ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 1, pp. 68-76. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © T. S. Sitnik, S. M. Avaeva, 2007, published in Biokhimiya, 2007, Vol. 72, No. 1, pp. 80-90.

Binding of Substrate at the Effector Site of Pyrophosphatase Increases the Rate of Its Hydrolysis at the Active Site

T. S. Sitnik and S. M. Avaeva*

Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3181; E-mail: avaeva@belozersky.msu.ru

Received June 30, 2006
Revision received September 11, 2006

Abstract—It is shown that in addition to the active site, each subunit of *Escherichia coli* inorganic pyrophosphatase (E-PPase) contains an extra binding site for the substrate magnesium pyrophosphate or its non-hydrolyzable analog magnesium methylenediphosphonate. The occupancy of the extra site stimulates the substrate conversion. Binding affinity of this site decreased or disappeared upon the conversion of E-PPase into a trimeric form or introduction of point mutations. However, when the slowly hydrolyzed substrate, lanthanum pyrophosphate ($LaPP_i$), is used, the extra site was revealed in all enzyme forms of E-PPase and of Y-PPase (*Saccharomyces cerevisiae* PPase), resulting in about 100-fold activation of hydrolysis. A hypothesis on the localization of the extra site and the mechanism of its effect in E-PPase is presented.

DOI: 10.1134/S0006297907010087

Key words: inorganic pyrophosphatase, effector site, activation by substrate

Soluble pyrophosphatases (PPases, EC 3.6.1.1) catalyze reversible hydrolysis of pyrophosphate and are the key components controlling the intracellular level of PP_i. The most studied representatives of this enzyme class are PPases from *Escherichia coli* (E-PPase) and *S. cerevisiae* (Y-PPase). The active site pocket of the proteins comprises 15 functional groups, which are conservative for the entire first family of soluble pyrophosphatases. The substrate is a complex of PP_i with magnesium ions, which have a very high affinity to the enzyme (K_S ~ 1 μM) [1]. Like the vast majority of enzymes involved in phosphate exchange, pyrophosphatases are metal-dependent and display maximal activity in the presence of Mg^{2+}. In the absence of substrate, two magnesium ions can bind at the sites M1 and M2. Upon substrate binding, both the first and second metal ions acquire an additional ligand, the oxygen atom of electrophilic phosphate in pyrophosphate. Metal ions at these sites are strictly necessary for substrate hydrolysis, since they activate a water molecule located between them.

In the mid 1980s the authors of study [2] assumed the existence of a regulatory site in Y-PPase. The assumption was based on the fact that hydrolysis kinetics did not follow the Michaelis–Menten equation, whereas the activation of substrate hydrolysis was observed in the presence of non-hydrolyzable analogs. The observed pattern was displayed only at very low concentrations of $MgPP_i$ (<1 μM), and could be detected using a highly sensitive phosphate detector. Further development of this work was limited by the lack of dimensional structure and knowledge of the enzyme mechanism.

At the turn of the centuries, the problem of describing $MgPP_i$ hydrolysis by the Michaelis–Menten equation arose again during the study of trimeric E-PPase form, and it was explained by the negative cooperativity associated with the occupancy of enzyme active sites [3]. However, it turned out that this feature is inherent not only to E-PPase trimer, but also to the hexamer upon the introduction of a mutational replacement in the zone of inter-trimer contact [4]. Activation of hydrolysis by methylenediphosphonate (PCP) at pH values below the optimum by 1.5-2 units (when the interactions between all the components are weakened) resulted in the assumption of the existence of E-PPase effector site, which activates the enzyme upon substrate binding. The existence of two sites in each enzyme subunit was confirmed by equilibrium dialysis data, indicating the involvement of two moles of PP_i in the presence of Ca^{2+} [5].

In the present work it has been determined that the effector site binds not free PP_i, but an additional $MgPP_i$ molecule. Mutational replacements in E-PPase change the properties of the effector site along with changes in

* To whom correspondence should be addressed.

other properties of the enzyme. The use of an alternative substrate, lanthanum pyrophosphate, clearly revealed the general principles of functioning of the effector site in the PPases from *E. coli* and yeast. The data suggest a possible site of effector binding.

MATERIALS AND METHODS

Recombinant *E. coli* inorganic pyrophosphatase and its mutant forms were obtained as described earlier [6, 7]. The mutant form containing the replacement of Lys112 with Gln, as well as two double mutants containing replacements of Lys112 and Lys115 with Gln and Ala, respectively, and Lys112 and Lys148 with Gln were provided by S. A. Kurilova and N. N. Vorobyeva, and the yeast enzyme was provided by A. A. Baykov. Enzyme suspension was stored in ammonium sulfate solution (90% saturation) and desalted prior to use by gel filtration on a column with Sephadex G-50 (fine) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. Other reagents of highly pure grade were from Sigma (USA) and Fluka (Switzerland). All solutions were prepared using double deionized water purified on a Milli Q column (Millipore, USA).

Protein concentration was determined spectrophotometrically using specific extinction coefficient $A_{280}^{0.1\%}$ (1.18 for E-PPase [8] and 1.45 for Y-PPase [9]). Enzyme concentrations given below were calculated per subunit. Activities of native and mutant E-PPases as well as Y-PPase were determined by the rate of P_i release from $MgPP_i$ or $LaPP_i$. A semiautomatic phosphate analyzer was used for quantitative determination of P_i [10]. Enzymatic reaction was performed at 20°C and instrument sensitivity of 10 μM P_i (recorder full scale). Here and further the enzyme activity is expressed in U/mg (μmol of substrate per mg of enzyme during 1 min).

To obtain the trimeric enzyme form, hexameric E-PPase (1.5 μM) was incubated in 0.1 M Mes-NaOH buffer, pH 5.3, for 1-2 h. The dissociation process was monitored by taking aliquots and measuring pyrophosphatase activity.

For calculation of required free concentrations of Mg^{2+} and $MgPP_i$ the following K_d values were used: 47.7 μM and 2.42 mM for $MgPP_i$ and Mg_2PP_i, respectively, and 170 μM for MgPCP (pH 7.5).

Initial hydrolysis rates of the substrate ($MgPP_i$) as a function of its concentration were determined in 0.05 M Tris-HCl buffer, pH 7.5, using several fixed concentrations of free Mg^{2+} (0.5-5 mM) and $MgPP_i$ concentrations in the range 3-400 μM.

The effect of MgPCP on hydrolysis of $MgPP_i$ catalyzed by E-PPase was elucidated by addition of 7.5 μM MgPCP to 2.5-15 μM $MgPP_i$ in the presence of 0.5 mM Mg^{2+} followed by determination of hydrolysis rate. The dependence of hydrolysis rate on the concentration of $LaPP_i$ in the case of E-PPase, its trimeric form, and Y-PPase was studied at pH 7.5 (0.05 M Tris-HCl buffer and variable concentration of free Mg^{2+} ranging from 1 to 15 mM). The concentration of $LaPP_i$ was varied between 10 and 150 μM. The maximal possible concentration of $LaPP_i$ is determined by its solubility, which is influenced by the presence of Mg^{2+}.

The dependence of hydrolysis of 50 μM $LaPP_i$ on the concentration of Mg^{2+} was determined at pH 7.5 and magnesium concentrations of 0.5-15 mM. Hydrolysis of $LaPP_i$ in the presence of 35 μM PCP was performed in 0.05 M Tris-HCl buffer, pH 7.5, in the presence of 2 mM Mg^{2+}.

The nature of the effector was identified based on the results of hydrolysis of 75 μM $LaPP_i$ in the presence of 10-60 μM MgPCP at the fixed PCP concentration of 2 μM, and also in the presence of 0.6-4.5 μM PCP at the fixed MgPCP concentration of 20 μM. Magnesium concentration was varied from 1 to 5 mM at pH 7.5 in both versions of the experiment.

The effect of MgPCP and PCP on hydrolysis of $LaPP_i$ by E-PPase, its trimeric form, Y-PPase, Lys112Gln-PPase, Glu31Ala-PPase, and Asp42Glu-PPase was examined in the following set of experiments: 50 μM $LaPP_i$ was hydrolyzed in 0.05 M Tris-HCl buffer, pH 7.5, at fixed concentration of $MgPCP_{free}$ (10, 20, 30, 40, and 50 μM). For each fixed MgPCP concentration a set of constant PCP_{free} concentrations was used (0.75, 1, 1.25, 1.5 μM), which was achieved by changing Mg^{2+} concentration from 1 to 13 mM.

As a result of conducting two series of experiments it has been demonstrated that there was no transition of lanthanum ions from $LaPP_i$ to PCP with the release of free and rapidly hydrolyzed PP_i. In the first series, the accumulation of phosphate (formed during the enzymatic hydrolysis of 20 μM $LaPP_i$ in the presence of 2 mM Mg^{2+} and 6.4 μM PCP_{tot}) with time was monitored. After 40 min, the content of P_i in the medium was 20 μM. If the formation of LaPCP and release of PP_i occurred, the maximal P_i concentration could not exceed 12.8 μM. In the second series, the behavior of two reaction mixtures was compared. One of them contained 50 μM $LaPP_i$, 30 μM PCP_{tot}, 2 mM Mg^{2+}, and the enzyme. The second also contained 20 μM $LaCl_3$, which was previously added to the PCP solution. Enzyme activity in both mixtures was the same and equal to 60 U/mg. Both series of experiments showed that PCP binds La^{3+} with much weaker affinity than PP_i.

RESULTS AND DISCUSSION

Hydrolysis of $MgPP_i$ by hexameric and trimeric E-PPase forms. Comparison of the rates of accumulation of P_i catalyzed by the two enzyme forms at pH 7.5 shows a significant difference between them (Fig. 1). First, as was pointed out above, a hyperbolic dependence and dramat-

ic increase in activity (resulting in that the enzyme displays the maximal activity at relatively low substrate concentrations) are observed for the rate of substrate hydrolysis by hexamer. In the case of trimeric E-PPase form the kinetics of $MgPP_i$ hydrolysis cannot be described in terms of the Michaelis–Menten equation, and very high substrate concentrations are needed for the achievement of maximal enzyme activity. Finally, for low substrate concentrations, the activity of hexamer is significantly higher than the activity of trimer. These dependences can be explained by the existence of the enzyme effector site, which is occupied with high affinity in hexameric, and with lower affinity in trimeric enzyme forms.

The existence of the additional binding site in hexamer, which promotes substrate hydrolysis in the active site, can be deduced from the table. It is seen that the addition of non-hydrolyzable substrate analog significantly influences the rate of product accumulation. High affinity to the effector reveals the effect of MgPCP only at low concentrations of substrate and metal ions, which was previously observed for Y-PPase [2].

It was important to determine what component of the reaction mixture is bound in the effector site and accelerates the hydrolysis of $MgPP_i$. There are three components in the mixture: $MgPP_i$, free PP_i, and free magnesium ions coupled by the equilibrium $MgPP_i \leftrightarrow PP_i + Mg$ with $K_d = 50 \cdot 10^{-6}$ M. To determine the contribution of each component in the activation of hydrolysis we have analyzed the dependence of the rate of $MgPP_i$ hydrolysis by trimeric E-PPase in three experiments (Fig. 2). In each of them fixed concentration of one of the components was maintained, whereas the concentrations of two other components were varied. At the constant concentration of PP_i (Fig. 2a), increase in Mg^{2+} concentration results in increase in $MgPP_i$ concentration, and a marked increase in the hydrolysis rate. As seen from Fig. 2b, in

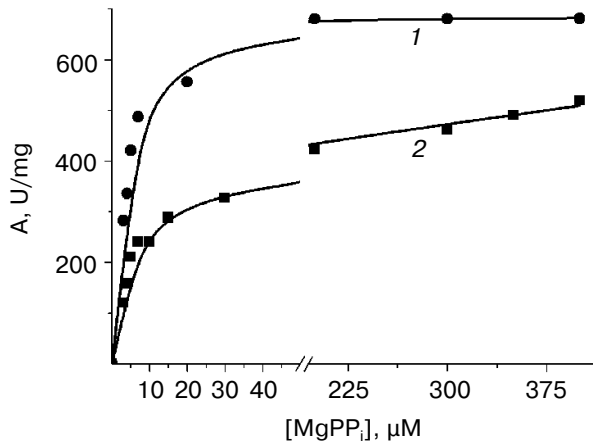


Fig. 1. Dependence of the rate of hydrolysis by WT-PPase (*1*) and trimeric E-PPase (*2*) at pH 7.5 on the concentration of $MgPP_i$ in the presence of 5 mM Mg^{2+}.

Effect of methylenediphosphonate (PCP) on $MgPP_i$ hydrolysis by hexameric E-PPase at pH 7.5 in the presence of 0.5 mM Mg^{2+}

[$MgPP_i$], μM	Activity, U/mg	
	– MgPCP	+ 7.5 μM MgPCP
2.5	82	142
5	178	232
10	268	317
15	360	360

the presence of 30 μM $MgPP_i$ (which is sufficient for occupation of the active site, as follows from Fig. 1), the increase in magnesium ion concentration was accompanied by decrease in PP_i concentration, but the enzyme activity is increased herewith. These data indicate that pyrophosphate has virtually no contribution to the activation of hydrolysis. The achieved activity is only slightly different from that of the trimer at low substrate concentrations, i.e. under conditions when the active site is saturated with the substrate and the occupancy of the effector site starts increasing. The studied concentration range corresponds to the occupancy of the effector site, thus indicating the crucial role of $MgPP_i$ in the activation of hydrolysis. This conclusion is confirmed by the results of simultaneous increase in $MgPP_i$ and PP_i concentrations at fixed concentration of Mg^{2+} of 5 mM (Fig. 2c). As can be seen, the achieved activity is not different from that shown in Fig. 2b, and is not increased in spite of the increase in PP_i concentration.

Comparison of these data unambiguously indicates that the activator of $MgPP_i$ hydrolysis in the active site is $MgPP_i$, which binds at the effector site. This feature is typical for both hexameric and trimeric forms of E-PPase. Therefore, E-PPase can be classified as a substrate-activated enzyme.

Significant difference in $MgPP_i$ affinity to active and effector sites in the trimer allows using the scheme of substrate activation and corresponding equation for the quantitative estimation of reaction parameters:

$$\begin{array}{ccccc} E + S & \xrightarrow{K_S} & ES & \xrightarrow{k_{cat}} & E + P \\ & & K_S' \updownarrow & & \\ & & ES_2 & \xrightarrow{\beta k_{cat}} & ES + P \end{array}$$

$$k_{eff} = k_{cat} \frac{(1 + \beta\,[S]_0 / K_S')}{K_S + [S]_0 + [S]_0^2 / K_S'}\,[S]_0\,,$$

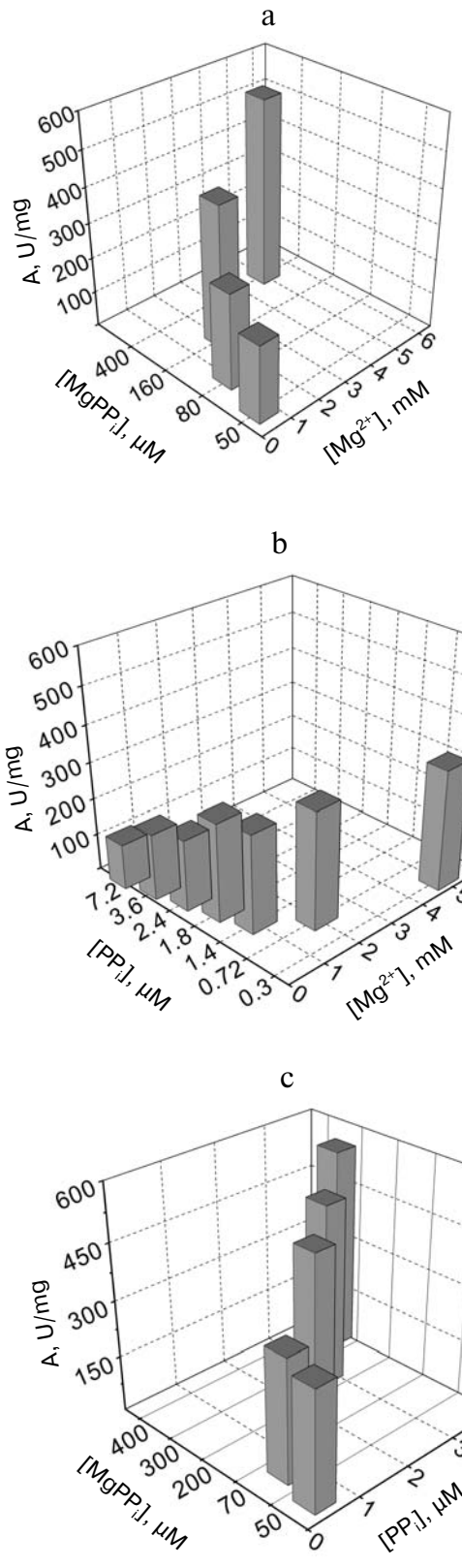


Fig. 2. Dependence of the rate of $MgPP_i$ hydrolysis by trimeric E-PPase on the concentration of Mg^{2+}, $MgPP_i$, and PP_i. a) Constant concentration of PP_i (4 μM); b) constant concentration of $MgPP_i$ (30 μM); c) constant concentration of Mg^{2+} (5 mM).

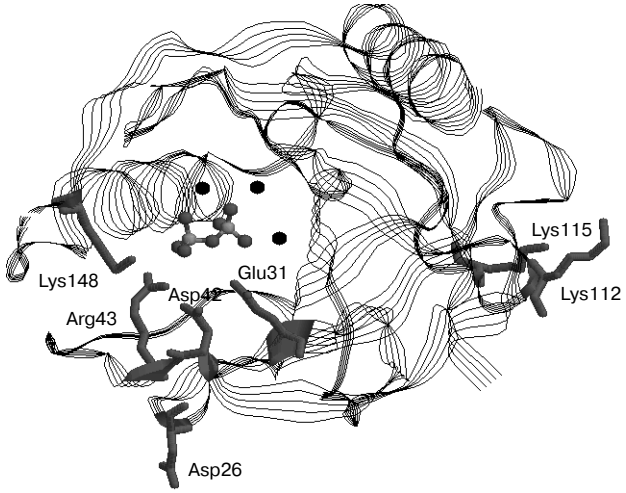


Fig. 3. E-PPase subunit. Substrate and metal ions in the active site are shown. Amino acid residues, the replacements of which are discussed in the text, are designated. The $E{-}Ca_2{-}CaPP_i$ complex structure (PDB code 1I40; http://www.rcsb.org/pdb/) was used to construct this drawing.

where E is the enzyme, S is the substrate ($MgPP_i$), P is the product (P_i), K_S and K_S' are substrate binding constants for the catalytic and effector sites, respectively, β is coefficient of acceleration of hydrolysis rate, k_{cat} and k_{eff} are rate constants in the absence and presence of effector [11]. The equation well describes the dependence of the rate of $MgPP_i$ hydrolysis on high effector concentrations, where k_{cat} = 250 U/mg, β = 2.5, and K_S' = 132 μM. Application of the equation to hydrolysis of $MgPP_i$ by hexameric E-PPase is limited by high affinity of the effector, and it allows estimating only K_m/K_S' ratio, equal to 2-3.

Introduction of mutational replacements in E-PPase influences the affinity of effector site. At present more than 40 E-PPase forms obtained by site-directed mutagenesis are known. The replacements have been introduced in different regions of the molecule. It is noteworthy that all replacements, including even the most conservative ones (Asp/Glu, Glu/Asp, Tyr/Phe, His/Gln, Gly/Ala) always result in the change of one or another property of the enzyme (kinetic characteristics, parameters of thermal and chemical denaturation, stability of oligomeric structure, etc.). All these results reflect structural lability of the molecule, which can be associated with the movement of functional groups and dynamic loops, as well as with rearrangement of a network of hydrogen bonds between a large number of water molecules retained by the protein globule.

The interest in functioning of the effector site in E-PPase stimulated the investigation of some mutant proteins, in particular, Asp26Ala-, Glu31Ala-, Asp42Glu-, Arg43Gln-, Lys112Gln-, Lys115Ala-, and Lys148Gln-E-PPase. The positions of replaced residues in the protein molecule are shown in Fig. 3.

Recently we demonstrated that along with the change in properties of Asp26Ala-, Arg43Gln-, Lys115Ala-, Lys148Gln-PPases, the decrease in affinity to the effector site occurs, and, as a consequence, the kinetics of substrate hydrolysis does not obey the Michaelis–Menten equation [12]. In the present work these dependences have been analyzed according to the scheme for substrate-activated enzymes, which allows determining the affinity to the effector and acceleration coefficient upon its occupancy. It turned out that a two-fold activation of hydrolysis is observed for Lys115Gln- and Lys148Gln-PPase, whereas the substrate molecule is bound at the effector site with $K_S' = 36$ and 142 μM, respectively.

Special attention is drawn to the mutants for which the kinetics of $MgPP_i$ hydrolysis is described by the classical Michaelis–Menten equation. This group includes Glu31Ala-, Asp42Glu-, and Lys112Gln-PPases. The role of mutated residues in enzyme functioning is not completely clear. However, it is known that Glu31 forms a bond with Asp42 [13]. At high concentrations of Mg^{2+}, when Mg_2PP_i binding to the enzyme occurs, Glu31 is included into the coordination sphere of the second metal ion of the substrate. Besides the bond with Glu31, Asp42 in EMg_2 forms a salt bridge with Lys29. The choice of Glu3Ala- and Asp42Glu-PPase mutants for the study of kinetics of $MgPP_i$ hydrolysis was random. However, the attention to Lys112Gln-PPase was due to the fact that Lys112, along with Lys115, Lys148, and Arg43, is incorporated into a cationic cluster, and is believed to play a crucial role in effector binding [14]. Nevertheless, a number of factors given below does not allow considering the cationic cluster as a site for effector binding. It turned out that the termination of effector binding during $MgPP_i$ hydrolysis is observed not only for the replacement of a component of the positive cluster, Lys112, but also for the replacements Glu31Ala and Asp42Glu. Mutational replacements have, to a greater or lesser extent, an influence on other enzyme properties, which indicates changes in protein structure. Thus, the introduction of a second replacement in Lys112Gln-PPase results in further changes in enzyme properties [15]. Activity of Lys112Gln/115Ala-PPase increases from 300 to 400 U/mg, and in the second double mutants it is decreased six-fold. It should also be taken into account that the formation of strong ion pairs on a protein surface is unlikely due to high desolvation energy. The dissociation constant is known for PP_i complex with cationic cluster, formed by two Lys residues and two Arg residues on the surface of interleukin-1 receptor antagonist, which is 2.9 mM, thus being tens and hundreds times higher than for effector binding in E-PPase trimer and hexamer [16].

It will be shown below that during the hydrolysis of $LaPP_i$ these mutant proteins greatly increase their activity upon the occupancy of the effector site by the substrate analog.

Pyrophosphatase hydrolysis of $LaPP_i$. The challenges in characterization of E-PPase effector site, associated with high affinity of $MgPP_i$, can to a certain extent be overcome by using a slowly hydrolyzing substrate, lanthanum pyrophosphate. This compound has a significant advantage compared to the native substrate, since the binding constant of La^{3+} with PP_i is 10^{17} M^{-1}, and, as a result, free PP_i is completely absent from the reaction medium [17].

Positive cooperativity in substrate binding is observed during the hydrolysis of $LaPP_i$ by E-PPase: occupancy of the active site in some subunits enhances the occupancy in the others (Fig. 4, curve *1*). The experimental data are well described by the Hill equation with $h = 2.0$, $K_m \sim 1$ mM, and $V_{max} = 16$ U/mg. It is most probable that dissimilar behavior of the active sites is due to mutual effect of the trimers on each other, since the concentration dependence of $LaPP_i$ hydrolysis by the trimer is a hyperbolic curve (Fig. 4, curve *2*). The rate of hydrolysis by both hexamer and trimer remains very low, and substrate saturation is not reached even at 200 μM $LaPP_i$.

The rate of $LaPP_i$ hydrolysis increases upon the increase in Mg^{2+} concentration in the reaction medium; however, even at 15 mM Mg^{2+} (Fig. 5, dependence *1*) it does not reach saturation. This implies that the dissociation constant for Mg^{2+} complex with the enzyme is higher than 15 mM, and this value is incomparable with Mg^{2+} affinity during $MgPP_i$ hydrolysis, and does not correspond to the value of dissociation constant for the M2 site of EMg_2MgPP_i complex (0.3 mM). Therefore, binding of $LaPP_i$ at the active site significantly changes the enzyme affinity to Mg^{2+} necessary for catalysis. Indeed, increasing the Mg^{2+} concentration in the medium from 2 to 15 mM dramatically changes the hydrolysis pattern (Fig. 4, curve *3*). First, some increase in hydrolysis rate is observed; second, the sigmoidal character of the dependence of $LaPP_i$ hydrolysis on concentration disappears;

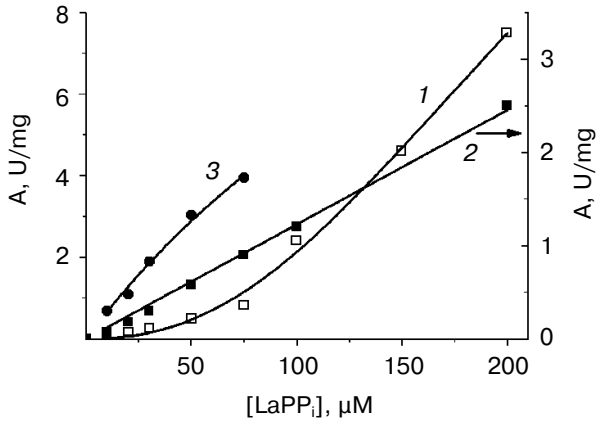


Fig. 4. Dependence of hydrolysis rate on concentration of $LaPP_i$ by hexameric (curve *1*) and trimeric (curve *2*) forms of E-PPase at pH 7.5 in the presence of 2 mM Mg^{2+}, and on concentration of $LaPP_i$ by E-PPase (curve *3*) at pH 7.5 in the presence of 15 mM Mg^{2+}.

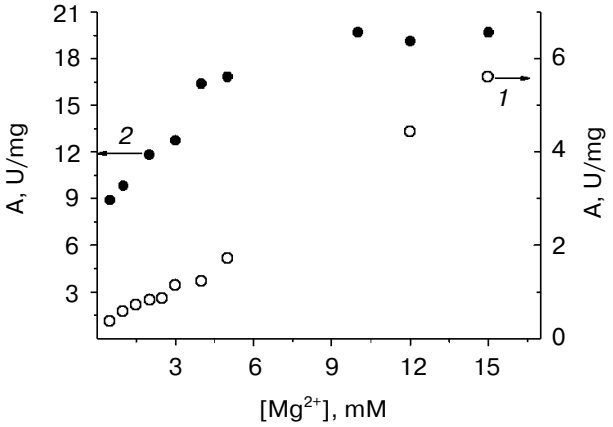


Fig. 5. Dependence of the rate of hydrolysis of 50 μM $LaPP_i$ on the concentration of magnesium ions for E-PPase (*1*) and Y-PPase (*2*) at pH 7.5.

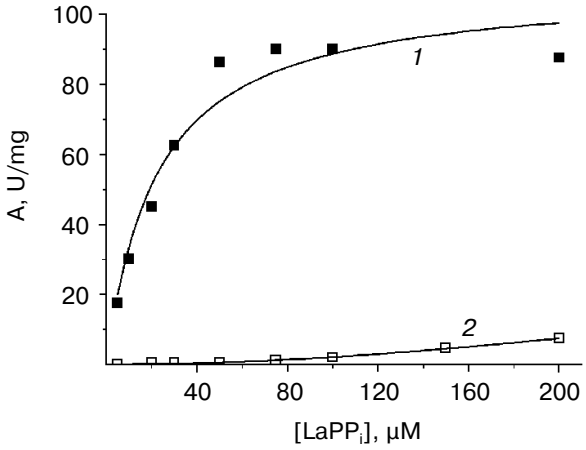


Fig. 6. Rate of $LaPP_i$ hydrolysis in the presence of 35 μM PCP (curve *1*) and without PCP (curve *2*) at pH 7.5 and in the presence of 2 mM Mg^{2+}.

and third, substrate affinity to the enzyme's active site is greatly increased.

Similar conclusions can be drawn for Y-PPase too, for which a sigmoidal dependence of $LaPP_i$ hydrolysis and diminished binding of Mg^{2+} are also observed. In the case of the yeast enzyme the binding constant of magnesium ion at the M2 site can be estimated. It is equal to 4.8 mM (Fig. 5, dependence *2*). Hence, higher affinity of Mg^{2+} to Y-PPase than to E-PPase upon $MgPP_i$ hydrolysis is also displayed during $LaPP_i$ hydrolysis. For this reason, disappearance of subunit asymmetry in the case of Y-PPase occurs with the increase in Mg^{2+} concentration from 0.5 to 2 mM. Since cooperativity disappears with the increase in magnesium ion concentration necessary for occupancy of the M2 site, apparently the state of this site defines the general architecture of the enzyme molecule.

The absence of free PP_i in the medium during the hydrolysis of $LaPP_i$ allows studying the effect of methylenediphosphonate and its magnesium complex in detail. The addition of 35 μM PCP_{tot} in the presence of different $LaPP_i$ concentrations causes the strongest activation of hydrolysis (Fig. 6). It can be seen that the rate of $LaPP_i$ hydrolysis increases by more than two orders of magnitude and approaches the rate of hydrolysis of native substrate, $MgPP_i$. The accumulation of hydrolysis product is described by the Michaelis–Menten equation, and K_S is improved by at least one order of magnitude and becomes equal to 25 μM.

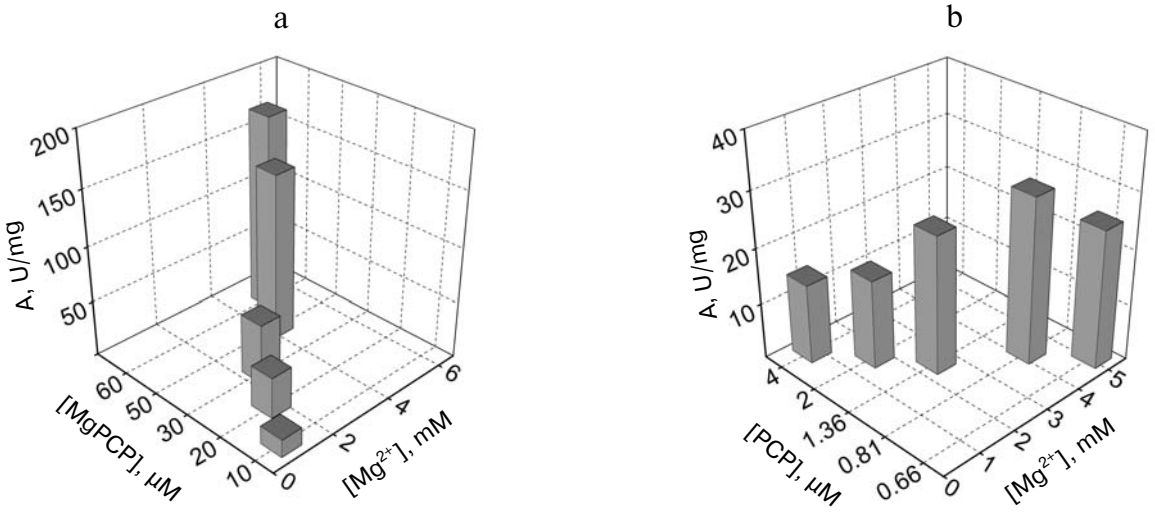


Fig. 7. Dependence of the rate of hydrolysis of 75 μM $LaPP_i$ by E-PPase on the concentration of Mg^{2+}, MgPCP, and PCP at pH 7.5. a) Constant concentration of PCP (2 μM); b) constant concentration of MgPCP (20 μM).

Dependences of the rate of $LaPP_i$ hydrolysis on concentrations of MgPCP and PCP are given in Fig. 7. As seen from Fig. 7a, an increase in hydrolysis rate occurs due to the effect of MgPCP; in this case, the concentration of free PCP remains constant at 2 μM. At the same time, in the presence of varied PCP concentration (which decreases with the increase in Mg^{2+} concentration), the rate changes slightly and corresponds to incomparably lower activity (Fig. 7b). In this case, the medium already contains 20 μM MgPCP and the increment of activity is due to its presence. As was pointed out before, the increase in Mg^{2+} content in the medium increases the rate of hydrolysis, but this effect is very insignificant compared to the action of MgPCP, and even with the change in Mg^{2+} concentration from 2 to 15 mM the activity does not exceed 6 U/mg (Fig. 4).

Thus, the activation of $LaPP_i$ hydrolysis is achieved due to the occupancy of the effector site by MgPCP complex. These data are in a complete agreement with the data obtained for hydrolysis of $MgPP_i$.

It could not be excluded a priori that non-hydrolyzable substrate analog (MgPCP) binds to the active site during $LaPP_i$ hydrolysis, and thereby changes the protein conformation, causing the increase in activity. Several facts contradict this assumption. First of all, MgPCP affinity to the active site is very low, the inhibition constant for $MgPP_i$ hydrolysis by E-PPase being 330 and by Y-PPase being 1100 μM [18]. Second, the increase in $LaPP_i$ concentration at the fixed concentration of MgPCP virtually does not decrease the influence of the effector, which could not be expected in the case of competition between $LaPP_i$ and MgPCP for the active site (Fig. 6). It was determined experimentally that no inhibition of hydrolysis of 50 μM $LaPP_i$ is observed with the increase in MgPCP concentration up to 200 μM (data not shown).

After the major contribution of MgPCP in activation of hydrolysis had been revealed, it allowed systematically comparing the effect of MgPCP (at different fixed concentrations of PCP) on E-PPase, Y-PPase, trimeric E-PPase, and a number of E-PPase mutants (Figs. 8 and 9). For all obtained dependences the increase in MgPCP concentration results in a dramatic increase in hydrolysis rate. With that, each enzyme has particular features. As follows from Fig. 8a, along with activation of E-PPase by MgPCP by more than 100-fold, the rate of hydrolysis is slightly increased in the presence of PCP. The extent of the increase in activity is higher for Y-PPase (Fig. 8b) than for E-PPase. These two enzymes are characterized by the same V_{max} values for $MgPP_i$ hydrolysis; however, Y-PPase displays higher affinity to Mg^{2+} in the M2 site, and higher rate of $LaPP_i$ hydrolysis. These features of Y-PPase can lead to a large scatter of points on the plot, probably due to weak activation by PCP at low MgPCP concentrations. The achieved maximal activity (420 U/mg) is similar to the rate of $MgPP_i$ hydrolysis. This fact is another confirmation of MgPCP binding at a site differing from the active site. If $LaPP_i$ was located only in the part of the active sites, such hydrolysis rate could not be reached.

Trimeric E-PPase hydrolyzes $LaPP_i$ with low rate (Fig. 4, curve *2*). Therefore, the achieved activity in the presence of MgPCP (Fig. 9) also indicates a very high degree of activation.

Special attention should be given to the data presented in Fig. 9. As shown above, $MgPP_i$ hydrolysis by a number of mutant forms of E-PPase, containing Asp42, Glu31, or Lys112 replacements, does not exhibit typical features of the occupancy of the effector site. However, the results obtained for $LaPP_i$ hydrolysis by Lys112Gln-, Glu31Ala-, and Asp42Glu-PPases clearly demonstrate a marked increase in hydrolysis rate upon MgPCP binding at the effector site. Similar dependence is also characteristic for Lys112Gln/Lys115Ala-PPase (data not shown).

Assumption about location of effector binding site and origins of its influence on PPase activity. Binding of sub-

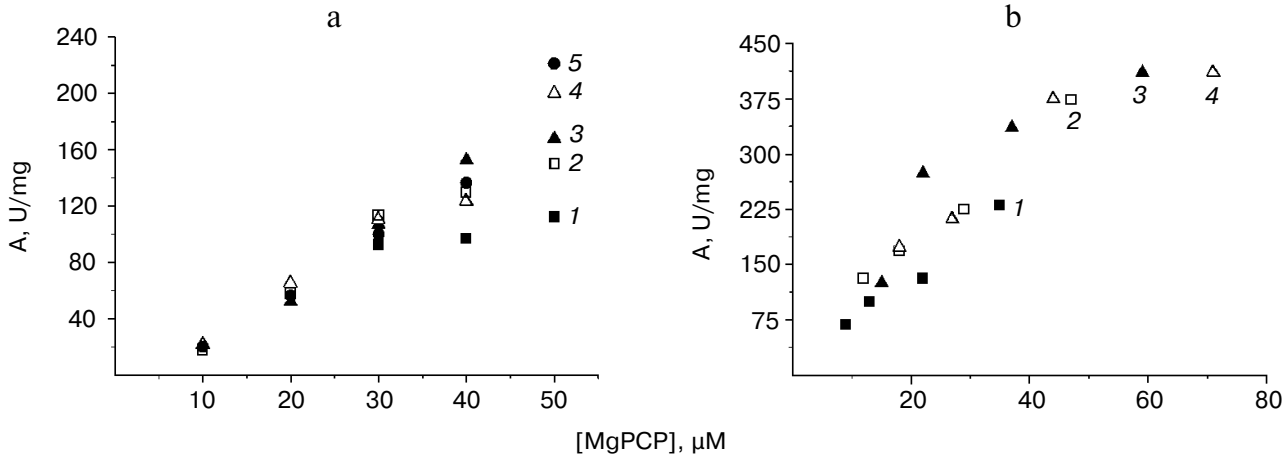


Fig. 8. Dependence of the rate of hydrolysis of 50 μM $LaPP_i$ by E-PPase (a) and Y-PPase (b) on the concentration of MgPCP at different concentrations of Mg^{2+} and fixed concentrations of PCP_{free}: 0.75, 1, 1.25, 1.5, and 2 μM (*1-5*, respectively).

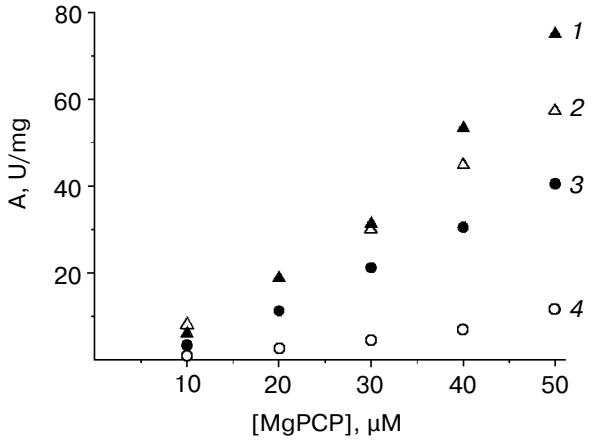


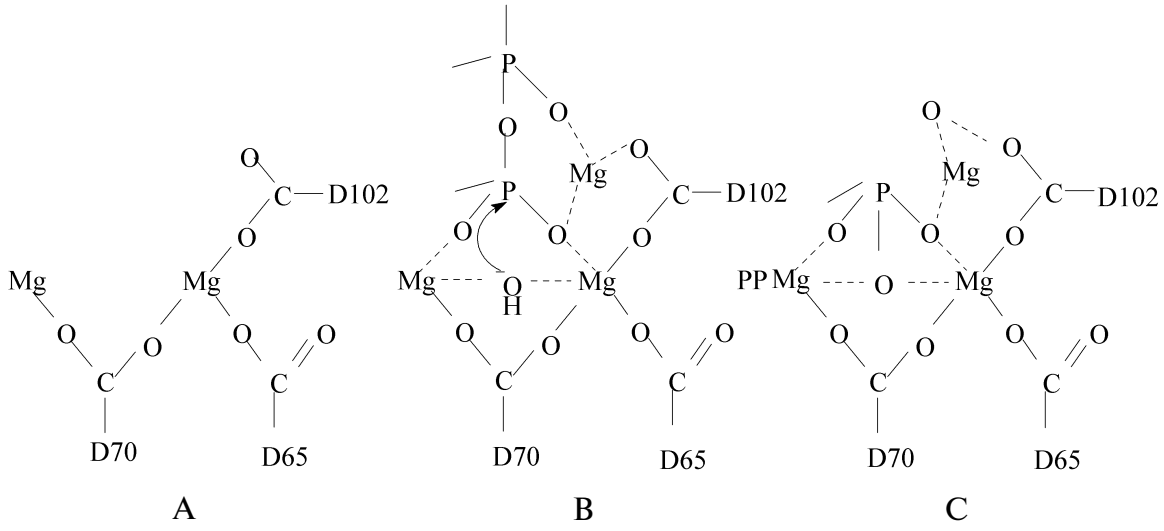
Fig. 9. Dependence of the rate of hydrolysis of 50 μM $LaPP_i$ by trimeric E-PPase (*1*), Lys112Gln-, Asp42Glu-, and Glu31Ala-PPases (*2-4*, respectively) on the concentration of MgPCP at different concentrations of Mg^{2+} and fixed concentration of PCP_{free} of 1.25 μM.

strate or its non-hydrolyzable analog by E-PPase and Y-PPase at a center that is different from the active site results in the increase in hydrolysis rate, which allows classifying these enzymes as substrate-activated. Only a small number of enzymes of such class are known in enzymology [19-21]. Indeed, it is hard to imagine the existence of an additional site in the protein globule, able to bind the substrate with high affinity without hydrolyzing it, and, in addition to this, having a certain influence on the events in the active site. At the same time, it is the most economic way of activation, which does not require the introduction of additional exogenous agent into the system. Regulation of gene expression for pyrophosphatases is not known. Therefore, the mechanisms for the regulation of activity of already synthesized proteins should exist for such constitutive proteins. Since PPases are the key enzymes in synthesis of most important biopolymers, different pathways for regulation of their activity are possible. The described substrate activation mechanism may be one of them.

The location of the effector binding site suggested in this work is specified, first of all, by a similar behavior of different PPases. For Y-PPases, the increase in the rate of $MgPP_i$ hydrolysis by PCP is known [2]. In the present work the increase in the rate of $LaPP_i$ hydrolysis by several hundred times was observed. The same features are typical for E-PPase and its mutant forms. The two enzymes differ in their tertiary structure (two and six subunits), subunit size (32 and 20 kD), and, as a consequence, by surface topology; but they have identical active site structure and similar mechanism. It is evident that the effector-binding site should have properties characteristic for all these proteins.

Among a large number of functional groups in the active site, there is a local region responsive to virtually all changes in the protein molecule. This is the binding site for the second metal ion, the M2 site. As mentioned before, the occupancy of the two sites, M1 and M2, is required for enzyme activity. Strength of Mg^{2+} binding in these sites is very different. As seen from the scheme shown below (structure A), three protein ligands enter the coordination sphere of Mg^{2+} in the M1 site, thus enabling high stability of the complex (K_d = 20 μM [13]).

The metal ion in the M2 site is retained due to a partial charge of only one protein ligand, which simultaneously coordinates the first Mg^{2+} also. The dissociation constant for this site is 5-6 mM [22, 23]. This means that



Scheme of location of Mg^{2+} in PPase active site (A) in the presence of substrate (B), in a complex with product (P_i), and with occupied effector site (C)

at the intracellular concentration of free Mg^2 of 1-1.5 mM [24] this site is only partially occupied. Substrate binding increases Mg^{2+} affinity (structure B), but hydrolysis of the substrate and release of products returns the enzyme to the initial state. The reason for evolutionary selection of such structure of the active site is not clear. It cannot be excluded that the role of Mg^{2+} in the M2 site is not limited only by the activation of a water molecule.

It is logical to assume that the effector ($MgPP_i$ or MgPCP), as well as Mg^{2+}, can bind to the M2 site (structure C in the scheme). The main consequence of the formation of such complex should be an increase in enzyme activity. One of the slowest stages in substrate hydrolysis is the release of product, accompanied by the disruption of bonds between oxygen atoms of the phosphate and magnesium ions. The lesser the positive charge on magnesium ion the easier this process is. Therefore, binding of $MgPP_i$ substrate at the M2 site instead of Mg^{2+} facilitates the occurrence of the slowest stage. As follows from the experiments performed in this work, the affinity of effector to protein is higher than the affinity of Mg^{2+} to the M2 site. As a consequence, the enzyme can be fully functional under conditions of lower Mg^{2+} content in the medium.

The quantitative ratio of the enzyme complexes with Mg^{2+} and $MgPP_i$ at the M2 site is ultimately determined by the concentrations of PP_i and Mg^{2+}. As follows from the scheme, at low concentrations of Mg^{2+} activation is possibly mediated by PP_i bound, apparently, to the metal in the M2 site. At medium concentration of Mg^{2+}, the most efficient is $MgPP_i$. At high concentrations, as, for instance, under crystallization conditions, when Mg^{2+} concentrations reaches 150-300 mM, the M2 site is always occupied with magnesium.

The appearance of $MgPP_i$ in the M2 site makes E-PPase and Y-PPase similar to PPases of the second family, which typically have a large number of ligands at the second metal ion. This leads to a decrease in affinity of substrate and products to the active site, and to high rate of hydrolysis.

This work was supported by the Russian Foundation for Basic Research (grant 03-04-48866) and the Program "Leading Scientific Schools" (grant NS-1706-2003-4).

REFERENCES

1. Baykov, A. A., Shestakov, A. S., Kasho, V. N., Vener, A. V., and Ivanov, A. N. (1990) *Eur. J. Biochem.*, **194**, 879-887.
2. Baykov, A. A., Pavlov, A. R., Kasho, V. N., and Avaeva, S. M. (1989) *Arch. Biochem. Biophys.*, **273**, 301-308.
3. Avaeva, S., Grigorjeva, O., Mitkevich, V., Sklyankina, V., and Varfolomeyev, S. (1999) *FEBS Lett.*, **464**, 169-173.
4. Sitnik, T. S., Vainonen, J. P., Rodina, E. V., Nazarova, T. I., Kurilova, S. A., Vorobyeva, N. N., and Avaeva, S. M. (2003) *IUBMB Life*, **55**, 37-41.
5. Vainonen, J. P., Vorobyeva, N. N., Rodina, E. V., Nazarova, T. I., Kurilova, S. A., Skoblov, Ju. S., and Avaeva, S. M. (2005) *Biochemistry (Moscow)*, **70**, 69-79.
6. Avaeva, S., Ignatov, P., Kurilova, S., Nazarova, T., Rodina, E., Vorobyeva, N., Oganessyan, V., and Harutyunyan, E. (1996) *FEBS Lett.*, **399**, 99-102.
7. Josse, J. (1966) *J. Biol. Chem.*, **241**, 1938-1947.
8. Harutyunyan, E. G., Oganessyan, V. Yu., Oganessyan, N. N., Terzyan, S. S., Popov, A. N., Rubinskii, S. V., Vainshtein, B. K., Nazarova, T. I., Kurilova, S. A., Vorob'eva, N. N., and Avaeva, S. M. (1996) *Kristallografiya*, **41**, 84-96.
9. Kunitz, M. (1952) *J. Gen. Physiol.*, **35**, 423-450.
10. Baykov, A. A., and Avaeva, S. M. (1981) *Analyt. Biochem.*, **116**, 1-4.
11. Berezin, I. V., and Klesov, A. A. (1976) *Practical Course of Chemical and Enzymatic Kinetics* [in Russian], MGU Press, Moscow, pp. 113-114.
12. Sitnik, T. S., and Avaeva, S. M. (2005) *Bioorg. Khim.*, **31**, 251-258.
13. Raibekas, A. A., Bures, E. J., Siska, C. C., Kohno, T., Latypov, R. F., and Kerwin, B. A. (2005) *Biochemistry*, **44**, 9871-9879.
14. Rodina, E. V., Vorobyeva, N. N., Kurilova, S. A., Sitnik, T. S., and Nazarova, T. I. (2007) *Biochemistry (Moscow)*, **72**, 100-108.
15. Sitnik, T. S. (2006) *Characterization of the Effector Site of Escherichia coli Inorganic Pyrophosphatase*: Candidate's dissertation (in Chemistry) [in Russian], MGU, Moscow.
16. Rodina, E. V., Vainonen, Y. P., Vorobyeva, N. N., Kurilova, S. A., Nazarova, T. I., and Avaeva, S. M. (2001) *Eur. J. Biochem.*, **268**, 3851-3857.
17. Smith, R. M., and Martell, A. E. (1976) *Inorganic Complexes. Critical Stability Constants*, Vol. 4, Plenum Press, N. Y., pp. 59-60.
18. Smirnova, J. N., Kudryavtseva, N. A., Komissarenko, S. V., Tarusova, N. B., and Baykov, A. A. (1988) *Arch. Biochem. Biophys.*, **267**, 280-284.
19. Masson, P., Schopfer, L. M., Bartels, C. F., Froment, M. T., Ribes, F., Nachon, F., and Lockridge, O. (2002) *Biochim. Biophys. Acta*, **1954**, 313-324.
20. Shiman, R., Xia, T., Hill, M. A., and Gray, D. W. (1994) *J. Biol. Chem.*, **269**, 24647-24656.
21. Baykov, A. A., Hyytia, T., Turkina, M. V., Efimova, I. S., Kasho, V. N., Goldman, A., Cooperman, B. S., and Lahti, R. (1999) *Eur. J. Biochem.*, **260**, 308-317.
22. Avaeva, S. M., Rodina, E. V., Kurilova, S. A., Nazarova, T. I., Vorobyeva, N. N., Harutyunyan, E. H., and Oganessyan, V. Yu. (1995) *FEBS Lett.*, **377**, 44-46.
23. Hyytia, T., Halonen, P., Salminen, A., Goldman, A., Lahti, R., and Coperman, B. S. (2001) *Biochemistry*, **40**, 4645-4653.
24. Alberts, B., Bray, D., Lewis, Z., Raff, M., Roberts, K., and Watson, Z. D. (1983) *Molecular Biology of the Cell*, N. Y., p. 286.