Identification of the structural similarity in the functionally related amidohydrolases acting on the cyclic amide ring

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The functionally related amidohydrolases, including D-hydantoinases, dihydropyrimidinases, allantoinases and dihydro-orotases, share a similar catalytic function of acting on the cyclic amide ring. We aligned 16 amidohydrolases by taking account of the conservative substitution and found a number of highly conserved regions and invariant amino acid residues. Analyses of the secondary structure and hydropathy profile of the enzymes revealed a significant degree of similarity in the conserved regions. Among the regions, the long stretched region I is of particular interest, because it is mainly composed of invariant amino acid residues, showing a similarity of 69 % for the enzymes. A search of the protein data bank using the sequence of the conserved region I identified a number of proteins possessing a similar catalytic property, providing a clue that this region might be linked with the catalytic function. As a particular sequence, one aspartic acid and four histidine residues are found to be rigidly conserved in the functionally related amidohydrolases. In order to investigate the significance of the conserved residues, site-directed mutagenesis was carried out typically for the D-hydantoinase gene cloned from *Bacillus stearothermophilus* SD1. These residues were found to be essential for metal binding as well as catalysis, strongly implying that these invariant residues play a critical role in other enzymes as well as in D-hydantoinase. On the basis of the similar catalytic function and existence of the rigidly conserved sequence, we propose a close evolutionary relationship among the functionally related amidohydrolases, including D-hydantoinase, dihydropyrimidinase, allantoinase and dihydro-orotase.

INTRODUCTION

The comparative study of enzymes that catalyse the same type reactions but have a different substrate specificity and kinetic properties is a useful approach to understanding the molecular mechanism of the enzyme reaction and evolutionary relationship when no structural data are available. In this context, comparison of the primary structure in the functionally related amidohydrolases including D-hydantoinases, dihydropyrimidinases, dihydro-orotases and allantoinases, all of which have a certain similarity in their actions on the cyclic amide bonds, seems very interesting, because basic aspects of the catalytic mechanisms are believed to be general, even though the substrates on which the enzymes acted are slightly different. Reactions catalysed by the four enzyme families are shown in Scheme 1. All reactions involve the hydrolysis of the cyclic amide bond (—CO—NH—) in either five- or six-membered rings. These enzymes are also known to catalyse reversible reactions.

Microbial D-hydantoinase catalyses the hydrolysis of a variety of hydantoins [1] and has been presumed to be a microbial counterpart [2] of the animal dihydropyrimidinase (5,6-dihydropyrimidine amidohydrolase, EC 3.5.2.2), which is involved in the catabolic degradation of pyrimidine [3]. These hydantoin-hydrolysing enzymes are produced by a wide variety of micro-organisms [4–6], but the majority of the studies have been focused on the characterization of the enzymes and biotechnological applications. Moreover, their physiological function in micro-organisms and the evolutionary relationship among the functionally related enzymes have not been elucidated. In an effort to understand the function of dihydropyrimidinase in the pyrimidine metabolism, biochemical properties of the enzymes from different mammalian sources have been revealed in detail [7,8]. Recently, cDNA sequences encoding dihydropyrimidinases of human liver [9] and rat liver [10] have been determined, and an evolutionary relationship between mammalian dihydropyrimidinase and microbial D-hydantoinase was suggested [9]. Dihydro-orotase catalyses the reversible cyclization of carbamyl aspartate to form dihydro-orotate, the third step in *de novo* pyrimidine biosynthesis. Characteristics of dihydro-orotases from mammalian multifunctional protein CAD [11] and *Escherichia coli* [12] have been reported. Allantoinase, the first enzyme involved in the degradation of allantoin, which is an intermediate in the catabolic pathway for purines, was studied from the eukaryotes [13,14] and micro-organisms [15]. However, little attention has been paid to the structure–function relationship and catalytic mechanism of the functionally related amidohydrolases at the molecular level.

Previously we cloned and sequenced the D-hydantoinase gene from *Bacillus stearothermophilus* SD1, and analysed its primary sequence [16]. In the present study we attempted to elucidate the structural and evolutionary relationship among the functionally related amidohydrolases, including D-hydantoinase, dihydropyrimidinase, dihydro-orotase and allantoinase. For this purpose, we analysed the primary and secondary structures of the 16 amidohydrolases and identified a number of conserved regions and invariant amino acid residues. To investigate further the functional significance of the conserved amino acid residues, site-directed mutagenesis was carried out for the D-hydantoinase from *B. stearothermophilus* SD1.

EXPERIMENTAL

Enzymes and reagents

Hydantoin and dihydrouracil were purchased from Sigma. Restriction enzymes and T_4 DNA ligase were from Boehringer-

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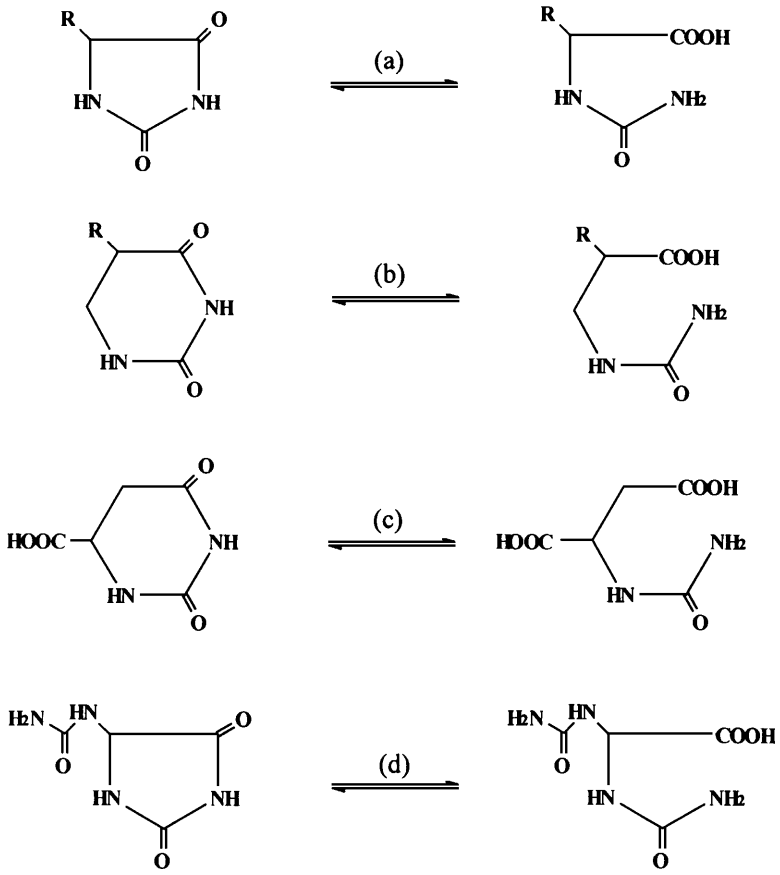

Scheme 1 Comparison of the reactions catalysed by the functionally related four enzyme families

(**a**) D-Hydantoinase; (**b**) dihydropyrimidinase; (**c**) dihydro-orotase; (**d**) allantoinase.

Mannheim. A thermophilic DNA polymerase for PCR was purchased from New England Biolabs, and radiolabelled materials were purchased from Amersham International. Oligodeoxynucleotides were obtained from DNA Int. (Lake Oswego, OR, U.S.A.). Prepacked Resource-Q, Phenyl-Superose, and Superose 12 columns were purchased from Pharmacia–LKB. All other reagents were of analytical grade.

Plasmids and strains

Thermophilic *B. stearothermophilus* SD1 isolated in our previous work [17] was used as the source of the hydantoinase gene. The *E. coli* strain JM109 was used to express the wild-type and mutated hydantoinase genes. A repair-minus strain of *E. coli* ES1301 *muts*, which was used as the host for mutagenesis reaction, was purchased from Promega. The pALTER-EX2 vector was also purchased from Promega. *E. coli* strains were grown in Luria–Bertani medium at 37 °C. Ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), and tetracycline (10 μg/ml) were added when needed.

Computer analysis

The amino acid sequences of the functionally related amidohydrolases obtained from GenBank were aligned using the program of the Blast Network service at the National Center for Biotechnological Information (Bethesda, MD, U.S.A.). Table 1 lists the sources of enzymes aligned in the present work. The initial alignment was performed using Clustal W software [18]. The initial alignment was further refined manually, taking into account the predicted secondary structure of the D-hydantoinase from *B. stearothermophilus* SD1. To improve the overall alignment through the entire sequence, gaps were introduced into the sequences to allow for blocks of divergence among four enzyme families. Unless otherwise stated, the numbering of amino acid residues is derived from that of the D-hydantoinase of *B. stearothermophilus* SD1. Analyses of the secondary structures of the enzymes were performed using the network software of EMBL (Heidelberg, Germany). Hydropathy profiles were obtained according to the method of Kyte and Doolittle [19].

Oligonucleotide-directed mutagenesis

The gene encoding the hydantoinase of *B. stearothermophilus* SD1 was inserted into the *Nco*I/*Pst*I site of the mutation vector pALTER-EX2 by using PCR, and the resulting plasmid, pALHYD, was used for oligonucleotide-directed mutagenesis with a kit from Promega based on the method of the supplier. The sequences of the synthetic oligonucleotides for site-directed mutagenesis are shown in Table 1. Mutated nucleotides are underlined. The mutated genes were cloned into the expression vector pTrc 99A (Pharmacia), and expressed in *E. coli* by the

Table 1 Enzymes used for alignment (a) and synthetic oligonucleotides used for site-directed mutagenesis (b)

(a)

Enzyme family	Source	Common name	Accession no.
D-Hydantoinases	*Bacillus stearothermophilus*	Bacterium	Our organism S73773
	Pseudomonas putida	Bacterium	L24157
	A thermophilic Gram-Positive	Bacterium	A13503
Dihydropyrimidinases	*Homo sapiens*	Man	D78011
	Rattus norvegicus	Black rat	D63704
Allantoinases	*Saccharomyces cerevisiae*	Baker's yeast	S48489
	Rana catesbeiana	Frog	U03471
Dihydro-orotases	*Cricetulus* sp.	Hamster	M33702
	Dictyostelium discoideum	Slime mould	X14634
	Bacillus subtilis	Bacterium	M59757
	Escherichia coli	Bacterium	X04469
	Salmonella typhimurium	Bacterium	X03928
	Saccharomyces cerevisiae	Baker's yeast	X07561
	Ustilago maydis	Rust fungus	X63181
	Pseudomonas aeruginosa	Bacterium	U73505

(b)

Mutation	Residue change	Oligonucleotide sequence
D56A	$Asp^{56} \rightarrow Ala$	TCCAGGCGGCATTGCTCCGCACACGCATTT
H58N	$His^{58} \rightarrow Asn$	GGCATTGATCCGAACACGCATYYAGAT
H60N	$His^{60} \rightarrow Asn$	GATCCGCACACGAATTTAGATATGCCG
H183N	$His^{183} \rightarrow Asn$	GTGATGGTTAATGCGGAAAAT
H239N	$His^{239} \rightarrow Asn$	CTTTACGTCGTTAACGTGACGTGTGCA

addition of isopropyl β-D-thiogalactoside. The mutations were confirmed by DNA sequencing as described elsewhere [20].

Enzyme purification

Wild-type and mutant enzymes were purified as previously reported [17]. All purification steps were conducted at room temperature in the presence of 1 mM $MnCl_2$. *E. coli* cells from a 1 litre culture were harvested by centrifugation. The cells were resuspended in 20 mM Tris/HCl buffer, pH 8.0, and disrupted by using a sonicator (Branson Sonic Power Co.). Cell debris was removed by centrifugation at 18000 ***g*** for 1 h, and supernatant was loaded on to a column of Resource Q equilibrated with 20 mM Tris/HCl buffer, pH 8.0, in an FPLC™ system (Pharmacia). The column was washed with 5 vol. of the same buffer and eluted with a linear gradient of 0–0.5 M NaCl. The wild-type and mutant hydantoinases were eluted at the same concentration, ranging from 0.2 to 0.25 M, and the active fractions were pooled and concentrated using a Centicon 100 apparatus (Amicon). The concentrated protein solution was loaded on to a Phenyl-Superose column equilibrated with 20 mM Tris/HCl buffer, pH 8.0, containing 1.8 M $(NH_4)_2SO_4$. The enzyme was eluted with a reverse linear gradient (1.8 M–0 M) of $(NH_4)_2SO_4$, and then dialysed against 20 mM Tris/HCl buffer, pH 8.0, containing 1 mM $MnCl_2$.

Metal content analysis

Metal contents of the wild-type and mutant enzymes were determined by using an ICP–IRIS Atomic Emission Spectrophotometer (Thermo Jarrell Ash). Double-distilled water was passed through a primary deionization unit and a 0.2 μM filter cartridge (Millipore), and the resulting water was found to contain negligible metal ions (0.15 ng/ml). No glassware was used during sample preparation, and plastic materials were acid-cleaned by using 2.5 % (v/v) HNO_3. Protein samples were prepared according to the method described elsewhere [21] and diluted 10-fold with metal-free water prior to analysis.

Assay of hydantoinase activity

The reaction mixture contained 1 mM $MnCl_2$, 2 μg of purified enzyme and 50 mM hydantoin in 1 ml of 0.1 M Tris/HCl buffer, pH 8.0. The enzyme reaction was carried out at 55 °C for 30 min with moderate stirring and nitrogen sparging to prevent oxidation of the substrate. The reaction was stopped by adding 0.5 ml of 12 % trichloroacetic acid, and precipitated proteins were removed by centrifugation. The amount of product formed was determined by using either HPLC or colour reagent (*p*-dimethylaminobenzaldehyde) [17]. One unit of hydantoinase activity was defined as the amount of enzyme required to produce 1 μmol of *N*-carbamyl-D-amino acid from hydantoin derivative/min under the specified conditions.

Analysis

Analytical SDS/PAGE in slab gels was performed according to the method of Laemmli [2]. Acrylamide gels were stained with either Coomassie Brilliant Blue G250 or $AgNO_3$. The protein concentration was determined by the method of Bradford [23].

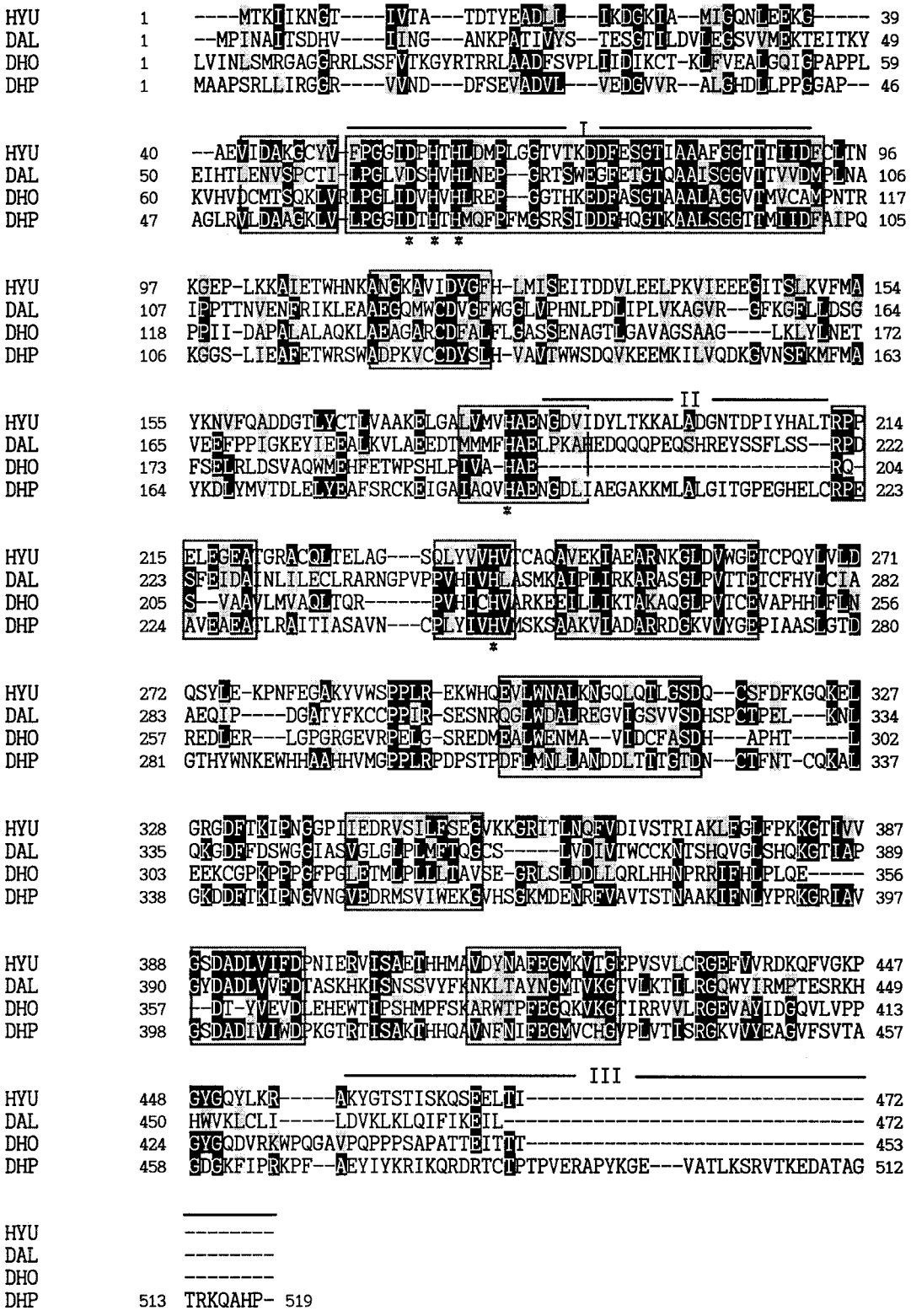

Figure 1 Multiple sequence alignment of D-hydantoinase, allantoinase, dihydro-orotase and dihydropyrimidinase

The alignment includes a typical sequence of microbial D-hydantoinase from *Bacillus stearothermophilus* SD1 (HYU), allantoinase from *Saccharomyces cerevisiae* (DAL), dihydro-orotase domain of hamster (DHO) and dihydropyrimidinase from human (DHP). Identified regions of conserved amino acid residues among the four enzyme families are shown in boxes. The number on the left indicate the amino acid position in each sequence. Roman numbers I, II and III define the highly conserved long stretch, internal mismatching region and C-terminal mismatching region respectively. Amino acid identities in the alignment are shown in black boxes with white letters. Relevant amino acid similarities are indicated by grey boxes. Residues mutated by site-directed mutagenesis are indicated by asterisks. Conservative amino acids were considered as follows [34]: Gly = Ala = Ser; Ala = Val; Val = Ile = Leu = Met; Ile = Leu = Met = Phe = Tyr = Trp; Lys = Arg = His; Asp = Glu = Gln = Asn; Ser = Thr; Pro = Gly.

RESULTS AND DISCUSSION

Alignment of amino acid sequences of the functionally related amidohydrolases

We aligned the amino acid sequences of 16 amidohydrolases including D-hydantoinases, dihydropyrimidinases, allantoinases and dihydro-orotases. Microbial D-hydantoinases [15,24–26] were revealed to share about 37–42 % amino acid identity with mammalian dihydropyrimidinases [9,10] and similarity increased up to 65 % when the conservative substitutions were considered, which implies that microbial D-hydantoinase is much more closely related to animal dihydropyrimidinase than allantoinase and dihydro-orotase. Thus this result strongly supports the suggestion that the microbial D-hydantoinase is a counterpart of animal dihydropyrimidinase. It has been already reported that the microbial D-hydantoinase also shows as high an affinity toward the dihydrouracil as the mammalian dihydropyrimidinase [5,6,17]. The amino acid identities of dihydropyrimidinases with allantoinases and dihydro-orotases were found to be about 19–26 and 7–16 % respectively. Allantoinases shared an identity of 9–20 % with dihydro-orotases. A relatively low identity among the enzymes despite the similar catalytic property raised a question about the evolutionary relationship. A close structural and evolutionary relationship among the four enzyme families might be proposed when there exists a high similarity in primary and secondary structures among the enzymes.

When the sequence similarity is lower than 35 %, current automated methods of alignment can not generate the accurate alignment of the sequences without the use of reliable anchor points [27]. Therefore we used the highly conserved regions among the enzymes as query sequences and introduced the rationale gaps for matching the appropriate regions. In this situation, when the conservative substitutions are taken into account, the similarity significantly increases.

For a clearer comparison, the sequence alignment was shown only for typical enzymes which represent each enzyme family. As a typical sequence in dihydro-orotase family, the core domain of dihydro-orotase in multifunctional CAD (a multifunctional polypeptide having carbamoyl-phosphate synthetase, aspartate transcarbamoylase and dihydro-orotase activities) has been predicted to have the amino acid residue from 66 to 399, corresponding to 334 amino acid residues [11]. However, the alignment of this sequence with the other three enzyme families revealed a low similarity ($\leqslant 25$ %) and required rather extensive insertion and deletion. Thus we tried to improve the overall similarity among the enzymes, and found an interesting result by using an extended sequence which has extensions on both the N- (65 residues) and C- (54 residues) termini of the core domain. From the alignment with this extended sequence of dihydro-orotase, sequence similarity of the four enzyme families was revealed to be about 33–46 % and several regions were found to be rigidly conserved in the same order over the entire sequences of four enzyme families as shown in Figure 1. The conserved regions were located throughout an entire sequence, except for the highly mismatched C-terminal region (region III in Figure 1). A fragment deletion at region II (26 amino acids) of dihydro-orotase caused an interruption of the alignment of this enzyme with the other three enzyme families. The similar size and high similarity were observed between the microbial D-hydantoinase and allantoinase. The conserved regions in each enzyme family represent a great percentage of the total sequence length of the enzymes, spanning approx. 75 %.

Sequence comparison not only provides evidence for an evolutionary relationship among the enzymes, but could also give an important clue for understanding the molecular function of the enzymes, by generating rigidly conserved amino acid residues and regions among the related enzymes. In this case, the longer the region conserved, the closer the evolutionary and functionary relationships provided. Among the conserved regions, the long streached region I was of particular interest because it consisted of 42 amino acid residues with 16 invariants and up to 29 identical plus highly conservative amino acid residues, showing a similarity of 69 %. In addition, this structural element was found to be localized at similar positions of the sequences among the enzymes. Analyses of the secondary structures for region I revealed to have a similar content in the four enzyme families, showing that loop, β-sheet/β-turn, and β-sheet/β-turn are arranged in the same order. From the analyses of hydropathy profiles, region I of the four enzyme families was shown to have a similar profile. On the basis of the above results,

Region I

I-I	I-II	I-III
LPGGI(V)DXHXHLD(N)	XPXXGT(S)XT(S)XD(E)DFXS(T)	GTXAAL(I)XGGXTXI(V)I(V)DM

Figure 2 Summary of SWISS-PROT search output file for the rigidly conserved region I derived from four enzyme families

Location of conserved region I is shown by a bar above the result of alignment in Figure 1. The total numbers of amino acids in query sequences are 42 amino acids, and the similarities are considered in three parts of the region:

Other proteins identified	Similarity (%)		
	I–I	I–II	I–III
Toad 64 protein (*Rattus norvegicus*)	66	50	74
Imidazolonepropionase (*Bacillus subtilis*)	83	43	47
Adenine deaminase (*Bacillus subtilis*)	66	36	50
Indole-3-pyruvate decarboxylase (*Azotobacter*)	33	43	31
Penton base protein (*denovirus*)	33	43	25
GTP cyclohydrolase (*Haemophilus influenzae*)	25	50	56
hUlip protein (*Homo sapiens*)	83	57	67

Conservative replacements of conserved amino acid residues in query sequences are considered. The capital X indicates the residues within the sequences that were not forced to match in the search. Random replacement of two or three amino acids in query sequences provided unrelated sequences to four enzymes families, and these unrelated proteins were excluded in the results.

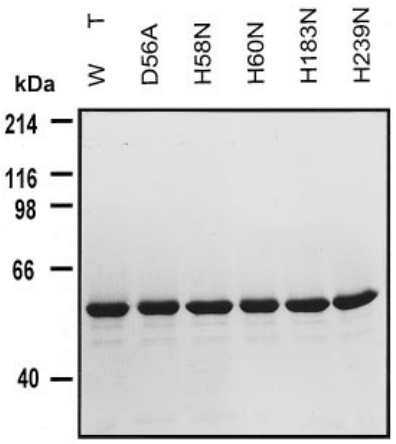

Figure 3 SDS/PAGE analysis of the wild-type and mutated D-hydantoinases

Purified wild-type and mutated enzymes (5 μg) were applied to electrophoresis on a 10% (w/v) polyacrylamide gel.

the conserved region I is thought to play an important role in these enzyme families. Recently, evidence has been reported for supporting that this region is catalytically essential for dihydro-orotase [28] and D-hydantoinase [16].

To investigate the significance and the distribution of the region I in other protein families, we searched for proteins from the SWISS-PROT data bank using the sequence of region I (Figure 2). The best scores were observed among the four enzyme families studied in this work, and some of other proteins were also aligned, showing a considerable similarity. One of the most interesting observations is that the aligned proteins share a similar catalytic function, acting on the substrates which have the cycle ring. These results strongly suggest that the highly conserved region I derived from the four enzyme families might be critical for the catalytic function of the enzymes. However, newly aligned proteins did not exhibit a significant similarity for the rest of the sequences. In other words, the enzymes possessing the conserved region I are closely related to each other with respect to the nature of the substrate on which they act, despite the low similarity and the location of the homologous region at different positions. Among the newly identified proteins, it was recently reported that the sequences of hUlip and Toad-64 revealed a considerable similarity to microbial D-hydantoinase [29].

Significance of the invariant amino acid residues in the conserved region

Alignment of the complete amino acid sequences of 16 amido-hydrolases revealed a number of invariant residues (see Figure 1), mainly clustered in region I. Interestingly, an aspartic acid residue and four histidine residues were found to be perfectly conserved in all of the aligned enzymes. The amidohydrolases mentioned above have been reported to require metal ions such as Mn^{2+} or Zn^{2+} for their activity [4–8,11,12,17]. In addition, the structure of the substrates on which the enzymes act is very similar, and limited identical residues were found. These observations led us to suggest that these conserved amino acid residues might play an essential role in metal binding, catalysis or structure of the enzymes. To confirm the functional significance of the conserved amino acid residues, site-directed mutagenesis was carried out typically for the D-hydantoinase gene cloned from *B. stearothermophilus* SD1 in our previous work [16]. Asp^{56} was replaced with Ala, and four histidine residues His^{58}, His^{60}, His^{183} and His^{239} were substituted for Asn. The mutated D-hydantoinase genes were subcloned into an expression vector, and mutant enzymes were purified to homogeneity as described in the Materials and methods section (Figure 3), and their activities were determined. As shown in Table 2, substitution of these residues resulted in an almost complete loss of enzymic activity, indicating that the conserved amino acid residues are critical for the catalytic activity of the D-hydantoinase of *B. stearothermophilus* SD1.

Table 2 Specific activities of mutated D-hydantoinases

Enzyme	Specific activity* (units/mg of protein)	Relative activity (%)
Wild-type	101.85 ± 2.54	100
$Asp^{56} \rightarrow Ala$	1.72 ± 0.26	1.7
$His^{58} \rightarrow Asn$	3.25 ± 0.23	3.2
$His^{60} \rightarrow Asn$	0.49 ± 0.06	0.5
$His^{183} \rightarrow Asn$	0.13 ± 0.01	0.1
$His^{239} \rightarrow Asn$	0.23 ± 0.03	0.2

* Three separate experiments, means ± S.D.

In the case of dihydro-orotases from mammalian sources, two histidine residues in region I, corresponding to His^{58} and His^{60} in the D-hydantoinase, were found to participate in metal binding [21,28]. These results were partly confirmed by comparison of zinc enzymes such as carbonic anhydrase for which the protein crystal structure is known [30]. Recently, Matsuda et al. proposed that the histidine residues of the conserved region I are involved in the zinc-binding motif of dihydropyrimidinase from rat liver [10]. The hydropathy profiles around the conserved sequence were shown to be almost the same for all amidohydrolases studied in the present work. From these observations, the conserved residue sequence Asp-Xaa-His-Xaa-His also seems to constitute a metal-binding site in both D-hydantoinase and alantoinase, although the required metal ion for D-hydantoinase was Mn^{2+} rather than Zn^{2+}.

In order to get some insights into the function of the conserved residues, the manganese content of the wild-type and mutant enzymes were determined by using an atomic-emission spectrophotometer. As a result, the metal contents of the mutant enzymes were found to be negligible compared with that of the wild-type enzyme (Table 3). This result confirms that the sequence

Table 3 Metal contents of the wild-type and mutant D-hydantoinases

Enzyme	Metal bound (mol fraction)*
Wild-type	1.08 ± 0.09
H56A	N.D.†
H58N	< 0.01
H60N	< 0.01
H183N	< 0.03
H239N	< 0.04

* Expressed as a mol fraction of the metal bound by wild-type enzyme.
† N.D., not determined.

Table 4 Specific activity of the D-hydantoinase for typical substrates of four amidohydrolases

Substrate	Specific activity* (unit/mg)	Relative activity (%)
Hydantoin	103.8 ± 2.4	100.0
Dihydrouracil	30.9 ± 0.9	29.8
Allantoin	3.3 ± 0.4	3.2
Dihydro-orotate	N.D.†	–

* Determined in triplicate and expressed as means ± S.D.
† N.D., not determined.

Asp-Xaa-His-Xaa-His participates in metal binding. The wild-type enzyme was observed to contain one Mn^{2+} ion per dimeric enzyme molecule. The mutant enzyme D56A ($Asp^{56} \rightarrow Ala$) was not analysed because this enzyme was unstable. Until now, the roles of His^{183} and His^{239} have not been clearly demonstrated, but these residues were presumed to constitute either a metal-binding site as an another ligand with other two residues in region I. The analysis of metal content revealed that the mutants H183N and H239N contain negligible metal ions, which strongly suggests that the His^{183} and His^{239} residues are also involved in metal binding. Four or six amino acid residues were reported to participate in the binding of Mn^{2+} ions [31–32], whereas co-ordination for Zn^{2+} ions was trigonal [28].

The amidohydrolases studied in the present work shared a relatively long conserved region starting from the N-terminus, but little similarity was observed in their C-terminal region. A recent report from our study has provided evidence that the C-terminal region is not involved in the catalytic function [16]. In other words, the C-terminal region of the D-hydantoinase corresponding to a non-similar region III could be artificially deleted without loss of the activity (results not shown).

The existence of at least a long conserved amino acid stretch and a number of shortly conserved regions among the four enzyme families, including D-hydantoinase, dihydropyrimidinase, allantoinase and dihydro-orotase, led us to suggest the existence of an evolutionary common ancestor in these enzyme families. This hypothesis was supported by the observation that these enzymes share similar structures of substrates and the rigidly conserved residues. Considering that a similar spatial positioning of the conserved regions and critical amino acid residues is a prerequisite for a particular residue to participate in catalysis, the four enzyme families seem to have a similar higher structure. Prediction of secondary structures and hydropathy profiles is consistent with this suggestion. Thus the structural similarities are likely to be significant and more than merely fortuitous.

Microbial D-hydantoinases were reported to exhibit the comparable affinity toward the substrates of other amidohydrolases. As shown in Table 4, the specific activity of D-hydantoinase from *B. stearothermophilus* SD1 was determined for the typical substrates of four amidohydrolases. The D-hydantoinase showed a considerable activity for dihydrouracil and allantoin, which are the substrates of dihydropyrimidinase and allantoinase respectively. It is noteworthy that dihydropyrimidinase and hydantoinase showing the activity for dihydro-orotate have not yet been reported. Similarly, no dihydro-orotase and allantoinase have been known to exhibit the activity toward dihydropyrimidines and structurally related hydantoins. In other words, the four enzyme families show different substrate specificities, but their primary and secondary structures are very similar to each other. Nowadays, it is believed that extant proteins have evolved from a limited number of ancestral proteins [33]. The driving force of protein evolution is a random mutagenesis of the amino acid sequence. Natural selection, the other phenomenon in evolution, modulates the rate of protein divergence by imposing constraints on changes in the amino acid sequence. It appears that the major restriction of natural selection is to maintain the overall structure of the protein in a family. Therefore it is likely that the divergently minor insertion and/or deletions within a given family (see Figure 1) have resulted in a slight change of the frame and structure. In this context, it is reasonable to suggest that a minor modification in the specific regions or residues may trigger a significant alteration in the substrate specificity under conditions where the overall protein scaffolds are maintained. From the evolutionary point of view, it can be inferred that clusters of similar residues with a minor variation of amino acid residues might enable the enzymes to possess different substrate specificities. In this regard it should be noted that the long stretched region I has been shown already to vary within related proteins acting on a cyclic ring. Resolution of the three-dimensional structure of the related enzyme is expected to provide a more definitive and evolutionary relationship among these enzymes families.

REFERENCES

1 Yamada, H., Takahashi, S., Kii, Y. and Kumagai, H. (1978) J. Ferment. Technol. **56**, 484–491
2 Vogels, G. D. and Van der Drift, C. (1976) Bacteriol. Rev. **40**, 403–468
3 Fritzson, P. (1957) J. Biol. Chem. **226**, 223–228
4 Runser, S. M. and Meyer, P. C. (1993) Eur. J. Viochem. **213**, 1315–1324
5 Takahashi, S., Kii, Y., Kumagai, H. and Yamada, H. (1978) J. Ferment. Technol. **56**, 492–498
6 Morin, A., Hummer, W., Schütte, H. and Kula, M.-R. (1986) Biotechnol. Appl. Biochem. **8**, 564–574
7 Jahnke, K., Podschun, B., Schnackerz, K. D., Kautz, J. and Cook, P. F. (1993) Biochemistry **32**, 5160–5166
8 Kautz, J. and Schnackerz, K. D. (1989) Eur. J. Biochem. **181**, 431–435
9 Hamajima, N., Matsuda, K., Sakata, S., Tamaki, N., Sasaki, M. and Nonaka, M. (1996) Gene **180**, 157–163
10 Matsuda, K., Sakata, S., Kaneko, M., Hamajima, N., Nonaka, M., Sasaki, M. and Tamaki, N. (1996) Biochim. Biophys. Acta **1307**, 140–144
11 Simmer, J. P., Kelly, R. E., Rinker, A. G., Zimmermann, B. H., Scully, J. L., Kim, H. S. and Evans, D. R. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 174–178
12 Backstrom, D., Sjoberg, R. M. and Lundberg, L. G. (1986) Eur. J. Biochem. **160**, 77–82.
13 Hayashi, S., Jain, S., Chu, R., Alvares, K., Xu, B., Erfurth, F., Usuda, N., Rao, M. S., Reddy, S. K. and Noguchi, T. (1994) J. Biol. Chem. **269**, 122269–12276
14 Buckholz, R. G. and Cooper, T. G. (1991) Yeast **7**, 913–923
15 Murke, M. S., Op den Camp, H. J., Semesi, A. K. and Van der Drift, C. (1995) Curr. Microbiol. **30**, 45–47
16 Kim, G. J., Park, J. H., Lee, D. C., Ro, H. S. and Kim, H. S. (1997) Mol. Gen. Genet. **255**, 152–156
17 Lee, S. G., Lee, D. C., Hong, S. P., Sung, M. H. and Kim, H. S. (1995) Appl. Microbiol. Biotechnol. **43**, 270–276
18 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) Nucleic Acids Res. **22**, 4673–4680
19 Kyte, J. and Doolittle, R. F. (1982) J. Mol. Biol. **157**, 105–132
20 Sanger, F., Nickeln, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
21 Zimmermann, M. K., Kemling, N. M. and Evans, D. R. (1995) Biochemistry **34**, 7038–7046
22 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
23 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
24 Lapointe, G., Viau, S., Leblanc, D., Robert, N. and Morin, A. (1994) Applied Environ. Microbiol. **60**, 888–895
25 Mukohara, Y., Ishikawa, T., Watabe, K. and Nakamura, H. (1994) Biosci. Biotechnol. Biochem. **58**, 1621–1626
26 Jacob, E., Henco, K., Marcinowski, S. and Schenk, G. (1990) U.S. Pat. 4,912,044

27 Thornton, J. M., Flores, T. P., Jones, D. T. and Swindells, M. B. (1991) Nature (London) **354**, 105–106
28 Williams, N. K., Manthey, M. K., Hambley, T. W., O'Donoghue, S. I., Keegan, M., Chapman, B. E. and Christopherson, R. I. (1995) Biochemistry **34**, 11344–11352
29 Gaetano, C., Matsuo, T. and Thiele, C. J. (1997) J. Biol. Chem. **272**, 12195–12201
30 Eriksson, A. E. and Liljas, A. (1993) Proteins **16**, 29–42
31 Ose, D. E. and Fridovich, I. (1979) Arch. Biochem. Biophys. **194**, 360–364
32 Stallings, W. C., Pattridge, K. A., Strong, R. K. and Ludwig, M. L. (1985) J. Biol. Chem. **260**, 16424–16432
33 Chothia, C. (1992) Nature (London) **357**, 543–544
34 Alconada, A., Flores, A. I., Blanco, L. and Cuezva, J. M. (1994) J. Biol. Chem. **269**, 13670–13679

Received 28 July 1997/24 October 1997; accepted 4 November 1997