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FLUORIDE INHIBITION OF INORGANIC PYROPHOSPHATASE

II. ISOLATION AND CHARACTERIZATION OF A COVALENT INTERMEDIATE BETWEEN ENZYME AND ENTIRE SUBSTRATE MOLECULE

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Summary

A presumed pyrophosphoryl-enzyme intermediate of the reaction catalyzed by bakers' yeast inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) has been isolated using fluoride-mediated inactivation of the enzyme during catalysis. The analysis of the F^--inactivated pyrophosphatase revealed the presence of one molecule of PP_i and one atom of fluoride per active site. The incubation of the inactivated enzyme at 25°C and pH 7.2 resulted in gradual recovery of catalytic activity and concomitant removal of PP_i by a first-order reaction with $\tau_{1/2}$ of 1 h. The digestion of the F^--treated pyrophosphatase with pepsin yielded phosphorous-containing peptides, which were reduced with $NaBH_4$ and gave homoserine and homoserine lactone after acid hydrolysis. This suggests that the PP_i residue is linked to the protein through a bond of an acyl phosphate type involving the β-COOH function of aspartic acid. Together with the results of the kinetic studies of fluoride inhibition of pyrophosphatase reported in accompanying papers, these findings strongly indicate that the enzyme-substrate compound stabilized by fluoride is a transient of the catalytic reaction.

Introduction

A considerable body of evidence suggests that the hydrolysis of PP_i catalyzed by bakers' yeast inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) involves formation of covalent intermediates between the enzyme and substrate. Thus, this protein catalyzes rapid P_i-H_2O exchange [1] and can be phosphorylated by incubation with P_i [2,3]. Preliminary experiments performed with partially purified pyrophosphatase showed that the

enzyme can also tightly bind the whole molecule of several substrates [3—5]. The isolation of the enzyme-substrate or enzyme-phosphate complexes required denaturation of the protein since otherwise they decomposed on removal of the excess of the substrates or P_i. There is, however, much uncertainty about the participation of these intermediates in catalysis because it was reported that they cannot be detected in course of PP_i hydrolysis [6].

In the present work, we demonstrates that fluoride stabilizes the covalent intermediate between pyrophosphatase and PP_i to such an extent that it can be isolated without protein denaturation and studied chemically. The results shed some light on the mechanism of pyrophosphatase functioning as well as the mechanism of its high sensitivity to fluoride and they are consistent with the kinetic pattern of fluoride action on this enzyme reported in two other papers of this series [7,8].

Materials and Methods

Materials

Inorganic pyrophosphatase was isolated from bakers' yeast by the methods of Cooperman et al. [9] or Braga et al. [10]. Specific activity of the preparations at 30°C, pH 7.2 was 980—1130 units/mg (61—70 Kunitz units/mg, ref. 11). Porcine pepsin with a specific activity of 52 units/mg with hemoglobin as substrate was a gift from M.V. Gonchar. δ-Chymotrypsin was prepared in this laboratory by N.N. Vorobjeva. Sodium [^{3}H]borohydride (320—340 Ci/mol) was from Medradiopreparat (Leningrad, U.S.S.R.), disodium [^{32}P]PP_i (40—50 Ci/mol) was from Amersham and homoserine was from Reanal. Homoserine lactone and α-amino-δ-hydroxyvaleric acid were prepared according to the method of Swallow and Abraham [12]. The latter compound was further purified by paper electrophoresis. Other reagents were obtained from usual commercial sources.

Preparation of F^--inactivated pyrophosphatase

Inactivation of the enzyme by fluoride was carried out at 20°C by mixing 0.45 ml of the solution containing 0.4—1.6 mg/ml pyrophosphatase, 0.1 M imidazole · HCl buffer (pH 6.2) and 2 mM $MgCl_2$ with an equal volume of the solution containing 0.2 M NaF and 1.5 micromol of radioactive PP_i per 1 mg of the enzyme. The mixture was applied to a 0.9 × 23 cm column of Sephadex G-50 medium which had been equilibrated with 0.02—0.05 M Tris · HCl buffer (pH 7.8). The column was eluted with the same buffer at 4°C at a flow rate of 20 ml/h. The void volume was discarded, 0.35-ml fractions were collected thereafter and their content of ^{32}P was measured. The fractions containing labelled pyrophosphatase were pooled and used in further experiments.

Measurement of radioactivity

The amounts of radioactive compounds were determined using a liquid scintillation counter (Nuclear Chicago model Mark II). ^{32}P was counted in water and ^{3}H was counted in a dioxane-based scintillator with efficiencies of 50 and 42%, respectively.

Measurement of free fluoride

The concentration of the fluoride ion in solutions was determined using a fluoride-sensitive electrode (Orion Research, Inc. model 96-09). Potentials were obtained with a Radiometer PHM26 pH meter at 25°C. The measurements were conducted in a small plastic beaker having a lid with a hole for the insertion of the electrode to prevent evaporation of its content. Sample solution (1 ml) was continuously stirred with a small magnetic bar and a magnetic stirrer.

Enzyme assay

Pyrophosphatase activity was calculated from the rate of PP_i hydrolysis in 0.1 M Tris · HCl buffer (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM PP_i. The liberation of P_i was followed continuously with an automatic phosphate analyzer as described earlier [7].

Results

Pyrophosphate content of F^--inactivated enzyme

It was shown earlier [7] that pyrophosphatase loses its activity if Mg^{2+}-stimulated hydrolysis of PP_i is carried out in the presence of NaF. The inactivation of the enzyme is accompanied by incorporation of PP_i which cannot be removed by gel filtration (Fig. 1). No radioactive material was eluted at the place of the protein when pyrophosphatase or fluoride was omitted from the inactivation mixture. Moreover, the amount of the bound PP_i was found to be proportional to the specific activity of the preparation of pyrophosphatase used. The residual activity of the inactivated enzyme after chromatography was less than 5% of the original value. Passing the enzyme over the same column for a second time did not decrease the amount of the bound PP_i. The quantitation of the first peak in Fig. 1 with a correction for uncompleteness of the inactivation gave a value of 2.0 ± 0.2 mol PP_i/mol protein. Since a molecule of bakers' yeast pyrophosphatase contains two active sites [13,14], the stoichiometry of enzyme-PP_i interaction is consistent with the binding of PP_i at the active site of the enzyme.

The concentration of pyrophosphatase in this and other experiments reported below was calculated assuming an extinction coefficient $E_{280}^{0.1\%} = 1.45$ [11] and a molecular weight of 65 000 [15—18].

Since incubation of F^--inactivated enzyme at 25°C after removal of the excess of PP_i and NaF results in splitting off the label (see below), it was possible to check if the P-O-P bonds of the substrate remained intact upon its binding. The incubation was performed in the presence of EDTA in order to protect the released PP_i from hydrolysis by the enzyme, which could be activated by traces of metals in the solutions. Ion exchange chromatography of the incubate revealed the presence of two labelled compounds (Fig. 2). The bulk of the radioactivity was eluted with authentic PP_i and it was concluded that the F^--inactivated pyrophosphatase contains the whole substrate molecule. The labelled compound eluted soon after void volume seemed to be part of the enzyme-substrate complex which had not decomposed during the incubation with EDTA.

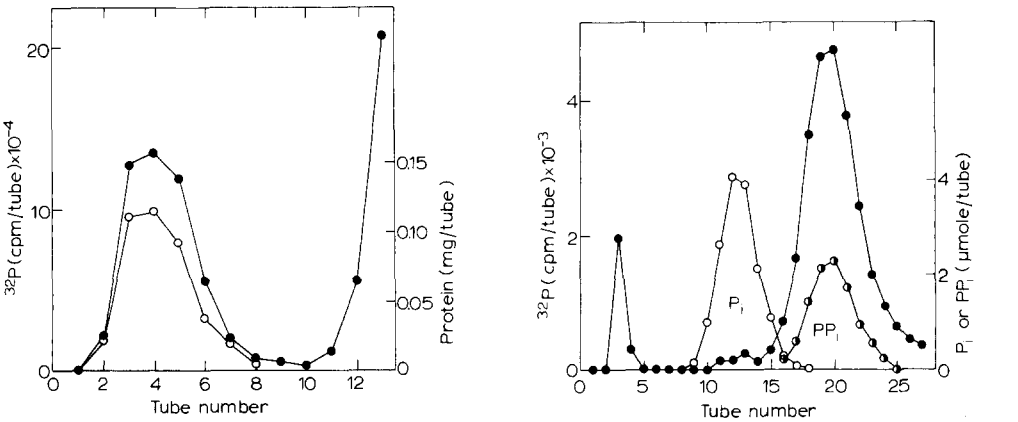

Fig. 1. Gel filtration of pyrophosphatase (0.4 mg) after inactivation with fluoride in the presence of radioactive PP_i (34 Ci/mol) and $MgCl_2$. Experimental details are given in Materials and Methods. Protein: o———o; cpm: •———•.

Fig. 2. Apparent chromatographic identity of the ^{32}P-labelled compound released by reactivation of F^--inactivated pyrophosphatase with authentic PP_i. A solution of 0.16 mg F^--inactivated enzyme (32 000 cpm) in 2 ml of 0.05 M Tris · HCl buffer (pH 7.3) obtained after gel filtration (see Materials and Methods) was made 2.5, 7.5 and 10 mM in PP_i, P_i and EDTA, respectively. The mixture was incubated for 30 min at 25°C, diluted 1 : 10 with water and fractionated at room temperature on a column (1 × 9 cm) of Dowex 1 x 8 resin in HCO_3^- form. The column was eluted with a linear gradient of 0.1—0.8 M $KHCO_3$ (2 × 50 ml) at a flow rate of 0.6 ml/min. The fractions (3.6 ml) were diluted 1 : 5 with water and aliquots were analyzed for radioactivity and orthophosphate content. To determine the elution profile for PP_i, the diluted fractions were mixed with an equal volume of 0.3 M imidazole · HCl buffer (pH 6.0) containing 20 mM $MgCl_2$ and 1 mg/l native pyrophosphatase, incubated for 20 min at 25°C to hydrolyze PP_i and analyzed for P_i. The amount of PP_i in the fractions was obtained as the difference between P_i content before and after treatment with native enzyme. P_i: o———o, PP_i: ◦———◦; cpm: •———•.

Stability of the E-PP$_i$

On removal of the excess of the substrate and inhibitor the F^--inactivated pyrophosphatase slowly recovered its activity by a first-order reaction with $\tau_{1/2}$ = 60 min (Fig. 3) with concomitant loss of PP_i. The rates of the two processes increased in the presence of 17 mM EDTA ($\tau_{1/2}$ = 23 min) and decreased in the presence of 1 mM $MgCl_2$ ($\tau_{1/2}$ = 135 min). It is seen that the curves for reactivation and splitting off the bound PP_i coincided in all cases, suggesting the interdependence of the two reactions.

Denaturation of the enzyme accelerated the decay of E-PP_i. Thus, the incubation of the F^--inactivated pyrophosphatase with 6 M guanidine hydrochloride or an iodinating mixture (1.5 mM I_2 plus 4 mM KI) for 30 min at 25°C resulted in almost complete disappearance of E-PP_i. The analysis was done in these experiments by the gel filtration method described in the legend of Fig. 3. The extraction of the protein with 88% aqueous phenol [19] also caused complete decomposition of E-PP_i judging by the fact that all radioactivity remained in the aqueous phase.

Isolation and characterization of ^{32}P-labelled peptides

The unusually high stability of the E-PP_i prepared in the presence of NaF can be explained by existence of a covalent bond between the enzyme and

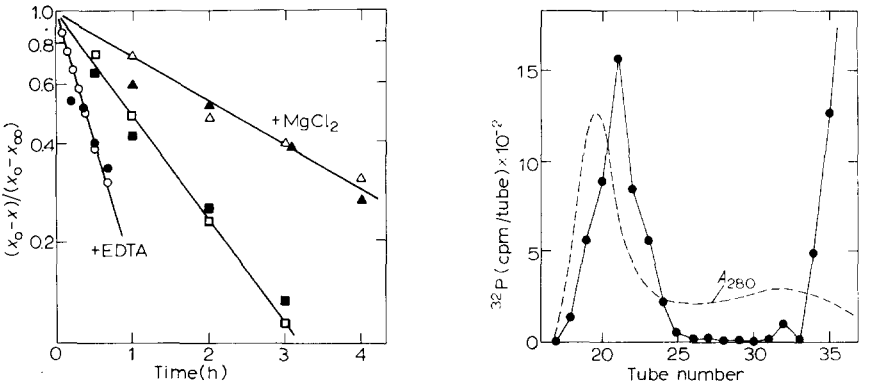

Fig. 3. Correlation of E-PP_i decay and enzyme reactivation. x_0, x and x_∞ refer to enzymic activity or the amount of the bound PP_i at time equal to zero, t and infinity, respectively. A solution of 0.35 mg F^--inactivated pyrophosphatase (75 000 cpm) in 1.4 ml of 0.05 M Tris · HCl buffer (pH 7.3) obtained after gel filtration (see Materials and Methods) was incubated at 25° C alone or in the presence of 1 mM $MgCl_2$ or 17 mM EDTA. Aliquots (0.2 ml) were withdrawn at fixed time, cooled down immediately to 0° C and assayed for enzymic activity and residual E-PP_i. The released PP_i was separated by gel filtration as described in Materials and Methods for purification of F^--inactivated enzyme and the amount of the bound PP_i was obtained from the radioactivity of the protein peak. Bound PP_i, filled symbols; enzymic activity, open symbols.

Fig. 4. Gel filtration of pepsin digest of F^--inactivated pyrophosphatase. A solution of 0.3 mg F^--inactivated enzyme (92 000 cpm) in 1.4 ml of 0.02 M Tris · HCl buffer (pH 7.3) obtained after gel filtration (see Materials and Methods) was mixed with a solution of 0.2 mg pepsin in 0.5 ml of 0.4 M acetate buffer (pH 3.9). After incubation for 30 min at 37° C the mixture was chromatographed on a 1.5 cm × 30 cm column of Sephadex G-25 medium equilibrated with water, at a flow rate of 1 ml/min; fraction size was 2 ml. A_{280} was monitored with an LKB model II Uvicord and shown in arbitrary units with a broken line.

substrate or by sequestration of the substrate into the protein globule. In order to distinguish between these possibilities the F^--inactivated pyrophosphatase was digested with pepsin and the hydrolyzate was analyzed by gel filtration. In the case of noncovalent binding, the fragmentation of the protein accompanied by distortion of its ternary structure is expected to release the buried substrate. The gel chromatography of the pepsin digest of the F^--inactivated enzyme gave the results shown in Fig. 4. It is seen that radioactivity was eluted in two peaks, one of which (tube 34 and above) contained ^{32}P as a low molecular weight compounds, most likely as P_i or PP_i. The other peak (tubes 17—25) containing about 20% of the original radioactivity of E-PP_i appeared later than that of the material absorbing light at 280 nm and was therefore retarded on the column. This means that the smaller peak of radioactivity is due to pyrophosphatase-originated peptides, since nonhydrolyzed pyrophosphatase is expected to elute with a void volume on the basis of its molecular weight. The ability of the fragments of pyrophosphatase to hold PP_i favours the hypothesis of the covalent binding of the substrate in the F^--inactivated enzyme.

Relatively low yield of ^{32}P-labelled peptides is accounted for by high lability of the bond between the enzyme and PP_i. It was shown above that even at 25° C, pH 7.3 pyrophosphatase loses about 30% of the bound PP_i on a 30-min incubation. In the conditions used for treatment with pepsin (37° C, pH 4.0)

the rate of the spontaneous decomposition of E-PP_i is expected to be even larger.

Studies of the reduction of the ^{32}P-labelled peptides with NaB^3H_4 gave further evidence for the covalent nature of the enzyme-substrate bond in the F^--inactivated pyrophosphatase. Fractions 19—23 of Fig. 4 containing ^{32}P were pooled, lyophylized, dissolved in dimethylsulfoxide and incubated with 25 mM NaB^3H_4 for 3 h at 37°C. The excess of the reducing agent was removed by gel filtration (Fig. 5). It was found that an appreciable amount of tritium remained associated with the peptides. Again, the radioactive peak was eluted

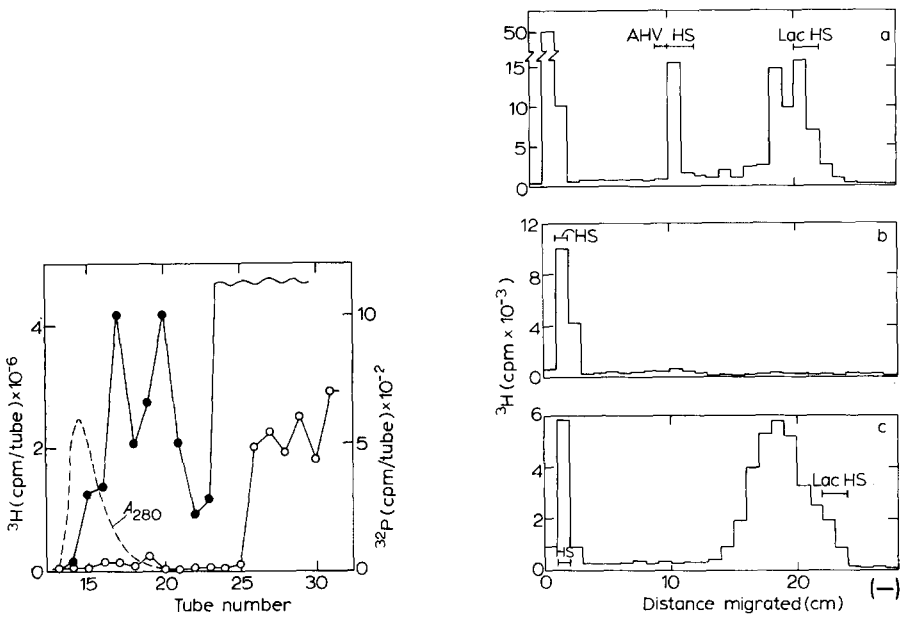

Fig. 5. Gel filtration of sodium borohydride-reduced ^{32}P-labelled peptides of pyrophosphatase. Fractions 19—23 of the first radioactive peak in Fig. 4 were combined and lyophylized. The residue was dissolved in 0.1 ml dimethylsulfoxide containing 25 mM NaB^3H_4 (320 Ci/mol). After incubation for 3 h at 37°C the mixture was diluted with water to 2 ml and chromatographed on a 1.5 × 50 cm column of Sephadex G-25 medium equilibrated with 0.05 M Tris · HCl buffer (pH 8.5). The column was eluted with the same buffer at a flow rate of 1.3 ml/min; fraction size was 2.6 ml. A_{280} was monitored with an LKB model II Uvicord and shown in arbitrary units with a broken line. ^3H: ●———●; ^{32}P: ○———○.

Fig. 6. High-voltage electrophoregrams of sodium borohydride-reduced ^{32}P-labelled peptides of pyrophosphatase after hydrolysis to amino acids. Fractions 14—18 in Fig. 5 were combined and the peptides were hydrolyzed in evacuated sealed tubes in 6 M HCl at 110°C for 24 h. The hydrolyzates were evaporated in vacuum using a rotor evaporator and the residues were taken up in 2 ml of water and dried repeatedly to remove exchangeable tritium. The final residue was taken up in 0.2 ml of water and aliquots (30 µl) were spotted on Whatman 2MM paper. Electrophoresis was carried out with a Shandon electrophorator for 2 h at 2500 V (4°C) after a desalting period of 30 min at 500 V. Strips of the dried electrophoregrams were treated with ninhydrin to localize the standards and radioactivity of 1-cm sections was counted with a liquid scintillation counter. For the repetition of the electrophoresis, the radioactive compounds were eluted from paper with water and concentrated using a rotor evaporator. A, electrophoresis of the hydrolyzate in acetic acid/formic acid/water medium (150 : 22 : 828, by vol.); B, repeated electrophoresis of homoserine of Fig. 6(a) (10—11 cm) in pyridine/acetic acid/water medium (1 : 10 : 989, by vol.); C, repeated electrophoresis of the sum of homoserine lactone and β-amino alcohols of Fig. 6(a) (16—23 cm) in the second of the above media after incubation for 1 h at 60°C, pH 12. HS, homoserine; AHV, α-amino-δ-hydroxyvalerate; Lac HS, homoserine lactone.

later than the peak of the material absorbing light at 280 nm. The reduction was accompanied by the removal of ^{32}P from the peptides. The tritiated fragments of pyrophosphatase (Fractions 14—18 of Fig. 5) were subjected to acid hydrolysis and after liberation from exchangeable label were analyzed electrophotetically. The results are shown in Fig. 6. Electrophoresis in two buffer systems (Fig. 6(a) and (b)) showed the presence of radioactive compounds with the mobilities of homoserine and homoserine lactone. The appearance of this amino acid in $NaBH_4$-reduced peptides means that PP_i is linked to them through the β-COOH function of an aspartic acid residue [20]. The product of glutamyl phosphate reduction, α-amino-δ-hydroxyvalerate, was not found on electrophoregrams. It is worth mentioning that the latter amino acid does not form a lactone [20,21]. The known fact of the conversion of homoserine lactone into homoserine in alkaline medium made it possible to further verify the identification. Fig. 6(c) shows that such conversion does occur.

The total yield of the products of aspartate reduction in three experiments was 11.5—13% of the theoretical value assuming that a molecule of pyrophosphatase contains two reducible residues. Fig. 6(c) was used to estimate the proportion of the homoserine lactone on the original electrophoregram (Fig. 6(a)) since its peak overlapped with the peak of β-amino alcohols, resulting from reduction of peptide bonds [20]. In control experiments, in which native pyrophosphatase was used instead of F^--inactivated one, the total yield of homoserine and homoserine lactone was 1.2%. One can conclude that about 50% of ^{32}P-labelled peptides were recovered as these compounds. The yield of the products of aspartate reduction dropped considerably when the pepsin digestion was omitted from the reaction scheme. This indicates that the acyl phosphate bond is inaccessible in this case because of steric limitations.

Search for transphosphorylation with F^--inactivated pyrophosphatase

The transfer of a phosphoryl or pyrophosphoryl group from the F^--stabilized pyrophosphoryl-enzyme intermediate to some nucleophiles such as fluoride, glucose, phenol and P_i was checked as a possibility. The F^--inactivated enzyme was purified by gel filtration and incubated for 3—15 h with 1 M acceptor at 20°C, pH 7.5 (with KF also at pH 5.5). The incubate was analyzed by ion exchange chromatography as described in Fig. 2. The only radioactive compounds found were P_i and PP_i with the first three nucleophiles and P_i with the last one. This means that no transfer of ^{32}P from E-PP_i to the nucleophiles occurs in these conditions.

Participation of fluoride in the stabilization of E-PP_i

The unusually high stability of the enzyme-substrate intermediate in the presence of NaF suggests also that fluoride is tightly bound to the protein and interferes with the normal course of reactions at the active site. To check this hypothesis, the inactive pyrophosphatase purified by gel filtration was analyzed for fluoride content using an ion selective electrode. Prior to the analysis, the enzyme was hydrolyzed to peptides with equimolar amount of δ-chymotrypsin. A multiple addition method [22] was accepted as the working procedure, and the data were analyzed graphically using a Gran plot [22] as shown in Fig. 7. The difference between fluoride contents of F^--inactivated and native

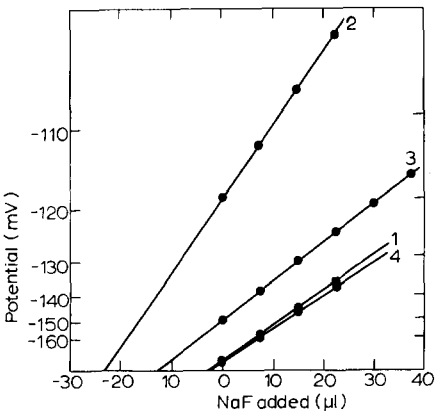

Fig. 7. Gran plot for the determination of fluoride content of pyrophosphatase with an ion selective electrode using multiple addition method [22]. Solid δ-chymotrypsin (approx. 0.3 mg) was added to 1-ml samples containing 0.05 M Tris · HCl buffer (pH 7.3) and the mixture was incubated for 24 h at room temperature. Portions (7.5 µl) of 1 mM NaF were added to the samples and equilibrium potentials were measured with the electrode. 10%-corrected Gran plot paper (Orion Research, Inc.) was used for graphical presentation of the results. The volume of the added titrant was plotted along the abscissa. The unknown original content of fluoride in the samples is given by abscissa intercept. 1, only buffer: 2, buffer plus 20 µM NaF; 3, 0.39 mg/ml F^--inactivated pyrophosphatase (16.5% residual activity) purified by gel filtration (see Materials and Methods); 4, 0.40 mg/ml native pyrophosphatase treated as the F^--inactivated one but omitting PP_i in the inactivating mixture.

pyrophosphatase was 2.1 ± 0.2 g equiv/mol protein with correction for incomplete inactivation.

The detection of stoichiometric quantities of fluoride in the preparations of F^--inactivated enzyme cannot be the result of a poor separation by gel filtration. This was confirmed by the fact that at least three 1-ml portions of the eluate following pyrophosphatase did not contain fluoride. Besides, essentially no protein-bound fluoride was found in a control experiment in which the enzyme was incubated with NaF in the absence of PP_i, purified by gel filtration, digested with δ-chymotrypsin and subjected to the analysis for F^- (Fig. 7, curve 4).

The stability of the enzyme-fluoride complex appears to be low in the absence of the hydrolytic reaction. To estimate the corresponding dissociation constant at 25°C, increasing amounts of pyrophosphatase were added to 20 µM solution of NaF in 0.1 M Tris · HCl buffer (pH 7.2). Free fluoride concentration was measured with the ion selective electrode by means of a calibration curve. The decrease in free fluoride at 10^{-5} M enzyme was less than 15% indicating that the dissociation constant for the E · F complex is higher than $1.5 \cdot 10^{-4}$ M. It should be noted that the inhibition constant for F^-, which describes the binding of the inhibitor to the enzyme in the presence of the hydrolytic reaction, is equal to $6.7 \cdot 10^{-6}$ M [7]. One may conclude that F^- and PP_i, are bound to the inactivated pyrophosphatase in such a manner that they mutually stabilize the links between them and the protein.

Discussion

Several lines of evidence lead to the conclusion that the enzyme-substrate complex isolated in the presence of NaF is an obligatory intermediate of the

catalytic reaction. First, the rate of fluoride inactivation of the enzyme and therefore binding of PP_i to it are strictly proportional to the rate of the catalytic process [7,8]. Second, the F^--treated pyrophosphatase loses its catalytic activity in PP_i hydrolysis and its recovery is correlated with the decay of E-PP_i. Third, the stoichiometry of PP_i binding by inactivation is 1 mol PP_i/mol active site.

The appearance of homoserine and homoserine lactone by reduction of F^--inactivated pyrophosphatase with $NaBH_4$ means that the substrate residue is linked to the protein through a covalent bond involving the β-COOH group of aspartic acid. The method of identification of an acyl phosphate on the protein used in this work was introduced by Degani and Boyer [20] for Ca^{2+}-dependent ATPase and subsequently applied to (Na^+, K^+)-dependent ATPase [21] and acetate kinase [23,24]. In the case of pyrophosphatase the site of attack by $NaBH_4$ was acyl pyrophosphate rather than acyl phosphate. Since acyl pyrophosphates of low molecular weight are unknown, this reaction could not be checked on a model compound. The possibility of such a reaction, however, seems quite likely. Besides, it is possible that the P-O-P bonds had been hydrolyzed by fragmentation of pyrophosphatase into peptides and $NaBH_4$ acted upon an acyl phosphate.

Our data lend much credit to an earlier suggestion [1—5] that covalent catalysis plays an important role in the mechanism of pyrophosphatase action. The overall reaction of PP_i hydrolysis appears to involve two covalent intermediates according to the following scheme:

$$E + PP_i \rightarrow E - PP_i \xrightarrow[P_i]{} E - P_i \rightarrow E + P_i$$

This mechanism, however, seems to be in disagreement with the data of Sperow et al. [6]. They arrested the enzymic hydrolysis of PP_i by addition of phenol, trichloroacetic acid or EDTA and found that no detectable incorporation of ^{32}P into the protein occurs. The reason for the discrepancy between the two studies is not apparent but two explanations can be offered. First, the stationary concentration of the covalent intermediates may be quite low compared to the total concentration of the enzyme if the steps at which they disappear are not rate-limiting. In case of E-P_i one can obtain an estimate of its stationary concentration assuming that the ^{18}O exchange between P_i and H_2O catalyzed by pyrophosphatase [1] occurs due to reversible formation of E-P_i. The rate for the decomposition of this intermediate is then at least $8 \cdot 10^5$ μmol/min · mg according to the data on ^{18}O exchange [1] while the rate constant for PP_i hydrolysis at a saturating concentration of the substrate is about 800 μmol/min · mg in similar conditions [25]. Basing on these values one can predict that only 0.1% of total protein will be present as the phosphoryl intermediate in the steady state of the hydrolytic reaction. This is less than could have been detected by the most sensitive method of those used by Sperow et al. [6]. It is possible that the rate constant for the breakdown of E-PP_i is similarly high and this transient also cannot be found by arresting the enzymic reaction. The second explanation of the discrepancy between our results and those of the literature is that the covalent intermediates might decompose during their isolation by the methods employed by Sperow et al. [6]. Really, the

complexation of metal activator by EDTA cannot give desirable stabilization since the covalent intermediates decay at a large rate even in the absence of metals [2,3]. On the other hand, the extraction with phenol makes E-PP_i quite unstable as was shown above.

The absence of the transfer of a phosphoryl or pyrophosphoryl group of E-PP_i to several nucleophiles indicates that the spatial organization of the active site prevents molecules of compounds other than water from their contact with the substrate. This conclusion agrees with the data of Sperow et al. [6] who did not observe any transfer of P_i from PP_i to ADP in course of the enzyme-catalyzed hydrolysis of PP_i. Steric limitations of the reactions at the active site of pyrophosphatase explains why the reduction of pyrophosphoryl as well as phosphoryl [26] pyrophosphatase with $NaBH_4$ needs preliminary disruption of the ternary structure by hydrolysis with pepsin.

While the fact of the binding of PP_i at the active site of the enzyme by its inactivation is evident, the same cannot be said about the binding of fluoride. The stoichiometry of the participation of the inhibitor in inactivation of pyrophosphatase, however, favours the mechanism of simultaneous binding of PP_i and F^- at the active site. This mechanism has merits of being simple and aprehensible. One can easily imagine that the presence of such a small ion as fluoride at the active side does not interfere with the binding of the substrate, especially if fluoride just substitutes a water molecule in the coordination sphere of Mg^{2+}. This substitution, however, will have large consequences for the step of the hydrolysis of the P-O-P bonds if one further assumes that the metal-bound water takes part in it.

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References

1 Cohn, M. (1958) J. Biol. Chem. 230, 369—379
2 Avaeva, S.M. and Nazarova, T.I. (1970) Khim. Prir. Soedinen. 243—247
3 Nazarova, T.I. and Avaeva, S.M. (1973) Biokhimiya 38, 169—173
4 Avaeva, S.M. and Mevkh, A.T. (1970) Biokhimiya 35, 552—555
5 Avaeva, S.M. and Mevkh, A.T. (1970) Mol. Biol. 4, 730—733
6 Sperow, J.W., Moe, O.A., Ridlington, J.W. and Butler, L.G. (1973) J. Biol. Chem. 248, 2062—2065
7 Baykov, A.A., Artjukov, A.A. and Avaeva, S.M. (1976) Biochim. Biophys. Acta 429, 982—992
8 Baykov, A.A., Artjukov, A.A. and Avaeva, S.M. (1977) Biochim. Biophys. Acta 481, 195—201
9 Cooperman, B.S., Chiu, N.Y., Bruckmann, R.H., Bunick, G.J. and McKenna, G.P. (1973) Biochemistry 12, 1665—1669
10 Braga, E.A., Baykov, A.A. and Avaeva, S.M. (1973) Biokhimiya 39, 344—350
11 Kunitz, M. (1952) J. Gen. Physiol. 35, 423—449
12 Swallow, D.L. and Abraham, E.P. (1959) Biochem. J. 72, 326—336
13 Ridlington, J.W. and Butler, L.G. (1972) J. Biol. Chem. 247, 7303—7307
14 Rapoport, T.A., Höhne, W.E., Heitmann, P. and Rapoport, S. (1973) Eur. J. Biochem. 33, 341—347
15 Schachman, H.K. (1952) J. Gen. Physiol. 45, 451—454
16 Ridlington, J.W., Yang, Y. and Butler, L.G. (1972) Arch. Biochem. Biophys. 152, 714—725
17 Hansen, G., Eifler, R. and Heitmann, P. (1972) Acta Biol. Med. Germ. 28, 977—988
18 Henrikson, R.L., Sterner, R. and Noyes, C. (1973) J. Biol. Chem. 248, 2521—2528
19 Feldman, F. and Butler, L.G. (1972) Biochim. Biophys. Acta 268, 698—710

20 Degani, C. and Boyer, P.D. (1973) J. Biol. Chem. 248, 8222—8226
21 Nishigaki, I., Chen, F.T. and Hokin, L.E. (1974) J. Biol. Chem. 249, 4911—4916
22 Liberti, A. and Mascini, M. (1969) Anal. Chem. 41, 676—679
23 Todhunter, J.A. and Purich, D.L. (1974) Biochem. Biophys. Res. Commun. 60, 273—280
24 Webb, B.C., Todhunter, J.A. and Purich, D.L. (1976) Arch. Biochem. Biophys. 173, 282—292
25 Braga, E.A. and Avaeva, S.M. (1972) Biochem. Biophys. Res. Commun. 49, 528—535
26 Avaeva, S.M., Bakulèva, N.P., Baratova, L.A., Nazarova, T.I. and Fink, N.Yu. (1977) Biochem. Biophys. Acta, in the press