Evolutionary Relationship and Application of a Superfamily of Cyclic Amidohydrolase Enzymes

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ABSTRACT: Cyclic amidohydrolases belong to a superfamily of enzymes that catalyze the hydrolisis of cyclic C—N bonds. They are commonly found in nucleotide metabolism of purine and pyrimidine. These enzymes share similar catalytic mechanisms and show considerable structural homologies, suggesting that they might have evolved from a common ancestral protein. Homology searches based on common mechanistic properties and three-dimensional protein structures provide clues to the evolutionary relationships of these enzymes. Among the superfamily of enzymes, hydantoinase has been highlighted by its potential for biotechnological applications in the production of unnatural amino acids. The enzymatic process for the production of optically pure amino acids consists of three enzyme steps: hydantoin racemase, hydantoinase, and N-carbamoylase. For efficient industrial application, some critical catalytic properties such as thermostability, catalytic activity, enantioselectivity, and substrate specificity require further improvement. To this end, isolation of new enzymes with desirable properties from natural sources and the optimization of enzymatic processes were attempted. A combination of directed evolution techniques and rational design approaches has made brilliant progress in the redesign of industrially important catalytic enzymes; this approach is likely to be widely applied to the creation of designer enzymes with desirable catalytic properties. © 2005 The Japan Chemical Journal Forum and Wiley Periodicals, Inc. Chem Rec 5: 298–307; 2005: Published online in Wiley InterScience (www.interscience.wiley.com) DOI 10.1002/tcr.20057

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Introduction

Proteins belonging to a superfamily are evolutionarily related, sharing more than 50% of sequence identity with each other.1 These proteins have common structural features and sometimes also have functional similarities, suggesting that they might have convergently or divergently evolved from common ancestral proteins. Identification of functional motifs and structure alignments as well as sequence comparisons has been carried out to search for proteins belonging to a superfamily. Many diverse superfamily proteins have been characterized with the help of an increasing number of three-dimensional structures and bioinformatics based on these structures.2 Cyclic amidohydrolases belong to a superfamily of enzymes that are mostly found in nucleotide metabolism. They
share the same TIM barrel scaffold, possessing similar catalytic mechanisms. They also have similar functions, catalyzing hydrolytic cleavage of cyclic amide bonds. These common properties suggest that these enzymes share intimate evolutionary relationships and provide critical clues to the structure–function relationship. Of the cyclic amidohydrolase superfamily of enzymes, hydantoinase has attracted much attention for its industrial applications. Hydantoinase-based enzymatic processes have been developed for the commercial production of unnatural amino acids that are used as important intermediates for many pharmaceuticals. With increasing demand for optically pure amino acids, much effort has been made toward the isolation and characterization of microbial hydantoinase with desirable properties. Various approaches to

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optimizing the reaction conditions have been investigated for efficient enzymatic processes. Recently, three-dimensional structures of several hydantoinases were determined, and this promoted extensive research to improve the intrinsic properties of hydantoinase.

In this report, the evolutionary relationship of the cyclic amidohydrolase superfamily of enzymes is presented. We describe the biochemical aspects of hydantoinase, its industrial use, and the laboratory evolution of this enzyme for practical applications.

**Evolutionary Relationship of Cyclic Amidohydrolase Superfamily**

Cyclic amidohydrolases (EC 3.5.2.–) are functionally and structurally related superfamily enzymes, and have evolved from a common ancestor. This superfamily includes hydantoinases, dihydropyrimidinases, allantoinases, and dihydroorotases, catalyzing the hydrolysis of cyclic amide bonds of five- or six-membered rings in the nucleotide metabolism (Fig. 1).

Allantoinase (EC 3.5.2.5) is found in the purine degradation pathway of plants and microorganisms, specifically hydrolyzing allantoin into allantoic acid. It shows diverse structural and functional properties such as variable subunit mass (30–50 kDa), metal requirement, substrate specificity, and L-, D-, or no favorable enantioselectivity. Dihydroorotase (EC 3.5.2.3) plays an important role in the *de novo* synthesis of pyrimidine nucleotide by catalyzing the reversible conversion of carbamoyl L-aspartate to L-dihydroorotate. In bacteria, it is a homodimeric and monofunctional enzyme. In contrast, mammalian dihydroorotase exists as a large polyfunctional protein (CAD) that consists of carbamoyl phosphate synthetase, aspartate transcarbamoylase, and dihydroorotase. It requires Zn\(^{2+}\) for catalytic activity and retains its activity even when substituted with other divalent metals such as Co\(^{2+}\), Mn\(^{2+}\), and Cd\(^{2+}\).

Dihydropyrimidinase (EC 3.5.2.2) is involved in the degradation of pyrimidine nucleotide and is found in diverse organisms such as bacteria, yeast, animals, and plants. All known dihydropyrimidinases have a oligomeric structure of a homodimer or homotetramer and also require metal ions such as Zn\(^{2+}\) for activity. It catalyzes the conversion of dihydropyrimidine into 3-ureidopropionate and also exhibits additional activities for hydantoin and 5′-monosubstituted hydantoin derivatives. This distinctive cross-reactivity has been observed in other superfamily enzymes. For example, some hydantoinases have comparable activities toward dihydrouracil and allantoin, which are substrates for dihydropyrimidinase and allantoinase, respectively.

Alignment of the amino acid sequences of the amidohydrolase enzymes revealed that microbial hydantoinases share about 40% identity with mammalian dihydropyrimidinases. Dihydropyrimidinases showed 19–26% and 7–16% amino acid identities with allantoinases and dihydroorotases, respectively. Comparison of the primary sequences and hydropathy profiles among these functionally related enzymes revealed a number of conserved regions and invariant residues. In particular, four histidine residues were found to be strictly conserved in these enzymes. Site-directed mutagenesis of conserved amino acid residues resulted in the complete loss of enzyme activity and metal ions. These results indicate that the conserved residues, including the four histidine residues, play

![Fig. 1. Schematic diagram showing reactions catalyzed by the cyclic amidohydrolase superfamily of enzymes. R indicates the substituted functional group.](image-url)
an essential role in the metal coordination, substrate binding, and catalysis of these functionally related cyclic amidohydrolases. Based on the conserved regions of these superfamily enzymes, two additional putative cyclic amidohydrolases were identified from *Escherichia coli*, in which no hydantoinase or allantoinase activity had been found previously. This was accomplished by the alignment of the conserved motifs with the complete genome sequence of *E. coli*. From biochemical characterization, they were identified as a tetrameric allantoinase that specifically catalyzes allantoin into allantoic acid and a new homotetrameric phenylhydantoinase showing high activity and stereospecificity toward D-phenylhydantoin. They showed relatively high sequence homology (22~41%) with other cyclic amidohydrolase enzymes, carrying common biochemical features of cyclic amidohydrolase family enzymes.

Recently, three-dimensional structures of several cyclic amidohydrolases including hydantoinases and dihydroorotase were determined. These enzymes possess the most prevalent fold, TIM barrel which consists of eight parallel β-sheets connected by eight α-helices. As observed in most of the TIM barrel fold, their active sites are located on the bottom of the pocket formed by the loops that connect the carboxyl end of each β-sheet with the amino end of the next α-helix. It was also confirmed that they share conserved metal ligands and catalytically important residues. These general structural features were also observed in previous reports on distantly related amidohydrolase superfamily enzymes such as urease and phosphotriesterase. Hydantoinase and dihydroorotase have two Zn ions as catalytic metals and the conserved metal ligands of four histidines and one carbamoylated lysine, which were thoroughly investigated in previous biochemical studies. When these two enzymes were structurally superimposed by using the DALI alignment program, despite low sequence homology (16%), they were well matched to each other within an rms deviation of 2.7 Å. These mechanistic properties and structural information clearly demonstrate that hydantoinase and dihydroorotase, as typical cyclic amidohydrolase superfamily enzymes, are evolutionarily closely related to each other.

Biochemical and structural studies have suggested that cyclic amidohydrolase enzymes have the following common characteristics. They have the most prevalent TIM barrel fold loaded with two divalent metal ions, such as Zn$^{2+}$ or Mn$^{2+}$. These metal ions play a critical catalytic role in the deprotonation of water molecules for hydrophilic attack on the substrate. In these superfamily enzymes, four histidines and one carbamoylated lysine are strictly conserved as ligand residues for the coordination of catalytic metal ions. In addition, aspartic acid is conserved, acting as a catalytic base in their active site. They have functional similarity in terms of the catalytic reaction they perform. That is, most of them are directly involved in nucleotide metabolisms and catalyze metal-assisted hydrolysis of cyclic amide bonds (Fig. 2). Despite a low level of homology in the amino acid sequence, these structural and mechanistic properties strongly imply that these superfamily enzymes have the following common characteristics.

**Pyrimidine synthesis**

- Glutamine + bicarbonate → Carbomoyl phosphate → N-carbamoylasparrtate → **Dihydroorotase** → Dihydroorotate → Orotate → Orotidylate → Uridylate (UMP)

**Purine degradation**

- AMP or adenosine → Adenosine deaminase → IMP or inosine → Hypoxanthine → Xanthine → Uric acid → Allantoin → Allantoinase → Allantoate → Urea → **Urease** → Ammonia + CO$_2$

**Pyrimidine degradation**

- Uridylate (UMP) → Uridine → Uracil → 5,6-dihydrouracil → **Dihydropyrimidinase (Hydantoinase)** → 3-Ureidopropionate → Alanine + ammonia + CO$_2$

Fig. 2. Catalytic functions of the amidohydrolase superfamily of enzymes in the metabolic pathway of purine and pyrimidine.
Enantiospecific Hydantoinase

Of the cyclic amidohydrolase superfamily enzymes, hydantoinase has attracted much attention due to its great potential for industrial application. Hydantoin-cleaving activities identified from animals, plants, and microorganisms were found to be dihydropyrimidinase-related activities. Because of the cross-reactivity of hydantoinase and dihydropyrimidinase as mentioned above, hydantoinase had been considered identical to dihydropyrimidinase. However, despite their similar structural, biochemical, and mechanistic properties, they are quite different from each other, performing separate metabolic functions. Dihydropyrimidinase plays an indispensable role in the reductive pathway of pyrimidine degradation in living organisms. In contrast, most hydantoinases have been screened by hydantoin-cleaving activities from microorganisms, and their exact metabolic function and physiological substrate have not yet been elucidated. In addition, unlike the strict substrate specificity of dihydropyrimidinase toward its physiological substrate, enzymes from the hydantoinase family display diverse substrate specificities and enantioselectivities. From the practical point of view, hydantoinases can be classified into D-, L-, and non-selective, depending on their enantioselectivity. Most hydantoinases are D-enantioselective but hydantoinase from *Arthrobacter aurescens* has unusual enantiomeric properties. It favorably converts some 5-mono-substituted hydantoins into L-amino acid and, in particular, the enantioselectivity of this enzyme is substrate-dependent.

Demands for unnatural amino acids that are critical building blocks for semisynthetic antibiotics, pesticides, and food additives have been increasing. Accordingly, many researchers have focused on the isolation and characterization of hydantoinases with favorable catalytic properties in an effort to develop hydantoinase-based enzymatic processes for the production of unnatural amino acids. For efficient screening of hydantoinase-producing microorganisms and cloning of hydantoinase-encoding genes, several rapid and reliable methods were developed. Screening of hydantoinase activity by using hydantoin derivatives as sole nitrogen and carbon sources and simple detection methods such as overlay assay on agar plates have been widely employed. We also developed a simple and effective method using a selective agar plate, which is based on the pH-dependent color change caused by the acidic N-carbamoyl compound produced from hydantoin derivatives by the action of hydantoinase. This method can be generally used for the detection of hydantoinase activity regardless of substrate specificity and enantioselectivity.

Several microbial hydantoinases with favorable catalytic properties were cloned and characterized. Most of them were found to be metal-dependent tetramers with broad substrate specificities. We previously isolated and characterized thermostable D-hydantoinases from thermophile *Bacillus stearothermophilus* SD1 and *Bacillus thermocatenulatus* GH2. The genes encoding hydantoinases were cloned and overexpressed in *E. coli* using a constitutive expression system. These hydantoinases have a molecular mass of 50–55 kDa and showed D-specific activities toward various hydantoin derivatives (Table 1). The optimal conditions for catalytic activity were found to be about pH 7–8 and 65°C. In particular, hydantoinase from *B. stearothermophilus* SD1 showed excellent thermostability with a half-life of 30 min at 80°C. Though the oligomeric structure of hydantoinase from *B. stearothermophilus* SD1 was found to be tetramer, it may exist as a dimer or tetramer *in vivo*. In addition, it was reported that the C-terminal region of hydantoinase determines its oligomeric status. Recently, a novel phenylhydantoinase with distinct substrate specificity toward hydantoin derivatives with aromatic exocyclic substituents was isolated from *E. coli* by alignment of the conserved regions with the genome sequence.

Three-dimensional structures of several hydantoinases with diverse substrate specificity and enantioselectivity have been reported. They were found to be (β/α)8 barrel fold with an additional β-sheet domain (Fig. 3A). The catalytic center is located at the bottom of the barrel fold where four

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Bst SD1[a]</th>
<th>Bth GH2[b]</th>
<th>Ph HYD[c]</th>
</tr>
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<tbody>
<tr>
<td>Hydantoin</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Isopropylhydantoin</td>
<td>5</td>
<td>11</td>
<td>230</td>
</tr>
<tr>
<td>Phenylhydantoin</td>
<td>72</td>
<td>230</td>
<td>792</td>
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<tr>
<td>Hydroxylphenylhydantoin</td>
<td>12</td>
<td>82</td>
<td>889</td>
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<tr>
<td>Dihydropyrazin</td>
<td>4</td>
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[a] D-hydantoinase from *Bacillus stearothermophilus* SD1.
[b] D-hydantoinase from *Bacillus thermocatenulatus* GH2.
[c] Phenylhydantoinase from *Escherichia coli*.

Table 1. Relative substrate specificity of hydantoinases.
histidines, carbamoylated lysine and hydroxide, are coordinated with catalytic metal ions. The crystal structure of D-hydantoinase from *B. stearothermophilus* SD1 resolved by our group has provided detailed information on the active site architecture: the active site is located at a 15-Å deep hydrophobic cleft, and post-translationally modified carbamoylated lysine 150 bridges the two Zn ions. Residues His58, His60 of 1st loop, His 183 of 5th loop, and H239 of 6th loop are involved in the metal binding (Fig. 3B). Asp315 acts as a catalytic base that assists the nucleophilic attack of the bridging hydroxide. However, because the potent competitive inhibitor of hydantoinase was not known and co-crystallization of the enzyme-substrate failed, detailed substrate binding regions were not elucidated. The substrate binding of hydantoinase was inferred by comparing it with a closely related superfamily enzyme, dihydroorotase, in which the mode of interaction with the substrate (dihydroorotate) and product (carbamoyl aspartate) were well demonstrated.\(^{18}\)

Based on sequence and structural comparisons, it was revealed that the recognition sites for the functional amide bond of the hydantoin ring are highly conserved among hydantoinases, but specific regions interacting with exocyclic side chains of hydantoin vary considerably. These interaction regions were considered to form a completely buried hydrophobic pocket at the site of the exocyclic side chain. In particular, stereochemistry gate loops (SGL)\(^{18}\) consisting of three specific loops, SGL1 (62–72 residues), SGL2 (93–100 residues), and SGL3 (153–162 residues) were thought to determine the substrate specificity and enantioselectivity of the hydantoinases.

**Hydantoinase-Based Synthesis of D-Amino Acids**

Optically pure D-amino acids are currently used as intermediates for the synthesis of pharmaceuticals such as semisynthetic antibiotics, peptide hormones, pyrethroids, and pesticides. In particular, D-p-hydroxyphenylglycine and D-phenylglycine, which are synthesized from phenylhydantoin and hydroxyphenylhydantoin, are essential building blocks of the semisynthetic antibiotics including amoxicillin, cephalixin, and cefadroxil.\(^{3,22}\) With the increasing demand for D-amino acids, the enzymatic process has been preferentially developed because of environmental and economic advantages over existing chemical synthetic processes.\(^{22,24}\) In the enzymatic process, the chemically synthesized 5-monosubstituted hydantoin is enantioselectively hydrolyzed into the corresponding N-carbamoyl-amino acid by hydantoinase, and this intermediate is further converted into optically pure D-amino acid by N-carbamoyl amino acid amidohydrolase (N-carbamoylase) or chemical decarbamoylation (Fig. 4).\(^{22,38,39}\) Spontaneous racemization of unreacted racemic hydantoin occurs under alkaline conditions and, theoretically, a 100% conversion is achieved. Because chemical decarbamoylation causes several problems such as low production yield and waste that is difficult to dispose of, much attention has been paid to the enzyme N-carbamoylase. In addition to hydantoinases with their diverse characteristics, as mentioned earlier, other enzymes essential for enzymatic processes, such as N-carbamoylases and hydantoin racemases, were also isolated from various microorganisms and characterized.\(^{40–44}\) Hydantoin racemases were found to
facilitate the efficient production of optically pure amino acids by fast racemization of hydantoin derivatives.

In the enzymatic process for the synthesis of D-amino acids, factors affecting efficiency include substrate specificity, enantioselectivity, functional expression, and thermostability of relevant enzymes. Enzyme thermostability is the most critical factor because of extreme reaction conditions caused by the low solubility of substrates. For this reason, thermostable enzymes from thermophilic microorganisms have been isolated, as mentioned earlier. Another crucial factor is substrate specificity, which should be favorable for the synthesis of commercially important D-amino acids. For example, hydantoinase from *B. stearothermophilus* SD1 is a versatile industrial enzyme with excellent catalytic properties such as strict enantioselectivity, high catalytic activity, easy overexpression, and high thermostability. However, its substrate specificity is biased toward nonsubstituted hydantoin, which is undesirable for the synthesis of commercially important D-amino acids with aromatic side chains, such as D-p-hydroxyphenylglycine and D-phenylglycine. This undesirable substrate specificity was modulated by the structure-based design of the active site loops, as mentioned below.45 As for the functional expressions of enzymes, it was reported that optimization of medium sources, such as nitrogen and carbon, could enhance hydantoinase production in microorganisms.38,46 With glycerol as the sole carbon source, we demonstrated mass production of D-hydantoinase from *B. stearothermophilus* SD1 by using a batch culture of *E. coli* with a constitutive expression system.47

In order to develop efficient hydantoinase-based processes, various experimental conditions were optimized, such as the use of whole-cell biocatalysts, control of reaction parameters, and immobilization of enzymes. One-pot enzymatic synthesis of D-hydroxyphenylglycine (HPG) from D-hydroxyphenylhydantoin (HPH) was attempted by using *Agrobacterium sp.* I-671, which possesses the catalytic activity of both D-hydantoinase and N-carbamoylase.30,48 However, in this process, N-carbamoylase had relatively low activity and stability compared with that of hydantoinase and was severely inhibited by accumulated ammonium ions. For a more efficient process, specific adsorbents for removal of ammonium ions were added, and the optimal ratio of hydantoinase and carbamoylase was determined to be 1:3.49 In addition, the conversion of HPH into N-carbamoyl-D-p-hydroxyphenylglycine (NC-HPG) was further investigated through immobilization of D-hydantoinase on DEAE-cellulose. The optimal reaction conditions of immobilized D-hydantoinase from *B. stearothermophilus* SD1 were revealed to be about 55°C and pH 9.50 Because of the low solubility of the substrates, a heterogeneous reaction system using mass-produced D-hydantoinase was employed and optimized for the synthesis of NC-HPG. In this case, the substrate concentration was increased to 300 g/L under heterogeneous reaction conditions with optimal temperature 45°C and pH 8.5: the resulting conversion yield approached 96%.51 For better understanding and optimization of the heterogeneous reaction system, a simulation model of this system was developed using critical kinetic parameters.52

The principal drawbacks in the enzymatic synthesis of D-amino acids lie in the differences in the catalytic activities and stabilities between D-hydantoinase and N-carbamoylase.53 In general, N-carbamoylase has unfavorable catalytic properties compared with those of hydantoinase. For efficient processing, we developed an *E. coli* whole-cell catalyst with separately expressed or coexpressed D-hydantoinase and N-carbamoylase for the synthesis of D-amino acid from D-, L-monosubstituted hydantoin (Fig. 5).54 The ratio of specific activity between hydantoinase and N-carbamoylase was found to be 1:1.2. In the coexpressed enzyme system converting HPH into HPG, the product yield and productivity were 98% and 6.47 mM/g-cell/h in 15 h, respectively. In addition, a kinetic model describing this whole-cell production was developed.55 Wilms et al. tested whole-cell biocatalysts consisting of L-hydantoinase, L-N-carbamoylase, and racemase for the one-pot synthesis of L-amino acids.56
Protein Engineering of Hydantoinase-Related Enzymes

Before the implementation of protein engineering for manipulation of enzymes, the main strategies to improve the efficiency of the enzymatic process have relied primarily on the isolation of talented enzymes from nature or the optimization of reaction conditions. The development of powerful protein engineering approaches enables alteration of the intrinsic properties of many potential enzymes. Essential properties such as catalytic activity, stability, stereospecificity, expression level, and substrate specificity have been improved or optimized by recombinant DNA technology and directed evolution techniques. In particular, the detailed revelation of mechanisms and the availability of three-dimensional structures of enzymes have enabled rational, structure-based design of enzymes. Recently, many successful results using directed evolution and structure-based rational design approaches have been reported.

For efficient synthesis of D-amino acid in a concerted fashion, we developed a bifunctional enzyme composed of N-carbamoylase from Agrobacterium radiobacter NRRL and D-hydantoinase from Bacillus steatothermophilus SD1 or Bacillus thermocatenulatus GH2.\(^{57}\) The functional fusion of two consecutive enzymes offers several advantages in the enzymatic process with respect to reaction kinetics and enzyme production. This novel fusion enzyme was constructed by end-to-end gene fusion of N-carbamoylase to the N-terminal region of D-hydantoinase. The resulting fusion enzyme displayed a distinct bifunctional activity, and converted monosubstituted hydantoin directly into corresponding D-amino acids. However, it exhibited structural instability resulting in extensive proteolysis \textit{in vitro} probably due to the low stability of the N-terminal fusion partner, N-carbamoylase. This unfavorable property of a fusion enzyme was improved by using DNA shuffling.\(^{58}\) Through three rounds of directed evolution, an evolved fusion enzyme with nine amino acid substitutions was obtained. This variant was found to possess enhanced structural stability, leading to a 6-fold increase in performance in the synthesis of D-amino acid.

Despite its industrial potential, N-carbamoylase demonstrates some undesirable properties, such as low oxidative and thermostability, and a high tendency to form an inclusion body under overexpression conditions. These undesirable properties are considered major drawbacks in the development of enzymatic processes for D-amino acids. In an attempt to resolve these inherent shortcomings, the oxidative and thermostability of N-carbamoylase isolated from \textit{Agrobacterium tumefaciens} NRRL B11291 was simultaneously improved by directed evolution using DNA shuffling.\(^{59,60}\) The best mutant, 2S3, with markedly increased oxidative and thermostability, was selected after two rounds of directed evolution and found to have six amino acid mutations. The temperature at which 50% of the initial activity remains after incubation for 30 min was 73°C for 2S3, whereas it was 61°C for its wild-type counterpart. As for oxidative stability, 2S3 retained 79% of the initial activity even after treatment with 0.2 mM hydrogen peroxide for 30 min at 25°C, whereas the wild-type completely lost its activity under the same conditions. Moreover, the functional expression of N-carbamoylase carrying low foldability in \textit{E. coli} was improved by using directed evolution.\(^{61}\) GFP was used as a reporter protein for the screening of variants showing higher levels of functional expression or correct folding. A library of N-carbamoylase mutants was generated by mutagenic PCR and screened by simple visual inspection of enhanced fluorescence intensity of fused GFP from the screening plate. Through this approach, insoluble fractions caused by aggregation were reduced, and the level of soluble expression of N-carbamoylase was enhanced about 4-fold when compared with the wild-type.

The enantioselectivity of hydantoinase was optimized by using directed evolution techniques.\(^{62}\) In this experiment, the optical preference toward L-enantiomer was increased along with catalytic activity by error-prone PCR and saturation mutagenesis. Interestingly, a single amino acid mutation, Ile95Phe, was sufficient to change the enantioselectivity in hydantoinase from D- into L-enantiomer. The production of L-amino acid was improved 5-fold, and the accumulation of unwanted intermediates was decreased 4-fold by using a whole-cell biocatalyst expressing this mutant.

The substrate specificity of hydantoinase was rationally manipulated toward a commercially important aromatic substrate based on three-dimensional structures. We previously suggested that the stereochemistry gate loops (SGLs) constituting the binding pocket of hydantoinase might determine substrate specificity and enantioselectivity.\(^{63}\) By using AutoDock3, the mode of substrate binding was simulated by fitting D-HPH as a target substrate into the active site pocket. From this simulated model, the hydrophobic and bulky residues of SGLs interacting with the exocyclic side chain of D-HPH were inferred.\(^{63}\) In particular, Leu65 of SGL1, and Phe152, and Phe159 of SGL3 were found to have close interactions with the exocyclic substituent of D-HPH (Fig. 6). Site-directed and saturation mutagenesis toward these residues were performed and induced remarkable changes in substrate specificity toward D-HPH.\(^{45}\) Surprisingly, the \(K_{\text{cat}}/K_{\text{m}}\) value of Phe159Ala mutant was enhanced toward D-HPH by ~200-fold. This seems to be mostly due to the removal of the steric hindrance between the exocyclic ring of D-HPH and the hydrophobic residues of the loops. These results indicate that the hydrophobic feature and the size of the binding pocket for the exocyclic group of the substrate in the SGLs are crucial factors for substrate specificity in D-hydantoinases. Thus, novel cyclic amidohydrolase enzymes with desirable substrates...
specificities are expected to be easily designed by manipulating these SGLs.

**Conclusion**

Cyclic amidohydrolases are an evolutionarily related superfamily of enzymes. Despite their low sequence homology, they share similar catalytic mechanisms and structural properties. Many enzymes involved in nucleotide metabolism have been revealed to belong to this superfamily. Expansion of this superfamily and revelation of functional diversity will provide more detailed insight into their evolutionary relationships. Hydantoinase is an industrially useful enzyme for the synthesis of unnatural D-amino acid, but it has some unfavorable properties for commercial application. Various approaches such as gene fusion, whole-cell biocatalysis, and directed evolution have been employed for the improvement of catalytic properties. Three-dimensional structures of several cyclic amidohydrolases including hydantoinase and N-carbamoylase have become available, providing an efficient way of manipulating the related enzymes in a rational manner. It is just a matter of time before a combination of directed evolution techniques and rational design approaches will be widely applied to the creation of designer enzymes with desirable catalytic properties.

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