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ATP as Effector of Inorganic Pyrophosphatase of *Escherichia coli*. The Role of Residue Lys112 in Binding Effectors

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Abstract—It has been shown that PP_i, methylenediphosphonate, and ATP act as effectors of *Escherichia coli* inorganic pyrophosphatase (E-PPase), and that they compete for binding at the allosteric regulatory site. On the basis of chemical modification and computer modeling of a structure of the enzyme–ATP complex, a number of amino acid residues presumably involved in binding effectors has been revealed. Mutant variants Lys112Gln, Lys112Gln/Lys148Gln, and Lys112Gln/Lys115Ala of E-PPase have been obtained, as well as a modified variant of wild type E-PPase (^{Ad}wt PPase) with a derivative of ATP chemically attached to the amino group of Lys146. Kinetic properties of these variants have been investigated and compared to the earlier described variants Lys115Ala, Arg43Gln, and Lys148Gln. Analysis of the data confirms the proposed location of an effector binding site in a cluster of positively charged amino acid residues including the side chains of Arg43, Lys146 (subunit A), Lys112, and Lys115 (subunit B). Lys112 is supposed to play a key role in forming contacts with the phosphate groups of the three studied effectors.

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Key words: pyrophosphatase, ATP, PP_i, binding site, effector, site-directed mutagenesis

This work continues an investigation of effector properties of the family I soluble inorganic pyrophosphatases (PPases). The object of this research, inorganic pyrophosphatase from *Escherichia coli* (E-PPase), is a constitutive enzyme that acts as a hexamer in the cell. Neither the ways of regulation of its catalytic activity *in vivo* nor the possible involvement of an oligomeric organization in these processes has been clarified yet. It has been found recently that metal-free pyrophosphate can act as an incompetitive activator of E-PPase, and that there is an allosteric regulatory site in the enzyme molecule [1, 2]. One of the supposed locations of the binding site for this effector is a cationic cluster formed by the side chains of Lys115, Arg43, and Lys148. Preliminary studies on mutant variants with single substitutions of the residues forming the cluster (Lys115Ala, Arg43Gln, and Lys148Gln) showed a decrease in affinity for effector in these variants [3]. In the previous paper [4], we demonstrated that ATP can also affect hydrolysis of $MgPP_i$ by E-PPase. According to the data of molecular docking and chemical modification, residues Lys112 and Lys146 were additionally proposed to be involved in binding effector [4]. In the present work, we describe mutant variants of E-PPase with the substitutions at position 112 (Lys112Gln, Lys112Gln/Lys148Gln, and Lys112Gln/Lys115Ala) as well as the properties of E-PPase where the amino group of Lys146 was chemically modified with the derivative of ATP (^{Ad}wt PPase [4]). Comparison of the kinetic behavior of these enzymes with the properties of the variants obtained earlier makes it clear that the effector binding site is situated in the cationic cluster, and reveals a key role of Lys112 in the enzyme–effector interaction. Comparison of the crystal structures of the

Abbreviations: E-PPase) *E. coli* inorganic pyrophosphatase; Y-PPase) *S. cerevisiae* inorganic pyrophosphatase; PCP) methylenediphosphonate; ^{Ad}wt PPase) pyrophosphatase chemically modified with the dialdehyde derivative of ATP at the amino group of Lys146.

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PPases from *E. coli* and *Saccharomyces cerevisiae* revealed the analogous cationic cluster in the eukaryotic enzyme, which suggests that the PPases of two branches share common mechanisms of regulation of their activity.

MATERIALS AND METHODS

Chemicals. Recombinant *E. coli* E-PPase was obtained as described in [5, 6]. The enzyme was stored at 4°C as a suspension in ammonium sulfate (90% saturation). Before use, it was gel filtered on a Sephadex G-50 (fine) column equilibrated with 0.05 M Tris-HCl, pH 7.5. E-PPase adenylated at the amino group of Lys146 (^{Ad}wt PPase) was obtained as described in [4]. The purity of enzyme preparations as characterized by gel electrophoresis in 12% polyacrylamide gel was at least 95%. Chemicals of high purity grade were purchased from Sigma (USA), Fluka (Switzerland), Merck (Germany), and Pharmacia Fine Chemical (Sweden). All solutions were made using deionized water additionally purified with MilliQ.

The concentration of Mg^{2+} solutions was determined by titration with Eriochrome Black B. The concentration of the enzyme solutions was determined by UV-spectrophotometry using the specific absorption coefficient $A_{1cm}^{1\%} = 11.8$ [7]. Hydrolytic activity of PPases was determined by the rate of P_i release from $MgPP_i$ at 25°C. The concentration of P_i was quantitatively measured using a semi-automatic phosphate analyzer [8] at a sensitivity of 10 μM P_i full scale. Hydrolytic activity was expressed as IU (1 U of PPase converts 1 μmol of $MgPP_i$ in 1 min).

Kinetic measurements. Concentrations of free Mg^{2+} and $MgPP_i$ were calculated using the values of K_d for $MgPP_i$ and Mg_2PP_i, respectively, of 47.7 μM and 2.42 mM at pH 7.5 [9]. $MgPP_i$ hydrolysis was studied in 0.05 M Tris-HCl, pH 7.5, at 2 mM Mg^{2+} and, if it was necessary, in the presence of 1 mM AMP or 0.5 mM MgATP. The concentration of $MgPP_i$ was varied in the range 3-300 μM. Effect of methylenediphosphonate (PCP) on the catalytic activity was studied in 0.1 M Mes-NaOH, pH 6.0, at 10 mM Mg^{2+} and 10 μM $MgPP_i$. The total concentration of PCP was varied in the range 0-100 μM. Kinetic parameters were determined as the best fit with the Michaelis–Menten equation or Eq. (1) using SigmaPlot 7.0.

Obtaining the trimeric form of E-PPase. Hexameric wild type E-PPase was dissociated to trimers by incubation of 25 μM enzyme solution in 0.1 M Mes-NaOH, pH 5.3, for 20 h at 25°C. Hexameric Lys112Gln was dissociated by incubation of 25 μM enzyme solution in 0.1 M Na-citrate buffer, pH 4.5, for 20 h at 25°C. Dissociation to trimers was confirmed by velocity sedimentation at 48,000 rpm at 20°C using a Spinco E ultracentrifuge (Beckman, USA) with scanning at 280 nm. Sedimentation coefficients were calculated using a standard protocol [10]. Enzyme concentrations were 5-10 μM.

RESULTS AND DISCUSSION

Experimental data on the kinetics of substrate hydrolysis by *E. coli* PPase can be best characterized with the following scheme [2]:

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

where E is PPase subunit; K_E is a dissociation constant of enzyme–effector (metal-free PP_i) complex; K_S is dissociation constant of enzyme–substrate ($MgPP_i$) complex; k is the rate constant for the catalytic step; β is an activation coefficient; upper indexes A or E indicate ligand binding at the active site or effector site, respectively.

According to this scheme, the rate of $MgPP_i$ hydrolysis at a fixed Mg^{2+} concentration is determined by Eq. (1):

$$v = \frac{V_1(1 + \beta K_d S/K_E[Mg^{2+}])S}{K_S + (1 + K_S K_d/K_E[Mg^{2+}])S + K_d S^2/K_E[Mg^{2+}]}, \quad (1)$$

where S is concentration of substrate ($MgPP_i$), V_1 is maximal rate of hydrolysis without effector, K_d is dissociation constant for the equation $MgPP_i \leftrightarrow Mg^{2+} + PP_i$ at given pH, and the remaining designations are the same as in the scheme.

It was shown earlier [2] that the allosteric effector site exists in each subunit of a hexameric molecule. When the catalytic activity is measured by a routine protocol under standard conditions (pH 9.0, 2-5 mM Mg^{2+}, 2-200 μM $MgPP_i$) allosteric activation is not apparent, and overall hydrolysis follows the classical hyperbolic kinetics. The major reason for this is a very high affinity of the allosteric site for effector, metal-free pyrophosphate. As a result, the allosteric site is filled at the lowest concentrations of $MgPP_i$ used in the experiment. To monitor the occupation of the effector site in progress, PPase activity should be measured under specially developed conditions when the affinity for effector is decreased and the metal-free PP_i concentration becomes comparable with the dissociation constant for the enzyme–effector complex. This proportion can be maintained at lowered pH and increased Mg^{2+} concentration. For the hexameric E-PPase at pH 6.0 and 10 mM Mg^{2+}, kinetics of hydrolysis was indeed found to deviate from hyperbolic. The value of the dissociation constant for the enzyme–effector complex determined under these conditions (K_E = 0.5 μM [2]) can be regarded as an upper bound for the value of K_E

at pH 7.5 and higher. Each case of a deviation from hyperbolic kinetics at pH 7.5 for the hexameric E-PPase is indicative of the decrease in the enzyme's affinity for effector. Such behavior was found to be a result of either disruption at the intertrimeric interface (trimeric form of E-PPase [11]; mutant variants Glu145Gln or Asp26Ala [1, 2]) or point mutations in the flexible loops flanking the active site [12].

Molecular docking of ATP to the E-PPase shows that one of the possible binding sites is located at a cationic cluster (Fig. 1) [4]. In this model, amino groups of Lys115 and Lys146 are H-bonded to OH-groups of the ribose ring while Lys112 is within H-bond distance from both an oxygen atom of γ-phosphate and the N3 nitrogen atom of adenine. Since, according to the equilibrium gel-filtration data, ATP can compete against pyrophosphate for binding at the effector site, the named residues as well as the nearby residues Lys148 and Arg43 are also probably involved in interaction in the case of PP_i.

Role of Lys112 for binding effectors. Figure 2 shows the rate profiles of $MgPP_i$ hydrolysis by the studied variants of E-PPase. In this figure, along with $MgPP_i$ concentration, proportionally varying concentration of a metal-free form of PP_i is shown on the additional abscissa axis. Two mutant variants, Lys112Gln and Lys112Gln/Lys115Ala, displayed hyperbolic kinetics (corresponding to the linear Lineweaver–Burk plots). Kinetic parameters determined from the data shown in Fig. 2a are collected in Table 1. Two other PPases, a mutant variant Lys112Gln/Lys148Gln and a chemically modified enzyme ^{Ad}wt PPase, displayed another type of kinetics that did not follow the Michaelis–Menten equation. The kinetic parameters in these cases were determined from Eq. (1) (Table 2). As mentioned above, deviation from hyperbolic kinetics found for these PPases indicates a decrease in affinity for the effector PP_i molecule (kinetics is nonlinear at 0.1-6 μM free PP_i). The values of V_2 are also shown for these PPases. This parameter according to the scheme corresponds to a maximal rate of hydrolysis when the enzyme is completely saturated with effector: $V_2 = \beta \cdot V_1$.

Table 1. Kinetic parameters of *E. coli* PPase and its mutant variants determined by linear regression using the Michaelis–Menten equation (pH 7.5, 2 mM Mg^{2+}, enzyme concentrations 0.1-1 nM)

PPase	V_{max}, IU	K_m, μM
wt	565 ± 15	3.2 ± 0.2
K112Q	282 ± 10	4.5 ± 0.5
K112Q (trimer)	291 ± 10	5.3 ± 0.6
K112Q/K115A	342 ± 6	3.2 ± 0.3
K112Q/K148Q*	54 ± 1	2.7 ± 0.2

* 10 mM Mg^{2+}.

The fact that the mutant variants of E-PPase Lys112Gln and Lys112Gln/Lys115Ala retain hyperbolic character of the rate profile can be attributed to the two alternative causes. The allosteric effector site in these variants may remain totally intact; or, quite the contrary, the allosteric site in these variants may be destroyed to such a degree that they are incapable of binding effector at the concentrations of free PP_i used in the experiment.

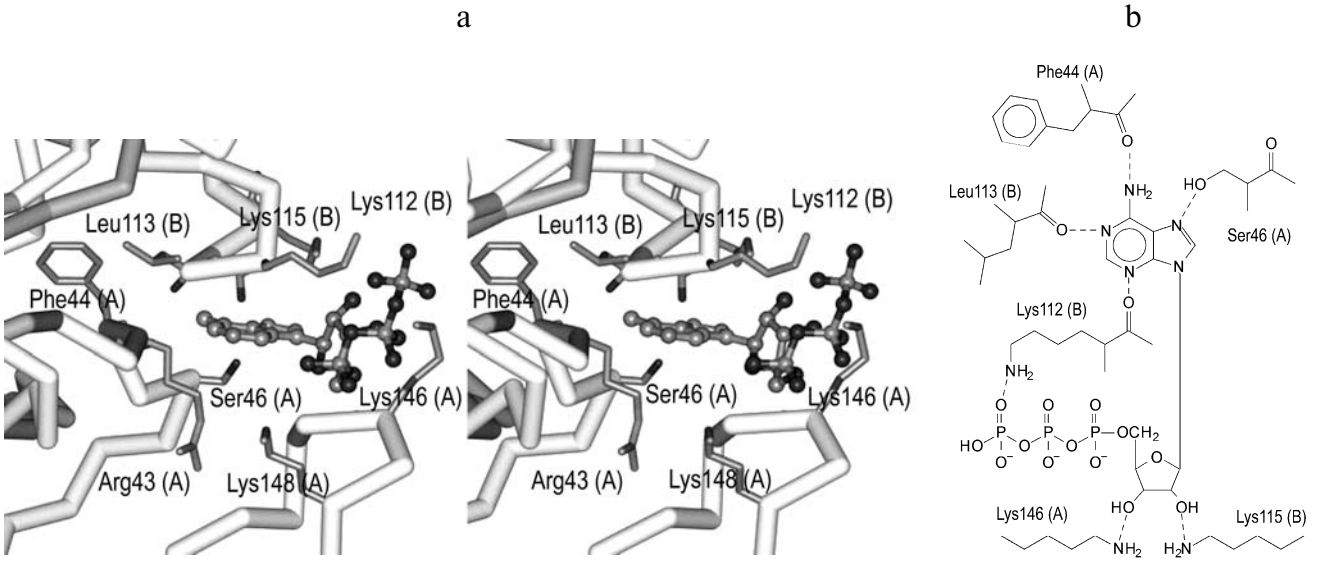

Fig. 1. Interaction of ATP with *E. coli* PPase at the allosteric site according to the results of molecular docking [4]. a) Stereo view of the binding region; b) schematic representation of possible interactions. The name of the subunit containing each residue is indicated in parentheses.

Further studies on the effector properties of the mutant PPases proved the second supposition.

The mutant PPase Lys112Gln/Lys148Gln had kinetic features that distinguished it from both the remaining two variants with the mutations of Lys112 and the adenylated enzyme ^{Ad}wt PPase. First, Lys112Gln/Lys148Gln had a maximal rate of hydrolysis <10% of V_{max} for the wild type PPase determined under the same conditions, while for the other three PPases there was only insignificant change in activity. Second, Lys112Gln/Lys148Gln displayed kinetics of $MgPP_i$ hydrolysis that depended on the concentration of free Mg^{2+}. At 2 mM Mg^{2+}, this double variant showed non-hyperbolic kinetics, but the data were not well described by Eq. (1). It was still possible to estimate K_E from this equation but this value was ~0.1 μM (Table 2), which did not indicate any significant decrease in affinity for effector, in contrast to the other mutant PPases showing non-hyperbolic kinetics. On the other hand, the single mutation Lys148Gln is known to lower the affinity of the active site for the activator magnesium ion M2 (measured in the presence of substrate, $K_d(M2)$ = 0.9 mM while the same value for the wild type enzyme was 0.3 mM [3]). To maintain the saturation of enzyme with activator, kinetics of substrate hydrolysis by Lys112Gln/Lys148Gln was also studied at 10 mM free Mg^{2+}. Figure 2b (inset) shows that at this concentration a double-reciprocal plot of the rate profile became linear (parameters are collected in Table 1). Free PP_i concentration in this experiment varied in the range 0.01-1.5 μM. If K_E were indeed about 0.1 μM, successive occupation of the effector site with metal-free PP_i would occur at these concentrations of $MgPP_i/PP_i$ and the deviation from hyperbolic kinetics would appear. This was not the case, however. Therefore, the deviation from hyperbolic kinetics displayed by Lys112Gln/Lys148Gln at 2 mM Mg^{2+} is most likely caused by a reason distinct from that for the other PPases.

The pyrophosphate analog with a CH_2 group between phosphate groups instead of a bridging oxygen atom is not hydrolyzed by PPases. This compound (PCP) is widely used as a competitive inhibitor of PPases. According to literature data, PCP is bound at the active site as a magnesium complex (MgPCP) with the inhibition constant K_I ~ 330 μM at pH 7.2 [13]. It has been found, however, that at some concentrations of PCP E-PPase activity increases almost two-fold [1, 2]. PCP can act as an activator only if it occupies the allosteric site.

In this work, we studied how PCP affected hydrolysis of $MgPP_i$ by the mutant PPases or ^{Ad}wt PPase, in order to characterize their effector properties. All three variants of PPase with Lys112 mutated were found to lack the ability to undergo PCP-induced activation (Fig. 3). The other studied enzymes (as well as Arg43Gln [3]) were found to be activated by PCP in the same way as the wild type PPase. This finding shows that intact Lys112 is important for binding the activator PCP molecule.

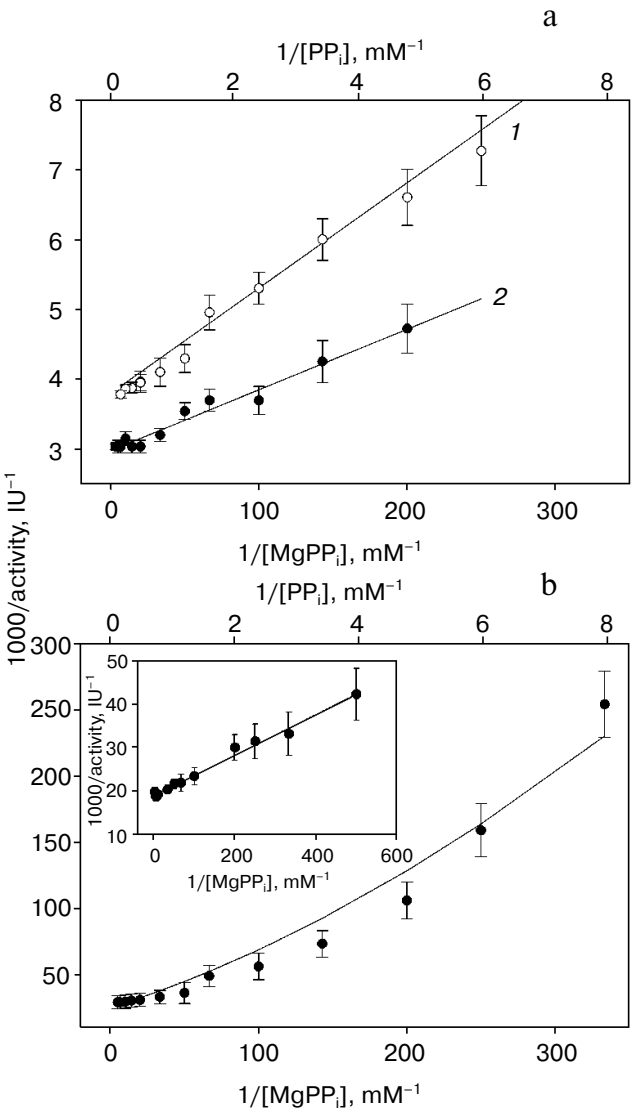

Fig. 2. Lineweaver–Burk plots of $MgPP_i$ hydrolysis by mutant E-PPases. PPases were assayed in 0.05 M Tris-HCl, pH 7.5, at 2 mM Mg^{2+}. The concentration of metal-free pyrophosphate (PP_i) is shown on the upper abscissa axis. All data here and below are given as mean values from 2-4 independent measurements. a: *1*) Lys112Gln (coefficient of linear correlation R = 0.9924); *2*) Lys112Gln/Lys115Ala (R = 0.9746). b) Lys112Gln/Lys148Gln. The line is drawn according to Eq. (1) using parameters shown in Table 2. Inset: data obtained at 10 mM Mg^{2+} (R = 0.9889).

This result probably indicates that Lys112 is involved in binding the activator pyrophosphate molecule as well. To verify this supposition, we determined kinetic properties of a trimeric form of Lys112Gln. Wild type E-PPase is known to sharply decrease affinity for effector PP_i upon dissociation of hexamers into trimers [11]. As a result, catalytic activity of trimer under standard conditions is only ~40% of this value for hexamer. It is only at high pyrophosphate concentrations that the allosteric site in the trimer is occupied (K_E = 60 μM, Table 2) and activity increases up to values typical for the hexamer.

Table 2. Kinetic parameters of E-PPase and its mutant variants calculated by nonlinear regression using Eq. (1) (pH 7.5, 2 mM Mg^{2+}, enzyme concentrations 0.1-1 nM)

PPase	[MgATP], mM	V_1, IU	β	K_S, μM	K_E, μM	V_2, IU*
wt	0.5	250 ± 15	2.6 ± 0.3	1.9 ± 0.2	1.4 ± 0.4	650 ± 13
wt (trimer)**	–	184 ± 5	2.0 ± 0.5	3.6 ± 0.5	60 ± 10	368 ± 7
wt (trimer)**	0.5	189 ± 5	2.0 ± 0.5	4.5 ± 0.6	60 ± 14	380 ± 8
K112Q/K148Q***	–	7	6	10	0.1	39 ± 2
^{Ad}wt	–	165 ± 6	2.1 ± 0.2	2.0 ± 0.2	0.6 ± 0.1	347 ± 7
K115A [3]	–	330 ± 15	1.8 ± 0.1	1.0 ± 0.2	3.7 ± 1.6	(594)
K115A	0.5	260 ± 10	3.5 ± 0.5	0.5 ± 0.2	1.0 ± 0.3	910 ± 18
K148Q [3]	–	123 ± 5	1.5 ± 0.1	3.5 ± 0.6	3.2 ± 1.2	(184.5)
K148Q	0.5	93 ± 6	2.0 ± 0.5	3.8 ± 0.4	13 ± 3	186 ± 4
R43Q [3]	–	7.2 ± 0.5	2.5 ± 0.2	6.2 ± 0.7	3.5 ± 0.7	(18)
R43Q	0.5	1.9 ± 0.3	6 ± 1	0.7 ± 0.5	3 ± 1	11.5 ± 0.3

* Maximal rate of substrate hydrolysis when enzyme is saturated with effector. In parenthesis: parameters calculated from literature data as $V_2 = \beta \cdot V_1$.
** pH 7.5, 1 mM Mg^{2+}.
*** Values used to draw the line in Fig. 2b.

The trimeric form of Lys112Gln was obtained after 20 h incubation of its hexameric form at pH 4.5. Dissociation of Lys112Gln was proved by ultracentrifugation (Table 3). In contrast to the wild type PPase, there was virtually no difference between maximal activities of trimeric and hexameric forms of Lys112Gln even at high concentrations of PP_i (Table 2). Besides, for the wild type E-PPase dissociation into trimers and the resulting impairment of the effector properties is known to cause deviation from hyperbolic kinetics [11], while for the trimeric Lys112Gln no deviation was found (Fig. 4). Hyperbolic kinetics was also typical for this variant in the presence of an alternative effector, ATP. Therefore, mutation of Lys112 made it impossible for the enzyme to bind effector molecules of PP_i, PCP, or ATP. This result confirms our supposition that the binding site for anionic effectors is located in the cationic cluster, and shows that Lys112 plays an important role in their complexing.

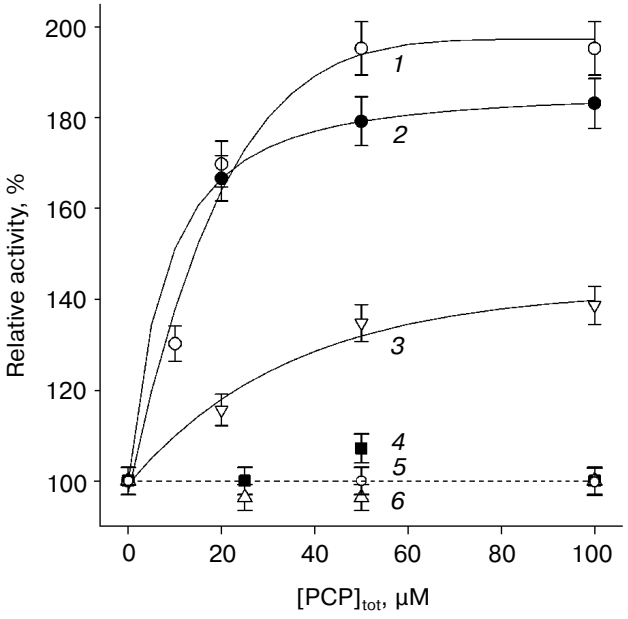

Fig. 3. Effect of PCP on $MgPP_i$ hydrolysis by wild type E-PPase (*1*) or its mutant variants Lys115Ala (*2*), Lys148Gln (*3*), Lys112Gln (*4*), Lys112Gln/Lys148Gln (*5*), and Lys112Gln/Lys115Ala (*6*). The PPases were assayed in 0.1 M Mes-NaOH, pH 6.0, at 10 mM Mg^{2+} and 10 μM $MgPP_i$. Lines are drawn as the best hyperbolic fit. The dotted horizontal line shows 100% activity.

ATP as an alternative effector of E-PPase. ATP is another compound capable of binding at the allosteric site. Being not a competitive inhibitor, it can affect kinetic parameters of $MgPP_i$ hydrolysis by E-PPase. Thus ATP is an alternative effector with respect to metal-free PP_i [4]. There is a number of another proteins known to function having ATP or PP_i bound alternatively at the same site. Kinase/phosphorylase HPr K/P, for example, catalyzes either of two reactions. The first reaction is phosphate transfer from ATP to Ser46 of protein HPr and the second reaction is pyrophosphorolysis, which occurs as reverse phosphate transfer from a phosphoserine residue of HPr, PSer46, onto inorganic phosphate yielding PP_i [14]. According to X-ray data the product of pyrophosphorolysis, PP_i, is arranged at the protein in the same position as β- and γ-phosphates of ATP in the reaction of phosphorylation.

For the hexameric form of the wild type E-PPase, competition between PP_i and ATP for binding at the allosteric site causes affinity of the enzyme for effector

Table 3. Sedimentation coefficients for hexameric and trimeric forms of wild-type E-PPase and Lys112Gln*

PPase	$s_{20,w}$, S	
	hexamer	trimer
wt	6.0 ± 0.1	3.6 ± 0.5
K112Q	6.4 ± 0.4	4.4 ± 0.1

* Sedimentation was performed at 20°C in 0.1 M Tris-HCl, pH 7.5, for the hexamers; or 0.1 M Mes-NaOH, pH 5.3, for the trimeric wild type E-PPase; or 0.1 M citrate buffer, pH 4.5, for the trimeric Lys112Gln. Enzyme concentration, 0.25 μM.

PP_i to appear lower than it is in the absence of ATP. As a result, kinetics of hydrolysis becomes non-hyperbolic and the Lineweaver–Burk plots are nonlinear at pH 7.5 [4]. Assuming that the wild type enzyme under these conditions has $K_E \leq 0.5$ μM (i.e. that K_E does not exceed the value determined at pH 6.0 and 10 mM Mg^{2+}), addition of ATP raises K_E almost two-fold (Table 2). Addition of ATP also results in some increase of V_2 and decrease in K_S, which technically is an analog of Michaelis constant K_m in hyperbolic kinetics. The last result implies the increase in affinity for substrate in the presence of effector, this fact providing additional support to the idea that in this experiment ATP does not compete against substrate at the active site. It should be noted that, in contrast to effector PP_i, in the case of ATP it is impossible to determine unequivocally which one of the forms of ATP coexisting in solution (metal-free ATP or its complexes with one or two Mg^{2+}) acts as effector. Under conditions used in the kinetic experiment, there were all three forms in the reaction mixture.

Those mutant variants that displayed effector properties but bound effector PP_i more weakly than the wild type E-PPase (Arg43Gln, Lys115Ala, Lys148Gln) were also found to be affected by ATP. Figure 5a shows the ATP-induced changes in kinetics of $MgPP_i$ hydrolysis, as exemplified by Lys148Gln. Most of the other "non-hyperbolic" variants of E-PPase were characterized by a similar effect of ATP on their kinetic parameters (Table 2). "Hyperbolic" variant Lys112Gln, quite the contrary, was not affected by ATP (Fig. 5c), which additionally confirms that the presumable allosteric site lost its ability to bind either of these two effectors as a result of mutation of Lys112. The trimeric form of wild type E-PPase was in the intermediate position. It is one of the "non-hyperbolic" at pH 7.5 E-PPases as it has lowered affinity for effector compared to the hexameric form. Its kinetic parameters, however, were not affected by ATP (Fig. 5b; Table 2). Similar behavior was found for the double mutant Lys112Gln/Lys148Gln (data not shown). In both intermediate cases, ATP appears not to bind at the allosteric site.

In the model structure of E-PPase–ATP complex (Fig. 1) the amino group of Lys112 forms an ionic pair with γ-phosphate of ATP. Involvement of this residue in binding ATP and/or PP_i at the allosteric site is indirectly supported by the kinetic features of Lys112Gln. On the basis of this theoretical structure, the lack of influence of ATP on $MgPP_i$ hydrolysis by Lys112Gln and Lys112Gln/Lys148Gln seems quite reasonable. It is harder to understand, however, why the allosteric site is so heavily disrupted in the trimeric E-PPase that it binds PP_i much more weakly than the hexamer, and it does not bind ATP at all at the concentrations used in the experiment.

Possible role of the allosteric site in PPase function. Analysis of effector properties of E-PPase indicates that its affinity for effector (PP_i, PCP, or ATP) and the state of intersubunit contacts in the oligomeric molecule are closely interconnected. Any impairment of the intersubunit contacts as a result of mutations in the trimer–trimer interface, or its complete destruction as a result of dissociation, weakens the enzyme–effector interaction. This has been shown by the examples of trimeric E-PPase [11]; Asp26Asn (carboxylic group of Asp26 is directly involved in the intertrimeric contact) [1]; Glu145Gln [2], Gly147Val [12], Lys148Gln [3], and PPase adenylated at the amino group of Lys146 (all the latter variants have mutations or modification in the region 144-149, which also contains the residues involved in the intertrimeric contact). These findings can be understood on the basis of the proposed position of ATP at the allosteric site (Fig. 1). First, involved in the binding site are the residues that belong to the two different subunits and do not interact in

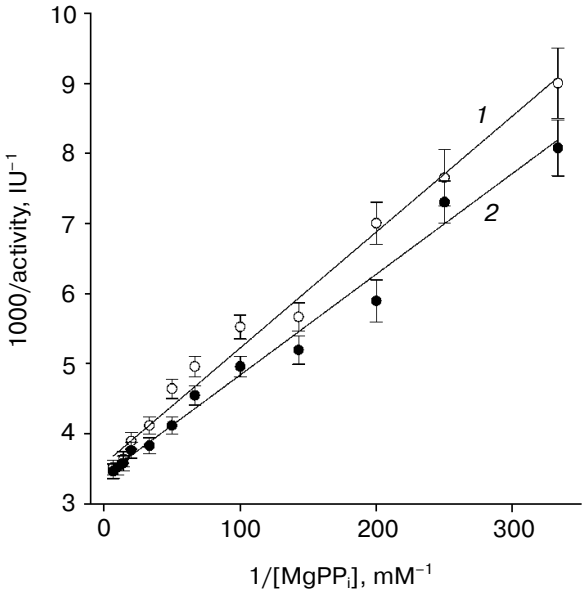

Fig. 4. $MgPP_i$ hydrolysis by hexameric (*1*) or trimeric (*2*) forms of Lys112Gln mutant E-PPase ($R = 0.9879$ and 0.9857, respectively). PPases were assayed in 0.05 M Tris-HCl, pH 7.5, at 2 mM Mg^{2+}.

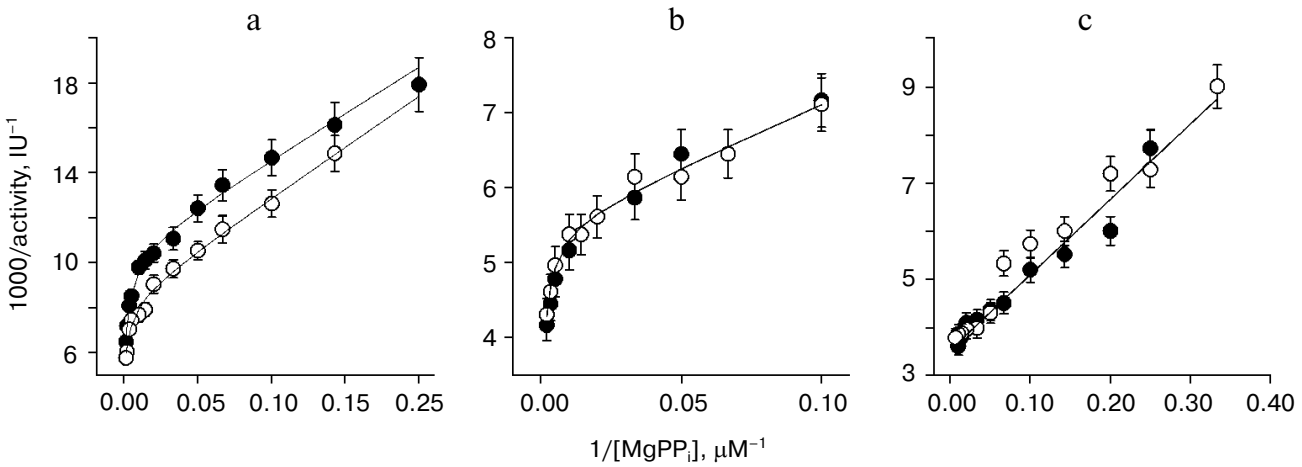

Fig. 5. Effect of ATP on $MgPP_i$ hydrolysis by Lys148Gln (a), trimeric form of wild type E-PPase (b), or Lys112Gln (c). Open symbols – without ATP, closed symbols – 0.5 mM MgATP. The PPases were assayed in 0.05 M Tris-HCl, pH 7.5, at 2 mM Mg^{2+}. In (a) and (b), lines are the best fit with Eq. (1), parameters are shown in Table 2. In (c), data are fitted with the same straight line, $R = 0.9746$ (no ATP) or 0.9749 (0.5 mM ATP).

the absence of effector. Second, adenine base of ATP in the model structure makes contacts with the peptide groups of Phe44, Leu113, and the OH group of Ser46. All these residues are part of the intertrimeric contact and their exact position is defined by the mutual orientation of subunits. On one hand, effector site with efficient binding can be formed only if the native intertrimeric contacts remain intact. On the other hand, according to the model, ATP binds in a way that results in strengthening of existing interactions and formation of new intersubunit contacts. Therefore, the proposed localization of allosteric site is quite consistent with the properties of the mutant E-PPases both described in literature and studied in this work.

It should be mentioned that effector properties were earlier found for the eukaryotic PPase from baker's yeast *S. cerevisiae*, Y-PPase [15]. This enzyme also displayed ATP-induced activation [16], but neither the exact binding site nor the mechanism of activation has been clarified. In contrast to the bacterial hexameric PPases, eukaryotic PPases are homodimers. The surfaces of Y-PPase and E-PPase globules show significant difference, and the intersubunit contact in the eukaryotic enzyme is formed with different residues. The arrangement of allosteric binding site at the subunit interface as in E-PPase is impossible in Y-PPase. Surprisingly, superposition of the crystal structures of these enzymes demonstrates that there is an analogous cationic cluster in Y-PPase. All its residues belong to the same subunit of Y-PPase and the cluster is formed by other structural elements than in E-PPase, but the arrangement of the cluster with respect to the active site is similar in the two enzymes (Fig. 6). Y-PPase has a longer polypeptide backbone that folds in the additional loops lacking in E-PPase.

The residues Asp42 and Arg43 (A subunit, E-PPase numbering) shown in Fig. 6 belong to the active site. The guanidine group of Arg43 forms an ion pair with substrate bound in the active site while the carboxylic group of Asp42 is in the second coordination sphere of the metal ion M4 [17]. Between the corresponding residues of Y-PPase, Asp71 and Arg78, there is an insertion loop Thr72-Leu77. Superposition of Y- and E-PPases (A subunit of the hexamer) by the active site residues reveals that, with the only exception of Asp42/71 and Arg43/78, the residues belonging to the insertion loop fall into the same position as a segment 110-116 of an adjacent subunit of E-PPase (B subunit in Fig. 6). In other words, the insertion loop of Y-PPase is arranged in the place corresponding to the intersubunit interface of E-PPase, namely, in the modeled ATP binding site. The best agreement is observed for the segments 73-75 of Y-PPase and 112-110 of E-PPase (for example, peptide carbon atoms of Gly75 and His110 are only 0.78 Å apart). The insertion loop in Y-PPase contains three lysine residues, Lys73, -74, and -76. Their amino groups form a positive cluster in the same region of Y-PPase as Lys112 and Lys115 do in E-PPase (Fig. 6). Besides, each peptide oxygen atom in E-PPase supposed to participate in binding ATP (residues Phe44, Lys112, Leu113; see Fig. 1b) has its counterpart in Y-PPase structure (Leu77, Lys76, and Thr72, respectively). Thus, based on the structural analysis we suppose that this cationic cluster is a possible site of binding anionic effectors in both prokaryotic and eukaryotic branches of family I PPases.

There are many examples described in literature where ATP affects oligomeric structure of the proteins or their complexing with the target high molecular weight ligands. This particular feature is caused by the particular structure of ATP binding sites. The typical mode of ATP

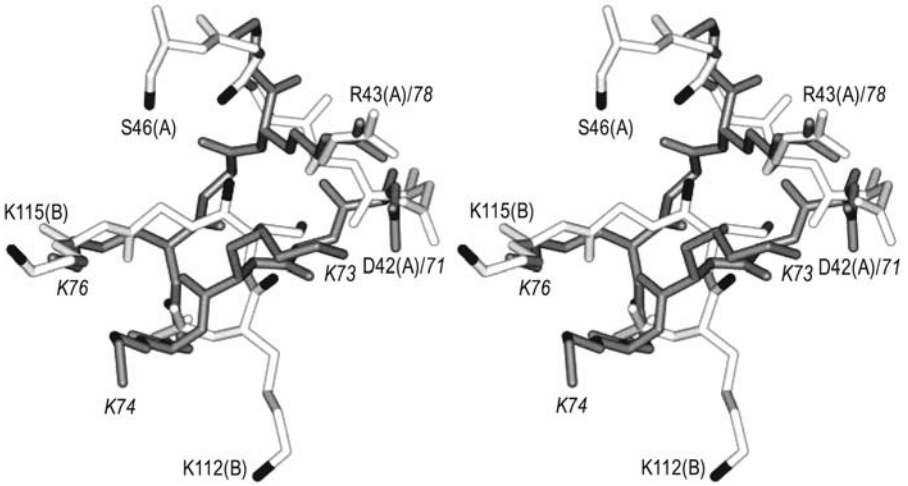

Fig. 6. Superposition of the crystal structures of PPases from *E. coli* and *S. cerevisiae*. Gray, fragment 71-79 of a Y-PPase backbone; light, fragments of E-PPase backbone 42-46 (A subunit) and 111-115 (B subunit); black, atoms involved in the interaction with ATP according to the molecular docking data are shown. Side chains are shown for residues Asp42/71 and Arg43/78 (indicating position of the active site), Ser46, and for the lysine residues in this region. The figure was made using WebLab Viewer Pro 3.7.

binding includes its interaction with the residues from different structural elements in a way that brings them closer and makes the contacts tighter. For example, kinase/phosphorylase HPr K/P mentioned above is a homohexamer. When it binds ATP, the adenine base of the nucleotide makes contacts with the flexible loops from two different subunits so that binding ATP changes the intersubunit interface. As a result, positive cooperativity is observed in the course of binding of ATP, its analogs, or allosteric effector [14].

Another example is protein Rad50, which belongs to ABC-transporters. In this protein, an ATP binding site is also formed by the residues of the adjacent subunits. Binding ATP induces dimerization of Rad50 that causes a conformational rearrangement of its membrane domains, which is necessary for its transport function [18]. Transport protein GlnK and apparently some other members of the P_{II} family [19] are capable of changing substrate specificity with respect to their target proteins in the presence of ATP or other nucleotide effectors. GlnK is a homotrimer where each subunit has a T-loop responsible for the interaction with the target proteins and for the overall molecular recognition. Binding sites for ATP are located between the pairs of subunits, apart from T-loops. Binding ATP brings the residues from the different subunits closer to each other, and this in turn causes delivery of the conformational changes to the T-loops that changes the protein-recognizing surfaces [20]. For many DNA-binding proteins, ATP is known to switch them into the active form, i.e. to stabilize a conformation with high affinity for DNA [21].

These examples demonstrate that the effector action of ATP on E-PPase may not only be the increase of its catalytic activity but also the changes in its ability to interact with possible protein targets. There is no literature data that PPases in the course of their functioning form complexes with the other cellular proteins. However, some experimental data collected lately make this supposition quite probable. Finding and investigation of such complexes is a subject of our further work.

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