Escherichia coli inorganic pyrophosphatase: site-directed mutagenesis of the metal binding sites

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Abstract Aspartic acids 65, 67, 70, 97 and 102 in the inorganic pyrophosphatase of *Escherichia coli*, identified as evolutionarily conserved residues of the active site, have been replaced by asparagine. Each mutation was found to decrease the k^{app} value by approx. 2–3 orders of magnitude. At the same time, the K_m values changed only slightly. Only minor changes take place in the pK values of the residues essential for both substrate binding and catalysis. All mutant variants have practically the same affinity to Mg^{2+} as the wild-type pyrophosphatase.

Key words: Inorganic pyrophosphatase; Aspartic acid; Site-directed mutagenesis; Kinetics; Differential spectrophotometry

1. Introduction

Inorganic pyrophosphatases (EC 3.6.1.1; PPase) hydrolyze inorganic pyrophosphate (PP_i) to inorganic phosphate (P_i). These enzymes are found in virtually any animal, plant or bacterial cell. The primary structures of 10 PPases have been identified [1–3]. Comparison of these sequences has demonstrated very little similarity, except for about 20 residues which are conserved in all known PPases. Of these, 15 are located in the PPase active site cavity. This indicates that the catalytic mechanisms for these enzymes are rather similar and evolutionarily conserved.

The three-dimensional structures are known for *Saccharomyces cerevisiae* PPase [4], *Escherichia coli* PPase [5–7] and *T. thermophilus* PPase [3]. PPases require divalent cations with Mg^{2+} conferring the highest activity. The hydrolysis of PP_i by *E. coli* PPase necessitates as many as 4 Mg^{2+} in the catalytic center. Two of them are bound to the enzyme in the absence of PP_i and the other 2 in the presence of the substrate [8]. All metal activators are grouped on the active site cavity. Metal ions are assumed to play specific roles in several steps of enzyme catalysis. Substrate activation, a strong specificity toward PP_i, acceptance of the leaving group, and activation of water molecules attacking PP_i are probably the most important among such functions. It has been suggested that the activated water molecule in the coordinating sphere of Mg^{2+} can be replaced by F^-. Therefore, fluoride inhibition represents a useful probe for the affinity of the water molecule to metal ion [9,10].

Determination of the catalytic mechanism of PPases would not be complete without elucidation of the roles of the active site metal ions and their associated ligands. Site-directed mutagenesis of the dicarbonic amino acid residues probably coordinating metal activators is one way to resolve this problem.

In this work, we have prepared five *E. coli* PPase mutants containing replacements of the active site dicarbonic amino acid residues by their amides and investigated their properties. The catalytic constants, affinity to Mg^{2+} in the presence and absence of the substrate, pH dependence of k_{cat} and K_m and F^- inhibition have been examined.

2. Materials and methods

2.1. Materials

Bacto-tryptone and Bacto-yeast were obtained from Difco Laboratories. Sequenase version 2.0 DNA sequencing kit was purchased from United States Biochemical. All enzymes used in the molecular biological experiments were purchased from BION (Moscow). [α-^{35}S]dATP (> 1000 Ci/mmol) was obtained from Amersham. All oligonucleotides used in this work were synthesized by phosphoramidite chemistry with an Applied Biosystems 380B DNA synthesizer using deoxyribonucleotide phosphoramidites from Pharmacia. All other chemicals were purchased from Sigma Chemical Co., Fluka Chemie AG or Pharmacia.

2.2. Methods

The recombinant *E. coli* PPase (WT PPase) was prepared as described previously [6]. The modified genes carrying D65N, D67N, D70N, D97N and D102N substitutions, the corresponding recombinant plasmids and mutant proteins were produced as described for D42N PPase [11].

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

The initial rates of PP_i hydrolysis were estimated from continuous recording of P_i liberation obtained with an automatic phosphate analyzer [12]. The reaction mixture of 5 ml total volume contained $MgPP_i$ (1.25–160 μM), Mg^{2+} (0.05–5 mM) and 0.05 M Tris-HCl, pH 9.1 at 25°C. The total concentrations of $MgCl_2$ and PP_i to maintain the desired levels of free Mg^{2+} and $MgPP_i$ (substrate) were calculated using dissociation constants for magnesium pyrophosphate and dimagnesium pyrophosphate equal to 0.0026 and 2.01 mM, respectively [13]. 1 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 in the presence of 5 mM free Mg^{2+}. The buffers used were MES, MOPSO, Tris, CAPSO, CAPS and Bistris-propane.

3. Results

3.1. Mutagenesis, expression and enzyme purification

The aspartic acids at positions 65, 67, 70, 97 and 102 in

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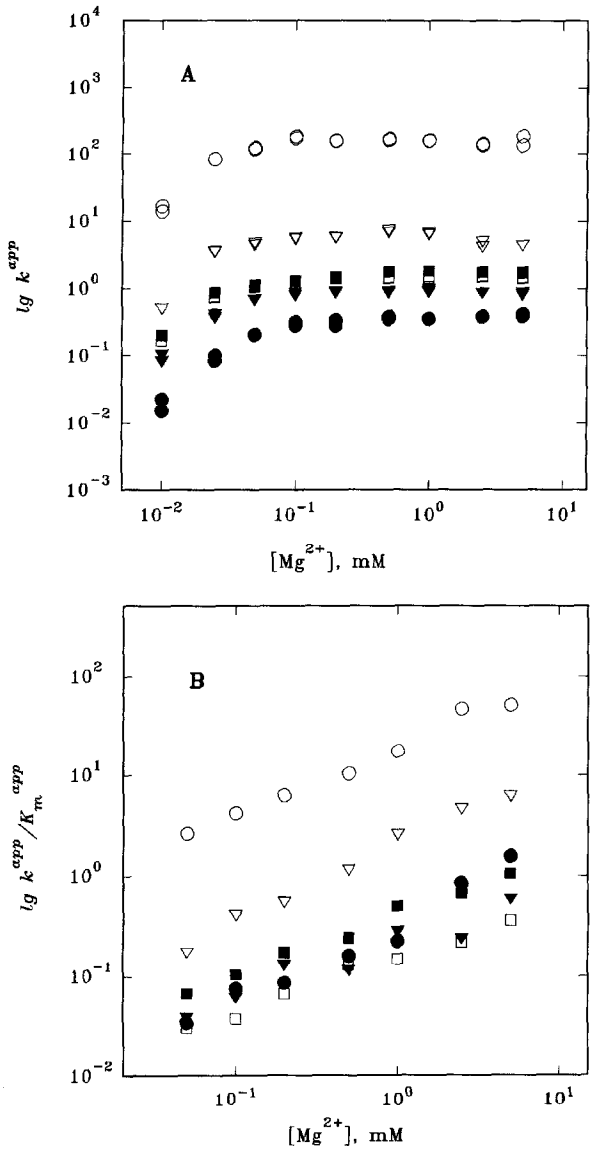

Fig. 1. Dependence of k^{app} (A) and k^{app}/K_m^{app} (B) for WT (○), D65N (●), D67N (▽), D70N (▼), D97N (□) and D102N (■) on Mg^{2+} concentration at pH 9.1.

E. coli PPase have been replaced by site-directed mutagenesis to their amides. The DNA sequences for the listed mutant variants contained no extraneous base changes. The total amount of mutant enzymes obtained from 2.5 l of cell culture varied from 35 to 90 mg. All mutant variants were prepared without the WT PPase impurity using the different affinity of the WT PPase and mutant variants to AH-agarose. The affinity of mutant proteins to AH-agarose increases after sulfate ammonium precipitation and becomes equal to that of the WT PPase.

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

Initial rates of the hydrolytic reaction were measured as a function of magnesium pyrophosphate concentration at several levels of free Mg^{2+} in the range 0.05–5 mM at pH 9.1 for the wild-type PPase and all variants. Apparent catalytic constants (k^{app}) and the ratio of k^{app} to apparent Michaelis constants (k^{app}/K_m^{app}) for magnesium pyrophosphate were calculated from the Michaelis-Menten equation using the computer program ENZFITTER (Fig. 1). Analysis of these data indicates that all mutant variants as well as the WT PPase had practically full activity at 100 μM Mg^{2+} and further increase in Mg^{2+} concentration up to 5 mM did not significantly change the k^{app} value (Fig. 1A). The influence of [Mg^{2+}] on the K_m^{app} value was observed to be similar for all mutant variants and the WT PPase (Fig. 1B). The difference between the K_m^{app} values for the WT PPase and all mutant variants was 2-fold smaller under identical conditions.

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

A spectrophotometric method was used to study Mg^{2+} interaction with the low affinity sites of *E. coli* PPase mutant variants [14]. Increasing amounts of Mg^{2+} were added to the enzyme and differential spectra were recorded vs. metal-free enzyme. The dissociation constant for Mg^{2+} binding to PPase could be determined from the hyperbolic dependence of the absorbance change at 243 nm on Mg^{2+} concentration. The effects of the mutations on the dissociation constants for enzyme-Mg^{2+} complexes at pH 8.5 are reported in Table 1. As can be seen, the affinity of mutants to Mg^{2+} is only slightly changed as compared to the WT PPase.

3.4. Dependence of the reaction rate on pH

The dependences of the reaction rate on pH were measured for the mutant variants and the WT PPase. The values of k^{app} and k^{app}/K_m^{app} for mutant variants and the WT PPase as a function of pH display maxima (Fig. 2), allowing one to calculate the apparent ionization constants for enzyme with substrate bound (pK_{ESH2} and pK_{ESH}) and in the absence of substrate (pK_{EH2} and pK_{EH}) as well as pH-independent values for k_{cat} and k_{cat}/K_m (Eqs. 1 and 2).

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

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

The results are presented in Table 2. All mutant variants have extremely low activities. At the same time, the Michaelis constant changes much less than the hydrolysis rates in all cases. The values of pK for free enzymes and enzyme-substrate complexes change slightly as compared to the WT PPase and no trend in these changes could be observed.

3.5. Fluoride inhibition

The initial rates of PP_i hydrolysis were measured as a function of $MgPP_i$ concentration at several levels of F^- and were analyzed using a Lineweaver-Burk plot. F^- inhibition corresponds to mixed-type inhibition and may be depicted by the corresponding scheme and equations [15].

The values of the dissociation constants for enzyme-inhibi-

Table 1
Dissociation constants of Mg^{2+}-enzyme complexes for the WT and mutant PPases at pH 8.5

Enzyme	K_d (mM)
WT PPase	1.46 ± 0.10
D65N	2.1 ± 0.2
D67N	4.49 ± 0.88
D70N	3.13 ± 0.37
D97N	5.56 ± 0.02
D102N	1.03 ± 0.13

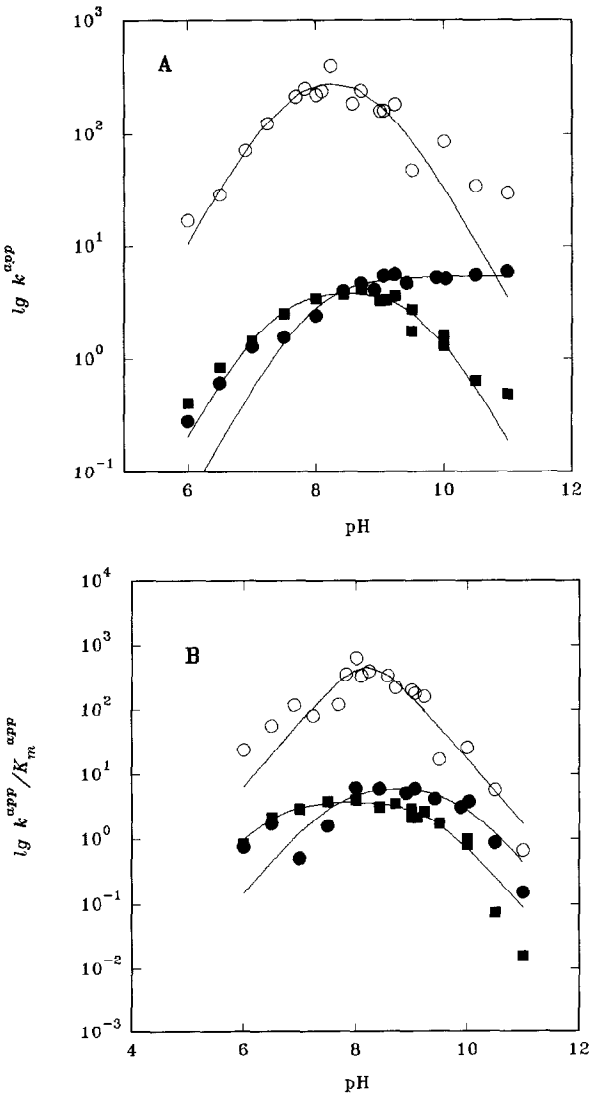

Fig. 2. Dependence of k^{app} (A) and k^{app}/K_m^{app} (B) for WT (○), D65N (●) and D102N (■) on pH.

tor complexes (K_i) and for enzyme-substrate-inhibitor complexes (K_i') are listed in Table 3. Data analysis shows that the values of K_i' for all variants except D65N and D67N are slightly different from those measured for the wild-type enzyme. Enzyme-substrate complexes of D65N and D67N PPases are characterized by a higher affinity to fluoride ion. The K_i values for all mutants except D67N are 2.5–6.0 times greater than that for wild-type PPase. In contrast, K_i for D67N is 3-fold lower.

4. Discussion

Site-directed mutagenesis is the most informative method for understanding of the role of individual amino acids in catalysis. Eight dicarbonic amino acid residues are constituents of the PPase active site [6]. Five of them (Asp-65, Asp-67, Asp-70, Asp-97 and Asp-102) were replaced by asparagine and 5 mutant variants were produced. Such transformation allows the group size to be maintained and the potential possibility of hydrogen bond formation, but decreases the total negative charge of the active site.

At the beginning of this investigation, we assumed that all dicarbonic amino acid residues in the active site are potential ligands of metal ions. In fact, recent studies show that Asp-65, Asp-70 and Asp-102 are ligands of metal ions in the high affinity site [7,16]. Simultaneously, the other oxygen atom of Asp-70 is very likely to be involved in metal ion binding with the low affinity site [14].

The common property of all mutant enzymes is extremely low activity. A plausible reason for this could be the decrease in Mg^{2+} affinity to the active site, however, this was not confirmed by the present data. The study of PP_i hydrolysis at different free Mg^{2+} concentrations shows (Fig. 1A) that the mutant variants as well as the WT PPase have practically full activity at pH 9.1 and 0.1 mM Mg^{2+}. Increasing the Mg^{2+} concentration up to 5 mM has no significant effect on the activity. The data presented provide some information about the binding of Mg^{2+} to enzyme-substrate complexes, but it is known that the Mg^{2+} affinity to PPase increases significantly in the presence of PP_i, and the filling of two magnesium sites proceeds before binding of the substrate [8]. The titration of mutant PPases by Mg^{2+} in the absence of substrate shows that filling of the low affinity site is not essentially changed with the substitution of aspartic acid residues by asparagine (Table 1). There is a possibility that amides could still be reasonably good ligands for the metal activator and the substitution of one dicarbonic amino acid residue by its amide may not change the affinity to Mg^{2+} significantly, especially if several carboxylate groups participate in the binding of metal ions.

Another reason for the low activity of mutant PPases could be a pK increase in the nucleophilic water molecule that decreases the effectiveness of PP_i hydrolysis. Such an assumption is based on the pH-rate dependences for mutant PPases with D↔E substitutions [17]. The most important result of the latter study was that the effect of the mutation was always to increase the pK_a of a basic group essential for both substrate binding and catalysis by 1–3 pH units. On the basis of this observation, the authors suggested that the most likely candidate for the essential group affected by all mutations is hydroxide ion stabilized by coordination to the essential Mg^{2+}

Table 2
Kinetic parameters of PP_i hydrolysis for WT PPase and mutant forms with 5 mM Mg^{2+}

Enzyme	pK_{EH2}	pK_{EH}	k_{cat}/K_m ($\times 10^6$) (M^{-1} s^{-1})	pK_{ESH2}	pK_{ESH}	k_{cat} (s^{-1})	K_m ($\times 10^{-6}$) (M)
WT	7.76±0.88	8.67±0.56	3038±800	7.56±0.25	8.96±0.21	389±78	0.13±0.06
D65N	7.66±0.23	9.83±0.21	6.93±0.97	6.33±0.91	8.21±0.19	5.44±0.15	0.78±0.13
D67N	8.24±0.41	8.29±0.38	80±50	6.93±0.16	8.62±0.12	15.2±1.9	0.19±0.13
D70N	7.38±0.13	9.33±0.13	2.67±0.27	7.13±0.08	9.35±0.07	2.67±0.14	1.00±0.14
D97N	6.40±0.18	9.27±0.13	1.54±0.13	7.12±0.15	9.58±0.13	1.74±0.13	1.13±0.18
D102N	6.52±0.13	9.25±0.09	4.15±0.27	7.30±0.10	8.93±0.21	4.29±0.27	1.03±0.13

Table 3
Dissociation constants of enzyme-fluoride complexes (K_i) and enzyme-substrate-fluoride complexes (K_i') for the TWT PPase and resulting mutants at pH 9.1

Enzyme	K_i (μM)	K_i' (μM)
WT	44.9 ± 25.6	117.7 ± 14.8
D65N	185.4 ± 56.1	51.0 ± 4.4
D67N	13.8 ± 5.3	18.7 ± 1.4
D70N	194.1 ± 96.7	101.0 ± 9.7
D97N	117.7 ± 14.8	117.1 ± 11.5
D102N	275.5 ± 41.1	107.5 ± 13.1

[17]. The data of the present investigation are inconsistent with this assumption (Table 2). In this work, studies of the reaction rate dependences on pH were carried out for the D65N, D67N, D70N, D97N, D102N PPases and the WT PPase (Fig. 2 and Table 2). The effect of carboxylate group mutations to amides results in a decrease of the k_{cat} value by approx. 2–3 orders of magnitude. At the same time, K_m is not greatly altered. The low activity is typical for mutant PPases with D→N substitutions as well as for mutant forms with D↔E substitutions. The only exception is the D42N PPase having 3-fold activity by comparison with the WT PPase [11]. However, the mutant PPases with D→N substitutions, in contrast to D↔E substitutions, are characterized by only minor changes of the pK values in the presence and absence of the substrate as compared to the WT PPase. Thus, the assumption of an obligatory increase in the pK of the water molecule has not been confirmed. Fluoride inhibition provides support for the retention of water molecule affinity to metal ion. The F^- affinity (K_i') to enzyme-substrate complexes for all mutant variants studied does not change or even improves in some cases. However, the affinity of inhibitor to the enzyme-magnesium complex (K_i) decreases (Table 3).

The extremely low activity of mutant forms is not surprising, since all the above-mentioned residues involved a branched net of non-covalent interactions in the active site cavity [6]. Undoubtedly, this unified system of functional group interactions is crucial to PPase functioning. The substitution of any of 5 dicarbonic amino acids by amides causes similar changes in the catalytic properties in mutant enzymes (a drastic reduction of the activity with concomitant retention of the K_m value and an affinity to Mg^{2+}). The changes of the detailed structure of the catalytic center could be invoked by the substitution of even one dicarbonic amino acid leading to changes in the orientation of the water molecule and/or to changes of a rate-limiting reaction step; for example, it could result in the dissociation of the products. This investigation suggests a tightly integrated active site in which the perturbation induced by conservative substitution at a single location can have widespread functional effects.

These problems could be clarified by studying the X-ray structure of *E. coli* PPase mutant variants and this work is now in progress.

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