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Structure-based mutational analysis of the active site residues of D-hydantoinase

Young-Hoon Cheon, Hee-Sung Park, Sang-Chul Lee, Dong-Eun Lee, Hak-Sung Kim*

Department of Biological Sciences, Korea Advanced Institute of Science and Technology, 373-1, Kusung-Dong, Yusung-Gu, Taejon 305-701, South Korea

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This paper is dedicated to Professor Joon-Shick Rhee on the occasion of his retirement

Abstract

We previously proposed the hydrophobic and bulky residues of the three loops, designated stereochemistry gate loops (SGLs), to constitute a hydrophobic substrate binding pocket of D-hydantoinase from *Bacillus stearothermophilus* SD1. Simulation of substrate binding in the active site of D-hydantoinase and sequence alignment of various D-hydantoinases revealed the critical hydrophobic residues closely located around the exocyclic substituent of substrate. To evaluate the roles of these residues in substrate binding pocket, site-directed mutagenesis was performed specifically for Leu 65, Tyr 155, and Phe 159. When Tyr 155 was mutated to Phe and Glu, both mutants Y155F and Y155E were totally inactive for nonsubstituted hydantoin and D,L-5-hydroxyphenyl hydantoin (HPH), which indicates that Tyr 155 is involved in substrate binding via a hydrogen bond with the hydantoinic ring. Furthermore, replacement of the hydrophobic residues Leu 65 and Phe 159 with Glu, a charged amino acid, resulted in a significant decrease in activity for nonsubstituted hydantoin, but not for HPH. The K_{cat} values of both mutants for nonsubstituted hydantoin also severely decreased, but a slight change in the K_{cat} values was observed towards HPH. These results suggest that the hydrophobic residues in SGLs play an essential role in substrate binding, and differentially interact according to the property of the exocyclic substituent.

Keywords: Mutational analysis; D-Hydantoinase; Stereochemistry gate loops

1. Introduction

Microbial hydantoinases are industrial enzymes for the production of optically pure D- and L-amino acids that are widely used in the pharmaceutical field as intermediates for the synthesis of semi-synthetic antibiotics, peptide hormones, and pesticides [1]. These enzymes are found in various microorganisms and commonly classified from an industrial viewpoint as D-, L-, and non-selective hydantoinases according to their stereospecificities.

The biological role of microbial hydantoinases remains to be elucidated, but it has long been assumed that D-hydantoinases are counterparts of mammalian dihyropyrimidinases catalyzing the reversible hydrolytic cleavage of six- or five-membered cyclic amide ring, such as dihydropyrimidines and 5′-monosubstituted hydantoins (Fig. 1) [2,3]. In recent reports, a superfamily of cyclic amidohydrolases, including hydantoinase, dihydropyrimidinase, allan-

* Corresponding author. Tel.: +82-42-869-2616; fax: +82-42-869-2610.
E-mail address: hskim76@kaist.ac.kr (H.-S. Kim).

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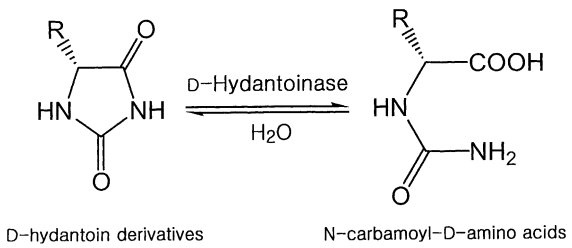

Fig. 1. Schematic reaction catalyzed by D-hydantoinase.

toinase, and dihydroorotase, has been proposed based on the functional and structural similarity of the related enzymes [4–6]. These superfamily enzymes are involved in the metabolism of pyrimidines and purines, and share the property of hydrolyzing cyclic amide bond of their respective substrates. A comparison of recently determined structures of the cyclic amidohydrolases revealed that they have a common structural core consisting of a $(\alpha/\beta)_8$-barrel with a similar active site architecture in which metal binding and catalytic residues are conserved at the C-terminal end of strands β1, β4, β5, β6, and β8, sharing a common reaction mechanism [4,7–10].

Despite the above-mentioned similarities, substrate specificity and enantioselectivity are very diverse among the cyclic amidohydrolase enzymes. Moreover, the diversities of substrate and stereo specificities are conspicuous even among hydantoinases. A non-selective hydantoinase from *Bacillus stearothermophilus* has an exclusive activity for aliphatic 5-substituted hydantoin derivatives [11], but L-hydantoinases from *Arthrobacter* sp. [12] and *Flavobacterium* sp. [13] show a preference towards aromatic or bulky 5-substituted hydantoin derivatives. Phenylhydantoinase from *E. coli* has a distinct activity for aromatic hydantoin derivatives, but little activity towards nonsubstituted hydantoin [14]. Interestingly, a hydantoinase from *Arthrobacter aurescens* shows a substrate-dependent enatioselectivity [15].

From a practical standpoint, it is important to elucidate the determinants governing substrate specificity and enantioselectivity in hydantoinases because natural hydantoinases are not optimized for the synthesis of industrially important non-natural amino acids. The structures of hydantoinases were recently determined, but biochemical studies on their active sites have not yet been implemented. Our previous analysis on the structure of the D-hydantoinase from *B. stearothermophilus* SD1 predicted three important loops; stereochemistry gate loops (SGLs), which constitute a hydrophobic substrate binding pocket [8]. Here, we attempted to identify the residues critical for substrate binding based on simulation of substrate binding to the enzyme and sequence alignment of various D-hydantoinases. Site-directed mutagenesis and characterization of the mutants were conducted to elucidate the role of the hydrophobic residues Leu 65, Tyr 155, and Phe 159 in the substrate specificity of D-hydantoinases. Details are reported herein.

2. Materials and methods

2.1. Materials

Nonsubstituted hydantoin and *N*-carbamoyl glycine were purchased from Sigma. D,L-Hydroxyphenyl hydantoin (HPH) was chemically synthesized by the method of Suzuki et al. [16]. Synthetic oligodeoxynucleotides were obtained from Bioneer (Taejon, Korea). Amylose resin was purchased from NEB. All other reagents for DNA manipulations and analyses were of analytical grade. Thermophilic *B. stearothermophilus* SD1 isolated in our previous work [17] was used as the source of the D-hydantoinase gene. *E. coli* JM109 was used as a host for expression of wild type and mutant enzymes. Plasmid pTrc99A for expression was obtained from Pharmacia and pMAL-c2x for fusion protein from NEB.

2.2. Docking simulation of substrate on the active site of D-hydantoinase

Structural coordination of substrate and enzyme was combined with the charge information by Vega program, and then substrate docking on the active site was simulated by AutoDock3.0 [18], which was run by feeding a substrate and D-hydantoinase from *B. Stearothermophilus* SD1. AutoDock3.0 consists of three major components; AutoGrid, AutoTors, and AutoDock3.0. As the first step, the grid map was created by running AutoGrid. AutoTors was then run by assigning the root position and the number of torsions. Finally, AutDock3.0 was run in order to investigate the mode of interaction between substrate and

enzyme. The structures of enzyme–substrate complex were analyzed by Swiss-PdbViewer [19].

2.3. Site-directed mutagenesis and saturation mutagenesis

Site-directed and saturation mutagenesis of D-hydantoinase were performed by the overlapping PCR method using two complementary oligonucleotides. The sequences of the mutagenic oligonucleotides used in this study are as follows: 5′-TAGAATTCATGACAAAAATTATAAAAAATG-3′ (N-terminal, *Eco*RI site) and 5′-TACTGCAGTTAAATGGTTAATTCCTCGCTC-3′ (C-terminal, *pst*I site) for cloning of D-hydantoinase gene; 5′-CATTTAGATATGCCGGAAGGCGGCACGGTGACAAAG-3′ (+), and 5′-CGGCATATCTAAATG-3′ (−) for mutation L65E; 5′-TCCCTCAAAGTGTTTATGGCGTTTAAAAACGTATTT-3′ (+), and 5′-CGCCATAAACACTTTGAGGGA-3′ (−) for mutation Y155F; 5′-TTTATGGCGTATAAAAACGTAGAACAGGCAGATGATGGA-3′ (+), and 5′-TACGTTTTTATACGCCATAAA-3′ (−) for mutation Y155E; 5′-TTTATGGCGTATAAAAACGTAGAACAGGCAGATGATGGA-3′ (+), and 5′-TACGTTTTTATACGCCATAAA-3′ (−) for mutation F159E. The plasmid pTrc99A containing D-hydantoinase gene was used as a template. N-terminal and C-terminal DNA fragments were amplified by PCR using mutagenic and cloning primers, and purified by gel-elution with GENECLEAN turbo from Bio101. The purified N-terminal and C-terminal DNA fragments were combined by overlapping PCR. The resulting DNAs were purified, digested with *Eco*RI and *Pst*I, and cloned into pMAL-c2x. The mutations were confirmed by DNA sequencing.

2.4. Enzyme purification and assay

Wild type and mutant enzymes were purified as MBP-fused forms using the pMAL-c2x vector system as previously reported [20]. The expression level and the quantity of the purified mutant enzymes are very similar to those of wild type in SDS-PAGE. The purified fusion proteins were treated with Factor Xa, and cleaved enzymes were purified by using the amylose resin column. Proteins were analyzed with SDS-PAGE and quantified by using the Bradford method.

The enzyme reaction mixture contained 0.5 mM $MnCl_2$, 50 mM nonsubstituted hydantoin or 10 mM HPH, and 0.2–100 μg purified enzyme in 1 ml of 0.1 M Tris–HCl buffer (pH 8.0). The enzyme reaction was carried out at 37 °C for 30 min with moderate stirring and nitrogen sparging to prevent oxidation of substrates. The reaction was stopped by adding 0.5 ml of 12% trichloroacetic acid, and precipitated proteins were removed by centrifugation. The amount of product was determined by using either HPLC [21] or color reagent, *p*-dimethylaminobenzaladehyde, according to the method of Takahashi et al. [2].

2.5. Determination of kinetic parameters

Kinetic constants of wild type and mutant enzymes were determined for nonsubstituted hydantoin and HPH by measuring the initial reaction rates at 37 °C for 10 min under specified conditions as described above. The concentration ranges of nonsubstituted hydantoin and HPH used were 50–400 and 1–10 mM, respectively. The maximum concentration of HPH for K_m analysis was 10 mM due to low solubility of the substrate.

3. Results and discussion

3.1. Substrate binding pockets of D-hydantoinases

Substrate binding pocket in the active site was investigated based on the structure to explain the difference of substrate specificity in hydantoinases. Three dimensional structures of several hydantoinases were recently determined [8–10], but the orientation of the substrate has not been resolved experimentally since no competitive inhibitor is known for hydantoinase. To predict the critical amino acid residues in the substrate binding pocket of the active site, the mode of substrate binding was first simulated by fitting the substrates into the active site of D-hydantoinase from *B. stearothermophilus* SD1 using “AutoDock3.0” [18]. While nonsubstituted hydantoin was not fitted in the active site, the docking simulation for D-HPH, which is an industrially important substrate for the building bock of semi-synthetic antibiotics [22], was well predicted because the large aromatic R-group has little freedom in the small pocket surrounded with hydrophobic

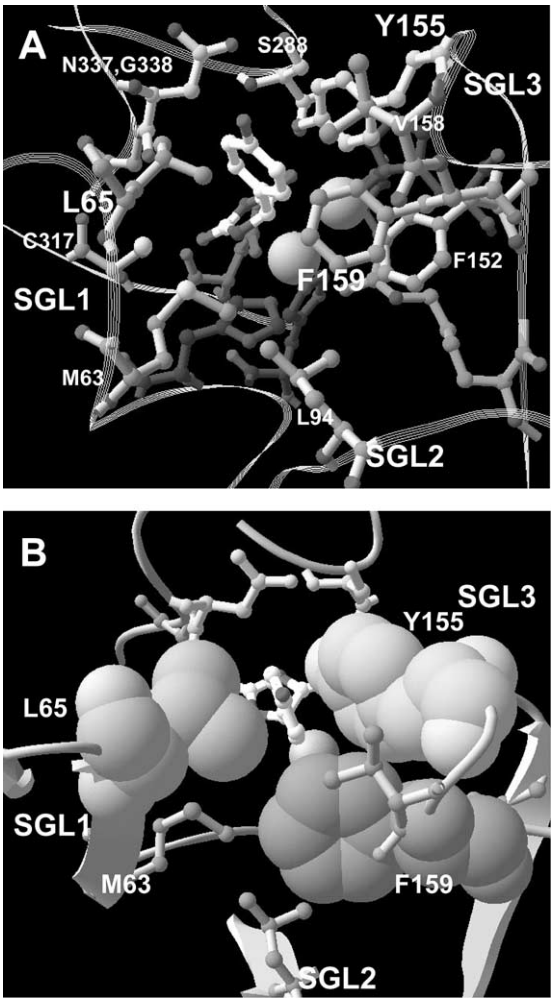

Fig. 2. Structure model of D-hydantoinase containing D-hydroxyphenylhydantoin. The residues interacting with substrate and three SGLs of substrate binding pocket (A) and the hydrophobic pocket composed of Leu 65, Tyr 155, and Phe 159 from the three SGLs (B) are presented.

side chains. From this model, the residues interacting with substrate was inferred. The substrate D-HPH was placed on the metal binding site of D-hydantoinase and was surrounded with the catalytic residues, metal binding residues, and substrate binding residues as expected (Fig. 2A).

As a next step, the amino acid sequences of various D-hydantoinases were compared. The sequences of D-hydantoinases from *B. stearothermophilus* SD1, *B. thermocatenulatus* GH2, *Agrobacterium* sp., and *Pseudomonas putida*, phenylhydantoinase form *E. coli*, and dihydropyrimidinase from *Human* were aligned (Fig. 3). The residues (four histidines, one lysine, and one aspartate) that are known as metal binding and catalytic residues in the cyclic amidohydrolases are strictly conserved at the identical positions. Besides, as shown in lower part of Fig. 3, high similarity is observed in the residues Ser 288, Cys 317, Phe 319, Asn 337 and Gly 338 for recognition of hydantoinic ring in the active sites of hydantoinases. On the other hand, the residues Met 63, Leu 65, Leu 94, Phe 152, Tyr 155, Val 158, and Phe 159 from the three loops constituting hydrophobic pocket for the exocyclic substituent of substrate show a different pattern (Fig. 3). Our previous study on structural comparison of dihydroorotase and D-hydantoinase also revealed that there is a remarkable difference in conformation of these stereochemistry gate loops [8]. In the model structure of D-hydantoinase with substrate, the side chains of the residues in SGL-1 and SGL-3 were predicted to completely seal the binding pocket, and particularly the hydrophobic and bulky residues Leu 65, Tyr 155, and Phe 159 are closely placed on the chiral exocyclic substituent of substrate (Fig. 2B). The hydrophobic nature originating from the three SGLs in the substrate binding pocket seems to be important determinants controlling the substrate specificity of D-hydantoinases.

3.2. Site-directed mutagenesis at the putative residues responsible for substrate specificity

Among the residues in the vicinity to the R-group of substrate, Tyr 155 is peculiarly well-conserved in all D-hydantoinases and its aromatic group is closely located in parallel with the exocyclic substituent of substrate in the inferred model (Fig. 2A). In addition, hydroxyl group of Tyr 155 was proposed to form a hydrogen bond with 4O of hydantoinic ring in the previous reports [8,9]. To identify the role of Tyr 155 in substrate binding, Tyr 155 was mutated into Phe, a non-polar hydrophobic amino acid, and Glu, a charged one. As a result, both mutants Y155F and Y155E were found to be totally inactive (Table 1), which indicates that the proposed hydrogen bond rather than inferred hydrophobic interaction of Tyr 155 primarily contributes to the substrate binding in the active site.

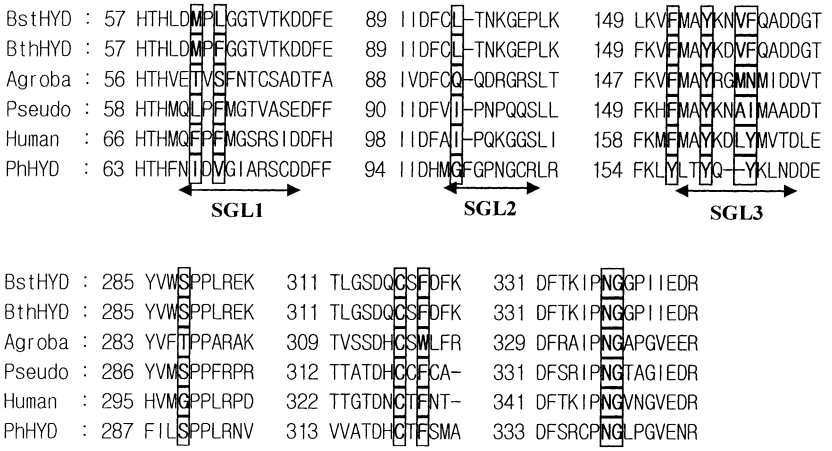

Fig. 3. Sequence alignment of various hydantoinases. The residues interacting with substrate are boxed. Metal binding and catalytic residues are not presented since they are strictly conserved in all cyclic amidohydrolases. The sequences of D-hydantoinases from *B. stearothermophilus* SD1 (BstHYD), *B. thermocatenulatus* GH2 (BthHYD), *Agrobacterium* sp. (Agroba), and *Pseudomonas putida* (Pseudo), phenylhydantoinase form *E. coli* (PhHYD), and dihyropyrimidinase from *Human* (Human) are aligned.

In the three SGLs of D-hydantoinase, Leu 65 and Phe 159 of D-hydantoinase take major part in the hydrophobic lid and interact with the exocyclic substituent of substrate in the neighborhood (Fig. 2B). To investigate the influence of the hydrophobic nature of the pocket on the activity, the hydrophobic residues Leu 65 and Phe 159 were replaced with a charged amino acid, and activities of the mutant enzymes towards nonsubstituted hydantoin and HPH were determined. When Leu 65 was changed to Glu, the activity for HPH significantly decreased to about 20% of wild type, and that for nonsubstituted hydantoin reduced more seriously to only 0.3% (Table 1). Likewise, mutation F159E also resulted in a decrease in the activity for HPH to about 20%, and for nonsubstituted hydantoin to less than 0.1%. Kinetic analysis shows that the low activity of these mutants for nonsubstituted hydantoin results from severely decreased K_{cat} value and affinity (Table 2). These results confirm that hydrophobic nature of substrate binding pocket near the exocyclic substituent of substrate plays an important role in the D-hydantoinase activity, which are consistent with the report that the activity of hydantoinase for diverse substrates is affected by the hydrophobic interaction of the exocyclic substituent and substrate binding residues [23]. According to the simulation model of HPH-embedded hydantoinase, though bulky hydrophobic side chains of Leu 65 and Phe 159 do not directly interact with 5′-hydrogen of nonsubstituted hydantoin, mutations

Table 1
Specific activities of the mutant enzymes for hydantoin and for HPH

Mutant	Specific activity (U/mg)	
	Hydantoin	HPH
WT	117	6.1
L65E	0.33	1.0
Y155E, Y155F	ND[a]	ND
F159E	0.067	1.1

These mutations did not affect on the enantioselectivity.
[a] ND, not detected under specified experimental conditions.

Table 2
Kinetic constants of the mutant enzymes for hydantoin and for HPH

	Hydantoin			HPH		
	K_{cat} (s^{-1})	K_m (mM)	K_{cat}/K_m	K_{cat} (s^{-1})	K_m (mM)	K_{cat}/K_m
WT	440	98	4.5	18	5.4	3.3
L65E	7.1	1100	0.0065	24	140	0.17
F159E	2.5	1900	0.0013	7.0	34	0.21

of these residues to Glu are thought to weaken the hydrophobic interaction between three SGLs constituting hydrophobic lid in the active site, consequently leading to a loose substrate binding pocket for the substrate with small R-group. For these reasons, hydrophobic environment seems to be important for the binding of substrate with hydrogen as R-group as well as substrate with bully aromatic group.

Interestingly, while the mutations L65E and F159E caused a dramatic decrease in activity for nonsubstituted hydantoin, the activity for more bulky substrate HPH was less severely affected (Table 1). As in the case of nonsubstituted hydantoin, the affinities of both mutants for HPH also significantly decreased, but only a slight change in the K_{cat} values was observed for HPH (Table 2). These results show that the hydrophobic residues in SGLs interact differentially according to the property of substrate, and also suggest that there is a good possibility of improving the activity for the desired substrates by changing the hydrophobic residues in the substrate binding pockets of D-hydantoinases.

Many reports have proposed the importance of the residues on the three SGLs in substrate and stereo selectivities. A recent study demonstrated that even a single mutation I95F in SGL-2 of hydantoinase from *Arthrobacter* sp. DSM 9771 can invert enantioselectivity toward L-methionine [24]. Difference in the substrate specificity and enantioselectivity between D-hydantoinase and L-hydantoinase are thought to come mainly from distinct conformation of the three SGLs, based on comparison of their structures [8–10]. As previously mentioned, three SGLs between dihydroorotase and D-hydantoinases also have significantly different conformation. In dihydroorotase, the exocyclic carboxylate of L-dihydroorotate is recognized by hydrogen bonds with the side chains of Asn 44, Arg 20, and His 254 [7] in comparison with hydrophobic residues in D-hydantoinases.

Based on our results and the previous reports, it can be concluded that the hydrophobicity of substrate binding pocket directly affects stereo/substrate specificity and therefore the three SGLs in substrate binding pocket can be a good target for engineering the stereo/substrate specificity in the cyclic amidohydrolases, including hydantoinases.

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