REACTION OF YEAST INORGANIC PYROPHOSPHATASE WITH INORGANIC PHOSPHATE LABELED WITH RADIOACTIVE PHOSPHORUS

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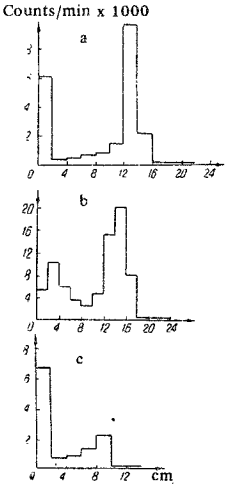
Inorganic pyrophosphatases are important enzymes of phosphorus metabolism; nevertheless, in not one of these enzymes is the nature of the functional groups present in the active center known at the present time.

One of the methods of investigating the active center which is widely used in the study of alkaline phosphates is the reaction of the enzyme with inorganic phosphorus labelled with P^{32}. Under very mild conditions these enzymes covalently bind phosphoric acid residues. In the elucidation of the nature of the functional groups participating in the binding of the phosphoric acid, acid hydrolysis of the phosphorylated protein is generally carried out and the hydrolysis products containing the radioactive phosphorus are analyzed. In the case of a number of alkaline phosphatases, [^{32}P]phosphoserine has been isolated from the acid hydrolysate, and this has permitted the conclusion that alkaline phosphates contain serine in their active centers [105].

Indirect information on the possibility of phosphorylating a pyrophosphatase has been obtained by M. Cohn [6] in a study of the exchange of inorganic phosphate with H_2O^{18} in the presence of the enzyme. The present paper gives the results of a study of the interaction of yeast inorganic pyrophosphatase with inorganic phsophate labelled with P^{32}. The enzyme was incubated at 0° C for 10 min with a large excess of monopotassium [P^{32}] phosphate. It was found that the protein treated in this way contained labelled phosphate. Since the inclusion of label can take place both by means of covalent and adsorption bonds, it appeared necessary to study the phosphorylated protein further. When high-voltage electrophoresis (1000 V, 3 hr, pH 5.6) was carried out, the protein remaining at the starting line retained radioactivity, and the partially sorbed acid migrated 14–16 cm from the start (figure, a). This gave grounds for assuming that the enzyme was bound to the phosphoric acid residue by a covalent bond. For confirmation, the phosphorylated protein was treated with 8 M urea or a 1% solution of sodium dodecyl sulfate and was again subjected to electrophoresis. In this case, the labelled phosphoric acid again remained bound to the protein. The results of the experiments performed are in favor of the assumption of a covalent bonding of the phosphoric acid residue with the inorganic pyrophosphatase. It is a fundamental fact that when the electrophoretic investigation of the phosphorylated protein eluted from the paper with sodium dodecyl sulfate solution was repeated, no additional separation of phosphorus was observed, and all the label remained bound to the protein. The inclusion amounted to about 0.4 mole per mole of protein.



Distribution of the radioactivity in the electrophoresis of the protein (a), in the chromatography of a hydrolysate of this protein (2 N HCl) (b), and in the electrophoresis of a hydrolysate of the phosphorylated protein (6 N HCl) (c).

A confirmation of covalent bonding was obtained in experiments where enzyme inactivated in 65% p-chloromercuribenzoate was brought into reaction with [P^{32}] phosphate, whereupon the inclusion of the label in the protein likewise decreased by 65%.

S. M. Avaeva and A. T. Mevkh have studied the action of yeast in organic pyrophosphatase with the methylamide of [P^{32}]-N-benzoyl-O-pyrophosphoserine [7]. In this case, likewise, a phosphorylated protein is formed. When enzymes with different specific activities were used, the inclusion of the label in the protein proved to be proportional to its specific activity, and a covalent bond of the enzyme with the substrate was formed.

What has been said above permits the conclusion that yeast inorganic pyrophosphatase binds firmly with the phosphate residue. The yield of phosphorylated enzyme does not vary when the time is increased from 10 min to 20 hr. The percentage inclusion remains constant if magnesium ions are added to the reaction mixture in a ratio of enzyme to the substrate of 1 : 1.

Since the reaction of inorganic phosphate with the pyrophosphatase takes place under very mild conditions (0° C, 10 min), it was desirable to assume that in this case, as in the case of alkaline phosphatases, one of the functional groups of the active center of the enzyme is phosphorylated. In order to determine the aminoacid that reacts with the phosphate, acid hydrolysis was carried out with 2 N hydrochloric acid at 105° C for 20 hr. However, when the hydrollysate was chromatographed on Dowex 50 × 8 resin and on paper in the trichloroacetic acid–isopropanol–ammonia–water system, no radioactive phosphoserine was found, and the only radioactive compound was phosphoric acid. When the time of hydrolysis was decreased to 10 hr, paper chromatography in the same solvent system showed that hydrolysis had not taken place completely: radioactive phosphorylated protein or its high-molecular-weight fragments remained at the starting line. Apart from labelled phosphoric acid, the mixture contained a whole series of radioactive compounds distributed over the whole front of the chromatogram and apparently consisting of phosphorylated peptides (figure, b).

An attempt was then made to hydrolyze the phosphorylated protein eluted from an electrophoregram with 6 N hydrochloric acid at 37° C. High-voltage electrophoresis (1000 V, 1 hr, pH 5.6) showed that the amount of phosphorylated protein at the starting line remained considerable even after hydrolysis for 42 hr (figure, c).

The results of the experiments performed confirmed once again that in the reaction of pyrophosphatase with inorganic phosphate a strong bonding arises, but they do not make it possible to determine the nature of the amino acid involved in the phosphorylation of the enzyme. It would appear that the unsuccessful attempt to isolate phosphoserine indicates the absence of this acid from the active center of the pyrophosphatase. Nevertheless, examples are known in which the phosphoric ester bond in phosphoserine becomes unstable under the influence of basic amino acids present in a protein molecule and is destroyed on acid hydrolysis [8]. Consequently, the absence of labelled phosphoserine from the acid hydrolysate still cannot, by itself, show that serine is not present in the active center of the enzyme. So far as concerns the phosphorylation of the carboxyl group of the dicarboxylic acids and the basic amino acids with the formation of a P—N bond, the derivatives obtained must be extremely unstable to acid. This contradicts the experiments described above on the hydrolysis of the phosphorylated protein with 6 N hydrochloric acid and 10-hr hydrolysis with 2 N hydrochloric acid, during which the protein retained its radioactivity. Apparently the probability of the phosphorylation of the enzyme at basic and acidic groups is extremely low. So far as concerns the thiol group of cysteine, it has been shown at Berlin University that it is not present in the active center of pyrophosphatase.

To answer the question of which amino acid in the enzyme is phosphorylated by the phosphate, it is proposed to perform a far-reaching enzymatic hydrolysis, the isolation of the phosphorylated fragment, and the determination of its structure.

EXPERIMENTAL

The work was carried out with a sample of yeast inorganic pyrophosphatase with a specific activity of 2–10 units obtained by Kunitz's method [9]. The concentration of protein was determined spectrophotometrically by Warburg's method. [10]. The P^{32}-labelled monopotassium phosphate had a specific activity of 1.5 μCi/mM.

Isolation of the phosphorylated protein (typical procedure). A) A mixture of 3 ml of protein solution (4–50 nM of enzyme, 10 μl (1200–15 000 nM) of monopotassium [P^{32}] phosphate, and 1 ml of 0.4 M acetate buffer, pH 5.0, was kept at 0° C for 10 min, the enzyme was inactivated by the addition of 1 ml of 2 N HCl, and the protein was precipitated with 20 ml of acetone, separated off by centrifuging, washed with acetone (4 × 10 ml), and dried in the air.

B) In the experiments with magnesium, 100 μl (1800 nM) of magnesium sulfate, 10 μl (1800 nM) of monopotassium [P^{32}]phosphate, and 1 ml of 0.4 M acetate buffer, pH 5.0, were added to the protein solution (6 nm of enzyme), and the solution was treated as described under A.

C) In a study of the dependence of the inclusion process on the time, samples each containing 3 ml of protein solution (4 nM of enzyme), 10 μl (1200 nM) of monopotassium [P^{32}]phosphate, and 1 ml of 0.4 M acetate buffer, pH 5.0, were kept at 0° C for 10 min, 1 hr, 4 hr, and 20 hr, respectively, and were then treated as described under A.

D) In a study of the dependence of inclusion on the activity of the enzyme, 720 μl of 5×10^{-4} M p-chloromercuribenzoate solution, 10 μl (1200 nM) of monopotassium [P^{32}]phosphate, and 1 ml of 0.4 M acetate buffer, pH 5.0 were added to 3 ml of protein solution (4 nM of enzyme), and the reaction was stopped as described under A. In parallel, an experiment was carried out without the addition of p-chloromercuribenzoate and also an experiment in which the protein was kept with solutions of p-chloromercuribenzoate and sodium pyrophosphate, and the activity was measured by Kunitz's method [11].

E) To purify the phosphorylated protein, the air-dried protein was dissolved in 50–200 μl of 1% sodium dodecyl sulfate solution, the solution was deposited on paper in the form of a band 2 cm wide, and vertical electrophoresis was carried out in a pyridine-acetate buffer, pH 5.6, and 1000 V for 1–3 hr. The dried electrophoregram was cut into strips 2 cm high and the radioactivities were measured on a "SELO" counter (Italy).

F) In a repeated electrophoretic study, the phosphorylated protein was eluted from the paper with a 1% solution of of sodium dodecyl sulfate, and electrophoresis was carried out as described under E.

Hydrolysis of the phosphorylated protein. 1) The dried phosphorylated protein, 2 mg of phosphoserine, and 3 ml of 2 N HCl were kept in a sealed tube at 105° C for 10–20 hr. The hydrolysate was dried over potassium oxide and phosphorus pentoxide, and the residue was dissolved in 0.5 ml of water. Part of the resulting solution was deposited on a chromatogram in the isopropanol–trichloroacetic acid–ammonia–water system, and the phosphorylated substances were revealed and the chromatogram was cut up as under E. The remainder of the solution was deposited on a column of Dowex 50 × 8 resin and was eluted with 0.01 N HCl, 2-ml fractions being collected. Their radioactivities were measured in 50-μl samples.

2) In the case of hydrolysis with 6 N HCl, the phosphorylated protein was eluted from the paper after electrophoresis with 6 N HCl, and the solution was kept in a sealed tube at 37° C for 42 hr and was subjected to electrophoresis as described under E.

CONCLUSIONS

The reaction of yeast inorganic pyrophosphatase with inorganic [P^{32}]phosphate forms a phosphorylated enzyme.

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