Manipulation of the Active Site Loops of D-Hydantoinase, a $(\beta/\alpha)_8$-Barrel Protein, for Modulation of the Substrate Specificity†

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ABSTRACT: We previously proposed that the stereochemistry gate loops (SGLs) constituting the substrate binding pocket of D-hydantoinase, a $(\beta/\alpha)_8$-barrel enzyme, might be major structural determinants of the substrate specificity [Cheon, Y. H., *et al*. (2002) *Biochemistry 41*, 9410−9417]. To construct a mutant D-hydantoinase with favorable substrate specificity for the synthesis of commercially important non-natural amino acids, the SGL loops of the enzyme were rationally manipulated on the basis of the structural analysis and sequence alignment of three hydantoinases with distinct substrate specificities. In the SGLs of D-hydantoinase from *Bacillus stearothermophilus* SD1, mutations of hydrophobic and bulky residues Met 63, Leu 65, Phe 152, and Phe 159, which interact with the exocyclic substituent of the substrate, induced remarkable changes in the substrate specificities. In particular, the substrate specificity of mutant F159A toward aromatic substrate hydroxyphenylhydantoin (HPH) was enhanced by ∼200-fold compared with that of the wild-type enzyme. Saturation mutagenesis at position 159 revealed that k_{cat} for aromatic substrates increased gradually as the size of the amino acid side chain decreased, and this seems to be due to reduced steric hindrance between the bulky exocyclic group of the substrate and the amino acid side chains. When site-directed random mutagenesis of residues 63 and 65 was conducted with the wild type and mutant F159A, the selected enzymes (M63F/L65V and L65F/F159A) exhibited ∼10-fold higher k_{cat} values for HPH than the wild-type counterpart, which is likely to result from reorganization of the active site for efficient turnover. These results indicate that the amino acid residues of SGLs forming the substrate binding pocket are critical for the substrate specificity of D-hydantoinase, and the results also imply that substrate specificities of cyclic amidohydrolase family enzymes can be modulated by rational design of these SGLs.

A number of enzymes have been used for medical and industrial applications, but in many cases, their substrate preferences are unfavorable for practical purposes. As demands for novel enzymes catalyzing new chemical reactions are continuously increasing, the design of novel enzymes has been extensively attempted in pursuit of improved catalytic properties, including altered substrate specificities (*1*−*5*). In general, rational redesign of certain enzymes with desirable functions requires a detailed understanding regarding the structure−function relationship and catalytic mechanism of the target enzyme. Successful results based on structural comparison and comparative analysis of homologous proteins have been reported (*6*−*9*). On the other side, when a limited amount of information is available for the target enzyme, region specific randomization, such as saturation mutagenesis at particular positions, has also been regarded as an effective way of modulating the substrate specificity (*10*−*13*).

The $(\beta/\alpha)_8$-barrel is the most prevalent fold which consists of eight parallel β-sheets connected by eight α-helices. Most of the $(\beta/\alpha)_8$-barrel enzymes have their active sites 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. In general, the substrate binding residues and the catalytic residues are located in separate regions of carboxyl ends of β-sheets and connecting loops, respectively. Because of these features, the $(\beta/\alpha)_8$-barrel enzymes have been a good target for reshaping the binding site for a new substrate or for grafting a new catalytic function.

The cyclic amidohydrolase superfamily, which includes dihydropyrimidinase, allantoinase, dihydroorotase, and hydantoinase, is part of the $(\beta/\alpha)_8$-barrel group of enzymes and has a common structural feature with conserved metal binding and catalytic residues at the end of β-sheets (*14*−*18*). Along with similar structural properties, these superfamily enzymes also have similar functions: they are involved in the metabolism of pyrimidines and purines, catalyzing the reversible hydrolytic cleavage of the cyclic amide bond of their substrates (*14*, *19*, *20*). Of them, the hydantoinase is considered a microbial counterpart of dihydropyrimidinase, but its precise function *in vivo* remains to be revealed. Microbial hydantoinases, catalyzing the cleavage of the cyclic amide bond of 5′-monosubstituted hydantoins (Scheme 1),

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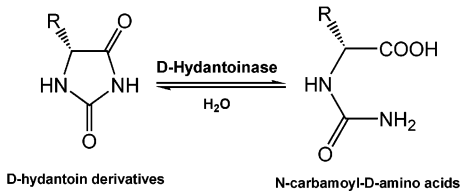
Scheme 1



are widely employed for the commercial production of optically pure D- and L-amino acids (*21*−*23*). D-Hydantoinase (BstHYD)[1] from *Bacillus stearothermophilus* SD1 is an especially versatile industrial enzyme with strict enantioselectivity, high activity, easy overexpression, and thermostability. Its substrate specificity, however, is biased toward nonsubstituted hydantoin, and therefore is undesirable for the synthesis of commercially important non-natural D-amino acids with aromatic side chains such as phenyl and hydroxyphenyl groups (*24*−*26*). Meanwhile, D-hydantoinase (BthHYD) from *Bacillus thermocatenulatus* GH2, the sequence of which is as much as 92% homologous with that of BstHYD, has high activity for aromatic hydantoin derivatives as well as small substrates such as hydantoin (*27*). Nonetheless, BthHYD was unlikely to be suitable for industrial use due to the difficulty in its overexpression (data not shown). Recently, cloned phenylhydantoinase (PhHYD) from *Escherichia coli* exhibited significantly different substrate specificity despite its structural and biochemical properties being similar to those of BstHYD (*28*, *29*). Interestingly, PhHYD exhibited ~10-fold higher activity toward an aromatic hydantoin derivative such as D,L-hydroxyphenylhydantoin (HPH) than toward hydantoin.

In our previous studies, we demonstrated that D-hydantoinase and dihydroorotase, despite their high degree of structural similarity, have major differences in their substrate binding pockets consisting of the stereochemistry gate loops (SGLs) (*16*). These loops were predicted to be crucial determinants of the substrate specificities of the cyclic amidohydrolases, and their importance for the activity and substrate specificity was partially proven by the site-directed mutagenesis study (*30*). Here, we attempted to modulate the substrate specificity of BstHYD for the synthesis of commercially important non-natural amino acids by rationally manipulating the SGLs on the basis of the structural analysis and comparison of the SGLs of the three different hydantoinases (BstHYD, BthHYD, and PhHYD). The relationship between the substrate specificity and conformation of the substrate binding pocket in hydantoinases was also presented. Details are reported herein.

EXPERIMENTAL PROCEDURES

Materials. Hydantoin and *N*-carbamoylglycine were purchased from Sigma. D,L-Hydroxyphenylhydantoin (HPH), D,L-phenylhydantoin (PH), and D,L-hydroxymethylhydantoin (HMH) were chemically synthesized by the method of Suzuki *et al*. (*31*). Synthetic oligodeoxynucleotides were obtained from Bioneer (Daejon, Korea). Amylose resin was purchased from NEB. All other reagents for DNA manipulations and analyses were analytical grade. Thermophilic *B. stearothermophilus* SD1 isolated in our previous work (*29*) was used as the source of the BstHYD gene. The PhHYD gene was cloned from the *E. coli* K-12 derivative in our previous work (*28*). *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.

Site-Directed Mutagenesis and Saturation Mutagenesis. Site-directed mutagenesis of wild-type and mutant BstHYD was performed by an overlapping PCR method using two complementary oligonucleotides. The sequences of the mutagenic primers used in this study were as follows: 5′-tagaattcatgacaaaaattataaaaaatg-3′ (N-terminus, *Eco*RI site) and 5′-tactgcagttaaatggttaattcctcgctc-3′ (C-terminus, *Pst*I site) for cloning of the D-hydantoinase gene; 5′-catttagatatgccggctggcggcacg-3′ (L65A), 5′-catttagatatgccggttggcggcacg-3′ (L65V), 5′-Catttagatatgccgtttggcggcacg-3′ (L65F), 5′-catttagatatgccgtggggcggcacg-3′ (L65W), and 5′-cggcatatctaaatg-3′ (−) for mutations at Leu 65; 5′-ggaattacatccctcaaagtgtatatggcgtataaa-3′ (F152Y), 5′-ggaattacatccctcaaagtggctatggcgtataaa-3′ (F152A), and 5′-cactttgagggatgtaattcc-3′ (−) for mutations at position 152; 5′-tttatggcgtataaaaacgtagctcaggcagatgat-3′ (Y159A) and 5′-tacgtttttatacgccataaa-3′ (−) for mutation at position 159; and 5′-cacacgcatttagatgggccgtggggcggcacggtg-3′ (M63G/L65W), 5′-cacacgcatttagatgcgccgtggggcggcacggtg-3′ (M63A/L65W), and 5′-atctaaatgcgtgtgcggatc-3′ (−) for double mutation at Met 63 and Leu 65. The pTrc99A plasmid containing the wild-type BstHYD or mutant F159A 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 DNA was purified, digested with *Eco*RI and *Pst*I, and cloned into pMAL-c2x. The mutations were confirmed by DNA sequencing.

Site-directed random mutagenesis at Met 63, Leu 65, and Phe 159 of BstHYD was carried out by using the same method described above. The following primers were used: 5′-tttatggcgtataaaaacgtannscaggcagatgat-3′ (Y159X) and 5′-tacgtttttatacgccataaa-3′ (−) for saturation mutagenesis at position 159 and 5′-cacacgcatttagatnnsccgnnsggcggcacggtg-3′ (M63X/L65X) and 5′-atctaaatgcgtgtgcggatc-3′ (−) for saturation mutagenesis at positions 63 and 65. In the sequences, N represents an equimolar mixture of A, C, G, and T and S represents that of G and C.

Screening of Clones Exhibiting High Activities for HPH. Each colony of the transformants was transferred onto new LB-amp plates in triplicate using toothpicks, and further cultured at 37 °C for 40 h. Primary screening of D-hydantoinase variants with enhanced activity for HPH was conducted using the activity staining method as previously described (*32*). The cultured colonies were transferred onto filter papers and washed with distilled water. Each filter was overlaid onto the activity staining plates with HPH. The activity staining agar plate contained 0.2% substrate (HPH), 0.003% phenol red, and 1.5% agar, and was adjusted to be purple by adding the appropriate amount of NaOH. D-Hydantoinase converts the substrate to the corresponding *n*-carbamoyl-D-amino aicd, decreasing the pH, and the purple

[1] Abbreviations: BstHYD, D-hydantoinase from *B. stearothermophilus* SD1; BthHYD, D-hydantoinase from *B. thermocatenulatus* GH2; PhHYD, phenylhydantoinase from *E. coli*; SGL, stereochemistry gate loop; HPH, D,L-hydroxyphenylhydantoin; PH, D,L-phenylhydantoin; HMH, D,L-hydroxymethylhydantoin.

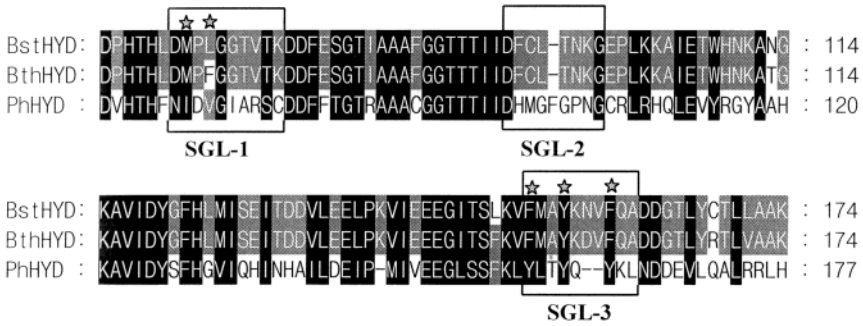


FIGURE 1: SGLs of various D-hydantoinases with distinct substrate specificities. The sequences of D-hydantoinases from *B. stearothermophilus* SD1 (BstHYD), *B. thermocatenulatus* GH2 (BthHYD), and phenylhydantoinase form *E. coli* (PhHYD) are aligned. The residues of SGLs interacting with the exocyclic group of the substrate are marked with stars.

color changes to yellow around the colonies with high activity. Colonies displaying a fast color change were primarily screened and confirmed by determining whole cell activity. The selected D-hydantoinase variants were sequenced and purified for further analysis.

Enzyme Purification and Assay. Wild-type and mutant enzymes were purified as MBP-fused forms using the pMAL-c2x vector system as previously reported (*33*). Purified fusion proteins were analyzed with SDS−PAGE and quantified with Bradford methods. The reaction mixture for the assay of enzyme activity contained 0.5 mM $MnCl_2$, 100 mM hydantoin, and 0.2−100 μg of the purified enzyme in 1 mL of 0.1 M Tris-HCl buffer (pH 8.0). In the case of other substrates, including HPH, PH, and HMH, 10 mM was used due to their low solubilities. 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 or color reagent, *p*-dimethylaminobenzaldehyde, according to the method of Takahashi *et al.* (*34*). One unit of activity was defined as the amount of enzyme required to produce 1 μmol of *N*-carbamoyl-D-amino acid per minute under specified conditions.

Determination of Kinetic Parameters. Kinetic constants for hydantoin and HPH of wild-type and mutant enzymes were determined with a double-reciprocal plot of the measured initial reaction rates and substrate concentrations. The concentration range of HPH was 1−10 mM, and that of hydantoins was 50−500 mM.

RESULTS

Analysis of Substrate Binding Pockets of D-Hydantoinases and Phenylhydantoinase. On the basis of the structure of D-hydantoinase, the amino acid sequences of SGLs constituting the substrate binding pockets of BstHYD, BthHYD, and PhHYD with distinct substrate specificities were compared (Figure 1). The mode of substrate binding was previously simulated by fitting D-HPH as a target substrate into the active site of BstHYD by using AutoDock3 (*30*, *35*). From this model, the amino acid residues interacting with the substrate could be inferred. In the active sites, the residues of the metal binding and recognition site for the hydantoin ring were highly conserved among these enzymes (*14*, *16*). While residues of BstHYD and BthHYD interacting with the exocyclic group of the substrate were identical except

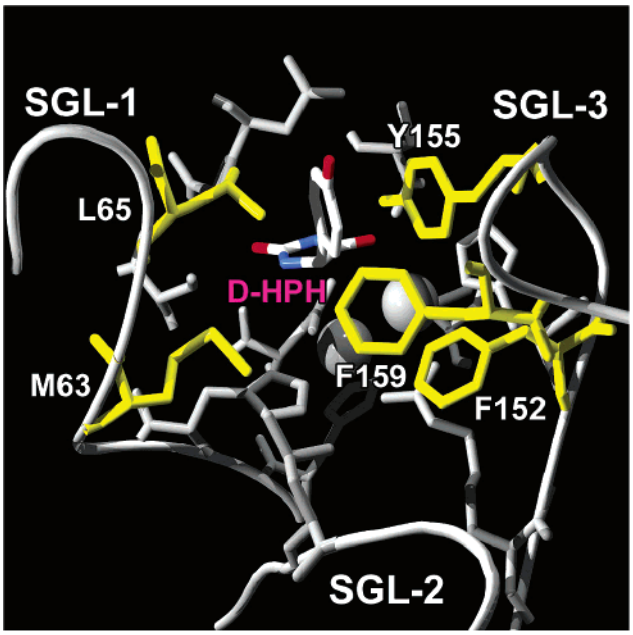


FIGURE 2: Active site of D-hydantoinase. D-Hydroxyphenylhydantoin (HPH) was fitted into the substrate binding pocket by using AutoDock 3. Met 63, Leu 65, Phe 152, Tyr 155, and Phe 159 of the SGLs constitute a hydrophobic lid interacting with the exocyclic substituent of the substrate.

one residue at position 65, their SGLs exhibited a pattern considerably different from those of PhHYD (Figure 1). In the case of BstHYD, the side chains of residues Met 63, Leu 65, Phe 152, Tyr 155, and Phe 159 in SGL-1 and SGL-3 seal the active site completely, forming the hydrophobic pocket for the exocyclic substituent of the substrate (Figure 2). The hydrophobic nature of the substrate binding pocket has been proposed to be critical for the activity and substrate specificity in hydantoinases (*16*, *23*, *30*). In particular, the hydrophobic and bulky side chains of Phe 152, Tyr 155, and Phe 159 in SGL-3 directed toward the active site of BstHYD were found to closely interact with the chiral exocyclic substituent of the substrate. On the other hand, a short SGL-3 of PhHYD seemed to be located away from the substrate binding site, and the amino acid residues in the SGLs of PhHYD interacting with the exocyclic group of the substrate are smaller and less hydrophobic than those of BstHYD. Consequently, PhHYD is likely to have a larger and less hydrophobic pocket for the exocyclic group of the substrate than BstHYD. Thus, the distinct substrate specificities of BstHYD and PhHYD seem to originate mainly from the difference in the SGLs.

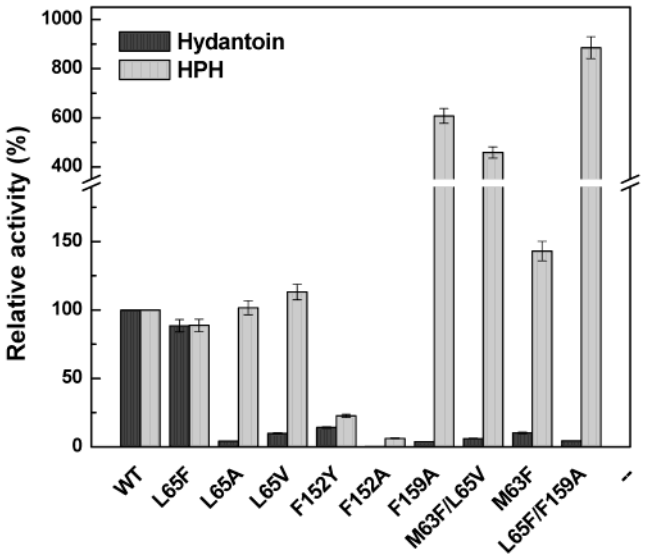


FIGURE 3: Relative activities of the D-hydantoinase variants for hydantoin and HPH.

Site-Directed Mutagenesis of the Residues Responsible for Substrate Binding. To investigate the influence of SGLs on the substrate specificity, Leu 65, Phe 152, and Phe 159 of the BstHYD were mutated to smaller or equivalent amino acids of other hydantoinases, and the activities of mutant enzymes for hydantoin and HPH were determined. These amino acid residues were found to be distinct in the SGLs of BstHYD, BthHYD, and PhHYD.

Leu 65 of SGL-1 in close contact with the exocyclic group of the substrate is the only different residue between the active sites of BstHYD and BthHYD as shown in Figure 1. First, this residue was changed to Phe, and mutation effect was examined. Contrary to our expectation, the L65F substitution has little effect on both the substrate specificity and activity of the enzyme. Additionally, when Leu 65 was further mutated to smaller amino acids, Ala and Val, to expand the substrate binding pocket, mutants L65A and L65V did not show any catalytic improvement for the aromatic substrate (Figure 3). Both mutations significantly lowered the activity for hydantoin.

When Phe 152 of SGL-3 constituting the hydrophobic lid of the substrate binding pocket was mutated to Tyr, an equivalent residue in PhHYD, it caused a noticeable impact on the activity. The activities of mutant F152Y for both hydantoin and HPH were reduced to ~20% of that of the wild type. Moreover, another replacement of Phe 152 with Ala, a smaller hydrophobic residue, also seriously lowered the activities for both substrates, resulting in only 6% for HPH and 0.4% for hydantoin when compared to the wild-type counterpart. These observations are consistent with our previous results in that the hydrophobic and bulky residues of the binding pocket are critical for the activity and substrate specificity (*30*). In the substrate binding pocket, Tyr 155 also closely interacts with the exocyclic substituent of the substrate. Tyr 155 has been proposed to play an important role in either the substrate binding or stabilization of the transition state (*16*, *17*), and mutations at this position led to a loss of activity in our previous work (*30*).

Phe 159 of BstHYD is one of the major constituents of the hydrophobic lid and has a strong interaction with the exocyclic substituent of the substrate in the neighborhood, whereas PhHYD is predicted to have a cavity in that region due to a short loop. Phe 159 was replaced with a smaller amino acid, Ala, to enlarge the substrate binding pocket in the vicinity of the exocyclic substituent. Surprisingly, mutant F159A displayed a remarkable change in the substrate specificity. The specific activities of mutant F159A were determined to be 4.5 units/mg of protein for hydantoin and 37 units/mg of protein for HPH, which correspond to ~5% and ~610% of that of the wild type, respectively (Figure 3). Even though mutation of Leu 65, Phe 152, and Tyr 155 to smaller amino acids had no positive effect on the activity for bulky substrates, the mutagenic study of residue Phe 159 indicated that enlargement of the binding pocket near the exocyclic substituent significantly modulates the substrate specificity toward aromatic hydantoin derivatives. This result strongly implies that enlargement of the substrate binding pocket at the precise position rather than random approach can lead to an increase in the activity for the specific bulky substrate.

Saturation Mutagenesis at Phe 159. To gain insight into a drastic alteration in the substrate specificity by a single mutation, F159A, saturation mutagenesis was performed at position 159, and mutants exhibiting higher activity for HPH were screened. Fifteen clones were selected form the activity staining agar plate as described in Experimental Procedures, and they were confirmed by determining whole cell activity. Of them, positive mutants displaying higher activity for HPH were found to be F159A, F159S, F159V, F159L, and F159I, and these mutant enzymes were purified for further studies.

Interestingly, as shown in Figure 4, as the size of the hydrophobic side chain at position 159 became smaller in the order of leucine, isoleucine, valine, serine, and alanine, the specific activities for small substrates such as hydantoin and HMH decreased gradually compared to that of the wild-type enzyme. On the other side, the opposite trend was observed for aromatic and bulky substrates such as HPH and PH. The activity increased as the size of the hydrophobic side chain of the amino acid decreased. To evaluate the alteration in the substrate specificity in more detail, the kinetic constants of the mutant enzymes for hydantoin and HPH were determined (Table 1). As a result, the k_{cat} value for HPH also increased with a decrease in the size of the amino acid side chain except for Leu, while no significant variation in the K_M values was observed except for mutant F159S. On the contrary, the k_{cat} value and the affinity for hydantoin decreased as the size of the side chain became smaller. In other words, mutants possessing the smallest side chains such as F159A and F159S showed the most dramatic change in substrate specificity. As for mutant F159A, the low activity for hydantoin seems to have resulted from both a decreased k_{cat} value (45 s^{-1}) and a decreased affinity (K_M value of 450 mM). In contrast to hydantoin, the increase in the k_{cat} value mainly accounts for the high activity for HPH. Consequently, mutant F159A displayed a remarkably altered substrate preference from hydantoin to HPH, which is supported by the fact that the ratio of k_{cat}/K_M values for HPH to that for hydantoin has changed from 0.74 to 150. Despite the similar side chain, because serine has a different hydrophobicity, mutant F159S exhibited slightly distinctive characteristics when compared with F159A. The k_{cat}/K_M of mutant F159S for HPH was lower than that of F159A, but

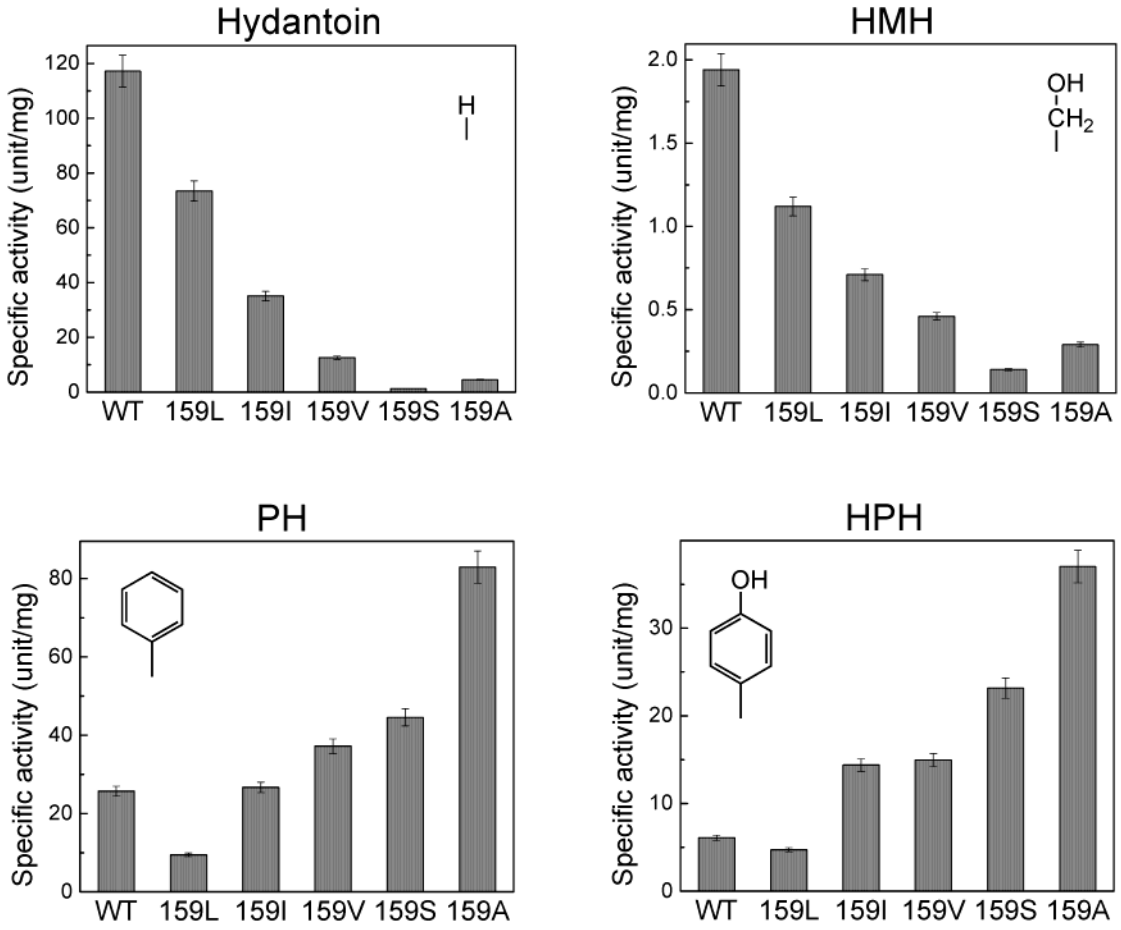


FIGURE 4: Specific activities of the BstHYD variants generated by saturation mutagenesis at position 159. In a mutant library, the enzymes with high activity for HPH were selected, and their specific activities for various substrates were determined.

Table 1: Kinetic Constants of the Wide Type and D-Hydantoinase Variants

	hydantoin			HPH			
	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M	$(k_{cat}/K_M)_{HPH}/(k_{cat}/K_M)_{Hyd}$[a]
wild type	440	98	4.5	18	5.4	3.3	0.74
F159L	280	89	3.2	11	2.2	5.0	1.6
F159I	270	190	1.4	46	3.5	13	9.3
F159V	97	160	0.61	50	3.6	14	23
F159A	45	450	0.10	110	7.4	15	150
F159S	21	400	0.053	280	27	10	190
M63F/L65V	42	260	0.16	180	24	7.5	47
L65F/F159A	48	470	0.10	200	13	15	150

[a] The ratio of k_{cat}/K_M for HPH to that for hydantoin.

the change in the substrate specificity (the ratio of k_{cat}/K_M for HPH to that for hydantoin) was more dramatic than for F159A due to the relatively low activity for hydantoin.

Site-Directed Random Mutagenesis of SGL-1. In the substrate binding pocket, the residues of SGL-1 and -3 closely interact with the exocyclic substituent of the substrate. The mutagenic study of Phe 159 revealed that the conformation of SGL-3 is important for activity and substrate specificity. Therefore, it is assumed that a conformation change in the SGL-1 could also yield a similar effect on activity or substrate specificity. As mentioned earlier, however, a single mutation at position 65 gave rise to a negligible effect on the activity for an aromatic substrate compared to the wild-type counterpart. Thus, double mutation at Met 63 and Leu 65 in SGL-1 of the hydrophobic pocket was attempted in a random manner. From a library of 10^4 colonies, mutants showing high activity for HPH were screened as described above, and of them, the best mutant (M63F/L65V) was selected (Figure 3). As shown in Table 1, mutant M63F/L65V possessed a significantly increased k_{cat} for HPH, showing an even higher value than mutant F159A. The substrate specificity of mutant M63F/L65V increased more than 60-fold compared to that of the wild type. Since a single mutation L65V did not enhance the catalytic activity for HPH in the site-directed mutagenesis study (Figure 3), we mutated Met 63 to Phe to investigate whether the M63F mutation has a major impact on the altered substrate specificity of mutant M63F/L65V. Interestingly, a single M63F mutation also had a negligible effect on the activity for HPH (Figure 3), which strongly implies that the M63F/L65V double mutation leads to a favorable conformation of SGL-1 for binding of the bulky substrate. The affinity of mutant M63F/L65V for HPH decreased ~4-fold, but the k_{cat} value increased drastically 10-fold compared with that of the wild type (Table 1); however, the catalytic efficiency of mutant M63F/L65V for hydantoin decreased severely.

On the basis of the above results, to develop mutant enzymes with higher activity for an aromatic substrate, Met 63 and Leu 65 of mutant F159A were subjected to random replacement. From a library of 6000 colonies, one mutant showing highest activity for HPH was selected. As can be seen in Figure 3, unexpectedly, the selected variant was found

to have a Phe at position 65. This mutation (L65F) in wild-type BstHYD was observed to cause a negligible effect on the catalytic property as mentioned above. From the kinetic analysis, the enhanced activity of mutant L65F/F159A for HPH seemed to be attributed to a 2-fold increase in the k_{cat} value when compared with mutant F159A, which corresponds to an ~11-fold increase compared to that of the wild type (Table 1). However, the ratio of k_{cat}/K_M of mutant L65F/F159A for HPH was almost similar to that of mutant F159A mainly due to the reduced affinity. The catalytic property of F159A for hydantoin was not affected by mutation L65F.

DISCUSSION

We have shown that the substrate specificity of D-hydantoinase, a $(\beta/\alpha)_8$-barrel enzyme, can be modulated by rationally manipulating the stereochemistry gate loops. Structural analysis and sequence comparison among hydantoinases with distinct substrate preference were effectively employed to reshape the stereochemistry gate loops and to construct a D-hydantoinase mutant exhibiting favorable substrate specificity for the synthesis of commercially important non-natural amino acids.

From the structural analysis and simulation regarding the substrate binding pocket using D-HPH as a target substrate, some specific residues of the SGLs were subject to manipulation. While mutation F159A resulted in a significant increase in the activity for HPH, mutation of M65 to smaller amino acids retained the activity for HPH comparable to that of the wild-type enzyme, even though the activity for hydantoin was lowered. Mutations at F152 and Y155 brought about reduced activities for both HPH and hydantoin. Replacement with alanine at position 152 also lowered the activity for hydantoin more drastically than that for HPH. Large side chains of L65, F152, and F159 were found to constitute the well-organized hydrophobic pocket interacting with the exocyclic group of the substrate without a gap as shown in Figure 2. Thus, mutations to smaller amino acids at these residues are expected to enlarge the substrate binding pocket, which might result in a loose binding of substrates. It was reported that substrate specificities of enzymes can be modified by changing the active site volume at critical positions. The mutations to smaller amino acids in the substrate binding pocket change the substrate preference of isocitrate dehydrogenase toward isopropylmalate (*12*) and that of methylamine dehydrogenase toward a long-chain amine (*36*), while mutations of lipoxygenase to introduce space-filling amino acids led to a gradual alteration of the positional specificity from 5S- toward 15S-lipoxygenation (*8*).

A single replacement of Phe 159 with serine resulted in a drastic change in the substrate specificity, and the ratio of k_{cat}/K_M for HPH to that for hydantoin increased up to 280-fold when compared with that of the wild type (Table 1). It is likely that enlargement of the substrate binding pocket alleviates the steric hindrance when the bulky substrate binds to the active site. However, in the cases of Leu 65 and Phe 152 which also closely interact with the exocyclic substituent of the substrate, mutations to small amino acids such as Ala or Val did not alter the activities for HPH. A clue to explain the drastic change in the substrate specificity was deduced from the structural comparison of the active sites between

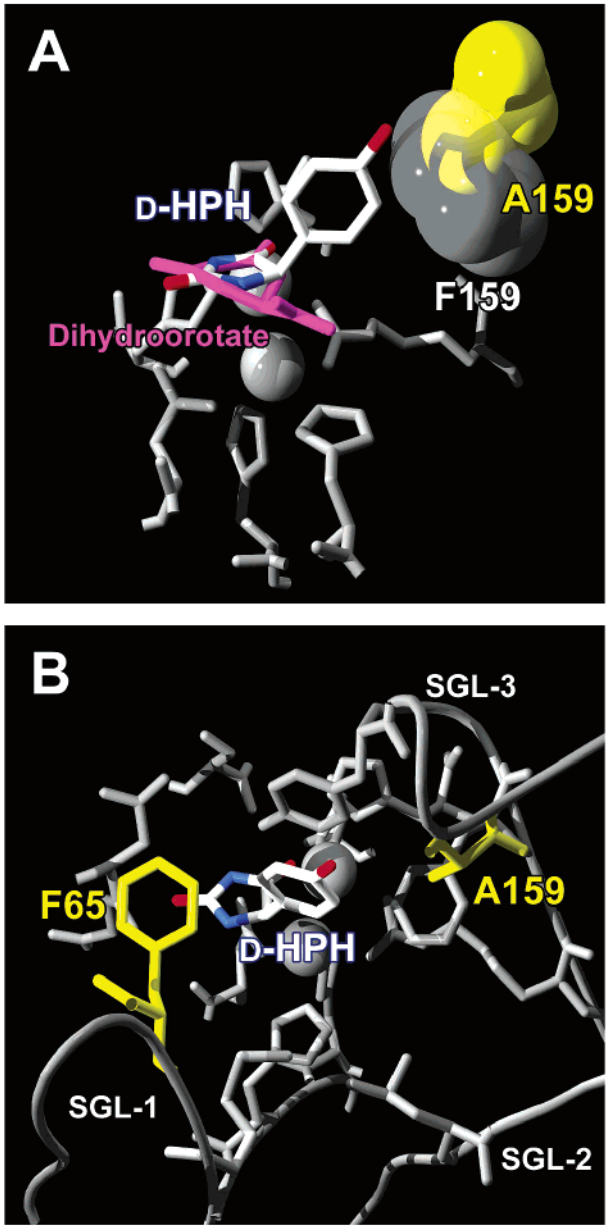


FIGURE 5: Proposed structural model of the active site of mutants F159A and L65F/F159A. (A) D-HPH was manually fitted into the active site of mutant F159A based on the position of the functional amide bond of dihydroorotate in dihydroorotase. (B) In mutant L65F/F159A, the bulky and hydrophobic side chain of Phe 65 seems to occupy the vacant space in the binding pocket and interact with the exocyclic substituent of the substrate.

BstHYD and dihydroorotase. As stated earlier, both enzymes share a very similar architecture of the active site, and the residues for metal binding and the catalytic center are almost completely aligned in their three-dimensional structures (*16*). In addition, BstHYD as well as dihyroorotase shows a catalytic activity for a substrate with a six-membered ring. In the simulated model, when HPH was oriented in the active site of BstHYD superimposed with dihydroorotase by manual substrate docking as in other reports (*16*−*18*), the bulky substituent of HPH was found to crash into the aromatic group of Phe 159, but not in the case of mutant F159A (Figure 5A). It is inferred that although the aromatic substrate HPH binds relatively well in the pocket of the wild type (Table 1), the orientation of the substrate is not favorable

for efficient turnover; that is, the functional amide bond of HPH might tilt away from catalytic metal ions due to steric hindrance between the aromatic exocyclic group of the substrate and the bulky side chain of Phe 159. On the other hand, in mutant F159A, the functional amide bond is almost aligned with that of dihydroorotate in superimposed dihydroorotase (Figure 5A), which means that the removal of this steric hindrance seems to induce the proper orientation of the bulky substrate suitable for catalysis, thus increasing the k_{cat} value. This explanation is further supported by the result that the k_{cat} value for HPH gradually increases as the size of the amino acid side chain at position 159 decreases in the order of phenylalanine, isoleucine, valine, serine, and alanine, while all these mutants possessed similar K_M values. Furthermore, the above result demonstrates the reason the substitution of L65 and F152 with smaller amino acids did not improve the activities for HPH.

In site-directed random mutagenesis at positions 63 and 65 of SGL-1, the resulting mutant M63F/L65V exhibited considerably higher k_{cat} and affinity for HPH than the wild type. In contrast, single mutations (M63F and L65V) did not change the activities for HPH, though they caused reduced activities for hydantoin. This result indicates that cooperative action of the double mutation (M63F/L65V) leads to an effective conformation change among the SGLs and consequently alters the substrate specificity toward HPH. It is interesting that while small amino acids (Ala and Ser) at position 159 induced higher activity for HPH, a bulky amino acid (Phe) at position 63 gave rise to a similar effect in mutant M63F/L65V. Met 63 of SGL-1 is located at the bottom of a well-organized substrate binding pocket, and therefore, mutation to a larger amino acid, Phe, is likely to cause repulsion among Phe 63 of SGL-1, Leu 94 of SGL-2, and Phe 152 of SGL-3, resulting in an overall conformation change in the SGLs. It seems that the conformational perturbation of the SGLs caused by the M63F mutation is well-adjusted further by secondary mutation at position 65. This result implies that simple enlargement of the substrate binding pocket does not lead to a proper binding of the bulky substrate. Instead, mutations such as F159A and M65F/L65V, enabling a favorable orientation of the exocyclic group of substrate in the active site, can eventually induce the desirable substrate specificity toward the bulky substrate. When random mutageneses at positions 63 and 65 were combined with mutation F159A, replacement of Leu 63 with Phe further increased the k_{cat} for HPH compared with the effect of a single F159A mutation. The enhanced activity of mutant L65F/F159A for the aromatic substrate can be explained by the following presumption. The larger side chain of Phe 65 might occupy the vacant space in the binding pocket created by a single F159A mutation; thereby, it further induced the bulky substrate to fit more properly for faster turnover (Figure 5B). As revealed in the Results, the activity and substrate specificity of D-hydantoinase were largely affected by the size of the amino acids constituting the SGLs in the substrate binding pocket. In addition to the size of the binding pocket, the hydrophobicity of the residues around the exocyclic substituent of the substrate also plays an important role in the enzyme activity. When hydrophobic residues Leu 65 and Phe 159 were changed to Glu in our previous work, the activities for hydantoin decreased dramatically (*30*). Also, it was observed that mutation F152Y gives rise to reduced activity for hydantoin, and likewise, mutant F159S shows significantly decreased affinity for HPH when compared with mutant F159A.

As proposed in our previous work (*16*), this mutagenic study demonstrated that both the hydrophobic feature and size of the binding pocket for the exocyclic group of the substrate in the SGLs are crucial factors for the substrate specificity in D-hydantoinases. Thus, it is reasonable that the role of SGLs in the substrate specificity might be expanded to cylclic amidohydrolase family enzymes such as dihydroorotase, dihydropyrimidinase, and hydantoinase. In dihydroorotase, the exocyclic carboxyl group of L-dihydroorotate is accommodated through hydrogen bonds with side chains of Arg 20, Asn 44, and His 254 (*15*). In BstHYD, the bulky residues, including Met 63, Leu 65, Phe 152, Phe 155, and Phe 159 in SGL-1 and SGL-3, make up the hydrophobic lid of the substrate binding pocket, and these loops form a smaller binding pocket than those of dihydroorotase (*16*). These differences in the pocket size and substrate binding interaction between D-hydantoinase and dihydroorotase seem to contribute to their distinct substrate specificities. Additionally, a recent report proposed that dihydropyrimidinases cannot accept dihydroorotate because the carboxyl group of dihydroorotate does not fit into the active site. It was suggested that only a limited number of mutations would be sufficient to convert them into dihydroorotases, and vice versa (*37*). The structures of D-hydantoinase (*16*, *17*) and L-hydantoinase (*18*) also revealed distinct differences in the conformation of their SGLs, and it was recently reported that a single I95F mutation in SGL-2 inverted the enantioselectivity of hydantoinase from *Arthrobacter* sp. DSM 9771 toward L-methionine (*38*).

On the basis of our previous results (*30*) and these reports, it is evident that substrate specificity as well as enantioselectivity in the cyclic amidohydrolase superfamily enzymes is primarily governed by the conformation and properties of the three SGLs. Thus, novel cyclic amidohydrolase enzymes with desirable substrate and stereospecificities are expected to be readily designed by manipulating these SGLs. From this study, it is inferred that rational design and molecular evolution based on structural comparison and sequence alignment would be valuable tools for creating enzymes with the desired catalytic property and understanding the structure−function relationship.

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