Characterization of zinc-binding properties of a novel imidase from *Pseudomonas putida* YZ-26

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**Abstract**

The imidase from *Pseudomonas putida* YZ-26 consisting of 293-amino acid residues is a novel imidase with four subunits as the holo-enzyme and low molecular weight which is significantly different from known mammalian imidase. This study measured the zinc-binding properties of the imidase using inductively coupled plasma-atomic emission spectrometry and competition assay combined with activity determinations. Results show that each subunit of the imidase binds the zinc ion by 1:1 stoichiometry with apparent binding constant of 9.5 × 10⁻⁸ M⁻¹. The activity of the apo-imidase (20 μM) was recovered with the addition of zinc in the lower concentration (0–20 μM), whereas the enzymatic activity is decreased in the presence of high concentration of zinc (above 100 μM). The site-directed mutagenesis of His247, His58 or Cys7, Cys108 in imidase resulted in loss of activity and zinc-binding abilities at different degrees, showing that these residues may critically affect both enzymatic activity and conformation.

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**Introduction**

Cyclic imide-transforming activity is widely distributed in bacteria, yeasts, and molds [1]. However, the activity differs from the cyclic ureide-transforming activity in bacteria. The two kinds of activities, performed by only one enzyme (dihydropyrimidinase) in mammalian, are catalyzed by hydantoinase (EC.3.5.2.2) and imidase (cyclic imide hydrolase, CIH, EC.3.5.2.16) in bacteria, respectively [2]. Ogawa et al. initially reported that imidase coexists in *Blastobacter* sp. A17p-4 [3]. The imidase has a broad substrate spectrum and accepts different imides, dihydropyrimidines, and hydantoin derivatives. In contrast to dihydropyrimidinase, the imidase hydrolyzes cyclic imides and rejects 5'-monosubstituted hydantoin as substrates [4]. The imidase that is involved in cyclic imide metabolism in bacteria catalyzes the ring-opening hydrolysis of cyclic imide to half-amide and the resulting half-amide is hydrolyzed by half-amidase to dicarboxylates, which then undergoes further transformation through tricarboxylic acid (TCA) cyclic reaction [3]. Both imidases from bacteria and mammalian have attracted increasing attention because they activate proteins and enzymes, or stabilize protein conformation, transcription factors. Generally, zinc can function as agents to either activate proteins and enzymes, or stabilize protein conformation, to maintain the physiological activities of cells [7]. Mammalian imidase, similar to hydantoinase with a subunit molecular mass of about 50–60 kDa, is a known tetramer containing four tightly bound zinc atoms [8–10]. The structural information and biochemical characteristics of bacterial imidase with a subunit molecular mass of about 30–40 kDa are not well documented except for *Blastobacter* sp. A17p-4 [3], *Alcaligenes eutrophus* 112R4, and *Pseudomonas putida* YZ-26 [11]. In this paper, we further investigated the relationship of zinc and imidase from *P. putida* YZ-26 using inductively coupled plasma-atomic emission spectrometry (ICP-AES), as well as through competition tests and gene mutation.

Our data show that imidase is a zinc-dependent enzyme. The molar ratio of zinc to its subunit is 1–2. Zinc has a dual effect on the imidase activity, whereas H₂₄⁷, H₅₈, and C₇,₁₀₈ may be involved in zinc-binding.

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1. Abbreviations used: ICP-AES, inductively coupled plasma-atomic emission spectrometry; CD, circular dichroism; PAR, 4-(2-pyridylazo) resorcinol; MBP, maltose-binding protein; Ëš, PAR zinc-binding constant; Ëš, imidase zinc-binding constant; DEPC, diethyl pyrocarbonate; IAA, iodoacetamide; 8-HQSA, 8-hydroxyquinoline; EDTA, ethylenediamine tetraacetic acid; DPA, pyridine-2,6-dicarboxylate; IP TG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.
Materials and methods

**Bacterial strains, plasmids and medium**

The PCR primers and restriction endonucleases were purchased from TaKaRa Co. (Dalian Branch, China), *Escherichia coli* BL21 (DE3), *E. coli* DH5α, vector pMAL-s, and recombinant plasmids pE-cih were stored in the laboratory. Water was purified by reverse osmosis, and then followed by passage through a Millipore Reagent Water System (Millipore Co., Bedford, MA), The ZnCl2, Colorimetric Zn-chelator PAR (4-(2-pyridylazo) resorcinol), and DL-hydantoin were purchased from Sigma–Aldrich Co. (St. Louis, USA). A UV-2010 spectrophotometer (Hitachi, Japan) was used to measure ultraviolet absorption spectra. Amylose Column, Superdex-200, and Phenyl-Sepharose FF were purchased from GE Healthcare (NJ, USA).

**Plasmid construction and site-directed mutagenesis**

The imidase mutants corresponding to Cys7,108→Gly, His151→Gly, His247→Ala, and His86→Ala (CC7,108/GG, H151/G, H247/A, H86/A) were introduced using the PCR method. The desired PCR products were recovered from 1% agarose gel and digested with EcoRI and HindIII. Digestion products were ligated into a pMAL-s expression vector, which were also digested with the same restriction endonucleases to generate constructs, namely, pMAL-s-cih, pMAL-s-cih86, pMAL-s-cih247, pMAL-s-cih151, and pMAL-s-cih7,108. Plasmid pE-cih containing the *P. putida* YZ-26 imidase gene was used as a template. The mutation primers used in this study are listed in Table 1.

**Expression and purification of imidase**

Both imidase and its mutants were fused with maltose-binding protein (MBP) tag at the N-terminus for expression. Recombinant plasmid was transformed into *E. coli* BL21 (DE3) host cells for large-scale protein production. One liter of bacterial culture of *E. coli* BL21 (DE3) supplemented with 100 μg/ml of ampicillin was grown to an optical density (OD600) of 0.7 before induction protein (MBP) tag at the N-terminus for expression. Recombinant large-scale protein production. One liter of bacterial culture of *E. coli* BL21 (DE3) supplemented with 100 μg/ml of ampicillin was grown to an optical density (OD600) of 0.7 before induction was performed for 4–6 h before centrifugation, the product in the supernatant, N-carbamyl-glycine, was measured at 430 nm, and then calculated in a standard calibration plot.

One unit of the activity was defined as the amount of enzyme catalyzing the hydrolysis of the substrate at a rate of 1 μmol/min under the assay conditions described above.

**Determination of metal ions**

Zinc-imidase was purified as previously described except for all buffers containing 50 μM zinc. Purified imidase or zinc-imidase was dialyzed in the solution 10 mM Tris–HCl, pH 7.5, at 4 °C for 48 h, and under four changes using fresh buffer. Protein concentration was determined by A280 (ε = 66,015 M–1 cm–1). Divalent metal contents of the imidase and zinc-imidase were measured by ICP-AES (Autum Scan 16) at the Shanxi Institute of Coal Chemistry of Chinese Academy of Sciences. The final external dialysate was used as the blank for metal ion determination. Each sample was tested thrice, and standard deviation was calculated.

**Dual effects of zinc on imidase**

The activity of the purified recombinant imidase was completely eliminated with the addition of 6 mM pyridine-2,6-dicarboxylate (DPA) at 4 °C for two days. The DPA imidase solution was dialyzed against a buffer of 10 mM Tris–HCl, pH 7.5, at 4 °C for two days, and through four changes of fresh buffer, to obtain the apo-imidase.

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**Table 1**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Residue change</th>
<th>The sequence of primers</th>
<th>Primer name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidase (WT)</td>
<td>None</td>
<td>5′-CGGAATTCTAGGGCAAGGAAATC-3′</td>
<td>FW</td>
</tr>
<tr>
<td>H247/A (mutant)</td>
<td>His247 → Ala</td>
<td>5′-CATTACATCGCAGCAGGATT-3′</td>
<td>FW247</td>
</tr>
<tr>
<td>CC7,108/GG (mutant)</td>
<td>Cys7 → Gly, Cys108 → Gly</td>
<td>5′-CAGTCCGAGGACTCGACCT-3′</td>
<td>FW108</td>
</tr>
<tr>
<td>H151/G (mutant)</td>
<td>His151 → Gly</td>
<td>5′-CAGTCCGAGGACTCGACCT-3′</td>
<td>FW151</td>
</tr>
<tr>
<td>H86/A (mutant)</td>
<td>His86 → Ala</td>
<td>5′-CGGTGAGCAGGCGATCAGGAC-3′</td>
<td>FW186</td>
</tr>
</tbody>
</table>
Zinc chloride with different concentrations (0.25 μM–1.5 mM) was added into the solution containing apo-imidase (20 μM), 10 mM Tris (pH 7.5). The mixture was incubated at 4 °C for 1 h and the activity of reconstituted enzyme was determined using standard assay.

**Determination of zinc-binding constant**

To estimate the binding affinity of imidase with zinc, a competition experiment was performed as follows: apo-imidase was titrated into 60 μM 4-(2-pyridylazo) resorcinol (PAR), a zinc particularly metallochromic indicator, and 4 μM ZnCl₂ in 20 mM Hepes (pH 7.5). Samples were equilibrated for 3 min at room temperature, and the absorbance was monitored at 500 nm. Equilibrium calculations were conducted using a PAR/zinc-binding constant (K_{app}) of 3.2 × 10^{11} M⁻² and a molar absorption of 61,500 M⁻¹ cm⁻¹ for the Zn(PAR)₂ complex. An equilibrium expression combining zinc binding to both imidase (K_{imidase}) and PAR (K_{app}) was used in the calculations (K_{imidase}:K_{app} = [Imidase/Zn][PAR]_{2}/[Zn(PAR)₂][Imidase]) [14]. The individual components of the expression can be calculated as follows:

\[
\frac{[\text{Zn(PAR)}₂]}{[\text{PAR}]} = \frac{A_{500}}{\Delta A_{500}} \text{ where } \Delta A_{500} = 61,500 \text{ M}⁻¹ \text{ cm}⁻¹
\]

\[
\text{[PAR]} = [\text{PAR}]_{\text{total}} - 2[\text{Zn(PAR)}₂]
\]

\[
[\text{Imidase}/\text{Zn}] = [\text{Zn(PAR)}₂]_{\text{imidase}} - [\text{Zn(PAR)}₂]
\]

\[
[\text{Imidase}] = [\text{Imidase}]_{\text{imidase}} - [\text{Imidase}/\text{Zn}]
\]

Absorbance and spectra were measured at 25 °C using a U-2010 spectrophotometer.

**Competition between modified or mutant imidase and PAR for zinc**

To test whether histidine and cysteine were involved in the activity site of the imidase, the enzyme was incubated with 1 mM diethyl pyrocarbonate (DEPC) or iodoacetamide (IAA) at 4 °C for 1 h. After modification by DEPC or IAA, the binding ability of zinc with imidase (DEPC) and imidase (IAA) was evaluated using a competition assay.

The apo-H₂₄₇/A, apo-H₁₅₁/G, apo-H₈₆/A and apo-CC₇,₁₀₈/GG were prepared similar to the apo-imidase, and then added the solution of 20 mM Hepes (pH 7.5) containing 60 μM PAR, 4 μM zinc to estimate the zinc-binding ability using a competition assay.

**Prediction of the structure of the imidase**

The homolog of the imidase was collected through a running PSI-BLAST with default parameters. Then the sequence-structure homology recognition software GUGUE was run with default parameters for the sequence of imidase. For homology modeling, the preliminary sequence alignment between imidase and the most potential template was submitted to the Swiss-Model server. Finally, the returned models were optimized by performing 100 steps of conjugated gradient energy minimization using Swiss-Pdb Viewer default parameters.

**Results**

**Expressing and purification of recombinant and mutant imidases**

Five engineered E. coli BL21 strains containing recombinant plasmids, namely, pMAL-s-cih, pMAL-s-cih₂₄₇, pMAL-s-cih₁₅₁, pMAL-s-cih₈₆, and pMAL-s-cih-cys, were cultured in 37 °C for 4–6 h to produce the corresponding enzymes. Thereafter, the recombinant imidase and its mutants were purified by amylose beads and hydrophobic columns to produce homogeneous proteins with about 25% yield (Fig. 1).

**Determination of metal ions**

The 8-hydroxyquinoline (8-HQSA), EDTA, and DPA, as normal metal chelators, usually inhibit the activities of metal or metal-dependent enzyme by depriving the enzyme of metal ions. The effect of metal chelators on imidase activity was investigated using 0.1–5 hydantoin as a substrate. A recommended amount of imidase was incubated at 4 °C for 3 h together with one of the following metal chelators: 8-HQSA (5 mM), EDTA (5 mM), and DPA (5 mM). Subsequently, the residual activity of the imidase was assayed under standard condition. The residual activities were calculated as 9.4%, 43.6%, and 20.8%, respectively. The metal content of imidase was determined by ICP-AES (Yontal Scan 16), as listed in Table 2. Growth conditions significantly affected the metal content of the enzyme. The purified imidase, less the addition of any metals, revealed 0.514 mol zinc, 0.102 mol cobalt, and 0.179 mol nickel per subunit. Meanwhile, enzyme purified from cells grown in zinc-supplemented medium contained an approximate stoichiometric amount of the metal (1.65 mol zinc per subunit). Neither cobalt nor nickel was detected in the enzyme. Each of the metal reconstituted-imidase contained approximately 1–2 atom of tightly bound metal per enzyme subunit. Therefore, zinc may be the main metal ion used for imidase from P. putida YZ-26.

**Dual effect of zinc on imidase**

A DPA was used for the removal of zinc from the active site of the imidase. The loss of its activity was coordinated with the decrease of zinc from the active site (Fig. 2A). The activity of the apo-imidase was reactivated with the addition of zinc (Fig. 2B). The stoichiometry of one zinc atom to each subunit was also

**Table 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zn⁺²</th>
<th>Co⁺²</th>
<th>Ni⁺²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc-imidase</td>
<td>1.650 ± 0.001b</td>
<td>0.000 ± 0.001</td>
<td>0.000 ± 0.001</td>
</tr>
<tr>
<td>Imidase</td>
<td>0.514 ± 0.001</td>
<td>0.102 ± 0.001</td>
<td>0.179 ± 0.001</td>
</tr>
</tbody>
</table>

*a* Equivalents of metal/subunit of imidase.

*b* Values are shown as means ± SD and each value was measured in triplicate.
The activity of the apo-imidase was recovered with the addition of zinc (0–20 μM), whereas enzymatic activity was decreased under high concentrations of zinc (above 0.1 mM) (Fig. 2C). The higher concentration of zinc might have caused the denaturation of protein due to conformational change; in turn, the protein was precipitated. Aggregation or precipitation produced by zinc was also observed in the mammalian imidase [23].

Determination of zinc-binding constant

The zinc-binding ability of the imidase was evaluated using a competition assay with PAR. At pH 7.5, the PAR and zinc can form a 2:1 complex under a binding constant of $3.2 \times 10^{11} \text{ M}^{-2}$ [14]. Incremental additions of apo-imidase to the zinc solution and an excess of PAR in 20 mM Hepes (pH 7.5) resulted to the decrease of a fraction of the PAR in the Zn(PAR)$_2$ complex, the forming zinc/imidase complex, thus, resulting in a diminution of the Zn(PAR)$_2$ signal observed at 500 nm (Fig. 3A). The binding constant for zinc/imidase complex was $9.5 \times 10^8 \text{ M}^{-1}$. The PAR is a special zinc-indicator that binds zinc in a 2:1 complex at a strong absorbance of 500 nm. This complex has no effect on the determination of imidase activity at 430 nm. After incubation under different concentrations of PAR at 4°C for 1 h, the imidase activity was determined using a standard assay. Results are shown in Fig. 3B. When PAR’s concentration reached 1 mM, residual activity was at 16.6% in relation to the recombinant imidase, confirming the imidase as a zinc-containing enzyme.
Competition between mutant imidases and PAR for zinc

When zinc appeared to play a critical role in the imidase, the study attempted to identify which sites or residues in the enzyme might contribute in zinc-binding even if there were no information available on any of its structure. Cysteine and histidine are commonly regarded as the most prevalent activity centers or the zinc-coordinating residues in most enzymes. The His and Cys residues of imidase were modified by DEPC and IAA, respectively. The DEPC specifically carbethoxylates the imidazole of histidines in neutral pH conditions and IAA, specifically alkylates the sulfhydryl group of the cysteines. Compared to the native enzyme, the modified enzyme decreased zinc-binding ability as shown in the PAR competition assay (Fig. 4A), indicating that the relative activities of the modified imidases were all approximately 25% compared to the wild type (Fig. 4B). It is suggested that histidines and cysteines in the imidase have an effect on zinc-binding. According to the modeling structure (see next section), we assumed that the histidines (86, 247) and cysteines (7, 108) may be involved in zinc-binding. In the competition with zinc assay, the decrease in absorbance at 500 nm of native imidase (53.1%) was more remarkable than in H247/A, H86/A and CC7,108/GG, which made the 500 nm absorbance decrease by 32.4%, 14.7%, and 12.8%, respectively. However, the native imidase’s ability of for zinc to compete with PAR is slightly lower than mutant H151/G (Fig. 5A). Accordingly,

\[ H_{247}/A, \text{H}_{86}/A, \text{and CC}_{7,108}/G \text{G have a relatively lower activity compared with native enzyme, except in H}_{151}/G, \] which was calculated as having 120% activity (Fig. 5B). These data suggest that two cysteine residues (C_{7} and C_{108}), H_{86} and H_{247}, are involved in zinc-binding. The secondary structure of wild-type imidase is more compact than apo-imidase based on circular dichroism, which is different from mammalian imidase [23]. In contrast, the secondary structure of the mutants of H_{86}/A and CC_{7,108}/GG were more relaxed than in the native imidase. Due to the observed decreasing zinc-binding capacity of H_{86}/A and CC_{7,108}/GG, we assume some conformational changes in the zinc-binding site in imidase. Moreover, H_{247}/A greatly decreased the regular structure of imidase, and probably not only for zinc-binding, but on imidase structure stability as well. It is surprising to note that the mutant of H_{151}/G made the secondary structure of imidase more compact than in the wild-type imidase. Therefore, it may be that H_{151}/G mutant could binds easily with zinc and has a relatively higher activity than native enzyme (Fig. 6). Based on collated data, the potential target sites for the evaluation of zinc-binding were His_{247}, His_{86}, Cys_{7}, and Cys_{108}.

Homology model of imidase

Information from FUGUE and PSI-BLAST server showed that carbohydrate esterase from Pseudomonas aeruginosa was the best homology structure with imidase (Z-score = 33.49; >99% Confidence) [15]. The sequence of imidase and the potential modeling templates were submitted to the Swiss-Model server, and then
YZ-26 is probably a zinc-dependent enzyme. It is notable that the chelators. Thus, we speculate that the imidase from metallochromic indicator of zinc, exhibited more inhibition than significant effect on activity[20–22]. The PAR, a sensitive high-affinity toinase wherein chelators and metal additions exhibit a more sig-

ificant role in imidase activity. Although the secondary structure of imidase underwent minor alteration following metal removal, the tetrameric structure of imidase was not affected as determined by gel filtration (data not shown). Thus, zinc may have focused on combining the enzyme and substrate.

The zinc-binding properties of imidase from P. putida YZ-26 is conferred by the histidine and cysteine residues since modification these residues by DEPC or IAA would abolish this activity. It is likely that this is true for the zinc coordination site in the imidase from P. putida YZ-26. This is based on the studies of mutants of his-
tidines and cysteines in imidase wherein H247, H68 are located in the internal part of the imidase, while H151 lies on the surface of enzyme from the Homology model of imidase. The H247, H68, and C7,108 may be directly involved in zinc-binding and play a key role in the catalysis of imidase, because their corresponding mutants, H247/A, H68/A and CC7,108/GG have shown significantly lower spe-
cific activity and zinc-binding capacity than in the wild type. Fur-
thermore, since the H151/G mutant with enhanced enzyme activity had a slightly higher affinity with zinc than the wild type of imidase, we speculate that H151/G may contribute to a more sta-
ble transition state that is more optimal for zinc-binding.

Discussion

Imidase, widely distributed in living organisms, catalyzes the hydrolysis of a variety of imides. To date, no structural data have been reported on imidase with a subunit molecular mass of about 30–40 kDa in bacteria, although structural information on dihydro-
pyrimidinase (hydantoinase), which belong to zinc enzymes or zinc-dependent enzymes, are sufficiently available [16–19].

To examine whether imidase activity is influenced by the metal chelators, the enzyme assay was performed in both the presence and absence of EDTA, 8-HQSA, and DPA. As expected, chelators inhibited the imidase activity, which is consistent with some allan-
toinase wherein chelators and metal additions exhibit a more signif-
ificant effect on activity [20–22]. The PAR, a sensitive high-affinity metallochromic indicator of zinc, exhibited more inhibition than the chelators. Thus, we speculate that the imidase from P. putida YZ-26 is probably a zinc-dependent enzyme. It is notable that the stoichiometry of imidase-zinc is 1:1 at mole ratio with a binding constant of $K_a = 9.5 \times 10^8 \text{ M}^{-1}$, which also have been confirmed by the analysis of ICP-AES. Imidase purified from the supplemental zinc in culture typically contains more stoichiometric levels of zinc. However, we could not detect any cobalt or nickel in this form of enzyme, which implies that too much zinc can replace cobalt and nickel in imidase, or limit cobalt and nickel transport. It also can be deduced from results that the imidase may contain another zinc-binding site, which has a relatively low affinity for zinc ions and is easily dissociated by dialysis.

Only one, tightly bound zinc is required for the imidase activity, because the isolated enzyme exhibits significant activity. Addition of extrinsic zinc ions does not enhance the enzyme activity. Upon addition of zinc to apo-imidase, an increase in the imidase activity was observed until one equivalent of added zinc was reached. However, above five equivalents of zinc caused a decrease in the imidase activity (Fig. 2C). Results suggest that zinc acted as an activ-
ator, as well as an inhibitor, for the enzyme activity. This is similar to the mammalian imidase, a zinc enzyme, which also loses its activity in high zinc concentration [10]. Previous investigations have postulated that metals (zinc, manganese, and cobalt) are involved in imide hydrolysis and act as a general base [17,23,24]. Results in this study demonstrate that zinc is necessary for the imidase from P. putida YZ-26, which is unlike in rat liver imidase [23]. Correlating with the analysis of ICP-AES, we speculate that zinc, which displays a high-affinity with imidase, plays a signifi-
cant role in imidase activity. Although the secondary structure of imidase underwent minor alteration following metal removal, the tetrameric structure of imidase was not affected as determined by gel filtration (data not shown). Thus, zinc may have focused on combining the enzyme and substrate.

Acknowledgments

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