Effect of D42N substitution in *Escherichia coli* inorganic pyrophosphatase on catalytic activity and Mg^{2+} binding

Svetlana M. Avaeva[a,*], Elena V. Rodina[a], Svetlana A. Kurilova[a], Tatjana I. Nazarova[a], Natalya N. Vorobyeva[b]

[a] *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Vorobeyevy Gory, Moscow 119899, Russian Federation*
[b] *Chemistry Department, Moscow State University, Vorobeyevy Gory, Moscow 119899, Russian Federation*

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Abstract Asp-42 located in the active site of *E. coli* inorganic pyrophosphatase (PPase) has been substituted by Asn by site-directed mutagenesis. This resulted in a 3-fold increase in hydrolytic activity measured under optimal conditions, a 15.5-fold increase in the K_m value and retention of the pK values of groups for enzyme and enzyme-substrate complex. The active site of the enzyme contains 4 metal binding centers (I–IV) [Harutyunyan et al. (1996) Eur. J. Biochem., in press]. Asp-42 is located near centers II and IV. The D42N replacement had no effect on Mg^{2+} binding with center II. At the same time, occupation of center IV eliminates the inhibition of inorganic pyrophosphate hydrolysis by high Mg^{2+} concentrations typical of wild-type PPase. It is proposed that the increase in activity and decrease in affinity for substrate of the D42N PPase results from changes in Mg^{2+} binding to center IV. The Mg^{2+} binding centers of *E. coli* PPase are lined up in filling order.

Key words: Inorganic pyrophosphatase; Site-directed mutagenesis; Metal binding center; Differential spectrophotometry

1. Introduction

Inorganic pyrophosphatases (EC 3.6.1.1; PPases) hydrolyze inorganic pyrophosphate (PP_i) to produce 2 phosphates (P_i). The major role of PPases is to control the level of PP_i in the cell, thus providing a driving force for the synthesis of important biopolymers. At the present time, the primary structures of 10 PPases are known [1–3]. Comparison of these amino acid sequences reveals little similarity except for 21 residues which are conserved in all known PPases. 15 of them are located in the PPase active site cavity as shown by X-ray crystallographic studies of *E. coli* PPase [4,5], *T. thermophilus* PPase [3] and *S. cerevisiae* PPase-Mn^{2+}-P_i complex [6]. These data are in accordance with the similarity of the catalytic mechanisms examined in detail on the *E. coli* and *S. cerevisiae* enzymes [7,8]. Catalysis by the *E. coli* PPase occurs via a pathway which requires the binding of metal activators to the sites of four types. The efficiency of cations as activators decreases in the order: $Mg^{2+} > Zn^{2+} > Co^{2+} > Mn^{2+} > Cd^{2+}$ [9].

Recent studies on the three-dimensional structure of the manganese-phosphate complex of *S. cerevisiae* PPase have located both two phosphates and four metal ions in the active site of each subunit [6]. Metal ion I has a maximal affinity to the enzyme and is associated with the protein before substrate binding [4]. It is suggested that metal ion binding to center II also does not require the presence of substrate in the active site [6,10], whereas metal ions III and IV are only revealed in the PPase active site simultaneously with PP_i and the filling order of these centers is not clear. Metal ions fulfil multiple roles in enzyme functioning, including activation of the water molecule involved in hydrolysis of the pyrophosphate bond. It was found earlier that replacing the activated water molecule in the coordination sphere of Mg^{2+} by fluoride ion led to enzyme inhibition [11]. Therefore, the determination of fluoride affinity to the enzyme-substrate complex is a useful probe for the ability of Mg^{2+} to activate the water molecule.

Previously, all conservative aspartic amino acid residues, except Asp-42, were replaced in PPase by amides (manuscript in preparation) and by homologous amino acids [12]. The most important result of these studies was the decrease in the k_{cat} value for all mutants investigated. In this work, the Asn substitution for Asp-42 was carried out for the first time. The binding of Mg^{2+} in the absence and presence of substrate was investigated as well as the dependences of k_{cat} on different parameters ($[Mg^{2+}]$, pH and $[F^-]$). Significant increases in catalytic activity and filling order of Mg^{2+} binding centers were established.

2. Materials and methods

The recombinant *E. coli* PPase was prepared as described previously [13]. The modified gene carrying the D42N substitution was obtained using a two-step PCR-based targeted mutagenesis technique [14]. The synthesized fragment was treated with *Bam*HI and *Eco*RI to produce a 0.8 kb fragment. The *Bam*HI-*Eco*RI fragment was ligated into plasmid pUC19. The resulting recombinant plasmid was transformed into *E. coli* strain JM109. The mutation was checked by sequencing using the dideoxy chain-termination method. The DNA sequence contained no extraneous base changes. The transformant was grown with vigorous stirring at 37°C in 2.5 l of M9 medium containing ampicillin (50 μg/ml). Cells were pelleted, resuspended in 50 ml of 50 mM Tris-HCl (pH 7.5) containing 10% sucrose and disrupted with lysozyme. Nucleic acids were precipitated by slow addition of a one-third volume of 5% streptomycin sulfate. After 20 min incubation at 0°C the precipitate was pelleted and discarded. The solution was placed on an AH-agarose column equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Mg^{2+}. The column was washed with the same buffer containing 0.2 M NaCl; elution was carried out with 0.3 M NaCl; wild-type PPase left the column at 0.4 M NaCl. D42N PPase was prepared without the WT PPase impurity using the different affinity of the WT PPase and D42N PPase to AH-agarose.

Difference spectra were recorded with a Hitachi 557 double-beam spectrophotometer. The sample and reference cells both contained a protein solution (0.4–1.2 mg/ml). $MgCl_2$ solutions (0.01–1 M) were added to a sample, and the same volumes of the buffer solution were added to a reference cell. Volume changes during titrations were negligible.

Initial rates of PP_i hydrolysis were estimated from continuous re-

*Corresponding author. Fax: (7) (095) 939 31 81.

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cording of phosphate liberation obtained with an automatic phosphate analyzer [15]. The reaction mixture of 5 ml total volume contained $MgPP_i$ (1.25–160 μM), Mg^{2+} (0.05–20 mM) and Tris-HCl (pH 8.5 or 9.1). The total concentrations of $MgCl_2$ and PP_i to maintain the desired levels of free Mg^{2+} and $MgPP_i$ (substrate) were determined using dissociation constants for magnesium and dimagnesium pyrophosphate equal to 0.0026 and 2.01 mM, respectively. Reactions were started by the addition of enzyme and were carried out at 25°C. The reaction progress curves were strictly linear for at least 3 min in all cases. One unit of enzyme activity is defined as the amount of enzyme hydrolyzing 1 μM PP_i per min. The inhibition of PP_i hydrolysis by fluoride ion was studied in the presence of 1 mM Mg^{2+}, $MgPP_i$ (1.25–80 μM) and NaF (50–500 μM) at pH 9.1. The pH-rate profiles were assayed over the pH range 6.0–11.0 at increments of 0.5 unit in the presence of 5 mM free Mg^{2+}.

3. Results

3.1. Mg^{2+} binding in the absence of substrate

A spectrophotometric method based on the decrease in pK of a single tyrosine residue accompanying Mg^{2+} binding was used to study the interaction of Mg^{2+} with the low-affinity center (center II) of the *E. coli* PPase [10]. Increasing amounts of Mg^{2+} were added to the enzyme and differential spectra were recorded versus the metal-free sample. The dissociation constant for the enzyme-Mg^{2+} complex could be determined from the hyperbolic dependence of the absorbance change at 243 nm on Mg^{2+} concentration. The effects of the substitution of Asp-42 by Asn on the dissociation constants of the enzyme (center II)-Mg^{2+} complex at pH 8.5 and 9.0 are reported in Table 1. It can be seen that the affinity of the D42N PPase to Mg^{2+} is close to that of the WT PPase.

3.2. PP_i hydrolysis at different Mg^{2+} concentrations

Initial rates of the hydrolytic reaction were measured at different free Mg^{2+} concentrations in the presence of 40 μM $MgPP_i$, at pH 9.1. At this pH value, Mg^{2+} binding to protein is sufficiently tight and the rate of PP_i hydrolysis is high. The resulting dependence of the apparent catalytic constant (k^{app}) on the free Mg^{2+} concentration is shown in Fig. 1 for both the WT and mutant enzymes. Analysis of these data indicates that the activity of the D42N PPase is equal to that of the WT PPase in the presence of up to 100 μM Mg^{2+}. Further increase in Mg^{2+} concentration results in some inhibition of the WT PPase. At the same time, the k^{app} value of the D42N PPase continues to increase with increasing [Mg^{2+}] up to 1 mM and does not further change when [Mg^{2+}] increases up to 20 mM. The Asp-42 → Asn mutation also results in a 3-fold increase in the rate of PP_i hydrolysis.

Table 1
Kinetic and thermodynamic parameters for WT and D42N PPases

Parameters	WT	D42N
k_{cat} (s^{-1})	389 ± 78	1153 ± 306
k_{cat}/K_m ($\times 10^6$) (M^{-1} s^{-1})	3038 ± 800	576 ± 144
K_m ($\times 10^{-6}$) (M)	0.128 ± 0.059	2.00 ± 1.03
pK_{ESH_2}	7.56 ± 0.25	8.11 ± 0.21
pK_{ESH}	8.96 ± 0.21	8.93 ± 0.21
pK_{EH_2}	7.75 ± 0.5	7.73 ± 0.18
pK_{EH}	8.67 ± 0.51	8.36 ± 0.18
K_D[a] (mM) (pH 8.5)	1.46 ± 0.1	1.55 ± 0.1
K_D[a] (mM) (pH 9.0)	0.71 ± 0.1	0.51 ± 0.08

[a]K_D, dissociation constant which characterizes the equilibrium $EMg_2 \leftrightarrow EMg+Mg$.

3.3. The dependence of the reaction rate on pH

The pH dependence of the reaction rate was measured for the D42N PPase in comparison with that for the WT PPase. The dependences of k^{app} and k^{app}/K_m^{app} on pH display maxima (Fig. 2), allowing one to calculate the apparent ionization constants for the enzyme with substrate bound (pK_{ESH_2} and pK_{ESH}) and in the absence of substrate (pK_{EH_2} and pK_{EH}) as well as pH-independent values of k_{cat} and k_{cat}/K_m. The measurements were carried out at 5 mM Mg^{2+}, i.e. saturation of the enzyme centers by Mg^{2+} was achieved. The values of pK_{EH_2} and pK_{EH} and the pH-independent value of k_{cat}/K_m were determined by fitting Eq. 1 to the pH profiles for k^{app}/K_m^{app}. The values of pK_{ESH_2} and pK_{ESH} and the pH-independent value of k_{cat} were determined by fitting Eq. 2 to the pH profiles for k^{app} (Table 1).

$$k^{app}/K_m^{app} = (k_{cat}/K_m)/(1 + [H^+]/K_{EH_2} + K_{EH}/[H^+]) \quad (1)$$

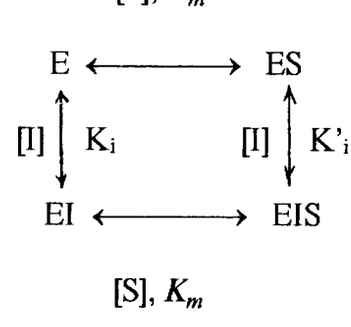
$$k^{app} = k_{cat}/(1 + [H^+]/K_{ESH_2} + K_{ESH}/[H^+]) \quad (2)$$

As mentioned above, the D42N PPase is more active than the WT PPase and exhibits a Michaelis constant value that is 15.5-fold higher than the K_m of the WT PPase (Table 1).

3.4. Fluoride inhibition

Initial rates of PP_i hydrolysis were measured as a function of $MgPP_i$ concentration at pH 9.1 in the presence of 1 mM Mg^{2+} and different concentrations of F^-. These data were analyzed using a Lineweaver-Burk plot. As seen in Fig. 3, the inhibition caused by F^- ion was non-competitive. On the other hand, the WT PPase exhibits a mixed-type inhibition (data not shown).

Since non-competitive inhibition can be considered as a special case of the more general case of mixed inhibition, the following scheme was used to depict these results (where E is a magnesium-enzyme complex and S is $MgPP_i$).



The values of the dissociation constants for the enzyme-inhibitor complex (K_i) and enzyme-substrate-inhibitor complex (K_i') were calculated from the corresponding equations [16]. The K_i' value for the D42N PPase was found to be equal to 160.4 ± 27.8 μM, differing slightly from that for the WT PPase (K_i' = 117.7 ± 14.8 μM). The K_i was equal to 836.1 ± 52.5 μM for the mutant variant, being 18.6-times greater than the K_i for the wild-type PPase (44.9 ± 25.6 μM).

4. Discussion

In this work, the properties of a mutant *E. coli* PPase containing the substitution of Asp-42 by Asn were examined. The

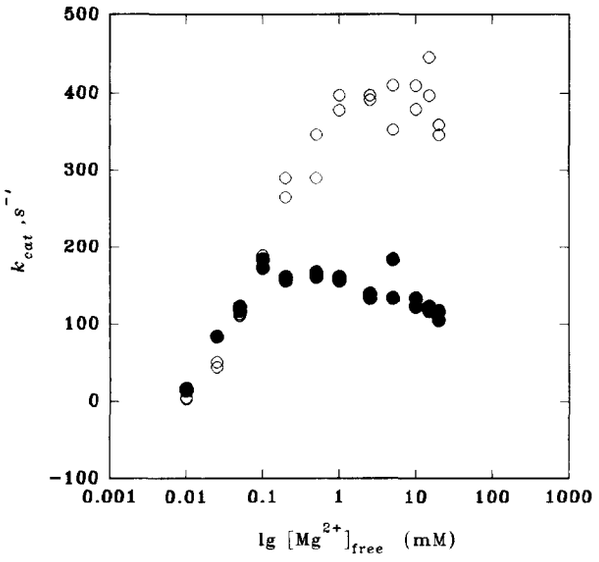


Fig. 1. Dependence of k^{app} of WT (●) and D42N (○) on Mg^{2+} concentration at pH 9.1.

aspartic acid residue in position 42 is one of the 15 amino acid residues forming the active site cavity, as demonstrated by the three-dimensional structures of the *E. coli* PPase [4,5], *T. thermophilus* PPase [3] and *S. cerevisiae* PPase-Mn^{2+}-P_i complex [6]. In spite of this, the participation of Asp-42 in catalysis has not yet been investigated. The Asp→Asn substitution does not markedly change the group size and its potential capability of hydrogen bond formation, but does decrease the total negative charge of the active site. The interaction of the D42N PPase with Mg^{2+} in the presence and absence of substrate was investigated, the pK values of essential groups and the values of k_{cat} and K_m were determined and F^- inhibition was examined.

The study of the reaction rate dependence on pH was carried out for the D42N PPase as well as for the WT enzyme. Comparison of the results obtained (Fig. 2, Table 1) demonstrates firstly a 3-fold increase in k_{cat}. At the same time, the K_m value increases 15.5-fold. The D42N PPase is characterized by only small changes in the pK values of the protein groups in both the presence and absence of substrate as compared to the WT PPase. Our studies of the reaction rate dependences on pH for the other mutant variants containing the substitutions D→N led to the same result (manuscript in preparation). These data differ from those observed earlier for the mutant variants containing the substitutions D↔E wherein the pK always shifted to basic pH values [12]. The authors believe that replacing amino acid residues could decrease the Mg^{2+}-OH_2 interaction, thereby raising the pK of the bound water. Our results are inconsistent with this assumption. We have also shown that F^-, believed to compete with OH^- for binding to Mg^{2+}, does not change its affinity. In fact, K_i' decreases 1.5-fold only on comparison with that for the WT PPase. Note that the affinity of fluoride to the enzyme-Mg^{2+} complex in the absence of substrate falls off 18.6-fold. Such behaviour of the mutant variant suggests that the only substitution breaks the hydrogen bond system in the active site but the substrate binding repairs it.

Asp-42 is located in proximity to the second and fourth centers of metal binding [6]. Mg^{2+} binding to center II is practically the same for the D42N PPase and WT PPase at 8.5 and 9.0, as is evident from the spectrophotometric titration data (Table 1). The dependences of the PP_i hydrolysis rate on free Mg^{2+} concentrations up to 100 μM show that the D42N and WT PPases have the same activities when the first two centers are saturated with Mg^{2+}, and the third metal ion is present as a constituent of substrate ($MgPP_i$, Fig. 1). The subsequent increase in Mg^{2+} concentration brings about significant differences between the wild-type and mutant PPases. The binding of metal ion to center IV inhibits the WT PPase, decreasing the reaction rate. At the same time, increasing k_{cat} is observed for the D42N PPase. The present data suggest that the substitution of Asp-42 by Asn alters the Mg^{2+} binding to center IV, eliminating its inhibitory effect. It also decreases the affinity of the enzyme to substrate and probably facilitates product release. All these factors contribute to the increase in catalytic activity of this mutant PPase.

It follows from these results that center IV binds the final Mg^{2+}. We can state that the filling order of Mg^{2+} binding

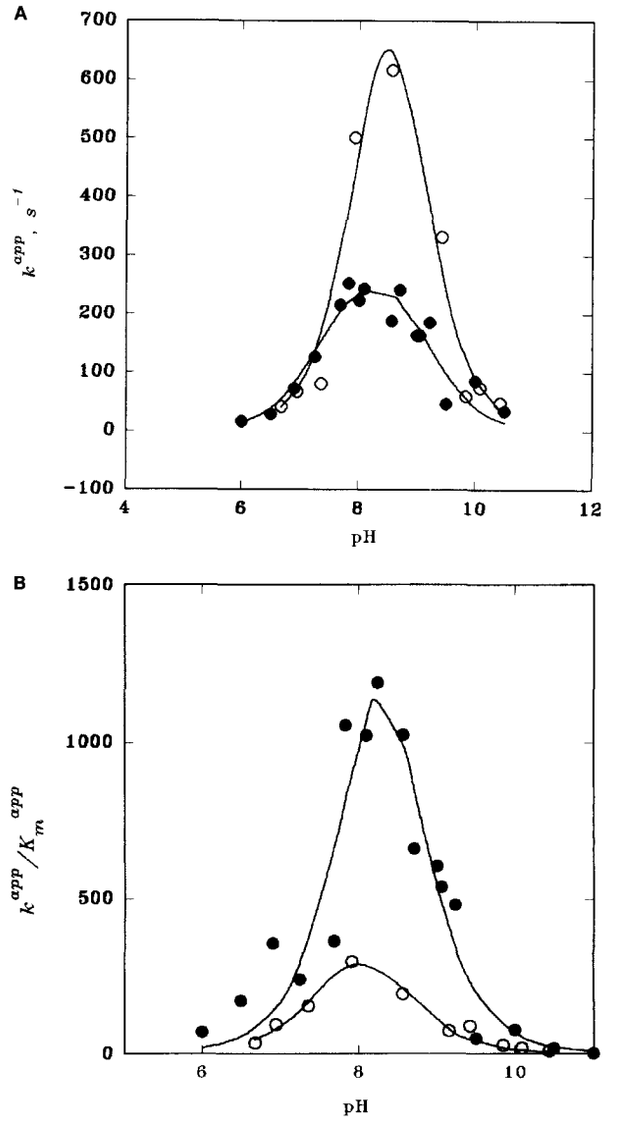


Fig. 2. pH dependence of k^{app} (A) and k_{app}/K_m^{app} (B) for WT (●) and D42N (○).

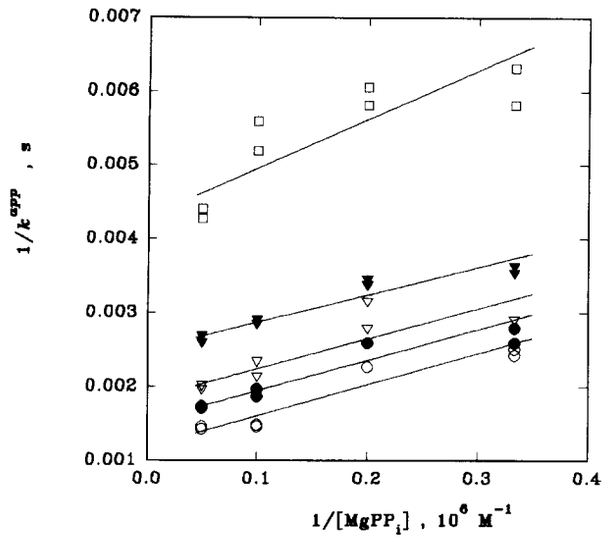


Fig. 3. Fluoride inhibition of D42N enzyme. F^- concentrations (μM): 0 (○), 50 (●), 100 (▽), 300 (▼), 500 (□).

centers is consistent with the presented numbering. The metal ion bound in center IV of the *S. cerevisiae* PPase has a single protein ligand, namely Glu-58 corresponding to Glu-31 in the *E. coli* PPase. In addition, metal ion IV is bound to two phosphates of the substrate [6]. The distance between O_2Asp-42 and O_1Glu-31 is equal to 3.1 Å [4], therefore a hydrogen bond is likely to occur between these residues. The substitution of Asp-42 by its amide evidently changes the relative positions of protein groups in center IV and association of metal ion IV with the substrate.

At present crystals of the D42N PPase have been obtained and X-ray analysis is in progress.

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