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Biochemical and Biophysical Research Communications 297 (2002) 1027–1032

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# The role of metal on imide hydrolysis: metal content and pH profiles of metal ion-replaced mammalian imidase☆

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Received 28 August 2002

**Abstract**

Imidase catalyzes the hydrolysis of a variety of imides. The removal of metal from imidase eliminates its activity but does not affect its tetrameric and secondary structure. The reactivation of the apoenzyme with transition metal ions $Co^{2+}$, $Zn^{2+}$, $Mn^{2+}$, and $Cd^{2+}$ shows that imidase activity is linearly dependent on the amount of metal ions added. $Ni^{2+}$ and $Cu^{2+}$ are also inserted, one per enzyme subunit, into the apoimidase, but do not restore imidase activity. Enzyme activity with different metal replaced imidase varies significantly. However, the changes of the metal contents do not appear to affect the $pK_a$s obtained from the bell-shaped pH profiles of metal reconstituted imidase. The metal–hydroxide mechanism for imidase action is not supported based on the novel findings from this study. It is proposed that metal ion in mammalian imidase functions as a Lewis acid, which stabilizes the developing negative charge of imide substrate in transition state.


*Keywords:* Imidase; pH profile; Metal replacement; Transition metal

Imidase is also known as dihydropyrimidinase (EC 3.5.2.2), dihydropyrimidine hydrase, or dihydropyrimidine amidohydrolase that catalyzes the hydrolysis of a variety of imides. Imide-hydrolyzing enzymes are widely distributed in living organisms and were first described in the 1940s as occurring in plants and animals [1,2]. In the 1950s, an imide-hydrolyzing enzyme isolated from calf liver was shown to use dihydropyrimidine (dihydrouracil and dihydrothymine) as a nature substrate and was known as dihydropyrimidinase [3]. In vitro data indicate that mammalian imidase prefers many xenobiotics to physiological substrates [4]. The substrate spectrum further extends to cyclic carbonate [5]. A recent finding shows that the substrate specificity of a thermophilic mammalian imidase is temperature dependent [6].

Mammalian imidase is found to be a tetramer that contains four tightly bound zinc atoms [7–9]. Significant variations are found in sequence, subunit composition, and metal content for enzymes catalyzed with similar reactions from different organisms. Imide-hydrolyzing enzyme from microorganism is normally known as hydantoinase which is a dimer, contains one manganese atom per dimer from *Bacillus stearothermophilus* SD-1 [10], but is a trimer from *Blastobacter* sp. A17p-4 [11]. Imide-hydrolyzing enzyme from *Pseudomonas putida* 77 requires ATP for the amidohydrolysis [12]. Hydantoinase from *Arthrobacter aurescens* DSM 3745 is a zinc enzyme [13], containing 10 mol zinc/mol enzyme [14]. Very recently, the crystal structures of bacterial hydantoinase from *Thermus* sp. [15] and *B. stearothermophilus* [16] have been reported. They contain a bridged dimetal ion per enzyme subunit. The structure of imide-hydrolyzing enzymes obtained from microorganism offers important information for the understanding of the role of metals in the function of these enzymes. However, it is intriguing to find that the number and the type of metals vary among imide-hydrolyzing enzymes from organism to organism. The mechanism for Imide-hydrolyzing enzyme is poorly understood.

☆ *Abbreviations:* EDTA, (ethylenedinitrilo)tetracetic acid; Bis–Tris propane, 1,3-bis(tris(hydroxymethyl)methylamino)propane; MES, 4-morpholine ethanesulfonic acid; ICP-MS, inductively coupled plasma-mass spectrometry; 8-HQSA, 8-hydroxy-5-quinolinesulfonic acid; DEPC, diethyl pyrocarbonate.

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PII: S0006-291X(02)02330-6

The substitution of zinc in imide-hydrolyzing enzymes has been carried out for cobalt [7], manganese [17], and cadmium [18]. It has been proposed that a single group should be protonated for the imidase activity [17] and that may be a metal–water complex ionization [18]. However, the differences in p$K_a$ values among different metal–water complex as indicative of the formation of the metal–hydroxide complex [19] have not been observed previously. Further analysis of the metallic effect on imidase is needed to reveal the role of metal on imidase action. We report here the divalent metals that bind, activate or inactivate imidase, and affect the activity of imidase.

## Experimental procedures

*Materials*. The resistance of the water used, which was purified by reverse osmosis and subsequently by the Millipore Reagent Water System (Millipore, Bedford, MA), was more than 18 MΩ. Octyl Sepharose CL-4B, DEAE Sephacel, chelating Sephacel (fast flow), gel filtration calibration kit, HiTrap desalting column, and Sephacryl S-300 (HR) were purchased from Pharmacia. Bis–Tris propane, PMSF, 8-hydroxy-5-quinolinesulfonic acid (8-HQSA), and phthalimide were purchased from Sigma (USA). EDTA, sodium chloride, sodium hydroxide, Tris–HCl, and zinc acetate were obtained from J.T. Baker (USA). All other chemicals were obtained commercially at the highest purity possible.

*Preparation of apo-imidase*. Pig liver imidase was purified as described previously [6] except that all the buffers used did not contain EDTA. The preparation procedures of apo-imidase followed with some modifications the published procedures [17,18]. Purified native imidase (1.5–2 mg/ml; 10–15 ml) was dialyzed against a chelating buffer (2.5 L) that contained MES (50 mM), 8-HQSA (15 mM), and EDTA (1 mM) at 25 °C, pH 6.5, for 4 days and two changes of fresh chelating buffer. Following the above treatment, the activity of imidase was completely eliminated. Metal and other small molecules were removed following incubation by a HiTrap desalting column eluted with potassium phosphate buffer (pH 7, 10 mM) at 4 °C. EDTA alone is found to be a poor chelator for removing zinc from imidase at pH 6–8.

*Metal derivatives of the reconstituted imidase*. The apo-imidase (4 mg in 4 ml) was dialyzed at 4 °C for 4 days in a HEPES buffer (4 L, 50 mM at pH 7) plus one of the following metals: $Ba^{2+}$ (5 mM), $Co^{2+}$ (5 mM), $Mn^{2+}$ (5 mM), $Zn^{2+}$ (0.1 mM), $Cd^{2+}$ (0.1 mM), $Mg^{2+}$ (5 mM), $Ca^{2+}$ (5 mM), $Ni^{2+}$ (5 mM), or $Cu^{2+}$ (0.05 mM). To prevent the precipitation of imidase, the concentration of some metal ions was lower than the others. A desalting column (HiTrap from Pharmacia) was used to separate excess metal ions and the metal-reconstituted imidase.

*Inductively coupled plasma-mass spectrometry*. Concentrations of zinc, barium, cobalt, manganese, cadmium, magnesium, calcium, nickel, and copper were determined by inductively coupled plasma-mass spectrometry (Elan 5000, Perkin–Elmer, USA). Each sample was placed in potassium phosphate buffer (10 mM) at pH 7. Water used in this study was found to contain $0.26 \pm 56\%$ ppb ($10^{-9}$ g/ml) zinc. Potassium phosphate buffer (10 mM) was found to contain $4.5 \pm 1\%$ ppb zinc and was used as background for zinc determination. The backgrounds for other metals, Ba ($1.0 \pm 10\%$ ppb), Mg ($6.5 \pm 1\%$ ppb), Ca ($180 \pm 2\%$ ppb), Ni ($1.4 \pm 4\%$ ppb), Mn ($0.4 \pm 40\%$ ppb), Cu ($1.7 \pm 5\%$ ppb), and Cd ($0.25 \pm 50\%$ ppb), were also determined. The background data indicated that a significant amount of calcium was contaminated in the potassium phosphate buffer. Calcium is not found in metal reconstituted imidase (Table 1). Our data show that the imidase activity measured in this study is not likely due to the contamination of zinc or other metals by the buffer or water used. Measurements for each sample were repeated five times and the standard deviation was calculated. Normally, two or more samples were used for each determination by ICP-MS. Less than 1 ml enzyme sample (0.05–0.2 mg/ml), imidase, apo-imidase, or metal-reconstituted imidase, was used for each metal determination. In a typical experiment, ICP-MS gave $278 \pm 2\%$ ppb of zinc for native imidase (0.142 mg/ml), the molar ratio of zinc to native imidase monomer being $1.01 \pm 0.02$.

*Enzyme assay*. A rapid spectrophotometric assay [4] was used as the standard assay. The decrease in absorbency at 298 nm was measured upon hydrolysis of phthalimide used as the substrate at 25 °C. To start the reaction, the purified imidase was added into a 1 ml solution, containing phthalimide (1 mM) and Bis–Tris propane (100 mM) at pH 7.0. Under these conditions, a change in $A_{298}$ of 2.26 represented the hydrolysis of 1 μmol of the substrate. The hydrolysis of imide was monitored with a UV/VIS spectrophotometer (Hitachi U 3300).

*Protein concentration*. The protein concentration of imidase was determined by $A_{280}$ ($\varepsilon = 44.25\ cm^{-1}\ mM^{-1}$). The extinction coefficient was calculated according to the amino acid content of rat liver [20] and human liver [21] according to a published method [22]. For the homogeneous imidase, 1 U of $A_{280}$ equals 1.2 mg/ml imidase.

Table 1
Metal content and specific activity of native imidase and metal-reconstituted imidase[a]

| Metal-reconstituted imidase | Metal per enzyme subunit (metal found) | Size of metal ion[b] (pm) | Specific activity (μmol/min $A_{280}$)[c] | | |
|---|---|---|---|---|---|
| | | | pH 7 | pH 8 (Bis–Tris propane) | pH 8 (phosphate buffer) |
| Native imidase | $1.01 \pm 0.02$ (Zn) | 74 | 7.9 | 9.4 | 6.5 |
| $Co^{2+}$-imidase | $0.91 \pm 0.01$ (Co) | 72 | 14.4 | 18.9 | 6.3 |
| $Mn^{2+}$-imidase | $0.90 \pm 0.01$ (Mn) | 80 | 1.8 | 2.3 | 1.4 |
| $Cd^{2+}$-imidase | $0.79 \pm 0.02$ (Cd) | 92 | 0.5 | 1.1 | 0.4 |
| $Ni^{2+}$-imidase | $0.84 \pm 0.02$ (Ni) | 69 | —[d] | | — |
| $Cu^{2+}$-imidase | $0.81 \pm 0.02$ (Cu) | 71 | — | | — |
| $Mg^{2+}$-imidase | $0.005 \pm 0.006$ (Mg) | 71 | — | | — |
| $Ca^{2+}$-imidase | $0.000 \pm 0.038$ (Ca) | 114 | — | | — |
| $Ba^{2+}$-imidase | $0.000 \pm 0.000$ (Ba) | 149 | — | | — |
| Apo-imidase | $0.037 \pm 0.002$ (Zn) | | — | | — |
| DEPC-imidase | $0.020 \pm 0.001$ (Zn) | | — | | — |

[a] Preparation of the metal-reconstituted imidase and the measurement of metal content by ICP-MS were described in Experimental procedures
[b] The datum are the radius of the metal ion with the lowest coordination number (4 or 6) and was obtained from [23].
[c] Specific activity was determined at least three times under standard assay condition with indicated buffer.
[d] — denotes no imidase activity that can be observed under the standard assay condition.

*The pH studies*. The initial rates of native and metal reconstituted imidase were determined over the pH range of 5.0–10.0. The reaction was buffered with 0.1 M MES and 0.1 M Bis–Tris propane in desired pH and imidase (about 10 μg) was added to start the reaction. The pH of buffers was determined at 25 °C. $K_m$ and $V_{max}$ were obtained by nonlinear regression (Enzyme Kinetics and SigmaPlot) using 11–15 measurements determined at substrate concentration range from 0.05 to 1 mM.

## Results and discussion

### *Characterization and reconstitution of apo-imidase with divalent metal ions*

Determined by ICP-MS, the de-activated imidase (apo-imidase) is shown to be free of zinc (Table 1). The tetrameric structure of imidase is not affected following metal removal as determined by gel filtration (data not shown). The secondary structure of imidase is mostly intact as observed by circular dichroism (Fig. 1) for apo-imdase and Zn-, Co-, and Ni-reconstituted imidase. Although the metal in DEPC treated imidase was also completely removed (Table 1), its secondary structure became fuzzy (Fig. 1) and its activity could not be restored with all the metals tested. This part of the experiment served as a control for the preparation of apo-imidse with a correct fold.

The insertion of a metal ion into imidase may be regulated by both the size and the property of the metal ions. The radii of these transition metal ions may vary within environment, particularly with a different coordination number [23]. As shown in Table 1, zinc can be replaced with other transition metals, such as $Co^{2+}$, $Mn^{2+}$, $Cd^{2+}$, $Ni^{2+}$, and $Cu^{2+}$, but not with alkaline earth metals such as $Mg^{2+}$, $Ca^{2+}$, and $Ba^{2+}$. Each of the metal reconstituted-imidases contains approximately one atom of tightly bound metal per enzyme subunit (Table 1 and Fig. 2A). It is intriguing that similar enzymes from microorganisms are found to contain two metal ions per protein subunits [13,14] and form a binuclear zinc center [15,16].

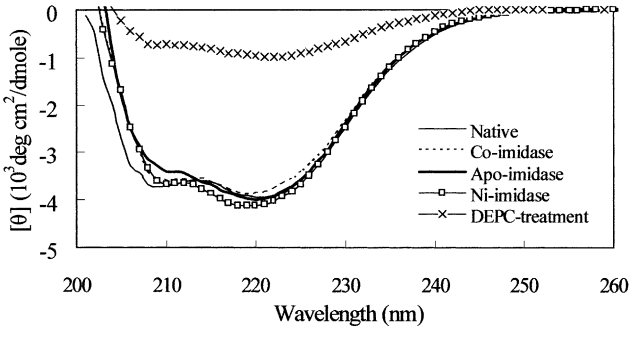


Fig. 1. Circular dichroism spectra of native, apo-, DEPC-treated, and metal-reconstituted imidase. The enzymes were prepared as described in Experimental procedures. The spectra were taken at room temperature by Jasco Spectropolarimeter J-715. Enzyme (0.3 mg in 3 ml) was in a potassium phosphate buffer (10 mM) at pH 7.

The activity of apo-imidase is reactivated upon the addition of zinc as shown in Fig. 2A and the stoichiometry of one zinc atom to one enzyme subunit is determined. Zinc becomes an inhibitor as soon as the first metal binding site is occupied (Fig. 2B). Too much zinc results in irreversible protein denaturation and the protein is precipitated. A similar inactivation of an enzyme by metal ions has been reported previously. Thermolysin is inhibited by an excess amount of a zinc ion, and it has been proposed that the second zinc ion interacts with histidine residue of the native thermolysin, blocking the active site for the substrate binding [24]. Dihydroorotase is also inhibited by an excess amount of zinc ions; however, upon removal of the metal ions, the enzyme activity is fully restored [25]. Aggregation or precipitation is observed when imidase becomes inactivated, as studied by gel filtration chromatography and scattering in UV–Vis spectrum (data not shown).

The activity of metal-reconstituted imidase is closely related to the size of metal ions. As listed in Table 1, the sizes of $Cd^{2+}$ and $Mn^{2+}$ are significantly larger than that

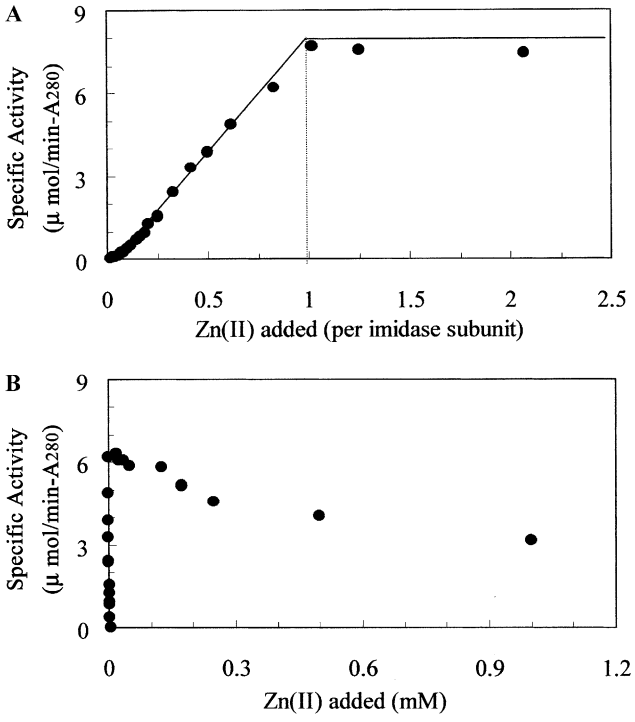


Fig. 2. Reactivation and inhibition of apo-imidase with zinc. An aliquot amount of $ZnCl_2$ (0.1 mM) was added into the solution containing apo-imidase (0.05 mg, prepared as described under Experimental procedures) and potassium phosphate (10 mM at pH 7) to 40 μl of final volume. The mixture was incubated at 4 °C for 3 days and the activity of reconstituted imidase was determined by standard assay. (A) Activity of imidase was proportional to the addition of the low concentration of $Zn^{2+}$ (0.25–20 μM). (B) High concentration of $Zn^{2+}$ (0.1–1 mM) inhibited activity of imidase.

of $Co^{2+}$ and $Zn^{2+}$. The specific activity of imidase (Table 1) follows the reverse trend that the larger the size of metal ions, the lower the specific activity of metal reconstituted imidase. The smallest metal ions ($Cu^{2+}$ and $Ni^{2+}$) reconstituted imidase do not exhibit enzymatic activity under our assay condition. The crystal structure of Co(II)-thermolysin suggests that the greater activity followed by metal replacement may be due to the enhancement of stabilization of the transition state than those of the native thermolysin [24].

*The pH profiles of metal-reconstituted imidase*

The pH profiles of V/K for the metal-reconstituted imidase (Co-, Zn-, Mn-, and Cd-imidase) determined in the pH range of 5.0–10.0 are presented in Fig. 3. Although the specific activities vary significantly with different metal-substituted imidases (Table 1), their pH dependencies are all similar; and p$K_a$ observed from these pH profiles would be alike (Table 2). These data show a similar pH dependency of activity for all the different metal-substituted imidases. The pH profiles of catalytic efficiency (V/K) for each metal-reconstituted imidase indicate that deprotonation of an acid with p$K_a$ around 6–6.3 and protonation of a base with p$K_a$ around 9.2–9.5 are important for imide hydrolysis (Fig. 3 and Table 2). A positively charged metal ion can stabilize developing negative charge in the transition state and serve as an electrophilic catalyst of the catalytic mechanism. The metal ion can also stabilize a highly reactive hydroxide ion, thereby ensuring that an activated nucleophile is available for catalysis at a physiological pH [19]. The later reaction mechanism is expected to depend on the acidity of the metal–water complex. The shift of pH profiles with different coordinated metals may reflect the ionization of an enzyme-metal aquo complex if it is important for the catalysis of imide hydrolysis. Since the change of metal ions does not alter the pH profiles, it suggests that metal–hydroxide does not play an important role for the catalysis of imide hydrolysis in imidase.

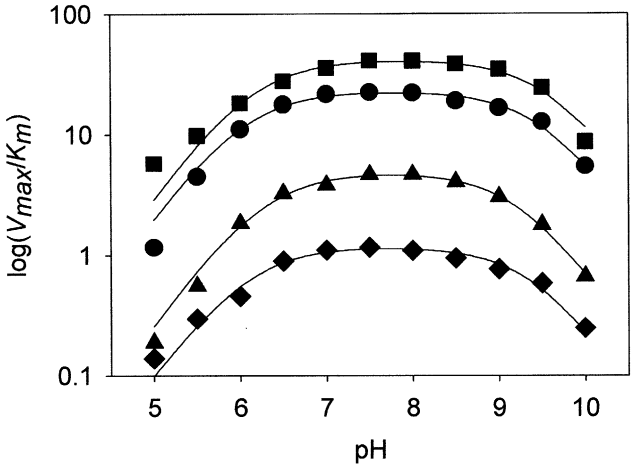


Fig. 3. The pH profiles of metal-reconstituted imidase. The pH profiles of $\log(V_{max}/K_m)$ are shown for Zn—(●), Co—(■), Mn—(▲), and Cd—(◆) imidase. The reaction condition and buffer for different pH are described under Experimental procedures. The unit of *Y*-axis is μmol/min/mg/mM.

Table 2
p$K_a$ values of metal derivatives of imidase[a]

| Metal-reconstituted imidase | p$K_{a1}$ | p$K_{a2}$ |
|---|---|---|
| $Zn^{2+}$-imidase | $6.03 \pm 0.06$ | $9.50 \pm 0.06$ |
| $Co^{2+}$-imidase | $6.13 \pm 0.06$ | $9.58 \pm 0.07$ |
| $Mn^{2+}$-imidase | $6.26 \pm 0.05$ | $9.23 \pm 0.05$ |
| $Cd^{2+}$-imidase | $6.05 \pm 0.08$ | $9.39 \pm 0.08$ |

[a] The p$K_a$ values were obtained by fitting catalytic efficiencies (V/K) of metal-reconstituted imidase at each pH value as shown in Fig. 3 by the program of Grafit with the equation of bell-shaped double p$K_a$ curve, $(y = y_{max}/(10^{-pH}/10^{-pK_{a1}}) + (10^{-pK_{a2}}/10^{-pH}) + 1)$ [34]. The experimental procedures were described in Experimental procedures.

The p$K_a$ values of the metal-bound water molecular in complexes of $Zn^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Ni^{2+}$, and $Mn^{2+}$ were 8.8, 8.9, 9.0, 10.6, and 10.6, respectively [26]. Whereas the p$K_a$ values of carbonic anhydrase containing $Zn^{2+}$, $Co^{2+}$, $Cd^{2+}$, and $Mn^{2+}$, were 6.9, 6.8, 9.1, and 8.2, respectively [27]. The p$K_a$ values of the metal-substituted phosphotriesterase were 5.8, 6.5, 8.1, 7.4, and 7.0 for Zn-, Co-, Cd-, Ni-, and Mn-enzyme, respectively [28]. It is clear that our data do not support the mechanism that metal-coordinated water plays a critical role as a nucleophile for imide hydrolysis catalyzed by imidase. Other metalloenzyme using water-metal ionization mechanism, such as phosphotriesterase, restores full enzymatic activity when zinc is replaced by nickel or copper ion [29]. In the case of imidase, there is no enzymatic activity observed for Ni-, and Cu-imidase, although copper and nickel can be incorporated into the enzyme (Table 1). Instead, these two metal ions inactivate the enzyme activity following incubation with native imidase (data not shown).

*Proposed metal function and reaction mechanism of imidase catalyzed imide hydrolysis*

A putative zinc-binding site [30] for imidase and related enzymes [31] has been proposed as a summary in Fig. 4. Bold type histidine indicates the proposed metal-binding ligand. The sequences of a group of functionally related amidohydrolases including imidase, dihydroorotase, and allantoinase from different species are compared. All of the enzymes have a certain similarity in their actions on the cyclic amide bonds, but total sequence homology is low (below 30% homology). However, the four histidine residues and the aspartic acid are perfectly conserved in these functionally related amidohydrolases. Site-directed mutagenesis was carried

| | Frame 1 | | | Frame 2 | | | Frame 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | 65 | 67 | 69 | 192 | | | 248 | | |
| Human imidase | P G G I | **D** T | **H** T **H** M | I A Q V | **H** A E N G D L | | I V | **H** V M S K S | |
| Rat imidase | P G G I | **D** T | **H** T **H** M | I A Q V | **H** A E N G D L | | I V | **H** V M S K S | |
| P. imidase | P G G I | **D** P | **H** T **H** M | V P T V | **H** A E N G E L | | V V | **H** I S S R E | |
| BSt. imidase | P G G I | **D** P | **H** T **H** L | L V M V | **H** A E G G D V | | V V | **H** V S C A Q | |
| Ag. Imidase | P G G I | **D** V | **H** T **H** V | L V M V | **H** A E N G D A | | I V | **H** L T C E E | |
| Ar. Imidase | P G V V | **D** D | **H** V **H** I | V I V V | **H** A E N E T I | | V L | **H** V S N P D | |
| M. DHOase | P G L I | **D** A | **H** V **H** F | P V T V | **H** C E N R D V | | I C | **H** L S T V K | |
| BC. DHOase | P G L I | **D** L | **H** V **H** L | A I V A | **H** C E D D T L | | V C | **H** I S T K E | |
| L. DHOase | P G L V | **D** I | **H** E **H** Y | A I C E | **H** I Q D D S L | | A C | **H** V S T K E | |
| E. ALLase | P G M V | **D** A | **H** T **H** I | P V L V | **H** C E N A L I | | V C | **H** V S S P E | |
| BSu. ALLase | P G V I | **D** C | **H** V **H** F | I L A L | **H** A E S D A I | | F V | **H** I S T A K | |

Fig. 4. Putative zinc-binding site of imidase. Bold type indicates the proposed metal-binding ligand. Arabic numbers indicate the number of amino acids from the N-terminal of human imidase. The protein sequences of human imidase (NP_001376, Protein number), rat imidase (Q63150), P. imidase (Q59699), B1. imidase (Q45515), Ag. imidase (Q44184), Ar. imidase (P81006), M. DHOase (O27199), B2. DHOase (P46538), L. DHOase (P48795), E. ALLase (P77671), and B3. ALLase (O32137) were obtained from NCBI. DHOase indicates dihydroorotase. ALLase indicates allantoinase. P, *Pseudomonas Putida*; Ag, *Agrobacterium radiobacter*; Ar, *Arthrobacter aurescens*; M, *Methanobacterium thermoautotrophicum*; L, *Lactobacillus leichmannii*; E, *E. coli*; BSt, *Bacillus sterothermophilus*; BC, *Bacillus caldolyticus*; BSu, *Bacillus subtilis*.

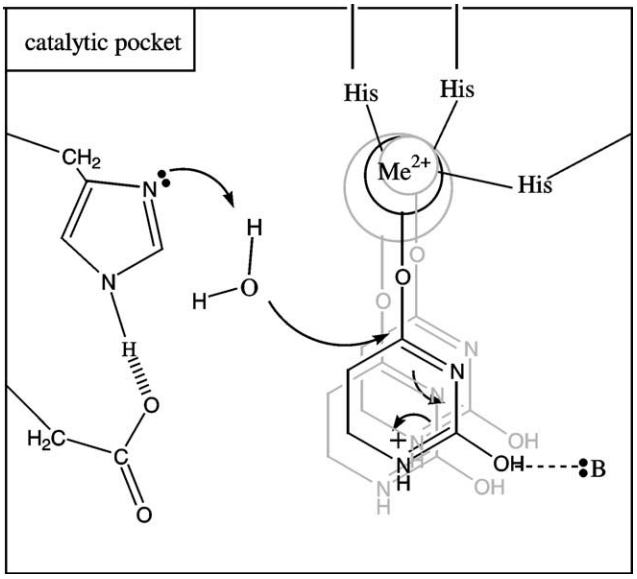


Fig. 5. Proposed mechanism for imidase catalyzed imide hydrolysis. The size of metal ion affects the activity and substrate specificity of imidase (Table 1 and Fig. 3). Site-directed mutagenesis studies on four perfect conserved histidine and aspartic acid residues indicate that they involve zinc binding or catalyzed function of imidase [31], and the removal of the imide proton is the driving force for imide hydrolysis catalyzed by imidase [4]. We sketch the mechanism of imidase action based on these observations.

out for the bacterial enzyme from *B. stearothermophilus* SD-1 and shows that these four perfectly conserved histidine residues are related to zinc binding [31]. Consistent with the above finding, a modification of imidase by DEPC removes zinc from imidase (Table 1) and eliminates enzyme activity. The pH profiles of the zinc removal (removal of zinc from imidase by 8-HQSA is found easier at pH 6.5) and enzymatic activity (Fig. 3, $pK_{a1}$ around 6–6.3) indicate that histidines may be involved in both the metal binding and water activating for imide hydrolysis.

It has been proposed previously that the removal of the imide proton forces the C4–N3–C2–N1 toward greater planarity in accommodating the delocalized electrons, which is the driving force for imide hydrolysis catalyzed by imidase [4]. This mechanism explains the fact that unlike that of peptidases or proteases, imidase does not catalyze hydrolysis of amide and ester. Based on the information reported in this study, we put forward an extended working mechanism for imidase action as shown in Fig. 5. The function of the metal ion in imidase is to coordinate substrate and maintain a suitable active site environment but not directly involve the ionization of a water molecule. Instead, a histidine may be responsible for the ionization of water because $pK_{a1}$ at around 6–6.3 are observed through the pH profiles of native and metal reconstituted imidases (Table 2). Conserved aspartic acid (Fig. 4) is also found to be important for imide hydrolysis [31] and may function as a proton donor/acceptor as shown in Fig. 5. Aspartic acid may be needed for the dehydration of *N*-carbamoyl acid (reverse reaction of the hydrolysis of dihydrouracil), which is catalyzed by imidase in acidic condition [4].

The structure of a zinc ion bound to three histidines has been observed in carbonic anhydrase, β-lactamase, DD-caboxypeptidase [32], and a Dnase [33]. It is found that in all catalytic active zinc sites, the fourth ligand is water [32], which may be replaced with a phosphate ion [33]. This observation may explain the inhibition of imidase activity by a phosphate buffer (Table 1) that phosphate ion may compete with substrate to coordinate with metal ions in the active site. The metal coordinated water may be activated by ionization, polarization, or poised for displacement [32]. Imidase metal ion may function as a Lewis acid for catalysis to stabilize the developing negative charge of imide in the transition state or to fix the orientation of the metal-bound substrate so that it is in position for nucleophilic attack by a water or a hydroxide ion activated by a histidine as proposed in Fig. 5.

The proposed active site configuration shown in Fig. 5 may be used to explain the correlation of enzyme activity versus the size of metal ions in imidase. As shown in Fig. 5, the position of nucleophilic attack on an imide substrate may fluctuate due to size of the coordinating metal ion. Imidase activity is decreased or eliminated if the size of metal ion in imidase is out of range. This explains why bigger metal-reconstituted imidase (Mn- and Cd-imidase) exhibits low activity and smaller metal-reconstituted imidase (Ni- and Cu-imidase) display no activity under standard assay condition.

## Acknowledgments

We thank Ms. Linyun W. Yang for reading the manuscript. This research is supported by grants from the National Science Council (89-2311-B-009-005 and 89-CPC-7-009-006), Taiwan.

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