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Catalytic analysis of a recombinant D-hydantoinase from *Agrobacterium tumefaciens*

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Abstract

The D-hydantoinase gene of a wild strain of *Agrobacterium tumefaciens* BQL9 had 99.78% nucleotide sequence identity with other available *Agrobacterium* genes. The resulting amino acid sequence showed two important substitutions affecting two α-helixes in the secondary structure of the protein. The union of Mn^{2+} to the protein was essential for activating the enzyme and was independent of the temperature. D-Hydantoinase only was inactivated in the presence of 70 mM EDTA and at over 40 °C. The enzyme showed both hydantoinase and pyrimidinase activities, but only with the D-enantiomers of the substrates. Activity was greater for substrates with apolar groups in the number 5 carbon atom of the hydantoin. The native structure of the *N*-terminal end of this D-hydantoinase proved to be indispensable to its enzymatic activity.

Introduction

Production of D-phenylglycine and D-*p*-hydroxyphenylglycine, intermediate D-amino acids in the chemio-enzymatic synthesis of the semisynthetic penicillins and cephalosporins, is the limiting factor in this process (Runser *et al.* 1990, Lee *et al.* 1994). D-Amino acids of industrial interest are synthesised from their corresponding D,L-5-monosubstituted hydantoins in a three-step process. In the first step, the D-5-monosubstituted hydantoins are opened by a D-hydantoinase to produce a D-carbamoyl derivative (Hartley *et al.* 1998). In the second step, at the same time as D-hydantoinase hydrolyses the enantioselective D-5-monosubstituted hydantoin, the chemical and/or enzymatic racemization of L-5-monosubstituted hydantoin starts (Martinez-Rodriguez *et al.* 2002). In the third step, the enantioselective D-*N*-carbamoyl amino acid is converted to the free D-amino acid catalysed by an *N*-carbamoyl-D-amino acid amidohydrolase (Grifantini *et al.* 1996).

Hydantoinases occur in a variety of microorganisms with slight differences in their relative substrate-specificity (Brooks *et al.* 1983). Due to the high affinity of microbial D-hydantoinase toward dihydrouracil, this enzyme is thought to be involved in the catabolism of dihydrouracil and dihydrothymine (Kim & Kim 1998a). The amino acid sequence identity between the dihydropyrimidinases from *Homo sapiens* and *Rattus norvegicus* and the hydantoinases from several microorganisms is high enough to suggest that all belong to the dihydropyrimidinase family. A recent structural study has shown the involvement of the *C*-terminal end of D-hydantoinase from *Bacillus stearothermophilus* in the maintenance of the oligomeric structure of the enzyme (Kim & Kim

1998b). However, no data is available on the involvement of the *N*-terminal end in the catalytic function or in the maintenance of the enzyme structure.

Different factors which may affect the catalytic mechanism of D-hydantoinase are studied in the present work. The structure of the D-hydantoinase amino terminal end was altered, and its effect on the enzyme's activity was studied. For this purpose, a new D-hydantoinase gene from *Agrobacterium tumefaciens* BQL9 was cloned and expressed. This was then fused with a β-galactosidase fragment from *Escherichia coli*. The performance of the resulting fusion enzyme was evaluated and compared with that of the wild one. The expressed D-hydantoinase was characterised biochemically, its divalent cation requirement was established and its amino acid sequence was compared to those of similar enzymes from other microorganisms. This enabled data to be obtained on the functional importance of the *N*-terminal end.

Materials and methods

Chemicals

Dihydrouracil, 2-thiouracil, hydantoin, 2-thiohydantoin, D,L-isopropylhydantoin, D,L-*p*-hydroxyphenylhydantoin, *N*-carbamoyl-D,L-valine and *N*-carbamoyl-D,L-*p*-hydroxyphenylglycine from Sigma.

Isolation of Agrobacterium tumefaciens *BQL9*

Agrobacterium tumefaciens strain BQL9 was isolated using selection medium containing hydantoin as sole nitrogen source. Qualitative hydantoinase activity of the isolated strain was carried out colorimetrically according to the method of Cecere *et al.* (1975).

Cloning of the D-hydantoinase gene from Agrobacterium tumefaciens *BQL9*

The pBluescript II SK (pBSK) vector (Stratagene) was used to express the product of the D-hydantoinase gene from the new isolated strain. Two sets of primers were designed to amplify the open reading frame. The primers used to amplify the D-hydantoinase gene were designed according to the *Agrobacterium radiobacter* NRRL B11291 sequence (Grifantini *et al.* 1998). One set was designed to amplify the gene and clone in pBSK to produce a fusion protein with a fragment of the *N*-terminal end of the β-galactosidase protein. *Eco*RI and *Hin*dIII restriction sites were introduced into the 5′ and 3′ ends of the gene respectively. The plasmid with new insert was named pBSH2. The other set included a STOP signal in the sequence of the 5′ primer for the expression of β-galactosidase *N*-terminal fragment and an RBS sequence for the translation of the D-hydantoinase native protein. This set also included *Eco*RI/*Hin*dIII sites for cloning in pBSK and creating the plasmid pBSH1.

After ligation, *E. coli* DH5α was transformed with both constructions following the $CaCl_2$ method (Dagert & Ehlrich 1979). The bacteria strains with recombinant construction were selected by blue/white assay using α-complementation. The recombinant plasmids were isolated by lysis alkaline method (Birnboim & Doly 1979) and sequenced (Sanger 1981).

Overexpression of different constructions of the D-hydantoinase gene from BQL9

The overnight preculture was prepared by inoculating a colony in LB medium with 100 μg ampicillin ml^{-1} at 37 °C in constant agitation. Cultures were prepared in LB medium with ampicillin at the concentration mentioned above, and 1% of the overnight preculture. They were grown at 37 °C and in constant agitation to a turbidity of 0.3–0.6. The cloned genes were then induced by adding 0.4 mM IPTG. Induction was for 3–6 h at 37 °C in constant agitation.

Preparation of cellular extracts

Cells were harvested by centrifugation at 8000 × *g* for 10 min at 4 °C. They were then resuspended in 100 mM sodium carbonate buffer pH 9.5, 0.2 mg lysozyme ml^{-1}, 2 mM $MnCl_2$, mixed for 20 min at 30 °C and sonicated. The crude extract was centrifuged at 10 000 × *g* for 20 min at 4 °C to remove the cellular debris. The resulting cellular extract was used to analyse the activity of both types of construction following the method described below.

Biocatalytic reaction with cellular extracts

D-Hydantoinase activity was measured by incubating 200 μl cellular extract in the presence of 50 μl of several compounds based on the hydantoin or pyrimidine ring for 30 min at 30 °C. The reaction was stopped with 0.5 ml 1 M HCl, and the sample was centrifuged for 10 min at 10 000 × *g*. The supernatant, 25 μl, was used to separate and quantify the products in an HPLC system (Waters Breeze) using a C18 Novapac column.

Mobile phase was 20 mM H_3PO_4 (pH 3.2)/methanol (85:15 v/v) at 1 ml min^{-1}. Detection was at 223 nm. Activity was defined as the μmol hydantoin transformed per min and g dry weight. To study the thermal stability of the D-hydantoinase the cellular extract was incubated for 30 min at each temperature and the reaction was carried out at 30 °C. In this way the effect of the temperature on the reaction rate is avoided.

Results and discussion

Isolation and identification of BQL9

Numerous bacterial strains were selected based on their ability to utilise hydantoin as sole nitrogen source. BQL9 strain showed significant hydantoinase activity and, by sequencing the 5.8S rRNA gene and the two internal transcribed spacers (Las Heras-Vazquez *et al.* 2003), it was genetically identified as *Agrobacterium* sp. According to standard assays based on physiological and biochemical characteristics (Holt 1984), the BQL9 strain was assigned to the species *Agobacterium tumefaciens*.

Analysis of the D-hydantoinase gene sequence from A. tumefaciens *BQL9*

Identification of the cloned fragment from *A. tumefaciens* BQL9 strain was carried out by comparing the sequence with that of X91070 from *Agrobacterium radiobacter* NRRL B11291. This comparison was carried out using the CLUSTALW 1.8 programme (Jeanmougin *et al.* 1998). The sequences showed 99.78% identity, with only three different nucleotides, two of which changed the amino acid sequence. These changes were: substitution of Ala^{105} by Valine and Pro^{140} by Alanine. The effects of these changes in the primary structure of both enzymes were analysed to predict the number and magnitude of the resulting changes in the secondary structure. For this study, Protein Sequence Analysis (PSA) server by BMERC was used (White *et al.* 1994, Stultz *et al.* 1997).

Taking into account the limitations of any mathematical prediction for macromolecular structures, both proteins seem to show a high probability for strand conformation. Also, seven regions clearly showed over 60% probability of forming α-helix structures. The primary structure changes found between the D-hydantoinase from BQL9 and that from NRRL B11291 affect two α-helixes. Ala^{105} substitution by Valine reduced the probability of forming the first α-helix from 83.3% to 61.6%. Furthermore, Pro^{140} substitution by Alanine drastically reduced the probability of a twist in this position, while increasing the probability of the second α-helix in BQL9 D-hydantoinase. The substitutions found do not appear in the highly conserved regions described for functionally related amidohydrolases by Kim & Kim (1998a). These changes in the amino acid sequence may not eliminate catalytic activity, but may modulate it.

Influence of the change in N*-terminal structure on enzymatic activity*

Two constructions were designed to obtain the enzyme (see Materials and methods), one with a native primary structure (pBSH1) and the other with an *N*-terminal peptidic tail (pBSH2) (Figure 1). These constructions were used to carry out an evaluation of the *N*-terminal end's influence on D-hydantoinase activity from *A. tumefaciens* BQL9. The signals which allow D-hydantoinase production with pBSH1 were recognised, and IPTG induction gave rise to a high activity of protein production. However, the majority of this protein precipitated, forming insoluble inclusion bodies. Furthermore, the stop signal in the insert prevents the formation of a fusion protein with the amino-terminal fragment of β-galactosidase. D-Hydantoinase expressed in *E. coli* BL21 cells transformed with pBSH1 vector showed twice as much activity as the wild strain. On the other hand, proteins obtained from pBSH2 vector showed no activity under any of the conditions assayed. The absence of the peptidic tail in the protein obtained from pBSH1 allows these molecules to have native conformation and, therefore, to show activity. These results agree with the hypothesis elaborated by Kim & Kim (1998a), according to which the *N*-terminal end from *Bacillus* hydantoinase is involved in catalytic activity.

Metal-dependence studies

The necessary presence of metallic ions for hydantoinase activity is widely reported (Morin *et al.* 1986, Runser & Meyer 1993, Syldatk *et al.* 1992). However, the biochemical characterization of various enzymes revealed that the type, amount or function of the ions has not been defined (Pietzsch & Syldatk 2002). Durham & Weber (1995) found *Agrobacterium* 47C D-hydantoinase to be independent of metallic ions for its activity. On the contrary, the BQL9 enzyme, although from *Agrobacterium* genus and showing high substrate specificity homology, was highly dependent

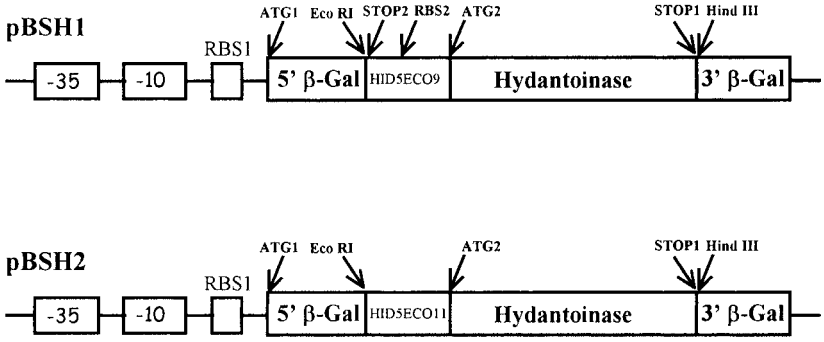

Fig. 1. Structure of each construction showing the sequences of their regions involved in the expression of the native D-hydantoinase (pBSH1), or bound to the *N*-terminal end of the β-galactosidase (pBSH2). These constructions were carried out in pBSK vector and overexpression was in *E. coli* BL21 (see text).

on Mn^{2+} (Figure 2A), and moderately dependent on Ni^{2+} (Table 1). The union of Mn^{2+} to the protein proved to be essential for activating the enzyme and was independent of the temperature increase from 20 °C to 50 °C during preincubation (Figure 2B). The metalloenzyme D-hydantoinase of *Arthrobacter aurescens* has been characterised in detail, and it contains 10 mol Zn^{2+} per mol of enzyme (May *et al.* 1998c). Treatment of this protein with EDTA chelating agent caused enzyme inactivation (May *et al.* 1998b). However, *A. tumefaciens* BQL9 D-hydantoinase was only inactivated in the presence of 70 mM EDTA and at over 40 °C (Figure 2C). The behaviour of D-hydantoinase from *Arthrobacter crystallopoites* in the presence of EDTA could be related to the results obtained in this work (Siemman *et al.* 1999) since it also remains unaffected by this chelating agent in the conditions assayed. This may indicate that both hydantoinases are strongly bound to their metallic ions. This would be the first *Agrobacterium* hydantoinase that was resistant to inactivation with EDTA. Other hydantoinases from *Agrobacterium* or *Pseudomonas* are easily inactivated by EDTA, even under milder conditions than those used in the present work (Buchanan *et al.* 2001). The greater affinity of apoprotein for the atoms of the metallic cofactor may be a consequence of the effect unleashed by the aforementioned mutations in the enzyme structure (Ala^{105} to Valine and Pro^{140} to Alanine).

The use of metallic cations by proteins is determined by the primary structure of the apoenzyme. Thus, enzymes from the hydrolase group keep the motive DXHXHXD, and it has been proved that both hystidines of this sequence have metallic ions such as Mg^{2+} or Zn^{2+} as ligands (May *et al.* 1998a, Kim *et al.* 2000). *A. tumefaciens* BQL9 D-hydantoinase shows the motive VHTHV (amino acids from 56 to 60), which may be the site of union to cations.

Table 1. Effect of some metallic cations on D-hydantoinase activity during protein folding. Cells transformed with plasmid pBSH1 were induced with 0.2 mM IPTG and incubated with eachcation for 6 h at 37 °C. They were then concentrated and broken as described in Materials and methods. The enzymatic reaction was carried out with 100 μl of cellular extract, adding 100 μl of D,L-*p*-hydroxyphenylhydantoin (1.5 mg ml^{-1}) at pH 7.5 and 37 °C. The reaction was stopped after 30 min by adding 400 μl of 1 M HCl. Results are expressed as μmoles of the carbamoyl-D,L-*p*-hydroxyphenylglycine (c-PHPG) per g dry weight (DW) per min, and are means of five experiments.

Sample	μmol c-PHFG/g DW · min
No ion	2.31 ± 0.07
Ca^{2+}	1.68 ± 0.28
Mg^{2+}	1.89 ± 0.07
Hg^{2+}	0
Fe^{2+}	0.63 ± 0.03
Fe^{3+}	0.98 ± 0.07
Pb^{2+}	1.33 ± 0.02
Cu^{2+}	1.54 ± 0.14
Mn^{2+}	8.47 ± 0.14
Zn^{2+}	0.35 ± 0.01
Co^{2+}	1.54 ± 0.14
Ni^{2+}	5.25 ± 0.21

Optimal pH and temperature for biocatalytic activity

The *A. tumefaciens* BQL9 D-hydantoinase expressed in *E. coli* showed its maximum activity using dihydrouracil as a substrate in the pH range from 8.71 to 9.72 (Figure 3A). Recombinant D-hydantoinase showed a linear decrease in activity as the temperature increased (Figure 3B). Similar studies have been car-

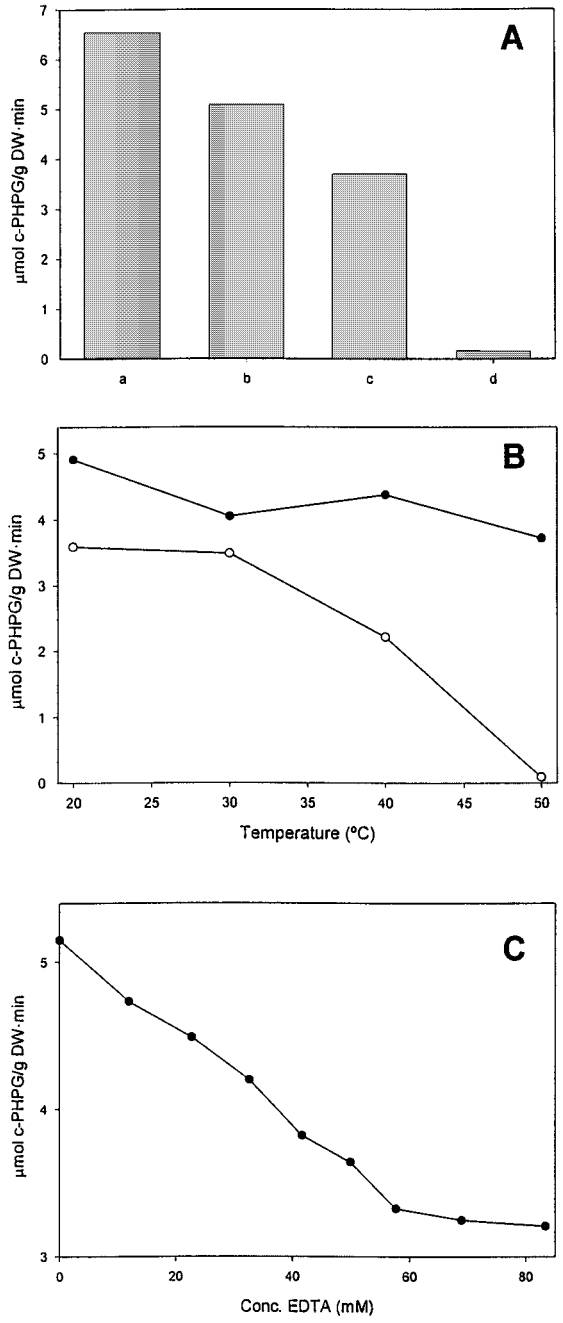

Fig. 2. $MnCl_2$ effect on D-hydantoinase activity. Results are expressed as μmoles of the carbamoyl-D,L-*p*-hydroxyphenylglycine (c-PHPG) per g of dry weight and min (g DW · min), and are means of five experiments. (A) Effect of $MnCl_2$ addition at different stages of expression of the D-hydantoinase gene: a) during induction; b) at the end of induction; c) induction without ion; d) cells grown without induction or ion. (B) Effect of temperature increase on D-hydantoinase activity in the presence (o) or absence (•) of 70 mM EDTA. Samples were preincubated for 5 min at different temperatures, but reactions were carried out at 20 °C, with cellular extracts produced in the presence of 2 mM $MnCl_2$. (C) Effect of EDTA concentration on D-hydantoinase activity. Reactions were carried out at 20 °C on cellular extracts produced in the presence of 2 mM $MnCl_2$.

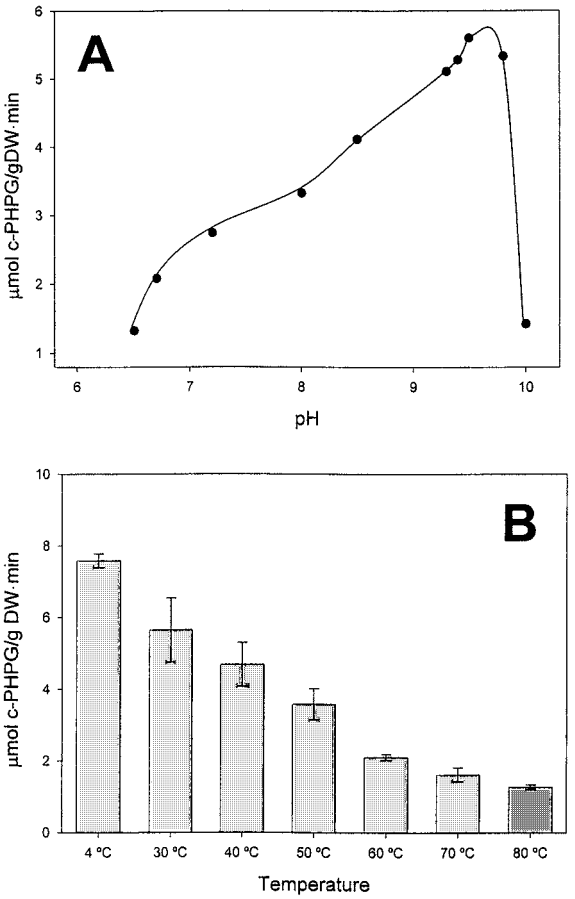

Fig. 3. Characteristics of D-hydantoinase of *A. tumefaciens* BQL9. (A) pH intervals as measured in the reaction medium. (B) Stability with temperature. Samples were preincubated for 30 min at each temperature and the activity was then measured at 30 °C. The carbamoyl-D,L-*p*-hydroxyphenylglycine (c-PHPG) was quantified by HPLC and results are the mean of five experiments.

ried out on strains NRRL B11291 and IP-671 (Olivieri *et al.* 1979, Kim & Kim 1995).

Optimal conditions for measuring the enzyme's K_m and V_{max} were at pH 9 and at 30 °C. D,L-*p*-Hydroxyphenylhydantoin was used as substrate as it is of industrial importance. Under these conditions the K_m value was 74 μM with a V_{max} value of 1.1 μM min^{-1}. This result agrees with those obtained by other researchers for different hydantoinases (Olivieri *et al.* 1981, Durham & Weber 1995, Meyer & Runser 1993).

Substrate specificity and characteristics

Knowledge of the substrate specificity of hydantoinase enzymes is fundamental for their industrial application since it defines the types of products which can be obtained. *A. tumefaciens* BQL9 D-hydantoinase was

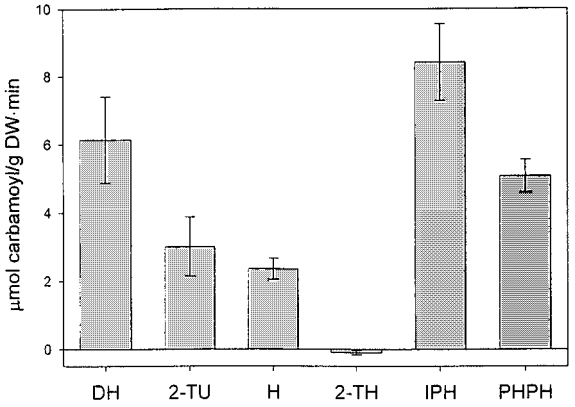

Fig. 4. Substrate specificity of D-hydantoinase of *A. tumefaciens* BQL9. The reactions were carried out for 30 min at pH 9.4 and 30 °C. DH, dihydrouracil; 2-TU, 2-thiouracil; H, hydantoin; 2-TH, 2-thiohydantoin; IPH, D,L-isopropylhydantoin; PHPH, D,L-*p*-hydroxyphenylhydantoin. The carbamoyl derived was quantified by HPLC and results are the mean of five experiments.

able to open both the hydantoin and the pyrimidine rings (Figure 4). Therefore, the enzyme's catalytic efficiency did not depend on the ring-type of the substrate. The presence of an aliphatic or aromatic group in C5 of the hydantoin-ring enhanced enzymatic activity. This may imply the presence of an apolar cavity in the tertiary structure of D-hydantoinase, where hydrophobic groups would fit perfectly.

BQL9 D-hydantoinase must be considered as a dihydropyrimidinase, but it shows important differences to other enzymes with similar activity. *Agrobacterium* sp. IP-671 has moderate activity over dihydrouracil, but high activity over hydantoin with large apolar groups at the C5 position (Runser & Meyer 1993). Furthermore, *Agrobacterium tumefaciens* 47C shows high levels of activity over dihydrouracil and low levels over 5-substituted hydantoins (Durham & Weber 1995). In comparison, BQL9 D-hydantoinase shows high levels of activity with both types of substrates, and only 2-thiohydantoin was not recognised as a substrate. This proves that this D-hydantoinase has a wider substrate specificity than those previously described.

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