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Catalytic and structural function of zinc for the hydantoinase from *Arthrobacter aurescens* DSM 3745

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Abstract

Metal dependency of the hydantoin amidohydrolase (hydantoinase) from *Arthrobacter aurescens* DSM 3745 has been analyzed based on kinetic studies of metal/chelator-caused enzyme inactivation, denaturation and reactivation, accompanied by the identification of specific metal binding ligands. The enzyme can be inactivated by metal chelating agents and—apart from the loss of its activity—completely dissociates into its subunits. Enzyme activity can be restored from recollected monomers by the addition of cobalt, manganese or zinc-ions, whereas nickel and magnesia remain ineffective. Subjection of the hydantoinase to metal analysis reveals a content of 10 mol zinc per mol enzyme. Zinc plays an essential role not only for the catalytic activity but also for the stabilization of the active quarternary structure of the hydantoinase. Histidine-specific chemical modification of the enzyme causes a complete loss of the catalytic activity and reveals histidine residues as putative zinc binding ligands. Both, the metal/chelator-caused enzyme inactivation as well as the metal-caused enzyme reactivation, can be reduced in the presence of the substrate. Therefore, it is very likely that at least one metal-ion acts specifically near or at the active site of the enzyme.

Keywords: Hydantoinase; Cyclic amidase; *Arthrobacter aurescens*; Metalloenzyme; Zinc

1. Introduction

Hydantoinases (Hydantoin amidohydrolases) are cyclic amidases (EC 3.5.2) [1] which catalyse a reversible ring-opening hydrolysis of hydantoin and their 5′-monosubstituted analogues. Based on high stereoselectivity and easy accessibility of their substrates, which already racemize under reaction conditions, hydantoinases have attracted much attention for the production of optically active amino acids [2].

Hydantoinases have commonly been classified as L-, non- or D-selective according to their stereoselectivity, which seems to be a rather unfavourable classification system as long as the stereoselectivity is depending on tested substrates [3]. A more expressive classification is based on the natural function of distinct hydantoinases. With the exception of *N*-methylhydantoinases (EC 3.5.2.14), which are involved in the degradation pathway of creatinine [4,5], hydantoinases are collected in the EC group 3.5.2.2 termed ‘dihydropyrimidinase’ or synonymously ‘hydantoinase’, although some hydantoinases are known to be absent of any

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dihydropyrimidinase activity [6]. Other studies confirm, that the group of hydantoinase is more diverse and versatile, which has recently been reviewed by Ogawa and Shimizu [7]. Holm and Sander [8] found, based on available sequence information that—despite to the above mentioned diversity—hydantoinases belong to a broad set of metal dependent amidohydrolases, which all are provided with the same active site architecture. Biochemical characterization of various hydantoinases (for review, see Ref. [2]) revealed that most hydantoinases are metal dependent but the function, type or amount has not been analyzed so far.

In this report, we describe the functions of the metal ions bound to the hydantoinase from *Arthrobacter aurescens* DSM 3745. Together with ongoing X-ray study of this enzyme [9], we expect an insight in the catalytic mechanism of this as well as other cyclic amidases.

2. Experimental

2.1. General remarks

3-*N*-methyl-5-(3′-indolylmethylene)hydantoin (*N*-3-IMH), 5-(3′-indolylmethylene)hydantoin (IMH), N-carbamoyltryptophan [N-CTrp] were obtained from Professor Krohn (Department of Chemistry, Gesamthochschule Paderborn, Germany). Unless otherwise stated, all reagents were of analytical grade and purchased from Fluka (Buchs, Switzerland). The solutions of salts and acids were prepared in deionized water purified with the MilliQ-system (Millipore, Bedford, MA, USA). *A. aurescens* DSM 3745 was cultivated under conditions as reported previously for *Arthrobacter* sp. DSM 3747 [10] using 0.3 g/l *N*-3-IMH as an inducer.

2.2. Enzyme purification

The hydantoinase from *A. aurescens* DSM 3745 was purified to homogeneity using a three-step chromatography procedure previously described [3]. Homogeneity of the enzyme was checked by standard SDS-polyacrylamide gel electrophoresis, isoelectric focusing and matrix-assisted UV-laser desorption/ionization mass spectrometry (MALDI-MS) [3].

2.3. Determination of the protein concentration

The protein concentration was determined according to the method of Bradford [11] using bovine serum albumin as a standard. The test-kit was purchased from BioRad, Munich, Germany.

2.4. Determination of hydantoinase activity (standard test)

Substrate solution: 0.050 g (0.218 mmol) L-IMH were suspended in 100 ml substrate buffer (0.1 M Tris, pH 8.5) Standard assay: 100 μl of the enzyme solution were added to 800 μl of the preincubated (37°C) substrate solution. The conversion of L-IMH was maintained below 10%. The reaction was stopped after 10 min by adding 400 μl TCA (12% in water (w/v)) and analyzed after centrifugation by HPLC as described previously [3].

2.5. Size exclusion chromatography

The molecular mass of the native and the deactivated hydantoinase was estimated by size exclusion chromatography using a Superose 12 column (Pharmacia, Freiburg, Germany) with 0.1 M Tris, pH 7.0 as an eluent at a flow rate of 0.25 ml/min at room temperature. The following proteins were used as molecular mass standards: catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa) and ovalbumin (43 kDa).

2.6. Inactivation and reactivation experiments

Chelator solution 1: a solution of 20 mM 8-hydroxyquinoline-5-sulfonic acid (8-HQSA) in buffer A (0.1 M Tris, pH 8.0) was prepared. Chelator solution 2: a solution of 10 mM ethylenediamine tetraacetic acid (EDTA) in buffer

A was prepared and the pH was adjusted to 8.0 (2 M NaOH). Metal–salt solutions: solutions of 50 μM and 500 μM $ZnCl_2$, $CoCl_2$, $MnCl_2$, and $NiCl_2$, respectively, in Tris-buffer (0.1 M, pH 7.0) were freshly prepared and used immediately.

2.6.1. Inactivation using 8-HQSA

A total of 5 μl of chelator solution 1 were added to 50 μl of the enzyme solution (2.5 mg/ml) and incubated at 37°C. Enzyme activity was measured immediately after addition (0 min) and subsequently after 7, 15, 25, 37, 50 and 120 min, respectively. The dissociation of the tetrameric hydantoinase into its subunits was analyzed by size exclusion chromatography (see Section 2.5). A total of 25 μl of the inactivation mixture containing 2.27 mg/ml hydantoinase and 1.82 mM HQSA were applied to the column after 120 min of incubation. The relative amount of tetrameric and monomeric form was calculated from peak areas. Monomer containing fractions were pooled (monomer fraction).

2.6.2. Reactivation of enzyme previously inactivated with 8-HQSA

For reactivation experiments, 10 μl of the metal–salts solutions were added to 100 μl monomer fraction obtained by size exclusion chromatography. Hydantoinase activity was assayed after 5.5, 21, 53 and 94 h of incubation at 4°C.

2.6.3. Inactivation using EDTA

A total of 400 μl of chelator solution 2 were added to 400 μl enzyme solution (5 g/l) and incubated at 37°C. Enzyme activity was measured immediately after addition and subsequently after 1, 2, 3 and 4 h, respectively. The dissociation of the tetrameric hydantoinase into its subunits was analyzed by size exclusion chromatography (see Section 2.5). After 1, 2, 3 and 4 h of incubation, respectively, 25 μl of the deactivation mixture containing 2.5 mg/ml hydantoinase and 5 mM EDTA were applied to the column. The relative amount of tetrameric and monomeric form was calculated from corresponding peak areas.

2.6.4. Chelator caused inactivation and reactivation in the presence of the substrate

Inactivation: 0.25 mg/ml hydantoinase in 0.1 M Tris pH 8.5 were inactivated for 5 min with 1.7 mM 8-HQSA at 37°C with and without addition of 1 mM DL-IMH. The enzyme activity was measured using the standard assay and was coordinated against controls estimating the spontaneous denaturation.

Reactivation: 0.8 g/l hydantoinase in 0.1 M Tris pH 8.5 were inactivated at 37°C using 1.7 mM 8-HQSA. After 15 min of incubation, the chelator containing buffer was exchanged using a PD-10 column (Pharmacia, Freiburg, Germany) equilibrated with 0.1 M Tris, pH 7.0. Reactivation was performed using 500 μM $CoCl_2$ with and without addition of 1 mM DL-IMH in 0.1 M Tris, pH 7.0 for 21.5 h at 4°C. Control experiments without $CoCl_2$ were performed to analyze the extent of inactivation.

2.7. Enzyme modifications

Histidine specific modifications were performed with diethylpyrocarbonate (DEPC). A total of 25 μl of 10 mM diethylpyrocarbonate (ICN, OH, USA) dissolved in ethanol (absolute) were added to 1 ml enzyme solution (1 mg/ml hydantoinase in 50 mM phosphate buffer, pH 7) and incubated at room temperature (20°C). Remaining enzyme activities were measured before and subsequently after 5, 10, 30 and 50 min of incubation with diethylpyrocarbonate, and the respective absorbance spectra were analyzed using a Beckmann DU-64 photometer. The influence of ethanol (absolute) was tested in control experiments under the same conditions as described above and found to be negligible. For metal analysis, buffers of the DEPC-modified and unmodified enzyme samples were ex-

changed using PD-10 columns (Pharmacia, Freiburg). These were equilibrated with either 10 ml 0.05 M phosphate buffer pH 7 (for modified sample) or 0.1 M Tris pH 8.5 (for unmodified sample). A total of 1 ml of the samples were added to the equilibrated columns and eluted within 5 ml of the respective buffer. Protein containing samples were pooled and concentrated by ultrafiltration to a final volume of about 1 ml.

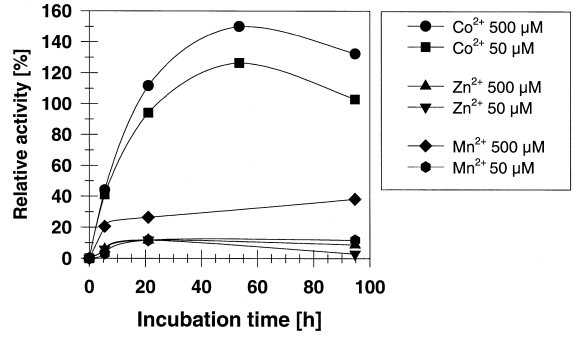

Fig. 2. Time dependent reactivation of inactivated hydantoinase with chlorides of cobalt, zinc and manganese at 4°C in 0.1 M Tris pH 7. The activity is related to the original activity of the non-treated enzyme (70 U/mg).

3. Results

3.1. Metal chelator caused inactivation

The native hydantoinase was incubated with EDTA or (alternatively) with 8-hydroxychinolinesulfonic acid (8-HQSA), which allowed—due to their metal chelating ability—the determination of metal requirements. Both treatments led to a time dependent loss of enzyme activity, which is demonstrated in Fig. 1A. Even a lesser amount of 8-HQSA was more effective than those compared to treatments with EDTA.

3.2. Restoration of enzyme activity

Enzyme activity could be restored from recollected monomers (see below) by the addition of $CoCl_2$, $MnCl_2$ or $ZnCl_2$, whereas $NiCl_2$ and $MgCl_2$ remained ineffective. Fig. 2 illustrates the time and concentration dependent fashion of this enzyme reactivation. When Co^{2+} was added to the monomers, enzyme reactivation was approximately 150%, Mn^{2+} resulted into 30%, whereas Zn^{2+} gave only 10% of the original activity.

However, since the inactivation was a fast event (40 min), reactivation occurred rather slowly (it was completed after approximately 60 h), and was even much slower than the tetramer declination (which was in a time scale of about 10 h; see also Fig. 1A).

3.3. Metal function

During inactivation of the enzyme, the hydantoinase completely denatured, by means of

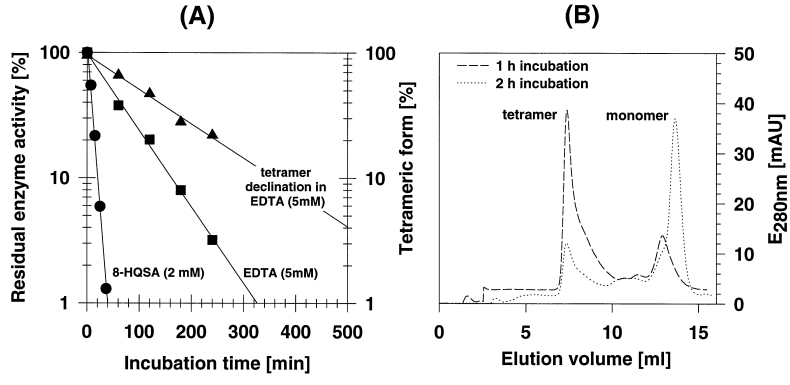

Fig. 1. (A) Time dependent loss of enzyme activity caused by metal chelation with 5 mM EDTA (■) or 2 mM 8-HQSA (●), respectively. Enzyme denaturation was caused by dissociation of the tetrameric hydantoinase into its subunits after treatment with 5 mM EDTA (▲). (B) Subunit (monomer) formation as a result of metal-chelation. Chromatograms from size exclusion separation onto a Superose 12 column of treated enzyme with 2 mM 8-HQSA. Separations were performed after 1 and 2 h incubation of the enzyme, respectively.

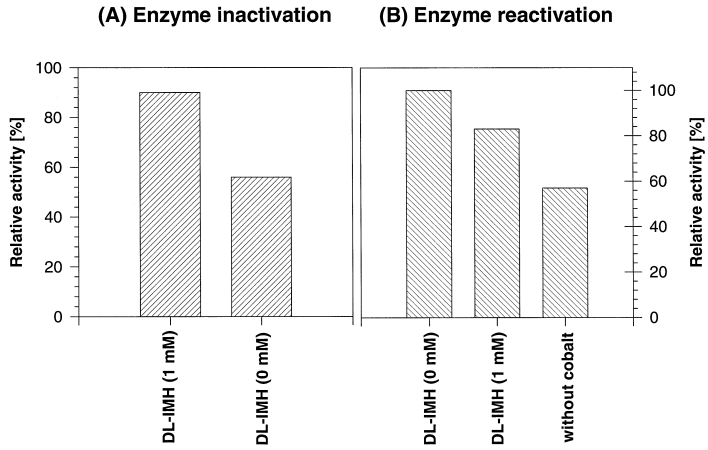

Fig. 3. Influence of substrate binding on metal/chelator-caused enzyme inactivation and reactivation. (A) The hydantoinase was inactivated with 8-HQSA in the presence of 1 mM DL-IMH or without any substrate, respectively. The enzyme activity was measured using the standard assay and coordinated against controls estimating the (metal/chelator independent) denaturation. (B) Reactivation was performed in the presence of 500 μM $CoCl_2$ with or without addition of 1 mM DL-IMH, respectively. Control experiments without $CoCl_2$ were performed to analyze the extent of inactivation. Relative activity in (A) and (B) is expressed as the percentage of the activity achieved without chelator dependent inactivation to this of the respective treatment.

dissociation into its subunits, which could be demonstrated by analyzing the amount of the formed subunits using a size exclusion chromatography. It is shown in Fig. 1B that this metal/chelator-caused enzyme inactivation was much faster than the dissociation of the enzyme into its subunits. Thus, metal removement features two separate events: it first causes a quick reduction of enzyme catalysis, and second, slow enzyme denaturation caused by tetramer declination and subunit formation.

Both, the metal/chelator-caused enzyme inactivation as well as the metal-caused enzyme reactivation, could be reduced in the presence of substrates, which is shown in Fig. 3. Therefore, it is very likely that at least one metal-ion acts specifically near or at the substrate-binding site of the enzyme.

In summary, these results indicate that the metal ions must have at least two different functions: a structural as well as a catalytic one.

3.4. Metal ligands

Preliminary sequence information of the hydantoinase [3] as well as studies on its molecu-

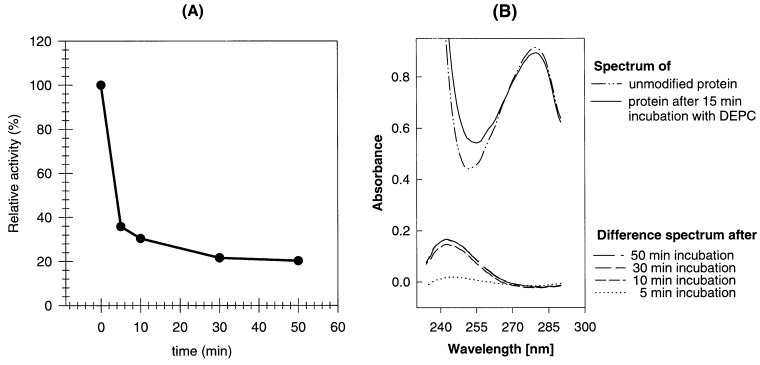

Fig. 4. Declination of hydantoinase activity (A) caused by enzyme modification using diethylpyrocarbonate (DEPC) and visualization by the change of absorbance spectra (B). The relative activity is expressed as the percentage of activity achieved with unmodified enzyme to the activity of the modified enzyme.

Table 1
Determination of zinc-concentrations of the unmodified homogeneous hydantoinase and the DEPC-modified enzyme using an inductive coupled plasma–atomic emission spectrometer (ICP–AES)

Probe no.	Probe description	Hydantoinase concentration (μm)	Zinc concentration measured (μM)	Mol metal per mol hydantoinase (mol metal per mol subunit)
1	unmodified hydantoinase	6.0	53.5	8.9 (2.2)
2	0.1 M Tris-buffer used for (1)	(–)	< 0.7	(–)
3	DEPC-modificated hydantoinase	4.0	33.6	6.1 (1.5)
4	50.0 mM phosphate-buffer used for (3)	(–)	9.2	(–)
5	water	(–)	< 0.7	(–)

Measurements were performed at the TU Clausthal, Department for Geochemistry, Germany.
Molar ratios of bound metal to the modified or unmodified enzyme were calculated according to the respective amounts of zinc measured in the used buffer.
The lower detection limit for zinc was 0.7 μM.
Preparation of enzyme samples: 250 μl of the respective enzymes were diluted to a final volume of 25 ml with metal-free water containing 500 μl of 65% suprapure HNO_3.
The instrument was calibrated against Zn^{2+}-standards for quantitative determinations.
All calculations were from double-measurements.
Other metals were analytically shown to be absent.

lar evolution indicate homologies to other metal dependent amidases, which share a common sequence motif involved in metal binding [12]. These are believed to have the same active-site architecture, all with histidine residues involved in metal binding [8].

Chemical modifications of histidine residues using diethylpyrocarbonate (DEPC) were performed in order to decide if these residues might play a similar function for the hydantoinase as well. It follows from Fig. 4A that the chemical modification leads to a fast inactivation of the enzyme. The change of the respect spectra during modification, which is shown in Fig. 4B, indicates by the increase of absorbance at about 240 nm (and the absence of other missing maxima) that histidine residues were exclusively modified.

Zinc was identified as a metal-ion bound to the hydantoinase by subjecting the enzyme to direct metal analysis, as recently reported by May et al. [13]. It could be calculated from inductive coupled plasma–atomic emission spectrometry, which is shown in Table 1, that the chemical modification significantly decreases the amount of zinc from 2.2 mol per subunit to 1.5 mol of around 1 mol since the native hydantoinase consists of four identical subunits [3,13].

4. Discussion

The homogeneous hydantoinase from *A. aurescens* DSM 3745 was used for experiments in order to obtain more information about its metal dependence. Incubation of the hydantoinase with EDTA or 8-HQSA resulted in a complete inactivation which could be restored by incubation with Mn^{2+}- or Co^{2+}-ions while Zn^{2+}-ions remained rather ineffective under identical conditions. An activating effect of Mn^{2+}- or Co^{2+}-ions is also reported for other microbial hydantoinases [14,15] but the metal content of the purified enzymes has not been analyzed so far. We found by atomic spectrometry that the hydantoinase from *A. aurescens* DSM 3745 contains between two and three atoms Zn^{2+}-ions per subunit. These results clearly indicate that reactivation experiments can not be a proof for actually bound metal ions as long as the reactivation conditions, like ion concentrations, temperature and pH-value strongly influence the

specificity of metal ligands. This might be an explanation for the obtained low reactivation with Zn^{2+}-ions compared to Co^{2+}- and Mn^{2+}-ions, which was also found for the zinc containing dihydropyrimidinase from bovine liver [16].

Zinc is very abundant in nature and a well known trace element in many proteins. Different functions of zinc ions like structural, catalytic, and/or regulatory functions in proteins are known [17]. The dissociation of the enzyme into its subunits caused by treatment with metal chelators suggests a structural function of the metal ion for the hydantoinase from *A. aurescens*. As long as the inactivation of the hydantoinase with EDTA was a faster event than the dissociation of the tetramer, it could be assumed that zinc also has a catalytic function. Further experiments supported the assumption that at least one metal ion plays a catalytic role at the active site, because substrate binding protected the enzyme from inactivation and decreased reactivation of the enzyme.

The function of the catalytic Zn^{2+} could be the activation of water, which is required for the hydrolytic cleavage of the hydantoin ring. Metal activated water molecules are involved in reaction mechanisms of other amidases, too. They play, for example, an important role in the reaction catalyzed by microbial ureases [18] and are proposed to be essential for a zinc-dependent dihydropyrimidinase from beef liver [19,20] and a dihydroorotase from *Escherichia coli*, which is reported to contain 3 mol zinc per subunit [21]. Together with other amidases, these enzymes are believed to have the same active-site architecture, all with histidine residues involved in metal binding [8]. Preliminary sequence information of the hydantoinase from *A. aurescens* DSM 3745 indicated homologies to this group of enzymes [3,12]. Therefore, we assumed that histidine residues might be potential zinc ligands in the hydantoinase. Our results show that selective chemical modification of histidine by diethylpyrocarbonate leads to a fast inactivation of the enzyme and a simultaneous loss of zinc. Therefore, it is very likely that histidine residues are involved in zinc binding at the active site of the hydantoinase.

Although the natural function of the hydantoinase from *A. aurescens* DSM 3745 seems to be different from all known cyclic amidases [3], our results indicate that the reaction mechanism might be very similar to those of other cyclic amidases: a metal activated water acts as a lewis base which attacks the C4-carbon atom of the hydantoin ring in a nucleophilic manner similar to the mechanism proposed for dihydropyrimidinase [20].

Our ongoing investigations are focused on determination of the X-ray structure which will give further information about the exact number of zinc ions and their specific functions for the enzyme. Furthermore, we expect a deep insight in the reaction mechanism and the understanding of the enantioselectivity and substrate specificity of this and other cyclic amidases.

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