Some features of hydrolysis of organic and inorganic substrates by *Escherichia coli* inorganic pyrophosphatase in the presence of various activator cations

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The kinetics of hydrolysis of the inorganic (PP_i) and organic (ATP) substrates by *Escherichia coli* inorganic pyrophosphatase (PPase) and its mutant forms with Asp42 replaced by Ala, Asn, or Glu was studied. The Mn^{2+} or Zn^{2+} ions were used as activators of the enzymatic reaction. The kinetic parameters of hydrolysis were determined. The inhibitory effect of these cations on substrate hydrolysis was investigated. The dissociation constants were calculated for the Mn^{2+}- and Zn^{2+}-binding activator and inhibitor subsites of *E. coli* PPase. The observed hydrolysis rate of PP_i increases in the series $Zn^{2+} < Mn^{2+} < Mg^{2+}$, whereas the potential efficiency of these cations decreases in this series. Hydrolysis of ATP by *E. coli* PPase occurs only in the presence of Mn^{2+}. The reasons for the observed differences in the substrate specificity of the enzyme are discussed.

Key words: inorganic pyrophosphatase, hydrolysis, activator cations, ATP, pyrophosphate, inhibition

Soluble inorganic pyrophosphatases (PPases) catalyze hydrolysis of pyrophosphate (PP_i) giving rise to two orthophosphate (P_i) molecules, thus regulating the level of pyrophosphate in cells. Since PP_i is formed in the synthesis of virtually all biopolymers of vital importance, pyrophosphatases play a very important role in metabolism. Like many other enzymes involved in phosphate exchange, PPases are metal-dependent enzymes. The Mg^{2+}, Mn^{2+}, Zn^{2+}, or Co^{2+} ions can serve as activators of PP_i hydrolysis.[1,2] The Mg^{2+} ions act as the most efficient physiological activator, whereas the Ca^{2+} ions efficiently inhibit all known PPases.[3]

Among the enzymes of this class, pyrophosphatases from *Saccharomyces cerevisiae* and *E. coli*. have received the most study. The former enzyme is a homodimer; the molecular weight of the subunit is 32 kDa.[3] The latter enzyme is a homohexamer built as a dimer of trimers (the molecular weight of the subunit is 20 kDa).[4]

Kinetic schemes of pyrophosphate hydrolysis in the presence of magnesium ions are known for both enzymes. It was established that the enzyme-substrate complex contains from three to four activator metal ions and the PP_i molecule. Splitting of the substrate and successive liberation of two phosphate molecules from the active site are the rate-determining stages of hydrolysis.[5,6]

For PPase from *S. cerevisiae* (baker's yeast), the structure of a complex with manganese ions and the enzymatic reaction product (P_i) were established.[7,8] This complex contains four Mn^{2+} ions and two P_i molecules. For PPase from *E. coli*, the structures of the complex with magnesium ions and the complex with pyrophosphate in the presence of calcium ions were established.[9,10] The active site of each subunit in the latter complex contains a PP_i molecule and three metal ions. Analysis of the kinetic data and the structures made it possible to compare the positions of PP_i and two phosphates in the enzyme active site, describe the ligands coordinated to the metal ions in all complexes under study, and identify the water molecule generating a nucleophile. Based on the data obtained, assumptions were made as to the role of each activator metal ion in catalysis and the order in which they bind to the enzyme active site. The metal ions occupying the M1 and M2 sites are bound to the enzyme prior to the substrate and produce an active conformation preceding the binding of pyrophosphate. The metal ion occupying the M3 site binds to the active site simultaneously with pyrophosphate. The metal ions in the M1—M3 sites neutralize the negative charges of the oxygen atoms of pyrophosphate, provide the required orientation of the substrate, and activate the attacking water molecule. The metal ion in the M4 site is apparently not directly involved in catalysis. The occupancy of this site leads to inhibition of the enzymatic activity.

Despite the similarity of the mechanisms of pyrophosphate hydrolysis, the enzymes from *S. cerevisiae*

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and *E. coli* differ in substrate specificity. In addition to pyrophosphate, tri- and tetraphosphates as well as their monoesters, for example, ADP, ATP, *O*-pyrophosphoethanolamine, and methyl pyrophosphate, are substrates of inorganic pyrophosphatase from *S. cerevisiae*.[11—13] Organic substrates are hydrolyzed by the enzyme in the presence of the Zn^{2+} or Mn^{2+} ions, but are not subjected to hydrolysis in the presence of the Mg^{2+} ions. The introduction of an organic radical into the molecule impairs binding of the substrate to the enzyme and decreases the rate of hydrolysis. Pyrophosphatase from *E. coli* hydrolyzes methyl pyrophosphate in the presence of Zn^{2+} or Mn^{2+}, whereas ATP is hydrolyzed only in the presence of Mn^{2+}.[12,14] It was suggested that changes in the substrate specificity of *S. cerevisiae* PPase upon the replacement of the activator metal ion are, most likely, associated with substantial differences in the arrangement of the organic and inorganic substrates.[15]

It should be noted that all investigations of the substrate specificities of *S. cerevisiae* and *E. coli* PPases have been carried out long ago. Numerous data on the structures and the mechanisms of functioning of these enzymes are currently available (these data are briefly considered above). The orientation of the inorganic substrate in the active site was described in detail based on the structures of the complexes of *S. cerevisiae* PPase with phosphates and of the complex of *E. coli* PPase with pyrophosphate. However, the understanding of the structure-function relationships for PPases is complicated by the fact that the three-dimensional structure of *S. cerevisiae* PPase was established for the complex in the presence of Mn^{2+}, whereas the structure of *E. coli* PPase was determined for the complex in the presence of inhibitory Ca^{2+} ions. However, the overall kinetic scheme for both enzymes was constructed in the presence of Mg^{2+} ions, which serve as the physiological activator. To close the gap between the structural and kinetic data, in the present study we examined hydrolysis of PP_i and ATP by *E. coli* inorganic pyrophosphatase and its Asp42Asn, Asn42Ala, and Asp42Glu mutants in the presence of Mn^{2+} and Zn^{2+}.

The residue Asp42 is located in the enzyme active site and is homologous in all known PPases. This residue is not directly involved in binding of the metal ions or of the substrate in the active site but plays an important role in the establishment of the required conformation of the enzyme-substrate complex.[16] In addition, the residue Asp42 exerts a substantial effect on the occupancy of the inhibitor site M4 by metal ions, thus affecting the release of the products.

Experimental

Tris(hydroxymethyl)aminomethane (Tris), *N*-(2-hydroxyethyl)-1-piperazine-1-ethanesulfonic acid (HEPES), $Na_4P_2O_7$, $CaCl_2$, imidodiphosphate (PNP), and *E. coli* alkaline phosphatase were from Sigma (USA), $MgCl_2$, $MnCl_2$, $ZnCl_2$, Methyl Green, adenosine 5′-triphosphate (ATP), and adenosine 5′-monophosphate (AMP) were from Fluka (Switzerland), Triton X-305 was from Merck (Germany), and Sephadex G-50 (fine) was from Pharmacia Fine Chemicals (Sweden). Other reagents of at least analytical purity grade were produced in Russia. In all experiments, solutions of $MgCl_2$ and $MnCl_2$ were titrated before use. All solutions were prepared using bidistilled water; ATP was twice recrystallized from ethanol before use.

Recombinant *E. coli* inorganic pyrophosphatase and mutant forms with Asp42 replaced by Asn, Ala, or Glu were prepared as described previously.[4] The enzymes were stored as suspensions in $(NH_4)_2SO_4$ solutions (90% saturation) and were desalted on a column with Sephadex G-50 (fine) equilibrated with 0.05 *M* Tris-HCl (pH 7.5).

The concentrations of the pyrophosphatase solutions were determined spectrophotometrically based on the absorption at 280 nm $A_{1\ cm}^{0.1\%} = 1.18$.[17] The absorption of the pyrophosphatase complexes with Mg^{2+} and Mn^{2+} was measured by differential UV spectroscopy on a single-beam Ultrospec 3000 spectrophotometer. Titration of the enzymes with solutions of $MgCl_2$ or $MnCl_2$ was carried out in 0.1 *M* Tris-HCl (pH 8.5) as described previously.[18]

The enzymatic activities of *E. coli* pyrophosphatases were determined from the rate of formation of inorganic phosphate at 25 °C. The quantitative determination of phosphate was carried out on a semiautomatic analyzer.[19] The activity of *E. coli* alkaline phosphatase was determined based on the rate of hydrolysis of *p*-nitrophenyl phosphate in 0.05 *M* Tris-HCl (pH 7.5) at the concentration of free Zn^{2+} equal to $1 \cdot 10^{-3}$ mol L^{-1}. The concentration of ZnATP or ZnAMP was $7 \cdot 10^{-4}$ mol L^{-1}. The total concentrations were calculated using the dissociation constants published in the literature.[20]

Hydrolysis of $MnPP_i$ was carried out in 0.05 *M* Tris-HCl (pH 7.5) at the Mn^{2+} concentration of $1 \cdot 10^{-3}$ mol L^{-1}. The dependence of the rate of $MnPP_i$ hydrolysis on $[Mn^{2+}]$ was studied at the substrate concentration of $1 \cdot 10^{-5}$ mol L^{-1}. Hydrolysis of $ZnPP_i$ was carried out in 0.05 *M* HEPES-NaOH (pH 7.2) at the substrate concentration of $1 \cdot 10^{-4}$ mol L^{-1}. Hydrolysis of MnATP was performed in 0.05 *M* Tris-HCl (pH 7.5) at the Mn^{2+} concentrations ensuring the highest activities of the enzymes. The dependence of the rate of MnATP hydrolysis on the Mn^{2+} concentration was studied in 0.05 *M* Tris-HCl (pH 7.5) at the substrate concentration of $7 \cdot 10^{-4}$ mol L^{-1}. Co-hydrolysis of MnATP and $MnPP_i$ was carried out in 0.05 *M* Tris-HCl (pH 7.5) at the Mn^{2+} concentration of $1 \cdot 10^{-3}$ mol L^{-1}. The concentration of MnATP was varied in the range of $(0.2—1) \cdot 10^{-4}$ mol L^{-1}; the ratio of the substrate concentrations $\alpha = [MnPP_i]/[MnATP]$ was kept constant (0.0005, 0.00075. or 0.001). Inhibition of MnATP hydrolysis with manganese imidodiphosphate was studied in 0.05 *M* Tris-HCl (pH 7.5); the concentration of Mn^{2+} was $1 \cdot 10^{-3}$ mol L^{-1} and the concentration of MnPNP was $1 \cdot 10^{-7}$ or $2 \cdot 10^{-7}$ mol L^{-1}. Inhibition of MnATP hydrolysis with zinc ions was examined in 0.05 *M* Tris-HCl (pH 7.5); the concentration of MnATP was $2 \cdot 10^{-4}$ mol L^{-1}, the concentration of Zn^{2+} was $5 \cdot 10^{-6}$ or $1 \cdot 10^{-5}$ mol L^{-1}, and the concentration of Mn^{2+} was varied from $5 \cdot 10^{-6}$ to $1.5 \cdot 10^{-5}$ mol L^{-1}.

Results and Discussion

Hydrolysis of pyrophosphate in the presence of Mn^{2+} and Zn^{2+}. The kinetic parameters of $MnPP_i$ hydrolysis by native PPase are summarized in Table 1. The pH-independent parameters of $MgPP_i$ hydrolysis are

Table 1. Kinetic parameters (k_{cat}/s^{-1}, $(k_{cat}/K_m)/\mu mol^{-1}$ L s^{-1}, and $K_m/\mu mol$ L^{-1}) of hydrolysis the substrates $MgPP_i$, $MnPP_i$,[b] and MnATP by *E. coli* PPase and its Asp42-Asn, Asp42-Ala, and Asp42-Glu mutant forms

PPase	Substrate								
	$MgPP_i$[a]			$MnPP_i$[b]			MnATP[b]		
	k_{cat}	$\frac{k_{cat}}{K_m}$	K_m	k_{cat}	$\frac{k_{cat}}{K_m}$	K_m	k_{cat}	$\frac{k_{cat}}{K_m}$	K_m
Native	390±80	3038±800	0.13±0.06	7.1±0.2	47±10	0.15±0.03	0.19±0.01	0.0011±0.0002	178±24
Asp42-Asn	1150±310	576±140	2.0±1.0	32.3±4.0	55±30	0.61±0.25	1.55±0.06	0.005±0.001	290±30
Asp42-Ala	58±5	35±7	1.7±0.5	0.97±0.03	0.24±0.08	0.23±0.07	0.051±0.002	0.0006±0.0001	80±15
Asp42-Glu	271±20	30±2	9.0±1.5	1.67±0.07	6±1	0.30±0.05	0.07±0.01	0.0010±0.0004	75±20

[a] pH-Independent parameters were determined at the Mg^{2+} concentration of 5 mmol L^{-1}.[29]
[b] The observed parameters were determined as described in the Experimental section.

given for comparison.[16] It is seen that the rate of $MnPP_i$ hydrolysis was only ~1% of the rate of $MgPP_i$ hydrolysis, whereas the constants K_m for these substrates are virtually equal. The dependence of the hydrolysis rate on the Mn^{2+} concentration was also studied (Fig. 1). The curve passes through a maximum at the Mn^{2+} concentration of $\sim 3 \cdot 10^{-5}$ mol L^{-1} followed by rapid inhibition to the residual activity of 20%. The results obtained fit Scheme 1 according to which the activator and inhibitory metal ions and the substrate S ($MnPP_i$) are bound to the enzyme possessing the occupied M1 site (EM).

Scheme 1

$$EM \overset{K_A}{\rightleftharpoons} EM_2 \rightleftharpoons EM_2S \overset{K_i}{\rightleftharpoons} EM_3S$$

According to this scheme, the observed hydrolysis rate is described by Eq. (1):

$$v = \frac{V_0[M^{2+}]}{K_A + [M^{2+}] + \frac{[M^{2+}]^2}{K_i}}, \quad (1)$$

where v is the observed hydrolysis rate, V_0 is the potential maximum rate of hydrolysis at a given concentration of the substrate in the absence of the inhibitory metal ion, and K_A and K_i are the dissociation constants of the activator and inhibitor M^{2+} binding sites, respectively. The results of calculations are presented in Table 2. For native PPase, the dissociation constant $K_d(1)$ for Mn^{2+} in the high-affinity site was determined by the spectrophotometric method and is equal to 1.5 μmol L^{-1}. Hence, it can be assumed that the high-affinity site (M1) is virtually completely occupied under the experimental conditions. Apparently, the constants K_A determined from Eq. (1) should be assigned to the low-affinity site (M2).

For native PPase, the dependence of the rate of hydrolysis of zinc pyrophosphate on the concentration of free Zn^{2+} ions was also obtained. The activities were

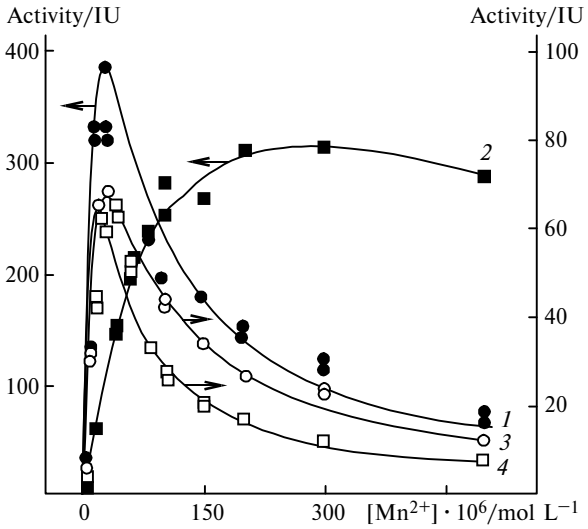

Fig. 1. Dependence of the rate of $MnPP_i$ hydrolysis by native PPase (*1*) and mutant PPases (*2*—*4*) on the concentration of free Mn^{2+} ions at pH 7.5: Asp42Asn (*2*), Asp42Ala (*3*), and Asp42Glu (*4*).

measured at pH 7.2 and at the saturating concentration of the substrate (10 μmol L^{-1}). This dependence (see Fig. 2) is similar to that obtained for Mn^{2+} ions. The dependence of the hydrolysis rate on the concentration of Zn^{2+} ions passes through a maximum at the Zn^{2+} concentration of 10—20 μmol L^{-1} and then the activity rapidly reduces.

Analysis of the dissociation constants K_A and K_i calculated for Mn^{2+}- and Zn^{2+}-activated PP_i hydrolysis using Eq. (1) (see Table 2) demonstrated that the affinities of these cations for the activator and inhibitor sites are substantially higher than those of Mg^{2+}. The maximum rates V_0 of Mn^{2+}- and Zn^{2+}-activated hydrolysis of PP_i are substantially higher than that of Mg^{2+}-activated hydrolysis, *i.e.*, Mn^{2+} and, particularly, Zn^{2+} are considerably more efficient activators of PP_i hydrolysis compared with Mg^{2+}. The reason is that the Mn^{2+} and Zn^{2+} ions exert a stronger polarizing effect than Mg^{2+}. Thus pK_a of the water molecule bound to

Table 2. Dissociation constants for the activator and inhibitory metal cations for the *E. coli* PPase and its Asp42Asn, Asp42Ala, and Asp42Glu mutant forms calculated according to Eq. (1)

Activator Substrate	PPase	V_0 /IU	K_A /μmol L^{-1}	K_i	K_d2 [a]
				mmol L^{-1}	
$Mg^{2+}/MgPP_i$	Native	594±15	200±40	16±2	1.5±0.1 [16]
	Asp42-Asn	>500	400±50	>200	1.5±0.1 [16]
	Asp42-Ala	190±20	780±200	>50	1.8±0.3
	Asp42-Glu	330±8	400±50	>150	0.9±0.1
$Mn^{2+}/MnPP_i$	Native	1070±50	25±6	0.029±0.005	0.020±0.005
	Asp42-Asn	595±15	124±6	0.647±0.083	—
	Asp42-Ala	190±10	51±5	0.017±0.001	—
	Asp42-Glu	257±20	15±7	0.020±0.002	—
$Zn^{2+}/ZnPP_i$	Native	3070±250	20±4	0.012±0.002	—
	Asp42-Asn	7000±1000	70±15	0.005±0.001	—
	Asp42-Ala	2100±200	105±15	0.0021±0.0002	—
	Asp42-Glu	2790±140	44±4	0.008±0.001	—
$Mn^{2+}/MnATP$	Native	2.6±0.4	21±10	1.5±0.8	—
	Asp42-Asn	6.0±0.7	30±10	4.5±2	—
	Asp42-Ala	0.30±0.07	85±45	0.9±0.4	—
	Asp42-Glu	0.26±0.03	28±9	1.1±0.3	—

[a] The constants K_d2 for all proteins were determined by differential spectroscopy at pH 8.5.

the metal ion in the hexahydrate are 11.4, 10.0, and 9.5 in the case of Mg^{2+}, Mn^{2+}, and Zn^{2+}, respectively.[21]

However, the experimental constants k_{cat} for these cations are smaller than that for the Mg^{2+} ions (see Table 1). Apparently, this is associated with the fact that the affinities of the inhibitor site for Mn^{2+} and Zn^{2+} are much higher than that for Mg^{2+} due to which deeper inhibition is achieved for these activators under the conditions used. These results are in agreement with the published data on higher affinities of PPase for these cations.[22,23] Previously, it has been demonstrated that lower catalytic activity of yeast PPase in the case of Mn^{2+} and Zn^{2+} ions results from the fact that the hydrolysis products leave the reaction site much more slowly.[24] Presumably, the higher affinities of Mn^{2+} and Zn^{2+} for the enzyme and phosphates in the case of *E. coli* PPase also slow down conformational rearrangements required for the products to leave the active site, whereas the ability of Mg^{2+} to undergo most rapid ligand exchange promotes these processes.

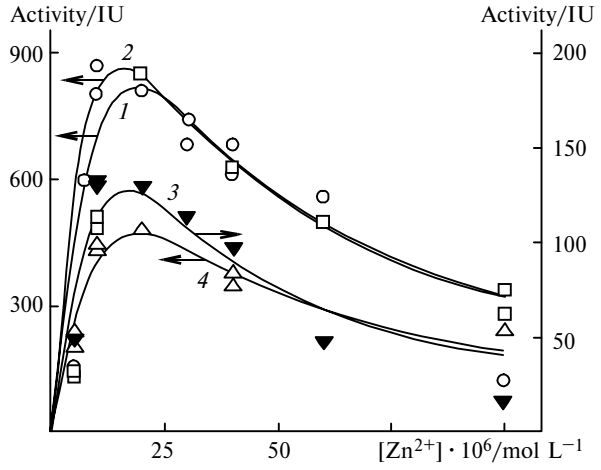

Fig. 2. Dependence of the rate of $ZnPP_i$ hydrolysis by native PPase (*1*) and mutant PPases (*2*—*4*) on the concentration of free Zn^{2+} ions at pH 7.2: Asp42Asn (*2*), Asp42Ala (*3*), and Asp42Glu (*4*).

Hydrolysis of ATP in the presence of Mn^{2+} and Zn^{2+}. Hydrolysis of the organic substrate ATP by native *E. coli* PPase was examined in the presence of Mn^{2+} and Zn^{2+} ions. Measurements of the rates of ATP hydrolysis by native PPase in the presence of Mn^{2+} and Zn^{2+} demonstrated that the enzyme activity is 2.4 and 0.022 IU, respectively. Previously, it has been established that Zn^{2+} activates ATP hydrolysis by yeast PPase, but these ions do not activate this reaction in the case of *E. coli* PPase.[2,13] Since insignificant activity was nevertheless observed in the presence of Zn^{2+} in the above-described experiment, it was necessary to ensure that the specimen contained no impurity of alkaline phosphatase. In the presence of Zn^{2+} or Mg^{2+}, this enzyme, unlike *E. coli* PPase, catalyzes hydrolysis both of ATP and AMP at approximately equal rates.[25] In the present study, it was demonstrated that AMP undergoes virtually no hydrolysis under the action of the PPase specimen used both in the presence of Mn^{2+} and Zn^{2+}, which excludes the presence of an impurity of alkaline phosphatase.

The characteristics of MnATP hydrolysis are given in Table 1. Analysis of these results and comparison with the results of $MnPP_i$ hydrolysis demonstrated that the constant K_m for the native enzyme is three orders of magnitude smaller compared with that for $MnPP_i$ hydrolysis. The maximum rate of ATP hydrolysis is 35 times lower than the rate of pyrophosphate hydrolysis.

Hydrolysis of MnATP by *E. coli* PPase demonstrated that this substrate binds to the enzyme active site. Apparently, the pyrophosphate fragment of ATP is located inside the active site, whereas the remaining fragment of the molecule is located outside this site. The metal ion in MnATP is coordinated by the β- and γ-phosphate groups and is also bound to the active site. However, the orientation of the pyrophosphate fragment of the organic substrate can differ from that of PP_i as evidenced by substantial deterioration of the parameters of MnATP hydrolysis compared with $MnPP_i$ hydrolysis. To elucidate this question, we studied co-hydrolysis of two substrates by *E. coli* PPase. The overall hydrolysis rate v was measured by varying the ratio of the substrate concentrations $\alpha = [MnPP_i]/[MnATP]$. The experimental data are adequately described by Scheme 2.

Scheme 2

$$E + S1 \underset{}{\overset{K_d(S1)}{\rightleftharpoons}} E\cdot S1 \xrightarrow{k_{cat,1}} \text{Products}$$

$$E + S2 \underset{}{\overset{K_d(S2)}{\rightleftharpoons}} E\cdot S2 \xrightarrow{k_{cat,2}} \text{Products}$$

Here, E is the active form of the enzyme (the complex of PPase with two activator metal ions), S1 and S2 are the hydrolyzable substrates $MnPP_i$ and MnATP, respectively. According to this scheme, the rate of co-hydrolysis of the substrates S1 and S2 is described by Eq. (2):

$$v = \frac{[S1](v_1 K_d(S1) + 2v_2\alpha / K_d(S2))}{[S1](\frac{1}{K_d(S1)} + \frac{\alpha}{K_d(S2)}) + 1}, \qquad (2)$$

where K_d(S1) and K_d(S2) are the dissociation constants for $MnPP_i$ and MnATP, respectively, and v_1 and v_2 are the hydrolysis rates for the substrates S1 and S2, respectively.

The effective Michaelis constants for co-hydrolysis of S1 and S2 are determined by Eq. (3):

$$K_m^{app} = \frac{1}{\frac{1}{K_d(S1)} + \frac{\alpha}{K_d(S2)}}. \qquad (3)$$

The experimental data are linearized in the coordinates (1/[S1], 1/v), which is indicative of the competitive binding of two substrates. Based on the secondary dependence of $1/K_m^{app}$ on α, the constant $K_d(MnPP_i)$ was estimated as 0.23±0.08 μmol L^{-1}. This value agrees well with the constant K_m determined for $MnPP_i$ hydrolysis in the absence of MnATP (see Table 1). The results obtained provide evidence that the β- and γ-phosphate residues of ATP are arranged analogously to the P1 and P2 sites of pyrophosphate, respectively (Fig. 3). Previously, it has also been demonstrated that

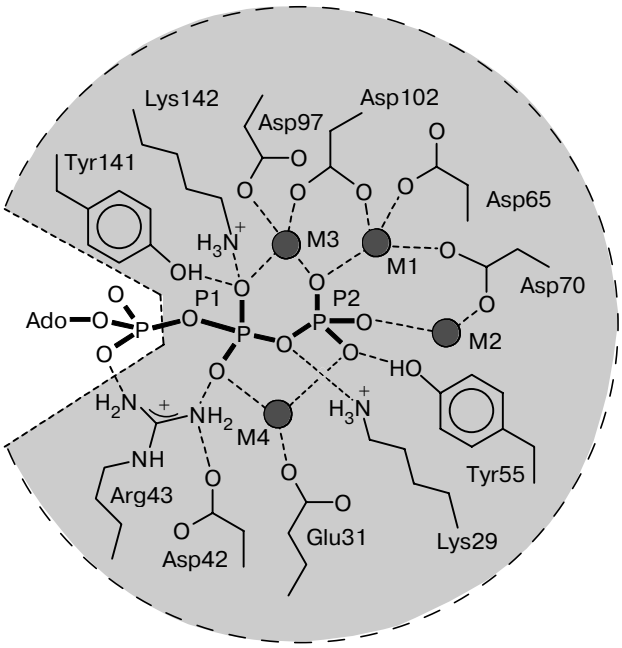

Fig. 3. Scheme of the assumed arrangement of the pyrophosphate fragment of the substrate in the active site of *E. coli* PPase (the shaded region); M1—M4 are the binding sites of the metal ions, P1 and P2 are the binding sites of the phosphate groups, and Ado is the adenosine residue. Possible contacts between the cations, the phosphate groups, and the residues involved in the active site are indicated by dashed lines. The coordination of the metal ions is completed with water molecule (omitted) to the coordination number of six. The proposed scheme is based on the data from X-ray diffraction analysis of the complex of *E. coli* PPase with $Ca^{2+}/CaPP_i$[10] and the complex of yeast PPase with Mn^{2+}/MnP_i.[8]

MnATP is a competitive inhibitor of $MnPP_i$ hydrolysis by yeast PPase.[26]

This conclusion was confirmed by the results obtained in the study of the effect of MnPNP on MnATP hydrolysis. In the presence of Mn^{2+} ions, PNP is a competitive inhibitor of ATP hydrolysis. At the same time, PNP is also a competitive inhibitor of PP_i hydrolysis in the presence of Mg^{2+} ions.[27] For MnPNP, the inhibition constant was $1\cdot10^{-7}$ mol L^{-1}, which is 50 times lower than the corresponding value for MgPNP. This result provides support for the above assumption that the affinity of the enzyme for Mn^{2+} in the substrate site is higher than the affinity for Mg^{2+}.

The dependence of the rate of MnATP hydrolysis on $[Mn^{2+}]$ (Fig. 4) is described by a curve with a poorly pronounced maximum at $[Mn^{2+}] = 0.2\cdot10^{-3}$ mol L^{-1}. No essential inhibition of the enzyme was observed as $[Mn^{2+}]$ was increased further to $2\cdot10^{-3}$ mol L^{-1}. The constants K_A and K_i calculated according to Eq. (1) are listed in Table 2. The dissociation constants for the activator Mn^{2+} binding site are equal in the case of PP_i and ATP hydrolysis. For the inhibitor site, this constant is two orders of magnitude lower in the case of an organic substrate. Binding of the activator Mn^{2+} ion

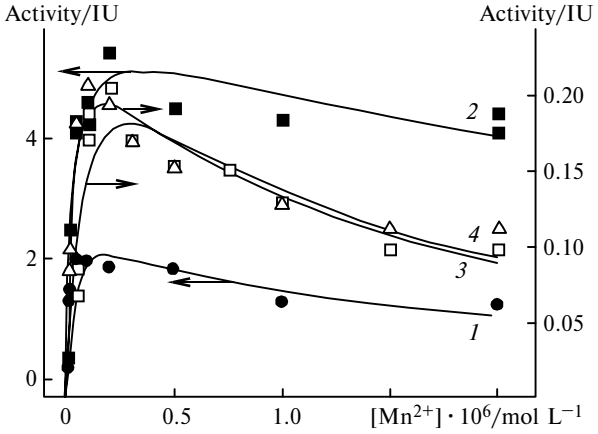

Fig. 4. Dependence of the rate of MnATP hydrolysis by native PPase (*1*) and mutant PPases (*2—4*) on the concentration of free Mn^{2+} ions at pH 7.5: Asp42Asn (*2*), Asp42Ala (*3*), and Asp42Glu (*4*).

precedes binding of the substrate, and K_A is independent of its nature. On the contrary, filling of the inhibitor site requires the presence of a substrate and, consequently, its nature influences, apparently, the orientation of the pyrophosphate group in the active site and has the determining effect on K_i.

The observed rate of MnATP hydrolysis is substantially lower than that of $MnPP_i$ hydrolysis. In the case of the organic substrate, the potential maximum rate V_0 is only 0.25% of the corresponding value for $MnPP_i$. This is attributable to the inadequate arrangement of ATP in the enzyme active site. In spite of the possible existence of additional contacts between the enzyme and the nucleotide group of the ATP molecule, the affinity of PPase for MnATP is several orders of magnitude lower than that for $MnPP_i$. It should be noted that, according to the published data, yeast PPase possesses equal affinity both for $MnPP_i$ and $MnPPP_i$ (manganese triphosphate).[3] Presumably, the reason is that pyrophosphate and ATP have different structures in the complexes with metals. It is known that in solutions, the transition metal ions, including Mn^{2+}, can coordinate not only the β- and γ-phosphate residues of ATP but also the N(7) atom of adenosine. As a result, a particular fraction of the molecules of MnATP have a "closed" conformation in a solution, which imparts the structural rigidity to MnATP.[28] It is unlikely that MnATP adopting this conformation can be bound at the active site and, hence, the formation of the "closed" conformation reduces the affinity of the active site of PPase for this substrate. It should also be taken into account that contacts between ATP and the residues of the enzyme beyond the active site may affect the positions of the groups of PPase involved in binding of PP_i.

Since the catalytic reaction proceeds according to the S_N2 mechanism, the exact orientations of the substrate and the attacking nucleophile are of great importance for efficient hydrolysis. Apparently, even insignificant differences in the arrangement of PP_i and the pyrophosphate fragment of ATP in the active site lead to a decrease in the rate of ATP hydrolysis. Hydrolysis of ATP by *E. coli* PPase can occur only in the presence of Mn^{2+}. This cation polarizes the attacking water molecule more efficiently than Mg^{2+} due to which it can activate hydrolysis of the organic substrate. In spite of the fact that Zn^{2+} ions exert an even stronger polarizing effect than Mn^{2+}, ATP hydrolysis in the presence of the former ions does not virtually take place. Most likely, this is due to the different, in principle, arrangement of the metal ions and the substrate in the active site.

Hydrolysis of PP_i and ATP by Asp42-replaced mutants of PPases. The residue Asp42 is located in the active site of *E. coli* PPase. This residue is not directly involved in binding of metal ions or the substrate. In the absence of PP_i, the carboxy group of Asp42 forms an ionic pair with the amino group of Lys29. In the presence of PP_i or P_i, the amino group of Lys29 is involved in coordination with their oxygen atoms, whereas contacts of the carboxy group of Asp42 are changed and this group is bound to the guanidine group of Arg43, which is also coordinated to PP_i or P_i. Thus, Asp42 stabilizes the groups of the protein which are required for the adequate orientation of the substrate in the active site. Previously, it has been demonstrated that the replacement of the residue Asp42 by Ala, Asn, or Glu impairs the binding of $MgPP_i$, which is manifested in higher K_m compared to that for native PPase.[25] It is significant that the Asp42Asn mutant possesses the anomalously high rate of hydrolysis (k_{cat} for this mutant is 3 times larger than that for the native enzyme) and it does not bind the inhibitory Mg^{2+} ion. It was suggested that high activity of the Asp42Asn mutant results from the fact that the products more readily leave the active site in the absence of the inhibitory metal ion.[16]

To elucidate the question of whether the replacements of Asp42 influence hydrolysis of the organic substrate, in the present study we determined the parameters of MnATP hydrolysis by Asp42Asn, Asp42Ala, and Asp42Glu mutant PPases. For comparison, we examined PP_i hydrolysis by these enzymes in the presence of Mn^{2+}. The results of this study are summarized in Table 1. For all enzymes, the rate of $MnPP_i$ hydrolysis is only ~1% of the rate of $MgPP_i$ hydrolysis. On going from Mg^{2+} to Mn^{2+}, the constants K_m for mutant PPases are changed substantially and this difference depends on the character of the replacement. As a result, the constants K_m for $MnPP_i$ hydrolysis are virtually the same for all four enzymes. In these cases, the sacrifice of binding of the pyrophosphate fragment of the substrate upon the replacements of Asp42 is apparently compensated by the higher affinity of PPase for the substrate Mn^{2+} ion compared to Mg^{2+}.

Comparison of the hydrolysis parameters for the inorganic and organic substrates in the presence of the same activator, *viz.*, the Mn^{2+} ion, demonstrated that

the maximum rate of ATP hydrolysis by mutant PPases, like that in the case of the native enzyme, is approximately 20 times lower than the rate of pyrophosphate hydrolysis. In both cases, Asp42Asn PPase is characterized by substantially higher activity compared with the native enzyme, as has been observed previously for $MgPP_i$ hydrolysis.[16] This indicates that the stage in which phosphate leaves the active site is the rate-determining stage of $MnPP_i$ and MnATP hydrolysis, whereas the Asp42Asn replacement leads to a decrease in the affinity for the inhibitory metal ion and facilitates dissociation of the products.

The constants K_m for MnATP hydrolysis by all enzymes are three orders of magnitude higher than those for $MnPP_i$ hydrolysis. As in the above-considered case, the replacements of the residue Asp42 have virtually no effect on K_m for MnATP hydrolysis. This is the common feature of hydrolysis of organic and inorganic substrates in the presence of Mn^{2+}. This fact provides support for the previous conclusion that the affinity of the substrate for the enzyme in the presence of Mn^{2+} depends more substantially on the metal ion than on the pyrophosphate group.

The inhibitory Mn^{2+} and Zn^{2+} ions in the active site of *E. coli* PPase. Previously, it has been demonstrated[16,29] that three Asp42-replaced mutants of *E. coli* PPase show the anomalous (compared with the native enzyme) dependence of the rate of PP_i hydrolysis on the Mg^{2+} concentration and possess substantially lower affinities for the inhibitory Mg^{2+} ions. This property of Asp42-replaced mutant PPases suggests that in the case of PP_i hydrolysis, the inhibitory Mg^{2+} ion is bound in the M4 site whose second coordination sphere involves Asp42. The order of binding in the active site is apparently identical for Mg^{2+} and Mn^{2+}. However, a number of facts, in particular, the difference in the substrate specificity in the presence of Mg^{2+}, Zn^{2+}, or Mn^{2+}, provide indirect evidence that there is the difference in binding of the metal ions. Because of this, we studied the effect of the replacement of Asp42 on the inhibitory properties of Mn^{2+} and Zn^{2+}. For this purpose, we determined the dependences of the rates of Mn^{2+}- or Zn^{2+}-activated PP_i and ATP hydrolysis by Asp42-replaced mutant enzymes on the concentration of free activator cations. The curves obtained for $MnPP_i$, $ZnPP_i$, and MnATP hydrolysis are shown in Figs. 1, 2, and 4, respectively. The data were processed by the non-linear regression method according to Eq. (1). The results of calculations are given in Table 1.

For native PPase and the Asp42Ala and Asp42Glu mutants, the dependences of the rate of $MnPP_i$ hydrolysis on the concentration of Mn^{2+} ions have similar shapes. In all three cases, the curves pass through a maximum at $[Mn^{2+}] = 30—40$ μmol L^{-1} after which inhibition takes place. In the case of Asp42Asn PPase, a completely different type of the dependence is observed and the enzyme is virtually not inhibited at the Mn^{2+} concentrations under study. The constants K_i for $MnPP_i$ hydrolysis by native PPase and by the Asp42Ala and Asp42Glu mutants are of the same order of magnitude, whereas the affinity of Mn^{2+} for the inhibitor site in the Asp42Asn mutant is much smaller. The results obtained allow the conclusion that the inhibitory Mn^{2+} ion, like Mg^{2+}, is bound in the M4 site. The Asp42Asn replacement also noticeably impairs the binding of Mn^{2+} in the low-affinity site.

In the case of $ZnPP_i$ hydrolysis, the dependence of the hydrolysis rate on the concentration of Zn^{2+} ions is common to all enzymes under study. The maximum is observed at $[Zn^{2+}] = 10—20$ μmol L^{-1} and then the activity rapidly reduces. Analysis of the dissociation constants K_i demonstrated that the affinities of Zn^{2+} for the inhibitor site are virtually the same for all four mutant PPases. The behavior of the Asp42Asn mutant, which differs sharply from native PPase in the case of Mg^{2+}- or Mn^{2+}-activated pyrophosphate hydrolysis, is similar to the behavior of the native enzyme in the case of Zn^{2+}-activated hydrolysis. Apparently, inhibition of PP_i hydrolysis in the presence of Zn^{2+} is not associated with the occupancy of the M4 site. According to the calculated constants K_A and K_i, the Zn^{2+} ion is more strongly bound in the inhibitor site than in the activator site due to which Zn^{2+} ions can occupy the M1—M4 sites in the order different from that observed for the Mg^{2+} and Mn^{2+} ions. The fact that the Zn^{2+} binding sites differ from those known for Mg^{2+} and Mn^{2+} must not be ruled out as well.

The dependences of the rate of MnATP hydrolysis on the Mn^{2+} concentration represent curves with poorly pronounced maxima at $[Mn^{2+}] = 0.2 \cdot 10^{-3}$ mol L^{-1} for native PPase and for the Asp42Asn and Asp42Ala mutants and at $[Mn^{2+}] = 0.1 \cdot 10^{-3}$ mol L^{-1} for the Asp42Glu mutant. As in the case of native PPase, no substantial inhibition of the mutants is observed. The calculated constants K_i for native PPase and its mutants are of the same order of magnitude. The fact that the filling of the M4 site is not responsible for inhibition must not be ruled out.

* * *

To summarize, the results of the present study allow the following conclusions. The pyrophosphate fragment of the organic substrate (as exemplified by ATP) is located in the active site of *E. coli* PPase analogously to PP_i in the P1 and P2 sites. However, the assumed small difference in the arrangement of these substrates leads to much more slow ATP hydrolysis compared with PP_i hydrolysis. As a result, ATP hydrolysis can be activated only by cations which are able to polarize the attacking water molecule to a greater extent than Mg^{2+}. Of the cations under study (Mn^{2+} and Zn^{2+}), only Mn^{2+} ions serve as an efficient activator of ATP hydrolysis. Apparently, Zn^{2+} ions are bound to the enzyme either in other sites or in another order than Mg^{2+} and Mn^{2+} resulting in the fundamental differences in the arrange-

ment of the substrate due to which ATP hydrolysis cannot occur. At high Mn^{2+} concentrations, inhibition of PP_i hydrolysis is observed due to the filling of the M4 site, as in the case of Mg^{2+}. At the same time, inhibition of ATP hydrolysis at the same Mn^{2+} concentrations occurs to a substantially lesser degree and arises apparently from other reasons.

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References

1. M. Kunitz, *J. Gen. Physiol.*, 1952, **35**, 423.
2. M. J. Schlesinger and M. J. Coon, *Biochim. Biophys. Acta*, 1960, **41**, 30.
3. B. S. Cooperman, *Methods Enzymol.*, 1982, **87**, 526.
4. V. Yu. Oganessyan, S. A. Kurilova, N. N. Vorobyeva, T. I. Nazarova, A. N. Popov, A. A. Lebedev, S. M. Avaeva, and E. H. Harutyunyan, *FEBS Lett.*, 1994, **348**, 301.
5. J. Käpylä, T. Hyytiä, R. Lahti, A. Goldman, A. A. Baykov, and B. S. Cooperman, *Biochemistry*, 1995, **34**, 792.
6. A. A. Baykov and A. S. Shestakov, *Eur. J. Biochem.*, 1992, **206**, 463.
7. E. H. Harutyunyan, I. P. Kuranova, B. K. Vainshtein, W. E. Höhne, V. S. Lamzin, L. Dauter, A. V. Teplyakov, and K. S. Wilson, *Eur. J. Biochem.*, 1996, **239**, 220.
8. P. Heikinheimo, J. Lehtonen, A. Baykov, R. Lahti, B. S. Cooperman, and A. Goldman, *Structure*, 1996, **4**, 1491.
9. E. H. Harutyunyan, V. Yu. Oganessyan, N. N. Oganessyan, S. M. Avaeva, T. I. Nazarova, N. N. Vorobyeva, S. A. Kurilova, R. Huber, and T. Mather, *Biochemistry*, 1997, **36**, 7754.
10. S. M. Avaeva, N. N. Vorob´eva, S. A. Kurilova, T. I. Nazarova, K. M. Polyakov, E. V. Rodina, and V. R. Samygina, *Biokhimiya*, 2000, **65**, 442 [*Biochemistry USSR*, 2000, **65** (Engl. Transl.)].
11. A. V. Kuznetsov, S. M. Avaeva, A. A. Baykov, and V. A. Sklyankina, *Biokhimiya*, 1977, **42**, 950 [*Biochemistry USSR,* 1977, **42** (Engl. Transl.)].
12. M. S. Mel´nik, T. I. Nazarova, and S. M. Avaeva, *Biokhimiya*, 1982, **47**, 323 [*Biochemistry USSR,* 1982, **47** (Engl. Transl.)].
13. S. A. Kurilova, A. V. Bogdanova, T. I. Nazarova, and S. M. Avaeva, *Bioorg. Khim.* 1984, **10**, 1147 [*Sov. J. Bioorg. Chem.*, 1982, **10** (Engl. Transl.)].
14. A. S. Shestakov, Ph. D. (Chem.) Thesis, Department of Chemistry, M. V. Lomonosov Moscow State University, Moscow, 1991 (in Russian).
15. M. S. Mel´nik, T. I. Nazarova, and S. M. Avaeva, *Bioorg. Khim.* 1984, **10**, 1483 [*Sov. J. Bioorg. Chem.*, 1984, **10** (Engl. Transl.)].
16. S. M. Avaeva, E. V. Rodina, S. A. Kurilova, T. I. Nazarova, and N. N. Vorobyeva, *FEBS Lett.*, 1996, **392**, 91.
17. J. Josse, *J. Biol. Chem.,* 1966, **241**, 1938.
18. S. M. Avaeva, E. V. Rodina, S. A. Kurilova, T. I. Nazarova, N. N. Vorobyeva, E. H. Harutyunyan, and V. Yu. Oganessyan, *FEBS Lett.,* 1995, **347**, 44.
19. A. A. Baykov and S. M. Avaeva, *Anal. Biochem.*, 1981, **116**, 1.
20. R. Phillips, *Chem. Rev.*, 1966, **66**, 501.
21. G. S. Baldwin, R. B. Sessions, S. G. Erskine, and S. E. Halford, *J. Mol. Biol.*, 1999, **288**, 87.
22. B. S. Cooperman, A. Panackal, B. Springs, and D. Hamm, *Biochemistry*, 1981, **20**, 6051.
23. S. E. Volk, A. A. Baykov, and S. M. Avaeva, *Biokhimiya*, 1981, **46**, 30 [*Biochemistry USSR*, 1981, **46** (Engl. Transl.)].
24. K. M. Welsh, A. Jakobyansky, B. Springs, and B. S. Cooperman, *Biochemistry*, 1983, **22**, 2243.
25. W. F. Bosron, R. A. Anderson, M. C. Falk, F. C. Kenhey, and B. L. Vallee, *Biochemistry*, 1977, **16**, 610.
26. E. A. Braga, A. A. Baykov, and S. M. Avaeva, *Biokhimiya*, 1973, **38**, 344 [*Biochemistry USSR*, 1973, **38** (Engl. Transl.)].
27. S. A. Kurilova, T. I. Nazarova, and S. M. Avaeva, *Bioorg. Khim.* 1984, **10**, 1336 [*Sov. J. Bioorg. Chem.*, 1984, **10** (Engl. Transl.)].
28. H. Sigel, R. Tribolet, R. Malini-Balakrishan, and R. B. Martin, *Inorg. Chem.*, 1987, **26**, 2149.
29. E. V. Rodina and Yu. P. Vainonen, *Tez. dokl. Mezhdunarodnaya Shkola-Konferentsiya "Gorizonty fiziko-khimicheskoi biologii"* [*Abstrs. of Papers, International School Conference on Prospects of Physicochemical Biology*], Pushchino, Moscow region, 2000, **2**, 103 (in Russian).

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