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Metal-Free PP_i Activates Hydrolysis of $MgPP_i$ by an *Escherichia coli* Inorganic Pyrophosphatase

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Abstract—Soluble inorganic pyrophosphatase from *Escherichia coli* (E-PPase) is a hexamer forming under acidic conditions the active trimers. We have earlier found that the hydrolysis of a substrate ($MgPP_i$) by the trimers as well as a mutant E-PPase Asp26Ala did not obey the Michaelis–Menten equation. To explain this fact, a model has been proposed implying the existence of, aside from an active site, an effector site that can bind PP_i and thus accelerate $MgPP_i$ hydrolysis. In this paper, we demonstrate that the noncompetitive activation of $MgPP_i$ hydrolysis by metal-free PP_i can also explain kinetic features of hexameric forms of both the native enzyme and the specially obtained mutant E-PPase with a substituted residue Glu145 in a flexible loop 144-149. Aside from PP_i, its non-hydrolyzable analog methylene diphosphonate can also occupy the effector site resulting in the acceleration of the substrate hydrolysis. Our finding that two moles of $[^{32}P]PP_i$ can bind with each enzyme subunit is direct evidence for the existence of the effector site in the native E-PPase.

Key words: inorganic pyrophosphatase, hexamer, trimer, effector site, substrate-induced activation, Glu145Gln substitution

The possible regulation of enzymatic activity originating from the allosteric binding of a substrate is a long-standing problem. Despite the common difficulty and ambiguity in the interpretation of data of that sort, several enzymes have been surely established to undergo allosteric activation by a substrate. This group of enzymes includes ribonucleotide reductase from *Escherichia coli* [1], phenylalanine hydroxylase [2], acetyl- and butyrylcholine esterases [3], pyruvate decarboxylase [4], and "malic enzyme" [5]. The present study places *E. coli* inorganic pyrophosphatase (E-PPase) among those enzymes. E-PPase is a highly specific enzyme converting a very simple compound and requiring no organic cofactors for the catalysis. These properties make E-PPase a suitable model for the study of substrate activation.

E-PPase is a member of the soluble inorganic pyrophosphatases (EC 3.6.1.1) and is one of the best-studied enzymes of this class. These enzymes catalyze hydrolysis of inorganic pyrophosphate (PP_i) producing two orthophosphates (P_i). Mg^{2+} is the best activator of E-PPase; Mn^{2+} or Zn^{2+} can also support the catalysis, but the enzymatic activity is much lower in those cases. For E-PPase, there are now a dozen X-ray structures including those of apo-form and a number of complexes with metal cofactors (Mg^{2+}, Mn^{2+}), inhibitor (Ca^{2+}), analog of the product of catalytic reaction (SO_4^{2-}), and non-hydrolyzable analog of the substrate ($CaPP_i$) [6-11]. The active site cavity can bind up to four metal ions of which three are necessary for catalysis. Two Mg^{2+} should fill subsites M1 and M2 of the apoenzyme with different affinities and provide the active conformation for the following substrate binding. Affinity of the enzyme for the metal ions is, in return, increased in the presence of a substrate. The third Mg^{2+} (M3) should be complexed with PP_i; together they form the active substrate $MgPP_i$. The last Mg^{2+}-binding subsite (M4) can be filled in the enzyme–substrate complex resulting in the inhibition of E-PPase [12].

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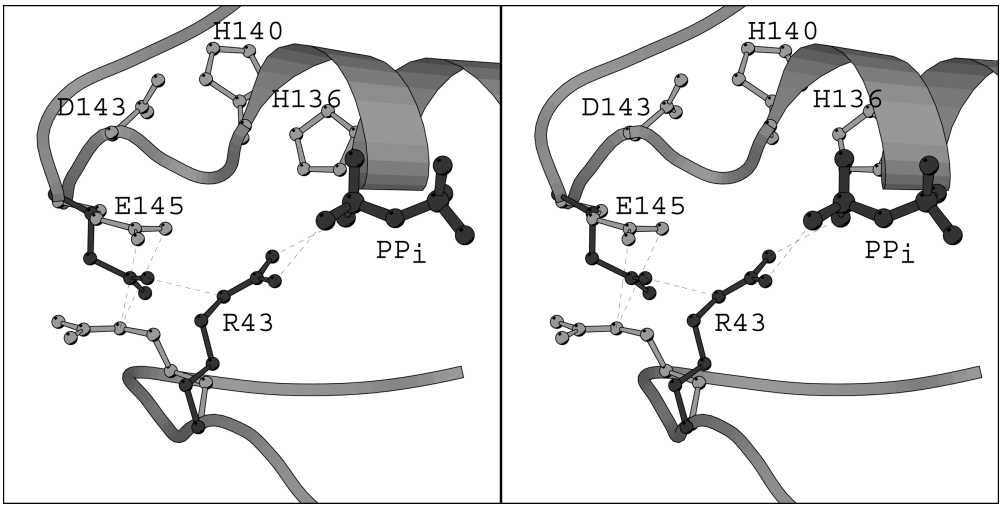

Fig. 1. Fragment of X-ray structure of E-PPase. Two structures superimposed are E-PPase complexed with Ca^{2+} (RCSB accession No. 1I6T) and E-PPase complexed with $CaPP_i$ (1I4O). The α-helix 128-143, the loop around Glu145, and the peptide backbone around Arg43 are shown. Positions of the side chains of Glu145 and Arg43 are shown in both structures (light gray, 1I6T; dark gray, 1I4O); dashed lines indicate ionic interactions. A pyrophosphate molecule bound in the active site is shown in ball-and-stick symbols. Side chains of the residues His136, His140, and Asp143 forming the intertrimeric interface are shown. This figure was prepared using the MOLSCRIPT program [16].

E-PPase is a homohexamer, each subunit having its own active site. The molecule of E-PPase is formed by two trimers, which lie in parallel plains like a sandwich and are several degrees twisted [6]. Intertrimeric contacts are formed by H-bonds and hydrophobic interactions [13], but the most essential part of the intertrimeric interface is represented by the two symmetry related ionic pairs Asp143–His136′ and the intertrimeric Mg^{2+} ion which is liganded by Asn24, Ala25, and Asp26 from two symmetry related subunits [6].

The trimeric form of E-PPase has been recently found to have an unusual kinetic property. Hydrolysis of $MgPP_i$ by this form did not obey the Michaelis–Menten equation, resulting in nonlinear Lineweaver–Burk plots. This finding had been first explained by a negative cooperativity of the two active sites within a trimer differing with their affinities with respect to $MgPP_i$ [14]. Analogous behavior was later observed for the trimeric and hexameric forms of the mutant E-PPase Asp26Ala with the substitution in the intertrimeric interface. To explain the observed kinetic features, a new scheme of allosteric activation of E-PPase by a metal-free PP_i has been proposed [15].

To advance the study of the effector subsite in E-PPase, we needed to ascertain if the native hexameric E-PPase possessed that property. In the present paper, by lowering pH and increasing $[Mg^{2+}]$, we have revealed the activation of $MgPP_i$ hydrolysis by a metal-free PP_i in the wild-type hexamer. Activation of $MgPP_i$ hydrolysis by a non-hydrolyzable substrate analog methylene diphosphonate (PCP) can also confirm the existence of the regulatory site in the native E-PPase, implying that PCP can substitute for PP_i in the effector site. We have determined the stoichiometry of PP_i binding with E-PPase and found that two PP_i are bound per enzyme subunit. This finding is direct evidence for the existence of an allosteric binding site for PP_i.

It is necessary to elucidate how a protein structure affects the properties of the effector site. For this purpose, the mutant E-PPase Glu145Gln has been obtained. The residue Glu145 came to a focus of our attention due to its location in the flexible loop 144-149. It does not participate directly in the coordination of a substrate or the metal ions, but sequence alignments of known PPases show rather high homology of this residue. The loop 144-149 follows directly the α-helix A, which forms the main part of intertrimeric interface, whereas another side of this helix forms the wall of the active site (Fig. 1). Besides, the side chain of Glu145 makes contact with Arg43, one of the ligands of PP_i. So, substitution of Glu145 could cause disorder in this region and as a consequence change kinetic properties of E-PPase. In fact, this mutation has been found to cause significant decrease in hydrolytic activity, impair affinity for activation by Mg^{2+}, and shift hexamer–trimer equilibrium toward trimer. Despite these alterations, trimeric form of Glu145Gln still retains a high affinity for the effector.

Therefore, in the present paper we have demonstrated the existence of an effector site in E-PPase by means of kinetic experiments as well as by a direct determination of

the stoichiometry of PP_i binding. Applicability of the proposed model has been shown for the explanation of the properties of both the native enzyme and its mutant variants.

MATERIALS AND METHODS

Chemicals. The recombinant *E. coli* inorganic pyrophosphatase and mutant enzyme were obtained as described previously [6, 17]. Enzyme suspensions were stored in ammonium sulfate (90% saturation) and desalted before use with a Sephadex G-50 (fine) column equilibrated with 0.05 M Tris-HCl, pH 7.5. $[^{32}P]Na_4P_2O_7$ of high quality was purchased from Izotop (Russia) and additionally purified by a thin-layer chromatography on PEI-cellulose using 0.7 M KH_2PO_4 as eluent. Labeled $[^{32}P]Na_4P_2O_7$ was diluted before use with the non-labeled $Na_4P_2O_7$ to a specific radioactivity required for each experiment. The other chemicals of high purity were purchased from Sigma (USA), Fluka (Switzerland), Serva and Merck (Germany), or Pharmacia Fine Chemicals (Sweden). All stock solutions were freshly prepared with high quality water purified in a MilliQ column.

The concentration of E-PPase solutions was determined by UV-spectroscopy using a specific absorption value $A_{1\,cm}^{0.1\%}$ of 1.18 [18]. Enzyme concentrations are calculated per enzyme subunit. Activities of the native and mutant E-PPases were determined by the rate of P_i release from $MgPP_i$. A semiautomatic phosphate analyzer was used for the quantitative P_i determination [19]. Enzymatic reaction was carried out at 25°C at the device sensitivity of 10 μM P_i for the full-scale. Hereinafter, enzyme activity was expressed in U/mg.

Dissociation of Glu145Gln E-PPase into trimers and their association. To obtain trimeric form of the wild-type or Glu145Gln E-PPases, hexameric enzymes (25 μM) were incubated for 0.5-2 h in 0.1 M Mes-NaOH buffer, pH 5.3, in the absence of Mg^{2+}. To reassociate trimeric forms, 1 M Tris-HCl, pH 9.0, was added to a solution of the trimers to pH 7.5. Both dissociation and association were carried out at 20°C.

Dissociation and association were monitored in progress by sampling aliquots and determining PPase activity. The PPase assay contained 0.05 M Tris-HCl, pH 7.5, 1 mM Mg^{2+}, and 20 μM $MgPP_i$. Rate constants for dissociation or association were calculated as described earlier [20].

Kinetic measurements. Total $MgCl_2$ and $Na_4P_2O_7$ concentrations required to keep fixed necessary concentrations of Mg^{2+} and $MgPP_i$ were calculated using K_d values for $MgPP_i$ and Mg_2PP_i of 47.7 μM and 2.42 mM (pH 7.5) or 696 μM and 16.23 mM (pH 6.0), respectively [21].

Apparent parameters of $MgPP_i$ hydrolysis, V_{max}^{app} and K_m^{app}, were determined in 0.05 M buffer of corresponding pH at 5 mM free Mg^{2+}, concentration of $MgPP_i$ being varied from 2.5 to 100 μM. The buffers used were Mes-NaOH (pH 6.0-7.0), Hepes-NaOH (pH 7.0-7.5), Tris-HCl (pH 7.2-9.0), and 2-amino-2-methyl-1,3-propanediol-HCl (pH 9.0-9.7). Activity of PPases at fixed pH did not depend on the nature or concentration of the buffer; thus, it was not necessary to keep the ionic strength constant. Apparent kinetic parameters k_{cat}^{app} and k_{cat}^{app}/K_m^{app} were calculated using Michaelis–Menten equation unless indicated otherwise. The pH-independent kinetic parameters of $MgPP_i$ hydrolysis (k_{cat} and k_{cat}/K_m) were determined from the best fit of Eqs. (1) and (2) to these data:

$$k^{app} = k_{cat}/(1 + [H^+]/K_{ESH_2} + K_{ESH}/[H^+]), \quad (1)$$

$$k^{app}/K_m^{app} = (k_{cat}/K_m)/(1 + [H^+]/K_{EH_2} + K_{EH}/[H^+]). \quad (2)$$

Initial rates of $MgPP_i$ hydrolysis as a function of free Mg^{2+} concentration were determined in 0.05 M Tris-HCl, pH 7.5, at 50 μM $MgPP_i$ and at free Mg^{2+} concentration varying in the range 0.01-40 mM. Initial rates of $MgPP_i$ hydrolysis as a function of $MgPP_i$ concentration were determined in 0.05 M Tris-HCl, pH 7.5, at several fixed free $[Mg^{2+}]$ from 0.2 to 5 mM and at 2.5-1000 μM $MgPP_i$. Analogous experiment was carried out in 0.1 M Mes-NaOH, pH 6.0, at 10 mM free Mg^{2+}. The rate of hydrolysis in the presence of methylene diphosphonate (PCP) was determined in 0.1 M Mes-NaOH, pH 6.0, at 10 mM free Mg^{2+} and 5 μM $MgPP_i$ for hexameric E-PPase or 10 μM $MgPP_i$ for trimeric E-PPase. Dissociation constant K_d for MgPCP at pH 6.0, for lack of literature data, was taken equal to K_d for $MgPP_i$ (0.7 mM [21]). All kinetic data in Figs. 2-4 are given as average from two or three independent measurements.

Differential UV-spectroscopy. The wavelength-scan absorption of Glu145Gln E-PPase complexed with Mg^{2+} was determined by differential UV-spectroscopy using an Ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech, Sweden). The enzyme was titrated with the $MgCl_2$ solution in 0.1 M Tris-HCl, pH 8.5, as described previously [22]. Dissociation constant K_d(M2) of E-PPase complexed with Mg^{2+} were calculated from the dependence of maximum absorption at 243-246 nm on the total $MgCl_2$ concentration according to [22].

Velocity sedimentation analysis. Sedimentation velocity measurements were carried out at 48,000 rpm, 20°C, in a Spinco E analytical ultracentrifuge (Beckman, USA) with scanning at 280 nm. Sedimentation coefficient was calculated with a standard procedure [23]. Enzyme concentrations were 5-10 μM.

Stoichiometry of PP_i binding with E-PPase. Stoichiometry of PP_i binding with enzyme was determined by equilibrium gel filtration using three variants of the experiment.

a) Reaction mixture (50 μl) containing E-PPase (59.7 μM), 363 μM $[^{32}P]Na_4P_2O_7$ (specific radioactivity

of 0.33 μCi/μmol), 0.9 mM $CaCl_2$, and 0.05 M Tris-HCl, pH 7.5, was gel-filtered on a Sephadex G-50 (fine) column (0.5 × 5 cm) pre-equilibrated with a mixture of the same components except for the enzyme. The same mixture was used as the eluent. Fractions of 50 μl were collected during elution. In all fractions, the protein concentration and radioactivity were determined (using Tracor Analytic 300 Liquid Scintillation System; Delta, USA), and the specific inclusion (PP_i/protein ratio) was calculated.

b) Reaction mixture (50 μl) containing E-PPase (61.5 μM), 200 μM $[^{32}P]Na_4P_2O_7$ (0.52 μCi/μmol), 200 μM $LaCl_3$, 1 mM $MgCl_2$, and 0.05 M Tris-HCl, pH 7.5, was separated and analyzed as in (a). In this modification of the experiment, free PP_i was absent because of the very high stability constant of $LaPP_i$ complex (~10^{17} M^{-1}).

c) Reaction mixture containing E-PPase (64.7 μM), 363 μM $[^{32}P]Na_4P_2O_7$ (0.27 μCi/μmol), and 0.05 M Tris-HCl, pH 7.5, was separated and analyzed as in (a).

RESULTS AND DISCUSSION

Properties of Glu145Gln E-PPase. The maximal rate of $MgPP_i$ hydrolysis determined for the hexameric Glu145Gln was 110 and 187 U/mg at 1 or 5 mM Mg^{2+}, respectively. Thus, Glu145Gln E-PPase had hydrolytic activity of only ~20% compared to the wild-type enzyme. To correctly compare kinetic properties of the mutant variant and the wild-type enzyme, pH-independent parameters of $MgPP_i$ hydrolysis were determined for Glu145Gln E-PPase (Table 1). The value of k_{cat} for the mutant enzyme decreased more than fourfold. The apparent Michaelis constant at pH 7.5 for the Glu145Gln E-PPase was found to be of the same order of magnitude as for the wild-type enzyme, but pH-independent values of K_m differed tenfold. Ionization constants of essential groups changed significantly for the free form of the mutant enzyme (pK_{EH_2} and pK_{EH}) but were virtually unchanged for the enzyme–substrate complex (pK_{ESH_2} and pK_{ESH}).

The dissociation constant of Mg^{2+} at the activating subsite M2, K_d(M2), was determined from UV-spectroscopic titration of Glu145Gln E-PPase. To determine the analogous constant in the presence of the substrate, hydrolysis of $MgPP_i$ by Glu145Gln variant was studied as a function of free Mg^{2+} concentration. Calculated values of K_d(M2) are collected in Table 1. It is that this mutation decreased affinity for Mg^{2+} of both the free enzyme and the enzyme–substrate complex.

Dissociation of the hexameric form of Glu145Gln E-PPase and association of trimers. Incubation of Glu145Gln E-PPase at pH 5.3 caused a decrease in the sedimentation coefficient from 6.6 to 3.6 S, paralleling the decrease in catalytic activity to ~10%. Hexameric Glu145Gln can be restored by increasing the pH from 5.3 to 7.5, as judged from the fully recovered catalytic activity. Inactivation and reactivation of Glu145Gln E-PPase followed first- and second-order kinetics, respectively. From these data, the rate constants for dissociation of hexamers, k_{diss}, and the association of trimers, k_{ass}, was calculated (Table 2). As can be seen, dissociation of the mutant enzyme is much faster compared to the wild-type PPase. The reverse process of association into hexamer is tenfold slower for the mutant trimers. Therefore, substi-

Table 1. Properties of the hexameric and trimeric forms of Glu145Gln and wild-type (WT) E-PPases

Enzyme	pH-independent parameters of $MgPP_i$ hydrolysis (5 mM Mg^{2+}, 25°C)					
	k_{cat}, sec^{-1}	k_{cat}/K_m, $\mu M^{-1}\cdot sec^{-1}$	pK_{EH_2}	pK_{EH}	pK_{ESH_2}	pK_{ESH}
E145Q hexamer	83 ± 3	64 ± 8	7.10 ± 0.06	9.71 ± 0.07	7.7 ± 0.2	9.2 ± 0.2
WT hexamer [12]	390 ± 80	3000 ± 780	7.8 ± 0.5	8.7 ± 0.5	7.6 ± 0.3	8.9 ± 0.2

Enzyme	K_d (M2), mM	
	free enzyme	enzyme–substrate complex
E145Q hexamer	5.5 ± 0.5	1.3 ± 0.4
E145Q trimer	not determined	0.9 ± 0.1
WT hexamer [12]	1.46 ± 0.02	0.20 ± 0.04
WT trimer [15]	not determined	1.2 ± 0.5

Table 2. Characteristics of trimeric forms of Glu145Gln and wild-type (WT) E-PPases

Trimeric E-PPase	$s_{20,w}$, S	Activity, U/mg	k_{ass}, $\mu M^{-1} \cdot min^{-1}$	k_{diss}, min^{-1}
E145Q	3.6 ± 0.1	15.0 ± 0.3	0.037 ± 0.002	0.26 ± 0.04
WT [20]	3.59 ± 0.05	280 ± 10	0.31 ± 0.02	0.102 ± 0.009

Note: Activities were determined at pH 7.5, 1 mM Mg^{2+}, and 20 μM $MgPP_i$. k_{ass}, rate constant of association of trimers at pH 7.5; k_{diss}, rate constant of dissociation of corresponding hexamers at pH 5.3.

tution of Glu145Gln causes a shift in the equilibrium between hexamers and trimers, making the latter species more preferable energetically than in the case of the native E-PPase. The observed features of the mutant E-PPase Glu145Gln is quite consistent with our expectations originating from the location of the mutated residue near the intersubunit interface (Fig. 1).

Kinetic properties of the trimeric form of Glu145Gln E-PPase. *Binding of Mg^{2+} at site M2.* The dissociation constant for Mg^{2+} at subsite M2 in the enzyme–substrate complex of trimeric form of Glu145Gln was determined (Table 1). Comparison of the constants K_d(M2) for the hexameric and trimeric forms of the mutant variant showed that, unlike for the wild-type E-PPase, these values did not differ much. This may indicate that the affinity for Mg^{2+} of subsite M2 is influenced much more by the substitution itself than by the following dissociation into trimers.

Non-Michaelis rate profiles. Hydrolysis of $MgPP_i$ by the trimeric form of Glu145Gln variant was studied at pH 7.5 over a wide range of concentrations of Mg^{2+} and $MgPP_i$ (Fig. 2a). The functions v versus [$MgPP_i$] obtained at the concentrations of free Mg^{2+} higher than 5 mM are not described by Michaelis–Menten kinetics. Plotting these data in double-reciprocal coordinates gave nonlinear dependence that could rather be treated as two lines corresponding to the low and high concentrations of substrate (Fig. 2a, inset). Nonlinear dependence flattened at lower Mg^{2+} concentration and became a straight line at ~0.2 mM Mg^{2+} (Fig. 2b). Trimeric forms of both mutant and wild-type enzymes showed similar dependencies of the initial rate of hydrolysis on the concentration of $MgPP_i$, though the absolute rates of hydrolysis differed more than tenfold.

The observed two-stage dependence could not result from the formation of a hexamer during the assay. Association of trimers is a second-order reaction, and the very low concentration of enzyme in the assay mixture makes it very slow. In special experiments, no changes in activity of trimers were found after 10-15 min incubation under the assay conditions, while one assay measurement took less than 5 min. In addition, the curves of phosphate accumulation were linear during PPase assay, confirming that hexamer–trimer interconversion does not occur in the course of activity measurement.

Non-Michaelis kinetics could be, in principle, interpreted in different ways. For the mutant E-PPase Asp26Ala, we recently proposed a model allowing the most adequate description of the whole set of observed

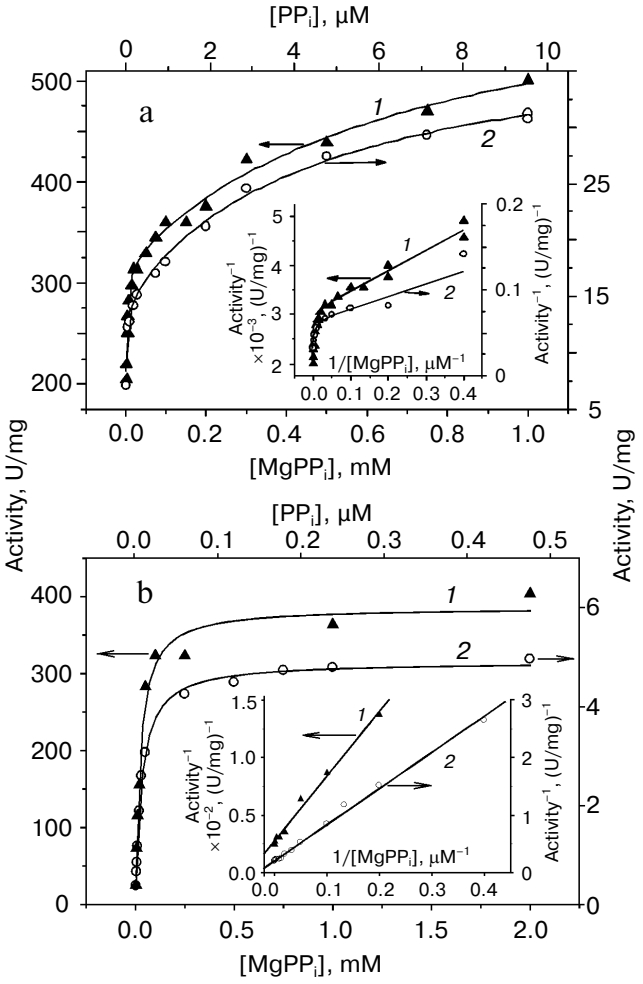

Fig. 2. Rates of $MgPP_i$ hydrolysis by the trimeric forms of wild-type (*1*) and Glu145Gln (*2*) E-PPases as a function of substrate concentration. Enzyme activity was assayed at pH 7.5, at free Mg^{2+} concentration of 5 (a) or 0.2 mM (b). Enzyme concentration was 0.5-1.25 nM for the wild-type and 5-10 nM for Glu145Gln E-PPases. Hereinafter, the upper abscissa axis shows concentration of free PP_i in a reaction assay. Inset, the same data in double-reciprocal coordinates. Lines are drawn according to Eq. (3) using parameters given in Table 3.

features [15]. In this paper, we have treated kinetic data for trimers in accordance with this model.

The proposed model assumes that each subunit of E-PPase, apart from the active site, has an extra binding site for the metal-free PP_i. Binding of PP_i at this allosteric site activates hydrolysis of $MgPP_i$ in the active site. A kinetic scheme has been developed for this model, where E is enzyme subunit, K_E is dissociation constant for the enzyme–effector (PP_i) complex, K_S is the dissociation constant for the enzyme–substrate ($MgPP_i$) complex, k is rate constant for the catalytic stage, β is activation coefficient, and superscripts A or E denote enzyme species with ligand bound in the active or effector sites, respectively:

$$\begin{array}{ccccc} & \overset{K_S}{} & & \xrightarrow{\quad k \quad} & \\ E & \Leftrightarrow & E(MgPP_i)^A & & \text{products} \\ \Updownarrow K_E & & & & \\ & \overset{K_S}{} & & \xrightarrow{\quad \beta k \quad} & \\ E(PP_i)^E & \Leftrightarrow & E(PP_i)^E(MgPP_i)^A & & \text{products} \end{array}$$

Scheme

According to this scheme, the rate of hydrolysis of $MgPP_i$ at any fixed concentration of Mg^{2+}, v, is described by Eq. (3), where S is the concentration of substrate ($MgPP_i$), V_{max} is the maximal rate of hydrolysis at saturating concentration of substrate, K_d is the equilibrium constant for the reaction $MgPP_i \Leftrightarrow Mg^{2+} + PP_i$ at given pH, and the other designations are the same as in the scheme:

$$v = \frac{V_{max}(1 + \beta K_d S / K_E[Mg^{2+}])S}{K_S + (1 + K_S K_d / K_E[Mg^{2+}])S + K_d S^2 / K_E[Mg^{2+}]} \quad (3)$$

If the concentration of Mg^{2+} is not too high, free PP_i is present in the reaction mixture along with the substrate $MgPP_i$. The ratio [free PP_i]/[$MgPP_i$] increases with decreasing [Mg^{2+}] and lowering pH. For example, at 5 mM Mg^{2+} and pH 9.0, 7.5, and 6.0 this ratio is equal to 0.00059, 0.0095, and 0.139, respectively. To demonstrate how activity increases as the concentration of effector increases, we have provided Figs. 2a and 2b with the additional abscissa axes that show the concentration of free PP_i under the conditions of the experiment. When the concentration of $MgPP_i$ in a reaction assay is lower than 50 μM, free PP_i is nearly absent and the rate of catalysis is close to that without activator. As the concentration of $MgPP_i$ increases, the concentration of free PP_i increases proportionally; the occupancy of the effector subsite increases according to the value of K_E, resulting in the activation of hydrolysis.

Kinetic parameters calculated according to the proposed scheme are given in Table 3. Data for the wild-type E-PPase are included for comparison. It can be seen from the obtained parameters K_E and K_S that the effector subsite in both trimeric enzymes has high affinity for an effector,

Table 3. Kinetic parameters of $MgPP_i$ hydrolysis by different oligomeric forms of Glu145Gln and wild-type (WT) E-PPases

E-PPase	Conditions	Kinetic parameters calculated according to a Michaelis–Menten equation	
		V_{max}, U/mg	K_m, μM
E145Q hexamer	pH 7.5, 5 mM Mg^{2+}	187 ± 4	3.7 ± 0.4
WT hexamer	pH 7.5, 1 mM Mg^{2+}	808 ± 15	6.3 ± 0.6

E-PPase	Conditions	Kinetic parameters calculated according to Scheme			
		V_{max}, U/mg	K_S, μM	β	K_E, μM
E145Q trimer	pH 7.5, 5 mM Mg^{2+}	14.5 ± 0.9	1.9 ± 0.5	2.6 ± 0.5	5 ± 1
	pH 7.5, 0.2 mM Mg^{2+}	2.32 ± 0.03	16 ± 3	2.1 ± 0.4	5 ± 2
WT hexamer	pH 6.0, 10 mM Mg^{2+}	48 ± 5	12 ± 2	2.00 ± 0.05	0.5 ± 0.4
WT trimer [15]	pH 7.5, 5 mM Mg^{2+}	319 ± 6	1.3 ± 0.1	2.0 ± 0.1	7 ± 2
	pH 7.5, 0.2 mM Mg^{2+}	192 ± 7	14 ± 8	2.0 ± 0.2	3 ± 6

which is comparable to affinity of the active site for substrate. This finding can provide an explanation why the regulatory subsite had not been earlier revealed in the hexameric E-PPases. Hydrolysis of $MgPP_i$ by the hexameric E-PPases, both the wild-type and a number of mutant variants including Glu145Gln, did not deviate from Michaelis–Menten kinetics [17, 24]. The only exception was a hexamer of Asp26Ala variant [15]. This fact could be understood under the assumption that the hexameric E-PPase had a significantly higher affinity of the regulatory subsite for PP_i compared to the trimeric form. As a consequence, this site is completely occupied with effector even at the lowest concentrations of $MgPP_i$ used in the assay.

Hydrolysis of $MgPP_i$ by hexameric E-PPase at pH 6.0 and 10 mM Mg^{2+}. Our proposal that the effector site has a very high affinity for PP_i was confirmed by the results of the following experiment. Hydrolysis of $MgPP_i$ was studied under specially designed conditions, in the presence of high concentration of Mg^{2+} (10 mM) and at pH lowered from 7.5 to 6.0. The hydrolytic rate as a function of substrate concentration is shown in Fig. 3. It can be seen that the rate profile under those conditions is two-staged and nonlinear in the double-reciprocal plot (Fig. 3, inset). Kinetic data were described according to the scheme (determined parameters are given in Table 3). As expected, dissociation constant K_E for the wild-type hexamer indicates a very high affinity of this species for effector even at pH 6.0. For both studied trimers, this constant was significantly higher (Table 3). It should be noted that affinity of the effector site for PP_i is influenced by the state of the intertrimeric interface, dissociation of hexameric enzyme into trimers being the most illustrative example.

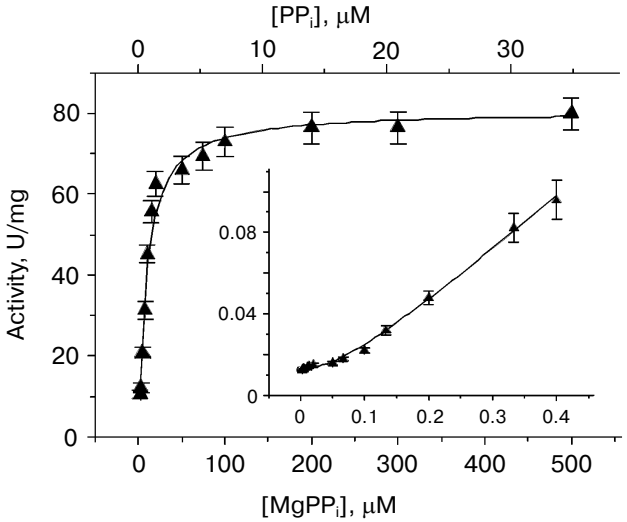

Fig. 3. Hydrolysis of $MgPP_i$ by hexameric wild-type E-PPase at pH 6.0, 10 mM Mg^{2+}. Inset, the same data in double-reciprocal coordinates. Enzyme assay was as given in "Materials and Methods". Enzyme concentration was 0.5-2 nM. Lines are drawn according to Eq. (3) using parameters given in Table 3.

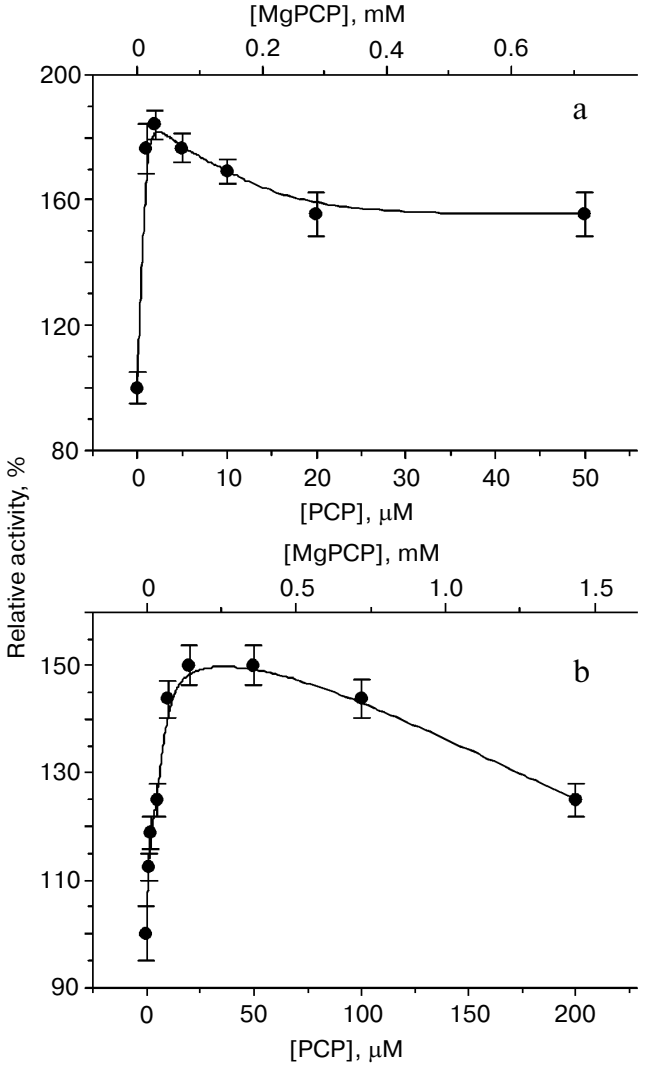

Fig. 4. Activation of hexameric (a) and trimeric (b) wild-type E-PPase by a metal-free PCP. Hydrolysis of $MgPP_i$ was assayed in 0.1 M Mes-NaOH, pH 6.0, at 10 mM Mg^{2+} and 5 (a) or 10 μM $MgPP_i$ (b). Concentration of metal-free PCP is on the bottom abscissa axis, and the contaminating increase in MgPCP concentration is on the upper abscissa axis. Lines are drawn according to a built-up regression.

Activation of $MgPP_i$ hydrolysis by methylene diphosphonate (PCP). Methylene diphosphonate is the analog of PP_i in which the bridging oxygen atom is replaced with a CH_2 group. Magnesium complex of this analog, MgPCP, is capable of binding in the active site of PPase and acts as a competitive inhibitor [25].

In the present paper, we have studied hydrolysis of $MgPP_i$ by the hexameric and trimeric forms of E-PPase in the presence of PCP at fixed concentrations of Mg^{2+} and $MgPP_i$. It has been shown that increasing total concentrations of PCP initially led to an increase in activity of E-PPase up to 180% for hexamer and up to 150% for trimer (Fig. 4). Because PCP is not hydrolyzed by a E-

PPase, PCP-induced activation of the enzyme can only be noncompetitive, i.e., an activating molecule of PCP must occupy the allosteric site rather than the active site. The most reasonable assumption is that the hydrolysis of $MgPP_i$ is activated by a metal-free form of PCP (in opposition to the inhibitory effect of MgPCP) and that the metal-free PCP mimics the effect of a metal-free PP_i. Anyway, this observation can serve as additional evidence of the allosteric effector site in hexameric E-PPase.

Maximal activation of E-PPase was observed in the micromolar range of PCP concentrations, which implies high affinity of the effector subsite for this analog. The estimated concentration of metal-free PCP corresponding to the maximal activity was significantly higher for the trimer (35 μM) than for the hexamer (2 μM). This finding is in good agreement with the assumption that the affinity of the effector subsite for its ligands is impaired in trimeric E-PPase.

Further increase in PCP concentration resulted in the inhibition of E-PPase, which was most likely caused by the competitive binding of MgPCP in the active site as the concentration of magnesium complex of PCP increased.

Stoichiometry of PP_i binding with E-PPase. Kinetic data indicate that pyrophosphate molecules of two types (hydrolyzable and effector) can interact with the enzyme with different affinities. The kinetic model does not enable to clarify in what way these PP_i molecules are bound in the hexameric enzyme. Two binding sites for PP_i can exist in the same subunit; alternatively, substrate and effector PP_i molecules can occupy the active sites of two different subunits. We managed to distinguish between those possibilities after gel filtration of the hexameric wild-type E-PPase with labeled pyrophosphate that allowed determination of stoichiometry of enzyme–PP_i complex. Three modifications of this experiment have been developed. In the first variant (experiment (a)), the enzyme was incubated in the mixture containing equilibrium amounts of a metal-free and metal-complexed labeled pyrophosphate (0.3 mM $[^{32}P]CaPP_i$, 63 μM metal-free $[^{32}P]PP_i$, and 0.6 mM free Ca^{2+}). After incubation, the mixture was gel-filtered and in all fractions the protein concentration and radioactivity were determined. Based on these data, total inclusion of PP_i in the hexameric E-PPase was calculated (Fig. 5). The resulting complex of E-PPase with PP_i included totally 12.0 ± 1.2 mol PP_i per mol of hexamer, or 2.0 ± 0.2 mol PP_i per mol of subunit. This finding univocally demonstrated that pyrophosphate can simultaneously occupy two different sites in each subunit of E-PPase. The non-hydrolyzable analog of substrate $CaPP_i$ occupies the active site (K_d = 0.6 μM [26]), whereas the second molecule of PP_i occupies the effector site.

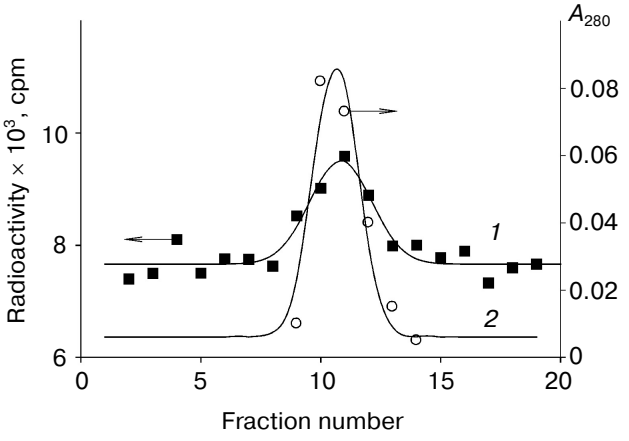

Fig. 5. Elution profile of E-PPase after equilibrium gel filtration with PP_i. Reaction mixture contained 59.7 μM E-PPase, 0.3 mM $[^{32}P]CaPP_i$, 0.6 mM Ca^{2+}, and 63 μM metal-free $[^{32}P]PP_i$ in 0.05 M Tris-HCl, pH 7.5 (see experiment (a), "Materials and Methods"). Fractions of 50 μl were collected after gel filtration and diluted with buffer to 300 μl. Radioactivity (*1*) and protein concentration (*2*) were determined as described in "Materials and Methods".

To rule out the possibility of nonspecific sorption of PP_i, two control experiments ((b) and (c)) were carried out. In variant (b), enzyme was incubated with 200 μM $[^{32}P]LaPP_i$ in the presence of free Mg^{2+}. Free PP_i under these conditions was absent in the mixture. The resulting enzyme–PP_i complex contained totally 6.0 ± 0.6 mol PP_i per mol of hexamer, or 1.0 ± 0.1 mol PP_i per mol of subunit. In the presence of Mg^{2+}, $LaPP_i$ is a poor substrate of E-PPase with K_m = 140 μM [26]. Therefore, under the conditions of the experiment, one molecule of PP_i included in the subunit of E-PPase corresponds to $LaPP_i$ bound in the active site of enzyme. In variant (c), the enzyme was incubated with $[^{32}P]PP_i$ in the absence of metal ions. Under these conditions, none of the PP_i was bound to E-PPase. These control experiments showed that the metal-complexed PP_i can occupy only the active site but not the effector site, and that the metal-free PP_i cannot occupy the effector site when the active site is empty. This is still a question whether filling of the effector site can occur when the active site is only occupied with the metal ions, or substrate binding is also necessary.

Thus, in this paper a number of arguments are presented for the existence of an effector site in E-PPase. First, they include the nonlinear kinetics of $MgPP_i$ hydrolysis typical for trimeric forms of the wild-type and the mutant E-PPases as well as for the hexameric form of Asp26Ala E-PPase. It is particularly important that the native hexamer under specially designed conditions also shows non-Michaelis kinetics of hydrolysis. Second, there is the fact of activation of $MgPP_i$ hydrolysis by the non-hydrolyzable analog of pyrophosphate, PCP. Finally, determination of the stoichiometry of enzyme–pyrophosphate complex after equilibrium gel filtration demonstrates that each subunit of a hexamer is capable of binding two PP_i molecules. This finding is direct evidence

that there is an allosteric binding site for PP_i in the subunit of E-PPase. Since the metal-complexed PP_i is unable to occupy the effector subsite, even when the active site is occupied with $LaPP_i$ (control (b)), we can conclude that the observed two molecules of PP_i corresponds to a metal-complexed species bound in the active site plus a metal-free species bound in the effector site.

The whole set of data obtained up to now by the kinetic and equilibrium methods can be most adequately described within the model (see Scheme) implying the activation of $MgPP_i$ hydrolysis by the effector molecule of metal-free PP_i bound at the allosteric site of the same subunit. It should be noted that this model does not claim to be the only model giving the real description of true mechanism, but it is not unreasonable, makes some sense, and allows satisfactory description of the experimental data, so it is acceptable as a working model until some contradictory facts might be found. Acceptance of this model in turn suggests a number of questions that still remain unclear.

One of these open issues is the location of the regulatory site in the E-PPase molecule. If the effector PP_i is bound on the surface of PPase globule, its preferable coordination in the metal-free form seems unreasonable since the PPase surface is rich with negatively charged residues (the p*I* of the enzyme is 5.0). Results obtained with the Glu145Gln variant demonstrate that the properties of the effector site are defined by the state of intersubunit contacts. Affinity of E-PPase for the effector is significantly decreased upon the destabilization of intertrimeric contacts resulting from this mutation. This effect becomes still more pronounced after hexamers dissociate [15]. The effector site is however unlikely to be located in the intertrimeric interface itself since trimeric form of E-PPase still retains effector properties. The fact that the affinity of PPase for the effector is influenced by mutation of Glu145 can also be explained by the alteration of the structure of the flexible loop 144-149 where this residue is placed. According to our preliminary studies, certain residues located in this loop may take part in formation of the effector site. In this case, communication between the active and effector sites may occur through residue Glu145, which is connected in the crystal structure of E-PPase with the key ligand of substrate/product, the guanidine group of Arg43 (Fig. 1).

It should be noted that we have not observed any additional molecules of PP_i in the 3D structure of hexameric E-PPase complexed with $CaPP_i$ [10]. This apparent contradiction may have resulted from the very high concentrations of Ca^{2+} and the precipitating salt excluding the metal-free PP_i in the course of crystallization.

Mechanism of the activating influence of effector on hydrolysis is still quite unclear. It is not improbable that the effector bound in the allosteric site facilitates release of phosphates after hydrolysis. We hope the disclosure of the effector site can provide the basis for clarification of some open questions concerning the hydrolytic mechanism.

Soluble PPases are constitutive enzymes, so our finding can be important in the further understanding of their *in vivo* regulation. PP_i is only one example of the numerous polyphosphates abundant in the cell, so probably the effector site of E-PPase can bind other phosphorus-containing ligands. Specificity of the effector site and its exact localization in the structure of E-PPase is the subject of our further investigations.

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