Biochemical characterization of a novel hydantoin racemase from *Agrobacterium tumefaciens* C58

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Abstract

A novel hydantoin racemase gene of *Agrobacterium tumefaciens* C58 (*AthyuA*2) has been cloned and expressed in *Escherichia coli* BL21. The recombinant protein was purified in a one-step procedure and showed an apparent molecular mass of 27,000 Da in SDS-gel electrophoresis. Size exclusion chromatography analysis determined a molecular mass of approximately 100,000 Da, suggesting that the native enzyme is a tetramer. The optimum pH and temperature for hydantoin racemase activity were 7.5 and 55 ºC, respectively, with L-5-ethylhydantoin as substrate. Enzyme activity was strongly inhibited by Cu^{2+} and Hg^{2+}. No effect on enzyme activity was detected with any other divalent cations, EDTA or DTT, suggesting that it is not a metalloenzyme. Kinetic studies showed the preference of the enzyme for hydantoins with short rather than long aliphatic side chains or hydantoins with aromatic rings.

1. Introduction

Hydantoin racemase is to be the key enzyme for the production of optically pure D- and L-amino acids, valuable intermediates for the synthesis of antibiotics, sweeteners, pesticides, pharmaceuticals and biologically active peptides [1,2]. In this cascade of reactions the chemically synthesized D,L-5-monosubstituted hydantoin ring is hydrolyzed by a stereoselective hydantoinase enzyme. The resulting enantiospecific N-carbamoyl α-amino acid is transformed into the corresponding free D- or L-amino acid catalyzed by highly enantiospecific *N*-carbamoyl α-amino acid aminohydrolase (*N*-carbamoylase). However, total conversion and 100 % optically pure D- or L-amino acid are only obtained when a hydantoin racemase racemizes the remaining non-hydrolyzed 5-monosubstituted hydantoin [3].

Hydantoin racemase enzyme allows the racemization of the 5-monosubstituted hydantoins under physiological conditions where chemical racemization is not favored. Chemical racemization of the 5-monosubstituted hydantoins proceeds via keto-enol-tautomerism under alkaline conditions [4]. The velocity of racemization is highly dependent on the bulkiness and electronic factors of the substituent in 5-position [5] and is usually a very slow process [6]. High velocities of chemical racemization have only been observed for D,L-phenyl and D,L-5-p-hydroxy-phenylhydantoin because of the resonance stabilization by the 5-substituent. The racemization of all other hydantoins is a very long process [7]. Increased racemization rates are obtained at alkaline pH values and higher temperatures [8].

Since several microorganisms have produced total conversion of optically pure amino acids from racemic mixtures of hydantoins [9-11], hydantoin racemase enzymes from different sources involved in the production of optically pure D- and L-amino acids have been purified and biochemically characterized [12-14]. Likewise, genetic

organization and genomic localization of the genes involved in the production of these amino acids have been reported with a hydantoinase, a carbamoylase and a hydantoin racemase gene, together with a putative hydantoin transport protein [15-17]. Here we describe the cloning, purification and biochemical characterization of a second hydantoin racemase enzyme encoded by a gene located on the linear chromosome of *Agrobacterium tumefaciens* C58. This is the first description of a second hydantoin racemase enzyme in a microorganism capable of producing optically pure D- and L-amino acids from racemic mixtures of hydantoins. The biochemical characteristics of this enzyme have been compared with those of hydantoin racemases present in *Arthrobacter aurescens* and *Pseudomonas* sp. as well as with those of the first hydantoin racemase detected in *Agrobacterium tumefaciens*.

2. Materials and methods

2.1. Microbes and reagents

Agrobacterium tumefaciens ATCC 33970 (named C58), from which the first hydantoin racemase gene was isolated [14], was used as the source for the second hydantoin racemase gene on the basis of *in silico* analysis. The 5-monosubstituted hydantoins used in this work, D- and L-5-benzylhydantoin (D- and L-BH), D- and L-5-ethylhydantoin (D- and L-EH), D- and L-5-methylthioethyl-hydantoin (D- and L-MTEH) and D- and L-5-isobutylhydantoin (D- and L-IBH) were synthesized according to the literature [18].

2.2. Cloning and sequence analysis of hydantoin racemase

The second gene of *A. tumefaciens* hydantoin racemase (*AthyuA*2) was amplified by PCR. The primers used were designed based on GenBank sequences accession number NC003063 [19]. These were Rac5 (5´-AATCTAGAGTGACAGGAAAGC-

TATTATGCGCATCCTCGTC-3′) and Rac3 (5′-AACTGCAGTTAATGATGATGAT-GATGATGTCTTCCTTCGATCGCCGTCATGCGGTCCGG-3), the latter including the factor Xa recognition sequence (Ile-Glu-Gly-Arg) and a polyhistidine tag (His_6 tag) before the stop codon. The 744 bp *Xba*I/*Pst*I fragment harbouring the hydantoin racemase gene was purified from agarose gel using QIAquick (Qiagen) and then ligated into pBSK, which was restricted with *Xba*I and *Pst*I to yield plasmid pSER22.

2.3. Expression of hydantoin racemase and enzyme assay

BL21 strain harboring plasmid pSER22 was grown in LB medium supplemented with 100 μg mL^{-1} of ampicillin. To induce expression of the hydantoin racemase gene, isopropyl-β-D-thiogalactosidase (IPTG) was added to a final concentration of 0.2 mM and the growth was continued at 37 °C for 4 hours. After centrifugation the collected cells were disrupted in ice by sonication, the pellet was removed by centrifugation and discarded. The supernatant was applied to a column with TALON™ metal affinity resin (CLONTECH Laboratories, Inc.), washed four times and eluted with an elution buffer (50 mM Imidazole, 100 mM NaCl, 0.02% NaN_3, 2 mM Tris, pH 8). The purified hydantoin racemase enzyme was dialysed against 0.1 M potassium phosphate buffer pH 7.5 and stored at 4 ºC before further analysis.

The enzyme reaction was carried out with the purified *AthyuA*2 together with 100 mM of optically pure 5-monosubstituted hydantoin dissolved in 100 mM phosphate buffer (pH 7.5) in a final reaction volume of 200 μL. The mixture was incubated at 40 ºC for 15 minutes and the reaction was stopped by adding 400 μL of 1M HCl. After centrifugation, the supernatant was analysed by high performance liquid chromatography (HPLC) as previously described [14]. The specific activity of the

enzyme was defined as the amount of enzyme that catalysed the formation of 1 mM of D- or L-5-monosubstituted hydantoin at 40 ºC per minute and per milligram of protein.

2.4. Molecular mass and protein characterization

Size exclusion chromatography-HPLC (SEC-HPLC) analysis was performed to calculate the molecular mass of the native enzyme. The HPLC System (Breeze HPLC System, Waters, Barcelona) was equipped with a Superdex 200 HR 10/30 column (Amersham Biosciences, Barcelona) and equilibrated and eluted with 0.1 M potassium phosphate pH 7.5, at a flow rate of 0.5 ml/min. The molecular mass of the monomeric form was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a low molecular weight marker kit (Amersham Biosciences, Barcelona).

The thermal stability of the hydantoin racemase enzyme was measured after preincubation of the enzyme 15 and 30 min at temperatures ranging from 30 to 80 ºC in 0.1 M potassium phosphate pH 7.5. The enzyme assay was then made at 40 ºC for 15 min with L-5-ethylhydantoin (L-EH) as substrate. To analyse the effect on the enzyme of dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), $CaCl_2$, $MgCl_2$, $HgCl_2$, $FeCl_2$, $ZnCl_2$, $NiCl_2$, $MnCl_2$, $CoCl_2$ and $CuCl_2$, 2 mM of each metal salt and 0.1 M of DTT and EDTA were incubated with the hydantoin racemase (8 μM) in 0.1 M potassium phosphate pH 7.5 (final volume 200 μl) at room temperature for 45 min. The effect of metals on activity was determined by the standard enzyme assay described above.

2.5. Nucleotide sequence accession number

The nucleotide sequence of a second hydantoin racemase gene of *Agrobacterium tumefaciens* C58 (*Athyu*A2) has been deposited in the GenBank database under accession number AY436504.

3. Results and discussion

3.1. Sequence analysis of the second hydantoin racemase from Agrobacterium tumefaciens

Agrobacterium tumefaciens C58 presented a DNA fragment carrying a second hydantoin racemase gene (*Athyu*A2). Compared to the GenBank database, AtHyuA2 was found to be homologous to other hydantoin racemase amino acid sequences originating from different sources. The highest amino acid sequence identity (50.42%) was found when it was compared with that of the first hydantoin racemase from *A. tumefaciens* C58 (AtHyuA1) [3]. When compared with *Agrobacterium* sp. IP I-67 (AspHyuA) [15], *Pseudomonas* sp. NS671 (PspHyuE) [16] and from *Arthrobacter aurescens* DM3747 (AaHyuA) [13] hydantoin racemase the identity percentage dropped to 46.96%, 46.37% and 42.80%, respectively. Alignment of the amino acid sequences revealed highly conserved regions, especially in the N-terminal ends. Moreover, two cysteine residues at positions 76 and 180, which are probably involved in the catalytic centre of the protein [13], were highly conserved within the studied hydantoin racemases.

3.2. Functional expression and purification of AtHyuA2 enzyme

The hydantoin racemase gene was expressed in *E. coli* BL21. AtHyuA2 activity was determined in crude extracts by HPLC using L-5-ethylhydantoin (L-EH) as substrate (see Materials and methods). A one-step purification procedure of the

recombinant hydantoin racemase fused to His_6 tag was employed using immobilized cobalt affinity chromatography followed by proteolytic digestion with factor Xa (see Materials and methods). SDS-PAGE analysis indicated that the purified enzyme was over 95% pure after elution of the affinity column. Specific activity was calculated for the purified enzyme including His_6 tag, and also after removing it by Xa dependent cleavage, showing no differences in enzymatic activity or biochemical characteristics (results not shown). The purified enzyme could be stored in 0.1 M potassium phosphate pH 7.5 at 4 ºC for 10 weeks and in the same buffer with 20% glycerol at –20 ºC over 3 months without noticeable loss of activity. It was also active after 10 freeze-thawing cycles (maintaining about 95% of its initial activity).

3.3. Molecular mass and subunit structure

Purified AtHyuA2 enzyme exhibited an apparent molecular mass of about 27,000 Da by SDS-PAGE, similar to that deduced from the amino acid sequence (26,106 Da). In contrast, the three previously described hydantoin racemases presented higher apparent molecular mass (31,000 to 32,000 Da) than the one deduced from the amino acid sequence (25,000 to 27,000 Da) [12-14]. These differences in apparent molecular mass may be due to changes in the conformational properties of AtHyuA2. The relative molecular mass of the native enzyme was estimated at 100,000 Da by SEC-HPLC on a Superdex 200 HR column. The combined results suggest that purified AtHyuA2 enzyme might have a homotetrameric structure. These results are in accordance with those reported for the HyuA1 enzyme of *A. tumefaciens* C58 [14]. However, they differ from those described for hydantoin racemases from *Pseudomonas* sp. NS671 and *A. aurescens* DSM 3747. The molecular weights of these enzymes were 190,000 and 175,000 Da, respectively, and they present a hexameric, heptameric or octameric structure [12,13].

3.4. Influence of pH and temperature on AtHyuA2 activity

The pH activity profile of purified AtHyuA2 was determined at values between 6.0 and 11.0 in 100 mM phosphate, Tris or glycine/NaOH buffer. Maximum enzymatic activity occurred at pH 7.5. At this pH it was determined that the optimum temperature for racemization of L-EH was 55-60 ºC. Thermal stability studies showed a gradual loss of activity at preincubation temperatures over 55 ºC. These parameters are the same as those obtained for HyuA1 of *A. tumefaciens* C58, showing higher thermal stability and optimum reaction temperature than *Pseudomonas sp.* NS671 and *A. aurescens* DSM 3747 hydantoin racemases [12-13]. However, the optimal pH was lower for both hydantoin racemases from *A. tumefaciens* C58 (7.5) than for *Pseudomonas sp.* NS671 and *A. aurescens* DSM 3747 hydantoin racemases (9.5 and 8.5, respectively). This suggests that hydantoin racemases may compensate higher optimum reaction temperatures with lower alkaline pHs or vice versa to avoid chemical racemization.

3.5. Influence of metals and chemical agents on AtHyuA2 activity

Activity of the purified hydantoin racemase enzyme was assayed in the presence of 2 mM of different metal ions and 0.1 M of DTT and EDTA. Most of the metal ions studied and the metal-chelating-agent EDTA had no significant effect on the AtHyuA2 enzyme, indicating that it is not a metalloenzyme. The inhibitory effect of Cu^{2+} and Hg^{2+} on the hydantoin racemase activity has also been reported in previous works [12-14].

3.6. Kinetic analysis and substrate enantioselectivity of AtHyuA2

Kinetic parameters for the four D- and L- isomers of 5-monosubstituted hydantoins (BH, EH, IBH, MTEH) were obtained from hyperbolic saturation curves by least-squares fit of the data to the Michaelis-Menten equation (Table 1). Reactions were carried out with different concentrations of D- or L-5-monosubstituted hydantoins at 40 ºC with a constant enzyme concentration of 3 μg/mL. AtHyuA2 showed high K_m values for both D,L-EH and D,L-BH, but k_{cat} values were 7 to 10 times higher for the former. Consequently the k_{cat}/K_m value was 10-fold higher for D,L-EH than for D,L-BH, indicating a higher affinity for hydantoins with aliphatic substituents than with aromatic ones at the 5-position. This affinity for aliphatic substrates was also observed for the first *A. tumefaciens* C58 hydantoin racemase (AtHyuA1), but the values of the apparent kinetic parameters K_m and k_{cat} were more favorable, with lower K_m values and higher k_{cat}, than for AtHyuA2. These results were corroborated by examining the ability of the purified AtHyuA2 to racemize the same D- and L-isomers of 5-monosubstituted hydantoins (Fig. 1). A high rate of hydantoin racemase activity was detected for the three aliphatic D- and L-hydantoins, EH, IBH and MTEH (Fig. 1A, 1B and 1C) compared to D- and L-BH (Fig. 1D), which racemized slowly. However, racemization of these D- and L-hydantoins took longer with AtHyuA2 than with the previously studied AtHyuA1.

The second *A. tumefaciens* C58 hydantoin racemase (AtHyuA2) has been recombinantly expressed in *E. coli*, purified and biochemically studied. This is the first observation of the presence of two hydantoin racemases in the same microorganism. AtHyuA2 has shown kinetic propierties very similar to AtHyuA1. However, after a hypothetical application of both hydantoin racemase enzymes in a multienzymatic system for production of optically pure D-amino acids, AtHyuA1 would be more viable

for industrial application than AtHyuA2, due to its higher substrate affinity and racemization velocity.

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Table 1

Kinetic parameters of AtHyuA2 for different 5-monosubstituted hydantoins. The parameters were determined at 40 ºC for 15 min at pH 7.5. The kcat was defined as the mmol of D- or L-5-monosubstituted hydantoin racemized per s and mmol of enzyme at 40 ºC.

Substrate [a]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
L-EH	19.42 ± 2.34	1.81 ± 0.01	0.09 ± 0.01
D-EH	12.54 ± 1.81	1.80 ± 0.10	0.14 ± 0.03
L-IBH	3.02 ± 0.78	0.48 ± 0.02	0.16 ± 0.05
D-IBH	6.79 ± 0.60	0.83 ± 0.03	0.12 ± 0.01
L-MTEH	10.90 ± 1.48	0.78 ± 0.07	0.07 ± 0.01
D-MTEH	6.31 ± 0.31	0.50 ± 0.01	0.08 ± 0.01
L-BH	18.42 ± 4.80	0.18 ± 0.02	0.01 ± 0.00
D-BH	20.77 ± 5.47	0.46 ± 0.07	0.02 ± 0.00

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[a] D- and L-EH: D- and L-5-ethyl-hydantoin; D- and L-IBH: D- and L-5-isobutyl-hydantoin; D- and L-MTEH: D- and L-5-methylthioethyl-hydantoin; D- and L-BH: D- and L-5-benzyl-hydantoin.

Fig. 1. Enzymatic racemization of different 5-monosubstituted hydantoins by AtHyuA2. The hydantoin racemase activity of the D-isomer (●) and L-isomer (○) was measured at 40 ºC and pH 7.5 by chiral-HPLC at the points shown in the graphs. Chemical racemization of the D-isomer (▼) and L-isomer (∇) of each substrate was also measured at the same intervals. (A) 5-ethyl-hydantoin; (B) 5-isobutyl-hydantoin; (C) 5-methylthioethyl-hydantoin; (D) 5-benzyl-hydantoin.

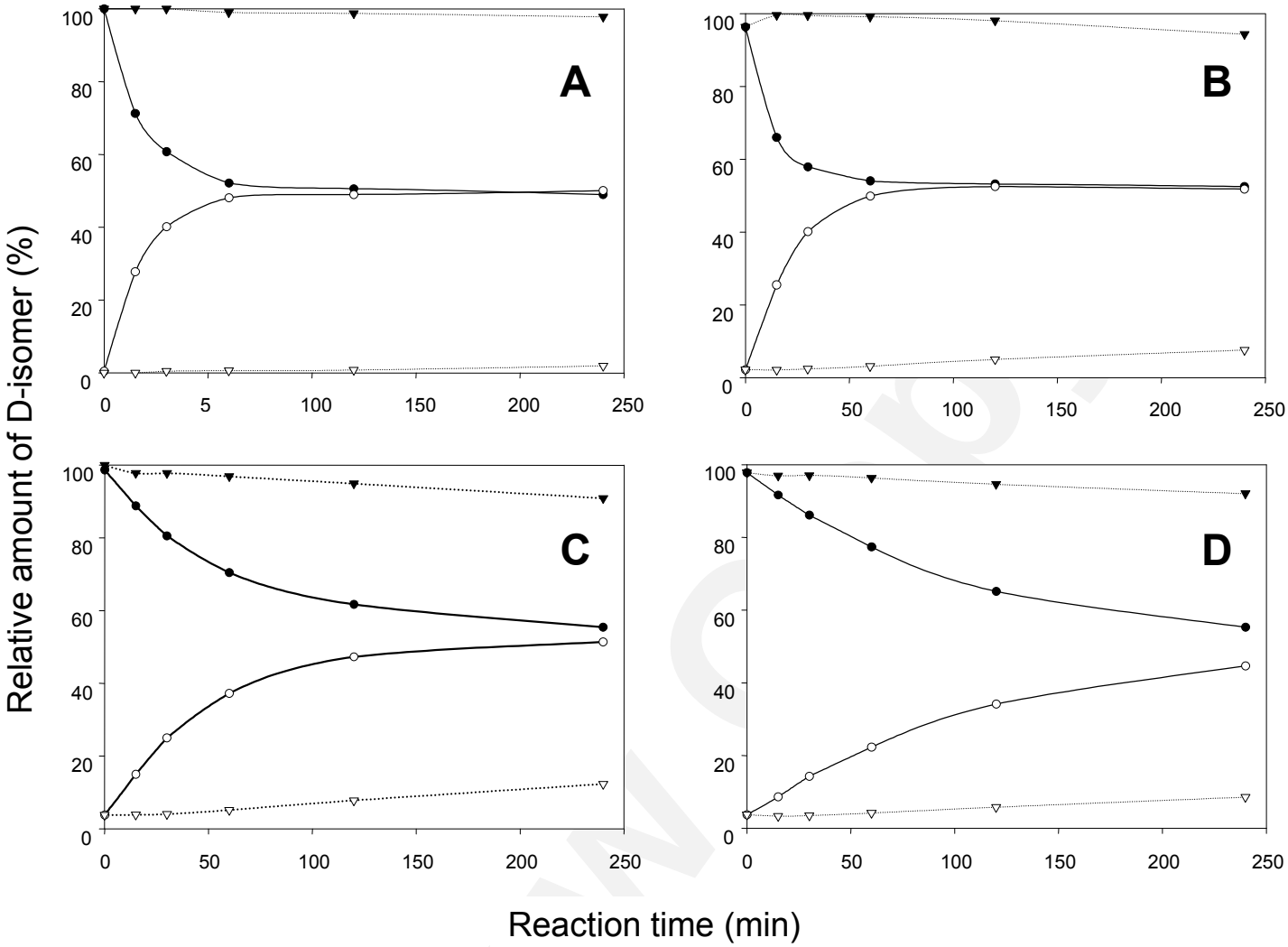
A
B
C
D
Relative amount of D-isomer (%)
Reaction time (min)
0
20
40
60
80
100
0
5
50
100
150
200
250

Figure 1