This is a postprint of an article published in
Marín, M., Pieper, D.H.
Novel metal-binding site of Pseudomonas reinekei MT1 trans-dienelactone hydrolase
(2009) Biochemical and Biophysical Research Communications, 390 (4), pp. 1345-1348.

Novel metal-binding site of *Pseudomonas reinekei* MT1 *trans*-dienelactone hydrolase

Macarena Marín[1] and Dietmar H. Pieper[1*]

[1]*Division of Microbial Pathogenesis, HZI – Helmholtz Centre for Infection Research Inhoffenstr. 7, 38124 Braunschweig, Germany*

*For correspondence: Phone: +49 (531) 61814200, Fax: +49 (531) 61814499 . E-mail: dpi@helmholtz-hzi.de

Abstract

Pseudomonas reinekei MT1 is capable of growing on 4 and 5-chlorosalicylate as the sole carbon source involving a pathway with *trans*-dienelactone hydrolase as the key enzyme. This enzyme uses 4-chloromuconolactone as substrate, which transforms to maleylacetate and thereby avoids the spontaneous formation of toxic protoanemonin. *trans*-dienelactone hydrolase is a Zn^{2+}-dependent hydrolase where activity can be modulated by the exchange of Zn^{2+} by Mn^{2+} in at least two of the three metal binding sites. Site directed variants of conserved residues of the $Q_{101}XXXQ_{105}XD_{107}XXXH_{111}$ motif and of H281 and E294 exhibit a two order of magnitude decrease in activity and a strong decrease in metal binding capability. As none of the variants exhibited a change in secondary structure, the analyzed amino acid residues can be assumed to be involved in metal binding, forming a novel trinulear metal binding motif.

Keywords: dienelactone hydrolase; zinc-dependent hydrolase; *Pseudomonas reinekei*; PF04199; trinulear metal binding motif; biodegradation

Introduction

Chlorocatechols are central intermediates in the degradation of chloroaromatics such as (chloro)benzoates, (chloro)naphthalenes, (chloro)salicylates, (chloro)benzenes and (chloro)phenols [1]. Increasing attention is being paid to these compounds as they are hazardous to the environment and to health. However, bacteria have developed strategies to degrade these chlorinated compounds and use them as energy and carbon sources. Most described bacteria mineralize these compounds via the widely distributed chlorocatechol pathway [1]. This pathway involves *ortho* (intradiol)-cleavage of the aromatic ring by a chlorocatechol 1,2-dioxygenase with high activity against chlorocatechols [2], a chloromuconate cycloisomerase with high activity against chloromuconates [3], a dienelactone hydrolase (DLH), active against both *cis*- and *trans*-dienelactone [3], and a maleylacetate reductase (MAR) [4]. However, more and more bacteria are being described to degrade chlorocatechols via alternative routes. One example is *Pseudomonas reinekei* MT1, which degrades 4-chlorocatechol via a route, which harbors a novel enzyme termed *trans*-dienelactone hydrolase (*trans*-DLH) instead of a typical DLH (Fig. 1) [5, 6].

Typically, DLHs belong to the well described dienelactone hydrolase family (PF01738), which presents an α/β hydrolase fold. Its members share a highly conserved cys-his-asp catalytic triad [7] and catalyze the transformation of *cis*- and/or *trans*-dienelactones to maleylacetate. In contrast, *trans*-DLH belongs to the PF04199 protein family in the Pfam database [8], which comprises putative cyclases or hypothetical metal-dependant hydrolases with no conserved catalytic triad. More importantly, *trans*-DLH has been proposed to catalyse the transformation of 4-chloromuconolactone, the cycloisomerization product of 3-chloromuconate to maleylacetate (Fig. 1).

The fold of proteins belonging to PF01499 is defined by the crystal structure of a protein of unknown function from *Bacillus stearothermophilum*. This protein contains the signature motif $H_{45}XGTH_{49}XD_{51}XPXH_{55}$ (PDBID=1r61, accession number P84132), which is replaced by the motif $Q_{101}XXXQ_{105}XD_{107}XXXH_{111}$ in the *trans*-DLH of *P. reinekei* MT1 [6]. It has recently been shown [6] that *trans*-DLH of *P. reinekei* MT1 is a zinc-dependant hydrolase and contains two Zn ions per monomer. Based on the 1r61 structure, where Zn ions are coordinated by residues H45, H49, D51, H155 and E167, it has been proposed that *trans*-DLH contains a binuclear zinc-binding site with Q101, Q105, and D107, and the conserved residues H281 and E294 as coordinating ligands.

However, whether this is true or not has yet to be proven. Moreover, whether the role of zinc is structural or catalytic remains to be determined. The approach taken to address these questions was to biochemically analyze variants of the putative metal-binding motif generated by site-directed mutagenesis.

Materials and Methods

Site-directed mutagenesis. The previously constructed plasmid pASKtdlh [6] was used as template to introduce site-directed mutations in the *trans*-DLH encoding *ccaC* gene. Mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions and primers tDLH_D107AF (5'-GGAAGTCAAATCGCCGGCTTGCGCCACATCGGCCATCC-3') and tDLH_D107AR (5'-GGATGGCCGATGTGGCGCAAGCCGGCGATTTGACTTCC-3'), tDLH_D107A_H111AF (5'-GGAAGTCAAATCGCCGGCTTGCGCGCCATCGGCCATCC-3') and

tDLH_D107A_H111AR (5'-GGATGGCCGATGGCGCGCAAGCCGGCGATTTGACTTCC-3'), tDLH_H111AF (5'-CGACGGCTTGCGCGCCATCGGCCATCC-3') and tDLH_H111AR (5'-GGATGGCCGATGGCGCGCAAGCCGTCG-3'), tDLH_Q101AF (5'-ATTTTTACACCGCGTACGGAAGTCAAATCG-3') and tDLH_Q101AR (5'-CGATTTGACTTCCGTACGCGGTGTAAAAAT-3'), tDLH_Q105AF (5'-CAGTACGGAAGTGCAATCGACGGCTTGCGC-3') and tDLH_Q105AR (5'-GCGCAAGCCGTCGATTGCACTTCCGTACTG-3'), tDLH_H281AF (5'-GCGGGCATCATGGCCCGAGCAATCATCCCTC-3') and tDLH_H281AR (5'-GAGGGATGATTGCTCGGGCCATGATGCCCGC-3'), tDLH_E294AF (5'-GCATGCCAATTGGTGCGCTCTGGGCCATTGATGC-3') and tDLH_E294AR (5'-GCATCAATGGCCCAGAGCGCACCAATTGGCATGC3'). Integrity of the mutants was verified by sequencing.

Expression and purification of strep-tagged *trans-DLH and variants*. *E. coli* DH5α(pASKtdlh) and *E. coli* XL1-blue carrying pASKtdlh encoding protein variants were grown at 37 ºC in LB medium containing 100 µg ampicillin mL^{-1}. Cells were induced with 0.2 µg/mL anhydrotetracyclin at an OD_{600}= 0.6 and harvested after 3 h of incubation at 30 ºC. The strep-tagged recombinant protein and variants were purified from cell extracts using a gravity flow Strep-Tactin Sepharose column (IBA). Cell extracts were applied to the column, the column washed using Tris/HCl (100 mM, NaCl 250 mM pH 8.0) and *trans*-DLH eluted with Tris/HCl (100 mM, NaCl 250 mM, 2.5 mM desthiobiotin pH 8.0).

Homogeneity was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) performed with a protean III mini gel system (Bio-Rad) as previously described [9] with acrylamide concentrations of 5 and 12.5 % w/v used

for the concentrating and separating gels, respectively. The proteins were stained with Coomassie brilliant blue (Serva). PageRuler Protein Ladder (Fermentas) was used as a marker.

Enzyme assay and protein concentration estimation. *trans*-DLH activity was determined by following the depletion of 50 μM *trans*-dienelactone (50 μM; ε_{280} = 15,625 M^{-1} cm^{-1}) at 280 nm in 50 mM Tris/HCl buffer (pH 7.5) [9], in this buffer supplemented with 2 mM $MnCl_2$ and in 50 mM Bistris buffer (pH 6.5). *trans*-dienelactone was prepared as described earlier [10]. A unit of activity (U) is defined as the amount of protein needed to transform 1 μmol of substrate per minute at 25ºC.

Protein quantification was performed by the method of Bradford [11], with the Bio-Rad protein assay reagent and bovine serum albumin as standard.

Biochemical studies. Metal-free strep-tagged apoenzyme was prepared by incubation of 400 μg of *trans*-DLH with EDTA (1 mM) for 5 h. EDTA was removed using a PD-10 desalting column (Amersham). Activities were assayed as stated above and compared to the activity of an untreated enzyme solution incubated under the same conditions. For reactivation, the apoenzyme (1.94 μg/mL) was incubated with $ZnCl_2$, $MnCl_2$, $MgCl_2$ and $NiCl_2$ (2.5 μM - 5 mM) for a period of 5 minutes, followed by activity determination. *trans*-DLH activity prior to EDTA treatment was used as the control (100%).

Analysis of metals. To determine the metal content of *trans*-DLH and its variants, proteins were concentrated using Vivaspin (500 ml) centrifugal filter units. To determine the metal content of *trans*-DLH after incubation with $MnCl_2$, 100 μg of *trans*-DLH was incubated for 15 min in a volume of 100 μL of Tris/HCl buffer (50 mM, pH 7.5, 2 mM $MnCl_2$). Samples were washed using metal free Tris/HCl buffer (50 mM, pH 7.5). All proteins samples (25 μg) and the eluate were analyzed by

inductively coupled plasma mass spectrometry (ICP-MS) (Perkin Elmer Life Sciences model PE ELAN 6100 DRC), after dilution of the enzyme with 3 or 5 ml of 0.5% (v/v) HNO_3 to hydrolyse the protein and release metal ions (this solution was used without any further manipulation). The trace metal ion concentrations of the buffer were subtracted from the metal ion concentrations in the *trans*-DLH sample, and the resulting sum was divided by the dimeric protein concentration to give the molar equivalents of metal ions bound to the purified protein.

Circular dichroism (CD) spectra. CD spectra of the proteins used in this study were collected using a Jasco J-815 spectropolarimeter (cell length = 1 cm). The samples contained enzyme (150 μg/mL) in phosphate buffer (50 mM, pH 7.3). CD data was collected from 195 to 250 nm with a band-width of 1.0 nm at 25 ºC.

Results and Discussion

trans-DLH is a zinc-dependant hydrolase, the activity of which is modulated by manganese

Previous studies had indicated that *trans*-DLH is a metal-dependent hydrolase where the metal free apoenzyme can be reactivated by Zn^{2+} and Mn^{2+} [6]. It could be now shown that besides $Zn^{2+}$and Mn^{2+}, Mg^{2+} can also reactivate this enzyme (Table 1). While at pH 7.5 incubation with Zn^{2+} only partially restored activity and concentrations of Zn >0.5 mM were inhibitory, incubation with Zn^{2+} at a pH of 6.5 completely restored activity and inhibition was observed only at concentrations >5mM (Table 1) as it has been reported for other enzymes [12]. As previously

reported [6], this pH-dependant behaviour may be explained by formation of inhibitory $ZnOH^+$ at basic pH [12].

Incubation of *trans*-DLH with Mn^{2+} not only restored the activity even at low concentrations, but also resulted in a 4 fold increase in activity compared to that of the untreated strep-tagged enzyme (Table 1). A similar increase in activity was observed when the activity of the enzyme was measured in buffer supplemented with 2 mM Mn^{2+}, compared to the activity in unsupplemented buffer. In accordance with previous analyses, which indicated that *trans*-DLH contains at least two Zn^{2+} ions and varying amounts of Ni^{2+} and Mn^{2+} summing up to a total of 3 metal ions per monomer, *trans*-DLH purified here showed a metal content of 2.7 moles of Zn^{2+} and 0.6 moles of Mn^{2+}. Incubation with Mn^{2+} resulted in a significant change in the metal content (Table 2), suggesting that some of the intrinsic zinc is not tightly bound and that at least two of the total three metal binding sites may be occupied by Mn^{2+} and thereby modulating the activity of *trans*-DLH.

Previous reports have shown that manganese can replace one of the two zinc ions in co-catalytic zinc-dependent enzymes and thereby enhance activity [13]. The crystal structures of native creatininase from *P. putida* and creatininase activated with manganese revealed that in the Mn-bound structure the catalytic water is closer to the metal in comparison to the Zn-bound structure, which probably leads to the increased activity [13].

Residues in putative metal binding site of trans-DLH are crucial for binding of divalent cations

Site directed variants of residues, which have been proposed to play a role in metal binding were generated. Variant Q101A exhibits only 2.5% of activity compared to

that of *trans*-DLH. Replacement of residues Q105, D107, D107_H111, H111, H281 and E294 by alanines resulted in an even more drastic decrease in activity with relative activities <1% (Table 2).

All mutants were significantly impaired in their metal-binding capability. Only variants Q101A and Q105A still contained 1 mole of Zn^{2+} per subunit. The metal content of variants D107A, D107A_H111A, H111A, H281A and E294A was below the limit of detection (Table 2). This indicated that all three metal ions are bound to the same region in *trans*-DLH, discarding the possibility of a distant metal binding site as previously discussed [6]. All together these results confirm the role of the $Q_{101}XXXQ_{105}XD_{107}XXXH_{111}$ motif and of residues H281 and E294 in metal binding.

Divalent metals play a catalytic role in trans-DLH

Far-UV spectra of *trans*-DLH and variants were compared in order to determine if the mutations cause a disruption to the protein structure. Comparison of the spectra show only minor changes revealing that the differences observed in activity are only due to the decreased metal binding ability and not in a loss of structure (Fig. 2). This result indicates that the divalent metals present in *trans*-DLH play a catalytic role.

Analysis of the structure 16r1 and its proposed binuclear binding site supports this notion. Binuclear metal binding sites are usually co-catalytic. Additionally, zinc ions in this structure are coordinated by a distorted tetrahedral coordination sphere, where one of the ligands is a water molecule, as characteristic for catalytic sites [14]. In contrast, in structural zinc sites, the metal ion is usually coordinated in a tetrahedral geometry by four amino acid side chains with cysteine the most frequently observed residue [14], which is absent from above described Zn^{2+} binding sites of *trans*-DLH.

Zinc/manganese trinuclear binding site

Based on the mutational analysis and on the structural information available from the 1r61 crystal structure, the metal binding site of *trans*-DLH can be proposed. The metal binding site in chain B of the 1r61 crystal structure contains two zinc ions located at a distance of 2.54 Å from each other. The Zn^{2+} at site 1 (Zn1) is coordinated by three protein ligands (imidazole nitrogen atom of H155, and carboxyl oxygen atoms of D51 and E167) and a water molecule. The Zn^{2+} at site 2 (Zn2) is also coordinated by three protein ligands (imidazole nitrogen atoms of H45 and H49 and carboxyl oxygen atom from E167) and a water molecule. In *trans*-DLH, Zn1 may thus be coordinated by H281, D107, E294 and a water molecule, and Zn2 by Q101, Q105, E294 and a water molecule (Fig. 3), with E294 acting as a bridging amino acid. In the 1r61 crystal structure, H55 which corresponds to H111 in *trans*-DLH does not seem to coordinate Zn^{2+} as the minimal distances between the closest imidazole nitrogen atom and Zn1 and Zn2 are 3.6 Å and 3.9 Å, respectively. However, a corresponding histidine residue is conserved among all members of the PF04199 protein family, suggesting a crucial role in enzyme function. Additionally, the absence of metals in the H111A variant along with the decrease in activity by two orders of magnitude compared to the wild type suggests an either direct or indirect role of H111 in metal binding.

In fact, even though no metal ion was localized in the proximity of H55 in the 1r61 structure, coordination of the third metal ion by this residue cannot be excluded. Various well-characterized enzymes contain co-catalytic sites comprising three metals, among them *B. cereus* phospholipase C [15] and *P. citrinum* nuclease P1 [16]

containing three Zn^{2+} ions, *E. coli* alkaline phosphatase [17] containing two Zn^{2+} ions and one Mg^{2+} ion and human inositol monophosphathase with two Mn^{2+} ions and one Mg^{2+} ion [18]. *trans*-DLH is a novel example of this diverse group of enzymes, and current efforts are directed towards elucidation of the role of the different metals for catalysis.

Acknowledgements

This work was supported by the DFG-European Graduate College 653. We thank Dr. Manuel Ferrer from CSIC (Madrid) for his precious collaboration in the ICP analysis and Dr. Melissa Wos-Oxley for critical reading the manuscript.

References

[1] D. Pieper, and W. Reineke, Degradation of chloroaromatics by Pseudomona(d)s, in *Pseudomonas*, in Ramos, J. L., (Ed.), Kluwer Academic / Plenum Publishers, New York, 2004, pp. 509-574.

[2] E. Dorn, and H.-J. Knackmuss, Chemical structure and biodegradability of halogenated aromatic compounds: Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown pseudomonad, *Biochemical J* 174 (1978) 73-84.

[3] E. Schmidt, and H.-J. Knackmuss, Chemical structure and biodegradability of halogenated aromatic compounds: Conversion of chlorinated muconic acids into maleoylacetic acid, *Biochemical J* 192 (1980) 339-347.

[4] S. R. Kaschabek, and W. Reineke, Maleylacetate reductase of *Pseudomonas sp. strain* B13: dechlorination of chloromaleylacetates, metabolites in the degradation of chloroaromatic compounds, *Arch Microbiol* 158 (1992) 412-417.

[5] B. Camara, P. Nikodem, P. Bielecki, R. Bobadilla, H. Junca, and D. H. Pieper, Characterization of a gene cluster involved in 4-chlorocatechol degradation by *Pseudomonas reinekei* MT1, *J Bacteriol* 189 (2009) 1664-1674.

[6] B. Camara, M. Marin, M. Schlomann, H. J. Hecht, H. Junca, and D. H. Pieper, *trans*-Dienelactone hydrolase from *Pseudomonas reinekei* MT1, a novel zinc-dependent hydrolase, *Biochem Biophys Res Commun* 376 (2008) 423-428.

[7] M. Schlömann, E. Schmidt, and H.-J. Knackmuss, Different types of dienelactone hydrolase in 4-fluorobenzoate-utilizing bacteria, *J Bacteriol* 172 (1990) 5112-5118.

[8] A. Bateman, L. Coin, R. Durbin, R. D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, M. Marshall, S. Moxon, E. L. Sonnhammer, D. J. Studholme, C. Yeats, and S. R. Eddy, The Pfam protein families database, *Nucleic Acids Res* 32 (2004) D138-141.

[9] P. Nikodem, V. Hecht, M. Schlömann, and D. H. Pieper, New bacterial pathway for 4-and 5-chlorosalicylate degradation via 4-chlorocatechol and maleylacetate in *Pseudomonas sp*. strain MT1, *J Bacteriol* 185 (2003) 6790-6800.

[10] W. Reineke, and H.-J. Knackmuss, Microbial metabolism of haloaromatics: Isolation and properties of a chlorobenzene-degrading bacterium, *Appl Environ Microbiol* 47 (1984) 395-402.

[11] M. M. Bradford, A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding, *Anal Biochem* 72 (1976) 248-254.

[12] K. S. Larsen, and D. S. Auld, Carboxypeptidase A: mechanism of zinc inhibition, Biochemistry 28 (1989) 9620-9625.

[13] T. Yoshimoto, N. Tanaka, N. Kanada, T. Inoue, Y. Nakajima, M. Haratake, K. T. Nakamura, Y. Xu, and K. Ito, Crystal structures of creatininase reveal the substrate binding site and provide an insight into the catalytic mechanism, *J Mol Biol* 337 (2004) 399-416.

[14] K. A. McCall, C. Huang, and C. A. Fierke, Function and mechanism of zinc metalloenzymes, *J Nutr* 130 (2000) 1437S-1446S.

[15] E. Hough, L. K. Hansen, B. Birknes, K. Jynge, S. Hansen, A. Hordvik, C. Little, E. Dodson, and Z. Derewenda, High-resolution (1.5 A) crystal structure of phospholipase C from *Bacillus cereus*, *Nature* 338 (1989) 357-360.

[16] A. Volbeda, A. Lahm, F. Sakiyama, and D. Suck, Crystal structure of *Penicillium citrinum* P1 nuclease at 2.8 A resolution, *Embo J* 10 (1991) 1607-1618.

[17] E. E. Kim, and H. W. Wyckoff, Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis, *J Mol Biol* 218 (1991) 449-464.

[18] R. Bone, L. Frank, J. P. Springer, and J. R. Atack, Structural studies of metal binding by inositol monophosphatase: evidence for two-metal ion catalysis, *Biochemistry* 33 (1994) 9468-9476.

Legends to figures

Fig. 1. 4-Chlorocatechol degradation pathway of *P. reinekei* MT1. Transformation of 4-chloromuconolactone and of the substrate analogue *trans*-dienelactone which is catalyzed by *trans*-dienelactone hydrolase (depicted in the box). SALOH, salicylate hydroxylase; C12O, catechol 1,2-dioxygenase; MCI, muconate cycloisomerase; *trans*-DLH, *trans*-dienelactone hydrolase; MAR, maleylacetate reducatase. The unstable intermediate 4-chloromuconolactone is depicted in brackets.

Fig. 2. Far-UV spectra of *trans*-DLH and variants. Data was obtained at 25 ºC in phosphate buffer (50 mM, pH 7.3). The protein concentration was 150 μg/mL.

Fig. 3. Proposed metal binding site of *trans*-DLH. The proposed metal binding site is based on the zinc binding pocket of crystal structure 1r61, which contains two zinc ions. In *trans*-DLH, the third metal ion is proposed to be coordinated by His111. Zinc ions are depicted in dark gray and water molecule in light gray.

Table 1

Reactivation of *trans*-DLH with divalent metals. The enzyme activity is expressed as % of the activity of a control prior to incubation with EDTA (1 mM).

Divalent metal Concentration (μM)	Zn^{2+}[a]	Mn^{2+}	Mg^{2+}	Ni^{2+}
0	7.9	9.6	9.6	9.6
2.5	32.8	381.6	27.7	21.7
5	30.6	405.3	ND[b]	ND
10	39.4	408.9	27.6	18.3
25	76.3	ND	ND	16.8
50	107.4	ND	280.1	ND
500	143.9	ND	ND	ND
5000	63.0	ND	ND	ND

[a]Activity was assessed in Bistris (50 mM, pH 6.5).

[b] ND, not determined.

Table 2

Metal content and enzymatic activities of *trans*-DLH and variants.

trans-DLH variants	Metal content (moles/monomer)		Specific activity (U/g)	Relative activity (%)
	Zn	Mn		
trans-DLH	2.7	0.6	15,672 ± 90	100
trans-DLH+Mn^{2+}	0.6	2.2	45,130 ± 90	280
Q101A	1.1	0.1	388 ± 37	2.5
Q105A	1.1	0.1	36 ± 18	0.2
D107A	<0.1	<0.1	65 ± 16	0.4
D107A_H111A	<0.1	<0.1	22 ± 6	0.1
H111A	<0.1	<0.1	35 ± 3	0.2
H281A	<0.1	<0.1	12 ± 7	0.1
E294A	<0.1	<0.1	59 ± 1	0.4