was formed and, as a consequence, the large unavoidable losses in the course of isolation and purification of the phosphorylated peptide must be borne in mind.

Modification of the noncatalytic center is also achieved by the use of certain phosphoric acid derivatives. A typical representative of such compounds is O-phosphoethanolamine. According to a proposed scheme, modification of the enzyme leads to the two-point binding of the inhibitor through the formation of acyl phosphate bond in the active center and an amide bond in another section of the protein molecule. It is assumed that the latter is realized in the specific noncatalytic center [3]. Proofs of this can be obtained by carrying out the tryptic hydrolysis of pyrophosphatase modified with O-phosphoethanolamine and comparing the composition of the peptide containing the bound inhibitor with that of the phosphorylated peptide isolated from a tryptic hydrolysate of the enzyme modified with P_i.

The solution of the problem posed is substantially facilitated by the observed possibility of isolating the phosphorylated enzyme by gel filtration. Such a phosphorylated enzyme contains 1 mole of P_i per 1 mole of enzyme. The ε-^{32}P-labeled product obtained was hydrolyzed with trypsin and the hydrolysate was investigated by the peptide map method (Fig. 1). As follows from the facts given, a labeled peptide was detected. Thus, in spite of the high lability of the acyl phosphate bond formed, tryptic hydrolysis can actually be used to prove the formation of a covalent between the protein and P_i.

M. V. Lomonosov Moscow State University. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 379-384, May-June, 1982. Original article submitted August 11, 1981.

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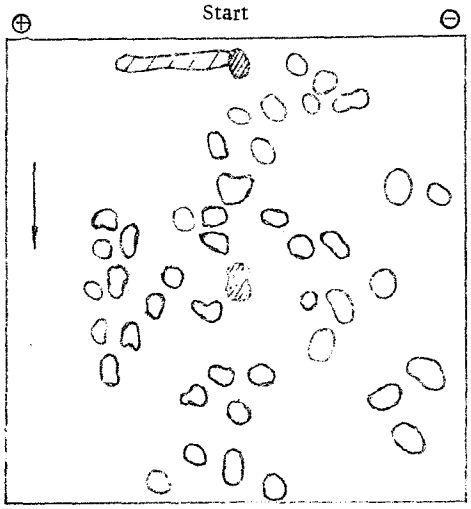

Fig. 1. Peptide map of a tryptic hydrolysate of phosphorylated pyrophosphatase. The ^{32}P-peptide is shown hatched.

The phosphorylated peptide was then isolated in the pure form. For this purpose, the phosphorylated protein was hydrolyzed with trypsin and the peptides were separated on a column of QAE-Sephadex A-50 (Fig. 2a). Three radioactive fractions were obtained. The amounts of radioactivity in two of them were extremely small, and the substance of the main radioactive fraction was investigated. The peptides of this fraction were separated in a high-performance column containing narrowly fractionated Sephadex G-50 (Fig. 2b). A single radioactive substance was detected in the eluate. The further purification of the labeled peptide was carried out by unidimensional chromatography on a cellulose plate. The radioactive fraction consisted of four peptides, only one of which contained phosphate. After rechromatography of the labeled peptide under the same conditions, the N-terminal amino acid was determined by the dansylation method. The peptide investigated was homogeneous and contained only one N-terminal amino acid — glycine. The primary structure of inorganic pyrophosphatase is known [4], and of the 35 possible peptides of tryptic hydrolysis only one begins with Gly, this occupying the position from residue 238 to residue 254 in the amino acid sequence: Gly-Ile-Asp-Leu-Thr-Asn-Val-Thr-Leu-Pro-Asp-Thr-Pro-Thr-Tyr-Ser-Lys. The results obtained permit the hypothesis to be suggested that the phosphate acceptor is the aspartic acid residue in position 240 or that in position 248. Unfortunately, the lability of the acyl phosphate bond made it impossible to isolate preparative amounts of the phosphorylated peptide necessary for determining its amino acid composition.

An analogous peptide was isolated from a tryptic hydrolysate of the pyrophosphatase inactivated with ^{3}H-labeled phosphoethanolamine, which was obtained as described by Cohen et al. [4]. The modified pyrophosphatase was subjected to cyanogen bromide cleavage. The hydrolysate was chromatographed on Sephadex G-50. The fractions containing radioactivity were combined and treated with trypsin. The tryptic hydrolysate was separated on Bio-Gel P-4 (Fig. 3). Two radioactive fractions were obtained. Further purification of the labeled peptides was carried out by two-dimensional chromatography on a cellulose plate.

The first radioactive fraction consisted of three peptides only one of which contained O-phosphoethanolamine. This peptide, just like the peptide isolated from the phosphorylated protein, had glycine at the N-end. Its amino acid composition corresponded to the structure of the peptide given below (the number of lysine residues is taken as unity):

Amino acid	Found	Literature data	Amino acid	Found	Literature data
Asp	2 93	3	Val	1,15	1
Thr	2,80	2	Ile	1,03	1
Ser	1,20	1	Tyr	0,83	1
Pro	1,84	2	Leu	2,0	2
Gly	2,05	1	Lys	1	1

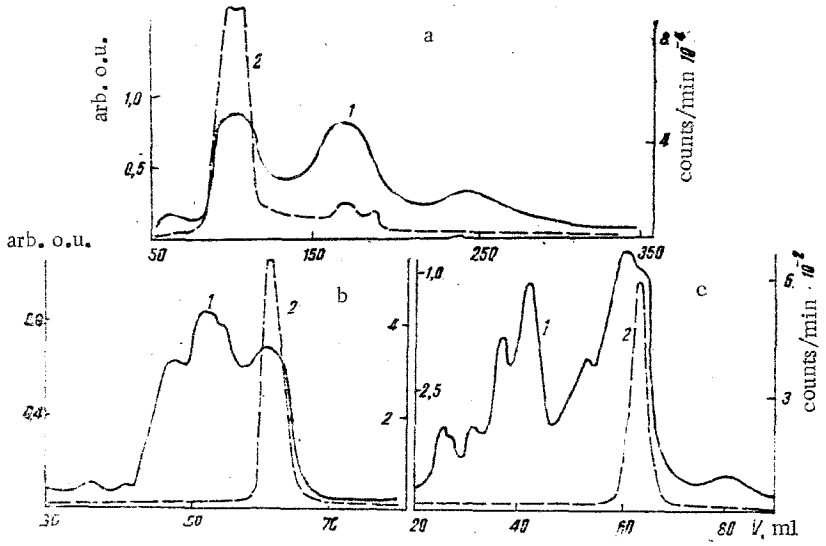

Fig. 2. Chromatography of a tryptic hydrolysate of the phosphorylated pyrophosphatase on QAE-Sephadex A-50 (a); gel filtration of the phosphorylated peptide on Sephadex G-50 (b) and of a tryptic hydrolysate of the phosphorylated pyrophosphatase kept at pH 13.0 (c): 1) absorption at 206 nm; 2) radioactivity; c) absorption at 280 nm.

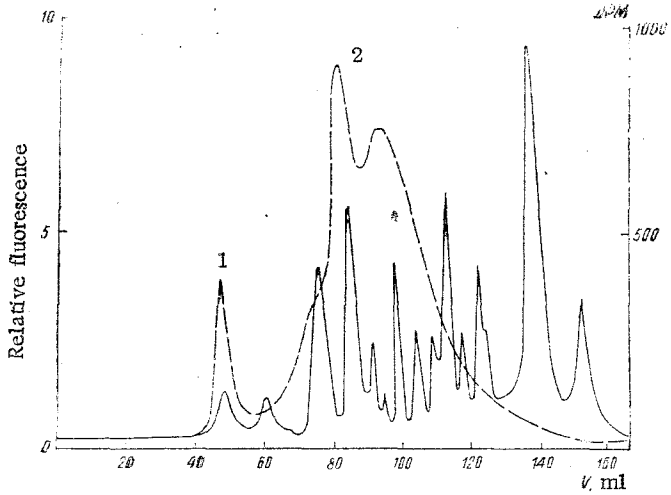

Fig. 3. Separation of tryptic hydrolysate of O-phosphoethanolamine-modified pyrophosphatase on Bio-Gel P-4. 1) Revelation with fluorescamine; 2) radioactivity.

In addition to the instability of the phosphopeptide formed on hydrolysis of the phosphorylated enzyme (E-P) with trypsin, there is another factor which complicates its isolation. In addition to phosphoaspartic acid, E-P contains phosphohistidine, formed as the result of the migration of a phosphate residue to histidine in an alkaline medium [5]. We have observed this process previously and have considered it in more detail in the present study. Phosphohistidine is formed already when the phosphorylated pyrophosphatase is kept at pH 7.2, but at pH 13.0 its amount rises considerably, reaching 10%. A subunit of the enzyme contains 6 histidine residues, and it appeared of interest to investigate whether selectivity exists in the migration process or whether several of the histidine residues of the protein molecule may be phosphate acceptors.

The phosphorylated peptide containing 1 mole of P_i per 1 mole of enzyme was kept at pH 13.0. The amount of phosphate in it fell to 0.1 mole per 1 mole of protein through the hydrolysis of the acylphosphate bond under these conditions. The phsophorylated enzyme was

hydrolyzed by trypsin, and the hydrolysate was separated on narrow-fractionated Sephadex G-50 (Fig. 2c). Only one radioactive peak was detected. Fractions containing radioactivity were chromatographed on a cellulose plate. The only fluorescamine-positive substance was subjected to acid hydrolysis, and its amino acid composition was determined. From a comparison of the results obtained with the amino acid sequence of the enzyme it is possible to assume that the fraction isolated consisted of a mixture of two heptapeptide located in the protein molecule from residues 183 to 189 and from 220 to 226 and having the following structures: Ala-Thr-Asp-Glu-Trp-Phe-Arg and Glu-Thr-His-Asn-Ser-Trp-Lys.

The method used in this investigation were based on the separation of peptides according to molecular weights and hydrophobicities. They proved inadequate to separate two peptides close with respect to the properties mentioned. It must be pointed out that the R_f values that we obtained and the positions of issuance of the peptides in gel filtration agree well with the results given for these peptides by Heinrikson et al. [14].

Since only one of these peptides contained a histidine residue, it may be assumed that is just this which is the phosphate acceptor on rearrangement. It is likely that in alkaline medium the histidine 222 residue is spatially close to the acylphosphate bond.

The results obtained are of interest in connection with the x-ray structural analysis of yeast inorganic pyrophosphate which is being carried on intensively at the present time [6].

EXPERIMENTAL

A homogeneous preparation of inorganic pyrophosphate from bakers' yeast was obtained by the method of Braga et al; it had an activity of 600 IU [7].

The following reagents were used: dithioerythritol, HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate), and trypsin free from chymotrypsin, from the USA; N-ethylmaleimide from the United Kingdom; cyanogen bromide from the GFR; carrier-free radioactive phosphoric acid with a specific radioactivity of 5-20 mCi/ml from the United Kingdom; and [^{3}H]-O-phosphoethanolamine with a specific radioactivity of 4 Ci/μmole obtained from the unlabeled compound at the Institute of Chemical Physics, Academy of Sciences of the USSR.

The phosphorylated inorganic pyrophosphate and the enzyme modified with O-phosphoethanolamine were obtained as described previously [1, 3]. N-Terminal amino acids were determined by the dansylation method followed by two-dimensional chromatography of the DNS-(amino acid)s in polyamide films [8]. The amino acid compositions of the peptides were determined on a LKB amino acid analyzer.

Tryptic Hydrolysis of the Phosphorylated Enzyme. Trypsin was added to a 10 μM solution of the phosphorylated enzyme in 0.2 M HEPES-NaOH buffer, pH 7.6 (1:50) and the reaction was carried out at 37°C for 60 min.

Peptide Map. The trypsin hydrolysate obtained in the hydrolysis of 1 mg of the phosphorylated protein was subjected to electrophoresis in the pyridine—acetic acid—water system, pH 6.76, under the following conditions: 30 min at a voltage of 0.5 kV and 4.5 h at 1.3 kV.

After drying, chromatography was carried out in the perpendicular direction in the butan-1-ol—acetic acid—pyridine—water (15:3:10:12) system.

The radioactive substances were detected with the aid of a recording apparatus, and the peptides were revealed with fluorescamine.

Separation of the Phosphorylated Peptides on QAE-Sephadex A-50. In 3.5 M ammonium bicarbonate solution, the mixture of peptides obtained in the hydrolysis of 20 mg of phosphorylated enzyme was deposited on a column (1.7 × 80 cm) of QAE-Sephadex A-50, and elution was carried out in a concentration gradient of NH_4HCO_3 from 0.35 to 1.2 M (2 × 280 ml). The optical density of the eluate at 206 nm was measured continuously on a Uvicord III. The radioactivities in the fractions were determined in a LKB liquid scintillation counter.

Separation of the Phosphorylated Peptides on Sephadex G-50. The mixture of peptides in a volume not exceeding 0.3 ml was deposited on a column (1 × 100 cm) of Sephadex G-50 (superfine) prepared as described by Gankina et al. [9] and equilibrated with 0.06 M ammonium acetate. Elution was carried out with 0.06 M ammonium acetate at the rate of 3.2 ml/h. The issuance of the peptides was monitored as described above.

Isolation of the Peptide Containing O-Phosphoethanolamine. The [^{3}H]-O-phosphoethanolamine-modified pyrophosphatase (17 mg) was incubated in 1 ml of 70% formic acid containing 60 mg of BrCN at 18°C for 7 h and at 4°C for 10 h. The reaction mixture was modified. The residue was kept in 1.2 ml of 4 M urea containing 0.05% of dithioerythritol for 30 min, and then 0.4 ml of a 0.1% solution of N-ethylmaleimide was added and the mixture was incubated at 30°C for 10 min. Gel filtration of Sephadex G-50 led to the isolation of peptides containing radioactivity, and hydrolysis with 0.1% trypsin was carried out for 4 h. The peptides were separated on Bio-Gel P-4 using as the eluting solvent a mixture of 10% formic acid and 10% n-propanol and then by two-dimensional chromatography on a cellulose plate. The single radioactive compound was extracted with 6 N HCl and the N-terminal amino acid in it and its amino acid composition were determined after hydrolysis at 105°C for 24 h.

Separation of the Peptides by Chromatography on Cellulose. The chromatography of the peptide was carried out on a plate (8 × 12 cm) coated with Whatman cellulose W 300 in the butan-1-ol–pyridine–acetic acid–water (15:3:10:12) system in one or two directions with subsequent revelation by ninhydrin or fluorescamine.

Formation of Phosphohistidine. The phosphorylated enzyme (15 μM) was kept at pH 7.2, 9.2, or 13.0 for 20 h. Then KOH was added to the reaction mixture to give a concentration of 3 N and the hydrolysate was separated on a column (1.5 × 30 cm) of Dowex 2 × 8 in the HCO_3^- form in a concentration gradient of potassium bicarbonate of from 0.2 to 1.6 M (2 × 150 ml).

Isolation of the Phosphohistidine-Containing Peptide. The phosphorylated enzyme (4.3 mg) in 5 ml of 0.05 M tris-HCl buffer, pH 7.2 was obtained as described above, the pH of the solution was brought to 13.0, and the mixture was left at room temperature for 20 h. Gel filtration of the reaction mixture was carried out in 0.02 M HEPES-NaOH buffer, pH 7.6, and then hydrolysis was carried out with trypsin. The peptides were separated on narrow-fractionated Sephadex G-50 and then by chromatography on a cellulose plate as described above. The only fluorescamine-positive compound was extracted with 6 N HCl and was subjected to hydrolysis at 105°C for 24 h.

The results of the amino acid analysis of the peptide obtained from a tryptic hydrolysate of the phosphorylated enzyme kept at pH 13.0 are given below (the number of lysine residues is taken as unity):

Amino acid	Relative amount	Amino acid	Relative amount
Asp	6	Tyr	2.4
Thr	4.9	Phe	4.8
Ser	1.2	His	1.6
Glu	4.5	Lys	1
Ala	4.1	Arg	3.7

SUMMARY

The reaction of pyrophosphatase with phosphate and O-phosphoethanolamine leads to the formation of a covalent bond with the carboxy group of an aspartic acid residue probably occupying position 240 or 248 in the amino acid sequence.

In an alkaline, medium, migration of the phosphate residue from the carboxy group to the imidazole ring of a histidine residue located under these conditions close to the acyl phosphate bond takes place. This residue is apparently the histidine-222 residue.

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CHARACTERISTICS OF THE MOLECULAR-MASS PARAMETERS AND VISCOSITIES OF SOLUTIONS OF THE LIPOPOLYSACCHARIDE–PROTEIN COMPLEX FROM *Yersinia pseudotuberculosis*

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UDC 576.851.45+576.809.7+541.24

The lipopolysaccharide–protein complex (LPPC) from *Y. pseudotuberculosis* isolated by extraction with trichloroacetic acid has been investigated by the methods of gel permeation chromatography, light scattering, and viscometry. The molecular-mass distributions of aggregates of the LPPC in water, 0.03 M Tris-HCl buffer (pH 8) and 0.1 M sodium chloride have been determined. A dependence of the polydispersity and dimensions of the aggregates on the concentration of the polymer and on the ionic strength of the solvent has been shown. It has been established that in water the LPPC has a high characteristic viscosity which falls with an increase in the ionic strength of the solution.

The lipopolysaccharide–protein complexes (LPPCs) localized in the outer membrane of Gram-negative bacteria are of interest as structural elements of the cell walls, endotoxins, and the complete antigens of these bacteria [1]. In the manifestation of a series of immunological properties of antigens, their physical characteristics are of fundamental importance [2]. The molecular dimensions of the endotoxins isolated from different bacteria vary over a wide range [3-5]. This is due to differences in the sources of isolation of the antigens and the methods for their extraction and the differences in the physicochemical methods of investigation used. The study of the physicochemical properties of LPPCs is difficult because of their high-molecular-mass nature, their considerable heterogeneity, and their capacity for forming aggregates in aqueous solutions [6].

An estimate of the dimensions of the LPPC isolated by means of the butanol–water system from *Yersinia pseudotuberculosis* has previous been made by the light-scattering method [7].

In the present investigation, some physicochemical properties of the LPPC obtained from *Y. pseudotuberculosis* by Boivin's method [8] and purified by gel filtration on Sepharose 2B – LPPC-A [9] – and by centrifugation in a cesium chloride gradient – LPPC-A_1 [10] – have been studied by the methods of gel-permeation chromatography (GPC), light scattering, and viscometry.

The molecular-mass parameters of the LPPC particles in solution were determined by the GPC method on macroporous glasses. Distilled water, 0.03 Tris-HCl buffer (pH 8) and 0.1 M sodium chloride were used as solvents. The molecular mass distributions (MMDs) of LPPC-A in aqueous solvents determined with the aid of GPC are given in Fig. 1. The relative area of each chromatographic peak (in percentages) and the corresponding values of the weight-average molecular mass (M_W) of the particles calculated from the chromatogram corrected for the instrumental broadening function are given in Table 1.

The multimodality of the MMDs (Fig. 1) shows the pronounced polydispersity of the LPPC. Such polydispersity is due to the fact that in aqueous solutions the LPPC forms aggregates of different dimensions. The molecular masses of these aggregates and the ratio of the components in the solution depend on the concentration of the polymer and on the nature of the

Pacific Ocean Institute of Bioorganic Chemistry, Far Eastern Scientific Center, Academy of Sciences of the USSR, Vladivostock. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 384-388, May-June, 1982. Original article submitted July 8, 1981.

0009-3130/82/1803-0355$07.50