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ATP as Effector of Inorganic Pyrophosphatase of *Escherichia coli*. Identification of the Binding Site for ATP

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Abstract—The interaction of *Escherichia coli* inorganic pyrophosphatase (E-PPase) with effector ATP has been studied. The E-PPase has been chemically modified with the dialdehyde derivative of ATP. It has been established that in the experiment only one molecule of effector ATP is bound to each subunit of the hexameric enzyme. Tryptic digestion of the adenylated protein followed by isolation of a modified peptide by HPLC and its mass-spectrometric identification has showed that it is an amino group of Lys146 that undergoes modification. Molecular docking of ATP to E-PPase indicates that the binding site for effector ATP is located in a cluster of positively charged amino acid residues proposed earlier on the basis of site-directed mutagenesis to participate in binding of effector pyrophosphate. Molecular docking also reveals several other amino acid residues probably involved in the interaction with effectors.

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Soluble inorganic pyrophosphatases (PPases, EC 3.6.1.1) catalyze hydrolysis of the phosphoanhydride bond in pyrophosphate (PP_i). The substrate molecule is bound in the active site of the holoenzyme as a magnesium complex, $MgPP_i$. Two more Mg^{2+} ions are involved in pre-formation of the holoenzyme. Prokaryotic and eukaryotic PPases have virtually the same architecture of the active sites as well as hydrolytic mechanism, but their oligomeric structure and the length of their polypeptide backbone are different [1, 2]. The object of this work, PPase of *Escherichia coli* (E-PPase), functions *in vivo* as a homohexamer.

Recent experimental data indicate that each subunit of *E. coli* PPase has an allosteric site for binding anionic effectors – PP_i and its analogs [3, 4]. First, the kinetics of hydrolysis of $MgPP_i$ by a trimeric form of wild type E-PPase as well as the trimeric or hexameric forms of a number of mutant variants cannot be described by the Michaelis–Menten equation. The double reciprocal plot of a reaction velocity versus substrate concentration has two linear segments. This indicates that there are two molecules of pyrophosphate involved in hydrolysis, one being a substrate and the other being an effector. The apparent affinity of the enzyme to effector depends on free magnesium concentration in a manner indicative of metal-free PP_i acting as effector rather than $MgPP_i$ [4]. Second, methylenediphosphonate (PCP), a structural analog of pyrophosphate, is capable of incompetitive activation of $MgPP_i$ hydrolysis by E-PPase. As there is no hydrolyzable phosphoanhydride bond in the molecule of PCP, activation can only be attributed to PCP bound at an allosteric site. Third, PPase is capable of binding 12 moles of PP_i per mole of hexameric enzyme under optimal conditions. This indicates directly that each subunit of E-PPase has an allosteric site capable of binding the second molecule of PP_i. It appears that its structural analog, PCP, occupies the same binding site.

Based on a proposed kinetic scheme of activation [3, 4], an activation coefficient, β, can be determined for each effector. In the cases of the hexameric or trimeric E-PPase as well as for all the mutant variants studied so far, activation of $MgPP_i$ hydrolysis induced by the second

Abbreviations: E-PPase) inorganic pyrophosphatase of *E. coli*; PCP) methylenediphosphonate.

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molecule of pyrophosphate increases the hydrolytic activity about two-fold ($\beta = 2.0 \pm 0.5$). Methylenediphosphonate is characterized by a similar activation coefficient [3, 4]. The question rises as to what physiological role this effector site might play *in vivo*.

Among the phosphorus-containing metabolites, a study of action of ATP as the possible effector of E-PPase is of a special interest as ATP is known to regulate a number of cellular processes and to interact with a wide spectrum of proteins. In the presence of magnesium, ATP is not hydrolyzed by pyrophosphatases, but it can be hydrolyzed in the presence of Mn^{2+} [5]. It has been found earlier that ATP activates hydrolysis of $MgPP_i$ by E-PPase [6] but neither the reasons for that nor the mechanism of interaction have been elucidated yet.

MATERIALS AND METHODS

Chemicals. Recombinant E-PPase was obtained as described in [7]. 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. [^{32}P]$Na_4P_2O_7$ and [α-^{32}P]dATP of high purity were purchased from Izotop (Russia). [^{32}P]$Na_4P_2O_7$ was additionally purified by thin-layer chromatography on PEI-cellulose, elution with 0.7 M KH_2PO_4. The labeled reagents were diluted before use with non-labeled pyrophosphate or ATP to maintain necessary specific radioactivity. All other chemicals were purchased from Sigma (USA), Fluka (Switzerland), Merck (Germany), and Pharmacia Fine Chemicals (Sweden). Aqueous solutions were made using deionized water purified with MilliQ.

Concentration of E-PPase solutions was determined by UV-spectrophotometry using the value of specific absorption coefficient $A^{1\%}_{1cm} = 11.8$ [8]. Hydrolytic activity of PPases was determined by the rate of P_i release from $MgPP_i$ at 25°C. Concentration of P_i was quantitatively measured using a semi-automatic phosphate analyzer [9] 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 [10]. $MgPP_i$ hydrolysis was studied in 0.05 M Tris-HCl, pH 7.5, at 2 mM Mg^{2+} and 0.5-3.0 mM effector (phosphoglycerate, citrate, glutamate, malonate, AMP, ADP, or ATP). Concentration of $MgPP_i$ was varied in the range 3-500 μM. Kinetic parameters were determined as the best fit with Michaelis–Menten equation or equation corresponding to the scheme of incompetitive activation of PPase by metal-free PP_i [4] using SigmaPlot 7.0.

Stoichiometry of ATP–E-PPase complex. Stoichiometry of ATP–E-PPase complex was determined by gel filtration using two approaches. In the first case, reaction mixture (50 μl) containing 58 μM E-PPase, 363 μM [^{32}P]$Na_4P_2O_7$ (specific radioactivity 0.33 μCi/μmol), 0.9 mM $CaCl_2$, and 0.82 mM ATP in 0.05 M Tris-HCl, pH 7.5, was gel filtered on a Sephadex G-50 column (0.5 × 5 cm) equilibrated with solution containing the same components excluding the enzyme. The same solution was used as eluent. Eluate was collected by fractions of 50 μl. Protein concentration and radioactivity were measured in each fraction using a Tracor Analytic 300 Liquid Scintillation System (Delta, USA), and the ratio PP_i/enzyme was calculated. A control experiment was performed analogously but the reaction mixture did not contain ATP. In the second case, the reaction mixture (50 μl) contained 65 μM E-PPase, 200 μM $Na_4P_2O_7$, 200 μM $LaCl_3$, 2.2 mM $MgCl_2$, 210 μM [α-^{32}P]dATP (specific radioactivity 0.76-1.1 μCi/μmol) in 0.05 Bis-tris-HCl, pH 6.5. The reaction mixture was gel filtered and the ratio PP_i/enzyme was determined as in the first experiment. A control experiment was performed analogously but the mixture did not contain $Na_4P_2O_7$ and $LaCl_3$.

Modification of E-PPase with oxidized ATP. Oxidized ATP was obtained by adding 15 mM KIO_4 to an equal volume of 15 mM ATP. The mixture was incubated for 1 h at 0°C, KOH being continuously added to keep pH at the fixed level (7.0). The reaction was stopped by addition of 10 μl of ethylene glycol [11]. To modify E-PPase, an aliquot of 250 μl of the solution obtained (2.3 mM oxidized ATP) was added to E-PPase solution (0.25-0.75 mg in 1 ml). The reaction mixture was incubated in 0.1 M Hepes-NaOH, pH 7.0, in the presence of 2.3 mM $MgCl_2$, and then gel filtered on a Sephadex G-50 column (0.5 × 5 cm) equilibrated with 0.05 M Tris-HCl, pH 7.5. Absorption at 260 and 280 nm (D_{260} and D_{280}) was measured in each fraction and inclusion of label into E-PPase (h, mol/mol subunit) was calculated using the following equations:

$$h = \frac{pA_{260}}{0.652\,pE_{280}},$$

$$pE_{280} = \frac{a-c}{a-b} D_{280},$$

$$pA_{260} = D_{260} - bpE_{280},$$

where pA_{260} is the contribution of adenine base in D_{260}; pE_{280} is the contribution of total protein in D_{280}; coefficients a, b, and c are the values of D_{260}/D_{280} for the oxidized ATP, protein sample before modification, and protein sample after modification, respectively. Factor 0.652 is the ratio of molar extinction coefficients of ATP at 259 nm and E-PPase at 280 nm.

Identification of a modified residue. Modified E-PPase was digested by trypsin (PPase/trypsin ratio was

30 : 1) for 12 h at 30°C in the presence of 5% isopropanol. Tryptic peptides were separated by HPLC using a Milichrome A-02 chromatograph (Econova, Russia). The sample mixture was chromatographed at 35°C on a Nucleosil C18 column (Macherey-Nagel, Germany), 2 × 75 mm, pore diameter 5 μm, equilibrated with 0.1% TFA, with elution by 0.1% TFA in a linear gradient of acetonitrile 0-70% for 20 min.

Reflectron MALDI-TOF mass spectra were acquired on a Reflex III mass spectrometer (Bruker, Germany) operating in the positive ions mode with a pulse laser emitting at 337 nm. Accelerating voltage and reflecting voltage were 25 and 28.7 kV, respectively. Mass accuracy was 0.01%.

Molecular docking. The structure of the enzyme–ATP complex was modeled using crystal structure 1I40 (E-PPase complexed with Ca^{2+} and $CaPP_i$ [2]) as a template of *E. coli* PPase with the occupied active site. Preparation of the target protein structure, building and optimization of ATP structure, and preliminary estimation of ligand–receptor interactions were performed using Tripos software (Sybyl 6.9; http://www.tripos.com). Further screening of possible ligand positions and orientations at a binding area was carried out automatically using AutoDock 3.0 [12]. Lamarckian genetic algorithm was applied for energy minimization as an evolutionary algorithm allowing an efficient search within all ligand-available space. Pre-calculated potential maps supplied with the program [13] were used in energy calculations (including solvation). One hundred independent runs were performed yielding docking solutions varying in free binding energy from −7.1 to −2.5 kcal/mol. The thirty best-ranked docking conformations were taken for subsequent analysis. One final model was eventually chosen on the basis of the chemical modification data. All calculations were performed on an SGI Octane R10000 workstation.

RESULTS AND DISCUSSION

We studied what cell metabolites, aside from PP_i and PCP, can act as effectors of E-PPase. A number of anionic compounds were tested including phosphoglycerate, citrate, glutamate, AMP, ADP, and ATP. In this series, only ATP was found to activate hydrolysis of $MgPP_i$. Figure 1 shows the Lineweaver–Burk plot for the hexameric E-PPase in the absence of adenine nucleotides, as well as in the presence of AMP or ATP. In the first case, metal-free form of PP_i acts as an effector of PPase activity. It has been shown earlier [4] that this effector has a very high affinity for the allosteric site. Under experimental conditions used in this work the allosteric site is occupied even at the low starting concentrations of a substrate ($MgPP_i$) so that the usual hyperbolic dependence is observed yielding a straight line on the double reciprocal plot (curve *1*). As ATP is added in the reaction mixture, it competes against effector PP_i for binding at the allosteric site, which breaks the linearity (curve *2*). It can be concluded that ATP competes against PP_i at the effector site rather than at the active site because otherwise competitive inhibition would be observed without a deviation from the classic hyperbolic dependence. In contrast to ATP, the adenine nucleotides with one or two phosphate groups do not compete against PP_i for binding at the allosteric site (curve *3* for AMP). ATP as effector of $MgPP_i$ hydrolysis shows the activation coefficient of 2.6 ± 0.3. ATP also activates hydrolysis of an alternative substrate of E-PPase, a tight complex of pyrophosphate with trivalent lanthanum ($LaPP_i$). For 5 μM $LaPP_i$, the rate of hydrolysis increases two orders of magnitude in the presence of ATP.

These results suggest that ATP can occupy the allosteric site of E-PPase. Its phosphate groups appear to mimic the effector PP_i. As shown below, either of the Mg^{2+}-complexed forms of ATP or its metal-free form can probably bind to E-PPase. The experimental data however do not indicate which form acts as activator.

The competition between ATP and PP_i at the effector site has been additionally proved by the equilibrium gel filtration. It was previously shown [4] that two molecules of PP_i were bound with each enzyme subunit after incubation of E-PPase with labeled calcium pyrophosphate, $[^{32}P]CaPP_i$. In the present work we have demonstrated that as a result of the subsequent incubation of this complex in the presence of CaATP, one of the two bound molecules of labeled PP_i is displaced, so that the total inclusion of $[^{32}P]CaPP_i$ decreases to one molecule per enzyme subunit. In this experiment, virtually the same

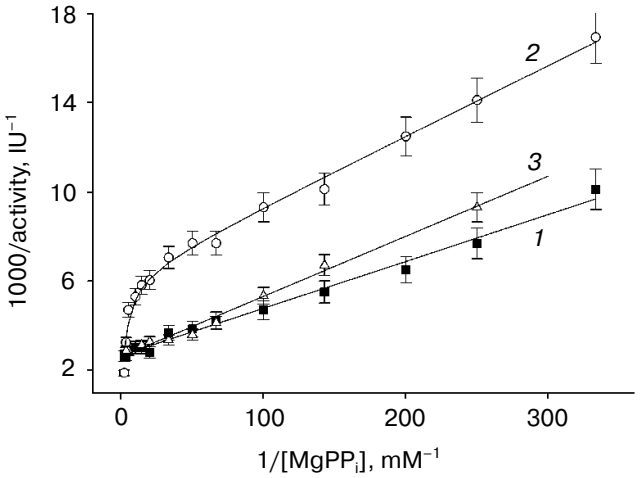
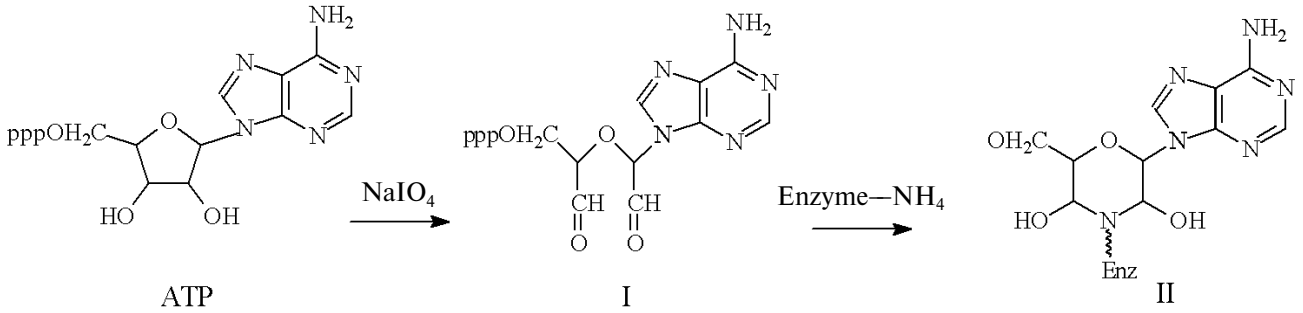


Fig. 1. Lineweaver–Burk plots of $MgPP_i$ hydrolysis by the wild type E-PPase: *1*) without additions; *2*) 0.5 mM MgATP; *3*) 1 mM AMP. PPase was assayed in 0.5 M Tris-HCl, pH 7.5, at 2 mM Mg^{2+}. Coefficients of linear correlation *R* for curves *1* and *3* are 0.9894 and 0.9904, respectively. Line *2* is drawn using an equation of incompetitive activation of PPase with metal-free PP_i [4].



Preparation of the dialdehyde derivative of ATP and modification of E-PPase

Scheme 1

concentrations of $CaPP_i$ and CaATP were used. Affinities of the active site for these compounds differ significantly. For $CaPP_i$, dissociation constant K_d (determined as the inhibition constant from the competitive inhibition of hydrolysis of $MgPP_i$) is $2.4 \cdot 10^{-9}$ M [14]. Affinity for CaATP appears to be much lower as can be judged from Michaelis constant for MnATP as a substrate, K_m = 178 mM [5]. This suggests that in the experiment described above the PP_i molecule is most likely displaced from the allosteric site rather than from the active site where $CaPP_i$ is tightly bound.

The same result, inclusion of one mole ATP per mole of E-PPase subunit with the occupied active site, was obtained after incubation of the enzyme in the simultaneous presence of [α-^{32}P]dATP (magnesium complex of dATP with α-phosphate labeled) and lanthanum pyrophosphate. $LaPP_i$ is a poor substrate of E-PPase and in the case of hydrolysis of $MgPP_i$ it acts as a competitive inhibitor. According to the inhibition data, it binds at the active site with K_d of about 10^{-8} M [14], while there is no data on whether it can occupy the allosteric site. Therefore, one molecule of ATP found in each subunit appears to be bound at the allosteric effector site while the active site is filled with $LaPP_i$.

To localize the site of binding of ATP in the structure of E-PPase, we have chemically modified the enzyme with oxidized ATP (Scheme 1). ATP was oxidized with potassium periodate to yield 2′,3′-dialdehyde derivative I. A hexamer of E-PPase was incubated in the presence of derivative I, then the mixture was gel filtered and the inclusion of a label into E-PPase was determined by UV-spectroscopy. At the various ratios of the enzyme/reagent concentrations, the extent of modification was 0.9-1.0 mol/mol of subunit. This indicates the specific modification of a single lysine residue (II) due to a reaction between its amino group and the aldehyde group(s) of ATP derivative I. According to the structure of derivative I, the modified lysine residue in E-PPase complexed with unoxidized ATP should form an H-bond with the 2′- or 3′-OH group of its ribose ring. Determination of this lysine residue was the next step in this work.

To identify the lysine residue modified with dialdehyde derivative of ATP, the adenylated enzyme was digested by trypsin. The tryptic peptides were separated by HPLC (Fig. 2) followed by MALDI-TOF mass determination of a target peptide (Fig. 3). Figure 2 shows the HPLC elution profile with additional detection at wavelengths 260 and 280 nm. As trypsin does not cleave the peptide bonds after lysine residues with the modified amino groups, the target tryptic peptide was supposed to

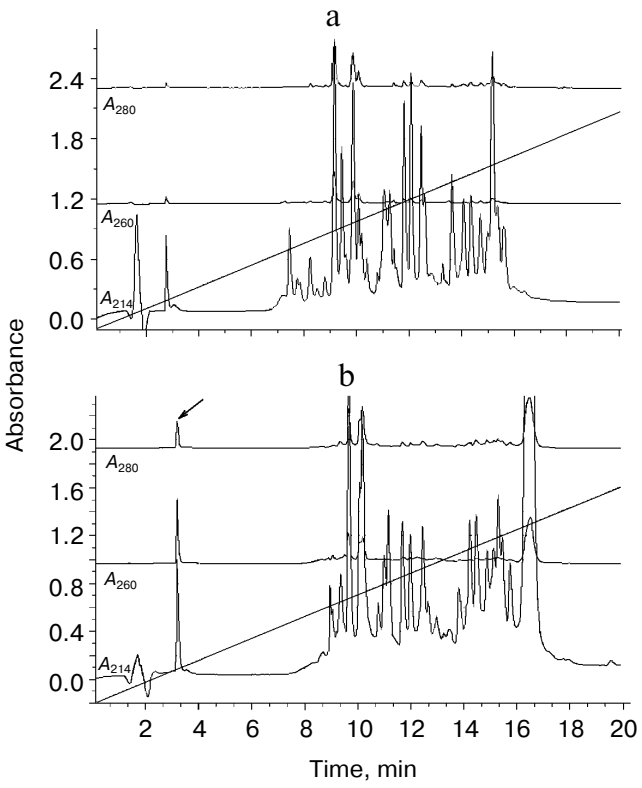


Fig. 2. HPLC elution profiles of E-PPase after trypsin digestion: a) native PPase; b) PPase modified with dialdehyde derivative of ATP. Both panels show absorbance profiles at 214, 260, and 280 nm. The target peak corresponding to a modified peptide is shown with an arrow.

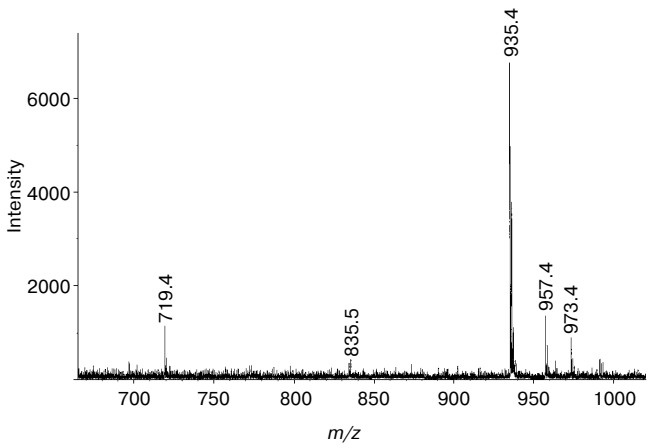


Fig. 3. MALDI-TOF mass spectrum of the modified peptide.

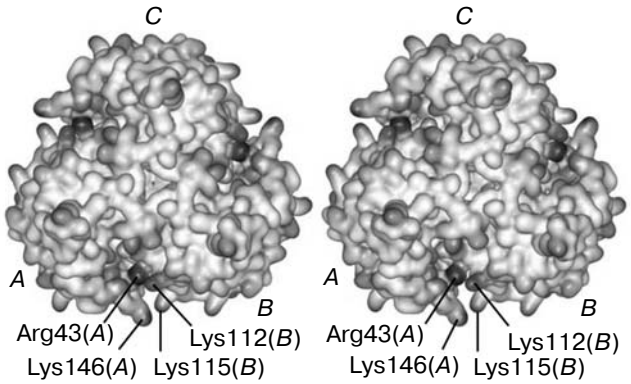
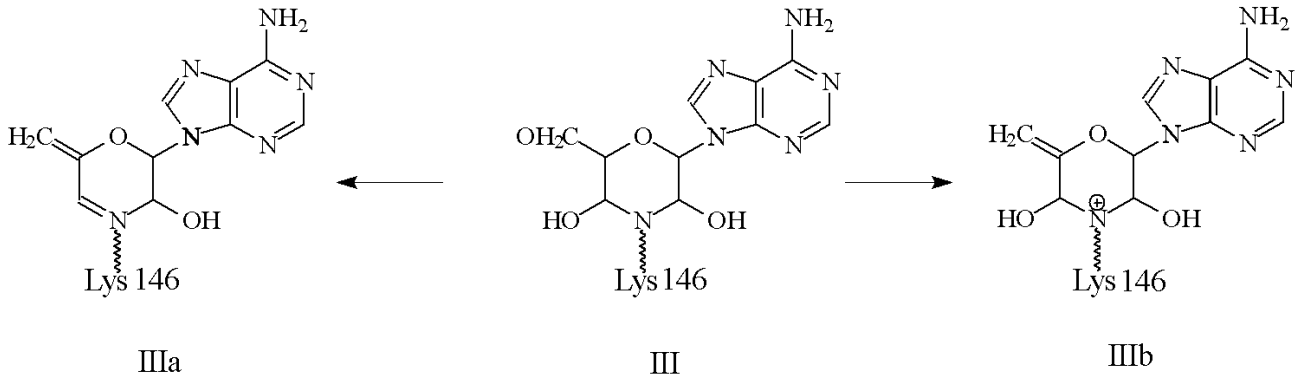


Fig. 4. Stereo view of one trimer of a hexamer of wild type *E. coli* PPase (protein data bank entry 1IGP) along a three-fold crystallographic axis. Globular surface is shown (subunits A, B, C) shaded by electrostatic potential (darker shade corresponds to a more negative charge). The figure was made and the potential calculated using WebLab ProViewer 3.7.

be a double peptide having ratio A_{260}/A_{280} significantly higher than the other peptides due to the inclusion of the adenine base. The only peptide that meets these expectations is marked with the arrow in Fig. 2. According to MALDI-TOF mass spectrometry, this peptide yields one major peak and several minor peaks (Fig. 3). The major peak corresponds by its mass to an adenylated derivative of a dehydrated form of fragment 129-148. This fragment is a double tryptic peptide with modified residue Lys146. According to literature data, in the course of mass determination by MALDI-TOF spectrometry the original peptide sample may produce derivatives in various dehydration states (Scheme 2). For the derivatives IIIa and IIIb their theoretical masses are 918.45 and 935.45 daltons, respectively. Experimental mass value for the major peak (935.4 daltons) corresponds to compound IIIb. None of the other possible tryptic peptides of E-PPase has similar mass. These results therefore demonstrate unequivocally that it was Lys146 whose amino group was modified with the dialdehyde derivative of ATP.

All compounds found to be E-PPase effectors are anionic, which implies that the effector-binding site should be cationic. Analysis of the distribution of the charged groups in E-PPase hexamer shows that there is only one site on the surface where positively charged groups are clustered [15]. This cluster consists of three amino groups of lysine residues and a guanidine group of Arg43 (Fig. 4). Lys146 and Arg43 belong to one subunit, while Lys112 and Lys115 belong to the adjacent subunit of the same trimer. In a hexamer, this cluster is symmetrically multiplied to a total number of six. Dissociation of the hexamer into trimers would leave these clusters intact, but they would be destroyed upon dissociation to the "two-deck" dimers. This consequence of supposed interfacial location of the effector site could account for the lack of effector properties found earlier for the dimeric form of E-PPase [16].

Possible structure of ATP–E-PPase complex was modeled by molecular docking. Crystal structure of E-



Possible dehydration forms of E-PPase modified with the dialdehyde derivative of ATP

Scheme 2

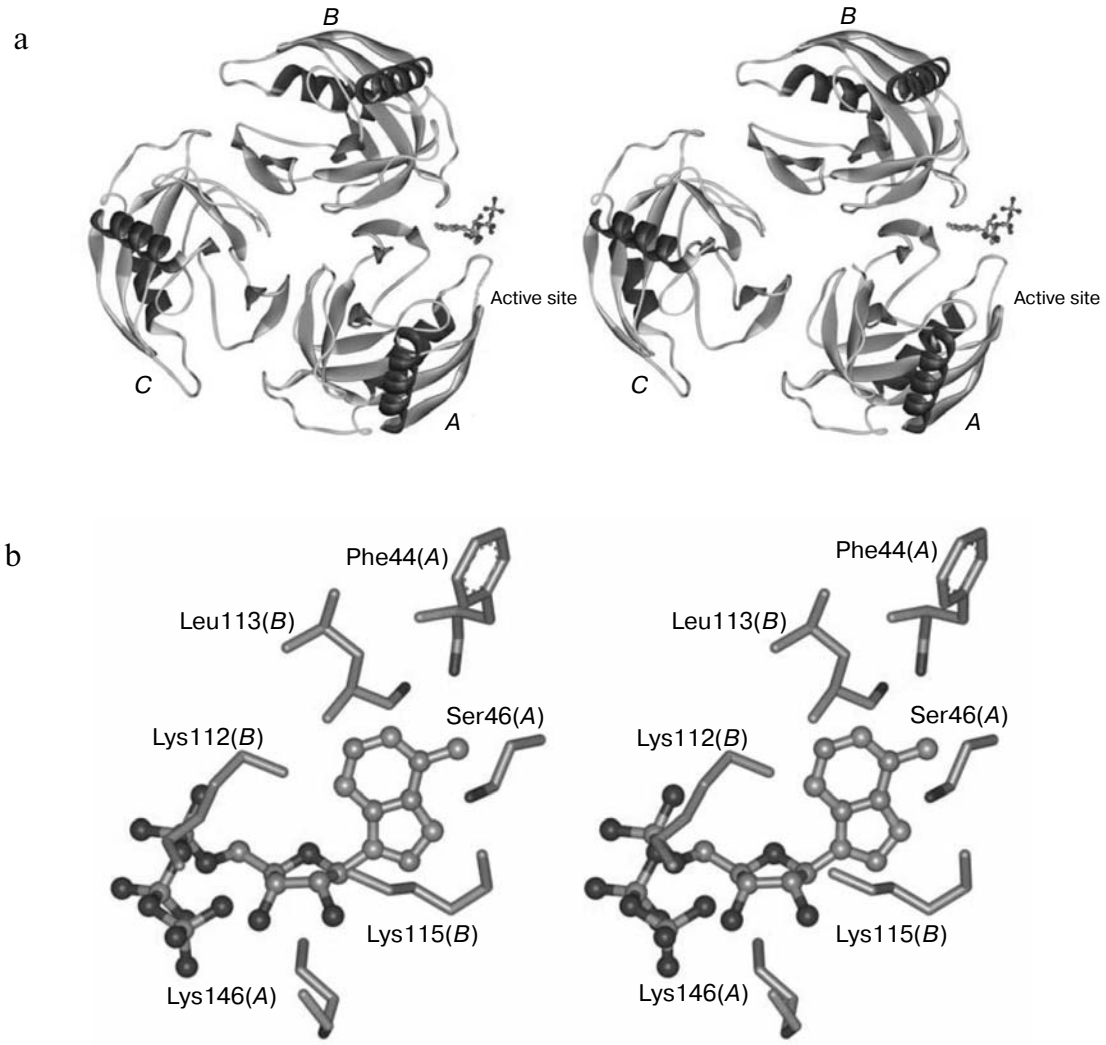


Fig. 5. Model structure of ATP–E-PPase complex according to the data of molecular docking. a) E-PPase, one trimer of a native hexamer. Secondary structure is shown for each subunit. An ATP molecule bound in one of three symmetry-related binding sites is shown in ball-and-stick. The position of the active site of an adjacent subunit is indicated. b) Possible ATP–enzyme contacts. The name of subunit containing each residue is indicated in parentheses. The figure was made using WebLab ProViewer 3.7.

PPase complexed with unhydrolyzable substrate analog, $CaPP_i$ (1I40 [2]), was used as a template of the enzyme with filled active site. From 30 docking solutions with the lowest free binding energy two models corresponded to ATP binding at the cationic cluster. Only one of these models had the orientation of ATP in which an OH group of its ribose ring was at the H-bond distance from an amino group of Lys146, as supposed from the data of chemical modification. This model structure of the ATP–E-PPase complex is shown in Fig. 5. The effector binding site is supposedly located at a subunit interface but in close vicinity to the active site. In Fig. 5a there is only one ATP molecule shown for clarity of three symmetry related molecules that could be seen in this projection. The heterocyclic base of ATP is arranged in the socket between three protein loops (segments 43-46 and 144-148 of subunit A, and 112-115 of subunit B) while its triphosphate tail is bound at the surface of the protein globule.

Figure 5b shows the protein side chain groups that fall within H-bond distance (3.2 Å) of the functional groups of ATP, implying that they supposedly form the ionic contacts or H-bonds with effector. In our model, amino groups of Lys112 and Lys115 are ionic partners of ATP phosphates. As ATP and PP_i were shown to compete for binding at the effector site, the same residues can be proposed to contact with the effector pyrophosphate molecule. To verify the supposition that the allosteric site is formed with the residues of a cationic cluster, we mutated these residues yielding a number of single or double mutant variants of E-PPase. Kinetic properties of these variants are described in detail in the next paper [17].

The possible participation of residues Arg43, Lys148, and Lys115 in binding effector PP_i was earlier tested using

site-directed mutagenesis [18]. According to the data of molecular docking, the first two residues do not contact directly with the ATP phosphates. It should be noted that in the protein structure used for molecular docking, E-PPase complexed with $CaPP_i$, the latter being bound in the active site cavity; as a result, a side chain of Arg43 is pointed into the cavity to form the ionic contacts with P1 phosphate of a substrate analog. However, in the absence of substrate or product molecules, the side chain of Arg43 rotates outside and falls within the cationic cluster as shown in Fig. 4. Therefore, its participation in binding effector cannot be ruled out. The same can be said about Lys148. This residue is arranged in loop 144-148 in close vicinity to the supposed binding site for ATP (Fig. 5a), its side chain having high conformational freedom. It is not unlikely that the amino group of Lys148 in one of its possible conformational states can make a contact to the effector molecule.

Based on the model described here, participation of Lys112 in binding the effector phosphate groups is proposed for the first time. According to this model, the amino group of Lys112 forms a salt bridge to the γ-phosphate group of ATP. Kinetic studies of the E-PPase mutant variants show [17] that Lys112 plays a key role in binding effectors. It can be concluded that the proposed model of the structure of the ATP–E-PPase complex is in good agreement with the experimental data.

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