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Primary Structure, sequence analysis, and expression of the thermostable *D*-hydantoinase from *Bacillus stearothermophilus* SD1

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Abstract The gene coding for the thermostable D-hydantoinase from the thermophilic bacterium *Bacillus stearothermophilus* SD1 was cloned and its nucleotide sequence was completely determined. The D-hydantoinase protein showed considerable amino acid sequence homology (20–28%) with other hydantoinases and functionally related allantoinases and dihydroorotases. Strikingly the sequence of the enzyme from *B. stearothermophilus* SD1 exhibited greater than 89% identity with hydantoinases from thermophilic bacteria. Despite the extremely high amino acid homology among the hydantoinases from thermophiles, the C-terminal regions of the enzymes were completely different in both sequence and predicted secondary structure, implying that the C-terminal region plays an important role in determining the biochemical properties of the enzymes. Alignment of the sequence of the D-hydantoinase from *B. stearothermophilus* SD1 with those of other functionally related enzymes revealed four conserved regions, and five histidines and an acidic residue were found to be conserved, suggesting a close evolutionary relationship between all these enzymes.

Key words D-Hydantoinase · Amidohydrolase · *Bacillus stearothermophilus* SD1

Introduction

Hydantoinase catalyzes the hydrolysis of hydantoin derivatives (Miller et al. 1988) and is assumed to belong to the class of amidohydrolases since it hydrolyzes the cyclic amide bond (Syldatk et al. 1990). Hydantoinases can be classified as D-, L- and non-stereospecific depending on their stereospecificity (Lapointe et al. 1995). D-Hydantoinase is considered to be the microbial counterpart of the animal dihydropyrimidinase (E.C 3.5.2.2) which plays an important role in the catabolism of dihydrouracil and dihydrothymine (Vogels and Van der Drift 1976). In the context of stereospecific catalysis, microbial D-hydantoinase has attracted much attention, and this enzyme is currently employed as a biocatalyst for the industrial production of optically pure D-amino acids which are intermediates in the manufacture of semi-synthetic antibiotics, peptide hormones, pyrethroids, and pesticides (Yamada and Shimizu 1988). L- and non-stereospecific hydantoinases have also been exploited for the production of optically pure L-amino acids (Yamashiro et al. 1988), but their functional roles in microorganisms have not been elucidated.

The comparative study of enzymes that catalyze the same type of reactions but have different substrate specificities and stereospecificities is a useful approach to understanding the molecular mechanism of the enzyme reaction and the evolutionary relationships between the various enzymes. From this point of view, a comparison at the molecular level of various amidohydrolases, such as hydantoinases, allantoinases (Buckholz and Cooper 1991) and dihydroorotases (Zimmerman et al. 1995), which act on the cyclic amide bond, seems very worthwhile because basic aspects of the catalytic mechanism are believed to be general, regardless of substrate specificity and stereoselectivity. However, this kind of study has rarely been done because insufficient information is available on the nucleotide sequences of genes encoding hydantoinases. To date, nucleotide sequences of D-hydantoinase genes from the gram-positive bacterium LU1220 (Jacob et al. 1990) and two *Pseudomonads*, DSM84 (Lapointe et al. 1994) and NS671 (Watabe et al. 1992) have been reported. Mukohara et al. (1994) recently determined the nucleotide sequence of the gene for a non-stereospecific hydantoinase from *B. stearothermophilus* NS1122A. Lapointe et al. (1994) have analyzed conserved segments of hydantoinase genes,

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including some amidohydrolases, to construct a DNA probe for the detection of D-hydantoinase-producing microorganisms.

Previously, we isolated and characterized a thermostable D-hydantoinase from *B. stearothermophilus* SD1 (Lee et al. 1995). In this paper, we report the cloning and expression in *E. coli* of the gene encoding the D-hydantoinase of *B. stearothermophilus* SD1, and have determined its nucleotide sequence. The putative amino acid sequence of the enzyme was aligned with those from other sources, and the possible significance of localized conserved regions of the gene for the biochemical properties of the enzyme is discussed.

Materials and Methods

Cells, plasmids, and culture conditions

B. stearothermophilus SD1 isolated in our previous work (Lee et al. 1995) was used as a source of the D-hydantoinase gene. The D-hydantoinase gene from *B. stearothermophilus* SD1 was first cloned into *E. coli* DH5α, and *E. coli* XL1-Blue was used as a host for subcloning. Plasmid pBluescript II SK was purchased from Stratagene, and pBR322 and pUC18 were from Takara Shuzo. *E. coli* strains were grown in Luria-Bertani medium at 37° C. Ampicillin (50 μg/ml) was added when necessary.

DNA preparation and manipulation

Total chromosomal DNA was extracted from *B. stearothermophilus* SD1 as described by Lewington et al. (1987), and plasmids were prepared by the alkaline lysis method (Sambrook et al. 1989). The nested deletion sets for the analysis of the minimum functional domain were constructed by using the Erase-a-Base system (Promega). The digested and serially deleted fragments of DNA were fractionated on agarose gels and purified by using Gene Clean II kit (Bio 101).

Nucleotide sequencing

Nested deletion sets of the cloned DNA were constructed from both ends by digestion with exonuclease III, and nucleotide sequences of both strands were determined according to the dideoxy chain-termination method of Sanger et al. (1977) using double-stranded DNA as a template with either the universal reverse primer or the M13-20 primer.

Computer analysis

The amino acid sequence of D-hydantoinase from *B. stearothermophilus* SD1 was compared with various amidohydrolases from other sources using the Blast Network service at the National Center for Biotechnological Information (Bethesda, Md.). The multiple sequence alignment of enzymes was performed with the CLUSTAL W program. Analyses of the secondary structure of the enzymes were performed using the network software at EMBL (Heidelberg, FRG). The average hydropathy of enzyme was obtained according to the method of Kyte and Doolittle (1982).

Assay of D-hydantoinase activity

D-Hydantoinase activity was determined by using HPLC as described in our previous paper (Lee et al. 1995). One unit of the enzyme activity was defined as the amount of enzyme required to produce one μmol of N-carbamyl-D-amino acid from the hydantoin derivative under the specified conditions. The protein concentration was determined by the Bradford (1976) method, using bovine serum albumin as a standard.

Results and discussion

Cloning and construction of the deletion derivatives

Total chromosomal DNA isolated from *B. stearothermophilus* SD1 was partially digested with *Sau*3A1, and the fragments of 3–10 kb were recovered from agarose gels. The resulting fragments were then ligated into the *Bam*H1 site of pBR322 and transformed into *E. coli* DH5α. Transformants carrying a D-hydantoinase gene were initially screened on selective plates containing 1% hydantoin and 0.005% phenol red in LB medium. The pH of the selective medium was adjusted to 7.7. Colonies that express D-hydantoinase convert hydantoin to hydantoic acid, thus lowering the pH, and a bright yellow color develops around the colony against a red background.

One clone showing high D-hydantoinase activity was selected, and the plasmid DNA isolated from this colony was analyzed with restriction endonucleases. The D-hydantoinase activity was found to be associated with a 10.5-kb insert, and this plasmid was designated pHBR5. The D-hydantoinase activity of pHBR5 was associated with the 2.9-kb *Eco*RI-*Sac*I fragment, and this fragment was subcloned in pUC18 to construct pHBU183. A plasmid containing the insert in the opposite direction to pHBU183 and subclones carrying the 2.9-kb *Eco*RI-*Sac*I insert produced similar levels of D-hydantoinase, indicating that this gene was constitutively expressed by its own promoter. The *Eco*RI-*Sac*I insert from pHBU183 was again subcloned into pBluescript II SK to determine the size required for activity minimal. The plasmid containing this insert was serially deleted from the *Sac*I site and then religated. A plasmid, pHBB183, containing a 1.8-kb insert expressed a D-hydantoinase having the same biochemical properties as the native enzyme.

To determine the minimum functional domain required for the enzyme activity and to analyze the nucleotide sequence, the 1.8-kb insert in pHBB183 was subcloned into the *Eco*RV site of pBluescript II SK by blunt-end ligation. The resulting plasmid pHBB184 was digested with exonuclease III from either the *Eco*RI or the *Not*I end of the multicloning site of pBluescript II SK and then religated. The detailed procedure for the construction of nested deletion sets is shown in Fig. 1. The shortest constructs retaining the D-hydantoinase activity after progressive deletion from either the *Not*I end or the *Eco*RI end were designated pHBBC450 and pHBBN 363, respectively. Further deletion of these mutants from either the N- or C-terminus resulted in a loss of the enzyme activity.

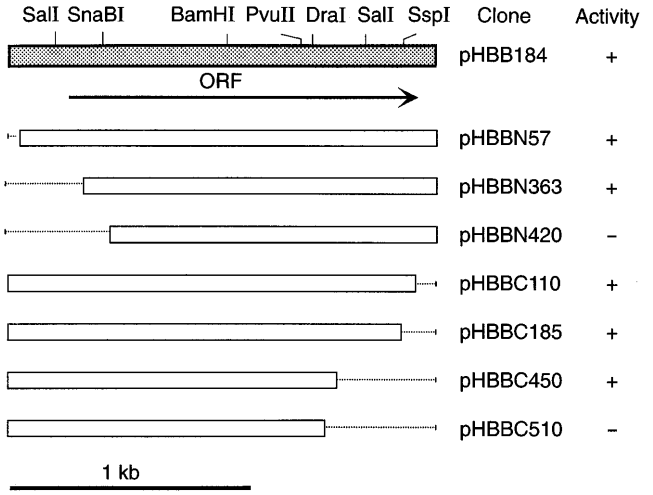

Fig. 1 Nested deletion sets of pHBB183 and selected deletion derivatives. *Open boxes* represent the insert DNA and the *dashed lines* the deleted fragments. Recognition sites for restriction endonucleases are shown at the top. Positive and negative signs indicate the presence and absence of hydantoinase activity

Nucleotide sequence of the D-hydantoinase gene

The nucleotide sequence of the 1.8-kb insert from pHBB183 was determined completely. The entire DNA sequence of the 1.8-kb fragment contained one open reading frame (ORF) of 1419 bp, starting with an initiation codon ATG and ending with the termination codon TAA (Fig. 2). This region encodes a protein of 472 amino acid residues with a calculated molecular weight of 51 819, in close agreement with that determined for the enzyme by SDS-polyacrylamide gel electrophoresis. The molecular weight of the enzyme was estimated to be 54 000 in our previous work (Lee et al. 1995). The ATG codon is in a favourable context for translation initiation because of its location near the putative ribosomal binding site (GGAGG) which is highly complementary to the 3′-end of 16 S rRNA. A possible consensus sequence similar to the -35 and -10 motifs of some *E. coli* promoters was found upstream of the ORF. The G+C content of the ORF was 43.5%. Both the N- and C-terminal sequences agreed completely with the amino acid sequences determined by Edman degradation and carboxypeptidase digestion of the purified enzyme from *B. stearothermophilus* SD1. Comparison of the experimentally determined amino acid composition of the enzyme and the composition predicted from its nucleotide sequence revealed a good correlation. Antiserum raised against the enzyme purified from the *B. stearothermophilus* SD1 also reacted with the enzyme produced by *E. coli* XL1-Blue (data not shown).

```
1    GTAACCTTGACCTTAATTATAAAGTGGTTGCCCGCATTAACACAGATTGTTTGCATTAAT
61   TGCAATAAATGTTATATCTCCTGTGAAGATGCTTCTCATCAATGCATTGATCGTTTAACA
121  GATGAAAATGGAAAAGAGTATTTAAAAGTACGCGAAGAAGATTGTGTAGGGTGTAATTTA
181  TGTTCGATCGTCTGTCCGGTGGATGGGGCGATTGACATGGTCGAAATTCCAAGCGAACAT
241  CCGCCGCTGACATGGAATGAGCGCCAGGCAGTCATTGGCGGGCTTAGCAGCTGTAACGTT
301  GACGTAAAATAAATAAACTAAAGGAGGATCTGGAAATGACAAAAATTATAAAAAATGGAA
                                   M  T  K  I  I  K  N  G
361  CGATTGTTACCGCGACAGATACGTATGAAGCGCACTTGCTCATTAAAGATGGGAAAATTG
     T  I  V  T  A  T  D  T  Y  E  A  H  L  L  I  K  D  G  K  I

421  CGATGATTGGCCAAAATTTAGAGGAAAAAGGCGCTGAAGTGATTGATGCAAAAGGCTGTT
     A  M  I  G  Q  N  L  E  E  K  G  A  E  V  I  D  A  K  G  C

481  ACGTATTTCCAGGCGGCATTGATCCCCACACGCATTTAGATATGCCGCTTGGCGGCACGG
     Y  V  F  P  G  G  I  D  P  H  T  H  L  D  M  P  L  G  G  T

541  TGACAAAGGATGATTTCGAATCCGGAACGATTGCGGCGGCATTTGGCGGGACAACGACAA
     V  T  K  D  D  F  E  S  G  T  I  A  A  A  F  G  G  T  T  T

601  TCATTGACTTTTGCTTAACGAATAAAGGGGAACCATTGAAAAAAGCGATTGAAACTTGGC
     I  I  D  F  C  L  T  N  K  G  E  P  L  K  K  A  I  E  T  W

661  ACAACAAAGCGAACGGAAAAGCGGTGATTGATTACGGCTTTCATTTAATGATTAGTGAAA
     H  N  K  A  N  G  K  A  V  I  D  Y  G  F  H  L  M  I  S  E

721  TTACGGATGACGTATTAGAAGAGCTGCCGAAAGTCATTGAAGAAGAAGGAATTACATCCC
     I  T  D  D  V  L  E  E  L  P  K  V  I  E  E  E  G  I  T  S

781  TCAAAGTGTTTATGGCGTATAAAAACGTATTTCAGGCAGATGATGGAACGTTATACTGTA
     L  K  V  F  M  A  Y  K  N  V  F  Q  A  D  D  G  T  L  Y  C

841  CATTACTGGCTGCTAAAGAACTTGGAGCGCTCGTGATGGTTCATGCGGAAAATGGTGATG
     T  L  L  A  A  K  E  L  G  A  L  V  M  V  H  A  E  N  G  D

901  TGATTGATTATTTAACGAAGAAAGCGCTCGCGGATGGGAATACGGATCCGATTTACCATG
     V  I  D  Y  L  T  K  K  A  L  A  D  G  N  T  D  P  I  Y  H

961  CATTAACAAGACCTCCAGAATTGGAAGGAGAAGCGACAGGGCGCGCCTGTCAATTAACAG
     A  L  T  R  P  P  E  L  E  G  E  A  T  G  R  A  C  Q  L  T

1021 AGCTTGCCGGTTCACAGCTTTACGTCGTTCACGTGACGTGTGCACAAGCGGTTGAAAAAA
     E  L  A  G  S  Q  L  Y  V  V  H  V  T  C  A  Q  A  V  E  K

1081 TTGCTGAGGCGCGCAATAAAGGGTTGGATGTATGGGGAGAAACGTGTCCGCAATATCTTG
     I  A  E  A  R  N  K  G  L  D  V  W  G  E  T  C  P  Q  Y  L

1141 TTCTTGACCAATCTTATTTAGAAAAGCCTAATTTTGAAGGTGCGAAATATGTTTGGTCAC
     V  L  D  Q  S  Y  L  E  K  P  N  F  E  G  A  K  Y  V  W  S

1201 CTCCGCTTCGTGAAAAATGGCATCAAGAAGTATTGTGGAATGCATTGAAAAACGGTCAGC
     P  P  L  R  E  K  W  H  Q  E  V  L  W  N  A  L  K  N  G  Q

1261 TGCAAACTCTCGGATCTGACCAATGTTCGTTTGACTTTAAAGGGCAAAAAGAACTTGGCA
     L  Q  T  L  G  S  D  Q  C  S  F  D  F  K  G  Q  K  E  L  G

1321 GAGGAGATTTTACAAAAATTCCAAATGGCGGACCGATCATTGAGGATCGGGTTAGCATTC
     R  G  D  F  T  K  I  P  N  G  G  P  I  I  E  D  R  V  S  I

1381 TCTTCAGTGAAGGAGTAAAGAAAGGAAGAATCACGTTAAATCAATTTGTTGACATTGTGT
     L  F  S  E  G  V  K  K  G  R  I  T  L  N  Q  F  V  D  I  V

1441 CGACAAGAATTGCCAAATTGTTCGGGTTGTTCCCGAAAAAAGGAACCATTGTAGTAGGTT
     S  T  R  I  A  K  L  F  G  L  F  P  K  K  G  T  I  V  V  G

1501 CGGACGCAGACTTAGTCATCTTTGACCCGAATATTGAACGGGTGATTTCGGCTGAAACGC
     S  D  A  D  L  V  I  F  D  P  N  I  E  R  V  I  S  A  E  T

1561 ACCATATGGCCGTTGACTACAATGCATTCGAAGGAATGAAAGTAACGGGTGAACCGGTAT
     H  H  M  A  V  D  Y  N  A  F  E  G  M  K  V  T  G  E  P  V

1621 CGGTTCTTTGTAGAGGCGAATTTGTCGTTCGCGATAAACAATTTGTCGGCAAACCAGGGT
     S  V  L  C  R  G  E  F  V  V  R  D  K  Q  F  V  G  K  P  G

1681 ACGGACAATATTTAAAACGAGCAAAATACGGAACTTCGACGATTTCCAAGCAGAGCGAGG
     Y  G  Q  Y  L  K  R  A  K  Y  G  T  S  T  I  S  K  Q  S  E

1741 AATTAACCATTTAACTTGTCTCCTAAAATGGCTATAAAAATGTAACATGAATT
     E  L  T  I
```

Fig. 2 Nucleotide and deduced amino acid sequences of the D-hydantoinase gene from the *B. stearothermophilus* SD1. The putative ribosome binding site is shown by the *dashed lines*. Sequences similar to the -10 and -35 consensus sequences for *E. coli* promoters are *underlined.* The amino acid residues underlined at the N- and C-terminus were determined by Edman degradation and carboxypeptidase digestion of the purified D-hydantoinase from *B. stearothermophilus* SD1

Sequence homology with other hydantoinases

The sequences of genes encoding D-hydantoinases from two thermophiles, one gram-negative (CBS30380) and the other gram-positive (LU1220), were previously reported (Jacob et al. 1990). However there were several stop codons within the sequence of the gene from CBS30380, implying a possible sequencing error, which makes it difficult to compare the precise nucleotide sequence with those from other microorganisms. The putative amino acid sequence of the D-hydantoinase reported in this study was compared with those of three hydantoinases (NS1122A, LU1220, DSM84), and the degree of sequence homology among these enzymes was found to be about 22%. However, inclusion of the hydantoinase from *P. putida* NS671 in the comparison resulted in a very low overall homology (< 7%), implying that the enzyme from *P. putida* NS671 has a different evolutionary origin. On the other hand, extremely high amino acid sequence homology (> 89%) was found among the three hydantoinases from thermophilic microorganisms (SDI, LU1220, NS1122A). In particular, the D-hydantoinase of SD1 was revealed to be almost identical to that of *B. stearothermophilus* NS1122A, showing about 93% identity. If conservative substitutions are taken into consideration, the overall homology increases to nearly 95%. This observation implies that the hydantoinases of thermophiles are closely related to each other.

However, despite the extremely high homology in the amino acid sequences of the hydantoinases from the thermophiles (SD1, NS1122A, LU1220), the C-terminal regions of the enzymes from SD1 and NS1122A are completely distinct (Fig. 3). When the C-terminal region of the LU1220 enzyme was compared with those of the two *Bacillus* species (SD1, NS1122A), approximately 20 amino acid residues were mismatched. Based on the overall homology in the amino acid sequences, hydantoinases from thermophiles were expected to possess similar biochemical properties. Characteristics of the enzyme from LU1220 have not been reported, but the biochemical properties of the non-stereospecific hydantoinase from *B. stearothermophilus* NS1122A have been investigated in detail (Mukohara et al. 1994). The enzyme from *B. stearothermophilus* SD1 is a homodimer (Lee et al. 1995), while the hydantoinase of *B. stearothermophilus* NS1122A has been reported to be composed of four subunits. With respect to stereospecificity, the SD1 enzyme exhibited no activity toward L-form hydantoin derivatives, but the NS1122A enzyme shows considerable activity on L-form and D-form substrates. Therefore, it is very interesting that two enzymes possessing almost the same amino acid sequence should exhibit quite different biochemical characteristics, and this strongly implies that the C-terminal region makes a critical contribution to the difference in the biochemical properties. The hydropathy profiles of two enzymes were also analyzed, and found to be almost identical, except for the C-terminal regions (data not shown): the average hydropathy index of the C-terminal region of the enzyme from *B. stearothermophilus* SD1 was negative, indicating that this region is hydrophilic, whereas that from *B. stearothermophilus* NS1122A was positive.

The secondary structures of hydantoinases from SD1, LU1220, and NS1122A were predicted by using an EMBL program (data not shown). As expected, these enzymes were found to share a similar secondary structure. The contents of α-helix, β-sheet, and loop were calculated to be about 24 ± 2, 33 ± 2, and $43 \pm 2\%$ for all three enzymes, respectively. The C-terminal region of the SD1 enzyme is predicted to form a loop, while that from *B. stearothermophilus* NS1122A is predicted to be helical. From its average hydropathy, the C-terminal region of the enzyme from *B. stearothermophilus* NS1122A is assumed to be hydrophobic. It is likely that the C-terminal region of NS1122A is buried in the interior of the enzyme, playing an important role as an interface between subunits. The hydantoinase from NS1122A was reported to be a tetramer, which also suggests that the C-terminal region of the hydantoinase from *B. stearothermophilus* NS1122A might be involved in the oligomerization of the enzyme.

Comparison with functionally related amidohydrolases

Allantoinases and dihydroorotases are also known to catalyze the ring-opening reaction on the cyclic amide bond, like hydantoinases, and it has been suggested that all belong to a amidohydrolase family (Lapointe et al. 1994). The putative amino acid sequence of the SD1 D-hydantoinase was aligned with those of three hydantoinases (NS1122A, LU1220, DSM84), two allantoinases (DAL1, FALAN), and five dihydroorotases (CAD, DIC, YST, ECO, BSU). The degree of sequence homology among these enzymes was about 20–29%, and four conserved regions were observed (Fig. 4). Five histidines and an acidic amino acid residue were strictly conserved among the enzymes.

```
SD1        421 VTGEPVSVLCRGEFVVRDKQFVGKPGYGQYLKRAKYG TSTISKQSEELTI  472
NS1122A        VTGEPVSVLCRGEFVVRDKQFVGKPGYGQYVKRAKYG ALMADQDVVKMS   471
LU1220         VTGEPVSVRAEANLLSVINNLSENQGTANI----------------------  452
               ********                    *
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Fig. 3 Amino acid sequences of the C-terminal regions of the hydantoinases from three thermophilic bacteria. SD1, *B. stearothermophilus* SD1; NS1122A, *B. stearothermophilus* NS1122A; LU1220, a gram-positive bacteria. Identical amino acid residues are marked by *asterisks*, and mismatches are boxed

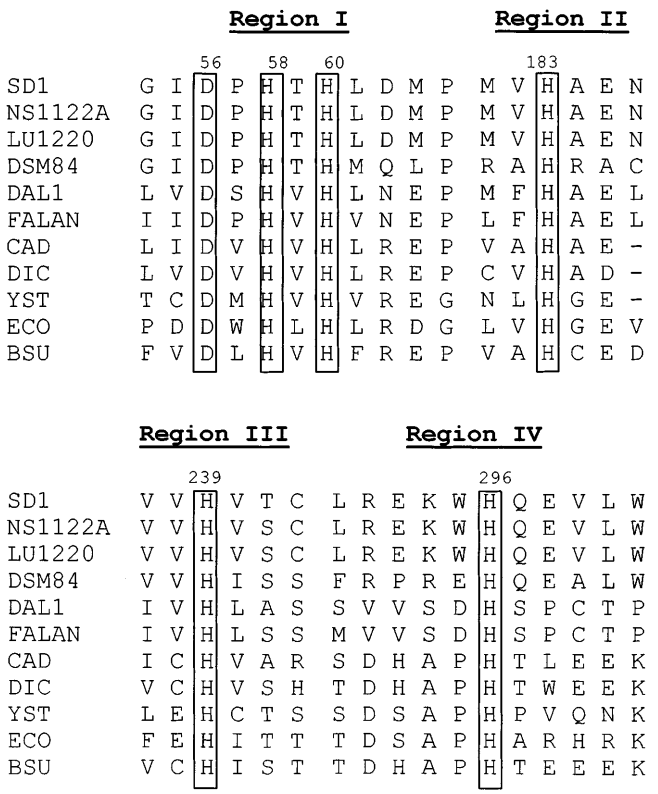

Fig. 4 Comparison of the amino acid sequences in the regions conserved between hydantoinases and amidohydrolases from various sources. SD1, D-hydantoinase from *B. stearothermophilus* SD1; NS1122A, non-stereospecific hydantoinase from *B. stearothermophilus* NS1122A; LU1220, D-hydantoinase from a gram-positive bacterium; DSM84, D-hydantoinase from *Pseudomonas putida* DSM84; DAL1, allantoinase from *Saccharomyces cerevisiae*; FALAN, allantoinase from *Rana catesbiana* (GenBank accession number U03471). CAD, dihydroorotase domain of the multifunctional CAD enzyme of hamster (GenBank accession number M33702); DIC, dihydroorotase from *Dictyostelium discoideum* (GenBank accession number X14634); YST, dihydroorotase from *S. cerevisiae* (GenBank accession number X07561); ECO, dihydroorotase from *E. coli* (GenBank accession number X04469); BSU, dihydroorotase from *B. subtilis* (GenBank accession number M59757). Conserved residues are boxed

Recently, Zimmerman et al. (1995) have investigated the role of the conserved histidines in catalysis and metal binding in mammalian dihydroorotase. It was suggested that the active-site zinc ion is coordinated by three histidines in regions I and II, and that the histidine of region IV is located at the active site and participates in catalysis. In the analysis of the deletion mutants of the D-hydantoinase gene from *B. stearothermophilus* SD1 (Fig. 2), enzyme activity was lost on deletion of region I (pHBBN 420), but the deletion mutant retaining region I (pHBBN 363) exhibited the enzyme activity. In addition, the enzyme from *B. stearothermophilus* SD1 is severely inhibited by diethylpyrocarbonate, which is known to modify histidine residues. From theses results, it would appear that region I of the SD1 D-hydantoinase is involved in catalysis, probably in metal binding as in dihydroorotase. This D-hydantoinase was also found to require divalent metal ions, such as manganese, for activity, and the three histidines of regions I and II are likely to participate in metal binding at the active site of the enzyme as suggested by Zimmerman et al. (1995). The amino acid residues which appear to constitute the active site seem to be conserved among the enzymes, which suggests a close evolutionary relationship between the amidohydrolases.

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