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trans-Dienelactone hydrolase from *Pseudomonas reinekei* MT1, a novel zinc-dependent hydrolase

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ABSTRACT

Pseudomonas reinekei MT1 is capable of growing on 4- and 5-chlorosalicylate, involving a pathway with *trans*-dienelactone hydrolase (*trans*-DLH) as a key enzyme. It acts on 4-chloromuconolactone formed during cycloisomerization of 3-chloromuconate by hydrolyzing it to maleylacetate. The gene encoding this activity was localized, sequenced and expressed in *Escherichia coli.* Inductively coupled plasma mass spectrometry showed that both the wild-type as well as recombinant enzymes contained 2 moles of zinc but variable amounts of manganese/mol of protein subunit. The inactive metal-free apoenzyme could be reactivated by Zn^{2+} or Mn^{2+}. Thus, *trans*-DLH is a Zn^{2+}-dependent hydrolase using halosubstituted muconolactones and *trans*-dienelactone as substrates, where Mn^{2+} can substitute for Zn^{2+}. It is the first member of COG1878 and PF04199 for which a direct physiological function has been reported.

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Catechols are central metabolites of aerobic metabolic pathways of aromatic compounds. Two distinct *ortho*-cleavage pathways have typically been described for the degradation of catechol and chlorocatechols: the catechol branch of the 3-oxoadipate [1] and the chlorocatechol pathway, respectively [2]. These pathways diverge after cycloisomerization of intermediary (chloro)muconates. While dienelactones, which are formed during the metabolism of chlorocatechols, are directly subjected to hydrolysis by dienelactone hydrolases [3], muconolactone, formed during the metabolism of catechol, undergoes a shift of the double bond catalyzed by muconolactone isomerase before it can be hydrolyzed by enol–lactone hydrolases [4].

The most thoroughly investigated dienelactone hydrolase is the enzyme from *Pseudomonas knackmussii* B13. This protein belongs to the α/β hydrolase fold enzymes [5] and is active against both the *cis*- and *trans*-isomer of dienelactone [6], a feature which is shared with other dienelactone hydrolases such as TfdEI of *Cupriavidus necator* JMP134 [7]. Protein sequence alignments have shown that these dienelactone hydrolases involved in chlorocatechol metabolism all share a conserved cys-his-asp triad and highly conserved flanking residues and all belong to the same α/β hydrolase fold family, termed dienelactone hydrolase family [7]. This protein family also comprises dienelactone hydrolases with substrate specificity restricted to the *cis*-isomer such as the ClcD1 and ClcD2 dienelactone hydrolases from *Rhodococcus opacus* 1CP [8,9]. Enol–lactone hydrolases share the α/β-hydrolase fold with dienelactone hydrolases, even though the sequences are quite dissimilar [3].

There is some evidence that lactone hydrolases, which are completely different in mechanism, could be involved in aromatic degradation. Recently, 4-sulfomuconolactone hydrolases of the sulfanilate metabolic pathway were suggested to belong to the amidohydrolase superfamily [10]. Also *cis*-DLH from *Burkholderia cepacia* [11] seems to employ a hydrolytic mechanism different from that of dienelactone hydrolases of chloroaromatic pathways.

In *Pseudomonas reinekei* MT1, which is able to degrade 4- and 5-chlorosalicylate, a dienelactone hydrolase capable of transforming the *trans*- but not the *cis*-isomer (*trans*-DLH) has been observed [12] (see Fig. 1). The enzyme was crucial for the degradation of chlorosalicylates by this strain, as it interacts with the muconate cycloisomerase-catalyzed transformation of 3-chloromuconate. It was proposed that *trans*-DLH hydrolyzes the cycloisomerization product 4-chloromuconolactone, and thereby prevents the formation of protoanemonin in favor of maleylacetate (Fig. 1), which can be further metabolized by maleylacetate reductase. The N-terminal sequence derived from the purified *trans*-DLH of strain MT1 showed no significant similarity with previously identified dienelactone hydrolases or enol–lactone hydrolases nor to any other

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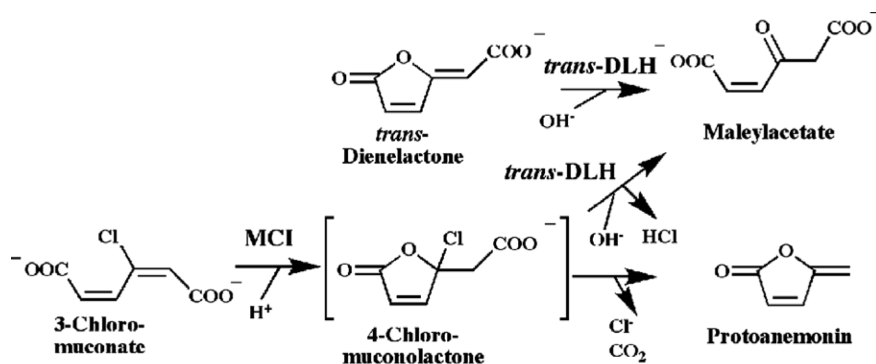

Fig. 1. Transformation of *trans*-dienelactone and 4-chloromuconolactone by *trans*-dienelactone hydrolase (*trans*-DLH). MCI, muconate cycloisomerase. 4-Chloromuconolactone as supposedly unstable intermediate is shown in brackets.

protein of known function reported in databases [12]. In the present study the gene encoding *P. reinekei* MT1 *trans*-DLH was localized and the protein overexpressed in *Escherichia coli*. Evidence is given to suggest that *trans*-DLH belongs to a poorly characterized protein family of putative metal dependent hydrolases.

Materials and methods

Bacterial strain and gene identification. Pseudomonas reinekei MT1 was grown and cell extracts prepared as previously described [12]. The gene fragment encoding the N-terminal protein sequence of *trans*-DLH was amplified by touchdown PCR (annealing temperatures of 60–50 °C) using primers YTNCCNACNTAYAAGCA and CCNGGNGGNGCRTCTTT, which were designed based on the previously determined N-terminal protein sequence (underlined) TDTSKSLPTYKQLLERKDAPPGSSWGLFGK [12]. A single 47 bp fragment was obtained, the deduced amino acid sequence of which matched that obtained by N-terminal amino acid sequencing. An extended fragment was amplified (annealing temperature of 55 °C) using primers AGTTGTTGGAGCGCAAAG and ACNGCR TCNCCNGGTTC, which were designed based on the DNA sequence obtained above and on the previously identified internal peptide sequence VSQGVTLEPGDAVLLR [12] (underlined). The deduced protein sequence of the obtained approximately 550 bp fragment matched those of the determined N-terminus and the internal peptide sequence. Regions flanking the obtained fragment were obtained by various cycles of arbitrary PCR [13].

Recombinant expression of trans-DLH. The *trans*-DLH encoding gene (*tdlh*) was amplified from genomic DNA of *P. reinekei* MT1 using primers GAAACCATGCGTGAGGACTT and ATTTGAGACCGCT GGTTTTG. The obtained 1305 bp fragment was ligated into pGEM-T Easy (Promega) and ligation products were transformed into ultracompetent *E. coli* XL10-Gold (Stratagene). A clone expressing high levels of activity against *trans*-dienelactone termed *E. coli* XL10-Gold (pTDLH16) was selected and the integrity of the insert verified by sequencing. The strain was grown at 37 °C in LB medium containing 200 μg ampicillin ml^{-1}. For induction, 0.5 mM IPTG was added when cultures reached an $A_{600nm} = 0.6$ and harvested after 3 h of incubation at 30 °C.

For preparing a strep-tagged fusion, the *tdlh* coding sequence was amplified using primers TACGTCGGTCTCAGCGCATGACTGACA CGTCCAAATCCC and TACGTCGGTCTCATATCATTATCTGATTGCGATG GCGTTTG, cloned via the BsaI restriction sites (underlined) into plasmid pASK-IBA7plus (IBA) and the ligation product transformed into *E. coli* DH5α (Invitrogen). A clone expressing strep-tagged *trans*-DLH (*E. coli* DH5α (pASKtdlh)) was selected and the integrity of the cloned gene verified by sequencing. The strain was grown at 37 °C in LB medium containing 100 μg ampicillin ml^{-1}. Cells were induced with 0.2 μg/ml anhydrotetracyclin at an $A_{600nm} = 0.6$ and harvested after 3 h of incubation at 30 °C.

Enzyme purification. Recombinant and wild-type *trans*-DLH were purified from cell extracts of *E. coli* XL10-Gold (pTDLH16) and from *P. reinekei* MT1 pregrown on 5-chlorosalicylate using a FPLC system (Amersham Biosciences) essentially as previously described for the wild-type protein by subsequent anionic exchange (MonoQ HR 10/10), hydrophobic interaction (SOURCE 15PHE PE 4.6/100), gelfiltration (Superose 12 HR10/10) and anionic exchange chromatography (MonoQ HR 5/5) [12]. The strep-tagged recombinant protein was purified from *E. coli* DH5α(pASKtdlh) cell extracts using a Strep-Tactin Sepharose column (IBA). After application of cell extracts, the column was 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) using a protean III mini gel system (Bio-Rad) as previously described [12]. The proteins were stained with Coomassie brilliant blue (Serva). PageRuler Protein Ladder (Fermentas) was used as a marker.

Biochemical studies. trans-DLH activity was determined by following the depletion of *trans*-dienelactone (50 μM; $\varepsilon_{280} = 15{,}625\ M^{-1}\ cm^{-1}$) at 280 nm in 50 mM Tris/HCl buffer (pH 7.5) [12]. *trans*-Dienelactone was prepared as described earlier [14]. 1U is defined as the activity transforming 1 μmol of *trans*-DLH per minute (substrate concentration of 50 μM). Standard protein quantification was performed using the method of Bradford [15]. Quantification of *trans*-DLH in purified fractions was performed by Ruthenium II tris (bathophenantroline disulfonate) staining as previously described [16].

The effect of EDTA on the enzyme activity was measured as previously described [12]. The effect of temperature on activity was assessed in Tris/HCl buffer (50 mM, pH 7.5) at temperatures from 20–68 °C. The effect of pH on enzyme activity was assayed at 20 °C in 10 mM succinate/NaOH (pH 4–5), histidine/HCl (pH 5.5–7), BisTris/HCl (pH 7), Tris/HCl (pH 7.5–9) and CAPS buffer (pH 9–10.5). For stability measurements, enzyme (0.4 μg/ml) was kept at 20 °C in the buffers mentioned above.

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). For reactivation, the apoenzyme (0.5–1 μg/ml) was incubated with divalent metals (2.5 μM–5 mM) for 5 min, followed by activity determination.

Analysis of metals. To determine the metal content, proteins were concentrated using Vivaspin (500 μl) filter units and washed using metal free Tris/HCl buffer (50 mM, pH 7.5). Proteins (25–300 μg) and the eluate from the final concentration step were both

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 hydrolyze the protein and release metal ions.

Sequence analysis and nucleotide sequence accession number. Protein similarity searches were performed by PSI-BLASTN using default parameters and public databases (http://www.ncbi.nlm.nih.gov/ and http://srs.ebi.ac.uk/). DNA and protein alignments were conducted with ClustalW. Phylogenetic analyses were performed with MEGA [17], by using the neighbor-joining method [18] and pairwise deletion of gaps. A consensus tree was inferred from a total of 100 bootstrap trees generated.

The nucleotide sequence reported in this study was deposited in the DDBJ/EMBL/GenBank databases under the Accession No. FJ184062.

Results

Identification of the trans-DLH gene

The gene encoding *trans*-DLH involved in the degradation of 4- and 5-chlorosalicylate by *P. reinekei* MT1 was identified. The deduced N-terminus (TDTSKSLPTYKQLLERKDAPPGSSWGLFGK) of the identified gene was identical to the N-terminus previously described [12]. The gene comprises 1011 bp and encodes a protein of 336 amino acids, with a calculated molecular mass of 36.935 kDa. The deduced protein did not show similarity with any protein of assigned function described in public databases. Similarity was observed with proteins belonging to COG1878 (http://www.ncbi.nlm.nih.gov/COG/) and PF04199 (http://www.sanger.ac.uk/Software/Pfam/), which are described as putative cyclases or putative metal-dependent hydrolases.

Expression and purification of the recombinant enzyme

To verify that the identified gene encodes an active *trans*-DLH, a fragment comprising *tdlh* was inserted into pGEM-T easy giving pTDLH16. Cell extracts of IPTG-induced *E. coli* XL10-Gold (pTDLH16) exhibited the presence of a prominent protein band with an apparent M_r of 37 kDa (Fig. 2), being in close agreement with that of the deduced *tdlh* gene product but also with the wild-type enzyme [12]. The respective protein was absent in cells not subjected to IPTG induction or devoid of pTDLH16. Activity determinations using *trans*-dienelactone as substrate in cell extracts of *E. coli* XL10-Gold (pTDLH16) showed that a functional *trans*-DLH was encoded, as evidenced by an activity of 8000 ± 500 U/g protein in IPTG induced cells, contrasting those observed in extracts not subject to IPTG induction (75 ± 7 U/g protein) or extracts of control *E. coli* XL10-Gold cells (<5 U/g protein).

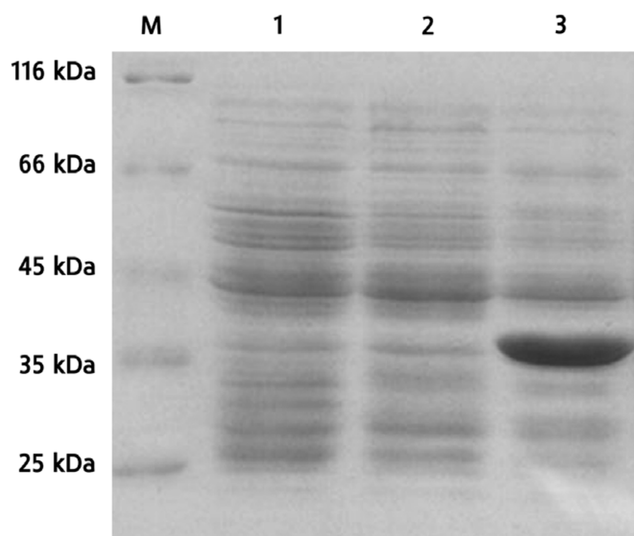

Fig. 2. SDS–PAGE of cell extracts of *E. coli* XL10-Gold expressing *trans*-dienelactone hydrolase. *Lane M*, molecular mass standard; *lane 1*, *E. coli* XL10-Gold; *lane 2*, *E. coli* XL10-Gold (pTDLH16); *lane 3*, *E. coli* XL10-Gold (pTDLH16) induced with IPTG.

The recombinant *trans*-DLH was purified to apparent homogeneity giving a preparation with a specific activity of 34,000 ± 3000 U/g protein. Kinetic measurements indicated a K_m value of 550 ± 50 μM as observed for the wild-type, indicating a k_{cat} value of 15,100 s^{-1}.

Biochemical properties and effect of chemical reagents on the recombinant enzyme

The pH optimum of recombinant *trans*-DLH was determined as 6.0 and highest activity was observed at temperatures of 55 ± 5 °C, 220% of that determined under standard conditions (20 °C). The enzyme was highly stable at pH 7 and >80% of activity could be recovered after storage for 24 h at 20 °C. As indicated for the wild-type enzyme [12], recombinant *trans*-DLH was inactivated by EDTA and almost quantitative inactivation was observed after treatment with 10 mM EDTA for 180 min (<2% of initial activity).

Metal dependence of trans-DLH

As *trans*-DLH showed similarity with putative metal-dependent hydrolases, the metal ion content of the purified recombinant enzyme was determined by ICP-MS. Of the metals analyzed (Mn^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}), only Zn^{2+} and Mn^{2+} were observed in amounts exceeding the control (Table 1) indicating each enzyme subunit to contain 2 Zn^{2+} and 1 Mn^{2+} ion.

The purified wild-type protein showed an activity of 65,000 ± 5000 U/g protein, slightly higher compared to that of the recombinant protein and contained, similar to recombinant *trans*-DLH, 2.4 moles of Zn^{2+} but 2.0 moles of Mn^{2+}, confirming that the protein also contains 2 Zn^{2+} ions per monomer (Table 1). However, significant amounts of Ni^{2+} and Mg^{2+} were also observed in purified wild-type *trans*-DLH.

To evaluate the metal dependence of *trans*-DLH, a strep-tagged protein was purified. The fusion protein exhibited an activity of 29,000 ± 500 U/g protein, similar to that of the untagged recombinant protein. ICP-MS analysis showed strep-tagged *trans*-DLH to be practically free of either Mn^{2+} (<0.0003 moles/mole of subunit) or Mg^{2+} (<0.04 moles/mol of subunit), indicating that the protein does not necessitate manganese ions for activity. However, the protein contained 1.9 ± 0.2 moles of Zn^{2+} as well as 1.1 ± 0.1 moles of Ni^{2+} per mol of subunit (Table 1). This proves that active *trans*-DLH contains 2 moles of Zn^{2+} per subunit but variable amounts of Ni^{2+} and Mn^{2+}.

To assess if Zn^{2+} is necessary for activity, metal-free apoenzyme was prepared by incubation of strep-tagged protein with EDTA. The obtained enzyme showed a residual activity <1% that of the native strep-tagged protein. According with the negligible activity, the respective enzyme preparation was shown to be practically free of Mn^{2+}, Mg^{2+} Zn^{2+} or Ni^{2+}, all being below the detection limit (<0.01 moles/mol of subunit).

Reactivation of metal-free apoenzyme was attempted by incubation of apoenzyme with Zn^{2+}, Mn^{2+}, or Ni^{2+}. Incubation in the presence of Zn^{2+} (2.5–10 μM) resulted in nearly complete reactivation (74 ± 7%). However, higher concentrations of Zn^{2+} were shown to be inhibitory, and only 30% of activity was recovered after incubation with 0.5 mM Zn^{2+}. Incubation with Mn^{2+} resulted in activities exceeding those of the native strep-tagged

Table 1
Metal content and activity of *trans*-dienelactone hydrolase

	Metal content (moles/mol of subunit)			Specific activity (U/g)
Enzyme source	Zn^{2+}	Mn^{2+}	Ni^{2+}	
Wild-type	2.4 ± 0.2	2.0 ± 0.2	1.1 ± 0.1	65,000 ± 5000
Recombinant	2.3 ± 0.2	0.8 ± 0.1	<0.1	34,000 ± 3000
Strep-tagged	1.9 ± 0.2	<0.1	1.1 ± 0.1	29,000 ± 500

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Q28QQ7_JANSC    60 ETLTVSTHNGTHLDAPYHHH    207 GHRASM-TTGYCHMEKLS
A3VAL7_9RHOB    65 EDLQITTHVGTHMDAPWHYH    212 GHKAGA-ETVYCHMEKLH
A7IHZ7_XANP2    65 ELLHVSAHNGTHMDAPWHYA    212 GHKAGR-DIGYGQMEKLT
Q65N24_BACLD    61 EKVKMSTHAGTHIDAPYHYR    208 GHYSGS-TIPYCQIEKLA
A8MJJ2_ALKOO    58 EFIKLSTHAGTHIDAPYHYY    205 GHRAGA-EKAYCHIEKLT
Q3INL7_NATPD    64 EEIEAITHTGTHMDAPYHYG    210 AHLAGR-EREYCQIEKMA
A5UTI1_ROSS1    54 DQVTIRAHAGTHVDAPWHYY    200 AHRVGR-DLEYCHIEKLA
O68500_STRPE    60 DRLTLTSHTGTHIDAPSHYG    220 AHVTGR-HREYCQIERLG
Q7MZT2_PHOLL    77 MYYRLTTHTGTHIDAPYHYG    221 SHFIGR-DHPYLQIERLC
Q39ZN2_GEOMG    40 TRITMTTHSGTHLDVPGHCR    158 VHRMLL-DAGVVILEGIT
Q3ZX16_DEHSC    41 SRLTMTLHNGSHIDAPHHFF    159 VHKHLL-SQGVVILETLQ
Q3Z8N2_DEHE1    41 GQLTMTLHNGSHIDAPLHFF    159 VHKHLL-SRGVVILESLQ
Q9HK78_THEAC    45 ETISGSTHSGTHIDAPAHML    168 VHKKLL-SKGIPFIEDLY
Q41RX4_FERAC    42 EKFETVTHTGTHIDAPYHMI    165 VHKALL-SKNMVFIEDLA
A4YGB6_METS5    42 ETISFATHTGTHIDAPYHFD    165 VHKKLL-SSGIVVIEDLA
P84132_1R61_A   38 SRIDMDVHTGTHIDAPLHMV    154 THKTLF-SAGVIIIEGLR
A1S0S8_THEPD    48 NLLMLVEHTGTHVDSPAHFI    170 GHKILL-PKGIVIFENLT
Q92NP5_RHIME    86 FELRVNEHTGTHVDAPLHFS    224 AHYAWL-PEGRWGLEAAA
Q8U6N8_AGRT5    54 HKLTIYEHTGTHIDAPFHFS    190 VHNSWL-PAGRYGIEGLN
A5USL5_ROSS1    87 SILTYWEHSGTHMDAPVHFA    226 VHYTIL-PTNRWGLENLA
Q1Q6J8_9BACT    74 GKYSTPEHLGTHIDAPNHFE    213 VHHIIN-GAGKYILENVA
Q8PT92_METMA    45 YKICMSNHIGTHIDAPAHFV    164 AHKEFA-GNGVVVIEGVN
Q020S6_SOLUE    55 DAIAMGSHLGTHIDALCHFS    201 VHVHLLVESGIHIIECLN
A0QZD9_MYCS2    58 EMLVLGGHVGTHMDALAHVA    209 VHRILLQDSGMNIIEAMN
Q1ATE3_RUBXD    55 GVIICMEHTGTHIDALCHQS    213 GHLILLARHGIYIIENLM
A4QR17_MAGGR    96 DMWTFNSQCSSQWDGFRHYA    273 LHEWLLAGWGMPIGELFE
Q2UAX8_ASPOR    95 DELHFNTQKSSQVDGLRHAA    275 YHEVLLAGWGCPIGELLW
Q2GX58_CHAGB    98 DELEFNTQFSSQWDSLCHVT    280 LHPWFLNQFGMSIGELWD
Q4IAL9_GIBZE   106 DEVSFNTQCSSQWDSLCHFQ    289 LHVYCLSLFGMPIGELWD
Q1DS23_COCIM   125 DKYELNTQSGTQWDGFRHFA    313 LHEYILALWGMPLGEMWD
Q0UE64_PHANO   121 DEVYINTQAGSQWDSLKHFA    293 LHEWLLVHWGTPIGEMWD
Trans-DLH       94 WLDNFYTQYGSQIDGLRHIG    280 MHRAIIPLLGMPIGELWA
Q0S164_RHOSR    87 YLDGFWPQAASHLDGLRHRR    264 MHQELIALLGLPIGELWK
Q2DBC1_ACICY   121 DLAILHLQYSTQWDALSHVG    316 LHEHCLFKNGIHLGELWH
Q395M9_BURS3    99 DAFCLHSQFSTQWDALSHVG    284 LHALCLFKLGIHLGELWH
Q13XF5_BURXL    99 DAVLLSTQYSTQWDSLAHVG    286 IHEHCLFKLGVPLGEQWL
A0PL48_MYCUA   116 DMIIMPLQAATQWDALSHVY    274 LHLLCLRDMGLMLGEYWD
Q93RT4_STRCO   103 DVIAMPLQCSTQWDGLGHIF    264 LHQVAIPHIGLLIGEMWD
A0QZJ6_MYCS2   111 DAVSMPLQCGTQWDALAHIF    271 LHLILLANAGMTIGEIFD
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Fig. 3. Multiple sequence alignment of protein regions of *trans*-DLH and selected members of PF04199 (TrEMBL entry names are given) comprising the conserved QXXXQXDXXXH or HXGTHXDXPXH signatures (left block) or conserved H and E residues (right block). Conserved residues are shown in bold.

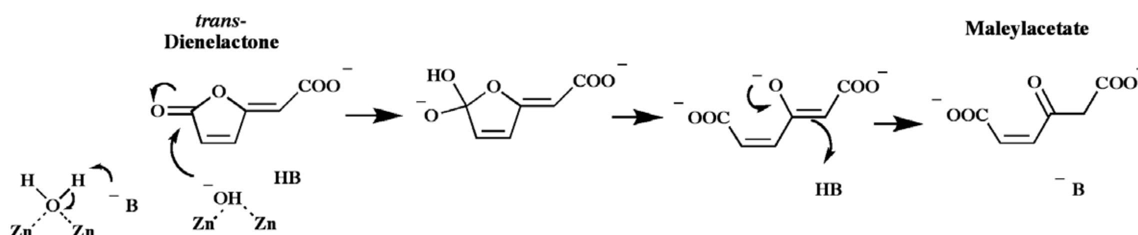

Fig. 4. Putative catalytic mechanism of *trans*-DLH assuming a general base acid catalysis (with B indicating the general base) and attack by zinc-bound water.

enzyme (380 ± 50%) while reactivation could not be achieved in the presence of Ni^{2+}.

Discussion

A novel type of hydrolase, which is involved in the degradation of chlorosalicylates [12] has been characterized from *P. reinekei* MT1. PSI-BLAST searches using default parameters starting with the *trans*-DLH protein converged to about 300 proteins, many of which were annotated as putative cyclases or putative metal dependent hydrolases (see Supplementary Material). Conserved Domain Database [19] searches suggested that these proteins form a family that is classified as COG 1878 in the Clusters of Orthologous Groups database [20] and as PF04199 in the Pfam database [21].

Information exceeding sequence information is only available for two proteins of this Pfam. One is the product of the *Streptomyces peucetius dpsY* gene, which is part of the doxorubicin biosynthesis gene cluster and was proposed to be a polyketide cyclase responsible for the formation of 12-deoxyaklanonic acid [22]. The second protein is one of unknown function from *Bacillus stearothermophilum* for which the structure was elucidated (Maderova et al. unpublished). The fold of this protein (PDBID=1r61, Accession No. P84132), and thus also of the other proteins belonging to PF01499, was found to be different from the αβ hydrolases and PF01499 proteins were shown to contain the signature motif HXGTHXDXPXH. In the protein from *B. stearothermophilum* this motif comprises residues 45–55 and partially occupied Zn ions are coordinated by H45 and H49 of this motif together with E167, and by D51 together with H155 and E167, respectively. Alignment of the sequences obtained by PSI-BLAST searches showed that in a subgroup of 67 sequences (including *trans*-DLH, see Supplementary Material) the HXGTHXDXPXH motif is replaced by QXXXQXDXXXH while D51, H155 and E167 are conserved almost exclusively in both groups, making it likely that they may have a functional role (Fig. 3).

The presence of two Zn ions per monomer in either wild-type, recombinant or strep-tagged protein, the loss of activity in the presence of EDTA, and its reactivation by Zn indicate that *trans*-DLH is a Zn-dependent hydrolase. Although aspartate and histidine residues predominate as ligands in cocatalytic zinc sizes [23], glutamate [23] and glutamine residues [24] have also been reported. Based on the 1r61 structure, it can thus be speculated that *trans*-DLH contains a binuclear zinc-binding site with Q101, Q105, D107, H281, and E294 as coordinating ligands. However, further experiments need to be performed to exclude a purely structural role of Zn and to verify metal ligating residues.

Metal-bound water molecules are known as good nucleophiles from many well-studied hydrolases, e.g. the zinc peptidases [25] and proteases, or carboanhydrases [26] supporting the notion that the nucleophile used by *trans*-DLH is a zinc-bound hydroxide ion which is stabilized by the Lewis-acidity of the zinc ion (Fig. 4).

Although two moles of Zn^{2+} per mole of *trans*-DLH subunit were observed in all protein purifications reported here, suggesting this metal to be natively incorporated, varying amounts of Mn^{2+} and Ni^{2+} were also observed indicating the presence of additional metal-binding sites in the protein. Such presence of additional metals is not unusual, and some zinc enzymes that catalyze phosphomonoester hydrolysis have cocatalytic zinc sites containing three metal atoms [23]. Analyses on leucine aminopeptidase of *Streptomyces griseus,* which contains two Zn^{2+} in its active site showed that the metal-free apoenzyme can be reactivated by Zn^{2+} and Mn^{2+} [27] and it is well established that Zn^{2+} can be replaced by many other transition ions in various metal dependent hydrolases [28]. However, the activity as well as the stability of *S. griseus* aminopeptidase was in addition modulated by Ca^{2+} [27], which is obviously due to two different Ca^{2+} binding sites [29]. Interestingly, like *trans*-DLH, also leucine aminopeptidase was inactivated by an excess of Zn^{2+} whereas excessive concentrations of Mn^{2+} do not inhibit [27]. No further information on the mechanism of Zn^{2+} inhibition is available. However, for various other zinc-dependent enzymes, inhibition by zinc due to binding to a low-affinity binding site has been reported [30,31]. To what extent the different metal binding sites and bound metals contribute to structure and activity of *trans*-DLH is currently under investigation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.006.

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