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THE ESSENTIAL ACTIVATED CARBOXYL GROUP OF INORGANIC PYROPHOSPHATASE

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Summary

1. A carboxyl group of high reactivity has been found in inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) from yeast. This group interacts with agents which react neither with carboxyl groups of low molecular weight compounds nor with other carboxyl groups of the protein.

2. The reaction of this activated carboxyl group with inorganic phosphate, hydroxylamine, *N*-methyl- and *O*-methylhydroxylamines, and glycine methyl ester has been studied.

3. Homoserine and homoserine lactone were found in the hydrolyzate of phosphorylated and $NaBH_4$-reduced pyrophosphatase, indicating that an aspartyl residue is phosphorylated.

4. Hydroxylamine and other nucleophilic agents cause inactivation of pyrophosphatase as a result of interaction with a carboxyl group. Both diaminobutyric and diaminopropionic acids were seen in the acid hydrolyzate of the protein treated with hydroxylamine and subjected to rearrangement in the presence of carbodiimide.

5. The ways in which the activation of a carboxyl group in the enzyme is achieved and the presumed mechanism of action of inorganic pyrophosphatase are discussed.

Introduction

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) was obtained from yeast in 1952 by Kunitz [1]. In the following 25 years considerable success has been achieved in the study of this enzyme: the subunit organization [2,3] and the regulatory role of metal ions in enzymatic activity [4—9] have been established, the primary structure [10—12] and X-ray analysis data [13] are under intensive study. The functional groups of the active site

have not yet been identified, and as a result the general mechanism of the enzyme action is not known.

The enzyme possesses a broad specificity and catalyzes not only the hydrolysis of inorganic pyrophosphate, but also that of nucleoside di- and triphosphates [14,15] and pyrophosphate monoesters [16]. Pyrophosphatase is a handy model for elucidating major properties of a number of other enzymes utilizing polyphosphates.

In this work, the presence of an activated carboxyl group in the yeast inorganic pyrophosphatase was ascertained. It was revealed by two independent methods, i.e. by the reaction of the enzyme with inorganic phosphate and several other nucleophilic agents.

Materials and Methods

Inorganic pyrophosphatase was prepared from bakers' yeast by the method of Braga et al. [17]. The specific activity of the enzyme was 750 I.U./mg (46 Kunitz units/mg). The concentration of the enzyme solution was determined assuming an absorbance coefficient $A^{0.1\%}_{280nm} = 1.45$ [1].

Pig pepsin with a specific activity of 52 units/mg, with hemoglobin as substrate, was obtained in Professor Stepanov's laboratory.

$^{32}P_i$, [^{3}H]$NaBH_4$ and [G-^{3}H]NH_2OCH_3 were obtained from Medradiopreparat Company (U.S.S.R.); [^{14}C]glycine methyl ester from Chemapol (Czechoslovakia). Homoserine and imidazole were the products of Reanal (Hungary), dimethylsulfoxide, guanidine hydrochloride and 2(*N*-morpholino)ethane sulfonic acid (MES) from Sigma (U.S.A.). Urea and hydroxylamine hydrochloride were used after three-fold crystallization.

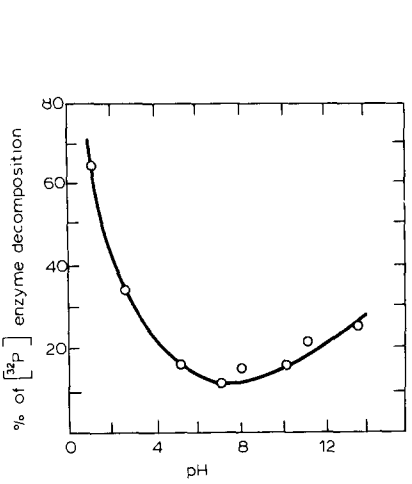
Synthesis of α-amino-δ-hydroxyvaleric acid was carried out by the method of Swallow and Abraham [18], that of $CH_3NHOH \cdot HCl$ and $NH_2OCH_3 \cdot HCl$ by the method of Semper and Lichtenstadt [19], that of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride by the method of Sheehan et al. [20] and of *N*(2-hydroxy-5-nitrobenzyl)-hydroxylamine hydrochloride according to Exner [21]. 1-Cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate was synthesized in the laboratory of Professor Knorre.

For measurement of the inorganic pyrophosphatase activity the reaction mixture (6 ml) containing 1.6 mM sodium pyrophosphate, 1.6 mM magnesium sulphate, and 16 mM ammonia/acetate or 8 mM imidazole/HCl buffer (pH 7.0) at 30°C was incubated with the enzyme (0.05—0.15 μg). 5—10 min later the enzymatic reaction was stopped by addition of 5% ammonium molybdate in 4 M sulfuric acid (1 ml) and the phosphate liberated was determined according to Weil-Malherbe and Green [22].

Results

Stability of the phosphate-protein bond in the phosphorylated enzyme

Inorganic pyrophosphatase was phosphorylated with $^{32}P_i$ as described previously; the reaction was stopped by dodecyl sulphate, and phosphoenzyme was isolated from the excess of reagent by gel filtration [23]. The lyophilized protein was incubated with a buffer at the required pH, and released P_i was



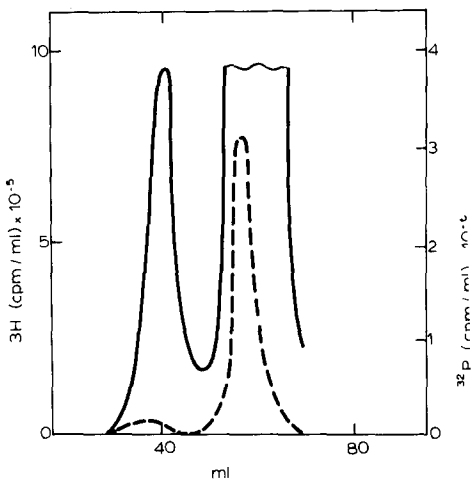


Fig. 1. pH stability profile of the phosphorylated yeast pyrophosphatase, 3 μM enzyme were incubated with 1.5 mM $^{32}P_i$ in 0.2 M acetate buffer (pH 5.0) for 10 min, at 5°C. Dodecyl sulphate was added to a 0.1% concentration and the mixture was kept overnight. The phosphorylated protein was isolated by gel filtration on a 1.25 X 25 cm Sephadex G-50 column, eluted with water and lyophilized [23]. ^{32}P-Labelled protein was incubated in buffer solutions for 2 h at 37°C; the temperature was lowered to 0°C, the solution was neutralized to pH 7 and the released phosphate was determined by the method of Suzuki et al. [24].

Fig. 2. Elution profile of reduced labelled peptides of the yeast inorganic pyrophosphatase. The enzyme (6 μM) was incubated with 1.8 mM solution of labelled phosphate in 0.2 M acetate buffer (pH 5.0) for 10 min at 3°C; then it was digested with 2.2 μM pepsin at pH 4.0, 37°C for 1 h and phosphorylated peptides were separated by gel filtration on a 1.5 X 30 cm Sephadex G-10 column. Lyophilized peptides were reduced with 25 mM [^{3}H]$NaBH_4$ (100 μCi/mmol) in dimethylsulfoxide at 37°C for 3 h, desalted on a 1.5 X 30 cm Sephadex G-25 column and the amount of ^{32}P (– – –) and ^3H (———) was determined in the fractions.

determined. The pH dependence of the dephosphorylation reaction is shown in Fig. 1. It was similar to those for low molecular weight acyl phosphates and other phosphoenzymes with a phosphorylated carboxyl residue [24–31].

Reduction of the phosphorylated enzyme with sodium [^{3}H]borohydride

The enzyme was phosphorylated with $^{32}P_i$ and partially digested by pepsin. Fractions containing ^{32}P-labelled peptides were lyophylized after gel filtration and treated with a solution of sodium [^{3}H]borohydride in dimethylsulfoxide. The reduction products were separated by gel filtration, ^{32}P and ^3H were determined in fractions. This gave us the ability to observe the tritium substitution for phosphorus in the sample (Fig. 2). The fractions containing reduced peptides were hydrolyzed for 24 h with 6 M HCl at 110°C. An enzyme sample given similar treatment but without phosphorylation served as a control.

The hydrolyzate was examined by high voltage electrophoresis at pH 1.9 as suggested by Degani and Boyer [32]. Homoserine, its lactone, and α-amino-δ-hydroxyvaleric acid were used as standards. To analyse the electrophoretograms, the latter were cut and extracted for 5 h in water at 37°C. Radioactivity was measured in a Nuclear Chicago model Mark-I or Mark-II liquid scintillation counter in a dioxan scintillator. The results are presented in Fig. 3. The sample corresponding to the phosphorylated enzyme was found to contain [^{3}H]homo-

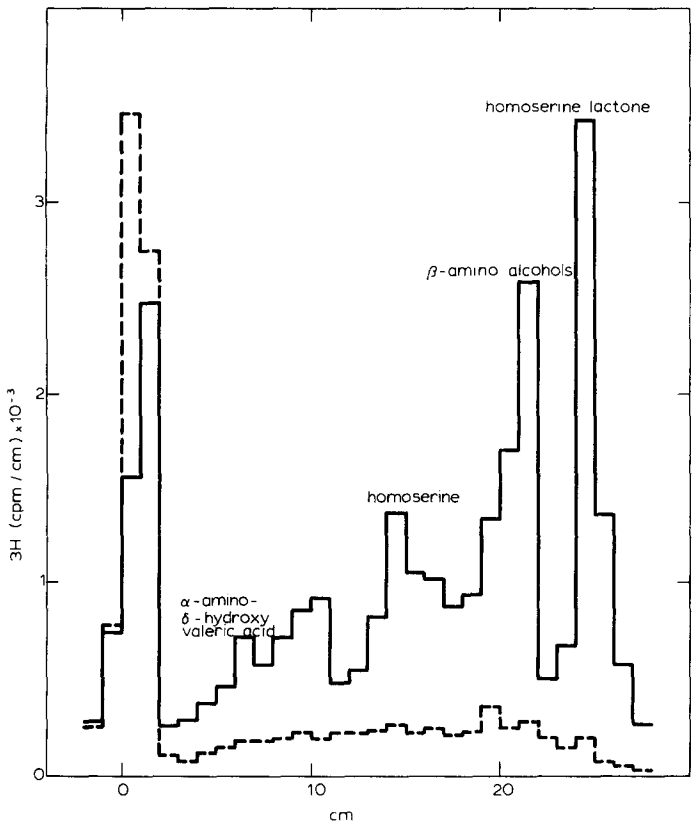


Fig. 3. High-voltage paper electrophoresis of the acid hydrolyzate of the reduced pyrophosphatase peptides. The ^{3}H-labelled peptides were hydrolyzed with 6 M HCl at 100°C for 24 h and the evaporated hydrolyzate was analyzed at pH 1.9 as described elsewhere [32]. Tritium radioactivity is presented for phosphorylated (———) and control (— — —) samples.

serine, its lactone, a small amount of α-amino-δ-hydroxyvaleric acid and amino alcohols. The latter compounds can be removed easily from the reaction mixture by using periodate oxidation [32]; under these conditions homoserine lactone converts into homoserine.

The yield of homoserine and its lactone was 40% with respect to the phosphorylated enzyme. The acyl phosphate bond is inaccessible to borohydride in the intact and dodecyl sulphate-treated protein.

Inhibition of pyrophosphatase with hydroxylamine, alkylhydroxylamines and glycine methyl ester

The enzyme was incubated with 0.1 M hydroxylamine at pH 7.0, 30°C, and assayed for activity (Fig. 4, curve 1). Fast inactivation took place and after 3 the enzymatic activity was less than 1% of the initial value. Reactivation was not observed after 500-fold dilution with 1 mM PP_i in 16 mM acetate buffer (pH 7) and incubation for 1, 2 or 24 h.

The substrate inorganic pyrophosphate completely protected the enzyme from inactivation (Fig. 4, curve 2). Mg^{2+} also displayed a protective effect, but

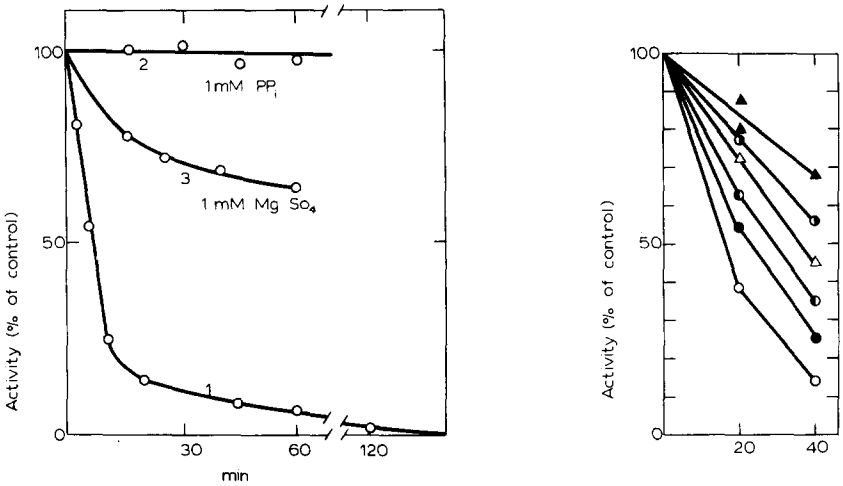


Fig. 4. Inactivation of pyrophosphatase (0.14 μM) by 0.1 M hydroxylamine at pH 7.0 and 30°C (curve 1); analogous reaction in the presence of 1 mM $Na_4P_2O_7$ (curve 2) or 1 mM $MgSO_4$ (curve 3).

Fig. 5. Dependence of the rate of inactivation of pyrophosphatase (0.14 μM) with 0.05 M hydroxylamine on the ionic strength at pH 7.0, 30°C. The values of the ionic strength were obtained by addition of NaCl: ○, 0.10; ●, 0.19; ◑, 0.39; △, 0.58; ◑, 0.77; ▲, 0.96.

their action was weaker than that of pyrophosphate (Fig. 4, curve 3).

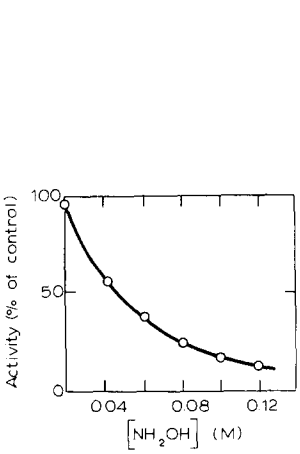
There exist two more factors affecting enzyme inactivation, i.e. ionic strength and protein concentration. Activity of pyrophosphatase, in the absence of inhibitors, strongly depends on the concentration and the nature of the salt present in the reaction mixture [33]. At a fixed concentration of NH_2OH, an increase in ionic strength masks the effect of the inhibitor (Fig. 5). In experiments with different concentrations of the inhibitor which was obtained by neutralization of $NH_2OH \cdot HCl$ with alkali, the amounts of hydroxylamine and the salt changed in parallel. Thus the change in the NH_2OH concentration from 0.02 to 0.1 M enhanced inhibition (Fig. 6); upon further increase of the inhibitor concentration the activity levelled off and then decreased. A high hydroxylamine concentration (e.g. 2 M) has no effect on the activity. The inhibiton was also very sensitive to the enzyme concentration. The effect of hydroxylamine was maximal when the enzyme concentration reached 1.5 μM and it was absent when the concentration of protein was higher than 10 μM, even when the concentration of NH_2OH was increased.

O-Methylhydroxylamine, *N*-methylhydroxylamine, *N*(2-hydroxy-5-nitrobenzyl)-hydroxylamine and glycine methyl ester are also inhibitors of inorganic pyrophosphatase. The change in the enzymatic activity in the presence of these compounds is shown in Fig. 7.

In the control experiments the inhibitor was omitted.

Interaction of pyrophosphatase with O-[^{3}H]methylhydroxylamine and [^{14}C]-glycine methyl ester

To determine the inhibitor incorporation into the protein molecule, the enzyme was incubated with *O*-[^{3}H]methylhydroxylamine or [^{14}C]glycine-



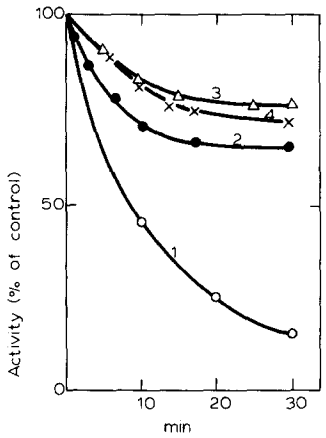


Fig. 6. Dependence of inactivation of pyrophosphatase on hydroxylamine concentration. 0.14 μM enzyme was incubated for 20 min at pH 7.0, in the presence of the indicated concentrations of hydroxylamine. The ionic strength of 0.1 was adjusted by addition of NaCl.

Fig. 7. Inactivation of pyrophosphatase (0.15 μM) by hydroxylamines. 1, 0.1 M CH_3NHOH in 0.05 M MES/NaOH at pH 7.0; 2, 0.1 M NH_2OCH_3 in 0.05 M MES/NaOH at pH 5.5; 3, 27 mM *N*(2-hydroxy-5-nitrobenzyl)-hydroxylamine in 0.05 M MES/NaOH at pH 7.0; 4, 0.5 M glycine methyl ester in 0.05 M MES/NaOH at pH 6.0.

methyl ester and its activity was assayed. Parallel experiments were carried out in the presence of pyrophosphate or with a high concentration of the protein. The results are summarized in Table I.

It may be inferred from these data that the loss of enzymatic activity (Expts. 1 and 3) corresponds with the incorporation of the label into the protein. However, in Expts. 2 and 4, where no inhibition was observed, the binding of the reagents was insignificant.

TABLE I

REACTION OF PYROPHOSPHATASE WITH [^{3}H]NH_2OCH_3 AND [^{14}C]$NH_2CH_2CO_2CH_3$

In experiments 1 and 2 the reaction mixture (1.5 ml) containing enzyme, 0.1 M [^{3}H]NH_2OCH_3 (1.75 μCi/mmol) and 0.1 M acetate buffer (pH 5.5) was incubated for 1 h. The mixture was separated on a column (1.4 × 22 cm) packed with Sephadex G-50 and 2-ml fractions were collected. The amounts of protein and radioactivity were measured in fractions. In experiment 2 sodium pyrophosphate was added to the reaction mixture. In experiments 3 and 4 the reaction mixture (1.5 ml) containing enzyme, 0.5 M [^{14}C]$NH_2CH_2CO_2CH_3$ (1 μCi/mmol) and 0.05 M MES/NaOH buffer (pH 6.0) was incubated for 1.5 h. The mixture was dialyzed against 0.05 M Tris · HCl buffer (pH 7.2), then separated on a column (1.4 × 22 cm) with Sephadex G-50 and 1-ml fractions were collected. In the fractions the amounts of protein and radioactivity were measured.

Expt. no.	Inhibitor	Enzyme (μM)	$Na_4P_2O_7$ (mM)	Inhibition (%)	Incorporation/mol inhibitor per mol protein subunit)
1	[^{3}H]NH_2OCH_3	2.0	—	35	0.38—0.42
2		2.0	1.0	0	0.07
3	[^{14}C]$NH_2CH_2CO_2CH_3$	1.1	—	35	0.45—0.50
4		13	—	0	0.05

Rearrangement of inorganic pyrophosphatase hydroxamate in the presence of carbodiimides

Our preliminary experiments on the rearrangement of acetylhydroxamic acid to methylamine by water-soluble carbodiimides showed that, in contrast to the literature data [34], the reaction is highly sensitive to the nature of carbodiimide. Thus the reaction did not occur in the presence of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate at 20°C or 50°C and at pH 4.0—6.0.

At the same time the rearrangement of acetylhydroxamic acid with 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide at pH 5.0, 20°C was completed within 20 min.

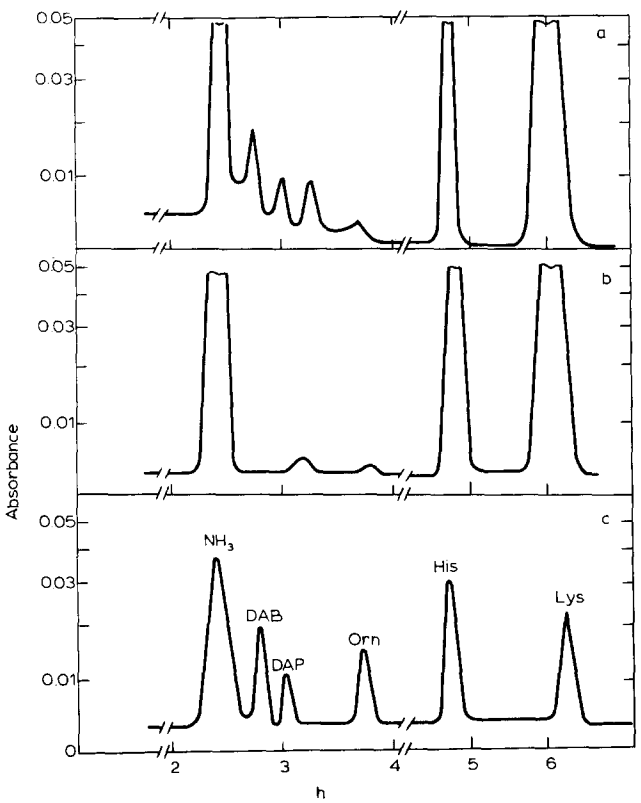


Fig. 8. (a) Composition of acid hydrolyzate of inorganic pyrophosphatase after the reaction with NH_2OH and carbodiimide. The reaction mixture (80 ml) containing enzyme (8 mg) and 0.1 M NH_2OH was incubated for 3 h at pH 7.0, 30°C, dialyzed for 40 h against 10 l of distilled water and lyophilized. The residue was dissolved in 7 ml of water (pH 5.0), 1-Ethyl(3-dimethylaminopropyl)-carbodiimide was added to 0.1 M concentration and kept for 2 h at 20°C, pH 4.5—5.0. The reaction mixture was dialyzed for 16 h against 5 l of distilled water and hydrolyzed for 24 h with 6 M HCl at 110°C. (b) Composition of the acid pyrophosphatase hydrolyzate after the reaction with carbodiimide. The reaction mixture contained, besides the components indicated in experiment a, 1 mM pyrophosphate. Treatment of the reaction mixture is similar to that in a. (c) Separation of the standard mixture containing diamino butyric acid (DAB), diaminopropionic acid (DAP) and ornithine (Orn) (1 mM each) and the rest of the components (0.25 mM each). Amino acid analysis was carried out as detailed in the Results.

TABLE II

MODIFICATION OF PYROPHOSPHATASE CARBOXYL GROUPS WITH GLYCINE METHYL ESTER IN THE PRESENCE OF WATER-SOLUBLE CARBODIIMIDES AND DENATURING AGENTS

The reaction mixture (0.6 ml) containing enzyme (0.55 mg), urea or guanidine hydrochloride, 0.1 M carbodiimide and 0.5 M glycine methyl ester hydrochloride was incubated for 2.5 h at 20°C, pH 4.75. Glycine methyl ester was completely removed by dialysis and protein was hydrolyzed with 6 M HCl at 105°C for 24 h.

Carbodiimide	Denaturing agent	Amino acid residues per mol of enzyme			Increase of glycine *
		Gly	Asp	Glu	
1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate	7.5 M urea	72.0	74.0	53.0	38.6
	5 M guanidine · HCl	72.6	78.0	58.8	39.2
1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride	7.5 M urea	127.4	74.2	52.0	94.0

* The increase in glycine was calculated using the known amino acid composition of the yeast inorganic pyrophosphatase [2].

Inorganic pyrophosphatase was incubated for 3 h with hydroxylamine, the protein was separated from the excess inhibitor and lyophilized. 1-Ethyl-3(3-dimethylaminopropyl)-carbodiimide at 0.1 M concentration was added to a water solution of protein and kept for 2 h at pH 4.5—5.0. After dialysis the protein was hydrolyzed and analyzed in an amino acid analyzer. In a control experiment, pyrophosphatase was treated with hydroxylamine in the presence of 1 mM PP_i.

Amino acid analysis was carried out in a 22-cm column packed with A-5 Aminex resin. Elution was performed with 0.38 M citrate buffer (pH 3.65) at 35°C, with a flow rate of 45 ml/h. In the protein hydrolyzate treated with hydroxylamine and carbodiimide (Fig. 8A) two new basic compounds were found, viz. diamino propionic (DAP) and diamino butyric (DAB) acids. In a control experiment, the rearrangement products were absent (Fig. 8B).

Determination of the amount of carboxyl groups in the yeast inorganic pyrophosphatase

The method of Koshland was used for this purpose [35]. The protein was incubated with glycine methyl ester and water-soluble carbodiimide in the presence of urea or guanidine hydrochloride. The hydrolysis of the protein was carried out after complete removal of the glycine methyl ester.

As can be seen in Table II, with 1-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate, 39 aspartyl and glutamyl residues per mol of the enzyme were modified independent of the nature of the denaturant, whereas the use of 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide resulted in the modification of 94 residues. This value seems to be close to the total amount of carboxyl groups in the protein. On this basis an approximate evaluation of the pyrophosphatase isoelectric point gives a value close to 5, which agrees well with the experimental data of Kunitz [1]. Thus less than 30% of protein carboxyl groups are present as amides.

Discussion

Cohn was the first (in 1958) to suggest the possibility of phosphorylation of the yeast inorganic pyrophosphatase in terms of incorporation of ^{18}O from water into phosphate in the presence of the enzyme [36]. However, the attempt of Butler et al. to obtain the phosphorylated enzyme in a reaction with inorganic pyrophosphate ended in failure [37]. Doubts about the possibility of phosphorylation of inorganic pyrophosphatase were expressed by Rapoport et al. [12]. Phosphorylated enzyme was first obtained in 1973 by some of the authors of this paper [23]. The study of the stability of the phosphate-protein bond (Fig. 1) allowed us to suggest that phosphate was accepted by a protein carboxyl group.

The direct proof of formation of the acyl phosphate has been given by reduction of phosphorylated protein with sodium borohydride. Recently, Degani and Boyer [32] introduced the borohydride reduction method to detect an acyl phosphate intermediate of ATPase from sarcoplasmic reticulum, and this method also has been applied to other enzymes [38,39].

Analysis of the experimental results leads us to the following conclusions. (1) The reaction of inorganic phosphate with the enzyme results in a covalent bond which has been formed between phosphate and the protein. (2) The product of the enzyme-phosphate interaction is an aspartyl phosphate. (3) Since it was previously shown that phosphate competes with the substrate in the phosphorylation reaction [23], it is quite possible that the aspartyl residue is located in the active center of pyrophosphatase. (4) Formation upon reduction of a small amount of α-amino-δ-hydroxyvaleric acid demonstrates partial formation of glutamyl phosphate. This fact deserves special attention and will be discussed below. (5) Formation of an energy-rich acyl phosphate bond indicates that an activated carboxyl group of the protein takes part in this reaction.

It was found thad such compounds as hydroxylamine and its *N*- and *O*-alkyl derivatives inhibited the activity of the yeast inorganic pyrophosphatase.

All the inhibitors are characterized by numerous common features. Inhibition is prevented by pyrophosphate, higher ionic strength or concentration of protein. Reaction of the enzyme with hydroxylamine and *N*-methylhydroxylamine leads to the complete loss of activity. The rest of the compounds are less active. The enzymatic activity drops sharply within 10—15 min and reaches a certain steady level which does not change afterwards. The fact is still far from being understood, but among possible explanations, special attention should be paid to the suggestion about the enzyme having two nonequivalent active sites.

More knowledge about inhibition processes was gained in the study of the labelled compounds and rearrangement of inactive enzyme into diamino acids. Since the rearrangement carried out with pyrophosphatase treated with hydroxylamine had led to the formation of diamino propionic and diamino butyric acids, the conclusion was made that hydroxylamine binds with the enzyme covalently and the site of attack, as in the reaction with phosphate, is a carboxyl group of the enzyme.

It should be emphasized that the total amount of carboxyl groups in inorganic pyrophosphatase is large, since it has been determined with glycine

methyl ester and carbodiimide in the presence of denaturing agents. It was found that out of 130 aspartyl and glutamyl residues and their amides per mol of enzyme, about 100 residues are carboxyl groups. Thus both phosphate and hydroxylamine in the reaction with carboxyl groups prove to be strictly selective.

So far there have been no direct experimental data proving that inorganic phosphate and hydroxylamine react with the same enzyme group, but this is quite possible, because the incorporation of ^{32}P into the protein molecule, by treatment of the enzyme simultaneously with $^{32}P_i$ and NH_2OH, decreases to 30% as compared to the control containing no hydroxylamine [40].

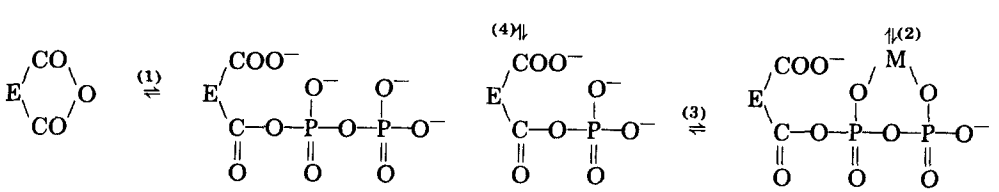
The appearance of an activated carboxyl group in the enzyme undoubtedly calls for further investigation. However, the available results allow certain assumptions to be made.

The formation of the high-energy bond between the enzyme and phosphate may be explained in thermodynamic terms. In 1975 Kanazawa [41] determined the changes in free energy, enthalpy and entropy associated with the binding of inorganic phosphate with sarcoplasmic reticulum ATPase. It turned out that it is the entropy alteration reflecting the changes in the enzyme conformation or hydration that contributes most to the reaction energetics. It is not quite clear, though, how such different compounds as phosphate, hydroxylamine and glycine methyl ester, on binding with the enzyme, cause structural changes leading to the same result, i.e. activation of the carboxyl group.

Another assumption for activation may be the existence in the protein molecule of an active carboxyl group derivative arising from chemical interaction of this carboxyl group with some functional protein groups, e.g. with another carboxyl, imidazole or SH-group. The most plausible is formation of an anhydride between aspartyl and glutamyl residues. In fact, after treatment of the protein with hydroxylamine and carbodiimide, two diamino acids are revealed at the same time. Upon reduction of the phosphorylated protein with borohydride, in addition to homoserine, a certain quantity of α-amino-δ-hydroxyvaleric acid is formed. However, in the former reaction almost equal amounts of diamino acids are formed, whereas the reaction of phosphate proceeds mainly with the aspartyl residue, apparently due to the affinity of the reagent. As described in ref. 42, the reaction with the substrate is more specific and results in the only product being formed by the aspartyl residue.

It should be emphasized that the mechanisms of both reactions are the same, since not only hydroxylamine, but also phosphate acts as a nucleophile which attacks the anhydride carbonyl atom. The reaction with phosphate is, in this case, similar to the preparation of acyl phosphates from carbonic anhydrides and phosphate [43].

With the suggestion of the existence in protein of anhydride, one may propose the following mechanism of action of inorganic pyrophosphatase:



According to this scheme, pyrophosphate reacts with the enzyme similarly to phosphate, i.e. the protein anhydride is attacked by the pyrophosphate oxygen atom (step 1). Support for the covalent intermediate with PP_i can be found in ref. 42. Further binding of the metal ion with pyrophosphate (step 2) makes hydrolysis of the pyrophosphate bond possible (step 3). It is essential that dephosphorylation of the phosphorylated intermediate (step 4) does not require water and proceeds under the action of a nucleophilic carboxyl group of the protein. It should be noted that E refers, in fact, to the complex of the enzyme with the metal activator in the above scheme.

Maintenance of the enzyme activity requires that both the anhydride and acyl phosphate should be buried in the enzyme and be inaccessible to water and other "accidental" nucleophiles. It is for this reason, apparently, that acyl phosphate does not react with borohydride in the intact enzyme.

It is evident that the presence in the enzyme of a labile, highly reactive bond makes it impossible to detect it by a standard primary structure study and calls for special experiments.

It should be stressed that inorganic pyrophosphatase is the first enzyme in which an activated carboxyl group has been revealed. However, one may believe that the existence of an activated carboxyl group is typical of a number of enzymes of phosphorus metabolism.

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