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Quantitative analysis and functional evaluation of zinc ion in the D-hydantoinase from *Pseudomonas putida* YZ-26

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Abstract D-Hydantoinase (HDT) is a metal-dependent enzyme that is widely used in industrial bioconversion to D-amino acids as valuable intermediates in the fields of food, pharmaceutical industry and agriculture. In this report, we prepared apo-HDT (metal-removed HDT) and Zn^{2+}-HDT (Zn^{2+}-added HDT) in vitro from a recombinant HDT (re-HDT) expressed in *E. coli*. The Zn^{2+}-HDT and re-HDT contain 2.17 and 0.95 mol Zn^{2+} per mol subunit, respectively, and they have comparable enzymatic activities. In contrast, the apo-HDT only retains 0.04 mol Zn^{2+} per mol subunit with less than 10% activity, compared with the re-HDT. When the apo-HDT was reconstituted with $ZnCl_2$, the enzymatic activity recovery was about 75%. Moreover, the fluorescence intensity, circular dichroism spectra and thermo-stability of the apo-HDT and Zn^{2+}-HDT are quite different from those of the re-HDT. These data suggest that the re-HDT may have two Zn^{2+}-binding sites, one is an intrinsic or tight-binding site (zinc-α) essential for its activity and the other is a vacant or loose-binding site (zinc-β) possibly non-essential for the activity.

Keywords D-Hydantoinase · Zn^{2+} analysis · Functional evaluation

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Introduction

D-Hydantoinase (HDT, EC 3.5.2.2) catalyses the substrate 5′-monosubstituted hydantoin to enantiomerical *N*-carbamoyl-amino acids which, in turn, can be chemically or enzymatically converted into the corresponding optically active amino acids (Altenbuchner et al. 2001). Hydantoinase is ubiquitously found in microorganisms, plants and animals (Pozo et al. 2002). The enzyme is also well known as dihydropyrimidinase in higher organisms and is responsible for dihydropyrimidine hydrolysis as the second step in the reductive catabolism of pyrimidine. Based on its chiral specificity for the substrate and product, HDT can be divided into three types: L-, D- and DL-configuration. A number of bacterial D-hydantoinases with different enantioselectivities and substrate specificities have been used in industrial bioconversion to optically active D-amino acids. As unnatural chiral products, D-amino acids are important intermediates in the synthesis of various β-lactam semisynthetic antibiotics, such as

ampicillin, amoxicillin, aspoxicillin and cefbuperazone, as well as of other products, such as antiviral agents, artificial sweeteners, pesticides, peptide hormones and pyrethroids etc. (Altenbuchner et al. 2001).

HDT, similar to other amidohydrolases, has been characterized as a typical metal-dependent enzyme, either in the fundamental research or in the industrial application (May et al. 1998a, b). It is demonstrated that divalent metal ions, especially Zn^{2+}, play an important role in the catalytic process of the enzyme (May et al. 1998a, b). In the last decades, some reports regarding the metal dependence of HDT have been mainly focused on the function of Zn^{2+} and its quantitative determination (Gojkovic et al. 2003; Cheon et al. 2003; Kikugawa et al. 1994; Brooks et al. 1983). Thereinto, most HDTs contain two Zn^{2+} ions per subunit (Gojkovic et al. 2003; Cheon et al. 2003). The first Zn^{2+} may bind to the carbonyl oxygen of hydantoin and subsequently polarize the carbonyl bond, while the second Zn^{2+} only activates the bridge water and does not bind to the substrate. In contrast, a few HDTs only contain one catalytic Zn^{2+} per subunit, such as the HDTs from bovine liver and rat liver (Kikugawa et al. 1994; Brooks et al. 1983). No matter how many Zn^{2+} per subunit HDT contains, they are all essential for the catalytic reaction. However, the potential structural and functional roles for Zn^{2+} have not been well established in hydantoinase family, although the role of Zn^{2+} has been well established in other amidohydrolases. Our previous work indicated that the D-hydantoinase from *Pseudomonas putida* YZ-26 was dissociated into monomer subunit from its dimer in the presence of 1 mM Zn^{2+} (Shi et al. 2006). To further explore the function of Zn^{2+} in the HDT, we prepared re-HDT, apo-HDT and Zn-HDT to study the relationship between its zinc content and catalytic activity. This report preliminarily demonstrates that the re-HDT has two different Zn^{2+}-binding sites, a tight one linked to its activity is filled with Zn^{2+} and a loose one related to its structure is vacant.

Materials and methods

Strain, plasmid, reagents and equipments

The host cell, *Escherichia coli* BL21 (DE3) and a recombinant plasmid pE-p479 used in this study were stored at −80°C in this laboratory. Hydantoin, 4-(2-hydroxyerhyl) piperazine-1-ethanesulfonic acid (HEPES, ultra pure grade) and Tris (Hydroxymethyl) aminomethane (Tris, ultra pure grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pyridine-2, 6-dicarboxylic acid (DPA) and EDTA were purchased from Alfa Aesar (Ward Hill, MA, USA). The protein purification equipment and separation materials were from Amersham Pharmacia Biotech. All reagents and buffer solutions used here were prepared using ultra-pure water with more than 18.2 MΩ m from a Milli-Q water purification system (Millipore Corporation, France). All other chemicals used were of the highest purity commercially available, unless otherwise indicated.

Expression and purification of the recombinant D-hydantoinase

Recombinant plasmid pE-p479 was introduced into *E. coli* BL21 and expressed in 1 l Luria-Bertani (LB) culture supplemented with ampicillin (100 μg/ml) at 30°C for 12–14 h without adding any inducers. The culture was harvested by centrifugation at 6,000*g* for 10 min at 4°C. Then pellets were suspended in chilled 50 mM Tris–HCl buffer, pH 8.0 (TB8) and sonicated. The resulting supernatant was purified by a two-step purification procedure to obtain the purified D-hydantoinase, as described previously (Zhang et al. 2008). The protein concentration was performed by the Bradford or Folin-phenol methods with bovine serum albumin as the assay standard.

Enzymatic assay

D-Hydantoinase activity was measured by a colorimetric method (Niu et al. 2007). An aliquot of the enzyme was added into the reaction mixture in a total volume of 1.5 ml, contained 100 mM hydantoin and 50 mM TB8. After being incubated at 37°C for 30 min, the reaction mixture was terminated by adding 0.25 ml trichloro-acetic acid (10% w/v), then 0.25 ml dimethylaminobenzaldehyde (DMBA) solution (10% w/v in 6 M HCl) and finally diluted with distilled water to 3 ml. After centrifugation, the product, *N*-carbamoylglycine, in the supernatant was measured at 430 nm wavelength and its amount was calculated from a standard calibration plot. One D-hydantoinase unit is equivalent to the formation of

1 μmol of *N*-carbamoylglycine per min under the above assay condition.

Apo-HDT and Zn^{2+}-HDT preparation in vitro

Apo-HDT was prepared by mixing re-HDT and 100 mM DPA to remove the intrinsic Zn^{2+} at 25°C for 8 h. Then the reaction mixture was thoroughly dialyzed against 10 mM TB8 at 4°C with buffer changes for four times to remove trace metal ions. Zn^{2+}-HDT was prepared with the re-HDT by addition of ten fold (ca. 0.3 mM) $ZnCl_2$. The reaction mixture was dialyzed as described above. Finally, the enzymatic activity, protein concentration and Zn^{2+} content of each sample were examined by DMBA, Folin-phenol and ICP-AES methods, respectively.

Determination of divalent metal ions

Divalent metal ions in re-HDT, apo-HDT and Zn^{2+}-HDT were determined by ICP-AES (inductively coupled plasma-atomic emission spectrometry, an ICP-AtomScan 16 apparatus from Thermo Jarrell Ash Co., USA) with a direct injection. All samples were prepared to be SDS-PAGE pure. Metals were analyzed at the following wavelengths: Zn^{2+}: 213.856 nm; Co^{2+}: 236.379 nm; Mn^{2+}: 257.690 nm; Ni^{2+}: 231.604 nm and Fe^{2+}: 259.640 nm. Data were calibrated against the standard of Zn^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+} and Ni^{2+}, respectively. Metal ion content and protein concentration were simultaneously performed each time to evaluate the reliability of metal: protein ratio. The last external dialyzed buffer was used as a blank in ICP-AES, and the equivalent amount of BSA was used as a control in the protein concentration assay.

Thermo-stability

The thermo-stability of re-HDT and Zn^{2+}-HDT was determined by pre-incubation in 50 mM TB8 at various temperatures (from 35 to 60°C) for 60 min. Samples were then chilled on ice for at least 5 min, and then the residual activity was performed under the standard assay condition.

Apparent binding constant of zinc-β site

The apparent binding constant of the zinc-β site in Zn^{2+}-HDT was estimated by a competition test with a colorimetric Zn^{2+}-chelator, 4-(2-pyridylazo) resorcinol (PAR). Re-HDT (1 μM) and Zn^{2+}-HDT (1 μM) in 10 mM HEPES buffer, pH 7.5 were used as the control and sample, respectively. Buffer alone was used as a blank. During the titration with PAR (1–35 μM), the data were collected at 500 nm (Shi et al. 2008), and then the optical absorbance was corrected by subtracting the trace from buffer blank.

The apparent binding constant of the vacant zinc-β site in re-HDT was estimated by a competition assay with a colorimetric Zn^{2+}-chelator, 2-carboxy-2-hydroxy-5-(sulfoformazyl) benzene (Zinco) (Armas et al. 2006). A typical assay is performed as follows: an amount of 20 μM Zinco and 10 μM $ZnCl_2$ in 10 mM HEPES buffer was added into a cuvette, and the spectrophotometer was blanked against this solution. Then the re-HDT was added to the cuvette step by step, and the absorbance value was acquired at 620 nm after sufficiently mixing.

Circular dichroism and fluorescence emission spectra

Circular dichroism (CD) spectra were carried out with a recording spectropolarimeter (Jasco 810) at 25°C under a nitrogen atmosphere. CD spectrum was conducted at an enzyme concentration of 0.2 mg/ml in 20 mM HEPES (pH 7.4) with a 1 mm path-length cell. The spectrum was recorded from 190 to 250 nm at a scan rate of 50 nm min^{-1}. A blank was also recorded in the absence of the enzyme using the same buffer as a control. The CD spectrum of each sample or control was measured at least three times.

Fluorescence emission spectra were recorded at 25°C using a spectrofluorometer (HITACHI, F-2500) with an excitation wavelength of 295 nm. The spectrum was scanned from 290 to 400 nm at a scan rate of 60 nm min^{-1}. Samples with an 8 μM enzyme concentration were measured in a 1 cm path-length fluorescence cuvette.

Results

Preparation of re-HDT, apo-HDT and Zn^{2+}-HDT

The re-HDT was purified by a two-step purification procedure from *E. coli* as described previously (Zhang et al. 2008). Zn^{2+}-HDT and apo-HDT was

prepared as described in "Materials and methods". To avoid the inactivation of the enzyme during the dialysis and ensure the reliability of the subsequent metal analysis, three samples (re-HDT, apo-HDT and Zn^{2+}-HDT) must be treated simultaneously for each test. All three species of HDTs show high purity and exhibit the same molecular weight on SDS-PAGE (Fig. 1). Moreover, the Zn^{2+}-HDT activity reaches a similar level as the re-HDT (9.5 U/mg), while the apo-enzyme has little activity compared with re-HDT.

Inactivation of re-HDT by metal chelators

The chelator such as EDTA or pyridine-2,6-dicarboxylate (DPA) is generally used as a reagent to remove metal ions in a protein, accompanied by losing the activity or changing the conformation. However, when the re-HDT was incubated with EDTA or DPA, its inactivation profile was quite different as shown in Fig. 2. In the presence of 100 mM EDTA, the re-HDT is very stable, and its activity retains more than 90% after 8 h incubation. The effect of other chelators, such as 1,10-phenanthroline or 8-HQSA on the re-HDT is roughly the same as that of EDTA (data not shown). This is likely due to that the intrinsic Zn^{2+} in the re-HDT is too tight to be removed by above chelators. However, the inactivation of the re-HDT obviously occurred in the presence of 100 mM DPA. The enzymatic activity gradually decreased to less than 10% after 8 h incubation, indicating that the inactivation was related to some metal ions. This result is similar to that of the hamster dihydroorotase domain, a member of the hydantoinase family, in which the metal chelating ability of EDTA is weaker than that of DPA (Huang et al. 1999).

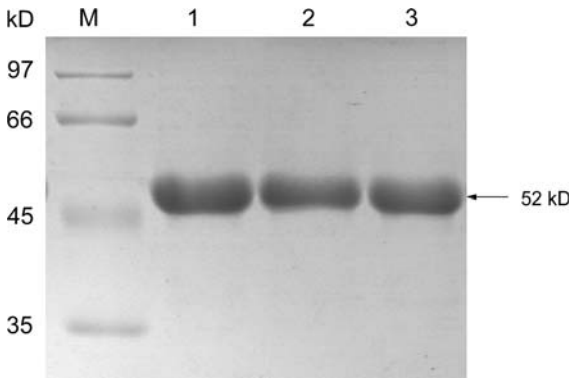

Fig. 1 SDS-PAGE analysis of re-HDT, apo-HDT and Zn^{2+}-HDT. *Lane M*: Molecular mass marker (phosphorylase 97 kDa, bovine serum albumin 66 kDa, ovalbumin, 45 kDa, carbonic anhydrase, 30 kDa); *Lane 1*: Re-HDT; *Lane 2*: Apo-HDT; *Lane 3*: Zn^{2+}-HDT

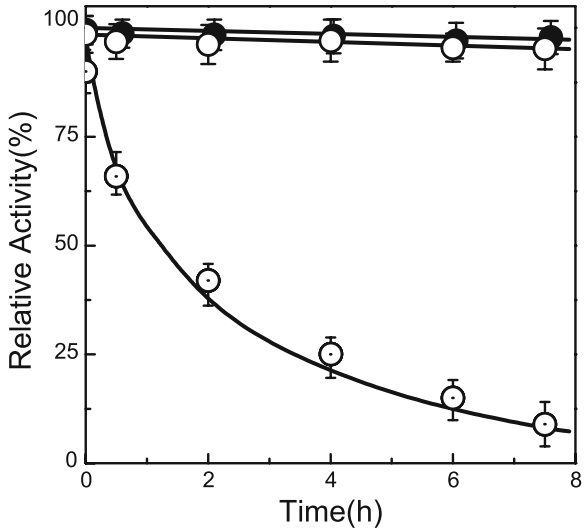

Fig. 2 Inactivation of re-HDT in the presence of chelator, EDTA or DPA. (●) Control, (○) EDTA, (⊙) DPA. The enzyme was incubated with 100 mM DPA or 100 mM EDTA at 25°C. At time intervals, aliquots were removed to measure the residual activity

Quantitative determination of divalent metal ions in the re-HDT

Based on our experiences, a repeated dialysis procedure was quite efficient to thoroughly remove free trace metal ions. Three samples of pure HDTs (re-HDT, apo-HDT and Zn^{2+}-HDT) were dialyzed simultaneously and extensively with 10 mM TB8 prepared by Milli-Q ultra-pure water and ultra-pure reagents as described in "Materials and methods". In order to confirm there is no metal ion contamination in samples, equal amounts of BSA as a control and the final external dialysate as a blank were measured by same procedures. ICP-AES analysis shows in Table 1 that the re-HDT contains 0.95 mol of Zn^{2+} per subunit, equivalent to a protein: Zn^{2+} ratio of 1:1. However, the apo-HDT treated with DPA only has 0.04 mol of Zn^{2+} per subunit, indicating that the intrinsic Zn^{2+} (so called zinc-α) was thoroughly removed by this dialysis procedure. On the contrary,

Table 1 Divalent metal ion content in various types of D-hydantoinases[a]

Enzyme or control	Metal ion (mol)/monomer enzyme(mol)				
	Mn^{2+}	Co^{2+}	Ni^{2+}	Zn^{2+}	Fe^{2+}
Re-HDT	0.09	0.06	0.01	0.95	0.08
Zn-HDT	0.03	0.03	0.02	2.17	0.02
Apo-HDT[b]	<0.01	<0.01	<0.01	0.04	<0.01
BSA[c]	<0.01	<0.01	<0.01	<0.01	<0.01
External dialysate[c]	<0.01	<0.01	<0.01	<0.01	<0.01

[a] The above data were carried out in triplicate by ICP-AES

[b] Apo-HDT is the re-HDT incubated with 100 mM DPA at 25°C for 8 h

[c] Blanks are the equal amount of BSA and the final external dialysate of different samples

the Zn^{2+} content of Zn^{2+}-HDT is up to 2.17 mol per monomer enzyme with no change on its activity. The result shows that Zn^{2+} may occupy other binding site (so called zinc-β), which may be nonessential for enzymatic activity. Moreover, the quantity of other metal ions, such as Co^{2+}, Ni^{2+}, Mn^{2+} and Fe^{2+} are negligible in comparison with Zn^{2+} as indicated in Table 1.

Reactivation of apo-HDT by divalent metal ion

To further explore the effect of Zn^{2+} on the activity of the apo-HDT it was titrated with $ZnCl_2$. As shown in Fig. 3, the activity of the apo-HDT is gradually increased until the ratio of Zn^{2+} to enzyme is 1:1. The recovery of enzyme activity is about 75% of the re-HDT. Beyond 1:1 ratio, the enzymatic activity no longer increases, even in the presence of excess Zn^{2+}.

In order to investigate reactivation of the apo-HDT, the above metal ions were added individually. As shown in Fig. 3, the reactivation capability of the apo-HDT seems in the sequence of $Co^{2+} > Mn^{2+} > Ni^{2+}$, implying that these three metal ions are different from Zn^{2+} not only on the activation level but also on the binding characteristics.

Thermo-stability of Zn^{2+}-HDT

The thermo-stability of Zn^{2+}-HDT (Fig. 4) is higher than that of the re-HDT under the identical condition. A possible explanation is that the zinc-β site filled with Zn^{2+} results in the conformational change of the protein to enhance its thermo-stability. In fact, it is not

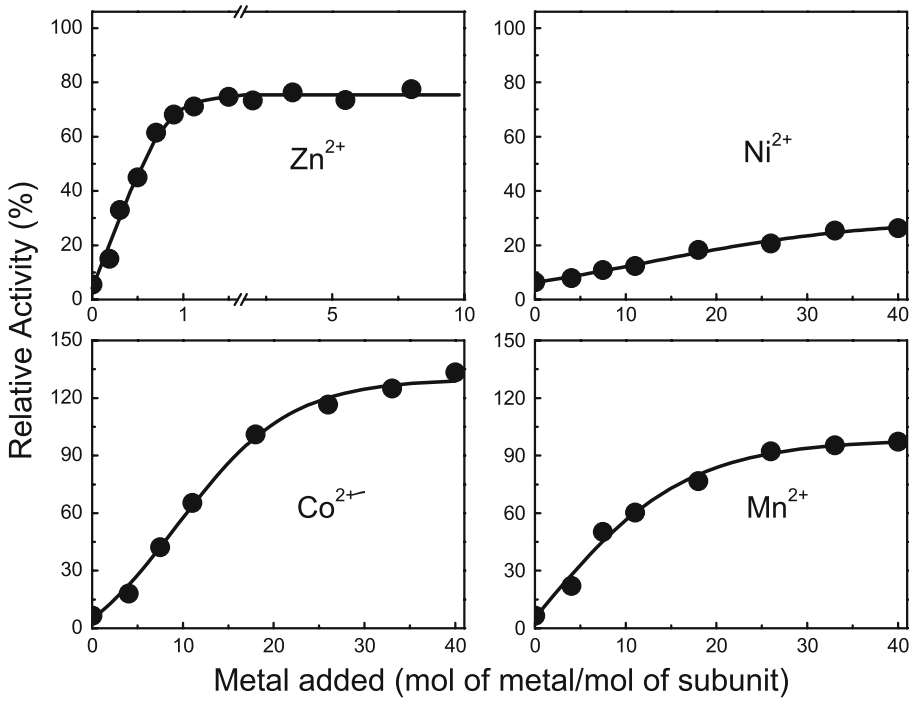

Fig. 3 Reactivation of apo-HDT in the presence of different metal ions. Apo-HDT was mixed with various molar ratios of Zn^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+}, respectively, at 4°C for 2 h, and then 20 µl each was added into 1.5 ml reaction system for the activity assay. The activity of re-HDT was normalized to 100%

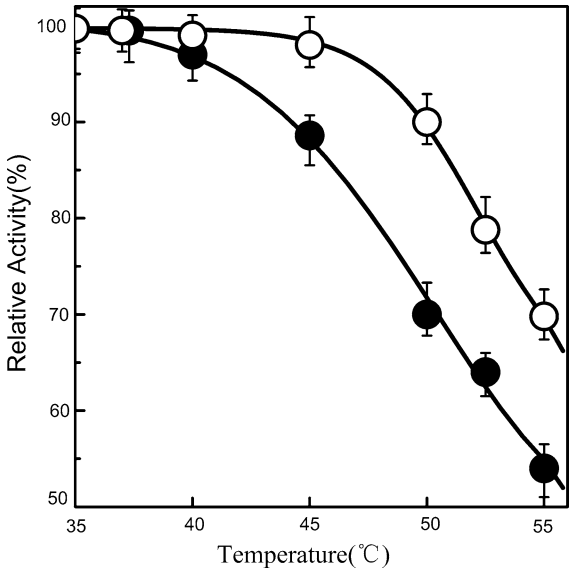

Fig. 4 Comparison of thermo-stability between re-HDT and Zn^{2+}-HDT. (●) Re-HDT, (○) Zn^{2+}-HDT. The enzyme was pre-incubated at different temperatures in a 50 mM Tris–HCl buffer, pH 8.0. An aliquot of the pre-incubated enzyme was removed and chilled on ice for 5 min. Then the residual activity was assayed as described in "Materials and methods"

unusual that the addition of metal ions to an enzyme solution results in changes of the protein conformation and thermo-stability. For example, the thermo-stability of the *Pho*ACY from *Pyrococcus horikoshii* was improved when it was incubated with Zn^{2+}, Mn^{2+} or Ni^{2+} and the presence of Mn^{2+} or Co^{2+} can increase the thermo-stability of the *Burkholderia pickettii* D-hydantoinase (Xu et al. 2003; Vieille and Zeikus 2001). Therefore, the enhancement of the Zn^{2+}-HDT thermo-stability may be dependent on the binding feature of zinc-β site.

Apparent binding constant of *zinc-β* site

The apparent binding constant between Zn^{2+} and a protein can be estimated by a competition test with a Zn^{2+} transferring reagent. The apparent Zn^{2+}-binding constant of the *loose zinc-β site in re-HDT* was estimated by the colorimetric Zn^{2+}-chelator, Zinco. The reagent, Zinco can form 1:1 complex with Zn^{2+}, which has a distinct absorption at 620 nm ($\varepsilon =$ 23,500 M^{-1} cm^{-1}) with a binding constant (K_{app}) of 7.9×10^4 M^{-1} (Armas et al. 2006). Therefore, the binding constant of the loose zinc-β site in the re-HDT can be calculated by resolving Eq. 1 for $K_{app(Zn-^{2+}HDT)}$.

$$\frac{K_{app(Zn-^{2+}HDT)}}{K_{app(Zn-Zinco)}} = \frac{[Zn-^{2+}HDT][Zinco]}{[re\text{-}HDT][Zn\text{-}Zinco]} \quad (1)$$

It is shown in Fig. 5a that the optical absorption at 620 nm is gradually decreased with increased concentration of the re-HDT. The absorption change (Δ620 nm) reflects the transfer of Zn^{2+} from the Zn^{2+}-Zinco complex to the loose zinc-β site in the re-HDT, therefore, the following Eqs. 2–7 would be produced.

$$[re\text{-}HDT] = [re\text{-}HDT]_{add} - [Zn^{2+}\text{-}HDT] \quad (2)$$

$$[Zn^{2+}\text{-}HDT] = \Delta[Zn\text{-}Zinco] = A_{620}/\varepsilon_{620} \quad (3)$$

$$[Zn\text{-}Zinco]_{original} = 10\ \mu M \quad (4)$$

$$[Zinco]_{total} = 20\ \mu M \quad (5)$$

$$[Zinco] = [Zinco]_{total} - [Zn\text{-}Zinco]_{original} + \Delta[Zn\text{-}Zinco] \quad (6)$$

$$[Zn\text{-}Zinco] = [Zn\text{-}Zinco]_{original} - \Delta[Zn\text{-}Zinco] \quad (7)$$

Finally, by the known binding constant of Zn-Zinco, the apparent zinc-β binding constant of Zn^{2+}-HDT can be calculated at the level of 5×10^4 M^{-1}. The result shows that Zn^{2+} in Zn-Zinco can be transferred into the vacant zinc-β site of the re-HDT, but its binding constant is not very high compared to the one for zinc-α site.

Similarly, the zinc-β binding constant of the Zn^{2+}-HDT was measured with the colorimetric Zn^{2+}-chelator, 4-(2-pyridylazo) resorcinol (PAR). The titration experiment (Fig. 5b) shows that the reagent, PAR only interacts with zinc-β site of the Zn^{2+}-HDT, but not with the zinc-α site of the re-HDT at the same concentration. It was reported that an excess PAR mainly became a complex with Zn^{2+} in a 2 to 1 stoichiometry ($Zn(PAR)_2$), which produces a distinctive absorption at 500 nm ($\varepsilon =$ 66,000 M^{-1} cm^{-1}) and has an apparent binding constant of 2×10^{12} M^{-1}(Shaw et al. 1991). Therefore, the apparent binding constant of the Zn^{2+}-HDT can be calculated by resolving the Eq. 8 for $K_{app(Zn^{2+}-HDT)}$, just as in the case of Zinco.

$$\frac{K_{app(Zn-^{2+}HDT)}}{K_{app(Zn(PAR)_2)}} = \frac{[Zn-^{2+}HDT][PAR]^2}{[re\text{-}HDT][Zn(PAR)_2]} \quad (8)$$

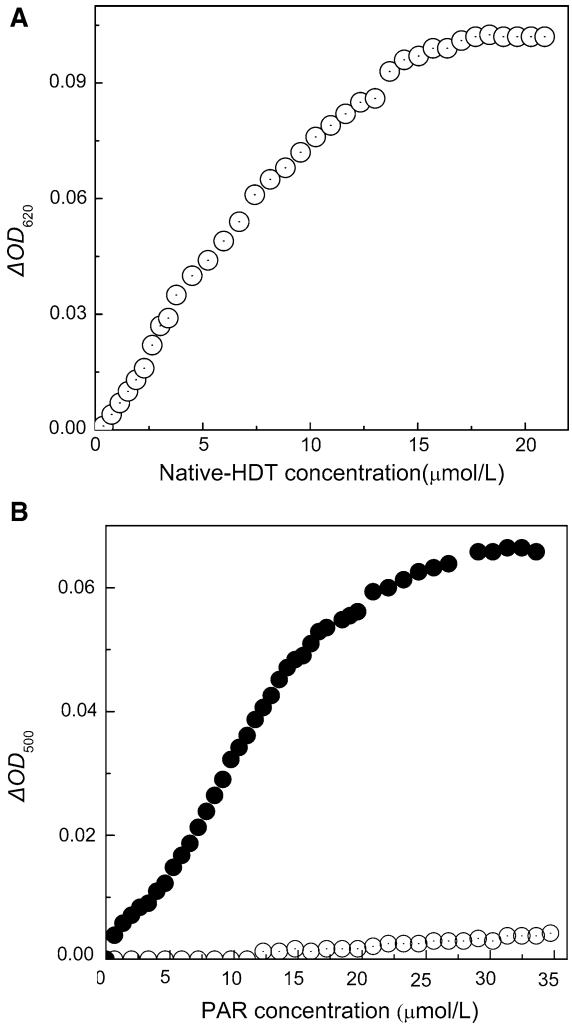

Fig. 5 **a** Determination of the binding constant at the vacant zinc-β site in re-HDT by Zinco. Zn-Zinco complex (20 μM Zinco, 10 μM $ZnCl_2$) was titrated with re-HDT (1–21 μM) in 10 mM HEPES, pH 7.5. The absorbent change at 620 nm is used as an index for Zn^{2+} transfer from Zn-Zinco to re-HDT. **b** Determination of the binding constant of the zinc-β site in Zn^{2+}-HDT by PAR. (●) Zn^{2+}-HDT, (⊙) Re-HDT. 1 μM Zn^{2+}-HDT or 1 μM re-HDT was titrated with PAR (1–35 μM) in 10 mM HEPES, pH 7.5, respectively. The increase of the absorbance at 500 nm was regarded as the formation of $Zn(PAR)_2$ complex

Figure 5b shows that the optical absorption at 500 nm is gradually increased with the addition of PAR due to the formation of $Zn(PAR)_2$ complex. The absorbance increment at 500 nm implies the transfer of Zn^{2+} at zinc-β site from the Zn^{2+}-HDT to PAR. The same equation can be obtained as follows:

$$[\text{re-HDT}] = \left[\text{Zn}(\text{PAR})_2\right] = A_{500}/\varepsilon_{500} \quad (9)$$

$$[\text{PAR}] = [\text{PAR}]_{\text{add}} - 2\left[\text{Zn}(\text{PAR})_2\right] \quad (10)$$

$$\left[\text{Zn}^{2+}\text{-HDT}\right]_{\text{original}} = 1\ \mu\text{M} \quad (11)$$

$$\left[\text{Zn}^{2+}\text{-HDT}\right] = \left[\text{Zn}^{2+}\text{-HDT}\right]_{\text{original}} - \left[\text{Zn}(\text{PAR})_2\right] \quad (12)$$

Finally, according to the known binding constant of $Zn(PAR)_2$, the apparent binding constant of zinc-β site in Zn^{2+}-HDT should be around $5 \times 10^{12}\ M^{-1}$. This experiment demonstrates that the zinc-β in the Zn^{2+}-HDT is very hard to be transferred into PAR. In other words, as soon as the vacant zinc-β site is filled with Zn^{2+}, the interaction strength between Zn^{2+} and the protein is too tight to be removed.

Circular dichroism and fluorescence spectra

Circular dichroism (CD) is often used to evaluate whether the secondary and tertiary structures of a protein are changed under various conditions. Figure 6a shows the far-UV CD spectra of the re-HDT in the absence and presence of Zn^{2+}. They display the characteristic spectra of a mainly β-sheet structure with a negative peak at 215 nm (Mishima et al. 2006). The data is consistent with our previous work that the three dimensional structure of the re-HDT is rich in β-sheet (Zhang et al. 2008). The negative peak is diminished by gradually adding Zn^{2+} ion, indicating that binding of Zn^{2+} to zinc-β site induces a modification of the re-HDT secondary structure, resulting in the decrease of β-sheet content in the enzyme.

Fluorescence spectra reflect the state of intrinsic fluorescent residues as a reliable indicator of protein conformation changes. Figure 6b shows that the fluorescence spectra of the re-HDT exhibit a maximum emission at about 337 nm, which is a characteristic of tryptophan residues buried inside a protein (Vlasova and Ugarova 2007; Zhao et al. 2004). Although there is no obvious shift (<5 nm) in maximum emission wavelength, significant differences in fluorescent intensity are observed among these three different species of HDTs. Fluorescence intensity of the Zn^{2+}-HDT is increased by 27%, while that of the apo-HDT is quenched by 28%, compared with the fluorescence intensity of the re-HDT. A possible explanation is that Zn^{2+}-binding on the re-HDT affects the structure of the

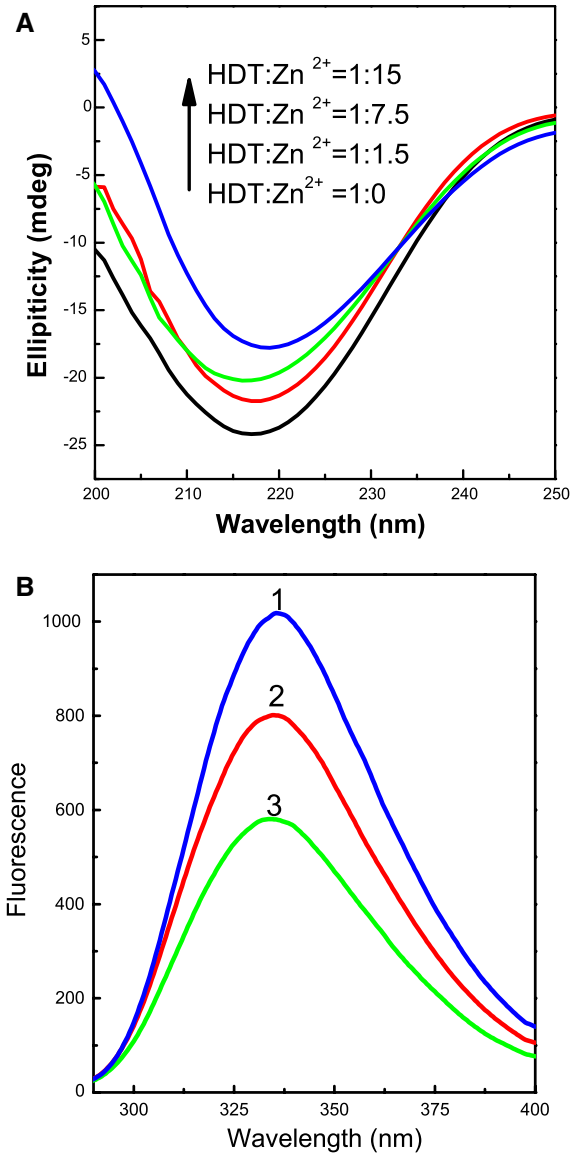

Fig. 6 **a** Far-UV circular dichroism spectra of re-HDT in the presence of Zn^{2+}. CD spectra of re-HDT (4 μM) in the presence of Zn^{2+} were scanned in 1 mm path-length cuvette at 25°C by a Jasco spectropolarimeter J-810 under a nitrogen atmosphere. The data were recorded from 190 to 250 nm at a scan rate of 50 nm min^{-1}. The final concentrations of Zn^{2+} are 0 mM (1:0), 0.01 mM (1:1.5), 0.05 mM (1:7.5) and 0.1 mM (1:15), respectively. **b** Fluorescent spectra of re-HDT, apo-HDT and Zn^{2+}-HDT. The concentration of all samples was 8 μM in 10 mM Tris–HCl, pH 8.0. The spectra were recorded at the excitation wavelength of 295 nm with 1 cm path-length cuvette and scanned from 290 to 400 nm at 60 nm min^{-1}. *1*, Zn^{2+}-HDT; *2*, Re-HDT; *3*, Apo-HDT

enzyme molecule, which may slightly alter the microenvironment surrounding tryptophan residues, resulting in the change of fluorescence quantum efficiency. However, the environment polarity of tryptophan residues may not be changed; therefore there is no obvious shift of the maximum emission wavelength.

Homology modeling

SWISS-MODEL server was used to construct the three-dimensional structure model of the re-HDT via the following steps. Human dihydropyrimidinase (PDB ID: 2VR2) was selected as the template by Swissmodel and Modeller programs from templates library. The primary sequence identity between the template protein and the re-HDT from *P. putida* YZ-26, is 52.1%, which is much higher than 30% limit generally considered to be the threshold limit for an accurate homology modeling (Marti-Renom et al. 2000). Then, the sequence of the re-HDT was aligned against the template structure, and the project file was submitted to SWISS-MODEL server. After the calculation, the resulting homology structure was evaluated employing the PyMOL program. It is found that the homology structure of the re-HDT is not only extremely similar to its template structure as expected, but also quite similar to the dihydropyrimidinase from yeast *Saccharomyces kluyveri* (r.m.s.d. = 1.3 Å), which has one tight zinc-α (five-coordinate) and one loose zinc-*β* (five-coordinate). The superposition (Fig. 7b) and the sequence alignment (Fig. 7a) demonstrate that the location of Zn^{2+}-binding residues in the dihydropyrimidinase is conserved in the re-HDT. Overlap of Zn^{2+} binding residues (H62, H64, H255, H199, D358 and K167) of the dihydropyrimidinase with the equivalent residues of the re-HDT (H59, H61, H239, H183, D316 and K150) yielded a r.m.s.d. value on the six Cα atoms of only 0.1 Å. That suggests that the six equivalent histidine residues in the re-HDT may also participate in Zn^{2+} binding.

Discussion

Microbial D-hydantoinase is one of the important enzyme sources in industrial bioconversion to D-amino acids and their derivatives (Altenbuchner et al. 2001). Although biochemists and chemists have contributed to the work on mechanisms and applications of this enzyme for several decades, some new features have being discovered from a variety of

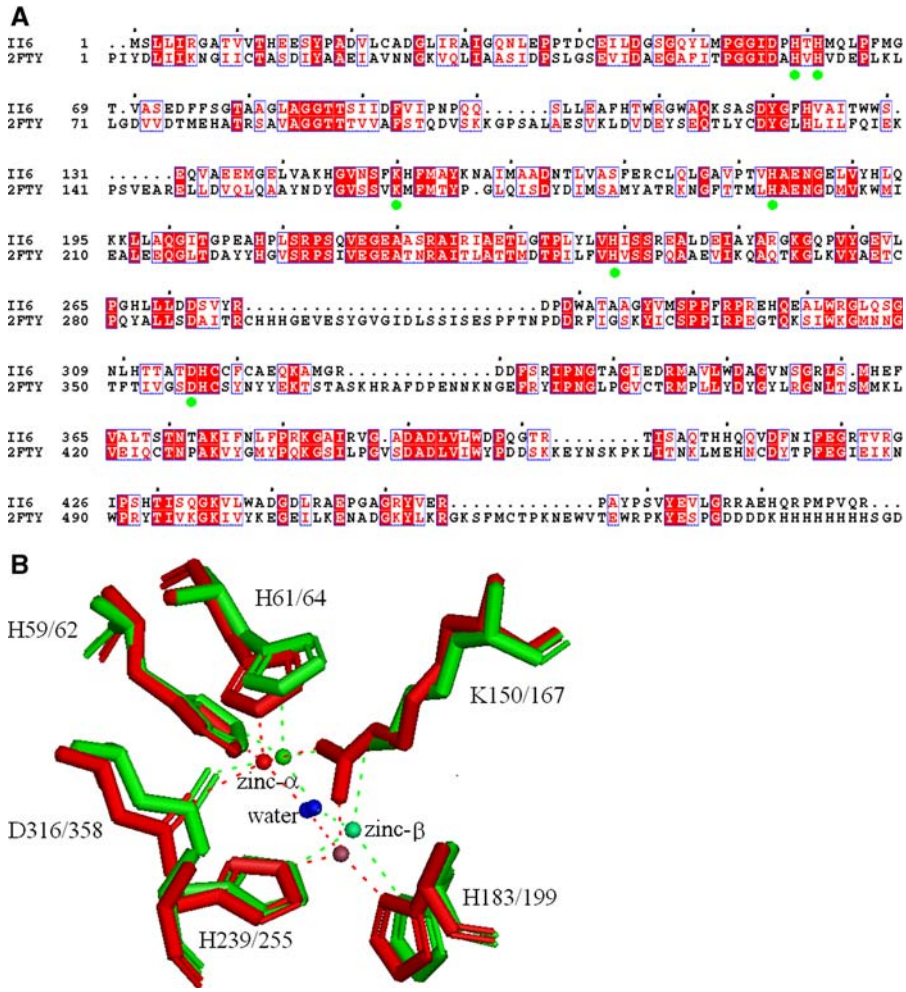

Fig. 7 Structural comparison between yeast dihydropyrimidinase and the re-DHT. **a** The sequence alignment of the dihydropyrimidinase from yeast *S. kluyveri* (2FTY) and the hydantoinase from *P. putida* YZ-26(II6). The Zn^{2+}-binding residues are indicated by green circular shapes. **b** Superposition at the active sites of yeast dihydropyrimidinase (*red*) and *P. putida* YZ-26 D-hydantoinase (*green*). The zinc atoms at zinc-α site and at zinc-β sites for the dihydropyrimidinase are shown as *red* and *raspberry spheres*, respectively. The putative zinc atom at zinc-α and zinc-β sites for the D-hydantoinase are show as *green* and *lime green spheres*, respectively. Water molecules are shown as *blue spheres* (colour online)

organisms, especially from microorganisms. Generally speaking, D-hydantoinase is thought to be a metal-dependent enzyme, which can be inhibited by addition of metal chelating reagents, for example DPA, to produce apo-enzyme. Similarly, it can also be re-activated by the addition of divalent metal ions to the apo-enzyme, such as Co^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} (May et al. 1998a, b). It is noteworthy that Zn^{2+} is the second most abundant transition metal in living organisms after iron, responsible for maintaining protein structure, regulating protein function and participating in catalysis. Approximately 9% structures in Protein Data Bank (PDB) belong to Zn^{2+}-containing enzymes. Previous reports have substantiated that divalent metal ions, especially Zn^{2+}, play an important role in the catalytic process of D-hydantoinase, and in fact most HDTs are Zn^{2+}-enzyme or Zn^{2+}-dependent enzyme (Jahnke et al. 1993; Abendroth et al. 2002). However, the data reported regarding Zn^{2+} content of D-hydantoinase were quite inconsistent. For example, the hydantoinase from bovine liver or rat liver contains only one Zn^{2+} per subunit, while the hydantoinase from *Bacillus stearothermophilus* or *Arthrobacter aurescens* has two Zn^{2+} ions per

subunit (May et al. 1998a, b; Cheon et al. 2002). The result herein from the quantitative analysis of Zn^{2+} content and the functional evaluation of the hydantoinase from *Pseudomonas putida* YZ-26 proves that this enzyme differs from those as described above. Based on our experimental data, one tight Zn^{2+} (zinc-α) per subunit is mostly responsible for the substrate catalysis and the other loose Zn^{2+} (zinc-β) per the same subunit is only essential for maintaining the protein conformation.

It is shown from the Zn^{2+} quantitative determination of the re-HDT and apo-HDT that the Zn^{2+} at the tight site is directly proportional to the enzymatic activity, while apo-HDT has little activity, indicating that the zinc-α site plays an important role in the catalytic process. Subsequently, being confirmed by a Zn^{2+} titration experiment of the apo-HDT, the activity recovery is related to Zn^{2+} amount and reaches a plateau when one Zn^{2+} is saturated for each subunit. This result demonstrated once again that only one Zn^{2+} in the re-HDT, so called the zinc-α, participates in the enzymatic catalysis. Obviously, the apo-HDT activity was not completely recovered to the level of the re-HDT (approximately 75%) by Zn^{2+} titration, suggesting that the removal of Zn^{2+} results in the conformational change or partly enzymatic inactivation. In addition to the zinc-α site, the re-HDT has another loose Zn^{2+}-binding site (zinc-β), which is different from the tight Zn^{2+} binding site. ICP-AES analysis shows if external Zn^{2+} is added to the re-HDT, the product, Zn^{2+}-HDT has two Zn^{2+} ions per subunit, which is different from the re-HDT carrying one Zn^{2+} per subunit. Moreover, the identical activity of the re-HDT and Zn^{2+}-HDT indicates that Zn^{2+} at zinc-β site is not essential for the enzymatic activity.

In addition, the presence or absence of Zn^{2+} for a protein is often accompanied by the protein conformational change (Erk et al. 2003). Golynskiy et al. (2005) reported that the addition of Mn^{2+} resulted in not only a significant stabilization of MntR, but also fluorescent quenching. This structural transformation can be manifested by obvious changes in the emission fluorescence intensity and by the special adsorption of circular dichroism (CD) spectrum. In our study, Zn^{2+}-HDT quenches the fluorescence intensity by 28% and apo-HDT increases the intensity by 27%, compared with that of re-HDT, indicating that there exists the corresponding protein structural change with respect to different Zn^{2+} content in enzymes. The result is consistent with CD spectrum that zinc-β site can influence the secondary structure of the re-HDT. These findings show that both zinc-α and zinc-β sites are all required to maintain the structure of the enzyme.

Interestingly, a phenomenon has been observed that during the titration of the apo-HDT with $ZnCl_2$, the enzyme activity was proportionally increased until Zn^{2+} to subunit ratio reached 1:1, and after that, the enzymatic activity was no longer enhanced (Fig. 3). The result indicates that when both zinc-α and zinc-β sites are vacant, the Zn^{2+} preferentially binds to zinc-α site and as soon as the zinc-α site is bound to Zn^{2+}, excess Zn^{2+} ions gradually bind to the zinc-β site. Obviously, the apparent binding constant of zinc-α site should be much higher than that of zinc-β site. Lohkamp et al. (2006) also found that at least one Zn^{2+} is loosely bound to the HDT from *S. kluyveri*, based on metal exchange experiments. The larger Zn^{2+}-binding capacity of the re-HDT may be from a longer time adaptation and evolution, as well as to ensure the preferential supply of Zn^{2+} for zinc-α site in Zn^{2+} deficient environment.

Lohkamp et al. (2006) suggested that one tight zinc-α and one loose zinc-β sites might be a general feature of HDT, which is consistent with our conclusion. Moreover, the homology comparison of the D-hydantoinase is not only similar to the gross structure of these two crystal structures as described above but also identical in their Zn^{2+} binding sites. It shows that the Zn^{2+} binding characteristics of this enzyme should be similar to that of these two known structures, which indicates that the binding affinity of the five-coordinate zinc-α site should be higher than that of the four-coordinate zinc-β site.

In conclusion, our present work predicts that the D-hydantoinase from the strain *P. putida* YZ-26, which is classified and identified by this laboratory, is a new-type metal-dependent or metalloenzyme involving one mole Zn^{2+} per one mole subunit, but it has two Zn^{2+} binding sites, a zinc-α and a zinc-β. The zinc-α site is essential for both activity and conformation, and the zinc-β site only contributes to the protein conformation.

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