The role of histidine-118 of inorganic pyrophosphatase from thermophilic bacterium PS-3

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Treatment of the inorganic pyrophosphatase from thermophilic bacterium PS-3 with diethyl pyrocarbonate resulted in the almost complete loss of its activity, which followed pseudo-first-order kinetics. The presence of Mg^{2+} prevented the inactivation. Enzyme inactivated with diethyl pyrocarbonate was re-activated by hydroxylamine. The inactivation paralleled the amount of modified histidine residue, and a plot of the activity remaining against the amount of modified histidine residue suggested that the modification of one of two histidine residues totally inactivated the enzyme. The site involved was found to be located in a single lysyl endopeptidase-digest peptide derived from the ethoxy[^{14}C]carbonylated enzyme. Amino acid analysis and sequence analysis of the peptide revealed that it comprised residues 96–119 of the inorganic pyrophosphatase from thermophilic bacterium PS-3. These results, when compared with those reported for the *Escherichia coli* and yeast enzymes, imply that His-118 of the inorganic pyrophosphatase from thermophilic bacterium PS-3 is located near the Mg^{2+} -binding site and thus affects the binding of Mg^{2+} .

INTRODUCTION

Inorganic pyrophosphatases (EC 3.6.1.1) play an important role in energy metabolism [1–3]. All so far studied, including that from thermophilic bacterium PS-3 [4], require bivalent metal ions, with Mg^{2+} being the most effective. Furthermore, the thermophilic enzyme is only thermostable in the presence of bivalent cations [5], the appearance of the stability accompanying a conformational change that can be detected by c.d. measurement: a positive c.d. band at 293 nm appears on the addition of bivalent cations. Shiroya & Samejima [6] found, in a study involving chemical modification through photo-oxidation in the presence of Rose Bengal, that histidine residues of the inorganic pyrophosphatase from *Bacillus stearothermophilus* may be located in the binding site for Mg^{2+} . The pH-dependence of the enzyme activity and the change in the c.d. band at 293 nm in the presence of Mg^{2+} indicated that a histidine residue is also important for the function of the inorganic pyrophosphatase from thermophilic bacterium PS-3. Recently we elucidated the primary structure of the inorganic pyrophosphatase from thermophilic bacterium PS-3 [7]. The enzyme is composed of three identical subunits, which each comprise 164 amino acid residues, and we found that two histidine residues are present at positions 118 and 125 per monomer chain. These observations prompted us to perform chemical modification of the histidine residues of the inorganic pyrophosphatase from thermophilic bacterium PS-3 in order to elucidate the amino acid residues included in the active site of the enzyme. Our results provide evidence that His-118 is essential for Mg^{2+} binding.

MATERIALS AND METHODS

Materials

The inorganic pyrophosphatase from thermophilic bacterium PS-3 was purified according to the method described previously [5]. The purity of the protein was confirmed by the appearance of a single band staining with Coomassie Blue in native PAGE [8,9] and SDS/PAGE [10]. Diethyl pyrocarbonate (ethoxy-formic anhydride) was purchased from Calbiochem. Diethyl

[^{14}C]pyrocarbonate (9.4 mCi/mmol) was obtained from Sigma Chemical Co. Lysyl endopeptidase (*Achromobacter* proteinase I, EC 3.4.21.50) was purchased from Wako Pure Chemicals. A BCA protein assay kit was purchased from Pierce Chemical Co. All others chemicals were of analytical grade.

Assays

The inorganic pyrophosphatase activity was assayed at 37 °C according to the procedure described previously [5]. Protein concentrations were determined with the Pierce BCA protein assay kit, with BSA as a standard.

Histidine modification

Ethoxycarbonylation of the enzyme was carried out by incubation of the enzyme in 20 mM-Mes/NaOH buffer, pH 6.1, at 25 °C with a freshly prepared solution of diethyl pyrocarbonate in ethanol. The final concentrations of the enzyme, diethyl pyrocarbonate and ethanol were 5.4 μM -monomer, 100 μM and 2.0 % (v/v) respectively. The control received an equal amount of ethanol. Samples of the reaction mixture were withdrawn at various times for determination of the enzyme activity. The pH-dependence of the modification was examined in the same way in 20 mM-Mes/NaOH buffer, pH 5.5–6.9, and 20 mM-Hepes/NaOH buffer, pH 7.1–7.5.

For preparation of the ethoxycarbonylated enzyme for isolation of a modified peptide, three 10 μl portions of a 5 mM-diethyl pyrocarbonate or -diethyl [^{14}C]pyrocarbonate solution were added, at 10 min intervals, to 0.5 ml of the inorganic pyrophosphatase (100 nmol of monomer) in 10 mM-Hepes/NaOH buffer, pH 7.0, and then the reaction mixture was incubated for another 10 min at 25 °C. The reaction was stopped by the addition of 0.5 ml of 10 mM-imidazole/HCl buffer, pH 6.1, and then the protein solution was concentrated with the use of a membrane filter (Molcut II; Millipore). The protein was dissolved in 0.5 ml of 10 mM-imidazole/HCl buffer, pH 6.1.

Peptide generation and purification

The diethyl pyrocarbonate- or diethyl [^{14}C]pyrocarbonate-modified inorganic pyrophosphatase (100 nmol of monomer)

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Fig. 1. (a) Effects of Mg^{2+} and iminobisphosphonate on inactivation of inorganic pyrophosphatase from thermophilic bacterium PS-3 by diethyl pyrocarbonate, and (b) effects of Mg^{2+} concentration on the rate of inactivation of the enzyme by diethyl pyrocarbonate and on the change in the c.d. band at 293 nm

(a) Effects of Mg^{2+} and the substrate analogue iminobisphosphonate, separately and in combination, on inactivation of the inorganic pyrophosphatase by diethyl pyrocarbonate. Inactivation of the inorganic pyrophosphatase ($5.4 \mu\text{M}$) with diethyl pyrocarbonate (0.1 mM) was carried out in 20 mM -Hepes buffer, pH 7.5, in the absence (●) and in the presence of 0.5 mM-Mg^{2+} (■), 1.0 mM-Mg^{2+} (△), 1.5 mM-Mg^{2+} (□), 1 mM -iminobisphosphonate (●), both 0.5 mM-Mg^{2+} and 0.5 mM -iminobisphosphonate (▲) and both 1 mM-Mg^{2+} and 1 mM -iminobisphosphonate (●). ○ shows the control experiment without diethyl pyrocarbonate. (b) Effects of the Mg^{2+} concentration on the rate of inactivation of the enzyme by diethyl pyrocarbonate (○) and on the change in the c.d. band at 293 nm (●). $\Delta[\theta]$ was measured with a Jasco J-600 automatic recording dichograph at room temperature and is expressed as a difference in ellipticity at 293 nm in the presence and in the absence of Mg^{2+} .

was denatured with 3 M -urea in 10 mM -imidazole/HCl buffer, pH 6.1, and then further digested with lysyl endopeptidase, which was carried out at 30°C for 4 h at an enzyme/protein molar ratio of 1:200. The digestion was stopped by cooling of the reaction mixture to 0°C , and then the peptides were fractionated by h.p.l.c. on a TSK-GEL ODS-80TM column ($4.6 \text{ mm} \times 250 \text{ mm}$) with a gradient of 40 mM -Mes/NaOH buffer, pH 6.1, to 10 mM -Mes/NaOH buffer, pH 6.1, containing 60% (v/v) acetonitrile at the flow rate of 0.6 ml/min . The column eluate was monitored at 225 nm.

Measurement of ^{14}C radioactivity

An LSC-1000 liquid-scintillation spectrometer (Aloka) was used. The ^{14}C radioactivities of $20 \mu\text{l}$ samples were counted in 3 ml of scintillation liquid. The results were corrected for quenching.

Amino acid analysis

Samples were hydrolysed in 6 M -HCl in evacuated sealed tubes at 110°C for 24 h. Amino acid analysis was performed with a Hitachi 835 amino acid analyser.

Automatic sequence determination

Automatic Edman degradation was performed with a gas-phase sequenator (Applied Biosystems model 477A) and identification of phenylthiohydantoin derivatives was accomplished as described previously [7].

U.v.-absorption spectra

The absorption and difference spectra were obtained with a Hitachi 330 automatic recording spectrophotometer at room temperature with 1 cm -path-length cells. The extent of ethoxy-carbonylation of histidine residues was determined from the increase in absorption at 242 nm. The number of modified histidine residues was calculated from the molar absorption coefficient, i.e. $\Delta\epsilon_{242} 3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [11].

C.d. spectra

C.d. spectra were obtained with a Jasco J-600 automatic recording dichograph at room temperature. A cell of 5 mm path length was used for the measurements. The mean residue weight was calculated to be 114.6 from the amino acid sequence [7].

RESULTS

Inactivation of the inorganic pyrophosphatase by ethoxycarbonylation

The incubation of inorganic pyrophosphatase with 0.1 mM -diethyl pyrocarbonate at 25°C resulted in rapid loss of its activity. The semi-logarithmic plot of residual activity versus time was linear, indicating that the inactivation follows pseudo-first-order kinetics. This inactivation was completely reversed on treatment with 20 mM -hydroxylamine at 25°C for 10 min. The treatment of inorganic pyrophosphatase with diethyl pyrocarbonate at different pH values in the range 5.5–7.5, showed that the loss of the enzyme activity was dependent on the pH. The rate of inactivation increased with increasing pH up to pH 7.3, with an inflexion point at about pH 6.7, which is close to the apparent pK_a value expected for a histidine residue in a protein.

The effects of Mg^{2+} and the substrate analogue iminobisphosphonate on the rate of inactivation on modification were examined in 20 mM -Hepes/NaOH buffer, pH 7.5. The results are shown in Fig. 1(a). On ethoxycarbonylation in the presence of Mg^{2+} at various concentrations, it was revealed that Mg^{2+} protected the enzyme against inactivation in a concentration-dependent manner. The rate of inactivation decreased in a hyperbolic manner with increasing Mg^{2+} concentration, as shown in Fig. 1(b). The Mg^{2+} -concentration-dependence of the change in the c.d. band at 293 nm is also shown in Fig. 1(b). The results indicate that they show good correlation. When ethoxy-carbonylation in the presence of 1 mM-Mg^{2+} was carried out at

Fig. 2. Relationship between the residual activity of inorganic pyrophosphatase from thermophilic bacterium PS-3 and the number of histidine residues modified by diethyl pyrocarbonate

The enzyme ($9.4 \mu\text{M}$) was incubated with $400 \mu\text{M}$ -diethyl pyrocarbonate at 25°C in 1.0 ml of 20 mM -Mes buffer, $\text{pH } 7.0$, containing 0.1 mM -EDTA. At appropriate times the residual activity was measured and a difference spectrum was obtained. The number of ethoxycarbonylated histidine residues was determined from the increase in absorbance at 242 nm by using the molar absorption coefficient, $\Delta\epsilon_{242} 3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [11].

different pH values the protection by Mg^{2+} against inactivation was evident in a higher pH region than the pK_a value of 6.7 . This correlates well with the pH-dependences of the enzyme activity and the change in the c.d. band at 293 nm [4,5], indicating that Mg^{2+} binds at pH values higher than 6.7 . On the other hand, the substrate analogue iminobisphosphonate afforded virtually no protection to the enzyme against ethoxycarbonylation when added alone. However, considerable protection was evident with

Fig. 4. Reverse-phase h.p.l.c. absorbance (a) and radioactivity (b) elution profiles of a lysyl endopeptidase digest of diethyl [^{14}C]pyrocarbonate-modified inorganic pyrophosphatase from thermophilic bacterium PS-3

For experimental details see the Materials and methods section.

the combination of Mg^{2+} (1.0 mM) and iminobisphosphonate (1.0 mM). No loss of enzyme activity was observed during incubation with diethyl-pyrocabonate, indicating the synergistic effects of these two factors.

Fig. 3. Reverse-phase h.p.l.c. absorbance profiles of lysyl endopeptidase digests of native (a) and diethyl pyrocarbonate-modified (b) inorganic pyrophosphatase from thermophilic bacterium PS-3

The inorganic pyrophosphatase (100 nmol of monomer) was modified with diethyl pyrocarbonate. The conditions for the modification and digestion and the details of the experiments are given in the Materials and methods section.

Table 1. Amino acid compositions of histidine-containing peptides from inorganic pyrophosphatase from thermophilic bacterium PS-3

Compositions are given relative to lysine. The numbers in parentheses were calculated from the primary structure of the inorganic pyrophosphatase from thermophilic bacterium PS-3 from residue 96 to 119 for peaks 10 and X, and from residue 122 to 131 for peak 11.

Amino acid	Amino acid composition (mol of residue/mol of peptide)		
	Peak 10	Peak X	Peak 11
Asx	2.9 (3)	3.0 (3)	
Glx	4.4 (4)	4.3 (4)	2.4 (2)
Ser	0.8 (1)	0.8 (1)	
Thr			
Gly	1.0 (1)	0.9 (1)	
Ala			0.9 (1)
Val	2.6 (3)	2.6 (3)	
Ile	1.7 (2)	1.7 (2)	1.1 (1)
Leu	1.8 (2)	1.7 (2)	
Phe	0.8 (1)	0.5 (1)	2.1 (2)
Tyr			0.4 (1)
Pro	2.7 (3)	2.8 (3)	
Met			
Cys			
Lys	1 (1)	1 (1)	1 (1)
Arg	1.9 (2)	2.0 (2)	1.1 (1)
His	1.0 (1)	1.0 (1)	1.1 (1)

The difference spectrum for the ethoxycarbonylated enzyme and the native enzyme showed the appearance of a new positive peak at 242 nm. It has been established that the formation of an ethoxycarbonylhistidine residue results in the appearance of this absorption peak at 242 nm. Since no change was observed around 278 nm, it seemed that no tyrosine residue was modified. Furthermore, none of the tryptophan residues reacted with diethyl pyrocarbonate during the inactivation, as judged by magnetic c.d. measurement [12]. Fig. 2 shows the relationship between the residual activity and the number of ethoxycarbonyl-histidine residues per subunit calculated from the increase in absorbance at 242 nm. It is evident that one histidine residue per subunit is essential for the enzyme activity.

Isolation of an active-site-histidine-containing peptide

The complete inactivation of the inorganic pyrophosphatase from thermophilic bacterium PS-3 by diethyl pyrocarbonate was accompanied by the modification of one histidine side chain per monomer. Two histidine residues are present at positions 118 and 125 per monomer chain of the enzyme, so we tried to determine which one is responsible for the enzyme activity. The native and diethyl pyrocarbonate-treated enzymes were digested with lysyl endopeptidase in 10 mM-imidazole/HCl buffer, pH 6.1, after the enzymes had been denatured with 3 M-urea, and the resulting peptides were fractionated by reverse-phase h.p.l.c. on a TSK-GEL ODS 80TM column. Fig. 3 shows the chromatographic profiles of the two enzymes. Since the enzyme subunit contains 11 lysine residues, one of which is located at the C-terminus, 11 peptide fractions were expected on the chromatography. Indeed, 11 peptide fractions were observed in the elution profile of the native enzyme. The chromatographic profile of the diethyl pyrocarbonate-treated enzyme was nearly identical with that of the native enzyme except for one new peak (designated as peak X) between peaks 9 and 10. The same approach was used for the diethyl [¹⁴C]pyrocarbonate-treated enzyme, and radioactivity was only observed for new peak X, as

shown in Fig. 4(b). This peak X was not observed when the modification was carried out in the presence of Mg²⁺. Amino acid analysis of each fraction was carried out, which revealed that only the materials in peaks 10, 11 and X contained histidine. Furthermore, as shown in Table 1, the amino acid compositions of the materials in peaks 10 and X were identical. This was probably because some of the ethoxycarbonyl group was hydrolysed from the histidine residues during enzymic digestion and purification of the peptides. Since the h.p.l.c. on the ODS column was carried out in Mes buffer, pH 6.1, some ionic interaction might have affected elution of the peptide fractions, the diethyl pyrocarbonate-modified peptide (peak X) being eluted before the unmodified peptide (peak 10). The amino acid compositions of these peak materials indicated that the peak 10 (and peak X) material is a peptide comprising residues 96–119 of the primary structure and the peak 11 material is a peptide comprising residues 122–131. Indeed, the amino acid sequences of the peak 10 and peak X materials obtained with the automated sequencer were both LIGVPVEDPRFDEVRSDLPQHK (corresponding to residues 96–119) and that of the peak 11 material was EIAHFFERYK (corresponding to residues 122–131). These facts led us to conclude that the peak 10 material is the target of the diethyl pyrocarbonate reaction and that His-118 is essential for the enzyme activity.

DISCUSSION

The inorganic pyrophosphatase from thermophilic bacterium PS-3 is a trimer of identical subunits and aggregates into a hexamer in the presence of Mg²⁺, which is essential for the enzyme activity, accompanying a conformational change that can be detected on c.d. measurement: a new positive c.d. band at 293 nm appears [5]. This c.d. band at 293 nm seems to reflect the hexamer state of the enzyme molecule, since it was also observed for the *Escherichia coli* inorganic pyrophosphatase, which is a hexamer irrespective of the presence or the absence of Mg²⁺ and which shows considerable amino acid sequence similarity to the enzyme from thermophilic bacterium PS-3 [7,13–15]. The Mg²⁺-induced change in the c.d. band at 293 nm depends on the pH of the solution: it exhibits a pK_a of 6.6, suggesting the participation of histidine residue(s) in the formation of aggregates [5].

Table 2. Putative active-site residues of inorganic pyrophosphatases

Proposed function	Yeast enzyme*	<i>E. coli</i> enzyme*	Thermophilic bacterium PS-3 enzyme
Metal ion binding	Glu-48	Glu-20	Glu-12
	Glu-58	Glu-31	Glu-22
	Tyr-89	Tyr-51	Tyr-42
	Tyr-93	Tyr-55	Tyr-46
	Asp-115	Asp-65	Asp-56
	Asp-117	Asp-67	Asp-58
	Asp-120	Asp-70	Asp-61
	Asp-147	Asp-97	Asp-88
	Glu-148	Glu-98	Ser-89
	Glu 150	Gly-100	Glu 91
	Asp-152	Asp-102	Asp-93
	Tyr-192	Tyr-141	Tyr-130
Substrate binding	Lys-56	Lys-29	Lys-20
	Arg-78	Arg-43	Arg-34
	Lys-154	Lys-104	Lys-94
	Lys-193	Lys-142	Lys-131
	Lys-193	Lys-148	Lys-136
No. of identical matches		16	16

* Data taken from ref. [18].

The results of this work indicated that ethoxycarbonylation almost completely inactivated the inorganic pyrophosphatase from thermophilic bacterium PS-3 and the amino acid residue modified by diethyl pyrocarbonate was identified as His-118. The Mg^{2+} -induced change in the c.d. spectrum was not observed for the diethyl pyrocarbonate-modified enzyme (results not shown). Mg^{2+} considerably protected the enzyme molecule against the inactivation by diethyl pyrocarbonate in both concentration-dependent and pH-dependent manners. The ethoxycarbonylated peptide was not observed on chromatography of a lysyl endopeptidase digest of the enzyme when the modification was carried out in the presence of Mg^{2+} . Furthermore, the Mg^{2+} -concentration-dependence of the protection against the inactivation by diethyl pyrocarbonate and that of the change in the c.d. band at 293 nm showed good correlation. The substrate analogue iminobisphosphonate did not affect the rate of inactivation when added alone. From these findings it might be expected that the active form of the enzyme molecule is a hexamer and that His-118 is included in the binding site for Mg^{2+} .

However, a histidine residue is not included among the 12 polar amino acid residues required for the binding of three Mg^{2+} ions per subunit of the yeast enzyme, which are predicted from the results of crystallographic studies [16,17]. Lahti *et al.* [18] found, on the alignment of the primary structure of the *E. coli* enzyme with that of the yeast enzyme, that all these polar residues required for the Mg^{2+} binding except one, as well as five basic residues that could plausibly interact with a substrate, are located at the corresponding positions in the *E. coli* enzyme. Lahti *et al.* [19] also revealed the importance of Asp-97 and Asp-102 for the catalytic activity and of Glu-98 and Lys-104 for the structural integrity of the *E. coli* enzyme through a study involving site-directed mutagenesis. When the amino acid sequences of the *E. coli* and thermophilic bacterium PS-3 enzymes were aligned optimally [7], all these residues, except Glu-98, which was replaced by Ser-89, were found to be conserved at the corresponding positions in the thermophilic bacterium PS-3, as summarized in Table 2. Furthermore, no histidine residue was observed at the position corresponding to His-118 in the *E. coli* enzyme. These facts might suggest that His-118 is not included in the binding site for Mg^{2+} , but it is located in the vicinity of the Mg^{2+} -binding site and thus affects the binding of Mg^{2+} to one of the polar residues listed in Table 2. As stated above, Glu-98 in the *E. coli* enzyme is replaced by Ser-89 in the thermophilic

bacterium PS-3 enzyme. If Ser-89 is really one of the Mg^{2+} -binding-site residues, then the hydroxy group of the serine residue must be activated. Thus we can suggest that His-118 and Ser-89 are in close proximity to one another, and that the imidazolyl nitrogen atom of His-118 acts as a hydrogen-bond acceptor for the Ser-89 hydroxy group, thereby facilitating interaction at the Ser-89 oxygen atom with Mg^{2+} . As a result of this relay system, Mg^{2+} may bind to Ser-89.

We thank Professor Y. Kagawa and Professor N. Sone of Jichi Medical School for donating the cell-free extract of thermophilic bacterium PS-3.

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Received 13 November 1990/18 March 1991; accepted 27 March 1991