Cyclohexane-1,2-dione hydrolase: A new tool to degrade alicyclic compounds

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ABSTRACT

Alicyclic alcohols are naturally occurring compounds which can be degraded by microorganisms via cleavage of the ring C–C bond. Denitrifying *Azoarcus* sp. strain 22Lin grows on cyclohexane-1,2-diol which serves as electron donor and carbon source. The diol is converted to cyclohexane-1,2-dione followed by hydrolysis to the corresponding semialdehyde and oxidation to adipate. The latter two reactions are catalyzed by the thiamine diphosphate-dependent flavoenzyme cyclohexane-1,2-dione hydrolase, the first α-ketolase known so far. Biochemical and structural properties of this new member of the thiamine diphosphate enzyme family will be presented.

1. Introduction

Alicyclic compounds, produced by plant cells as secondary metabolites and occurring in fossil fuels, are widespread in nature. They are also natural intermediates in the anaerobic degradation of aromatic compounds. In chemical industries they serve as insecticides, herbicides, and as intermediates or solvents in many chemical reactions. Microorganisms can convert these xenobiotics to cellular metabolites under oxic or anoxic conditions. The biodegradation of these compounds may proceed via a carbon–carbon bond ring cleavage to form aliphatic intermediates which can be further degraded by β-oxidation [1]. With the isolation of the denitrifying bacterium *Azoarcus* sp. strain 22Lin on cyclohexane-1,2-diol as sole electron donor and carbon source, and nitrate as electron acceptor, a novel degradation pathway for α-diketones was discovered [2]. The diol is oxidized to the α-diketone cyclohexane-1,2-dione (CDO). Subsequently, CDO is hydrolytically cleaved to 6-oxohexanoate and finally oxidized to adipate in an NAD^+-dependent reaction (Scheme 1).

The latter two reactions are catalyzed by the ThDP-dependent flavoenzyme cyclohexane-1,2-dione hydrolase (CDH), the first α-ketolase known so far. In this contribution we will discuss several structural and biochemical aspects of CDH, and we will propose a reaction mechanism for this new member of the ThDP enzyme family.

2. Experimental

Azoarcus sp. strain 22Lin was cultivated as described previously, with cyclohexane-1,2-diol (4 mM) as electron donor and carbon source, and nitrate (10 mM) as electron acceptor [2]. CDH was purified using a modified protocol of Steinbach; ThDP, FAD, and Mg^{2+} were quantitated by standard procedures; CDH activity was assayed monitoring the reduction of NAD^+. Typically, 30 mg of pure CDH were obtained from 10 g cells, with a specific activity of $900 \pm 100\,mU\,mg^{-1}$ [3,4].

3. Results and discussion

3.1. Molecular architecture and cofactors

On SDS-PAGE, pure CDH exhibits a single band at 59 ± 1 kDa; from the amino acid sequence the mass of one monomer amounts to 64.5 kDa (including one FAD, one ThDP, one Mg^{2+}). According to size exclusion chromatography, CDH appears to be a homodimer in solution, in contrast to the homotetrameric architecture found in the crystalline state [3,4]. Each subunit harbors one noncovalently but tightly bound FAD, a less tightly bound ThDP, and one Mg^{2+} ion for anchoring the diphosphate moiety of ThDP to the protein matrix [4]. Solutions of CDH are yellow, with optical spectra typical for flavoproteins; as isolated, major absorption maxima at 371 and 434 nm are observed. The yellow cofactor was released upon denaturation of CDH with trichloroacetic acid; it

Abbreviations: CDO, cyclohexane-1,2-dione; CDH, cyclohexane-1,2-dione hydrolase; ThDP, thiamine diphosphate.

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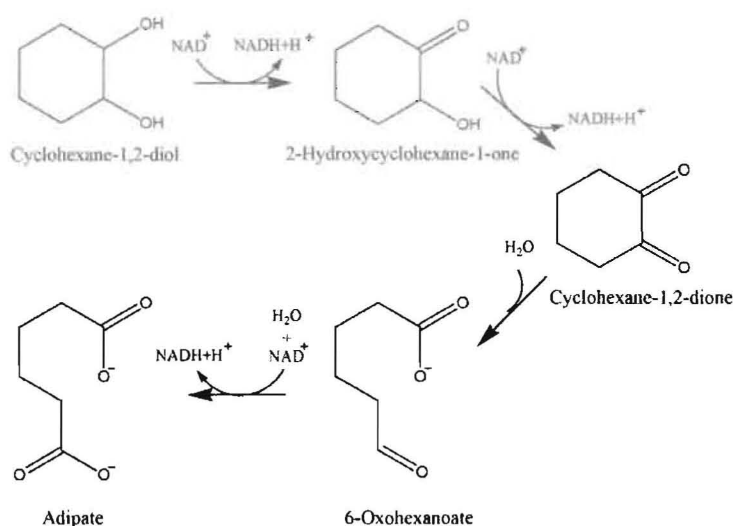
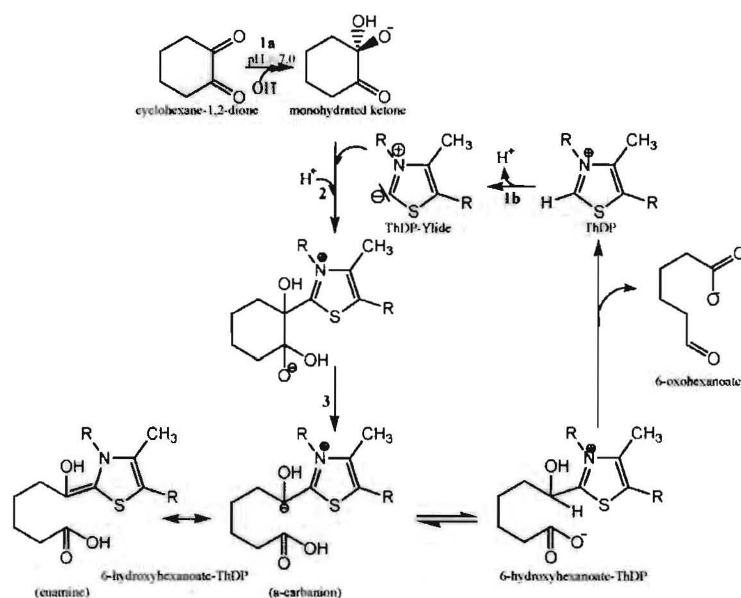


Scheme 1. Degradation of cyclohexane-1,2-diol in *Azoarcus* sp. strain 22Lin. The two reactions depicted in black are catalyzed by cyclohexane-1,2-dione hydrolase.

was identified and quantitated as FAD by HPLC analysis. Similarly, ThDP was determined by HPLC and the thiochrome assay [5]; quantitative analysis revealed 0.8 ThDP/CDH monomer, when purified in buffer pH 6.5 containing $MgCl_2$ (1.0 mM) and ThDP (0.5 mM). CDH purified at pH 8.3, and without ThDP and $MgCl_2$ added, had only 0.23 ThDP/CDH monomer. Attempts to fully reduce the FAD cofactor of CDH with Na^+dithionite or Ti(III)-citrate were not successful, maximum reduction (45%) was achieved photochemically with the 5-deazaflavin/oxalate system ($E' = -0.65$ V) [6]. A similar behavior was reported for other ThDP-dependent flavoenzymes, such as glyoxylate carboligase and acetolactate synthases/acetohydroxyacid synthases [7,8]. Reoxidation of CDH with $K_3[Fe(CN)_6]$, in the absence of dioxygen, proceeded via formation of the blue neutral flavin radical as indicated by the characteristic absorption maxima around 575–625 nm.

3.2. Catalytic properties

The specific activity of CDH depended on the enzyme concentration, with a pH optimum at 8.0. The specific activity of CDH increased with increasing enzyme concentration, up to 0.02 mg ml^{-1} CDH. Higher concentrations led to saturation, indicating that the active form of CDH must be a multimer. The enzyme appears to convert the monohydrated ketone form of cyclohexane-1,2-dione, rather than the monoenol form, with a k_{cat} of 1.3 s^{-1} and a K_M value of 12.3 μM [4]. For the substrate NAD^+, a slower conversion (k_{cat} 0.86 s^{-1}) and a K_M value of 88.5 μM were found. CDH has a similar catalytic efficiency by comparison to other ThDP-dependent enzymes. NaCl (>50 mM) led to a significant decrease of specific activity due to binding of the chloride anion in close neighborhood to the ThDP cofactor (Fig. 1) [4].



Scheme 2. Mechanism of the cleavage of cyclohexane-1,2-dione to 6-oxohexanoate catalyzed by cyclohexane-1,2-dione hydrolase [4].

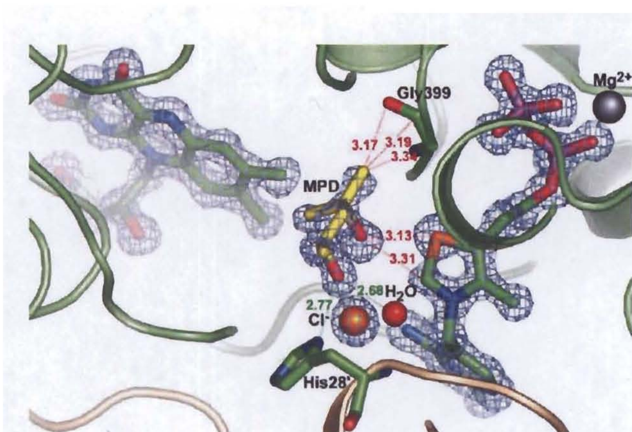


Fig. 1. Active site of cyclohexane-1,2-dione hydrolase with bound chloride anion (orange) and one molecule of the cryoprotectant 2-methyl-2,4-pentane-diol (MPD, yellow); distances in Å [4]; RCSB protein data bank code 2pgo.

3.3. Mechanism of ring cleavage

We propose a first reaction mechanism for the cleavage of cyclohexane-1,2-dione to 6-oxohexanoate (Scheme 2) based on mechanistic ideas developed for pyruvate oxidase [9], starting with the monohydrated ketone of CDO (1a) [10] and the ThDP-ylide (1b) which undergoes a nucleophilic attack on the carbonyl group of CDO (2). The subsequent cleavage of the C–C bond yields an α-carbanion (3), which is in equilibrium with its corresponding enamine. In the following step, the carbonic acid protonates the α-carbanion yielding 6-hydroxyhexanoate-ThDP. Finally, the product 6-oxohexanoate is released.

Acknowledgements

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References

[1] P.W. Trudgill, in: D.T. Gibson (Ed.), Microbial Degradation of Organic Compounds, Marcel Dekker Inc., New York, 1984, pp. 131–180.
[2] J. Harder, Arch. Microbiol. 168 (1997) 199–204.
[3] S. Fraas, Dissertation, Universität Konstanz, 2009.
[4] A.K. Steinbach, Dissertation, Universität Konstanz, 2005.
[5] J. Bontemps, P. Philippe, L. Bettendorff, J. Lombet, G. Dandrifosse, E. Schoffeniels, J. Chromatogr. 307 (1984) 283–294.
[6] V. Massey, P. Hemmerich, Biochemistry 17 (1978) 9–17.
[7] D.M. Chipman, Z. Barak, J.V. Schloss, Biochim. Biophys. Acta 1385 (1998) 401–419.
[8] K. Tittmann, K. Schroder, R. Golbik, J. McCourt, A. Kaplun, R.G. Duggleby, Z. Barak, D.M. Chipman, G. Hübner, Biochemistry 43 (2004) 8652–8661.
[9] K. Tittmann, R. Golbik, S. Ghisla, G. Hübner, Biochemistry 39 (2000) 10747–10754.
[10] R. Bakule, F.A. Long, J. Am. Chem. Soc. 85 (1963) 2309–2312.