Biochimica et Biophysica Acta, 321 (1973) 336–347

BBA 66974

POLYPHOSPHATE PHOSPHOHYDROLASE FROM *ENDOMYCES MAGNUSII*

TATIANA P. AFANASIEVA AND IGOR S. KULAEV

A. N. Bakh Institute of Biochemistry of the USSR Academy of Sciences, Moscow (USSR)

(Received February 9th, 1973)

SUMMARY

In cell-free extracts from *Endomyces magnusii*, a polyphosphate phosphohydrolase (EC 3.6.1.11) activity has been revealed. This enzyme catalyzes the cleavage of terminal phosphate groups from inorganic polyphosphate molecules. The enzyme has a pH optimum at 7.1 and displays a marked thermal lability.

Polyphosphate phosphohydrolase is a metalloenzyme which requires the presence of Mn^{2+} or Co^{2+} in the incubation medium. Mg^{2+}, Zn^{2+}, Fe^{2+} and Ni^{2+} are less effective.

When dialyzed, the enzyme preparation loses its activity. The reaction is arrested if EDTA is added to the medium. The enzyme is inhibited by Cu^{2+}, Ca^{2+}, Al^{3+} and by the heavy metal ions Ag^{+}, Hg^{2+}, Cd^{2+}, Pb^{2+}, UO_2^{2+}. Enzymic hydrolysis of polyphosphates is also inhibited by *p*-chloromercuribenzoate, NaF and KCN.

The kinetics of the action of polyphosphate phosphohydrolase does not obey the classical Michaelis–Menten law. The dependence of reaction rate on equimolar substrate–activator concentrations is described by a sigmoid curve with a maximum. There is no direct dependence of the reaction rate on the concentration of the enzyme.

The results obtained have given grounds to suggest that the role of divalent metals involved in the polyphosphate phosphohydrolase reaction is that of activating the enzyme molecule and of forming the true reaction substrate, *i.e.* a complex between the cations and polyphosphate molecules, the effect resulting in specific modifications in their conformation and labilization of the phosphoranhydride linkages.

Evidence has been obtained that orthophosphate, a product of enzymic hydrolysis of polyphosphates, may be one of the regulators of activity of the enzyme in the cell.

INTRODUCTION

At present the cytoplasmic membrane of the fungal cell is considered to be the site for the *de novo* synthesis and localization of inorganic polyphosphates of highest molecular weight[1–4]. This is somehow associated with the physiological role of these compounds, which has not yet been fully elucidated. Van Steveninck[5], the author of

one of the hypotheses, suggested, on the basis of experimental data, that inorganic polyphosphate serves as a source of energy and phosphorus in the transport of carbohydrates across the cell membrane of yeasts. This seems to be an attractive point of view because polyphosphate phosphohydrolase, an enzyme capable of cleaving terminal phosphate residues from this substrate molecule[6,7], is localized on the outer membrane. By analogy with various phosphatases from animal and microorganism cells[8], one may suggest that polyphosphate phosphohydrolase is a polyfunctional enzyme which can, in certain conditions, catalyze the transfer of phosphate groups from polyphosphates not to water but to some other acceptors. This fundamental problem requires a detailed study of the properties, mechanism of action and regulation of the activity of this enzyme.

In the literature there is some, although far from exhaustive, evidence that polyphosphatase is found in some microorganisms[9–14], but there is no unanimity as to the function of polyphosphatase in the cell.

The present work is a part of the investigation of the metabolism and physiological role of inorganic polyphosphates in *Endomyces magnusii*, a yeast-like organism[4]. The paper deals with the isolation and properties of polyphosphate phosphohydrolase (EC 3.6.1.11) in the species.

MATERIAL AND METHODS

Material

E. magnusii cells were grown at 28 °C on a solid medium of the following composition: $(NH_4)_2SO_4$ (0.3%), K_2HPO_4 (0.01%), KH_2PO_4 (0.1%), $Ca(NO_3)_2$ (0.04%), NaCl (0.05%), $MgSO_4$ (0.07%), sucrose (2%), yeast extract (0.3%), malt must (5%), agar-agar (2%).

16-h cultures were used in the experiments, which corresponded to the end of the logarithmic phase. The cells were washed free from the medium with water and promptly used for preparing cell-free extracts.

Preparation of cell-free extracts

The biomass (5 g) in $5 \cdot 10^{-2}$ M Tris–maleate–NaOH buffer at pH 7.1 (5 ml) was placed in a special chamber cooled to −50 °C (ref. 15) in which the cells were disintegrated under a pressure of 120 atm. The homogenate was then diluted with several volumes of buffer and centrifuged for 30 min at 18 000 × *g* at 0 °C. The sediment was discarded and the supernatant was dialyzed against the buffer for 6–8 h at 4 °C to remove salts and low molecular weight organic substances. The resulting preparation was used for measuring enzymic activity. Sometimes, as a result of fractionation with $(NH_4)_2SO_4$, in the fraction precipitating between the 60 and 80% saturation an enzymic activity 2.5 times as high as that of the original extract was obtained.

Determination of polyphosphate phosphohydrolase activity

The enzymic activity of the preparation was assayed by the increase of orthophosphate in the incubation mixture. In most experiments a sodium salt of inorganic polyphosphate with an average degree of polymericity $\bar{n}$ 290 was used as a substrate. The compound was synthesized in the laboratory of Professor U. Schülke (Institute of Inorganic Chemistry of the Academy of Sciences of the German Democratic

Republic). In some experiments polyphosphates with $\bar{n}$ 40, 72 and 180 were also used. The admixture of orthophosphate in the polyphosphate preparations did not exceed 0.2%.

A number of preliminary experiments were made in order to choose the standard composition of the incubation mixture: 50 μmoles of Tris–maleate–NaOH buffer, pH 7.1; 1 μmole $MnCl_2$; 1 μmole acid-labile phosphate as the sodium salt of inorganic polyphosphate ($\bar{n}$ 290). The mixture was cooled in an ice bath and supplemented with 0.1 mg of the protein from the enzymic preparation. The total volume of the sample was 0.5 ml. Incubation was carried out for 5 min at 37 °C.

The reaction was arrested by adding 0.5 ml cold 1 M $HClO_4$, and the test tubes were quickly placed in the ice bath. In 30 min the samples were neutralized with 1 M NaOH, the total volume was adjusted to 5 ml and the quantity of the liberated orthophosphate was determined. Samples to which the substrate was added only after incubation and acidification with $HClO_4$ were used as control. Incubation of the substrate in the absence of the enzymic preparation did not result in the hydrolysis of the former, and no orthophosphate was observed in the samples.

The activity of polyphosphate phosphohydrolase was expressed in standard units of activity (*E*), *i.e.* in μmoles of orthophosphate liberated per min. Specific activity was expressed as *E* per 1 mg protein of the enzyme preparation.

Analytical methods

Orthophosphate was determined by the modified method of Weil-Malherbe and Chain[16]. The content of acid-labile phosphate in the initial solutions of polyphosphates was determined after 10 min of hydrolysis at 100 °C in 1 M HCl. The protein content in the enzymic preparations was determined by the method of Lowry *et al.*[17].

Reagents

The following salts were used: chlorides of Mn^{2+}, Mg^{2+}, Co^{2+}, Ca^{2+}, Ba^{2+}, Al^{3+}, K^+, Na^+, Li^+, NH_4^+; sulphates of Zn^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Cd^{2+}; acetates of Hg^{2+}, Pb^{2+}, UO_2^{2+} and nitrate of Ag^+.

RESULTS

The effects of pH and of temperature on the rate of the polyphosphate phosphohydrolase reaction, and also the effect of some metal ions and inhibitors, were studied. In addition, some kinetic characteristics of the enzyme were elucidated.

The effects of pH

The effects of the H^+ concentration on the enzymic activity was studied in 0.1 M acetate buffer, pH 4.0–5.4, and in 0.1 M Tris–maleate buffer, pH 5.8–8.4 (Fig. 1). It was found that the optimum of the reaction lies in the region of neutral pH. pH 7.1 was employed in the later experiments.

The effects of temperature on the stability of the enzyme

The temperature dependence of the reaction rate was studied within the range of 25 to 40 °C (Fig. 2). The enzyme displayed highest activity at 37 °C. The temperature coefficient of the reaction, Q_{10}, is 1.8.

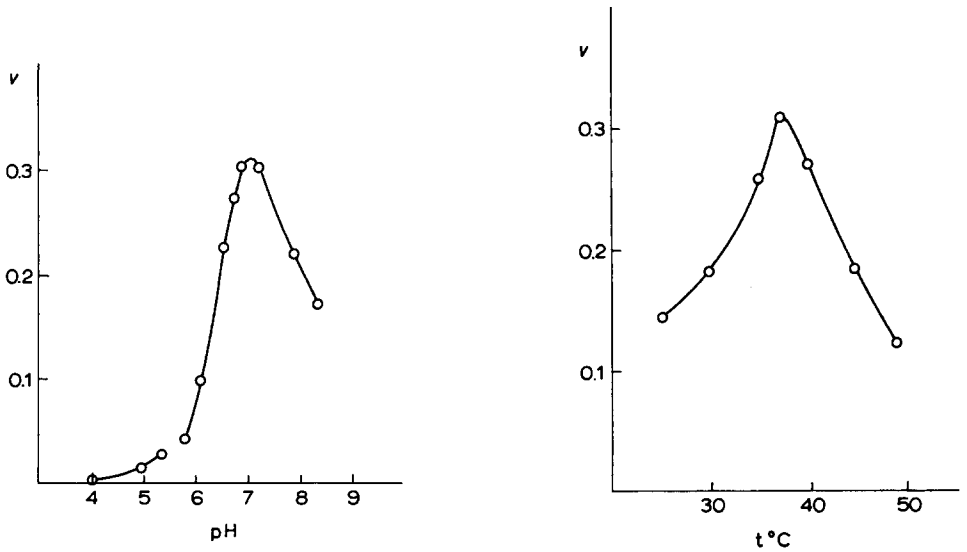


Fig. 1. The effects of pH on polyphosphate phosphohydrolase activity. The standard reaction mixture contained: 2 mM $MgCl_2$, polyphosphate ($\bar{n}$ 290, 2 mM P_i), 0.1 mg of protein from the enzyme preparation, 0.1 M acetate or Tris–maleate–NaOH buffer in a final volume of 0.5 ml. The enzyme activity was measured after a 5-min incubation at 37 °C. The initial reaction rate (v) is expressed as μmoles of P_i liberated/min per mg of protein from the enzyme preparation.

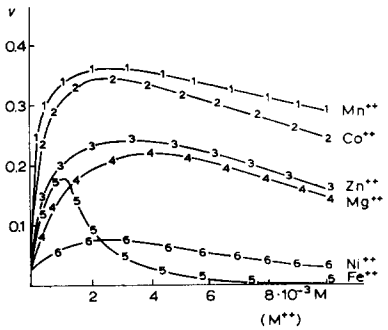
Fig. 2. The effects of temperature on the enzyme activity. The reaction mixture contained: 0.1 M Tris–maleate–NaOH buffer (pH 7.1), 2 mM $MnCl_2$, polyphosphate ($\bar{n}$ 290, 2 mM P_i), 0.1 mg of protein from the enzyme preparation in a final volume of 0.5 ml. The reaction rate is expressed as in Fig. 1.

At temperatures above 37 °C the activity of the enzyme rapidly decreases, apparently due to denaturation of its protein. The thermal lability of the enzyme was also confirmed when the preparations were preheated at different temperatures.

Incubation at 40 °C for 15 min inactivated the enzyme by 70%, and heating at 60 °C even for 2 min caused complete loss of enzyme activity. Addition of the substrate to the enzyme preparations did not prevent it from being denatured by heating. The process was only slightly decelerated (by 10%) when $MnCl_2$ was added at a concentration of $1 \cdot 10^{-3}$ M. On the other hand, storage of the enzyme preparations at −20 °C for a month did not markedly affect their activity. This was to a large degree facilitated by preliminary dialysis and the addition of Mg^{2+} ($1 \cdot 10^{-3}$ M) and cysteine ($1 \cdot 10^{-3}$ M) to the buffer.

The effects of cations

Only trace amounts of enzymic activity were revealed in the dialyzed cell-free extracts. A 10–20-fold increase in activity was achieved by adding ions of some bivalent metals (Fig. 3). It follows from the results obtained that Mn^{2+} at a concentration $2 \cdot 10^{-3}$ M is the strongest activator of polyphosphate phosphohydrolase. Co^{2+} ($2 \cdot 10^{-3}$ M) is almost as active. The effects of Zn^{2+} ($3 \cdot 10^{-3}$ M) and Mg^{2+} ($4 \cdot 10^{-3}$ M) are meagre and amount, respectively, to 70 and 60%, of the effect of Mn^{2+}. Ni^{2+} ($2 \cdot 10^{-3}$ M) produces only a slight stimulating effect. The effect of all the above ions decreases when the concentration is raised to $1 \cdot 10^{-2}$ M. Fe^{2+} at a concentration



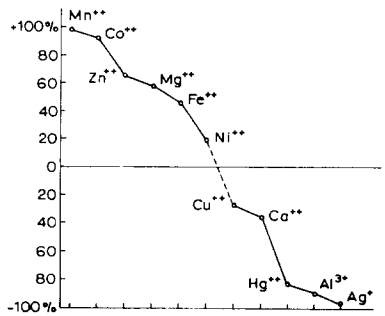


Fig. 3. The activating effects of metal ions on the enzyme activity. The reaction mixture contained: 0.1 M Tris–maleate–NaOH buffer (pH 7.1), polyphosphate ($\bar{n}$ 290, 2 mM P_i), 0.1 mg protein from the enzyme preparation, various concentrations of metal ions in a final volume of 0.5 ml. The reaction rate was determined as in Fig. 1.

Fig. 4. The diagram of the effect of cations on polyphosphate phosphohydrolase activity.

of $1 \cdot 10^{-3}$ M is an activator, and at a concentration of $1 \cdot 10^{-2}$ M is an inhibitor, Fe^{3+} displays no activating effect.

From the point of view of their activating effect the above cations fall into the following sequence: $Mn^{2+} > Co^{2+} > Zn^{2+} > Mg^{2+} > Fe^{2+} > Ni^{2+}$.

It is noteworthy that, when the Mn^{2+}-containing incubation medium is supplemented with some other cation, in some cases the activating effect of Mn^{2+} becomes much stronger, whereas in other cases the ions appear to be totally incompatible. It was shown that Co^{2+} enhances the effect of Mn^{2+} by 20%, Mg^{2+} does not affect the reaction but Ni^{2+} and Zn^{2+} decrease the activating effect of Mn^{2+}. Fe^{2+} at a concentration of $4 \cdot 10^{-3}$ M completely arrests the reaction. Cu^{2+}, Ca^{2+}, Hg^{2+}, Al^{3+}, Ag^{+} and also Cd^{2+} and UO_2^{2+} produce an inhibiting effect regardless of whether or not Mn^{2+} is present in the medium. Ag^{+}, Al^{3+} and Hg^{2+} are the most potent inhibitors; they completely arrest the reaction if present at a concentration of $2 \cdot 10^{-3}$ M.

Fig. 4 shows a diagram which summarizes the action of some cations on the polyphosphate phosphohydrolase activity of the preparations isolated from *E. magnusii*.

In later experiments only Mn^{2+}, which has the most pronounced activating effect, was used. It was established that the optimal activator–substrate ratio (Mn^{2+}/P_i in the polymer form) is from 1.0 to 1.5.

The effects of complexing

EDTA, if added in a concentration equal to that of the activating cation, inhibited the reaction by 32%, but if the concentration was 1.5 times higher than that of the activator, the reaction was completely inhibited.

Specificity of the enzyme

The enzyme preparations from *E. magnusii* were shown to hydrolyze to orthophosphate not only polyphosphates with $\bar{n}$ 290 but other, less polymeric molecules of this group of phosphorus compounds. The specific activity of the enzyme was inversely proportional to the chain length of the molecule which served as the reaction substrate (Table I).

TABLE I

THE EFFECTS OF THE CHAIN LENGTH OF INORGANIC POLYPHOSPHATE MOLECULES ON THE ACTIVITY OF POLYPHOSPHATE PHOSPHOHYDROLASE FROM *E. magnusii*

	$\bar{n}$			
	290	*180*	*72*	*40*
Relative specific activity (%)	100	110	130	160

The hydrolysis of pyrophosphate under the reaction condition employed was insignificant, and was apparently due to the presence of pyrophosphatase in the enzyme preparation. No orthophosphate was liberated when β-glycerophosphate was used as substrate.

Inhibiting effects of some agents on the reaction rate

Monovalent cations. Li^+, Na^+, K^+, NH_4^+ at a concentration of $5 \cdot 10^{-3}$–$1 \cdot 10^{-2}$ M inhibit the reaction with the non-dialyzed cell-free extract from *E. magnusii* by 30–60%.

NaF. NaF, which is a very potent phosphatase inhibitor, produced a weak effect on polyphosphate phosphohydrolase of *E. magnusii.* 0.2 M NaF inhibited the reaction by 65% at the most. It should be noted that this effect of NaF may be regarded as a result of a non-specific inhibiting action of a high concentration of this salt in the incubation medium.

Other inhibitors. The experiments performed have given grounds for concluding that *p*-chloromercuribenzoate and KCN ($2 \cdot 10^{-2}$ M), which inhibited enzymic hydrolysis by 50 and 40%, respectively, are polyphosphate phosphohydrolase inhibitors.

The effects of enzyme concentration

A study of the concentration dependence of the initial rate of polyphosphate phosphohydrolase reaction has shown that it has a non-linear character (Fig. 5). The curve most likely means that the enzyme preparation contains a small admixture of the activator which was not removed during dialysis[18], although other reasons are not impossible. In our work one and the same quantity of the enzyme was used (0.1 mg protein per sample), so that the results obtained should be comparable.

The effects of substrate concentration

In view of the fact that enzymic hydrolysis of polyphosphates involves an modifier, a divalent metal ion, the effect of the substrate concentration on the rate of the process was studied in the presence of various amounts of metal. Fig. 6A shows a plot of the initial reaction rate (v) *versus* the concentration of polyphosphate (S), the concentration of the modifier (M) being constant and rather high. The curve is hyperbolic. The same data presented as double-reciprocal curves (Fig. 6B) give a straight line with an intercept of $-1/K_m$ on the ordinate indicating that, under the conditions employed, the apparent K_m is equal to $3.5 \cdot 10^{-4}$ M of phosphate (polymer form with $\bar{n}$ 290).

In Figs 7A and 7B the initial reaction rate is plotted against equimolar concen-

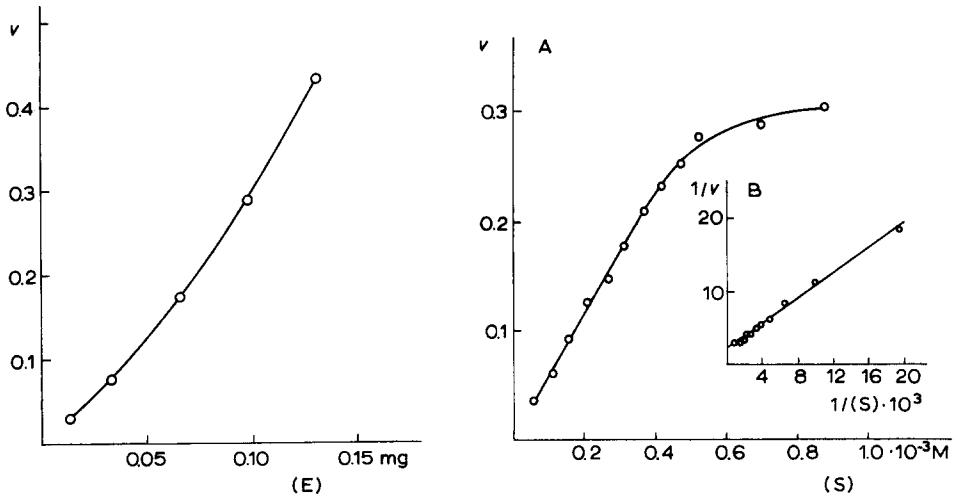


Fig. 5. The initial rate of enzymic hydrolysis of polyphosphate as a function of enzyme concentration. The reaction mixture contained: 0.1 M Tris–maleate–NaOH buffer (pH 7.1), 2 mM $MnCl_2$, polyphosphate (2 mM P_i), various concentrations of enzyme in a final volume of 0.5 ml. [E] was expressed in mg of protein from the enzyme preparation. The reaction rate is expressed as in Fig. 1.

Fig. 6. (A) The initial rate of polyphosphate phosphohydrolase reaction as a function of the substrate concentration at a constant concentration of the modifier (Mn^{2+}). The reaction mixture contained: 0.1 M Tris–maleate–NaOH buffer (pH 7.1), 3 mM $MnCl_2$, various concentrations of polyphosphate ($\bar{n}$ 290), 0.1 mg protein of the enzyme preparation in a final volume of 0.5 ml. (B) The double-reciprocal plot of the data in A.

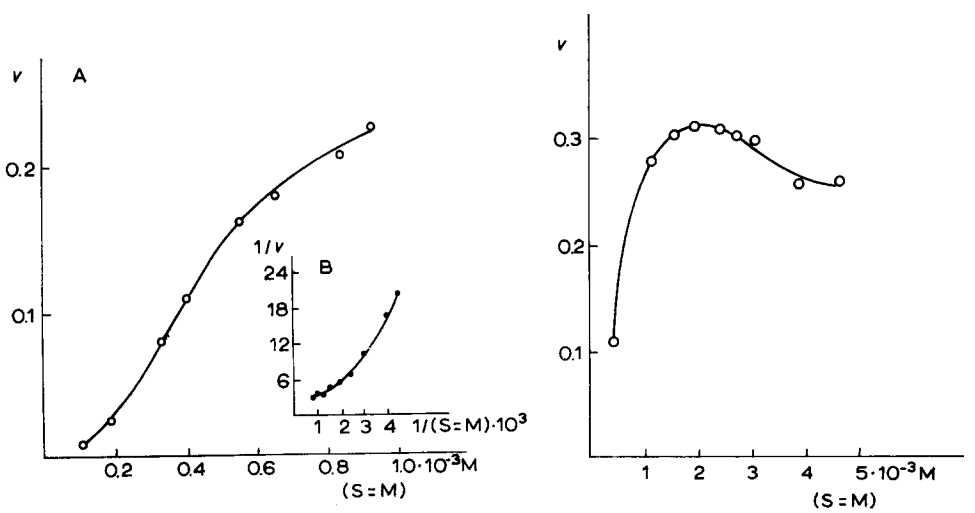


Fig. 7. (A) The plot of the initial reaction rate against the equimolar concentrations of the substrate (polyphosphate) and modifier (Mn^{2+}). The reaction mixture contained: 0.1 M Tris–maleate–NaOH buffer (pH 7.1), various equimolar concentrations of polyphosphate ($\bar{n}$ 290) and $MnCl_2$; 0.1 mg of protein from the enzyme preparation in a final volume of 0.5 ml. (B) The double-reciprocal plot of the data in A.

Fig. 8. The plot of the initial rate of the polyphosphate phosphohydrolase reaction against high equimolar concentrations of polyphosphate and Mn^{2+}. For details see the legend to Fig. 7A.

trations of the substrate and modifier. The *v versus* $[S = M]$ plot is sigmoidal, and the $1/v$ *versus* $1/[S = M]$ dependence is non-linear.

Fig. 8 shows how equimolar concentrations of the substrate and modifier exceeding $2 \cdot 10^{-3}$ M markedly inhibit the rate of digestion of polyphosphate.

The kinetic data obtained allowed the conclusion to be made that the mechanism of the reaction is of a complex nature which requires further studies for elucidation.

Therefore, the next step was to use the method of London and Steck[19] which helps to clarify the role of the modifier in the polyphosphate phosphohydrolase reaction. To this end, the dependence of the initial reaction rate on various concentrations of the substrate in the presence of some constant concentrations of the modifier (the *S* profiles, Fig. 9) and, secondly, the effect of increasing concentrations

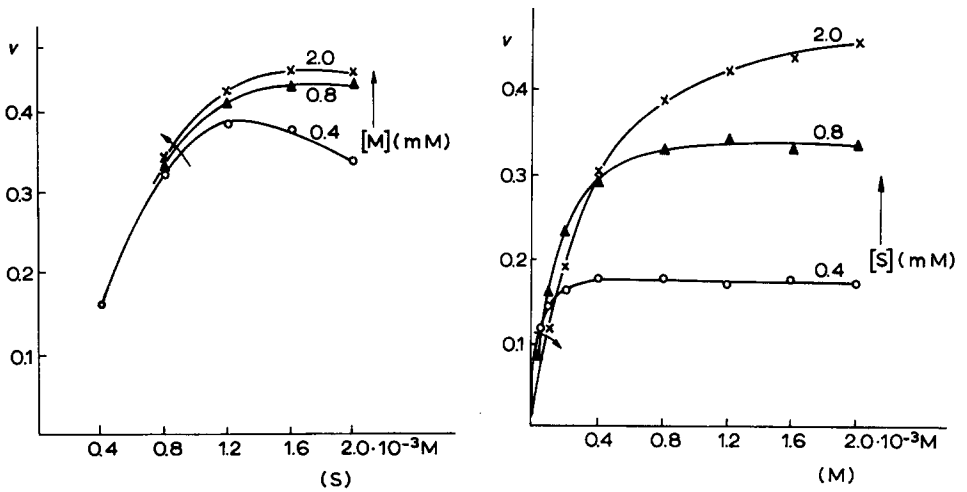


Fig. 9. *S* profiles. The plots of the initial rate of the polyphosphate phosphohydrolase reaction against the concentration of polyphosphate at various constant concentrations of the modifier (Mn^{2+}). The reaction mixture contained: 0.1 M Tris–maleate–NaOH buffer (pH 7.1), various concentrations of polyphosphate ($\bar{n}$ 290), 0.4, 0.8 or 2.0 mM $MnCl_2$, 0.1 mg of protein from the enzyme preparation in a final volume of 0.5 ml.

Fig. 10. *M* profiles. The plot of the initial reaction rate against the concentration of the modifier (Mn^{2+}) at various fixed concentrations of the substrate. The reaction mixture contained: 0.1 M Tris–maleate–NaOH buffer (pH 7.1), various concentrations of $MnCl_2$, 0.4, 0.8 or 2.0 mM of polyphosphate ($\bar{n}$ 290), 0.1 mg of protein from the enzyme preparation in a final volume of 0.5 ml.

of the modifier with constant substrate concentrations in the incubation medium (*M* profiles), have been studied (Fig. 10). It was revealed that the shift of the *S* profiles with increasing concentrations of the modifier is to the left (see the arrow in Fig. 9) and the shift of the *M* profiles under the same conditions is to the right (the arrow in Fig. 10). In terms of the London–Steck[19] scheme, such a tendency in the relative localization of the *S* and *M* profiles should be interpreted as pointing to a double role of the cation as an activator of both the enzyme and the substrate.

The effects of orthophosphate

It has been established that the reaction product produces an inhibiting effect

on the rate of cleavage of polyphosphate. $1 \cdot 10^{-3}$ M orthophosphate in the incubation medium inhibited the activity of the enzyme by approximately 10%; if the concentration was raised to $2 \cdot 10^{-3}$ M, the inhibition amounted to over 30%. According to the literature data[13], $5 \cdot 10^{-2}$ M of orthophosphate completely arrests polyphosphate hydrolysis. However, we failed to experiment with such high concentrations of phosphate, as its excess in the incubation medium interfered with determinations of the degree of polyphosphate hydrolysis by the method we employed.

DISCUSSION

The pH optimum and effects of cations

The data we have obtained show that polyphosphate phosphohydrolase from *E. magnusii* is active within the neutral pH range, and requires the presence of some bivalent metal ions. On the other hand, monovalent cations inhibit the enzymic reaction. These features of the enzyme may be accounted for by the structure and properties of its substrate, inorganic polyphosphates.

Polyphosphates are polyanions readily binding metal ions in the environment. It has been previously established by Van Wazer[20] and Thilo[21] that the molecules of these compounds display a considerable stability in solution in the presence of monovalent cations. However, polyvalent metal ions are capable of catalysing non-enzymic hydrolysis of polyphosphates, with a rate which increases as the dielectric constant of the environment decreases.

It is most interesting that, at neutral pH, it is mostly terminal phosphate residues which break off, whereas in the acidic medium the predominant process is depolymerization of polyphosphates with the chain being ruptured in the middle[20]. This explains the different pH optima of the two types of enzymes which catalyze the hydrolysis of inorganic pyrophosphates in the presence of some bivalent metals.

Polyphosphate polyphosphohydrolase, which breaks the molecules into low molecular weight fragments, is operative at pH 3–5 (refs. 9 and 22), whereas polyphosphate orthophosphohydrolase, has a pH optimum at 7.1.

We have also found that cations differ greatly in the strength and direction of the effect they produce on the course of the enzymic digestion of polyphosphate (Fig. 4). We know now, owing to the above cited works of Van Wazer[20] and Thilo[21], that polyvalent metal ions and polyphosphates form complex compounds of the chelate type in which the structure and stability depend on the valency of the complex-forming agent, its charge and radius, and on the chain length of the ligand.

A study of the catalytic effect of the metal ions with respect to polyphosphates has shown that the more firmly the metal is bound in the complex, the more actively it catalyzes the non-enzymic hydrolysis of polyphosphate molecules[21,23]. However, for enzymic reactions the dependence is just the opposite, as was established by Williams[24]: the metals which are the most effective catalysts of non-enzymic reactions are, as a rule, inhibitors of enzymic processes (the more stable the complex of the cation with a compound, the less active is the metal as a catalyst of the corresponding enzymic reactions).

The results of our study (Fig. 4) and the data of other authors[9,10,14,22,25] have lent additional support to the empirical rule of Williams[24] that it is the transient group metal ions which are activators of polyphosphate phosphohydrolase (as well

as polyphosphate polyphosphohydrolase); these ions form rather readily-dissociating complexes with polyphosphates. The activating effect of the metal increases as the chain length of polyphosphate decreases, *i.e.* as the stability of its complex with cations decreases (Table I). And *vice versa*, among the inhibitors of the enzymic hydrolysis of polyphosphates there are cations of great charge and valency which form stable complexes with several chains of polyphosphate molecules[20,21], in which all the coordinating capacity of the cation is mobilized, the effect making impossible the enzyme–substrate binding.

Thus, all the data available at present give grounds to conclude that the activating effect of some metals on polyphosphate phosphohydrolase is, at least in part, an outcome of the complex formation between the cations and polyphosphate molecules. This interaction may result, first of all, in a specific change in the conformation of the polyphosphate molecule and, secondly, in the labilization of the P–O–P linkage. Both these processes may facilitate the attack of the nucleophylic groups of the enzyme on the terminal phosphate residues. *In vivo* not only water, but other molecules may play the role of phosphate acceptors, for example, specific carriers involved in the transport of different substances from the environment. Some authors[26,27] have confirmed this by demonstrating that divalent metal ions may, under certain conditions, catalyze non-enzyme phosphotransferase reactions. In addition to their function as activators of the substrate, the cations may affect the enzyme–substrate binding.

However, the effects of cations should not be confined to activating the substrate of the enzymic reaction. The ability of cations to react with some protein molecules which are typical multidentant ligands should be taken into consideration. Such a reaction may entail some changes in the enzyme molecule, which may eventually result in either activation or inhibition of the reaction. It is noteworthy that among the inhibitors of the enzyme there are cations readily binding with the SH groups of proteins. This evidence, and also the data on the inhibiting effect of *p*-chloromercuribenzoate and the stimulating action of cysteine, point to the significant role of SH groups in the functioning of polyphosphate phosphohydrolase.

Also, attention should be paid to the fact that metal may be localized in the active site of the enzyme, and the effect of the cation with some groups of the enzyme protein may have a character of an interaction.

Kinetics

We have found that the dependence of the rate of enzymic hydrolysis of polyphosphates upon the concentration of the enzyme is not a strictly linear function. This is likely to be due to the presence of a dissociating activator in the system[18]. This suggestion was been confirmed by relevant experiments.

The further study of the kinetics of polyphosphate phosphohydrolase was more difficult due to the participation in the enzyme–substrate interaction of an additional factor, a metal ion. Both the free substrate and the free modifier inhibited the reaction when used in certain concentrations, therefore one could not alter their amount in the system at random.

The study of the effect of the concentration of the substrate on the reaction rate involves several steps. At first this dependence was followed with the modifier concentration being saturating and constant. The resulting curve was a hyperbola

(Fig. 6A), which makes it a typical Michaelis–Menten reaction. However, when elucidating the dependence of the reaction rate on the initial equimolar concentrations of the substrate and modifier, we obtained a sigmoid curve (Fig. 7A). At higher concentrations of substrate and modifier, the *v versus* $[S = M]$ function had a maximum at $2 \cdot 10^{-3}$ M $[S = M]$ (Fig. 8).

A sigmoid dependence of the reaction rate on the substrate concentration is believed to be characteristic of allosteric enzymes having at least two active sites in the molecule and a positive cooperativity, or consisting of several subunits[28]. In the case of polyphosphate phosphohydrolase, such a dependence should indicate an allosteric interaction between the spatially distant sites of the enzyme which bind with the substrate and cation. However, it would be more logical to suggest that we are dealing with a seeming cooperativity[19,29,30] the reason for which is the specific character of the action of the enzyme, the true substrate of which is the polyphosphate–cation complex.

To clarify this question, which is of fundamental importance, we used the method of London and Steck[19] for studying enzymic reactions in which the substrate and modifier (cation) react both with the enzyme and with each other. To this end, reaction rates were plotted, first against the substrate concentration, with the concentrations of the modifier being low and constant (Fig. 9), and secondly against the concentrations of the modifier with some fixed substrate concentrations (Fig. 10). This evidence led to the conclusion that the enzyme reacts with the metal ions, the result of which is a modified or stabilized state of the enzyme with a catalytic activity exceeding that of the initial state of the enzyme. This is accompanied by the formation of an intermediate active complex with the true reaction substrate, which is a complex of polyphosphate with the activating cation (Model 3 by London and Steck[19]). It should be noted, however, that our data do not completely fit the London–Steck scheme, since with polyphosphate phosphohydrolase the plot of the reaction rate *versus* equimolar concentrations of the substrate and modifier has a distinct maximum which is usually not the case with the London–Steck model. This fact does not agree with the above scheme. Apparently, no final conclusion about the character of the enzyme and its mechanism of action can be made until the kinetics of reactions catalyzed by a highly purified polyphosphate phosphohydrolase preparation has been studied in detail.

The effects of orthophosphate

The inhibiting effect of the reaction product on the activity of polyphosphate phosphohydrolase may point to a particular way of regulating the enzyme action *in vivo*. This, still a preliminary conclusion, is supported by the data on the regulatory role of orthophosphate on the activity of another enzyme which is also to be found on fungal cytoplasmic membranes[31]. This enzyme catalyses the depolymerization of high molecular weight polyphosphates[22]. All these data should be interpreted as signifying a universal regulatory role of orthophosphate in polyphosphate metabolism.

ACKNOWLEDGEMENT

The authors wish to thank Professor U. Schülke for the preparations of inorganic polyphosphates.

REFERENCES

1 Kulaev, I. S. and Belozersky, A. N. (1962) *Izv. Acad. Nauk SSSR Ser. Biol.* 3, 354–369
2 Kulaev, I. S. and Belozersky, A. N. (1962) *Izv. Acad. Nauk SSSR Ser. Biol.* 4, 502–522
3 Kulaev, I. S., Krasheninikov, I. A. and Kokurina, N. K. (1966) *Biokhimiya* 31, 850–859
4 Kulaev, I. S., Afanasieva, T. P. and Belikova, M. P. (1967) *Biokhimiya* 32, 539–547
5 Van Steveninck, J. (1963) *Biochem. J.* 88, 387–388
6 Kulaev, I. S., Krasheninikov, I. A. and Afanasieva, T. P. (1970) *Dokl. Akad. Nauk SSSR* 190, 1238–1240
7 Kulaev, I. S. and Konoshenko, G. I. (1971) *Biokhimiya* 36, 1175–1182
8 Nordlie, R. C. and Arion, W. J. (1965) *J. Biol. Chem.* 240, 2155–2164
9 Krishnan, P. S. (1952) *Arch. Biochem. Biophys.* 37, 224–234
10 Mattenheimer, H. (1956) *Hoppe-Seyler's Z. Physiol. Chem.* 303, 107–114
11 Muhammed, A., Rodgers, A. and Hughes, D. E. (1959) *J. Gen. Microbiol.* 20, 482–495
12 Grossman, D. and Lang, K. (1962) *Biochem. Z.* 336, 351–370
13 Harold, F. M. and Harold, R. L. (1965) *J. Bacteriol.* 89, 1262–1270
14 Felter, S., Dirheimer, G. and Ebel, J.-P. (1970) *Bull. Soc. Chim. Biol.* 52, 433–446
15 Lyubimov, V. I. and Lvov, N. P. (1968) *Prikl. Biokhim. Microbiol.* 4, 592–593
16 Weil-Malherbe, H. and Green, R. H. (1951) *Biochem. J.* 49, 286–292
17 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
18 Dixon, M. and Webb, E. C. (1964) *Enzymes*, 2nd edn, pp. 61–63, Longmans, London
19 London, W. P. and Steck, T. L. (1969) *Biochemistry* 8, 1767–1779
20 Van Wazer, J. R. (1962) *Phosphorus and its Compounds*, Vol. I, pp. 360–364, Russ. edn, Moscow
21 Thilo, E. (1962) in *Adv. Inorganic Chem. Radiochem.* (Emeléus, H. J. and Sharpe, A. G., eds), Vol. 4, pp. 1-75, Academic Press, New York
22 Kritsky, M. S., Chernysheva, E. K. and Kulaev, I. S. (1972) *Biokhimiya* 37, 983–989
23 Van Steveninck, J. (1966) *Biochemistry* 5, 1998–2002
24 Williams, R. J. P. (1959) in *The Enzymes* (Boyer, P. D., ed.), Vol. I, pp. 391–441, Academic Press, New York
25 Malmgren, H. (1952) *Acta Chem. Scand.* 6, 16–26
26 Mansurova, S. E., Skryabin, K. G. and Kulaev, I. S. (1971) in *Kurzreferate für das Symposium der Biochemischen Gesellschaften der UdSSR und DDR*, p. 13, Leipzig
27 Le Port, L., Etaix, E., Godin, F., Leduc, P. and Buvet, R. (1971) in *Chemical Evolution and the Origin of Life* (Buvet, R. and Ponnamperuma, C., eds), pp. 197–206, North-Holland Publ. Co., Amsterdam
28 Kurganov, B. I. (1971) in *Allosteric Regulation of Enzyme Action* (Kretovitch, W. L., ed.), pp. 57–98, Moscow
29 Denburg, J. and DeLuca, M. (1968) *Biochem. Biophys. Res. Commun.* 31, 453–460
30 Sweeny, J. R. and Fisher, J. R. (1968) *Biochemistry* 7, 561–565
31 Kulaev, I. S., Konoshenko, G. I., Chernysheva, E. K. and Kritsky, M. S. (1972) *Dokl. Akad. Nauk SSSR* 206, 233–235