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Active Dimeric Form of Inorganic Pyrophosphatase from *Escherichia coli*

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Abstract—A dimeric form can be obtained from native hexameric *Escherichia coli* inorganic pyrophosphatase (E-PPase) by destroying the hydrophobic intersubunit contacts, and it has been shown earlier to consist of the subunits of different trimers. The present paper is devoted to the kinetic characterization of such a "double-decked" dimer obtained by the dissociation of either the native enzyme or the mutant variant Glu145Gln. The dimeric form of the native inorganic pyrophosphatase was shown to retain high catalytic efficiency that is in sharp contrast to the dimers obtained as a result of the mutations at the intertrimeric interface. The dimeric enzymes described in the present paper, however, have lost the regulatory properties, in contrast to the hexameric and trimeric forms of the enzyme.

Key words: inorganic pyrophosphatase, dimer, activity, regulation

The object of this study is *Escherichia coli* inorganic pyrophosphatase (E-PPase). This enzyme, like other PPases, catalyzes the hydrolysis of pyrophosphate producing orthophosphates and thus pulling many biosynthetic reactions toward synthesis. PPases are metal-dependent enzymes. Three to four metal ions are required for their catalytic activity [1], Mg^{2+} being the physiological cofactor. Two metal ions bound at subsites M1 and M2 activate the water molecule necessary for the catalysis [2]. The third metal ion is a part of the substrate molecule, $MgPP_i$.

Thorough investigation of E-PPase has led to the description of the structure of the active site and establishment of some catalytic steps and the role of the cofactors in the functioning of the enzyme. A number of aspects, however, remain unclear, including the role of oligomeric structure in E-PPase functioning and the regulation of its activity. PPases are constitutive enzymes, so they are presumably regulated at the level of expressed protein.

E-PPase is a hexamer organized as a dimer of two trimers. The trimers are arranged in parallel plains and twisted several degrees relative to each other. Each subunit makes contacts with the four neighboring ones: two subunits within one trimer and two subunits from the other trimer. Intersubunit contacts within the trimer are formed by hydrophobic interactions and by the net of H-bonds. Ion pairs and the binding sites for intertrimeric Mg^{2+} contribute mainly to the contacts between trimers [3, 4].

Obtaining the different oligomeric forms of the enzyme and studying their kinetic properties is one of the possible ways to clarify the role of quaternary structure in E-PPase functioning.

Trimeric and dimeric forms of E-PPase can be obtained from the hexamer either by changing the medium composition or by mutations at the intersubunit interface. Trimers of both the native E-PPase and the mutant variants have been studied in detail; their most interesting feature was the disclosure of an effectory site where the metal-free pyrophosphate could bind resulting in the activation of substrate hydrolysis [5, 6].

E-PPase dimers described earlier have been obtained by insertion of three or four mutations at the intersubunit interface [7]. Those dimers had a very low catalytic efficiency due to the significant (two or three orders of magnitude) increase of Michaelis constant and simultaneous two- or three-fold decrease in the rate of hydrolysis. The question had to be solved what exactly was the reason for that sharp change of enzymatic properties, insertion of multiple mutations or destruction of intersubunit contacts. Since even a single mutation is known to greatly

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change the properties of the enzyme [8], comparison of the mutant dimers with the native hexamers requires certain care.

We recently developed a method for obtaining a dimeric form of native E-PPase by destruction of hydrophobic interactions. Such a dimer has been shown to be "double-decked", i.e., there still was the ionic contact in the dimer between amino acid residues that belong to the different trimers [9]. The rate constants have been determined for the dissociation of hexamer into dimers and for the association of dimers back into hexamer. Dimers associated into hexamer very slowly, so it was possible to study in detail their kinetic properties.

In the present paper, along with dimer of the native enzyme, dimer of the E-PPase mutant variant Glu145Gln has been obtained and studied. The residue Glu145 is located in the flexible loop 144-149 that follows immediately the intersubunit region; therefore, mutation of this residue was expected to influence the state of intersubunit contacts, this fact being actually observed.

Dimers obtained by this method retain high catalytic efficiency. The rate of $MgPP_i$ hydrolysis has been studied at pH 7.5 as a function of the substrate and cofactor concentration, and pH-independent parameters of $MgPP_i$ hydrolysis have been determined. It has been shown that, as opposed to the opinion prevalent in the literature, dissociation of the hexamers into dimers resulted in the decrease of the catalytic constant whereas the affinity for a substrate remained high.

MATERIALS AND METHODS

Chemicals. Recombinant E-PPase and its mutant variant were obtained as described earlier [3]. The protein solutions were stored as a suspension with $(NH_4)_2SO_4$ and desalted directly before use on the Sephadex G-50 (fine) column equilibrated with 50 mM Tris-HCl, pH 7.5.

The concentration of the E-PPase solutions was determined by UV-spectrophotometry using $A_{280}^{0.1\%} = 1.18$ [10].

All the chemicals used were of high purity grade purchased from Sigma, Fluka (USA), Serva, Merck (Germany), or Pharmacia Fine Chemicals (Sweden). The solutions were always prepared fresh using bidistilled water.

Obtaining dimeric forms of E-PPase. The dimeric form of the native E-PPase was obtained as described earlier [9]. The dimeric form of Glu145Gln E-PPase was obtained by the incubation of the hexameric form in 50 mM Tris-HCl, pH 7.5, with 1 mM Mg^{2+} and 20% isopropanol for 15 min at 20°C. Dimers reassociated into hexamers after decreasing the isopropanol concentration in the mixture at least tenfold by the dilution of the dimer solution with 50 mM Tris-HCl, pH 7.5, at 20°C.

Dissociation and association were monitored in progress by measuring the activity in aliquots of enzyme mixture; the enzyme was assayed in 50 mM Tris-HCl, pH 7.5, at 20 μM $MgPP_i$ and 1 mM Mg^{2+}.

Sedimentation analysis. Sedimentation velocity measurements were carried out as described earlier [9].

Kinetic measurements. Activities of the native and mutant E-PPases were determined by the rate of product release from $MgPP_i$ using a semiautomatic phosphate analyzer [11]. All measurements were carried out at 25°C and at the device sensitivity of 10 μM P_i for the full-scale. Reaction mixture of 10 ml contained buffer, Mg^{2+}, and substrate. The enzymatic reaction was started by the addition of an enzyme aliquot. To calculate required Mg^{2+} and pyrophosphate concentrations, K_D values for $MgPP_i$ and Mg_2PP_i used were 47.7 μM and 2.42 mM at pH 7.5 and 696 μM and 16.23 mM at pH 6.0, respectively [12].

The pH dependence of the kinetic parameters of $MgPP_i$ hydrolysis was determined at 5 mM free Mg^{2+} using 50 mM buffer solutions of the following composition: Mes-NaOH for the pH range 6.0-7.0; Hepes-NaOH for pH 7.0-7.5; Tris-HCl for pH 7.2-9.0; and 2-amino-2-methyl-1,3-propanediol-HCl for pH 9.0-9.7. Substrate concentration was varied in the range 2.5-100 μM. The pH-independent parameters of $MgPP_i$ hydrolysis were calculated as described in [13].

Dependence of the rate of $MgPP_i$ hydrolysis on free Mg^{2+} concentration was studied at pH 7.5 (50 mM Tris-HCl) and 50 μM $MgPP_i$. The Mg^{2+} concentration was varied in the range 0.03-10 mM.

Dependence of the rate of $MgPP_i$ hydrolysis on the substrate concentration was studied at pH 7.5 (50 mM Tris-HCl) and several fixed concentrations of free Mg^{2+} (0.05-1 mM). Substrate concentration was varied in the range 2.5 μM-1 mM.

RESULTS AND DISCUSSION

Preparation of the dimeric form of Glu145Gln E-PPase. The dimeric form of E-PPase can be formed upon the destruction of hydrophobic intratrimeric contacts under the action of 20% isopropanol, and it consists of two subunits from the different trimers [9]. The rate constant of this process k_{diss} is 0.03 min^{-1} for the native E-PPase, and the resulting dimers have activity 20% of the hexamer under the standard conditions.

Under the same conditions, Glu145Gln E-PPase forms dimers so quickly that the course of dissociation cannot be followed. Resulting dimers have activity 5% of the original hexamer. To slow down the dissociation, it was carried out in the presence of 1 mM Mg^{2+}. Even under these conditions, however, dimers were formed almost 100 times faster than from the native hexamer in the absence of Mg^{2+} (Fig. 1, Table 1). This easy dissociation of Glu145Gln E-PPase in the organic medium indicates that the inserted mutation significantly weakens the hydrophobic contacts within the trimer, despite the fact

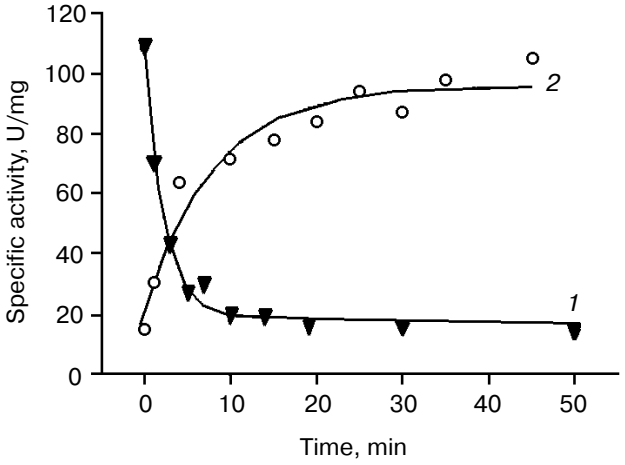

Fig. 1. Dissociation of the hexamer of Glu145Gln E-PPase into dimers (*1*) and association of dimers into hexamer (*2*). Concentration of the dimer during the association was 3.33 μM.

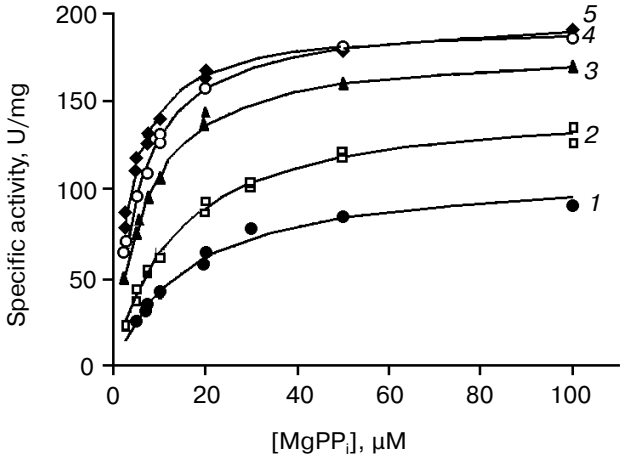

Fig. 2. Rate of $MgPP_i$ hydrolysis by the dimeric form of native E-PPase as a function of substrate concentration at pH 7.5 and several fixed Mg^{2+} concentrations: *1*) 0.05; *2*) 0.1; *3*) 0.25; *4*) 0.5; *5*) 1 mM.

that residue Glu145 is rather close to the intertrimeric interface.

Decrease of the concentration of isopropanol in the reaction mixture to 2% results in the restoration of the hydrophobic contacts and the reassociation of dimers back into hexamer with accompanying full recovery of activity. The rate constant of association is given in Table 1. Formation of hexamer was verified by determination of the sedimentation coefficient.

An analogous attempt has been made to obtain the dimeric form of the E-PPase mutant variant Asp26Ala. The residue Asp26 together with Asn24 and Ala25 take part in important intertrimeric contact, the binding site for the intertrimeric Mg^{2+} ion [4]. This mutant variant however turned out not to dissociate into dimers upon the isopropanol treatment. This finding shows that the intersubunit contacts change as a result of this mutation.

Table 1. Sedimentation coefficients and the characteristics of hexamer ↔ dimer transitions for native and Glu145Gln E-PPases

E-PPase	$s_{20,w}$, S	k_{diss},* min^{-1}	k_{ass}** × 10^{-3}, $\mu M^{-2} \cdot min^{-1}$
WT [9]	2.62 ± 0.23	0.030 ± 0.002	1.0 ± 0.1
Glu145Gln	2.42 ± 0.05	3.6 ± 0.3	21 ± 2

* k_{diss} is the rate constant of dissociation of hexamer into dimers.
** k_{ass} is the rate constant of the reverse association.

Properties of dimeric forms of native and Glu145Gln E-PPases. *Determination of the affinity of Mg^{2+} for the active site.* To characterize the state of the active site of the E-PPase dimeric forms, we determined the affinity of Mg^{2+} for the M2 binding site of these forms in the presence of the substrate at pH 7.5. Kinetic parameters V_{max} and K_m depend on the free Mg^{2+} concentration (Fig. 2). This dependence can be described by the scheme proposed earlier for the hexameric form of the enzyme where only the two-magnesium complex of the enzyme subunit is active [14]. The dissociation constant of Mg^{2+} at the M2 site is 0.17 ± 0.04 mM for the dimer of the native E-PPase, this value being about the same as obtained earlier for the native hexamer (K_d = 0.20 ± 0.04 mM [13]). For Glu145Gln E-PPase, the dimeric form has dissociation constant at site M2 of 2.2 ± 0.2 mM while the hexameric form has this constant of 1.1 ± 0.1 mM. Therefore, the mutation Glu145Gln causes fivefold increase of K_d for Mg^{2+} at site M2, whereas the affinity for Mg^{2+} is much less affected by the following dissociation of this mutant variant into dimers.

The native hexamer is inhibited by an excess of free Mg^{2+} (at concentrations higher than 5 mM) [13]. The inhibition appears to take place when Mg^{2+} fills the M4 binding site in the active site of E-PPase. Dimeric forms of the enzyme do not display this property, nor does the hexameric form of Glu145Gln E-PPase. This may be caused by impairment of the affinity for Mg^{2+} of the M4 site due to the mutation or dissociation into dimers.

Kinetics of the hydrolysis of $MgPP_i$. Investigation of the kinetic properties of trimeric forms of native and

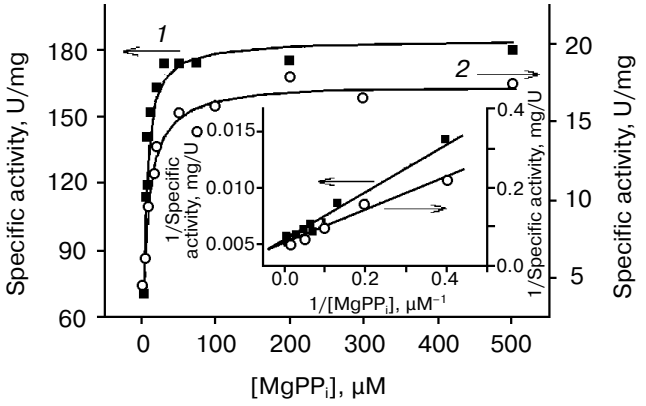

Fig. 3. $MgPP_i$ hydrolysis by the dimeric forms of native (*1*) and Glu145Gln (*2*) E-PPases over a wide range of substrate concentration at pH 7.5 and 5 mM Mg^{2+}.

mutant E-PPases revealed that the rate of $MgPP_i$ hydrolysis as a function of the substrate concentration did not obey the Michaelis–Menten equation but could be adequately described by another scheme implying the activation of the hydrolysis by the metal-free pyrophosphate bound at the effectory site [6]. Activation can be observed when studying the rate of hydrolysis over a wider range of substrate concentrations, up to 1 mM. As the substrate concentration rises, at fixed pH value and Mg^{2+} concentration, the concentration of the effector increases proportionally; this can account for the observed phenomenon. These findings raise a question if the effectory site exists in the hexamer, too. Kinetic experiments at pH 6.0 and equilibrium gel filtration of the hexamer with the isotopically labeled pyrophosphate in the presence of Ca^{2+} (unpublished data) confirmed the existence of the effectory site in the hexamer. Kinetic experiments have also shown that the hexameric form of E-PPase has very high affinity for the effector. For this reason, the effectory site affecting the kinetics of hydrolysis by the hexameric enzyme cannot be revealed at pH 7.5 because this site is filled with pyrophosphate in the whole studied range of substrate concentrations. A comparison of the affinities of ligands for the effectory site showed that the dissociation of the hexamer into trimers leads to significant impairment of the effector binding capacity.

These data forced us to investigate the rate of hydrolysis of the substrate by the dimeric form of E-PPase over a wide range of $MgPP_i$ concentrations. The dependence obtained in this case turned out to obey the Michaelis–Menten equation, as was in the case of the hexameric form (Fig. 3). This finding may be explained by two alternative possibilities. Since the dissociation of the enzyme into trimers caused significant impairment of the affinity of the effectory site for its ligands, the dissociation into dimers may be supposed to fully destroy the effectory site. Another possibility that cannot be excluded is that the effectory site is not impaired upon the dissociation into dimers and retains the same affinity for the effector as in the hexamer. The hyperbolic character of the kinetics of $MgPP_i$ hydrolysis by the dimeric form of E-PPase does not allow distinguishing between those possibilities, so we studied the rate of hydrolysis by the dimeric form of native E-PPase as a function of $MgPP_i$ concentration at pH 6.0 and 10 mM Mg^{2+}. Under these conditions, the affinity of the effectory site for its ligands is expected to be lower, and at the same time the concentration of the metal-free pyrophosphate is reduced, so the influence on the kinetics of $MgPP_i$ hydrolysis of the effectory site being filled in progress can be observed for the hexameric E-PPase. Besides, methylenebisphosphonate, which is an unhydrolyzable analog of pyrophosphate, activates $MgPP_i$ hydrolysis by the hexamer at pH 6.0.

The character of the kinetics of $MgPP_i$ hydrolysis by the dimeric enzyme was found to remain the same at pH values of 7.5 and 6.0 (data not shown). Methylenebisphosphonate did not activate $MgPP_i$ hydrolysis by the dimeric enzyme. These findings support the conclusion that the binding capacity of the effectory site is fully destroyed upon the dissociation of E-PPase into dimers.

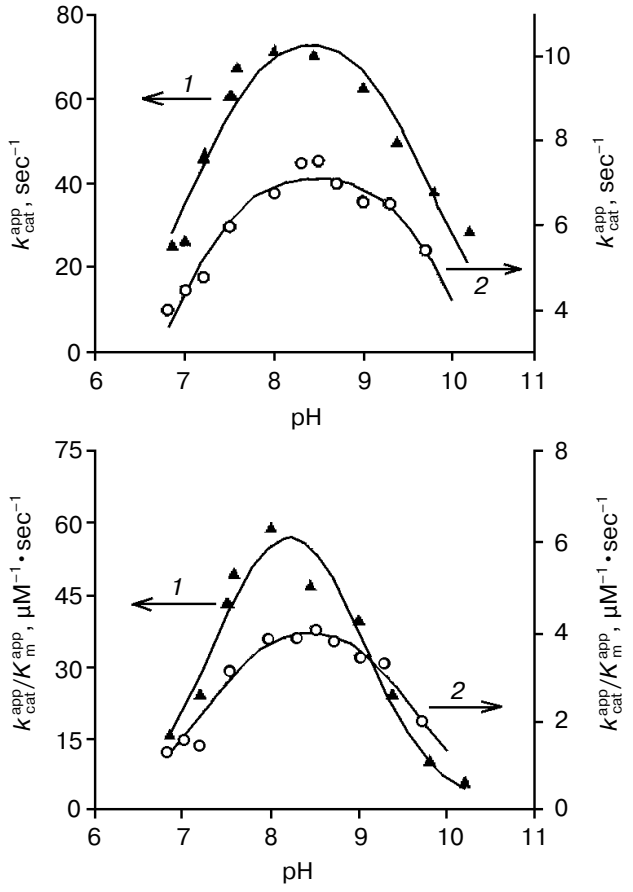

Fig. 4. pH dependence of the rate of $MgPP_i$ hydrolysis by the dimeric forms of native (*1*) and Glu-145Gln (*2*) E-PPases.

Table 2. pH-independent parameters of $MgPP_i$ hydrolysis by the dimeric and hexameric forms of native and Glu145Gln E-PPases

Parameter	WT E-PPase dimer	WT E-PPase hexamer [13]	Glu145Gln E-PPase dimer	Glu145Gln E-PPase hexamer
k_{cat}, sec^{-1}	76 ± 5	390 ± 80	7.7 ± 0.2	83 ± 3
K_m, μM	0.8 ± 0.1	0.13 ± 0.06	1.8 ± 0.2	1.3 ± 0.2
pK_{EH2}	7.1 ± 0.1	7.8 ± 0.5	6.92 ± 0.08	7.10 ± 0.06
pK_{EH}	9.8 ± 0.1	8.7 ± 0.5	10.0 ± 0.1	9.71 ± 0.07
pK_{ESH2}	7.58 ± 0.09	7.6 ± 0.3	7.2 ± 0.1	7.7 ± 0.2
pK_{ESH}	8.90 ± 0.09	9.0 ± 0.2	9.5 ± 0.2	9.2 ± 0.2

pH dependence of the substrate hydrolysis. To characterize the dimeric forms of E-PPase and compare them with the hexameric ones, pH-independent parameters of $MgPP_i$ hydrolysis were determined at 5 mM Mg^{2+}. Hydrolysis of $MgPP_i$ was studied over a wide range of pH allowing the determination of k_{cat} and K_m for the dimeric forms of E-PPase, as well as pK_a of catalytic groups of the enzyme and enzyme–substrate complex (Fig. 4, Table 2). It can be seen that the mutation Glu145Gln causes a 4.5-fold decrease in k_{cat} for the hexamer and the impairment of K_m about an order of magnitude. Dissociation of the hexamers into dimers results in further impairment of the catalytic properties. The value of k_{cat} for the dimer of the native enzyme decreased fivefold, K_m increased sixfold, whereas for the dimer of Glu145Gln E-PPase k_{cat} decreased more than 10 times while its K_m changed only slightly. As a result, dimer of the native enzyme has catalytic efficiency of 95 $\mu M^{-1}\cdot sec^{-1}$, which is more than 5000 times higher than the catalytic efficiency of the dimer of the triple mutant variant Tyr77Asp/Gln80Glu/His140Gln of E-PPase described in the literature [7].

Determination of the pK_a of the groups involved in catalysis revealed a general rule. According to it, dissociation into dimers, like mutations do, significantly changes the pK_a of the groups in the free enzyme but influences only a little the pK_a of the groups in the enzyme–substrate complex. This can be because the intersubunit contacts play an important role in the stabilization of the protein structure, while the formation of the enzyme–substrate complex gives a more compact structure.

Thus, in the present work kinetic properties of the dimeric forms of native E-PPase and its mutant variant Glu145Gln have been studied in detail. The studied dimers have high catalytic efficiency but, in contrast to the hexameric and trimeric forms, lose the ability of binding pyrophosphate in the effectory site and so lose the regulatory properties. This distinction should be taken into account in further research aimed at the localization of the effectory site.

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