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A D-specific hydantoin amidohydrolase: properties of the metalloenzyme purified from *Arthrobacter crystallopoietes* [1]

Martin Siemann, Ádrian Alvarado-Marín, Markus Pietzsch, Christoph Syldatk *

Institut für Bioverfahrenstechnik, Universität Stuttgart, Allmandring 31, D-70569 Stuttgart, Germany

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Abstract

A D-specific hydantoinase has been purified to homogeneity from *Arthrobacter crystallopoietes* DSM 20117 with a yield of 5% related to the crude extract. The active enzyme is a tetramer of 257 kDa consisting of four identical subunits, each with a molecular mass of 60 kDa. Incubation of the enzyme with the metal-chelating agent EDTA had no inhibitory effect, while 8-hydroxyquinoline-5-sulfonic acid resulted in a complete and irreversible inactivation. The purified enzyme contains zinc as cofactor, which could be detected by subjection to direct analysis using inductive/coupled plasma-atomic emission spectrometry. The hydantoinase has a wide substrate specificity for the D-selective cleavage of 5-monosubstituted hydantoin derivatives with aliphatic and aromatic side chains. The V_{max}-value for phenylhydantoin is 217 U/mg, the K_m-value is 8 mM. Dihydrouracil was found to be a natural substrate (V_{max} = 35 U/mg). The N-terminal amino acid sequence of the enzyme shows distinct homologies to other metal-dependent cyclic amidases involved in the nucleotide metabolism especially to dihydropyrimidinases as well as to ureases, L- and unselective hydantoinases. Due to these findings, this enzyme has to be considered as a possible link in the evolution to related L-selective and unselective hydantoinases from the genus of *Arthrobacter*.

Keywords: Hydantoinase; Dihydropyrimidinase; Urease; Cyclic amidase; Protein purification; Hydantoin; Amino acid

1. Introduction

Hydantoinases are valuable enzymes for the production of optically pure D- and L-amino acids. They catalyse the reversible hydrolytic ring cleavage of hydantoin or 5-monosubstituted hydantoins and therefore are classified in the EC-nomenclature as cyclic amidases (EC 3.5.2 group) [1]. Hydantoinases have been classified into D-, L-, unselective or ATP-requiring enzymes due to their substrate specificity, stereoselectivity and cofactor dependency. From recent findings based on protein sequence data all hydantoin cleaving enzymes, with the exception of the ATP-dependent *N*-methylhydantoinases, belong to a protein superfamily of 'amidohydrolases related to urease' [2] and seemed to have evolved from a common ancestor in a divergent evolution [3].

An important and well examined member of this EC 3.5.2 group of cyclic amidases is the dihydropyrimidinase (EC 3.5.2.2). It is known since the early 1950s that this widely dis-

* Corresponding author. Tel.: +49-711-685-5157; Fax: +49-711-685-5164; E-mail: syldatk@ivbt.uni-stuttgart.de

[1] Dedicated to Professor Hideaki Yamada in honor of his 70th birthday.

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tributed, inducible catabolic enzyme plays an important role in the pyrimidine metabolism [4–10]. Its natural substrates are 5,6-dihydrouracil and 5,6-dihydrothymine. The dihydropyrimidinase is described to have a wide substrate specificity and to be strictly D-specific for the cleavage of 5-monosubstituted hydantoins [11]. The group of Yamada et al. in Japan was the first who studied intensively the D-selective cleavage of 5-monosubstituted hydantoins in microorganisms [12]. They postulated the identity of microbial D-hydantoinases with dihydropyrimidinases and proved this hypothesis for the enzyme from *Pseudomonas striata* [13]. In the meantime, several publications describe various similar D-selective microbial hydantoinases from microorganisms as *Pseudomonas fluorescens* DSM 84 [14], *Pseudomonas* sp. AJ-11220 [15,16], *Agrobacterium* sp. IP-I 671 [17,18,29], several *Bacillus* spp. [19–21] and even from anaerobic microorganisms [22]. However, recently a hydantoinase from *Agrobacterium* was identified which exhibits no dihydropyrimididase activity [23].

In case of the genus *Arthrobacter*, only the occurrence of L-selective, unselective [24] and ATP-dependent *N*-methylhydantoinases [25] has been reported so far. In this paper we describe the isolation and characterization of a D-specific hydantoinase with dihydropyrimidinase activity from *Arthrobacter crystallopoietes* DSM 20117. It is possible that this D-specific enzyme is an important link among the evolution of L-selective and unselective hydantoinases and thus contributes an important tool for understanding the structural reasons for the different substrate specificities and stereoselectivities of these enzymes.

2. Experimental

2.1. Microorganism and cultivation

The Gram-positive, strictly aerobic bacterium *A. crystallopoietes* DSM 20117 was obtained from the Deutsche Stammsammlung für Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSMZ). A semi-synthetic medium was used for enzyme production (in g/l): D,L-sodium lactate, 20; citric acid, 0.75; yeast extract, 1; $FeSO_4 \cdot 7H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.5; $CaSO_4 \cdot 2H_2O$, 0.22; $MnSO_4 \cdot H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.005; $(NH_4)_2SO_4$, 6; L-methionine, 0.05; unsubstituted hydantoin, 1; K_2HPO_4, 3.8. The pH was adjusted to 7.0. For batch-cultivation in a 50-l scale, a first preculture of the same composition (100 ml) was inoculated with a loopful of cells and cultivated at 30°C for 45 h, while agitated at 100 rpm. A second preculture (500 ml) was inoculated with 10 ml of the first preculture and again cultivated under the same conditions for 42 h. A 100-l bioreactor (Bioengineering, Swiss) was used for batch cultivation (final volume was 50 l). Forty-five liters of the semi-synthetic medium was inoculated with 5 l of the second precultures. The agitation was 100–200 rpm, the aeration rate was 11–15 nl/min, and the pH was controlled at 7.0.

2.2. Analytical methods

High performance liquid chromatography (HPLC) and chiral HPLC analysis of hydantoins and *N*-carbamoyl-amino acids were performed as described elsewhere [24]. Protein concentration was determined according to the method of Bradford [31] using a test-kit purchased from Biorad, Munich, FRG.

2.3. Enzyme purification

Unless otherwise stated, all purification steps were performed at room temperature (20°C) using either an FPLC- or a Biopilot-system (Pharmacia, Freiburg, Germany). The elution profile of proteins was determined at a wavelength of 280 nm. Ultrafiltration modules (cutoff 10 kDa) were purchased from Pall Filtron Technology, Northborough, USA. After expanded bed chromatography and hydrophobic interac-

tion chromatography (HIC), an Ultrasette-module was used at 4°C and an Alphacell-module after step 4.

Step 1: Cell disintegration: Cell disintegration of 1000 ml ice-cooled suspension of wet cells (30% w/v) in 0.1 M phosphate buffer was carried out as described [26] using a Dyno-Mill KDL (Willy A. Bachofen, Basel, Switzerland).

Step 2: Protamine sulfate treatment (PST): 10 ml of a solution of protamine sulfate (2.0 g in 10 ml phosphate buffer, pH 6.5) was added to 1000 ml crude extract. The suspension was stirred for 12 h at 4°C and centrifuged ($5380 \times g$, 45 min). The supernatant was separated and the pH was adjusted to pH 6.5 (920 g, PST supernatant).

Step 3: Expanded bed adsorption: 460 g of the PST supernatant were diluted with buffer A (50 mM phosphate buffer, pH 6.5) to a final volume of 2.0 l and applied to a Streamline 50 column, containing 600 ml of Streamline-DEAE in the expanded bed modus (50 ml/min, Biopilot-System), previously equilibrated with buffer A. After washing with buffer A, bound protein was eluted (100 ml/min) in the packed-bed modus through a linear gradient between buffers A and B (0–100% B, while B contained 1 M NaCl in buffer A, within 10 l). Fractions of 0.5 to 1.0 l were taken and analyzed for activity. Active fractions (2550 ml) eluted between 30 and 40% of buffer B and were pooled and concentrated by ultrafiltration to 1 l (streamline fraction).

Step 4: Ammonium sulfate fractionation (ASF): While permanently stirring, 530 ml of a solution of ammonium sulfate in buffer A (2.0 M) was added to the streamline fraction (4°C). The suspension was centrifuged after 12 h of incubation ($5300 \times g$, 45 min) and the obtained supernatant was filtered using an 0.45 μm membrane filter (ASF fraction).

Step 5: Hydrophobic interaction chromatography (HIC): 1055 ml of the ASF fraction were applied to a Super Flow 500 column (ICT, Bad Homburg), which contained 500 ml of High-Load Phenyl Sepharose HP (50 ml/min), previously equilibrated with buffer C (50 mM phosphate buffer, 1.0 M $(NH_4)_2SO_4$, pH 6.5). After washing the column with buffer C, bound protein was eluted (75 ml/min) through a linear gradient between buffers C and A (100–0% C within 5.0 l). Fractions of 0.5 l were taken and analyzed for activity. Active fractions (1500 ml), which eluted between 65 and 55% of buffer C, were pooled. Active fractions were concentrated by ultrafiltration to 200 ml, diluted with 2.0 l buffer A and concentrated again to 230 ml (HIC fraction).

Step 6: Ion exchange chromatography: 230 ml of the HIC-fraction were applied to a MonoQ 16/10 column (5.0 ml/min), previously equilibrated with buffer A. After washing with buffer A, bound protein was eluted through a linear gradient between buffers A and B (0–100% B, within 400 ml). Fractions of 3.5 to 5 ml were taken and analyzed for activity. Active fractions (13.5 ml) eluted at 40% of buffer B were pooled and ultrafiltrated to 7 ml (MonoQ fraction No. 1).

Step 7: Gel filtration: The MonoQ fraction No 1 was applied to a XK 26/100 column (0.25 ml/min) filled with Sephadex G 200, previously equilibrated with buffer D (50 mM phosphate buffer, 150 mM NaCl, pH 6.5). Fractions of 5 ml were taken and analyzed for activity. Active fractions (50 ml) eluted between 200 and 250 ml and were pooled and diluted with 100 ml of buffer A (gel filtration fraction).

Step 8: Ion exchange chromatography: Under the conditions described above (step 4), ion exchange chromatography was used to concentrate and fractionate the gel filtration fraction. One-hundred-forty-seven milliliters of this fraction were applied to a MonoQ 16/10 column. Active fractions (10 ml) eluted at 40% of buffer B were pooled and used for the characterization experiments (MonoQ fraction No. 2).

2.4. Size exclusion chromatography

Determination of the molecular mass of the enzyme by gel-filtration chromatography was

performed under non-denaturing conditions. Hydantoinase and standard proteins were individually chromatographed on Superose 12HR using buffer A. The molecular mass was calculated from standard elution profile with respect to the distribution coefficient K_{av}, which is defined as $(V_e - V_o)/(V_i - V_o)$ with V_e, V_i, V_o being the elution volume, the column volume (23.6 ml) and the void volume (7.3 ml), respectively. Flow rate was 0.25 ml/min. Protein standards were albumin (67 kDa; $K_{av} = 0.31$), aldolase (158 kDa; $K_{av} = 0.25$), catalase (232 kDa; $K_{av} = 0.26$) and ferritin (440 kDa; $K_{av} = 0.17$); hydantoinase ($K_{av} = 0.23$).

2.5. N-terminal amino acid sequencing

The N-terminal amino acid sequence of the hydantoinase was determined by automated Edman degradation using protein sequencer model 491 (Applied Biosystems, Weiterstadt, Germany), and standard programs supplied by the manufacturer. For sequencing, the non-homogeneous enzyme fraction (Gel filtration step 7) had been supplied to SDS-PAGE. For transferring the separated proteins onto a polyvinylidene difluoride (PVDF)-membrane (ICN, Eschwege, Germany), a standardized protein transfer technique has been applied using a Fast-blot system (Biometra, Germany), following the suppliers manual. After transfer, the membrane was stained for 0.3 h using a Coomassie Brilliant Blue R 250 solution (BioRad, München, Germany). Destaining was performed in a solution of 90 ml methanol/water (1:1 vol/vol) and 10 ml acetic acid (puris). The main band was cut out of the filter and supplied for N-terminal sequencing.

2.6. Determination of enzyme activity, substrate- and stereospecificity

One hundred microliters of enzyme solution were added to 800 μl of prewarmed substrate solution (5 g/l D,L-5-phenylhydantoin (D,L-PH) in 50 mM glycine/NaOH-buffer, pH 9.5) and incubated at 50°C. The reaction was stopped after 5 min by addition of 200 μl of 12% trichloroacetic acid (TCA). After centrifugation (Eppendorf centrifuge, 14.000 rpm) and dilution, the concentrations were measured by HPLC. For the determination of the substrate specificity, all compounds listed in Table 3 were dissolved in a concentration of 2.0 g/l in 50 mM phosphate buffer, pH 8.0. For the determination of the stereospecificity, a substrate solution consisting of 2.18 mM D,L-5-indolylmethylhydantoin (D,L-IMH) in 1.0 M Tris-buffer, pH 8.5 was used and the reaction was carried out at 37°C. The enzyme activity was expressed in Unit (U), while 1 U is defined as the conversion of 1 μmol of substrate per minute.

2.7. Determination of metal dependency

Inactivation using 8-hydroxychinoline-sulfonic acid (8-HQSA) and ethylenediaminetetraacetic acid (EDTA): stock solutions of 15 mM 8-HQSA and EDTA in 0.05 M phosphate buffer were prepared and the pH was adjusted to 7.0 using 2 M NaOH. One-hundred-fifty microliters (2 mM) to 750 μl (10 mM) of chelator stock solutions were added to 75 μl of the homogeneous enzyme solution (step 8, 0.25 mg/ml), prediluted with phosphate buffer to a final volume of 1 ml, and incubated at 26°C. Enzyme activity was measured immediately after addition (0 min) and subsequently after 1, 2, 3, 5, 7 and 19 h.

2.8. Metal analysis

Inductive/coupled plasma-atomic emission spectrometry (ICP-AES, Spectroflame Modula) was performed at the Technical University of Clausthal, Department for Geochemistry, Germany, as described previously [28]. For metal analysis, 250 μl of purified hydantoinase (0.2 g/l), dissolved into 50 mM phosphate buffer, pH 8.0, was diluted to 25 ml with metal-free water, containing 500 μl of 65% suprapure HNO_3.

3. Results

3.1. Cultivation

A. crystallopoietes DSM 20117 was batch-wise cultivated in a 50 l scale as described in the experimental section. During the exponential growth phase, a maximum growth rate μ_{max} of 0.15 h^{-1} was obtained. The culture was harvested after 27 h at a concentration of 2.8 g/l (dry weight), which corresponds to a total biomass amount of 585 g (wet weight). Probes, which were taken during the exponential growth phase (8 h) as well at the beginning of the stationary phase (24 h) were analyzed according to their enzyme activity. The specific hydantoinase activity during the exponential growth phase was slightly higher (17.9 U/g dry weight) than compared to the very beginning of the stationary phase (12.2 U/g dry weight) and at the time of harvest (12.9 U/g dry weight).

3.2. Purification, molecular mass and quaternary structure of the hydantoinase

The hydantoinase from *A. crystallopietes* DSM 20177 was purified according to the protocol described in Section 2. The respective results are summarized in Table 1. The overall activity yield of the hydantoinase was only 5%, and the final purification factor (in relation to the crude extract) was 20, as determined through the standard assay.

The MonoQ fraction was analyzed by SDS-PAGE, which revealed a main protein band of a molecular mass of 60 ± 5 kDa (Fig. 1A). After electro blotting (Fig. 1B) and subsequent N-terminal sequencing, this main band was identified as hydantoinase (see Section 3.3).

For calculating the molecular mass of the native enzyme, a size exclusion chromatography was performed using a Superose 12-column. The active hydantoinase eluted as a single and symmetric peak. In relation to the used reference proteins the molecular mass of the native enzyme was calculated to be 257 ± 30 kDa and thus suggested a homotetrameric structure of the enzyme. The isoelectric point (pI) of the enzyme was determined to be at 4.8 ± 0.2 (data not shown).

3.3. N-terminal amino acid sequence analysis and alignment

Using the purified protein it was possible to determine its N-terminal amino acid sequence up to first 30 amino acid residues without any interference (Table 2). Sequence alignment performed in databases of PIR, Swiss Prot and GenBank, which is illustrated in Table 2, revealed significant, but in all cases distant sequence homologies to all kinds of hydantoinases, like the L- (*Pseudomonas putida*) and D-specific (*Bacillus sp.*) or unselective (*Arthrobacter aurescens*) hydantoinases and to

Table 1
Summary of the purification of the hydantoinase from *A. crystallopoietes* DSM 20117

Purification step	Volume [ml]	Protein concentration [g/l]	Specific activity [U/mg]	Purification factor	Yield [%]
Crude extract	1010	17	5.2	0	100
Protamine sulfate precipitation	910	18	4.8	0.9	90
Streamline (DEAE)	2550	1.8	6.4	1.2	63
Ammonia sulfate precipitation	1055	2.9	12.3	2.4	84
HIC (radial flow)	1515	0.3	51.2	9.8	41
MonoQ	13.5	0.4	157.4	30.1	20.4
Gel filtration	47	0.1	126.5	24.2	9.7
MonoQ	10	0.2	100	20	5

Yields were calculated assuming that the total volume of the crude extract was purified according to the respective eight purification steps.

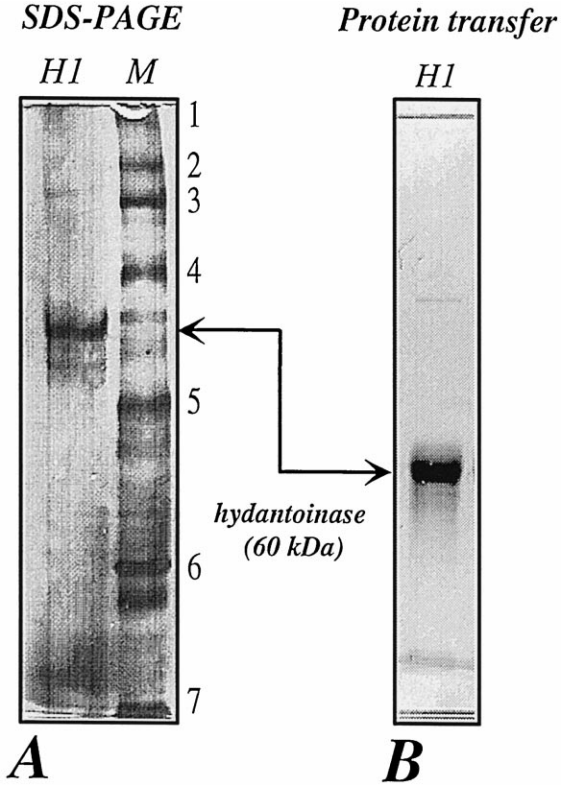


Fig. 1. (A) SDS gel electrophoresis of the purified D-hydantoinase from *A. crystallopoietes* DSM 20177. SDS-PAGE was carried according to the method of Laemmli [27]. Gels were stained with the silver staining method from BioRad (BioRad, München, Germany). Lane H1: hydantoinase fraction (MonoQ-fraction, see also Table 1). Lane M: marker proteins: (1) myosin (200 kDa), (2) ß-galactosidase (116 kDa), (3) phosphorylase 97 kDa, (4) bosine serum albumine (66 kDa), (5), ovalbumine (45 kDa), (6) carboanhydrase (31 kDa), (7) trypsin inhibitor (22 kDa). (B) Electro-blot of the respective hydantoinase fraction (H1) used for N-terminal sequencing.

dihydropyrimidinases [EC 3.5.2.2] derived from different organisms. Above that, either parts of the β- and α-subunit of ureases [EC. 3.5.1.5] from *Streptococcus* and *Heclicobacter*, were as well found to be homologous.

3.4. Biochemical characterization

3.4.1. Determination of pH-optimum and stability

Both parameters were determined by using D,L-5-phenylhydantoin as substrate. In case of the pH optimum, four different buffers were used (acetate, phosphate, Tris and carbonate-buffers), each of a molar concentration of 50 mM, in order to investigate a proper pH range of pH 4 to 10.5. Independent of the used buffer system, the hydantoinase occurred to have a pH-optimum at pH 8.0.

In order to establish proper storage conditions for the purified enzyme, its pH-stability, depending on either the pH, as well as the kind of used buffer system was investigated. It occurs that the pH-stability of the enzyme strongly depended on the used buffer system. In case of the phosphate buffer, the enzyme remained to be stable (half life time $t_{0.5}$ of 400 h), independent from the used pH-value. In contrast, when Tris-buffer was used, only a small change in the pH value from 7.5 to 7.0 drastically destabilized the enzyme. In this case the estimated half life time decreased to a minimum of 20 h (Tris-buffer, pH 7.5). Thus, the dissolved free enzyme can be stored under weakly alkaline conditions using phosphate buffer at 5°C for at least several weeks.

3.4.2. Determination of temperature optimum and stability

For optimizing biotransformation conditions, the influence of the temperature on reaction kinetics as well as on the enzyme stability was investigated. Using D,L-5-phenylhydantoin as substrate and realizing the above described optimal pH—and buffer system conditions (50 mM phosphate buffer, pH 8.0), the optimum temperature occurred to be at 50°C. Initial investigation revealed that freezing of the enzyme lead to its subsequent inactivation (data not shown). Thus, thermal stability of the enzyme was further analyzed within a temperature scale of 4°C to 70°C. When the enzyme was incubated at 50°C, only 50% of its initial activity remained after 6 h and its activity decreased within minutes by incubating at 60°C.

3.5. Substrate- and stereospecificity

Substrate specificity and stereoselectivity of the hydantoinase were investigated with respect

Table 2
N-Terminal sequence and alignment (Database: Non-redundant GenBank CDS translations + PDB + Swiss Prot + SPupdate + PIR; release date: January 9, 1998) of the *A. crystallopoietes* DSM 20117 hydantoinase

Enzyme; species	N-terminal sequence/alignment	Identity	Source
Hydantoinases			
A. crystallopoietes	1 MPAKLLVGGT IVSETGKVRA DVLIENGKVA 30	100%	this work
Bacillus thermoglucosidasius	1 MTKIIKNGT IVTATDTYEA DLLIKDGKIA 29	48%	Burtscher et al., 1996 [40]
Bacillus sp. LU 1220	TKIIKNGT IVTATDTYEA DLLIKDGKIA 29	48%	Jacob et al., 1990 [41]
Bacillus stearothermophilus	1 MTKLIKNGT IVTATDIYEA DLLIQDGKIA 29	48%	PIR: JC2310
Agrobacterium radiobacter	1 MDIIIKNGT IVTADGISPA DLGIKDGKIA 29	42%	GNL: PID: e197826
Arthrobacter aurescens	1 MFDVIVKNCR MVSSDGITEA DILVKDGKVA 30	36%	EMBL: P81006
Consensus:	m liknqt iVt g A D li #Gk!a		
Dihydropyrimidinases			
Rattus norvegicus	4 QERLLIRGGR VVNDDFSQVA DVLVEDGVVR 33	44%	PIR: S70581
Homo sapiens	4 PSRLLIRGGR VVNDDFSEVA DVLVEDGVVR 33	36%	GNL:PID:d1011851
Homo sapiens	15 DRLLIKGGR IINDDQSLYA DVYLEDGLIK 43	36%	GNL:PID:e313140
Consensus:	rlLi GGr!!n#d s A DVl E#G!		
Ureases			
Streptococcus salivarius (α subunit)	70 VDTIITGAT IIDYTGIIKA DIGIRDGKIV 98	40%	Swiss Prot P50047
Helicobacter heilmannii (β subunit)	66 LDLVLTNAL IVDYTGIYKA DIGIKDGKIA 93	40%	Swiss Prot P42823
Consensus:	d itgat I!dyTGi kA D!gI #Gkia		

The alignment was performed using the Program Manual for the Wisconsin Package, version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, WI, USA.
Consensus sequence: capital letters: 100% conserved. Small letters: conservation between 50 and 90%. !: I or V; #: N or D.

to the obtained homologies (see Table 2). The results are shown in Table 3.

Significant activity was found for dihydrouracil, which is described to be a natural substrate for dihydropyrimidinase, while no activity was detectable with dihydroorotic acid, the natural substrate of dihydroorotase.

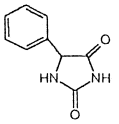
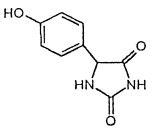
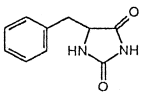
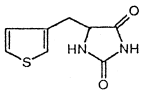
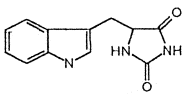
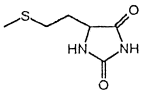
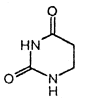
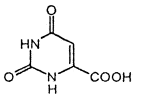
The K_m-value for D,L-5-phenylhydantoin, which commonly serves as a model substrate for D-hydantoinases in literature, was 7.9 mM, the V_{max}-value was 216 U/mg. A fivefold higher activity was detected for unsubstituted hydantoin. The enzyme from *A. crystallopoietes* DSM 20117 was shown to be strictly D-selective for the conversion of D,L-5-indolylmethylhydantoin (Fig. 2) and D,L-5-methylthioethylhydantoin (Table 3, V_{max} for D-MTEH was 101 U/mg).

3.6. Cofactor dependency

For investigation on metal dependency, the enzyme was incubated with either weak (EDTA) or strong (8-HQSA) metal-chelating agents. Only the treatment with 8-HQSA led to a time and concentration dependent loss of enzyme activity. Even high amounts of EDTA (10 mM) did not effect the enzyme activity at all. All attempts for restoration of enzyme activity failed for the hydantoinase from *A. crystallopoietes*.

According to a procedure previously reported for the hydantoinase from *A. aurescens* [28], the hydantoinase was subjected to metal analysis using ICP-AES to confirm the kind and quantity of the metal bound to this enzyme. Molar ratios of bound metal to the enzyme can be calculated according to the respective amount of zinc measured in the used phosphate buffer and assuming that the a molar mass of the hydantoinase is 247 kDa. The determined zinc concentration of the purified enzyme-fraction was 1.2 mg/l, while the used buffer contained 0.65 mg/l. Thus, the hydantoinase contained 8 mol zinc in average, which corresponds to 2 mol zinc per mol subunit, presuming that the

Table 3
Substrate specificity of the hydantoinase from *A. crystallopoietes* DSM 20117

Substrate	Formula	Substrate concentration (mM)	Relative activity (%)
D,L-5-Phenyl-hydantoin		14.1 11.3 8.5	100 99.7 83
D,L-5-p-Hydroxy-phenyl-hydantoin		10.4	44.3
D,L-5-Benzyl-hydantoin		10.6	35.6
D,L-5-Thienylmethyl-hydantoin		8.2	22.7
D,L-5-Indolylmethyl-hydantoin		2.1	1.7
D-5-Methylthioethyl-hydantoin		20.0	74.5
Hydantoin		249.8	512
Dihydrouracil		35.0	27.1
Dihydroorotic acid		12.1	0.0

The specific enzyme activity for D,L-5-phenylhydantoin was 216 U/mg.

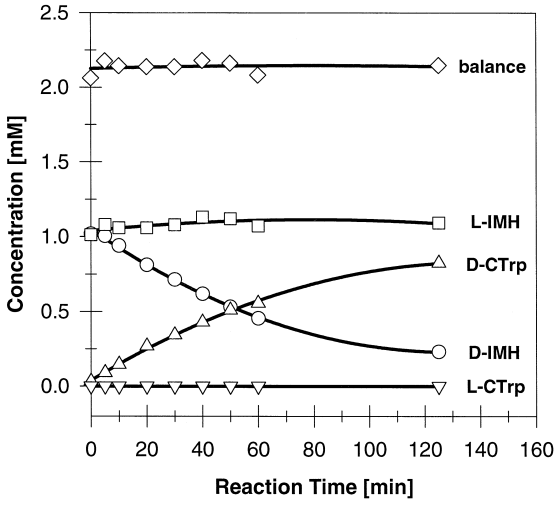


Fig. 2. Enantioselectivity of the hydantoinase from *A. crystallopoietes* DSM 20177 for either D- and L-indolylmethylhydantoin (IMH). CTrp: *N*-carbamoyltryptophan.

native hydantoinase consists of four identical subunits. Only traces of Mg^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} were determined, or were absent at all.

4. Discussion

Up to date, three types of hydantoinases from the genus of *Arthrobacter* have been described, which all differ with respect to substrate specificity and stereoselectivity: The ATP- and Mg^{2+}-dependent L-selective *N*-methylhydantoinase [33,34], several L- or non-selective enzymes [35,36], and including the one isolated from *A. aurescens* [37], which was recently characterized in our group [24,28]. The third one is the D-specific hydantoinase from *A. crystallopietes* DSM 20117, which is object of this paper. Originally, this strain was isolated through a screening procedure for microorganisms for producing aliphatic and aromatic D-amino acids from corresponding D,L-5-monosubstituted hydantoin derivatives. It occurred that this enzyme accepts a wide range of substrates being converted in a strictly D-specific manner [38].

Recently we reported on the complete amino acid sequence of the hydantoinase from *A. aurescens* [3]. This was the first ever reported amino acid sequence of a non-ATP-dependent hydantoinase, which L-selectively hydrolyses 5′-monosubstituted aryl-hydantoin derivatives like indolylmethylhydantoin, but was even D-specific for other alkyl-hydantoin derivatives, like methylthioethylhydantoin. Homology search performed in protein and nucleic acid databases retrieved only distantly related proteins, which all are members of the recently described protein superfamily of amidohydrolases related to ureases [2]. Phylogenetic analysis revealed that this hydantoinase forms a new branch separate from other hydantoin cleaving enzymes like dihydropyrimidinases (EC 3.5.2.2) and allantoinases (EC 3.5.2.5). The enzymes of this protein superfamily have evolved from a common ancestor and therefore are the product of divergent evolution. Above that, the enclosed gene families have developed very early in evolution, probably prior to the formation of the three domains, Archaea, Eukarya and Bacteria. It was of surprise that other hydantoinases, which are related to ATP-dependent *N*-methylhydantoinases (EC 3.5.2.14) or 5-oxoprolinases (EC 3.5.2.9) do not belong to this superfamily. The N-terminal amino acid sequence of the D-specific hydantoinase from *A. crystallopoietes* shows distinct homologies to other cyclic amidases involved in the nucleotide metabolism —especially to dihydropyrimidinases—as well as to ureases, and non-selective hydantoinases. In particular, this enzyme offers 36% identity to the non-selective hydantoinase from *A. aurescens* DSM 3745. Since these enzymes are from the same genus of *Arthrobacter*, the D-specific hydantoinase from *A. crystallopoietes* might be considered as a possible link in the molecular evolution of L-selective, non- and D-specific hydantoinases.

Despite to the significant sequence homology, the enzyme from *A. crystallopoietes* and *A. aurescens* are completely different with respect to their substrate specificity and stereoselectivity, since the enzyme from *A. aurescens* has no detectable activity for D,L-5-phenylhydantoin or unsubstituted hydantoin [24]. The

same enzyme shows a substrate-dependent enantioselectivity for the cleavage of D,L-5-indolylmethylhydantoin and D,L-5-methylthioethylhydantoin [24], which is in contrast to the enzyme from *A. crystallopoietes*, too. This was shown to be highly active for D,L-5-phenylhydantoin and unsubstituted hydantoin and to be strictly D-selective for the conversion of D,L-5-indolylmethylhydantoin and D,L-5-methylthioethylhydantoin. Thus it occurs that neither a methylene group between the hydantoin ring and the 5-substituent nor an aromatic or bulky 5-substituent are structurally necessary for catalysis, which is in contrast to the enzyme from *A. aurescens*, that irretrievably needs the respective methylene bridge for conversion.

Both enzymes from *A. aurescens* [30] and *A. crystallopoietes* DSM 20117 are dependent on Zn^{2+}. Up to now, this has only been reported for the related animal dihydropyrimidinases (for a review see Ref. [32]). While the native enzyme from *A. aurescens* contains 10 mol Zn^{2+}, which corresponds to 2.5 mol Zn^{2+} per subunit, and can be reversibly inactivated by EDTA [28], the enzyme from *A. crystallopoietes* contains 8 mol Zn^{2+} in total and 2 mol per subunit. In contrast to the latter enzyme it is not inactivated by EDTA, but by treatment with 8-HQSA. Thus, it occurs that these metal ions are more tightly bound to the enzyme, since the stability constants are considered to be higher for 8-HQSA than for EDTA. This is as well in contrast to other D-selective hydantoinases from different strains from *Agrobacterium* spp. [unpublished results], which are under identical conditions rather easily inactivated by EDTA. For the enzyme from *A. aurescens* it was shown that incubation with EDTA caused apart from the loss of enzyme activity a complete dissociation into its subunits [30]. Zinc, which was identified as metal ligand [28], does either play a catalytic and structural role for this hydantoinase. It is not yet clear if the same is true for the hydantoinase from *A. crystallopoietes*.

Based on general biochemical similarities and striking N-terminal sequence homologies between the hydantoinases from both *Arthrobacter* strains described above, it is worthwhile to elucidate their molecular structures by X-ray studies for understanding the molecular reasons for their different substrate- and even reciprocal stereoselectivities. These investigations are ongoing for the enzyme from *A. aurescens* DSM 3745 [39].

In contrast to the hydantoinase from *A. aurescens*, which could be purified in only three steps to a very high yield of 80% [24], the procedure for the purification of the hydantoinase investigated in the present paper turned out to be very complex and was accomplished with a low yield of 5%. In order to simplify the purification procedure and to produce the enzyme for further biochemical and molecular studies, the cloning and expression of the gene becomes of relevance and is under investigation.

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