Interaction of *Escherichia coli* inorganic pyrophosphatase active sites

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Abstract ***Escherichia coli*** **inorganic pyrophosphatase (PPase) is a hexamer of identical subunits. This work shows that trimeric form of PPase exhibits the interaction of the active sites in catalysis. Some trimer subunits demonstrate high substrate binding affinity typical for hexamer whereas the rest of subunits reveal more than 300-fold substrate affinity decrease. This fact indicates the appearance of negative cooperativity of trimer subunits upon substrate binding. Association of the wild-type (WT) trimer with catalytically inactive, but still substrate binding mutant trimer into hexameric chimera restores the high activity of the first trimer, characteristic of trimer incorporated in the hexamer of WT PPase. Interaction of PPase active sites suggests that there are pathways for information transmission between the active sites, providing the perfect organization and concerted functioning of the hexameric active sites in catalysis.**

Key words: Inorganic pyrophosphatase; Hexameric form; Trimeric form; Kinetics; Cooperativity

1. Introduction

Inorganic pyrophosphatase (EC 3.6.1.1.; PPase) catalyzes the hydrolysis of inorganic pyrophosphate (PP_i) to two molecules of orthophosphate (P_i), shifting towards synthesis the equilibrium of many biosynthetic processes with PP_i as by-product. All known cytoplasmic PPases are oligomeric proteins. *Escherichia coli* PPase is a hexamer of chemically identical subunits. The hexamer is arranged as a dimer of two trimers [1]. Each subunit in the PPase molecule interacts with four neighboring subunits, i.e. it has two intratrimeric and two intertrimeric contacts. The intertrimeric contacts in the enzyme molecule are rather weak and can be destroyed in mild acidic medium. On the other hand, the subunit contacts in the trimer are very strong. *E. coli* PPase spatial structure is united into a system due to the contacts between subunits as well as mobile network of non-covalent bonds in each subunit. Catalysis exhibits an absolute requirement for divalent metal ions, with Mg^{2+} conferring the highest activity. Each active center has four metal binding subsites. Two of them have different affinities for Mg^{2+} and are filled up before the substrate binding. The third subsite accepts metal ion simultaneously with the substrate whereas the fourth Mg^{2+} is detected after PP_i hydrolysis.

To date, much experimental material has been amassed concerning the interaction between the subunits in PPase hexamer while binding the ligands in the active site. In particular, it was found that methylphosphate, the affinity inhibitor of the enzyme, reacts in such a way that the phenomenon of 'half site reactivity' appears [3]. The complex of PPase with sulfate, which is an analog of both P_i and methylphosphate, is shown to be a dimer composed of two conformationally different trimers [4]. Binding of Mg^{2+} to the active site also induces the asymmetry of subunits in both trimers [5]. However, the possibilities of the active site interactions initiated by the substrate binding and its implication for catalysis are questions that remain to be answered.

This work describes the comparison of the catalytic properties for various *E. coli* PPase variants, namely the trimer, hexamer and the chimeric hexamer in which active trimer associated with inactive mutant trimer. Our results showed the cooperativity of the subunits in the trimer and the necessity of a concordant functioning of the hexamer active sites for providing the most efficient catalysis.

2. Materials and methods

Wild-type *E. coli* PPase (WT PPase) and its three mutant variants with substitutions in the active site (D70N, D97N, K104Q) were obtained and courteously supplied by our colleagues [6,7]. The enzymes were stored as suspension in ammonium sulfate solution and desalted directly before use, on a column with Sephadex G-50 (fine) equilibrated by 50 mM HEPES/NaOH, pH 7.5.

The trimers of WT and mutant PPases were prepared by the enzyme incubation in 25 mM MES/NaOH, pH 5.3, as described earlier [8]. The trimeric WT PPase was obtained also by incubation of PPase (0.1–0.5 μM) with 30 mM *O*-phosphoethanolamine (Sigma, USA) in 50 mM HEPES/NaOH, pH 7.2, for 20 min at 25°C.

The chimeras were prepared by mixing trimeric WT and mutant PPases in 25 mM MES/NaOH, pH 5.3, in the ratio 1:10 (on a masses). Then, pH of the solution was brought to 7.2 by slow addition of 0.4 M Tris–HCl, pH 7.5, and the mixture was incubated for several hours. Final concentrations of WT PPase and the mutant trimers were 0.15–1.5 μM and 1.5–15 μM, respectively.

The enzyme in solution was quantified spectrophotometrically using $A_{280}^{0.1\%} = 1.18$ [9]. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μM PP_i per minute. For chimeras and trimer obtained by the treatment of WT PPase with phosphoethanolamine, the calculation of specific activity takes into account only the active part of PPase, i.e. it was half from the spectrophotometrically detected amount of protein.

Gel chromatography was performed on a column (44×2 cm) with Toyopearl HW-55 (Toyosoda mFG, Japan) equilibrated with 50 mM HEPES/NaOH, pH 7.2, containing 0.15 mM NaCl. *E. coli* PPase (M_r 120 000) and *Saccharomyces cerevisiae* PPase (M_r 60 000) were used as markers.

The initial rates of PP_i hydrolysis were estimated from continuous recording of P_i liberation using an automatic phosphate analyzer [10]. The reaction mixture of 5 ml total volume contained 1–2000 μM $MgPP_i$, 0.1–20 mM Mg^{2+} and Tris–HCl, pH 7.2. The total concentrations of $MgCl_2$ and sodium pyrophosphate for maintaining the desired levels of free Mg^{2+} and $MgPP_i$ were calculated using dissoci-

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ation constants for magnesium pyrophosphate and dimagnesium pyrophosphate equal to 0.0026 mM and 2.01 mM, respectively [7]. The enzyme concentration in kinetic studies was 0.1–0.5 nM.

The kinetic parameters of PP_i hydrolysis by various forms of the enzyme giving best fits of the data to Eqs. 1 and 2 were determined using the method of non-linear regression.

3. Results

3.1. Interaction between WT PPase and O-phosphoethanolamine

O-phosphoethanolamine is affinity inhibitor of *E. coli* PPase [3]. In the present study, it was shown that modification of half of the PPase subunits by this reagent leading to 50% inactivation of the enzyme is accompanied by dissociation of the hexameric WT PPase into trimers. This conclusion is based on gel chromatography analysis of the reaction mixture demonstrating a 2-fold decrease of protein molecular mass (Fig. 1).

Thus, modification of WT PPase by *O*-phosphoethanolamine entails a full shift of hexamer ↔ trimer equilibrium towards the latter and as a result, PPase is a mixture of the equal parts of catalytically active and inactivated trimers. This fact was used in our work on obtaining the trimeric PPase as the alternative to the earlier described method based on the incubation of WT PPase at low pH [8].

3.2. Kinetics of PP_i hydrolysis by trimer

The initial rates of substrate hydrolysis by trimeric PPase were measured at the concentrations: 1–2000 μM for $MgPP_i$ and 0.1–8 mM for Mg^{2+} (Fig. 2A). Trimers derived by two different procedures were used in the experiments. For comparison, Fig. 2B presents the kinetic curves obtained in this research for hexameric PPase. The dependencies for trimer principally differed from those for hexamer. The trimer catalytic reaction had a two step pattern and could not be described in terms of the Michaelis–Menten kinetics. The first step was observed at substrate concentrations < 200 μM and at any Mg^{2+} concentration. In these cases, about half of the limited activities were reached. The second step occurs at notably higher substrate concentrations.

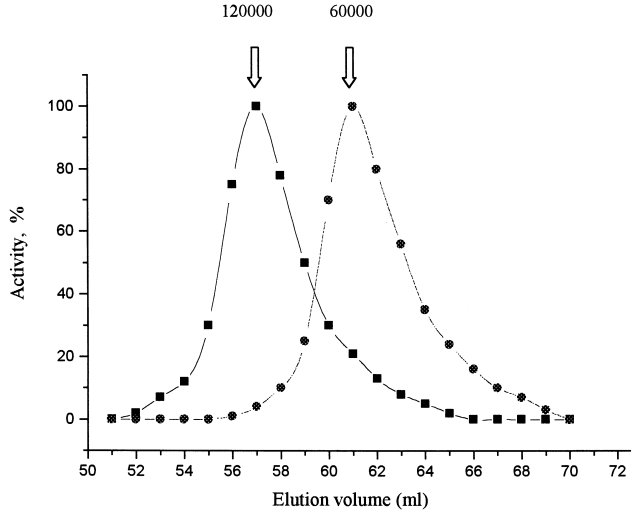


Fig. 1. The profiles of WT PPase elution before (■) and after (●) modification by phosphoethanolamine. Arrows point to the sites of markers release and their M_r.

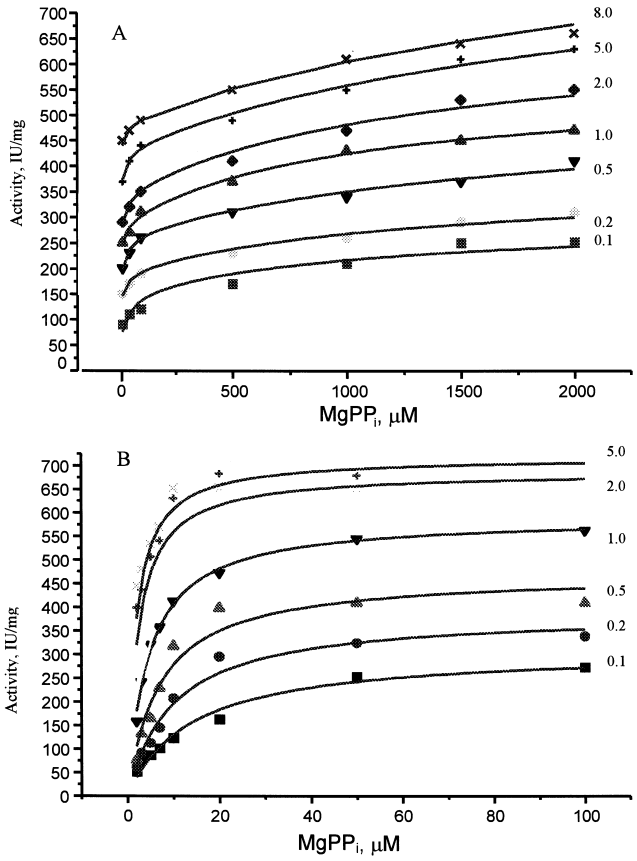
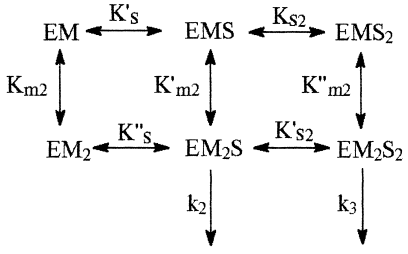


Fig. 2. The activities of trimeric (A) and hexameric (B) PPase as functions of concentrations of substrate ($MgPP_i$ complex) and magnesium ions. The Mg^{2+} concentrations (mM) are shown at relevant curves.

The results obtained cannot be explained by presence of the hexameric contamination since the substrate hydrolysis kinetics do not differ for trimers, obtained by various procedures. It was noted above that after reaction with *O*-phosphoethanolamine, WT PPase presents in the trimeric form only. No conversion of the trimer to hexamer was observed during assay as evidenced by nearly linear product formation curves. The linearity also persisted up to a 10-fold increase at the used enzyme concentration. Hence, the observed kinetic peculiarities of the substrate hydrolysis are an inherent feature of the trimeric PPase form. The necessity of high substrate concentrations for reaching the maximal activity makes the



where E = enzyme, M = Mg^{2+}, S = $MgPP_i$

Scheme 1. Minimal scheme for trimeric PPase catalysis.

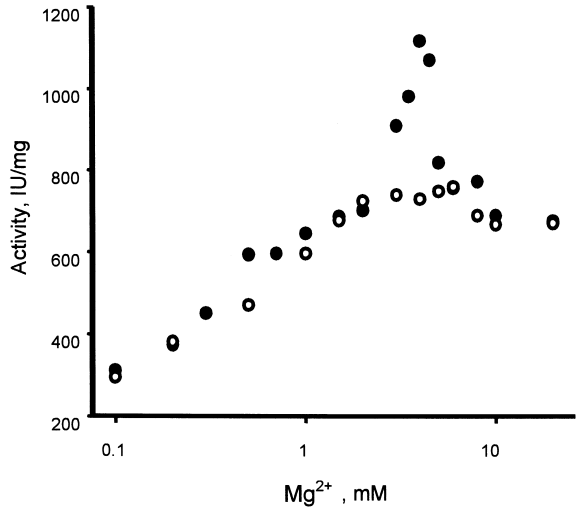


Fig. 3. The activities of WT/K104Q (●) and WT PPases (○) as functions of Mg^{2+} concentration in the conditions of saturation with substrate.

supposition about appearance of subunits asymmetry logical and greatly decreased affinity for the substrate in some subunits. For this reason, the kinetic scheme of PP_i hydrolysis by trimer involves the attachment of the second substrate molecule to the enzyme with another dissociation constant (Scheme 1).

To calculate kinetic parameters, the following assumptions: $K_{s2} = K'_{s2}$; $K'_s = K''_s$; $K_{m2} = K'_{m2} = K''_{m2}$ were used.

$$v/[E_0] = \frac{k_2[M][S_0](1 + \alpha[S_0]/K_{s2})}{(K_{m2} + [M])(K'_s + [S_0] + [S_0]/K_{s2})} \quad (1)$$

where $\alpha = k_3/k_2$

The lines are drawn on Fig. 2A according to Eq. 1 with the parameter values outlined in Table 1.

The obtained results show that the trimeric PPase contains at least two centers of substrate binding differing in affinity more than 300-fold (Table 1). The admitted equivalence of all subunits in the starting trimer leads to the inference about a negative cooperativity of active sites in the trimer under substrate binding. Yet, the rate of substrate hydrolysis at the first step is twice lower than at the second, i.e. a positive cooperativity of active sites is observed in the trimer at the step of substrate conversion.

Thus, the kinetic study of substrate hydrolysis by the trimeric PPase disclosed interaction of the trimer active sites and the development of subunit asymmetry in the catalytic process.

Comparison of properties of the trimer, obtained in this work from WT PPase with the trimers obtained earlier [11,12], shows that lower affinity for substrate is their common property (Table 1). However, the cooperativity of the trimer subunits in the catalytic reaction has not been discussed earlier. It is to be noted that all previously known trimers contained 1–2 mutations, which could probably determine their properties.

3.3. Preparation of chimeras (WT/D70N, WT/D97N, WT/K104Q PPases)

Chimeras, the hexameric variants of PPase, were composed from WT PPase trimer and the trimer of some mutant PPases. For chimeras, preparation D70N, D97N and K104Q PPases were chosen. All the mutants had very low catalytic activity ($k_{cat} < 1\%$), but retained the capacity to bind the substrate and magnesium ions [7]. Solutions of WT PPase and mutant variants in equal concentrations were incubated at pH 5.3 to convert them into the trimeric state. Then, WT and mutant PPase trimers were mixed in a 1:10 ratio and incubated at pH 7.2. In these conditions, hexameric WT PPase formation is excluded due to a very low concentration of WT trimer and large excess of the mutant trimer. The presence of hexamer mutant protein did not affect the result of kinetic experiments for their very low enzyme activity.

3.4. Kinetics of PP_i hydrolysis by chimeras

The initial rates of PPi hydrolysis by chimeras were measured over the wide ranges of substrate and Mg^{2+} concentrations. The dependences of the hydrolysis rate on substrate concentration for all studied chimeras had the same pattern as for WT hexamer (not shown). It is important that in all instances, the maximal activity was reached at a low $MgPP_i$ concentration (< 100 μM). For calculation of the catalytic and equilibrium constants, Scheme 2 and corresponding Eq. 2 were used. They comprise a subsequent binding of two metal ions and formation and hydrolysis of two enzyme-substrate complexes containing one or two metal ions (in addition

Table 1
Kinetic parameters of PP_i hydrolysis by different forms of PPase (pH 7.2, 25°C)

	Trimeric PPase	Hexameric WT PPase	Hexameric chimeras PPase			Trimeric H136Q PPase [12]	Trimeric H140Q PPase [12]	Trimeric H136, 140Q PPase [11]
			WT/D70N[a]	WT/D97N[a]	WT/K104Q[a]			
k_1 (s^{-1})	106 ± 7	45 ± 5	23 ± 3	59 ± 6	66 ± 12			< 40
k_2 (s^{-1})		212 ± 20	210 ± 22	207 ± 23	220 ± 40	178 ± 10	300 ± 20	227 ± 12
K_s (μM)		46 ± 3	30 ± 3	110 ± 12	35 ± 5			
K'_s (μM)	3.6 ± 0.3	5.5 ± 0.5	6.9 ± 0.9	14.3 ± 2.2	7.5 ± 2.8			
K''_s (μM)		0.7 ± 0.2	0.8 ± 0.3	1.4 ± 0.4	1.1 ± 0.5	198 ± 6	4200 ± 300	> 1000
K_{m1} (μM)		160 ± 30	266 ± 52	183 ± 42	95 ± 36			
K'_{m2} (μM)		20 ± 2	61 ± 15	23 ± 4	19 ± 9			
K_{m2} (mM)	0.31 ± 0.02	2.4 ± 0.3	2.5 ± 0.3	3.7 ± 0.5	2.1 ± 0.5	77 ± 18	10 ± 4	8 ± 2
K'_{m2} (mM)		0.29 ± 0.04	0.30 ± 0.05	0.4 ± 0.05	0.31 ± 0.15			0.85 ± 0.09
K_{s2} (μM)	985 ± 70							
k_3 (s^{-1})	210 ± 15							

[a]Hexameric chimeras PPase are composed from trimers of WT and mutant PPase (D70N, D97N or K104Q PPase).

to substrate metal).

$$v/[E_0] = \frac{\dfrac{k_1[M]K'_{m1}(1+\gamma[M]/K'_{m2})}{1+[M]/K'_{m1}(1+[M]/K_{m2})}}{[S_0]+K_s\dfrac{1+[M]/K_{m1}(1+[M]/K_{m2})}{1+[M]/K'_{m1}(1+[M]/K'_{m2})}} \quad (2)$$

where $k_2 = \gamma k_1$; $K'_{s1} = K_s K'_{m1}/K_{m1}$; $K''_s = K'_s K'_{m2}/K_{m2}$

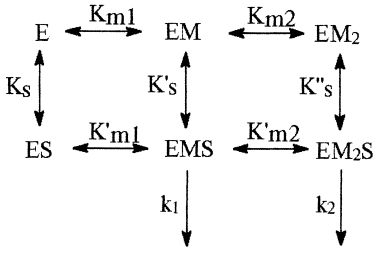
The kinetic parameters for chimeras are summarized in Table 1.

The results demonstrate that the catalytic constants, affinity for the substrate and metal ions are practically similar for WT and chimeric PPases. Thus, the association of the WT trimer with a devoid of activity mutant trimer leads to restoration of activity of the former. As a result, the properties of the trimers incorporated either into WT hexamer or into chimeric PPases become identical.

Some mutation effect on the catalytic properties of chimeric proteins (Table 1) should be noticed and it is best seen in chimera WT/K104 (Fig. 3). In this protein, unlike WT PPase and other chimeras, the bell-shaped dependence of the maximal rate on Mg^{2+} concentration in the range 3–5 mM was observed. In these concentrations, the last, fourth, center of metal binding in the active site is filled up [2]. The pattern of the rate dependence on Mg^{2+} concentration shows two binding centers in the chimeric protein. The filling of one of them activates the hydrolysis whereas the filling of the other one inhibits the process. Since this effect is absent in the trimer, the described phenomenon is presumably a reflection of the interaction of two metal binding centers present in different trimers. This is one more demonstration of the subunit interactions in PPase hexamer.

4. Discussion

This work found out that the catalytic properties of the trimeric and hexameric forms of *E. coli* PPase are quite different. The trimer typical property is the negative cooperativity of the active sites upon binding of the substrate. It reveals in fact that the K_m value for substrate binding is 3.6 μM for some subunits and 960 μM for other subunits (Table 1, Fig. 2). Requirement of high substrate concentrations for reaching the maximal activity results in a low catalytic efficiency of not in contact trimer (k_{cat}/K_m). The association of this trimer with another trimer, obtained from catalytically, inactive, but still substrate, binding mutant PPase, into hexameric chimera restores the high activity of the first trimer, characteristic of trimer incorporated in hexamer (Table 1). The fact of trimer active site interactions in catalysis reveals earlier unknown features of *E. coli* PPase functioning.



where E = enzyme; M = Mg^{2+}; S = $MgPP_i$

Scheme 2. Minimal scheme for chimeras PPase catalysis.

To explain a decreased trimer catalytic efficiency compared with the hexamer activity, one may assume that the destruction of trimer-trimer contacts causes notable conformational changes of subunits in the trimer. However, this proposal was not confirmed. Really, the properties of a portion of trimer subunits were found to differ only slightly from those of hexamer subunits (K_m 3.6 μM and 0.7 μM, respectively) (Table 1). In addition, at the substrate concentration >1 mM, the trimer activity reaches values indistinguishable from that of the hexamer. It is very important that if the structural changes occurred, the consequences would have been identical for all trimer subunits. It is appropriate to recollect a notable discrepancy in the properties of the isolated trimer and of the mutant PPases with substitutions of the active site groups [7,13]. In both cases, the catalytic efficiency is low at the assay conditions when the WT PPase is fully active. However, the activity of mutant PPases cannot be stimulated by increasing the substrate or metal ion concentration, although changes of their three-dimensional structure are rather small [14].

Earlier, we have shown that interdependence of PPase active sites upon binding of Mg^{2+} or reaction product analogs leads to asymmetry in the enzyme molecule [3–5]. The present work demonstrates non-equivalence of the trimer active sites in catalysis and their identity when trimer is transformed into hexamer. This property of hexameric PPase might be a result of the substrate, as well as metal or product, binding. However, this work shows that the efficient trimer functioning in chimeric PPase reduces the number of supposed variants. The interaction of hexamer subunits in catalysis is revealed upon the substrate binding, since the mutated trimer is actually inactive and can bind the substrate but not convert it to the product.

Complicated PPase responses to the binding of different ligands (metals, substrate, product) point to the existence of pathways for information transmission between the active sites of neighboring trimers, providing a perfect organization and concerted functioning of hexameric active sites in catalysis. This process may include some rearrangements of a network of non-covalent interactions in oligomeric enzyme molecule. It should be stressed that these informational pathways are not identical to the trimer-trimer contacts which play a crucial role in maintenance of the quaternary structure. Such a conclusion follows, for example, from the properties of H136Q and H140Q PPases [12]. The proteins have a higher activity than the WT PPase in spite of the absence of important trimer-trimer contacts. On the other hand, the subunits asymmetry of the enzyme-metal complex is conserved although neither one of the trimer-trimer contacts is broken [5].

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