Appl Biochem Biotechnol (2008) 144:237–247
DOI 10.1007/s12010-007-8004-3

The Flexibility of the Non-Conservative Region at the C Terminus of D-Hydantoinase from *Pseudomonas putida* YZ-26 is Extremely Limited

Xue-Yao Zhang · Li-Xi Niu · Ya-Wei Shi · Jing-Ming Yuan

Published online: 18 August 2007

Abstract We previously reported that a deletion mutant (P478) with a residue Arg deleted at the C terminus of D-hydantoinase (P479) from *Pseudomonas putida* YZ-26 was dissociated into the monomer from its dimeric state. Based on the above result, a series of mutants of the enzyme with the C-terminal residues either deleted or substituted were prepared. The size-exclusion chromatography and bioactivity assay show that a C-terminal-substituted enzyme (R479D) and several truncated mutants (P478, P477, P476, and P475) are dissociated into the monomeric state as well, but their activities are largely retained. In contrast, two other mutants (R474 and R479A) are expressed in the form of random aggregates without any activity. Our experiments demonstrate that only the last four amino acids (-PVQR) at the C terminus of the enzyme can be deleted without seriously affecting its activity, although the enzyme is dissociated from a dimer into a monomer. These mutants also reveal some unique properties such as the enzymatic activity in vivo or in vitro, the effect of divalent metal ions, and the thermostability etc. in comparison to wild-type enzyme (P479). In addition, the three-dimensional structural modeling shows that the intact structure of the enzyme is essential, and the flexibility of the non-conservative region at the C terminus of the enzyme is quite limited.

Keywords D-Hydantoinase · C-terminal deletion and substitution · Subunit dissociation · Structural modeling · Properties

Introduction

Hydantoinase (EC 3.5.2.2), presented in a wide variety of bacteria, fungi, and plants, as well as a few animals, catalyzes the substrate 5′-monosubstantial hydantoin to enantionmerical

X.-Y. Zhang · L.-X. Niu · Y.-W. Shi · J.-M. Yuan (✉)
Key Laboratory of Chemical Biology and Molecular Engineering of National Ministry of Education, Institute of Biotechnology, Shanxi University, 92 Wu-cheng Road, Taiyuan 030006, People's Republic of China
e-mail: jmyuan@sxu.edu.cn

N-carbamyl-amino acids [1, 2]. Based on different enantioselectivities for various substrates, hydantoinases can be classified into three types: D-, L- or DL-configuration [3, 4]. However, the D-hydantoinase from microorganisms has attracted much more attentions because it is used for the production of optically pure D-amino acids that are key intermediates in the synthesis of commercial products such as β-lactam semisynthetic antibiotics, peptides, hormones, pyretroids, and pesticides [5]. In addition, extensive studies of hydantoinases from various microorganisms such as strain screening, gene cloning, protein purification, dependence of metal ions, reaction mechanism, and three-dimensional conformation have been reported [6–8]. As one of the effective ways to study the relationship between protein structure and function, the site-directed mutagenesis and deletion mutation have been widely used [9, 10]. Recently, Cheon et al. [11] reported that some stereochemistry gate loops in the D-hydantoinase from *B. stearothermophilus* SD1 constituted a hydrophobic substrate binding pocket, and its mutants (Y155F, Y155E, L65E, and F159E) located at these loops might cause a dramatic decrease in enzymatic activity for two different substrates, non-substituted hydantoin and hydroxylphenyl-hydantoin (HPH). Kim and Kim [12] analyzed the relationship between the structure and function of D-hydantoinases as well as dihydropyrimidinases from various sources, and they found that the amino acid sequence at the N-terminal region of the enzyme is highly conserved, whereas that at the C-terminal region is flexible and non-homologous. Therefore, they knocked 11 (LMADQDVVKMS) and 12 (STISKQSEELTI) residues from the C terminus of D-hydantoinases originated from *B. thermocatenulatus* GH2 and *B. stearothermophilus* SD1, respectively. As a result, these two deletion mutants are dissociated into monomer without a significant change of the bioactivity [12].

Our previous work has indicated that a mutant enzyme (P478) with the C-terminal Arg residue deleted is only dissociated into monomer from the native dimeric state and retains about 45% activity [3]. In this study, we further substituted the C-terminal residue Arg (positively charged) of the wild-type D-hydantoinase to Asp (negatively charged) and Ala (neutral), respectively, to generate mutants R479D and R479A. Meanwhile, by analyzing the C-terminally truncated mutants P477, P476, P475, and P474, we found that the flexibility of the non-conservative region at the C terminus of the enzyme is quite limited, implying that the intact enzyme structure is essential for its activity. The mutant enzyme R479D and C-terminally truncated mutants P477, P476, and P475 retain their catalytic activity, although they are dissociated into monomer. Meanwhile, the higher pH stability of these mutants and their positive response of bioactivity with Mn^{2+} concentration have a significant implication for these mutants to be used in industrial processes.

Materials and Methods

Enzymes, Reagents, and Strains

All restriction endonucleases were obtained from New England Biolabs (Beverly, MA, USA). *Escherichia coli* BL21 (DE3), *E. coli* DH5α, vector pET-3a, recombinant plasmids pE-p479 and pE-p478 used in these experiments were stored in this laboratory. DL-Hydantoin was purchased from Sigma-Aldrich (St. Louis, USA). The protein purification equipment and chromatography columns were from Amersham Pharmacia Biotech (Uppsala, Sweden). Unless otherwise stated, all reagents were of analytical grade.

DNA Manipulation and Recombinant Plasmid Construction

The open reading frame encoding D-hydantoinase mutants were amplified by polymerase chain reaction from a recombinant plasmid pE-p479 as the template using following primers:

FW (public): 5′-CCC CAT ATG TCC CTG TTG ATC CGT-3′
RV_1 R479D: 5′-CCC GGA TCC TCA GTC CTG AAC TGG CAT-3′
RV_2 R479A: 5′-CCC GGA TCC TCA CGC CTG AAC TGG CAT-3′
RV_3 P477: 5′-GG CGGA TCC TCA AAC TGG CAT CGG CCG CTG -3′
RV_4 P476: 5′-CCC GGA TCC TCA TGG CAT CGG CCG CTG-3′
RV_5 P475: 5′-CGG GGA TCC TCA CAT CGG CCG CTG ATG-3′
RV_6 P474: 5′-CCC GGA TCC TCA CGG CCG CTG ATG CTC-3′
RV_7 P473: 5′-CCC GGA TCC TCA CCG CTG ATG CTC GGC -3′

Each amplified product was inserted into restriction sites (*Nde*I/*Bam*HI) of the vector pET3a to produce the corresponding recombinant plasmid. The truncation or substitution bases of mutant genes were confirmed by DNA sequencing.

Expression and Purification of Mutants

Recombinant plasmids were introduced into *E. coli* BL21 (DE3) and expressed in 1-l Luria–Bertani culture supplemented with 100 mg/l of ampicillin at 30 °C for 12–14 h without adding any inducers. Each culture was harvested by centrifugation at 6,000×*g* for 10 min at 4 °C. The pellet was resuspended in chilled 50 mM Tris–HCl pH 8.0 (TB8) as well as sonicated 15 times for 8 s each time in an ice bath and centrifuged at 20,000×*g* for 30 min. To improve the protein recovery and specific activity of the enzyme, we developed an efficient purification procedure by a two-step chromatography, an ion exchange chromatography, and followed by a hydrophobic chromatography. After the determination of the enzymatic activity and protein concentration (Bradford method), the above supernatant was directly applied to a Q-Sepharose Fast Flow (30/8) column pre-equilibrated with TB8. The enzyme was eluted from the column with TB8 including 0.35 M NaCl. The fractions containing the target enzyme were collected and mixed with equal volume of 2 M ammonium sulfate. Then, the mixture was applied to a Phenyl-Sepharose Fast Flow column (16/10) pre-equilibrated with TB8 containing 1 M ammonium sulfate. After the column was washed with 0.2 M ammonium sulfate, the enzyme was eluted with 50 mM Tris–HCl pH 8.0. The purified enzyme was stored at 4 °C for following experiments. The protein concentration was determined by the Bradford assay using bovine serum albumin as the assay standard.

Enzyme Activity Assay

D-Hydantoinase activity was determined by the colorimetric method [2, 3]. Briefly, the enzyme reaction mixture contained 0.2 ml cultured cells or 10–100 μg purified enzyme in 1.5 ml of TB8 with 100 mM non-substituted hydantoin as the substrate. After being incubated at 37 °C for 30 min with moderately stirring, the reaction mixture was stopped by adding 0.25 ml of 10% trichloroacetic acid and 0.25 ml 10% dimethylaminobenzaldehyde solution and then diluted with distilled water to a volume of 3 ml. The precipitate was removed by centrifugation at 10,000×*g* for 10 min. The amount of product, *N*-carbamyl-glycine in the

supernatant was measured at 430 nm, and its amount was calculated from a standard calibration plot. One unit of D-hydantoinase activity is defined as the amount of enzyme required to produce 1 μmol *N*-carbamyl-D-amino acid from hydantoin derivative per minute under the assay conditions.

Molecular Weight Measurement of Wild-Type Enzyme and Its Mutants

The molecular weight of the monomer enzyme and its mutants was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis SDS–PAGE [13], and the molecular weight of their native forms were determined by size-exclusion chromatography using a pre-packed Superose12 column (10/30 cm) equilibrated with TB8 on an AKTA purifier. The eluting buffer used was the same buffer at the flow rate of 1 ml/min. The appropriate standard proteins used are: α-lactalbumin 18 kDa; trypsin inhibitor 20.1 kDa; albumin chicken egg 45 kDa; bovine serum albumin (dimer 134 kDa, monomer 67 kDa).

Effect of pH and Temperature on Enzymatic Activity

An aliquot of enzyme solution was incubated in buffers with various pH values (0.05 M K_2HPO_4–KH_2PO_4 buffer pH 6.0–7.0, 0.05 M Tris–HCl buffer pH 8.0–9.0, and 0.05 M $NaHCO_3$–NaOH buffer pH 10–11.0) at 37 °C for 1 h, and then 10 μl of incubated solution was taken to the standard reaction system for the activity assay. For thermal stability study, samples were incubated at 60 °C for 10 min and chilled on ice for 5 min, and then the enzymatic activity was measured under the standard assay conditions.

Three Dimensional Structure Modeling

The Swiss-Model Protein Modeling Server was used to select optimal homology model structures (Swiss-Model at http://swissmodel.expasy.org//) [14]. A search for three-dimensional structure templates was performed by the ExPASy Molecular Biology Server. SWISSPDBVIEWER 3.7 (http://www.expasy.ch/spdbv/) program is used for investigating and analyzing the derived structure.

Results

Enzymatic Activity of D-Hydantoinase Mutants In Vitro and In Vivo

Each engineered strain (*E. coli*) containing stepwise truncation gene of the D-hydantoinase C terminus was expressed under the same conditions, and the bioactivity in cells was assayed as described in "Materials and Methods". Table 1 shows that mutants P478, P477, P476, P475, and R479D are not only expressed in the soluble form but also basically retain their activities, whereas P474 and R479A are expressed as an aggregate with no activity, implying that the C-terminal residues of the enzyme are related to expression forms of various recombinants in *E. coli*. Next, we purified each soluble enzyme, P478, P477, P476, P475, and R479D by the purification procedure as described in "Materials and Methods". A single band of approximately 54 kDa is observed on SDS–PAGE (Fig. 1). Moreover, the activity of the various mutants purified is consistent with the activity measured in cells (Table 1). These data suggest that first, a polar group at the C-terminal residue, such as Arg or Asp residual, is essential for both expression and activity of the D-hydantoinase;

Table 1 Comparison of enzymatic activities in vitro or in vivo for mutant D-hydantoinases[a].

		In cells				Free enzyme	
Samples	Residues of C terminus	Total activity (U)	Specific activity (U/ml)	Relative activity (%)	Exp. prod.[b]	Specific activity (U/mg)	Relative activity (%)
P479	-PMPVQR	1752	2.12	100.0	sol	10.75	100.0
R479D	-PMPVQD	1452	1.73	81.6	sol	8.41	78.2
P478	-PMPVQ	1256	1.33	62.7	sol	9.32	86.7
P477	-PMPV	1050	1.32	62.2	sol	6.60	61.4
P476	-PMP	1180	1.48	69.8	sol	8.10	75.3
P475	-PM	1162	1.40	66.0	sol	6.13	57.0
P474	-P	6	0.01	0.4	ppt	–	–
R479A	-PMPVQA	50	0.06	2.8	ppt	–	–

[a] Data were from one liter culture expressed by various engineered strains. P479 is the recombinant wild-type enzyme, and others are mutants.

[b] *Exp. prod.* Expression product, *sol* soluble, *ppt* precipitate

otherwise, the enzyme cannot be functionally expressed as occurred in R479A; second, only the last four residues can be deleted without significantly affecting the enzyme activity, indicating that the flexibility of the non-conservative region at the C terminus is very limited, and these four residues may not be a part of the hydrophobic core structure of the enzyme; third, the penultimate fifth residue (Met) at C terminus is a critical amino acid for both quaternary structure and enzymatic activity.

Oligomeric State of Mutant Enzymes

In our previous work [1], the wild-type enzyme P479 has been confirmed to be a dimer as its functional unit. However, the oligomeric structure of mutant enzymes was obviously different from P479, as monitored by size-exclusion chromatography and native-PAGE (data not shown). The retention volume of the wild-type enzyme (P479) is at 11.41 ml on Superose 12 column, whereas that of various mutants is 12.25 ml as shown in Fig. 2. In view of the plot of Kav with log *Mr*, the molecular mass of the wild-type enzyme and mutants should be 110 and 54 kDa, respectively. These data indicate that wild-type enzyme P479 is a dimer, whereas mutant enzymes R479D, P478, P477, P476, P475, and P474 are dissociated into monomer. Therefore, the results pointed out that when a few residues at the

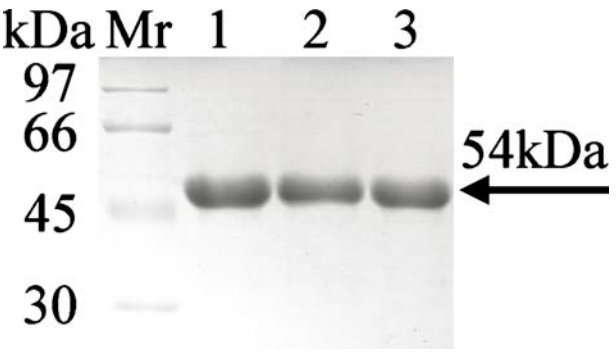

Fig. 1 SDS–PAGE analysis of the purified wild-type D-hydantoinase and its mutants. *Mr* Molecular marker: Phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa. *1* P479, *2* P477, *3* R479D. Other mutants were omitted due to same bands as indicated in Fig. 1

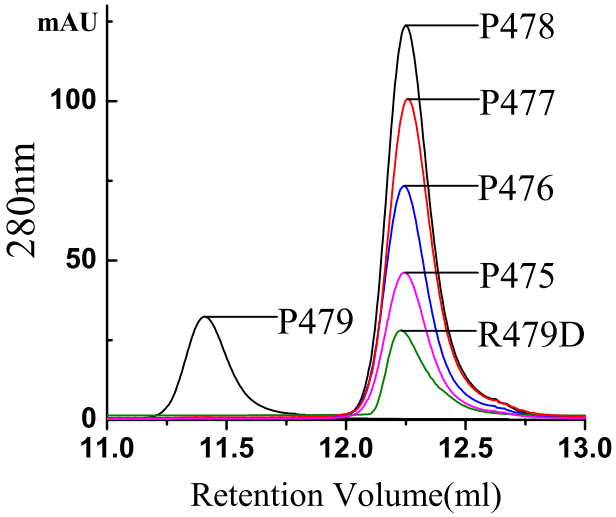

Fig. 2 The molecular mass determination of the D-hydantoinase and its mutants using size-exclusion chromatography. The D-hydantoinase purified was loaded on a pre-packed column Superose 12 (10/30) and eluted with 50 mM Tris–HCl (pH 8.0) containing 100 mM NaCl at a flow rate of 0.5 ml/min. All samples were conducted at the same conditions

C-terminal region of D-hydantoinase are truncated, it may cause the dissociation of oligomeric structure without significant loss of its enzymatic activity. Meanwhile, the data also indicates that if the truncation at the C terminus of D-hydantoinase is more than four residues, the enzyme can be only expressed as an aggregate without any activity.

Tertiary Structure Modeling of the Enzyme

Although the tertiary structure of the D-hydantoinase from *P. putide* YZ-26 has not been determined so far, the data from X-ray crystallography and nuclear magnetic resonance spectroscopy of highly homologous proteins in the same superfamily of cyclic amidohydrolases can be used as a modeling reference [15]. The three-dimensional structure of some relevant homologous enzymes has been determined, such as Dihydropyrimidinases from *Dictyostelium discoideum* [16] and *Thermus* sp. [8]; D-Hydantoinases from *Bacillus stearothermophilus* [17], *Burkholderia pickettii* [7], and *Bacillus* sp. AR9 [18]. They reveal a similar tertiary structure that the closed, parallel β-sheet structure of the $(\alpha/\beta)_8$-barrel is formed from eight parallel (α/β)-units linked by hydrogen bonds that form a cylindrical core. To investigate whether these data could be used to model a putative structure for the enzyme from its primary structure, the amino acid sequence of D-hydantoinase from *Pseudomonas putida* YZ-26 (1-479) was sent to the Swiss-Model Automated Protein Modeling Server (http://swissmodel.expasy.org//) [14]. We selected the Dihydropyrimidinase from *D. discoideum* (*E* value=$3e^{-128}$, PDB ID: 2FTW) [16] as the best homology for deriving a putative tertiary structure of the D-hydantoinase as shown in Fig. 3. In this model, a few residues at C terminus seem to be out of the core region and to form an extended structure. Moreover, the last seven residues of the model are in random coil nearby the terminal α-turn, meaning that the flexibility of the C terminus is quite limited. Thus, the over-truncation in C-terminal residues, such as the mutant P474, will destabilize the protein structure with complete loss of the activity. In view of the sequence at the C terminus of the enzyme (-PMPVQR), it is undoubtedly that the polar residue Arg is a crucial one for the quaternary structure and activity of the enzyme. This may explain why the polar residue Arg can be substituted by a similarly polar residue Asp, but cannot be substituted by the non-polar residue Ala, as shown in Table 1.

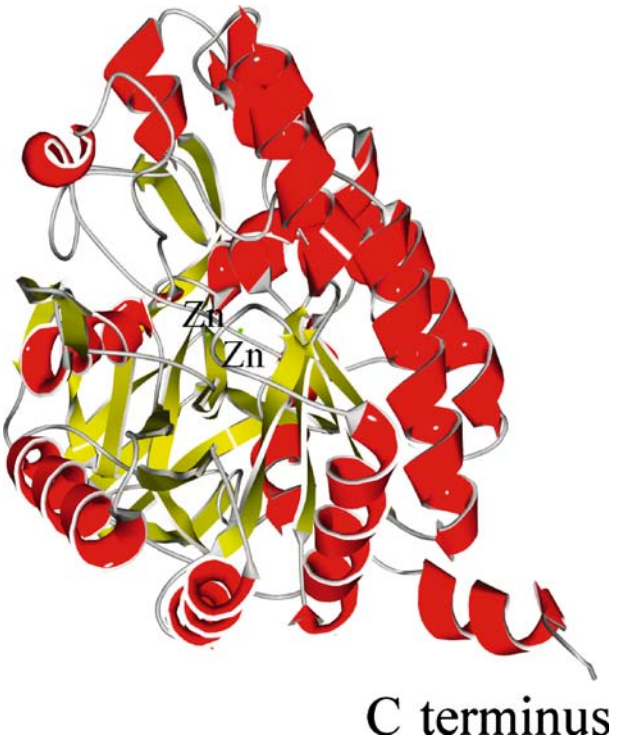

Fig. 3 The tertiary structure modeling of the D-hydantoinase monomer. The amino acid sequence homology of the D-hydantoinase was compared with that of Dihydropyrimidinase monomer from *D. discoideum*. β-Sheets are presented in *yellow*, α-helices in *red*, and coil in *gray*. Zn^{2+} ion sites in the enzyme are as indicated by *two green spheres*. The C-terminal residues are depicted

pH Stability of Mutant Enzymes

The pH stability of mutant enzymes is significantly higher than that of the wild-type enzyme at both acidic and basic sides. The relative activity of mutants is about twice as that of the wild-type enzyme at pH 6.0 and pH 10.0. However, the influence of pH, either on the wild-type enzyme or on mutants, is close to the optimal pH (from pH 8.0 to 9.0) as shown in Fig. 4. A possible explanation is that some titratable groups of the D-hydantoinase in acid or basic environment will exhibit different pKa values on the monomer and dimer, resulting in altering the interaction among the enzyme, substrate, and solvent in the reaction system.

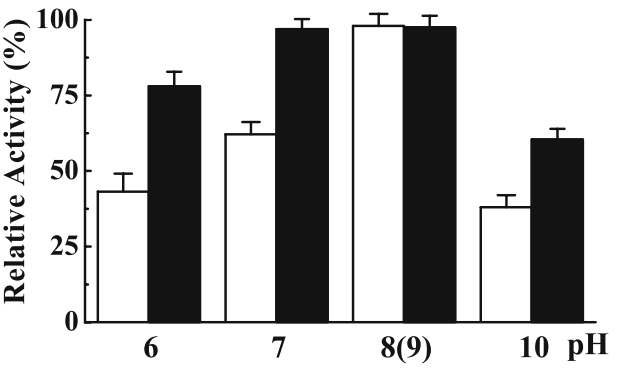

Fig. 4 pH stability of the wild-type D-hydantoinase (*open square*) and its mutants (*filled square*). Conditions are as follows: enzyme (100 μg/ml) was incubated in various buffers (0.05 M K_2HPO_4–KH_2PO_4 buffer pH 6.0–7.0, 0.05 M Tris–HCl buffer pH 8.0–9.0, 0.05 M $NaHCO_3$–NaOH Buffer pH 10.0–11.0) at 37 °C for 1 h, then taking 20 μg each to 1.5-ml reaction system for activity assay as described in "Materials and Methods"

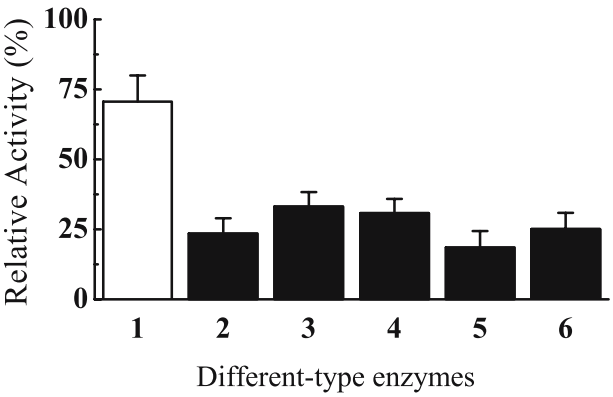

Fig. 5 Comparison of thermostability between wild-type D-hydantoinase (*filled square*) and its mutants (*open square*). *1* P479, *2* R479D, *3* P478, *4* P477, *5* P476, *6* P476. Each kind of enzyme (100 μg/ml) was incubated at 60 °C for 10 min and then an aliquot of solution was taken to assay the enzymatic activity as described in "Materials and Methods"

Usually, the pH dependence in a protein is mainly from electrostatic interaction from charges in the protonation of acidic and basic groups [19]. Thus, it is easily understood that the higher pH stability of mutants occurred at both acidic and basic sides.

Thermal Stability of Mutant Enzymes

The thermal stability of P479 is obviously higher than that of all mutants under the same conditions (Fig. 5). Mei et al. [20] reported that the thermal stability of oligomer protein could be higher than that of monomer due to the interaction of more hydrogen bonds and electrostatic bonds between subunits. In this case, the oligomeric D-hydantoinase may be less flexible and resist the thermal inactivation. On the contrary, the monomer enzyme has less these interaction forces that may affect its stability under the externally environmental factors.

Effect of Zn^{2+} and Mn^{2+} on Enzyme Activity

Our present work shows that the D-hydantoinase from *P. pudita* YZ-26 is a Zn^{2+}-binding enzyme (data unpublished). However, the effect of Zn^{2+} and Mn^{2+} at the same concentration of 1 mM on mutant enzymes was quite different from that on the wild-type enzyme P479. As seen in Table 2, 1 mM Zn^{2+} ion attenuates the enzymatic activity down to 60% for P479 and to more than 90% for all of mutants, whereas 1 mM Mn^{2+} ion can

Table 2 Effect of Zn^{2+} or Mn^{2+} on wild-type D-hydantoinase and its mutants.

Enzymes	Metal ions					
	None		Mn^{2+} (1 mM)		Zn^{2+} (1 mM)	
	U/mg	%	U/mg	%	U/mg	%
P479	10.75	100.0	21.0	196	6.45	60.0
R479D	8.41	78.2	49.4	459	0.93	8.7
P478	9.32	86.7	45.8	426	0.53	4.9
P477	6.60	61.4	50.4	468	0.61	5.7
P476	8.10	75.3	71.5	665	0.71	6.6
P475	6.13	57.0	47.9	446	0.41	3.8

enhance the activity up to 200% for P479 and to 400–500% for its mutants (Table 2). Moreover, the activation of Mn^{2+} is largely increased, and the inhibition of Zn^{2+} still retains 90% or so if the activity of each mutant in the absence of Zn^{2+} or Mn^{2+} is calculated as 100% (comparison of each mutant by itself). The result implies that the binding sites of Zn^{2+} and Mn^{2+} may locate at different regions on the enzyme. We have mentioned in the previous report that the effect of Zn^{2+} or Mn^{2+} on the D-hydantoinase is similar to D-aminoacylase as a TIM (Triose phosphate isomerase) barrel-dependent hydrolase, which has αβ-binuclear subset to bind essential metal ions [1, 21]. Therefore, the external Mn^{2+} ion as an activator may loosely bind to the β-subsite so as to enhance its substrate transformation, and the external Zn^{2+} ion as an inhibitor may tightly hold the α-subsite to attenuate the activity.

Discussion

For several decades, D-hydantoinase has been extensively investigated for its efficient application in bioconversion of D-amino acids [5]. Although the action substrate of D-hydantoinases from different sources is as the same as non-substituted DL-hydantoin or its derivatives, their primary structures reveal the obvious difference. The amino acid sequence homology of D-hydantoinases from various sources is generally low, reflecting that the conservative region is located at the N terminus and the variable region is at the C terminus [12]. The cloning and sequencing of the D-hydantoinase gene from *P. putida* YZ-26 indicated that the amino acid sequence homology of the enzyme was 90.4 and 58.4% compared with those from strains *P. putida* CCRC 12857 and *P. putida* DSM84, respectively [1, 2]. The relationship between the structure and function of this novel enzyme (Genbank AY387829) has been the focus of our research. In addition, understanding the structure and function of the C-terminally truncated mutants as well as the effect of Mn^{2+} and Zn^{2+} on the hydantoinase may facilitate understanding of the catalytic mechanism of the enzyme and enhancing the efficiency of bioconversion.

The enzymatic activity in vitro and in vivo shows that the C-terminal flexibility is only confined in the last four amino acids (-PVQR). If the penultimate fifth amino acid (Met) is deleted, both the correct conformation and enzymatic activity are completely abolished. The observation is consistent with the 3D model as shown in Fig. 3 that only seven extended residues at C terminus are out of the terminal α-helix, implying that these residues may be far from the active site and not essential for the enzyme conformation. Our data are quite different from that of Kim's report regarding the deletion of C-terminal residues of D-hydantoinases from strain *B. thermocatenulatus* GH2 and *B. stearothermophilus* SD1 [12]. Meanwhile, we also found that the polar residue, either positive or negative, at C-terminal residue of the enzyme is absolutely essential for both the conformation and bioactivity, and otherwise, the enzyme may form an aggregate without any activity if the C-terminal residue is substituted with a non-polar neutral amino acid.

Structurally, hydantoinase belongs to the urease superfamily and contain a catalytic TIM-barrel domain where the metal binding site is present on the C-terminal end of the β-strands. The β-strands locate nearby the α-helixes such as α4, α5, α7, and α8, which dominate the interface between subunits. It has been suggested that metal ions in hydantoinase may be involved in the inter-subunit interaction, and removal of them will cause dissociation of tetramer into the monomer [4]. However, in all crystal structure of urease family, the metal ions are inside TIM-barrel domain, and no metal is found in the inter-subunit surface. Therefore, it is speculated that there might be some direct or indirect interaction between metal binding site and inter-subunit surface. Obviously, it is not

surprising that the dissociation of oligomeric enzyme can result in the microenvironmental change around the metal ion binding sites and, in turn, change the effect of Zn^{2+} or Mn^{2+} on the D-hydantoinase.

In conclusion, our work demonstrates that the D-hydantoinase from *P. putida* YZ-26 has some unique features which are different from those reported in literatures, although their common characters are dominant. Further work on site-directed mutagenesis, 3D structure, metal ion dependence, and oligomeric state of the enzyme are ongoing in our laboratory.

Acknowledgments The authors wish to thank the National Science Foundation of Shanxi Province (NSFSX, 031042), China for this research support. We gratefully acknowledge the valuable discussion with Dr. Tao Yuan from Sanofi Pasteur, Canada.

References

1. Shi, Y. W., Niu, L. X., Feng, X., & Yuan, J. M. (2006). Purification, enzymatic properties of a recombinant D-hydantoinase and its dissociation by zinc ion. *World Journal of Microbiology and Biotechnology, 22*, 675–680.
2. Shi, Y. W., Zhao, L. X., Niu, L. X., & Yuan, J. M. (2005). Gene sequence, soluble expression and homologous comparison of a D-Hydantoinase from Pseudomonas putida YZ-26. *Chemical Research in Chinese University, 21*, 552–557.
3. Niu, L. X., Zhang, X. Y., Shi Y. W., & Yuan, J. M. (2007). Subunit dissociation and stability alteration of D-Hydantoinase deleted at the terminal amino acid residue. *Biotechnology Letters, 29*, 303–308.
4. May, O., Siemann, M., Siemann, M. G., & Syldatk, C. (1998). Catalytic and structural function of zinc for the hydantoinase from Anthrobacter aurescens DSM 3745. *Journal of Molecular Catalysis B, Enzymatic, 4*, 211–218.
5. Ogawa, J., & Shimizu, S. (1999). Microbial enzymes: new industrial applications from traditional screening methods. *Trends in Biotechnology, 17*, 13–20.
6. Huang, C. Y., Chao, Y. P., & Yang, Y. S. (2003). Purification of industrial hydantoinase in one chromatographic step without affinity tag. *Protein Expression and Purification, 30*, 134–139.
7. Xu, Z., Liu, Y. Q., Yang, Y. L., Jiang, W. H., Arnold, E., & Ding, J. P. (2003). Crystal Structure of D-Hydantoinase from Burkholderia pickettii at a resolution of 2.7 angstroms: Insights into the molecular basis of enzyme thermostability. *Journal of Bacteriology, 185*, 4038–4049.
8. Abendroth, J., Niefind, K., & Schomburg, D. (2002). X-ray structure of a Dihydropyrimidinase from Thermus sp. at 1.3 Å resolution. *Journal of Molecular Biology, 320*, 143–156.
9. Abe, I., Abe, T., Lou, W. W., Masuola, T., & Noguchi, H. (2007). Site-directed mutagenesis of conserved aromatic residues in rat squalene epoxidase. *Biochemical and Biophysical Research Communications, 352*, 259–263.
10. Villadsen, D., & Nielsen, T. H. (2001). N-terminal truncation affects the kinetics and structure of fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase from Arabidopsis thaliana. *Biochemical Journal, 359*, 591–597.
11. Cheon, Y. H., Park, H. S., Lee, S. C., Lee, D. E., & Kim, H. S. (2003). Structure-based mutational analysis of the active site residues of d-hydantoinase. *Journal of Molecular Catalysis. B, Enzymatic, 26*, 217–222.
12. Kim, G. J., & Kim, H. S. (1998). C-Terminal Regions of D-Hydantoinases are nonessential for catalysis, but affect the oligomeric structure. *Biochemical and Biophysical Research Communications, 243*, 96–100.
13. Laemmli, U. (1970). Cleavage of structural proteins during the assembly of bacteriophage T4. *Nature, 227*, 680–685.
14. Schwede, T., Kopp, J., Guex, N., & Peitsch, M. C. (2003). SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Research, 31*, 3381–3385.
15. Gerlt, J. A., & Raushel, F. M. (2003). Evolution of function in (β/α)8-barrel enzymes. *Current Opinion in Chemical Biology, 7*, 252–264.
16. Lohkamp, B., Andersen, B., Piskur, J., & Dobritzsch D. (2006). The crystal structures of dihydropyrimidinases reaffirm the close relationship between cyclic amidohydrolases and explain their substrate specificity. *Journal of Biological Chemistry, 281*, 13762–13776.
17. Cheon, Y. H., Kim, H. S., Han, K. H., Abendroth, J., Niefind, K., Schomburg, D., et al. (2002). Crystal structure of D-Hydantoinase from Bacillus stearothermophilus: Insight into the stereochemistry of enantioselectivity. *Biochemist, 41*, 9410–9417.

18. Radha Kishan, K. V., Vohra, R. M., Ganesan, K., Agrawal, V., Sharma, V. M., & Sharma, R. (2005). Molecular structure of D-Hydantoinase from Bacillus sp. AR9: Evidence for Mercury inhibition. *Journal of Molecular Biology, 347*, 95–105.
19. Skoulakis, S., & Goodfellow, J. M. (2003). The pH-dependent stability of wild-type and mutant Transthyretin Oligomers. *Biophysical Journal, 84*, 2795–2804.
20. Mei, Y. Z., He, B. F., & Ouyang, P. K. (2005). Progress in study on thermostability and substrate specificity of hydantoinase. *Chinese Journal of Bioprocess Engineering, 3*, 24–28.
21. Holm, L., & Sander, C. (1997). An evolutionary treasure: Unification of a broad set of Amidohydrolases related to urease. *Proteins: Structure, Function and Genetics 28*, 72–82.