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C-Terminal Regions of D-Hydantoinases Are Nonessential for Catalysis, but Affect the Oligomeric Structure

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Most microbial D-hydantoinases have been reported to have catalytic properties similar to those of mammalian dihydropyrimidinases. Comparison of the primary structures of microbial D-hydantoinases with mammalian dihydropyrimidinases revealed that the amino acid homology is about 37% and functionally important residues are rigidly conserved at identical positions. Interestingly, however, the C-terminal regions were found to be completely mismatched with each other. In order to investigate the possible role of the C-terminal regions, we deleted the C-terminal regions of the D-hydantoinases from two thermophilic *Bacilli* and compared the catalytic and structural properties of the mutant enzymes with those of wild-type enzymes. As a result, the C-terminal region was found not to be essential for catalysis, but it does affect the oligomeric structure of the enzyme.

Microbial hydantoinases catalyze the stereospecific hydrolysis of hydantoin derivatives and are known as a kind of amidohydrolase which hydrolyzes the cyclic amide bond(1). Three kinds of hydantoinases, D-, L- and non-stereospecific, are classified according to their stereospecificity. Among them, D-stereospecific hydantoinase has attracted much attention because this enzyme is currently employed as a biocatalyst for the production of optically pure D-amino acids which are intermediates for semi-synthetic antibiotics, peptide hormones, pyrethroids, and pesticides(2,3).

With the increasing demand for optically pure D-amino acids, much effort has been made toward isolation(4,5) and characterization of the enzymes(6-8). This hydantoin- hydrolyzing enzyme is known to be distributed in a wide variety of microorganisms even though the relative substrate-specificity is somewhat different, which leads us to speculate that D-hydantoinase might play a physiologically important role in microorganisms. From high affinity of microbial D-hydantoinase toward the dihydrouracil, this enzyme has been considered to be the microbial counterpart(9) of the animal dihydropyrimidinase(E.C 3.5.2.2) which is involved in the catabolism of dihydrouracil and dihydrothymine(10). However, physiological role of D-hydantoinase in microorganisms, structure-function and evolutionary relationships between D-hydantoinase and dihydropyrimidinase have not been elucidated, mainly because of insufficient information on the comparative study at the molecular level and no structural data. Recently, cDNA sequences encoding dihydropyrimidinases of human liver and rat liver have been determined(11,12).

Previously, we cloned, sequenced, and analyzed the D-hydantoinase gene from *Bacillus stearothermophilus* SD1(13), and identified the essential histidine residues which were deduced from the comparative analysis of the functionally related enzymes(14). Recently, D-hydantoinase from *Bacillus thermocatenulatus* GH2 has been isolated and characterized(15), and nucleotide sequence of the open reading frame(ORF) of the gene encoding the D-hydantoinase from *B. thermocatenulatus* GH2 was determined to be identical with that of *Bacillus stearothermophilus* NS1122A(16).

In this paper, in an effort to understand the structure-function relationship of microbial D-hydantoinase with mammalian dihydropyrimidinase, we analyzed their primary structures and found that the C-terminal regions of the enzymes are non-homologous. To get some insight into possible role of the C-terminal region, we constructed the C-terminal region-deleted mutants from two thermophilic *Bacilli*(15,17) and compared with those of wild-type enzymes.

MATERIALS AND METHODS

Enzymes, reagents, and strains. A thermophilic DNA polymerase for PCR was purchased from New England Biolabs, and radiolabelled

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materials were purchased from Amersham. Oligodeoxynucleotides were obtained from DNA Int. Inc. DL-5-monosubstituted hydantoin derivatives were chemically synthesized(18). Prepacked Resource-Q, Phenyl Superose, and Superose 12 columns were purchased from Pharmacia LKB. All other reagents used were of analytical grade.

Two thermophilic *B. stearothermophilus* SD1 and *B. thermocatenulatus* GH2 previously isolated in our laboratory were used as the source of the D-hydantoinase gene. *E.coli* XL1-Blue was used as a host for the expression of the genes encoding the C-terminal region-truncated mutant enzymes. Plasmid pBluescript II SK was purchased from Stratagene, and pGEM-7Zf(+) from Promega, respectively. *E. coli* strains were grown in Luria-Bertani medium at 37 °C.

Computer analysis. Amino acid sequence alignment of microbial D-hydantoinases with mammalian dihydropyrimidinases was performed using Clustal W software(19). The initial alignment was further refined manually, taking into account the predicted secondary structure of the D-hydantoinases from two *B. stearothermophilus* SD1 and *B. thermocatenulatus* GH2. Analyses of the secondary structures of the enzymes were performed using the network software of EMBL (Heidelberg, FRG). Hydropathy profiles were obtained according to the method of Kyte and Doolittle(20).

Deletion of mismatching C-terminal regions between two D-hydantoinases. Oligonucleotide-directed deletion of mismatching C-terminal regions was carried out using PCR. The oligonucleotide, 5′-CTCGAGCTCCATGACAAAAATTATA-3′, corresponding to the N-terminal region of the enzymes, was used as a primer. On the other hand, two oligonucleotides were used for the C-terminal region of each enzyme: 5′-CGAATTCTTAAGTTCCGTATTTTGCTCGTT-3′ for the enzyme from *B. stearothermophilus* SD1 and 5′-CGAATTCTT-ACGCCCCATATTTCGCGCGTT-3′ for *B. thermocatenulatus* GH2, respectively. The amplified DNA subcloned into the multicloning sites(*Eco*RI/*Sac*I) of pGEM-7Zf(+). Deletion of the C-terminal region was confirmed by DNA sequencing(21).

Purification of enzymes. The purification of wild- and mutant enzymes was conducted at room temperature in the presence of 1mM $MnCl_2$. *E. coli* cells were harvested by centrifugation. The cells were resuspended in 20 mM Tris-HCl buffer (pH 8.0) and disrupted by sonication. The extract was heated at 50 °C for 25 min to denature the thermally labile proteins, and denatured proteins were removed by centrifugation, and supernatant was applied to a Resource Q column equilibrated with 20 mM Tris-HCl buffer(pH 8.0) in FPLC system (Pharmacia). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 0-0.5 M NaCl. The active fractions were concentrated, and the resulting solution was loaded onto a Phenyl Superose column equilibrated with the same buffer containing 1.8 M ammonium sulfate. The enzyme was eluted with a reverse linear gradient (1.8 M- 0 M) of ammonium sulfate, and then dialyzed against 20 mM Tris-HCl buffer(pH 8.0).

Determination of the oligomeric structure. The gel filtration was performed by using a Superose 12 HR 10/30 column equilibrated with 20 mM Tris-HCl(pH 8.0) containing 0.1 M NaCl and 1 mM $MnCl_2$. Standard proteins and their molecular masses were as follows: blue dextran, 2000 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; Fab fragment, 50 kDa.

Cross-linking of enzymes was conducted using 1-ethyl-3-[3-(dimethylamino)propenyl] carbodiimide hydrochloride(EDC) as a cross-linking agent. Purified enzymes(5-7 μg) were treated with 20 mM EDC at various temperatures for 1 h in 20 mM phosphate buffer(pH 7.0). The cross-linking state of the enzymes was analyzed by using a SDS/PAGE system(22).

Enzyme assays and activity staining. D-Hydantoinase activity was determined by the same method as described(17). One unit of hydantoinase activity was defined as the amount of enzyme required to produce one μmol N-carbamyl-D-amino acid from hydantoin derivative per minute under the specified conditions. Analytical gel electrophoresis and activity staining were performed according to the method of our previous paper(23).

Circular dichroism and fluorescence spectra. CD measurement was carried out with a recording spectropolarometer (Jasco, J-700) at 25 °C with a 1mm pathlength cell. The CD spectra were obtained at the protein concentration of 2.5 μM in the Far-UV region(190 nm-260 nm) under a nitrogen atmosphere. Fluorescence emission spectra were observed at 25 °C using a spectrofluorometer(Jasco, FP770) with an excitation wavelength of 285 nm. The protein samples were prepared as for the CD measurement. The protein concentration was determined by Bradford's method(24).

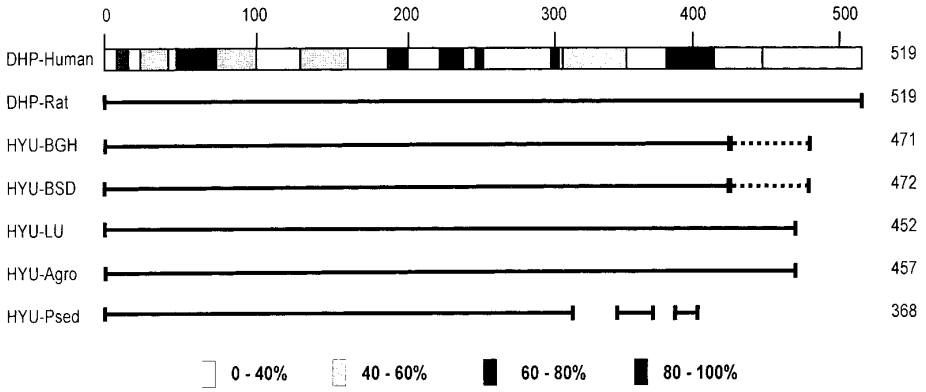
RESULTS AND DISCUSSION

Amino Acid Sequence Homology between D-Hydantoinases and Dihydropyrimidinases

We first analyzed the molecular relationship of the functionally related D-hydantoinases and dihydropyrimidinases. From the alignment of the amino acid sequences, microbial D-hydantoinases were revealed to share about 37-42 % amino acid homology with mammalian dihydropyrimidinases, and similarity was increased up to 65 % when the conservative substitutions were considered. These results suggest that the microbial D-hydantoinase is closely related with mammalian dihydropyrimidinase at the molecular level, and these genes have divergently evolved from a common ancestor. It has been already reported that most of microbial D-hydantoinases exhibit high affinity toward the dihydrouracil and share the similar catalytic properties, such as quaternary structure, molecular weight, and optimum pH(6-8,16). In addition, several regions in primary and secondary structures were found to be rigidly conserved in the same order over the entire sequences (Fig 1), consisting of 61 identical residues and up to 160 invariant plus highly conservative amino acid residues. Among the conserved regions, a region was revealed to possess the essential residues which appear to participate in catalysis or metal binding(14). The conserved regions in this enzyme family represent a great percentage of the total sequence length of the enzymes, spanning approximately 82 %, but, interestingly, the C-terminal regions were non-homologous.

Construction of the C-Terminal Region-Truncated Mutant Enzymes

To analyze the functional significance of the non-homologous C-terminal regions, deletion mutants were constructed for the enzyme from *B. thermocatenulatus* GH2. The 1.8-kb gene encoding the D-hydantoinase amplified by PCR and subcloned into the *Eco*RV site of pBluscript II SK. The resulting plasmid was digested with exonuclease III from the *Not*I end of the multicloning site, closely located at the C-terminal region of the enzyme, and then religated. From the analysis of the deletion mutants, the shortest construct retaining the enzyme activity was revealed to be deleted 40 amino acid residues from the C-terminus, strongly implying that the non-homologous C-terminal region is non-es-



DHP-Human	55	PGGIDTHTHM	64
DHP-Rat	54	PGGIDTHTHM	63
HYU-BGH	52	PGGIDPHTHL	61
HYU-BSD	52	PGGIDPHTHL	61
HYU-LU	52	PGGIDPHTHL	61
HYU-Agro	51	PGGIDVHTHV	60
HYU-Psed	53	PGGIDPHTHM	62

FIG. 1. Amino acid sequence homology among the enzymes belonging to the dihydropyrimidinase family; Human, dihydropyrimidinase from *Homo sapiens*(11); Rat, dihydropyrimidinase from *Rattus norvegicus*(12); BGH, D-hydantoinase from *B. thermocatenulatus* GH2 which has the identical sequence of that from *B. stearothermophilus* NS1122A(16); BSD, hydantoinase from *B. stearothermophilus* SD1(13); LU, hydantoinase from a gram positive bacterium(GenBank accession number A13503); Agro, hydantoinase from *Agrobacterium sp.* KNK712(25); Psed, hydantoinase from *P. putida* DSM84(26). Dotted lines represent the dispensable regions which are artificially deleted without significant loss of enzyme activity. The conserved sequences possessing the catalytically important aspartic acid and histidines are shown in the box.

sential for the enzyme catalysis. Similar result was also observed in the case of the D-hydantoinase from *B. stearothermophilus* SD1(data not shown).

In order to get some insight into the biochemical role of the non-homologous C-terminal region, the D-hydantoinases from two *Bacilli* were further investigated because these two enzymes have quite different catalytic properties and oligomeric structures. Concerning the oligomeric structure, the enzyme from *B. stearothermophilus* SD1 was a dimer(17). On the other hand, the enzyme from *B. thermocatenulatus* GH2 was a tetramer and showed higher affinity toward hydantoin derivatives with a aromatic group at 5′-position(15) when compared with that of *B. stearothermophilus* SD1. From the comparison of the primary structure, the amino acid homology between two enzymes was found to be about 92 %, however, the C-terminal regions were completely mismatched with each other. These observations led us to speculate that different properties between two enzymes might be resulted from the non-homologous C-terminal regions. Based on this assumption, we constructed the C-terminal region-truncated mutant enzymes by PCR. Eleven(LMADQDVVKMS) and twelve(STISKQSEELTI) amino acid residues were deleted from the C-terminus of the enzyme originated from *B. thermocatenulatus* GH2 and *B. stearothermophilus* SD1, respectively, and the resulting mutant enzyme from the former strain was designated BT460, and that from the latter one BS460. Corresponding wild-type enzymes were called BT471 and BS472, respectively. The wild and mutant enzymes were purified to homogeneity as described in experimental section.

Structural Properties of the Truncated Mutant Enzymes

Gel filtration chromatography was performed to examine the change in oligomeric structure of the C-terminal region-deleted enzymes. As seen in Fig. 2, BS460 was eluted at the same time corresponding to a dimeric position as wild-type BS472. However, the elution time of BT460 was quite different from that of BT471. In other words, BT460 was eluted at a dimeric position, whereas BT471 appeared at the time corresponding to a tetramer. Even when BT460 and BT471 were mixed and eluted together, the elution time was clearly separated. The effects of pH, salts, and temperature on this result were negligible. As an another approach, the cross-linking result also supports that the truncated-mutant enzyme BT460 is a dimeric form, which was not detected the protein band corresponding to tetrameric position as wild type BT471(data not shown). Mean-

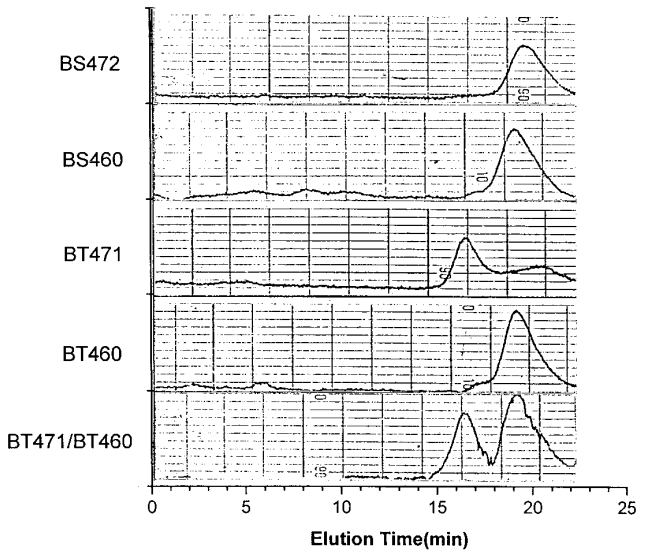


FIG. 2. Gel filtration chromatography of the wild-type and truncated mutant enzymes using Superose 12 column. Purified enzyme was loaded on the column pre-equilibrated with 20 mM Tris-HCl(pH 8) containing 100 mM NaCl and 1 mM Mn^{2+}.

while, cross-linked of the mutant enzyme BS460 appeared at the identical band positions as those of BS472.

To exclude the possibility that the oligomeric structure of BT460 might be changed during purification, native gel electrophoresis and activity staining of BT460 were conducted at each purification step(Fig. 3). The enzyme activity was observed at the same position as the enzyme band on the native gel at each purification step, and this result indicates that the oligomeric structure of BT460 was not changed during purification.

From the comparison of the secondary and tertiary structures using CD and fluorescence spectra, the difference between the mutant and wild-type enzymes was observed to be minor(data not shown), suggesting that the secondary and tertiary structures of the mutant enzymes were not significantly changed. From these results, it is likely that secondary and tertiary structures of the C-terminal region-deleted enzymes were maintained as native ones.

Catalytic Properties of the Mutant Enzymes

The D-hydantoinases described in this work were found to require manganese ions for activity(15,17). The C-terminal region of each enzyme contains two negatively charged amino acid residues. Therefore, if the C-terminal region was involved in the metal binding domain, removal of this region might affect the metal binding property and consequently activity of the enzyme. When the C-terminal region-deleted enzymes, BS460 and BT460, were treated with EDTA and dialyzed against a metal free buffer, activities of the enzymes were lowered to about 30-40 % of the control. But, addition of manganese ions almost fully restored the activities of the enzymes(data not shown).

The specific activities of the wild and mutant enzymes were determined toward the hydantoin as a typical substrate. Since BT460 is a dimerized enzyme, the specific activity was calculated based on the molar concentration of the enzyme. The mutant BS460 was found to have the specific activity of 5.0 unit/μ mole enzyme, which corresponds to about 95 % of the activity of the wild-type enzyme BS472, and this result strongly implies that the C-terminal region is not closely associated with the catalytic domain. However, the specific activity of BT460 was determined to be 4.1 unit/μ mole enzyme, exhibiting about 36 % of the activity of the wild enzyme BT471. When the activity of BT460(dimerized enzyme) was compared with those of BS472 and BS460(dimeric enzyme), a similar value in the specific activity was observed. In other words, the specific activity of BT471(tetrameric enzyme) was lowered to the level of BS472(dimeric enzyme) upon dimerization by the deletion of the C-terminal region. From these observations, it is likely that the substrate specificity of the D-hydantoinase might be closely linked with the oligomeric structure of enzyme. The structure-activity relationship was remained as further study.

Comparative studies described in this work provides an evidence that the microbial D-hydantoinase is closely related with mammalian dihydropyrimidinase and its C-terminal region affects the oligomeric structure of the enzyme. A three-dimensional structure of the enzyme is crucial for demonstration of structure-function relationship, and resolution of the crystal structure is under progress.

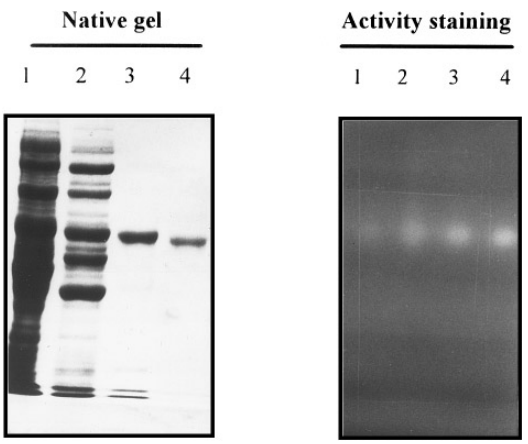


FIG. 3. Native gel electrophoresis and activity staining of the C-terminal region-truncated enzyme BT460 at each purification step. Lanes are: 1, crude extract; 2, heat treatment; 3, Resource Q; 4, Phenyl Superose. Acrylamide gel overlaid with agarose gel was incubated for 30 min at 55 °C.

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