Biochemistry (Moscow), Vol. 70, No. 8, 2005, pp. 858-866. Translated from Biokhimiya, Vol. 70, No. 8, 2005, pp. 1041-1050.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-049, July 24, 2005.

Substitutions of Glycine Residues Gly100 and Gly147 in Conservative Loops Decrease Rates of Conformational Rearrangements of *Escherichia coli* Inorganic Pyrophosphatase

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Received February 28, 2004
Revision received November 12, 2004

Abstract—*Escherichia coli* inorganic pyrophosphatase (PPase) is a one-domain globular enzyme characterized by its ability to easily undergo minor structure rearrangements involving flexible segments of the polypeptide chain. To elucidate a possible role of these segments in catalysis, catalytic properties of mutant variants of *E. coli* PPase Gly100Ala and Gly147Val with substitutions in the conservative loops II and III have been studied. The main result of the mutations was a sharp decrease in the rates of conformational changes required for binding of activating Mg^{2+} ions, whereas affinity of the enzyme for Mg^{2+} was not affected. The pH-independent parameters of $MgPP_i$ hydrolysis, k_{cat} and k_{cat}/K_m, have been determined for the mutant PPases. The values of k_{cat} for Gly100Ala and Gly147Val variants were 4 and 25%, respectively, of the value for the native enzyme. Parameter k_{cat}/K_m for both mutants was two orders of magnitude lower. Mutation Gly147Val increased pH-independent K_m value about tenfold. The study of synthesis of pyrophosphate in the active sites of the mutant PPases has shown that the maximal level of synthesized pyrophosphate was in the case of Gly100Ala twofold, and in the case of Gly147Val fivefold, higher than for the native enzyme. The results reported in this paper demonstrate that the flexibility of the loops where the residues Gly100 and Gly147 are located is necessary at the stages of substrate binding and product release. In the case of Gly100Ala PPase, significant impairment of affinity of enzyme effector site for PP_i was also found.

Key words: inorganic pyrophosphatase, structure, activity, conformational flexibility, glycine residues, dissociation constant

Soluble inorganic pyrophosphatases (PPases) catalyze hydrolysis of inorganic pyrophosphate (PP_i) giving two orthophosphates (P_i). These enzymes necessary for a biosynthesis of the most important cell biopolymers have been isolated from quite different organisms. *Escherichia coli* PPase is one of the best-studied enzymes of this class. Catalysis by inorganic pyrophosphatases proceeds in a number of stages [1, 2]. A key scheme of catalysis includes the following stages: binding of two Mg^{2+} ions, binding of a substrate ($MgPP_i$), hydrolysis itself, and sequential release of two phosphates:

$$E + 2Mg^{2+} \leftrightarrow EMg_2 \leftrightarrow EMg_{2+n}PP_i \leftrightarrow$$

$$\leftrightarrow EMg_{2+n}P_{i2} \leftrightarrow EMg_{1+n}P_i \leftrightarrow EMg_2.$$

Globular proteins in solution are now assumed to consist of a number of conformational states coexisting in equilibrium. Some proteins form only a few significantly different conformations, and some others form many similar conformations that can differ in fine structure of the local parts of a chain including the interactions between residues. Inorganic pyrophosphatase is a proteins of the second type. Generally, such minor rearrangements simply accompany functioning of the

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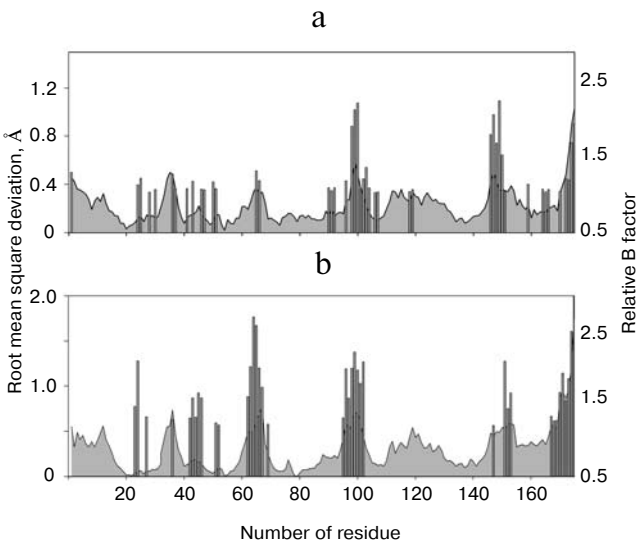

Fig. 1. Root mean square deviation for the C_α atoms of a polypeptide backbone calculated from the superposition of the following structures: a) apo- versus holoform of E-PPase (1IGP and 1OBW, respectively); b) E-PPase complexed with Ca^{2+} versus E-PPase complexed with $CaPP_i$ (1I40 and 1I6T). Filled area (right ordinate axe) shows B-factor for C_α atoms normalized by the mean value of B-factor ((a) mean B for 1IGP of 26.8 Å^2 was used; (b) 19.58 Å^2 for 1I40).

proteins, being a waste of energy. In some cases, however, conformational transitions are necessary for proteins and enzymes to exhibit their biological activities. In these cases glycine residues, even though they are not generally considered as catalytic residues, may provide for a high rate of transitions between conformations and thus may play an important role in catalysis.

To date, three-dimensional (3D) structures are known for pyrophosphatase, its mutant variants, and for its complexes with a number of ligands [3-10]. Superposition of 3D structures mimicking successive catalytic intermediates has revealed the structural rearrangements PPases undergo in the course of their catalytic reaction [11]. It has been shown that at some stages of catalysis not only side chains shift but some parts of the polypeptide backbone move as well (Fig. 1). For example, after binding two Mg^{2+} ions, loops I and II mostly respond, while at the stages of substrate binding and product release loops II and III are the most affected. These segments of the backbone have high relative B-factors, yet their movement is not unregulated but rather well ordered [11]. Superimposition of 3D structures of *E. coli* PPase versus *Saccharomyces cerevisiae* PPase, which represent two different families, has shown that the architecture of these segments is also very similar. Conservation of disordered regions in the course of evolution is a rare phenomenon and might reflect the importance of those regions in catalysis. To reveal a possible role of these loops in *E. coli* PPase catalysis, mutant variants have been designed with substitutions Gly100Ala and Gly147Val in conservative loops II and III, respectively. In the present paper, the catalytic properties of these mutants are described.

MATERIALS AND METHODS

All chemicals used in the work were of the highest purity available: Tris, MES, H_3PO_4, and methyl green (Fluka, Switzerland); Mops, ATP-sulfurilase, luciferin–luciferase mixture, adenosine-5′-phosphosulfate, $MgCl_2$, and $Na_4P_2O_7{\cdot}10H_2O$ (Sigma, USA); CAPSO (3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid) (ICN, USA); Triton X-305 (Merck, Germany). Other chemicals were of at least chemical grade. Solutions of $MgCl_2$ were titrated; all stock solutions were freshly prepared using high quality water purified in a MilliQ column. Solid H_3PO_4 was dissolved and the concentration of phosphate was determined using a semi-automatic phosphate analyzer.

ATP-sulfurilase (50 units in 750 μl H_2O), luciferin–luciferase mixture (one standard vial dissolved in 5 ml H_2O), and 1 mM water solution of adenosine-5′-phosphosulfate were divided into portions, frozen, and stored at −12°C. Required portions of each reagent were unfrozen just before use. A portion of a mixture for the determination of pyrophosphate was freshly prepared for each 20 measurements and stored at 4°C for not more than 6 h. The required aliquot (300 μl) of a mixture was incubated for 5 min in a thermostat at 25°C immediately before each measurement.

Recombinant *E. coli* inorganic pyrophosphatase (EC 3.6.1.1) and its mutant variants Gly100Ala and Gly147Val were obtained as described previously [3]. Enzyme suspensions were stored at 4°C in ammonium sulfate and desalted before use with a Sephadex G-50 (fine) column equilibrated with 50 mM Tris-HCl, pH 7.5.

The concentration of pyrophosphatase solutions was determined by UV spectroscopy at 280 nm using the specific absorption $A^{1\%}_{1cm}$ of 11.8 [12].

Measurements of hydrolytic activity. Activities of the wild-type *E. coli* pyrophosphatase and the mutant variants were determined by the rate of P_i release from $MgPP_i$ by quantitative P_i determination using a semi-automatic phosphate analyzer [13]; enzymatic reaction was carried out at 25°C at the sensitivity of 5-10 μM P_i for full-scale.

Mutant variants Gly100Ala and Gly147Val (3-10 mg/ml) were preincubated in 50 mM Tris-HCl, pH 7.5, in the presence of 10 mM Mg^{2+} for 15-20 h at 4°C. Stock solutions were diluted before use to the necessary enzyme concentration. Concentration of Mg^{2+} added to a reaction mixture from the aliquot of enzyme did not exceed 1 μM, so this amount was not included in calculations.

To calculate total concentrations of magnesium and pyrophosphate to be added, required concentrations of free Mg^{2+} and $MgPP_i$ were preset. All the species containing Mg or PP_i were included in the calculations. Dissociation constants of the corresponding species were used [14].

The rate of hydrolysis of $MgPP_i$ as a function of free Mg^{2+} was studied in 50 mM Tris-HCl, pH 9.0, at fixed substrate concentration 40 μM. Mg^{2+} concentration was varied from 10 μM to 30 mM. Experimental data were treated by nonlinear regression using Eq. (1):

$$A = \frac{A_0\,[Mg^{2+}]}{K_A + [Mg^{2+}] + [Mg^{2+}]^2 / K_i}, \qquad (1)$$

where A_0 is maximal potential level of the hydrolytic rate under the assumption of the absence of inhibition; K_A is dissociation constant for the enzyme–Mg^{2+} complex in the activator subsite (usually M2); K_i is the dissociation constant for the enzyme–Mg^{2+} complex in the inhibitor subsite M4.

Determination of pH-independent parameters of $MgPP_i$ hydrolysis. The pH dependence of kinetic parameters was studied at 5 mM free Mg^{2+} in the pH range 5.8-10.5. The total concentrations of the components of the reaction mixture were calculated using pH dependence of the stability constants of $MgPP_i$ and Mg_2PP_i. The buffers (50 mM) used for the necessary pH values were: MES-KOH (5.8-6.8), Mops-NaOH (6.8-7.5), Tris-HCl (7.5-9.0), and CAPSO-HCl (9.0-10.5). PPase activity at any fixed pH did not depend on the nature of the buffer. The pH-independent kinetic parameters were calculated using Eqs. (2) and (3) [15]:

$$k^{app} = k_{cat}/(1 + [H^+]/K_{ESH2} + K_{ESH}/[H^+]), \qquad (2)$$

$$k^{app}/K_m^{app} = (k_{cat}/K_m)/(1 + [H^+]/K_{EH2} + K_{EH}/[H^+]), \qquad (3)$$

where k^{app} and K_m^{app} are values of the catalytic constant (k_{cat}) and Michaelis constant (K_m), respectively, at given pH value; K_{ESH2}, etc. are ionization constants of titrable groups of the enzyme and enzyme–substrate complex.

Determination of the amounts of enzyme-bound pyrophosphate. The equilibrium amount of pyrophosphate bound in the active site of PPase or its mutant variants was determined using a modified version of a procedure described earlier [1]. Reaction mixture of total volume 50 μl containing 50-150 μM enzyme (1-3 mg/ml) in 0.2 M Tris-HCl, pH 8.0, 50 μM EGTA, and 10 mM $MgCl_2$ was incubated for 30 min at 25°C in the presence of magnesium phosphate. The reaction was stopped by addition of 10 μl of trifluoroacetic acid (TFA). After 10 min, the precipitate was separated by centrifugation for 10 min at 10,000 rpm. In the supernatant, the amount of pyrophosphate was measured. The following mixture was prepared (calculated for about 20 measurements): 60 μl 100 mM dithiothreitol (DTT), 100 μl 0.8 mg/ml BSA, 40 μl solution of ATP-sulfurilase, 200 μl luciferin–luciferase mixture, and 60 μl 1 mM adenosine-5′-phosphosulfate were added to 6 ml 0.2 M Tris-HCl, pH 8.0, containing 30 μM EGTA. An aliquot of the supernatant (5 μl) was added to 300 μl of the mixture, and luminescence was immediately measured using a BioOrbit 1250 luminometer (LKB, Finland). For each concentration of MgP_i, a blank experiment was done where the mixture contained all the components except the enzyme.

The concentration of MgP_i in the synthetic reaction mixtures was varied from 0 to 35 mM. Calculated amounts of titrated solutions of $MgCl_2$ and H_3PO_4 were mixed, the pH was brought to 8.0 by addition of 1 M Tris-HCl, and H_2O was added to the required volume. Total concentrations of $MgCl_2$ and H_3PO_4 to set required concentrations of free Mg^{2+} and MgP_i were calculated using the value of $K_d(MgP_i) = 6.36$ mM [16].

To determine the rate of accumulation of enzyme-bound PP_i, a set of synthetic reaction mixtures was made with identical concentrations of all components including MgP_i (30 mM). The reaction in the different samples was stopped in different time intervals (1-10 min).

Amount of enzyme-bound PP_i was normalized to total enzyme concentration, and EPP/E_t was plotted as a function of MgP_i concentration. Experimental data were treated by non-linear regression using Eq. (4) (wild-type PPase) or (5) (mutant PPases):

$$EPP/E_t = 1 / (1 + K' + (K' \cdot K_{P2} / [MgP_i] + K' \cdot K_{P2} \cdot K_{P1} / [MgP_i]^2), \qquad (4)$$

$$EPP/E_t = \frac{n_{max} \cdot [MgP_i]^h}{K_P^h + [MgP_i]^h}, \qquad (5)$$

where K' is the ratio of the rate constants for the hydrolysis and the synthesis of PP_i; K_{P1} and K_{P2} are dissociation constants for two phosphates; n_{max} is maximal level of EPP/E_t; h is the Hill coefficient.

RESULTS AND DISCUSSION

Catalytic properties of the mutant PPases Gly100Ala and Gly147Val. *Binding of activating Mg^{2+} ions at subsites M1 and M2.* For inorganic pyrophosphatase to be catalytically active, its active site must be preliminary saturated with magnesium ions. Mg^{2+} occupies the binding subsites M1 and M2. This results in significant conformational rearrangements of the protein molecule [17]. Dissociation constants of resulting complexes EM and EM_2 are collected in Table 1. In the case of the native enzyme the equilibrium is quickly achieved, i.e., it is suf-

Table 1. Effect of pre-incubation with Mg^{2+} on kinetic parameters of $MgPP_i$ hydrolysis by mutants Gly100Ala and Gly147Val (PPases were assayed in 50 mM Tris-HCl, pH 9.0, 1 mM free Mg^{2+}, at variable concentration of substrate in the range 0-40 μM)

Mutant variant	Conditions of pre-incubation	V_{max}, U/mg	K_m, μM
Gly100Ala	without pre-incubation	11.8 ± 0.7	27 ± 3
	2 h, 5 mM Mg^{2+}	28.8 ± 0.5	1.4 ± 0.2
	15-20 h, 10 mM Mg^{2+}	66 ± 1	1.3 ± 0.2
Gly147Val	without pre-incubation	18 ± 4	34 ± 15
	2 h, 5 mM Mg^{2+}	49.1 ± 0.6	1.8 ± 0.1
	15-20 h, 10 mM Mg^{2+}	305 ± 5	2.1 ± 0.2

ficient to add Mg^{2+} into the reaction mixture together with substrate and enzyme. The further binding of the substrate (magnesium pyrophosphate, $MgPP_i$) is also a quick process. Therefore, the measurements of enzymatic activity of the native PPase do not require its pre-incubation with Mg^{2+}.

Pyrophosphatase activity of the mutant variants Gly100Ala and Gly147Val, being determined in the same way (pH 9.0, 1 mM Mg^{2+}, substrate $MgPP_i$ concentration 40 μM), turned out to be very low (Fig. 2) and was of about 10 and 20 U/mg, respectively. For comparison, the native PPase under the same conditions has an activity of about 600 U/mg. The values of K_m for both variants were very high (Table 1). At the same time, pre-incubation of Gly100Ala and Gly147Val PPases with Mg^{2+} notably improved their catalytic properties, the extent of these changes being dependent on the time of pre-incubation as well as on the concentration of Mg^{2+}. The rise in maximal rate of hydrolysis and the decrease in K_m occurred after incubation of these enzymes with 5 mM Mg^{2+} for 2 h, but the best results were observed after incubation with 10 mM Mg^{2+} for 15-20 h (Fig. 2, Table 1). Further incubation of Gly100Ala and Gly147Val PPases with Mg^{2+} did not lead to any notable changes. Therefore, further kinetic measurements were carried out after pre-incubation of these mutant PPases with 10 mM Mg^{2+} for 15-20 h at 4°C. Pre-incubated enzymes retained their catalytic activity for two weeks. The maximal rate of hydrolysis and K_m of both mutants, however, did not reach the values typical for the native enzyme even after their pre-incubation.

This anomalous behavior of the mutant enzymes might be caused by a decrease in affinity of M1 and M2 subsites for Mg^{2+}. A great variety of cases are described in the literature when flexible loops around binding sites are necessary just for providing the desired affinity of enzymes for ligands (see, for example, [18, 19]). Dissociation constants K_d(M2) of enzyme–Mg^{2+} complex in the absence of substrate determined for the native PPase by UV spectrophotometry [15] could not be measured for Gly100Ala and Gly147Val variants because of a very slow attainment of the equilibrium after adding each new portion of Mg^{2+}. Dissociation constants of enzyme–Mg^{2+} complexes at the sites M1 and M2 for these variants were evaluated in the presence of substrate. For this purpose, hydrolytic activity was studied as a function of Mg^{2+} concentration at a fixed concentration of $MgPP_i$. An aliquot of enzyme pre-incubated with 10 mM Mg^{2+} was added to reaction mixture containing the desired concentration of Mg^{2+}. As a result, the enzyme solution was diluted at least 1 : 10,000, so only a negligible amount of Mg^{2+} was in excess in the mixture. The values of K_d(M1) and K_d(M2) determined in this way did not increase as compared with the native PPase (Table 2). Quite the contrary, a certain decrease in these parameters was observed indicating an apparent rise in affinity for Mg^{2+}. This fact may be explained by the slow dissociation of Mg^{2+} after dilution of enzymes, due to which the real concentration of activator may probably slightly exceed the calculated concentration.

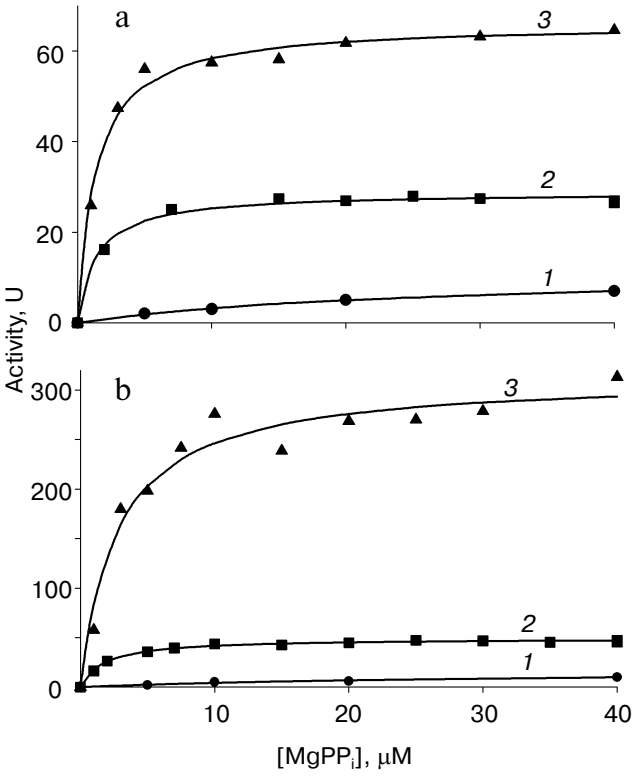

Fig. 2. Hydrolytic activity of mutant Gly100Ala (a) and Gly147Val (b) as a function of substrate concentration. Profiles were measured in 0.05 M Tris-HCl, pH 7.5, 10 mM Mg^{2+} without pre-incubation of enzymes with Mg^{2+} (*1*), or after 2 h pre-incubation with 5 mM Mg^{2+} (*2*), or after 15-20 h pre-incubation with 10 mM Mg^{2+} (*3*).

Table 2. Dissociation constants of enzyme–Mg^{2+} complexes in activator and inhibitor subsites for the mutant PPases Gly100Ala and Gly147Val (PPases were assayed in 50 mM Tris-HCl, pH 9.0, at 40 μM $MgPP_i$)

PPase	V_0, U/mg	K_a, μM	K_i, mM
WT [23]	594 ± 15	200 ± 40	16 ± 2
Gly100Ala	57.7 ± 2.6	23.2 ± 5.4	43.5 ± 1.34
Gly147Val	196.9 ± 5.2	94 ± 11	126 ± 37

Therefore, the studied mutant PPases are capable of binding Mg^{2+} at sites M1 and M2 with affinities comparable to that for the native enzyme, but longer time is required for them. Perhaps the conformational rearrangements accompanying Mg^{2+} binding are significantly slower in the case of Gly100Ala and Gly147Val variants. None of some twenty earlier studied mutants of *E. coli* PPase behaved in such a way. This unique feature of mutant enzymes with substituted glycine residues suggests that the rate of structural rearrangements accompanying activator Mg^{2+} ions binding is determined by the flexibility of the loops containing Gly100 and Gly147.

Non-Michaelis kinetics of $MgPP_i$ hydrolysis. Hydrolysis of $MgPP_i$ by the mutant PPases Gly100Ala and Gly147Val in a wide range of substrate concentration does not follow the classic Michaelis scheme. For both variants, dependence {[S]; *v*} plotted in double-reciprocal coordinates can be divided into two linear segments (Fig. 3, inset). This feature has been earlier found for the trimeric form of the native PPase [20], and for the trimeric or hexameric forms of several mutants [21]. Such a deviation from Michaelis kinetics has been explained as the incompetitive activation of PPase by metal-free pyrophosphate anions [21]:

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

where E is PPase subunit; K_E is the dissociation constant for the enzyme–effector (PP_i) complex; K_S is the dissociation constant for the enzyme–substrate ($MgPP_i$) complex; k is rate constant for the catalytic step; β is the activation coefficient; superscripts A or E denote ligand bound at the active or effector site, respectively.

According to this model, metal-free PP_i is bound at the allosteric site of each subunit and causes the activation of $MgPP_i$ hydrolysis at the active site. As a result, dependence of the hydrolytic rate on the substrate concentration has two stages. When the concentration of $MgPP_i$ is low, Michaelis kinetics are observed because the level of metal-free PP_i is negligibly low under those conditions. Further increasing concentration of $MgPP_i$ is accompanied by a rise in the concentration of metal-free PP_i, resulting in progress of filling the allosteric site and the approximately twofold activation of hydrolysis. Hexameric form of the native PPase is characterized by an extremely high affinity for the activator, so it reveals non-Michaelis kinetics only under the conditions of low level of metal-free PP_i in the reaction mixture (e.g., at acid pH, or at high levels of free Mg^{2+}). The location of the allosteric site in the PPase molecule is still unknown, the study of non-Michaelis kinetics of the mutant PPases being one of the possible approaches to solve this problem.

For the hexameric PPases Gly100Ala and Gly147Val, kinetics of $MgPP_i$ hydrolysis deviates from the Michaelis scheme at pH 7.5, though the wild-type hexamer under these conditions still shows Michaelis behavior. The {[S]; *v*} dependences obtained for Gly100Ala and Gly147Val variants have been treated according to the scheme above. One could hardly correctly compare parameters of activation (Table 3) to the analogous parameters of the wild-type hexamer since they were determined at different pH, while affinity for the effector is greatly affected by pH. Nevertheless, the presented data allows conclusion to be made that the affinity for the effector (K_E) was not influenced much by the mutation Gly147Val. At the same time, for the mutant Gly100Ala significant impairment of the effector site is observed, as judged by an increase in K_E. Dissociation constants of enzyme–substrate complexes (K_S, analog of K_m in the Michaelis scheme) for both mutant variants have the same order of magnitude as

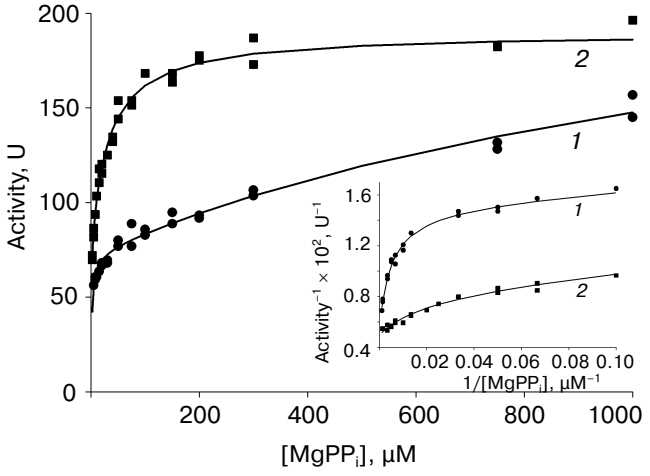

Fig. 3. $MgPP_i$ hydrolysis by mutant PPases Gly100Ala (*1*) and Gly147Val (*2*) over a wide range of substrate concentrations. Inset: the same data plotted in double-reciprocal coordinates. PPase activity was assayed in 0.05 M Tris-HCl, pH 7.5, 10 mM Mg^{2+}, after 15-20 h pre-incubation with 10 mM Mg^{2+}.

Table 3. Kinetic parameters of $MgPP_i$ hydrolysis by mutants Gly100Ala and Gly147Val calculated within the above-mentioned scheme [21] (pH 7.5, 5 mM free Mg^{2+})

PPase	V_{max}, U/mg	K_S, μM	K_E, μM	β
WT*	48 ± 5	12 ± 2	0.44 ± 0.38	2.00 ± 0.05
Gly100Ala	65.43 ± 1.27	0.92 ± 0.14	5.24 ± 1.02	2.75 ± 0.17
Gly147Val	83.59 ± 21.21	0.71 ± 0.81	0.32 ± 0.12	2.27 ± 0.55

* Data for the hexameric WT PPase obtained at pH 6.0 and 10 mM Mg^{2+} [21].

expected for the wild-type hexamer at pH 7.5. Therefore, affinity for the substrate of the active sites of Gly100Ala and Gly147Val was not greatly impaired. The Gly100Ala variant is also characterized by an increase in activation coefficient β, whereas for Gly147Val it is not the case. We can thus summarize that the mutation of Gly100 results in significant destruction of the effector region, while the mutation of Gly147 affects the activation of PPase by a metal-free PP_i to a lesser degree.

Binding of inhibitory magnesium ion at site M4. The wild-type *E. coli* pyrophosphatase is inhibited at high concentrations of free Mg^{2+}. We have earlier demonstrated that this inhibition is caused by an additional Mg^{2+} ion bound at subsite M4 within the active site [15, 22, 23]. According to the X-ray data, this metal ion is liganded with the both phosphate groups of PP_i, or with both product phosphates after hydrolysis [10, 24]. Thus, the hampered release of products is the most reasonable explanation of the inhibiting action of this Mg^{2+}.

The dissociation constant of the enzyme–inhibitor Mg^{2+} complex, K_i, can be determined from the dependence of the hydrolytic rate on the concentration of free Mg^{2+} in the reaction mixture. Inhibition is well pronounced for the wild-type PPase, K_i being about 16 mM (Table 2) [15]. The mutant variants Gly100Ala and Gly147Val are also inhibited at Mg^{2+} concentrations higher than 1 mM but the activity of both variants decreased only slightly. The value of K_i for Gly100Ala is three times higher compared with the wild-type PPase, whereas for the Gly147Val variant K_i can only be roughly estimated. This fact might be regarded as a decrease in affinity for the inhibitor Mg^{2+} ion as a result of the mutations. On the other hand, the slow saturation of the M4 subsite, analogous to M1 and M2 subsites, cannot be ruled out. In this case, the decrease in flexibility of loops II and III resulting from mutations of Gly residues might lead to the release of phosphates after hydrolysis occurs before binding the inhibitory Mg^{2+}.

It should be noted that lack of inhibition has also been observed for the trimeric form of the wild-type PPase, as well as for the hexameric forms of some mutants. Moreover, a definite correlation exists between the lack of inhibition at high concentrations of Mg^{2+} and impairment of the effector properties, as judged from the non-Michaelis kinetics of hydrolysis. The reasons for this observation are not still clear, but this feature is now extended to the mutant PPases Gly100Ala and Gly147Val. Thus, the lack of inhibition by Mg^{2+} for these variants may not be directly related to the inserted mutations.

pH-independent kinetic parameters. To reveal the role of mutated loops at the stages of substrate binding and the product release, it is necessary to estimate the possible alterations of the acid–base properties of the catalytically important groups of the enzyme as a result of mutations Gly100Ala and Gly147Val, and finally to determine pH-independent hydrolytic constants k_{cat} and k_{cat}/K_m. For this purpose, pH-dependence of the maximal rate of hydrolysis and K_m for Gly100Ala and Gly147Val at 5 mM free Mg^{2+} has been studied. The pH profiles of k_{cat} and k_{cat}/K_m are, as well as for the wild-type PPase, bell-shaped with virtually the same location of maxima (Fig. 4). Ionization constants of titrable groups for both the free enzyme (pK_{EH_2} and pK_{EH}) and the enzyme–substrate complex (pK_{ESH_2} and pK_{ESH}) have been determined for the mutant PPases. The calculated values (Table 4) do still differ for the wild-type and the mutant enzymes. The most influenced parameter is pK_{ESH} for Gly147Val. The possible reasons for the observed differences may be both the shift in the location of titrable groups resulting in the alterations of H-bonds length or angles, and the change in microenvironment of these groups. Thus, the found differences demonstrate the violation of the fine architecture of the active site as a result of the mutations.

The main result of the substitution of Gly residues is a decrease in the rate constants for $MgPP_i$ hydrolysis. The value of k_{cat} is about 4% for Gly100Ala and about 25% for Gly147Val compared to the wild-type PPase. Catalytic efficiency, k_{cat}/K_m, is decreased two orders of magnitude for both mutants. The pH-independent K_m value for Gly147Val PPase increased tenfold, whereas for Gly100Ala this parameter is only slightly affected, so the inactivation of PPase as a result of the mutations can only partly be attributed to an impairment of affinity for sub-

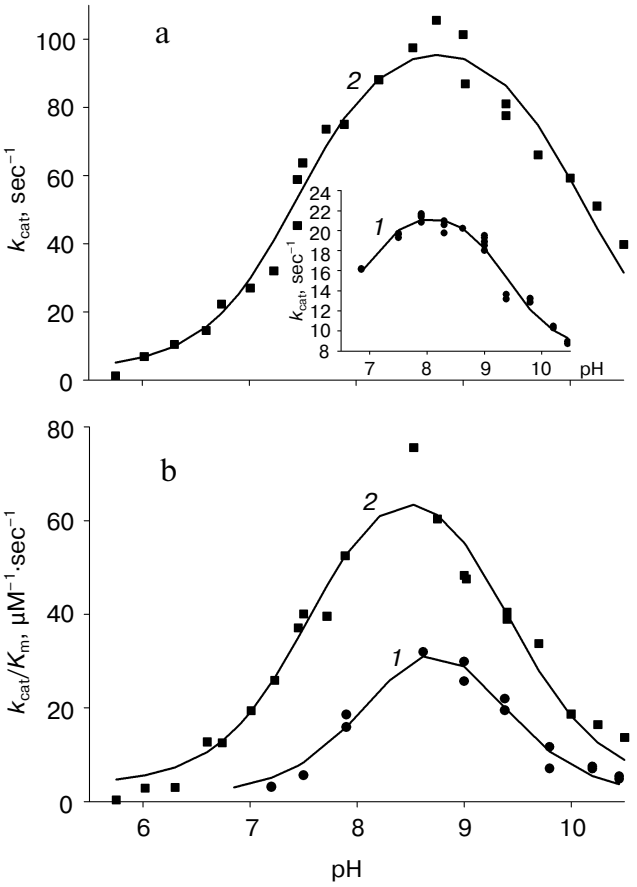

Fig. 4. The pH profiles of k_{cat} (a) and k_{cat}/K_m (b) for the mutant PPases Gly100Ala (*1*) and Gly147Val (*2*).

strate. Therefore, a decrease in the rate characteristics of catalysis, k_{cat} and k_{cat}/K_m, for these mutant PPases indicates that the flexibility of loops II and III is necessary at the stages of substrate binding and product release.

Synthesis of pyrophosphate in the active site of PPase. Aside from the hydrolysis of pyrophosphate, in the presence of Mg^{2+} PPase also catalyzes the reverse reaction of formation of inorganic pyrophosphate (PP_i) from two phosphates (P_i). In the active site of the enzyme, this process occurs with notable velocity (rate constant k_{synth} is 140 sec^{-1} at 20 mM Mg^{2+} [25]) and competes with the dissociation of phosphates. Synthesized PP_i however is held in the active site much stronger than phosphates, so its release into solution (rate constant k = 20 sec^{-1}) is much slower than the release of phosphates (k is about 400 sec^{-1} [25]). As a result, virtually all of the synthesized PP_i remains bound in the active site of the PPase. When the concentration of P_i in the medium is increased, the equilibrium amount of PP_i bound with the enzyme rises as well and approaches the maximal level dependent on the ratio of velocities of PP_i synthesis and P_i dissociation.

Figure 5 shows the amount of synthesized and bound with PPase pyrophosphate as a function of concentration of magnesium phosphate, MgP_i, in a mixture, for the wild-type PPase and the two mutants Gly100Ala and Gly147Val, obtained after the equilibrium P_i/PP_i was reached in the active sites of the enzymes. It can be seen from the presented data that at the concentration of MgP_i < 20 mM the equilibrium amount of synthesized PP_i in the active sites of the mutant variants is comparable with the analogous value for the wild-type PPase. At higher concentrations of phosphate, significant difference is found. The maximal level of equilibrium concentration of synthesized PP_i is twofold higher, for Gly100Ala, or fivefold higher, for Gly147Val, than for the native enzyme. This difference shows that the studied variants are characterized by higher ratio of velocities of PP_i synthesis and P_i dissociation than the wild-type enzyme. Since the release of phosphates after hydrolysis is known to be accompanied by large structural rearrange-

Table 4. The pH-independent parameters of $MgPP_i$ hydrolysis by mutants Gly100Ala and Gly147Val (PPases were assayed at 5 mM Mg^{2+})

Parameter	WT [15]	Gly100Ala	Gly147Val
k_{cat}, sec^{-1}	389 ± 78	14.3 ± 0.6	101 ± 5
K_m, μM	0.13 ± 0.06	0.27 ± 0.07	1.4 ± 0.2
k_{cat}/K_m, μM^{-1} · sec^{-1}	3038 ± 800	54 ± 10	75 ± 6
pK_{EH2}	7.75 ± 0.50	8.31 ± 0.20	7.59 ± 0.12
pK_{EH}	8.67 ± 0.51	9.13 ± 0.20	9.38 ± 0.13
pK_{ESH2}	7.56 ± 0.25	6.78 ± 0.10	7.45 ± 0.08
pK_{ESH}	8.96 ± 0.21	9.38 ± 0.08	10.09 ± 0.09

Table 5. Synthesis of PP_i by wild-type PPase and mutants Gly100Ala and Gly147Val (pH 8.0, 10 mM Mg^{2+}; parameters are calculated by Eqs. (4) (WT PPase) or (5) (mutant PPases))

Parameter	WT	Gly100Ala	Gly147Val
K', mM	0.10 ± 0.01	—	—
K_{P1}, mM	2.66 ± 0.16	—	—
K_{P2}, mM	>10	—	—
n_{max}, mol/mol E	—	0.7 ± 0.1	1.5 ± 0.6
h	—	6 ± 2	5 ± 1
K_P, mM	—	25 ± 2	30 ± 5

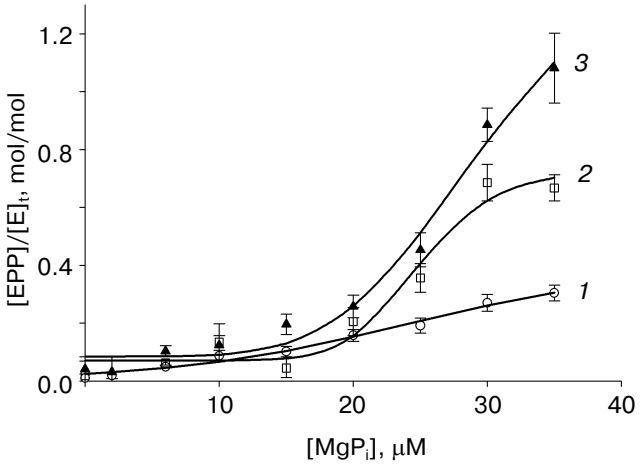

Fig. 5. Equilibrium amount of enzyme-bound PP_i as a function of MgP_i concentration for the wild-type (*1*) and the mutant PPases Gly100Ala (*2*) and Gly147Val (*3*).

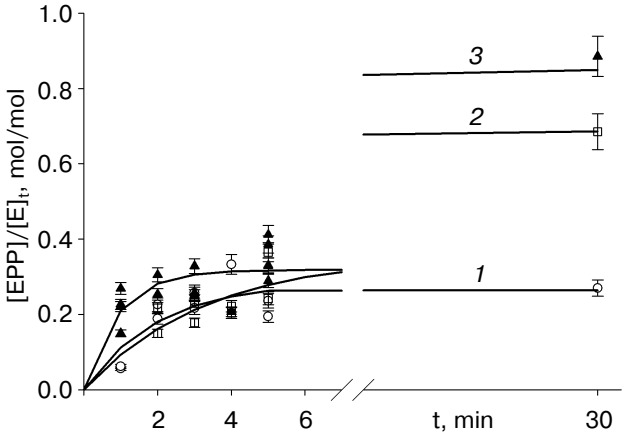

Fig. 6. Accumulation of enzyme-bound PP_i as a function of incubation time for the wild-type PPase (*1*) and mutants Gly100Ala (*2*) and Gly147Val (*3*).

ments [25], the key reason for the observed increase in the maximal level of PP_i is, most probably, the slowed release of phosphates as a result of the mutation of the Gly residues.

The synthesis of PP_i is possible only after both phosphates are bound in the active site, so the hyperbolic dependence obtained for the wild-type PPase allows estimation of the dissociation constant for the low-affinity phosphate. For the mutant PPases Gly100Ala and Gly147Val, this dependence is clearly sigmoidal, indicating cooperative binding of two phosphates with similar K_d values. These profiles were treated using the Hill model (Table 5). Calculated values of K_P are not dissociation constants of two phosphates but rather the mean value between those constants, so they still characterize the mean affinity of two phosphates for the enzyme. It can be seen that there is no significant difference in K_P for the wild-type and the mutant PPases, though mutation of Gly147 still slightly affects this parameter.

Figure 6 shows the accumulation of synthesized pyrophosphate in the course of incubation at a fixed concentration of MgP_i in the solution. For the mutant variant Gly100Ala, the rate of accumulation is comparable with the wild-type PPase; in the case of Gly147Val the rate is some higher. Since the study of synthetic properties of Gly100Ala and Gly147Val has been carried out after pre-incubation of enzymes with free Mg^{2+}, these results can be regarded as indirect evidence for the following consideration. After pre-incubation these enzymes adopt the competent conformation for binding P_i and the synthesis of PP_i, and these two steps themselves do not require the high flexibility of mutated loops II and III.

This work was carried out under financial assistance of the Russian Foundation for Basic Research (grant Nos. 03-04-48866 and NSh. 1706-2003-4).

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