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The hydantoin amidohydrolase from *Arthrobacter aurescens* DSM 3745 is a zinc metalloenzyme

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Abstract

The hydantoin amidohydrolase (hydantoinase) from *Arthrobacter aurescens* DSM 3745 was purified to homogeneity and subjected to metal analysis under atomic absorption spectrometry (AAS) and inductive coupled plasma–atomic emission spectrometry (ICP–AES). Three independent preparations of homogeneous enzyme indicated that 1 mol of the active enzyme contains 10 mol zinc ions. This corresponds to 2.5 mol zinc per mol subunit, since the hydantoinase consists of four identical subunits. Only trace amounts of manganese, magnesia, nickel and cobalt were detected. Other metals were either absent or existed below detection levels.

Keywords: Hydantoinase; Amidase; *Arthrobacter aurescens*; Zinc metalloenzyme

1. Introduction

Hydantoin amidohydrolases (hydantoinases) are cyclic amidases (EC 3.5.2) [1], which catalyze a reversible ring-opening hydrolysis of 5′-monosubstituted hydantoins. They are used as biocatalysts for the stereospecific production of optically active non-proteinogenic amino acids, which are valuable synthons for the synthesis of antibiotics, sweeteners, pesticides, pharmaceuticals and biologically active peptides [2]. The biochemical properties and substrate specificities of various hydantoinases, mainly originated from bacteria, have been characterized in detail [2]. In particular, hydantoinase from *Arthrobacter aurescens* DSM 3745 has recently been crystallized and is currently undergoing X-ray studies [3]. Information about the kind and quantity of metal bound to the enzyme are both necessary for understanding the mechanism of enzyme catalysis. Other microbial hydantoinases are reported to be dependent on Co^{2+}, Fe^{2+}, Mg^{2+} or Mn^{2+} [2,4,5]. However, these assumptions are based on enzyme inactivation experiments, wherein metal chelating agents are used to induce metal-caused reactivation, without direct measurement of the respective metals, which are presumably bound to the enzyme. Hence, we decided to subject purified hydantoinase to metal analysis using two different kinds of direct detection systems, namely atomic

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absorption spectrometry (AAS) and inductive coupled plasma–atomic emission spectrometry (ICP–AES).

2. Experimental

2.1. General remarks

Unless otherwise stated, all reagents were of analytical grade and purchased from Fluka (Buchs, Switzerland). The salt and acid solutions were prepared in deionized water purified with the MilliQ system (Millipore, Bedford, MA, USA). *A. aurescens* DSM 3745 was cultivated under conditions reported previously for *Arthrobacter* sp. DSM 3747 [6].

2.2. Enzyme purification

The hydantoinase from *A. aurescens* DSM 3745 was purified to homogeneity using hydrophobic interaction and anion exchange chromatography, previously described by May et al. [1]. Enzyme homogeneity was verified by standard SDS-polyacrylamide gel electrophoresis, isoelectric focusing and matrix-assisted UV-laser desorption/ionization mass spectrometry (MALDI-MS) [1]. Successful crystallization [3] and protein sequencing using these kinds of enzyme fractions have recently been published [1,7]. Enzyme activity was measured, using L-indolymethylhydantoin as standard substrate, under conditions previously described [1].

For metal analysis, the purified enzyme was brought, through ultrafiltration, to a concentration of 10 g/l using an Omegacell membrane (Filtron) with an exclusion limit of 30 kDa.

2.3. Metal analysis

Atomic absorption spectrometry (AAS) with a Perkin Elmer AAS 2100 and inductive coupled plasma–atomic emission spectrometry (ICP–AES, Spectroflame Modula) were both performed at the Technical University of Clausthal, Department for Geochemistry, Germany, while a Perkin Elmer AAS 3030 was used at the Research Center (KFA) Jülich, also in Germany. Samples of the hydantoinase supplied from three independently performed purification passages were applied for metal analysis. For AAS-based determination at KFA-Jülich, 50 μl of the homogeneous hydantoinase (ultrafiltrated fraction; protein concentration 10 mg/ml) were freeze-dried and, afterwards, resuspended for analysis into 300 μl of metal-free water. The metal content was calculated from three independent measurements with a relative standard error of $\pm 10\%$. The instrument was calibrated against standards of Zn^{2+}, Mg^{2+}, Mn^{2+} and Ni^{2+}. For AAS-based determinations at TU-Clausthal, the hydantoinase (ultrafiltrated fraction; protein concentration 10 mg/ml) was diluted to a final concentration of 0.625 mg/ml (the total volume was 1 ml) and was directly analyzed according to its zinc-content. For Mn determinations, a further enzyme dilution step was omitted. The instrument was calibrated against standards of Zn^{2+} and Mn^{2+} and the respective metal contents of the enzyme was calculated from three independent measurements. For analysis based on ICP–AES, 250 μl of ultrafiltrated hydantoinase (1.2 mg/ml) was diluted to 25 ml with metal-free water containing 500 μl of 65% suprapure HNO_3. For quantitative determinations, the instrument was calibrated against Co^{2+} and Zn^{2+} standards. All calculations were made with double measurements.

3. Results and discussion

The molecular mass of the native enzyme was previously calculated to be 200 ± 20 kDa using size-exclusion chromatography [1]. Mass spectrometry studies (MALDI-MS) revealed a molecular mass of 49.68 kDa, which represented the mass of a single charged monomer [1]. These measurements confirmed the homogeneity of the enzyme and furthermore sug-

gested its homotetrameric structure without any covalent linkage between the subunits.

3.1. Metal content

Using methods to be published elsewhere, the native hydantoinase was incubated with EDTA or with 8-hydroxychinolinesulfonic acid, which allowed—due to their metal chelating ability—the determination of metal requirements. Both treatments led to a time-dependent loss of enzyme activity (data not shown). In order to confirm the kind and quantity of the metal bound to the native enzyme, homogeneous enzyme fractions, received from three independent purification passages, were subjected to metal analysis. The active hydantoinase contained an average of 10 mol zinc, which can be calculated from the values presented in Table 1. This corresponds to 2.5 mol zinc per mol subunit, since the native hydantoinase consists of four identical subunits. The above data comes from three independent enzyme preparations and two basically different kinds of spectrometric analytical methods (AAS and ICP–AES), which were performed in at least two different laboratories. Only trace amounts of Mg^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} were detected (Table 1). Other than the four, no other metals were detected, mainly because ICP–AES-based analysis allows the simultaneous detection of trace elements at detection levels far below AAS standards (e.g., for zinc it is approximately 100-fold).

Zinc is a well known trace element in many proteins, and can have structural, catalytic and/or regulatory functions [8]. Catalytic zinc ion, for instance, can activate water, which is required for the hydrolytic cleavage of the hydantoin ring. Metal-activated water molecules appear to be similarly involved in other amidases. They, for example, play an important role in the reaction catalyzed by microbial ureases [9], and are thought to be essential in zinc-dependent dihydropyrimidinase from beef [10,11] or bovine liver [12] and in dihydroorotase from *Escherichia coli* [13]. In this case, it was found that the enzyme binds three zinc ions per subunit, the first is thought to be essential for catalytic activity, while the other two serve structural purposes [13].

Enzyme evolution studies [7] on the hydantoinase from *A. aurescens* DSM 3745 revealed homologies to dihydropyrimidinases and other metallo-dependent amidases, such as the tetrameric D-hydantoinase (dihydropyrimidinase) from *Bacillus stearothermophilus*, which, however, is thought to be Mn^{2+}-dependent [5].

In general, zinc is reported to be tightly bound to enzymes [8]. The same seems to be true for the hydantoinase from *A. aurescens*,

Table 1
Metal analysis of *A. aurescens* DSM 3745 hydantoinase

Metal	Metal concentration (μM)	Hydantoinase concentration (μM)	Mol metal per mol hydantoinase
Zn	101.90[a]	8.3	12.3
	29.20[b]	3.1	9.3
	53.50[c]	6.0	8.9
Mg	1.37[a]	8.3	0.16
Mn	0.36[a]	8.3	0.04
	2.80[b]	50.0	0.06
Ni	1.53[a]	8.3	0.18
Co	< 0.10[c]	6.0	< 0.02

[a] Performed at the Technical University of Clausthal, Department for Geochemistry, Germany, with a Perkin Elmer AAS 2100.
[b] Performed at the Research Center Jülich (KFA), Germany, using a Perkin Elmer 3030 instrument.
[c] Measured using ICP–AES (Inductive Coupled Plasma–Atomic Emission Spectrometer, Spectroflame Modula), at the TU Clausthal, Germany.
Buffers were analytically shown to be metal-free and the instruments were calibrated against Zn^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Co^{2+} standards. Other metal ions were below detection limits.

since the enzyme was never exposed to any zinc-containing solutions during the purification procedure and was even not removed during ultrafiltration or size-exclusion chromatography (data not shown). Using the metal-content detection methods documented in this paper, we are currently conducting studies focusing on the kinetics of metal/chelator-caused enzyme inactivation, denaturation and reactivation, as well as the identification of the specific metal-binding ligands involved.

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