ElaC encodes a novel binuclear zinc phosphodiesterase

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Running title: ElaC encodes a novel binuclear zinc phosphodiesterase
SUMMARY

ElaC is a widespread gene found in eubacteria, archaebacteria, and mammals with a highly conserved sequence. Two human ElaC variants were recently associated with cancer (Tavtigian, S. V. et al. (2001) Nat. Genet. 27, 172-80; Yanaihara, N. et al. (2001) Genomics 72, 169-79). Analysis of the primary sequence indicates homology to an arylsulfatase and predicts a metallo-β-lactamase fold. At present, no ElaC gene product has been investigated. We cloned the *Escherichia coli* ElaC gene and purified the recombinant gene product. An enzymatic analysis showed that ElaC does not encode an arylsulfatase, but rather a phosphodiesterase which hydrolyzes bis(p-nitrophenyl)phosphate with a $k_{cat}$ of 59 s$^{-1}$ and $K'$ of 4 mM. Kinetic analysis of the dimeric enzyme revealed positive cooperativity for the substrate bis(p-nitrophenyl)phosphate with a Hill coefficient of 1.6, whereas hydrolysis of the substrate thymidine-5’-p-nitrophenylphosphate followed Michaelis-Menten kinetics. Furthermore, the enzyme is capable of binding two zinc or two iron ions. However, it displays phosphodiesterase activity only in the zinc form. The metal environment characterized by zinc K-edge X-ray absorption spectroscopy was modeled with 2 histidine residues, 1 carboxylate group, and 1.5 oxygen atoms. This corresponds to the coordination found in other metallo-β-lactamase domain proteins. Phosphodiesterase activity is strongly dependent on the presence of zinc. These results identify the currently unassigned gene product ElaC to be a novel binuclear zinc phosphodiesterase (ZiPD).

**Key Words**: metallo-β-lactamase, binuclear zinc enzyme, phosphodiesterase, EXAFS, PIXE, substrate screen
INTRODUCTION

The rapidly increasing number of proteins identified as containing a metallo-β-lactamase fold catalyze a variety of diverse reactions. The members of this group which have been functionally and structurally characterized are the hydrolytic enzymes β-lactamase (1) and glyoxalase II (2), as well as the redoxenzyme rubredoxin:oxygen oxidoreductase (ROO) from Desulfovibrio gigas (3). Whereas β-lactamases were shown to be active with one or two zinc atoms (4, 5), active glyoxalase II requires two zinc atoms (2) or a mixed zinc/iron binuclear active site (6). In contrast to the zinc β-lactamase domain enzymes, ROO contains two iron atoms in its β-lactamase domain and an additional flavodoxin domain (3). A large number of hypothetical enzymes found in GenBank (7, 8) might also share this fold and bind one or two metals, but most of the enzymes are not yet functionally or structurally characterized.

One of the hypothetical metallo-β-lactamase domain proteins is encoded by the ElaC gene (third open reading frame in the ela-locus) in Escherichia coli (GenBank accession number U58768). Based on weak sequence homology (35 % with 32 % gaps) to a functionally characterized arylsulfatase from Alteromonas carrageenovora (9), it was suggested that ElaC could also exert this function (10). Stronger sequence homology, up to 50 %, was found to two human genes, named ElaC1 and ElaC2. Genetic studies revealed an association with cancer for both human genes. ElaC2 has been identified as a prostate cancer susceptibility gene (11, 12) and ElaC1 (named D29 in that study) as a candidate lung tumor suppressor (13). ElaC2 appears to be unique to eukaryotes, whereas ElaC1 orthologs were found in eukaryotes, eubacteria, and archaeabacteria (12). A study of mRNA expression revealed that both human proteins were expressed in all tissues analyzed (12, 13). Gene disruption experiments in Saccharomyces cerevisiae indicated that ElaC2 is an essential gene (12). The
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Evolutionary conservation of the ElaC family, together with its ubiquitous expression and the essential function of *S. cerevisiae* ElaC2, emphasizes that the functions of the gene products are of fundamental biological interest.

No ElaC gene product has previously been characterized. As the first member of this widespread and highly conserved group we purified and functionally characterized the *E. coli* ElaC gene product. We have shown that this protein is not an arylsulfatase, but a phosphodiesterase. The phosphodiesterase activity is strongly correlated with the zinc content of the enzyme. X-ray absorption spectroscopy provided the first structural model of the active site, which is in agreement with the metal coordination found in metallo-β-lactamase domain proteins. Since no explanation has been published for the abbreviation ElaC, we name this novel type of binuclear metallohydrolase ZiPD (zinc phosphodiesterase).

**EXPERIMENTAL PROCEDURES**

**Materials**

Except when stated otherwise, all proteins and fine chemicals were purchased from Sigma (Deisenhofen, Germany). Restriction enzymes were from New England Biolabs (Frankfurt, Germany). Oligonucleotides were synthesized by Genset (Paris, France). DNA-sequencing was performed by MWG (Ebersberg, Germany).

**Cloning and expression of ZiPD**

ZiPD was cloned from *E. coli* DH5α genomic DNA by polymerase chain reaction (PCR) using primers derived from the ElaC gene (5ZiPD: 5’-GAACATCATGAACCTAATTATTTTTTGGTACT-3’, 3ZiPD: 5’-TAATAACTCGAGTTAACCCTAAACCAGGT-3’) utilizing the translation initiation and termination sites specified in GenBank entry U58768 and introducing restriction sites for
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BspHI and XhoI (underlined), respectively. The PCR was performed with Pfu-polymerase (Promega, Heidelberg, Germany). The PCR product was digested with BspHI and XhoI and cloned into the NcoI and XhoI sites of a modified pET24d vector (Novagen), which contains an N-terminal 6xHis-tag and a TEV cleavage site, yielding pETM-ZiPD. DNA sequencing confirmed that the sequence was identical to that of the gene identifier b2268 under GenBank accession number NC_000913. For expression of ZiPD, BL21(DE3) cells harboring pETM-ZiPD were grown in Luria-Bertani medium to an OD_{600} = 0.6 followed by induction with 0.1 mM isopropylthiogalactopyranoside at 25°C for 16 hours. The recombinant protein was purified with Ni-NTA agarose (Qiagen, Hilden, Germany). The eluted fraction from the Ni-NTA column was further purified by gelfiltration using a Superdex 200 26/60 column (Amersham Pharmacia Biotech, Freiburg, Germany) equilibrated with 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM DTT. The main peak of the recombinant protein eluted at a volume corresponding to a dimer of approximately 70 kDa. The pooled fractions from the gel filtration column yielded the enzyme as isolated (a.i.-ZiPD). The concentration of purified ZiPD was determined using the calculated extinction coefficient of E_{280 nm}^{1%} = 6.7 and a molecular mass of 36.04 kDa.

**Metal incubation**

The apoenzyme (apo-ZiPD) was obtained after incubation of a.i.-ZiPD with 10 mM EDTA for 1 hour at room temperature and subsequent dialysis of the EDTA-metal complex and excess EDTA. Apo-ZiPD was saturated with metals by addition of 0.2 mM Zn^{2+} or Fe^{2+}. Iron incubation was performed in degassed buffer and the presence of 2 mM dithionite. After one hour incubation at room temperature, excess metal was removed by extensive dialysis against metal free buffer (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM DTT). Buffers were treated with Chelex100 (Biorad, München, Germany) to remove trace metals. This yielded the zinc
ElaC encodes a novel binuclear zinc phosphodiesterase and iron saturated ZiPD preparations, Zn-ZiPD and Fe-ZiPD respectively, which were used for subsequent analysis.

**Metal analysis**

*Proton induced X-ray emission*

The metal content of different ZiPD samples was determined by detection of the proton induced X-ray emission (PIXE) at the Hamburg 2 MeV proton microprobe (14). Using this technique, all elements apart from those lighter than sodium can be detected in a single scan at a minimum limit of about one to ten parts per million by weight. Its application to protein analysis was reviewed by Garman (15). A particular advantage of this method is the ability to measure the sulfur content, which serves as an internal standard to determine the protein content of the samples. For the ZiPD samples the number of sulfur atoms was derived from the encoded sequence in pETM-ZiPD which contains 6 cysteine and 6 methionine residues. Protein samples were extensively dialyzed against Chelex100 treated buffer composed of 20 mM Tris/acetic acid pH 7.4, 100 mM NaNO₃ to remove chloride and sulfur compounds which disturb the protein sulfur signal. The samples (10 – 15 µg) were dropped onto sample holders covered with a 1.5 µm polycarbonate backing foil. With the proton microbeam, sample areas of a few mm² were scanned and the characteristic X-rays were detected using a Si(Li) detector. A proton current of a few hundred picoamperes was applied for about one hour, ensuring that no elemental loss (sulfur or metals) due to thermal stress occured which would otherwise change the X-ray signal intensity.

The unknown number of metals per molecule was calculated from their calibrated signals with reference to the calibrated sulfur signal. The overall accuracy was estimated taking into account the statistical errors of the X-ray yield and the uncertainty in the correction for X-ray absorption within the samples.
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**Inductively coupled plasma mass spectrometry**

The metal content of Zn-ZiPD was additionally analyzed using an inductively coupled plasma mass spectrometer (ICP-MS HP 4500, Hewlett-Packard, Waldbronn, Germany), equipped with a PFA µ-flow nebulizer (100 µL/min self-aspirating) and a cooled Scott spray chamber. Four isotopes ($^{64}\text{Zn}$, $^{66}\text{Zn}$, $^{67}\text{Zn}$, and $^{68}\text{Zn}$) were simultaneously monitored in order to recognize any spectral interference, but no deviations between the isotopes could be observed. Release of the metal ions from Zn-ZiPD took place by acidification with concentrated nitric acid. The total zinc content was determined by the standard addition method (N=3). In order to check the zinc distribution in the protein sample, the ICP-MS was on-line coupled to size-exclusion chromatographic equipment (TSK Gel 3000PWXL 150 mm x 4.6 mm i.d., TosoHaas, Stuttgart, Germany). The chromatographic conditions (1 ml/min Tris/HCl, pH = 7.5) in combination with the element-selective detection enables a separation of protein-bound and free zinc.

**X-ray absorption spectroscopy**

The metal coordination was determined by X-ray absorption spectroscopy. The extended X-ray fine structure (EXAFS) provides element specific local structural information (16, 17). Zn-ZiPD in 50 mM Tris/HCl pH 7.4, 15 % glycerol was concentrated to 1-2 mM using Millipore concentrators with a 10,000 molecular weight cut off. Sample holders covered with Kapton windows were filled with this protein solution and were frozen in liquid nitrogen. During measurement the sample was cooled to about 30 K. X-ray absorption spectra at the Zn K-edge were recorded at the EMBL bending magnet beamline D2 (DESY, Hamburg, Germany) utilizing a Si(111) double crystal monochromator, a focusing mirror and a 13 element Ge solid-state fluorescence detector (Canberra). The energy axis of each scan was calibrated by using the Bragg reflections of a static Si(220) crystal in back reflection geometry (18). Data reduction, including averaging of the 30 scans, was performed with the
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EXPROG software package (C. Hermes and H. F. Nolting, EMBL-Hamburg) using $E_0, Zn = 9660 eV$. The EXAFS spectrum was analyzed with EXCURV98 applying constrained refinement (19). Ligand types as well as coordination numbers were varied manually, whereas the distances, the Debye-Waller factors, and the Fermi energy offset were refined.

Enzymatic assays

Screen with $\beta$-lactamase domain protein substrates

Activity tests were performed in 200 µl buffer (20 mM Tris pH 7.4 or sodium acetate pH 5.0, 150 mM NaCl) with the a.i.-, apo-, Zn, and Fe-ZiPD (50 ng) preparations. Hydrolysis of Nitrocefin (100 µM) (Calbiochem, Schwalbach, Germany), a $\beta$-lactamase substrate, was detected by a color change to red. Liberation of $p$-nitrophenol from $p$-nitrophenylsulfate and $p$-nitrophenylphosphate (10 mM) was detected by a yellow color, hydrolysis of $p$-nitrophenylsulfate (10 mM) by a color change from yellow to red. Glycosulfatase activity was probed with glucose-6 sulfate as previously described (20). Glyoxalase II activity using the substrate S-D-lactoylglutathione (0.5 mM) was monitored at 240 nm. In order to verify the correct setup of the tests, positive controls were conducted with $\beta$-lactamase from Bacillus cereus, arylsulfatase from Aerobacter aerogenes (both from Sigma), and glyoxalase II-2 from Arabidopsis thaliana, kindly provided by C. Makaroff (Miami University, Ohio). All reactions were carried out at room temperature and monitored for two hours.

Random substrate screen

A random substrate screen was performed using Taxa Profile E plates (Merlin, Bornheim-Hersel, Germany). These plates, originally designed for microorganism classification and identification, contain different chromogenic substrates and were used in this study to identify the enzymatic function of ZiPD. Each reaction was set up twice on the top and bottom halves of a 384-well plate, enabling two identical screens on one plate. Each well was filled with 25 µl of either Zn-ZiPD or Fe-ZiPD solution (0.004 mg/ml in 150 mM NaCl). A negative control
ElaC encodes a novel binuclear zinc phosphodiesterase using the buffer without enzyme was performed to locate nonspecific reactions. After 2 hours incubation at 22°C the reactions were stopped and the color developed according to the manufacturer’s recommendations (Merlin).

**Substrate screen with physiological phosphodiesters**

Enzymatic tests with diverse physiological phosphodiesters were performed with Zn-ZiPD in 20 mM Tris /HCl pH 7.4, 150 mM NaCl at room temperature for at least one hour. Positive reaction controls with small amounts of either commercial enzyme or reaction product were carried out for all tests. Hydrolysis of DNA (single stranded M13 DNA, double stranded λ-DNA) was inspected by analyzing reaction products by agarose gel electrophoresis. Hydrolysis of RNA (yeast RNA Type II) was analyzed by following the absorption change at 260 nm for 20 min after addition of ZiPD. Hydrolysis of single stranded oligonucleotides (3-, 7-, and 9-mers of oligo-dA) was tested by incubating the reaction products with shrimp alkaline phosphatase (SAP, Roche, Mannheim, Germany) and subsequent detection of the inorganic phosphate using molybdate/malachite green reagent (21). Similarly, hydrolysis of cyclic mononucleotides was probed. In this test the reaction products were incubated with 5'-nucleotidase (Sigma) to liberate phosphate from open nucleoside monophosphates. Detection of the inorganic phosphate took place with molybdate/malachite green reagent. Reactivity with pyrophosphate, AMP, ADP, and ATP was investigated by detection of released phosphate using molybdate/malachite green reagent. Phospholipase activity towards phosphatidylcholine was investigated via detection of choline. This makes use of choline oxidase (Sigma) and chromogenic detection of the produced H₂O₂ analogous to the glycosulfatase assay described in (20). To distinguish between phospholipase C and D activity, alkaline phosphatase was added to the former assay to convert phosphocholine to choline and phosphate.
Phosphodiesterase assay for kinetic analysis

Phosphodiesterase activity was determined using the substrate bis(p-nitrophenyl)phosphate (bpNPP). Standard reaction conditions were 20 mM Tris/HCl pH 7.4, 14 mM bpNPP. Release of p-nitrophenol (pNP) was continuously monitored for 2 min at 405 nm. The reaction rate was determined from initial slopes using $\varepsilon_{pNP}$ (pH 7.4) = 11,500 M$^{-1}$ cm$^{-1}$. Activity towards thymidine-5'-monophosphate-p-nitrophenylester (TpNPP) was determined analogously. The background activity of a zinc solution in the range between 0.1 and 100 mM towards bpNPP and TpNPP were negligible under the conditions used. One unit of activity corresponds to 1 µmol of pNP liberated per min at 22°C. The kinetic parameters $K'$, $k_{cat}$ and the Hill coefficient, $n_H$, were extracted from the dependence of the initial reaction velocities on the substrate concentration applying the Hill equation (22)

$$v = V_{max} \frac{c_S^{n_H}}{K' + c_S^{n_H}}$$

by nonlinear regression analysis implemented in the program Origin 5.0 (Microcal, Northampton, USA). For the substrate TpNPP, an $n_H$ of 1 was obtained, which converts the Hill equation to the Michaelis-Menten equation. Each measurement was performed in triplicate for each of the three different enzyme preparations.

RESULTS

Expression and characterization of ZiPD

Expression of ZiPD as a His-tagged fusion protein in E. coli yielded soluble recombinant protein in amounts of 20-50 mg/l. After the first purification step using a Ni-metal chelating column, the eluted recombinant protein was almost pure as shown by SDS-PAGE analysis (Figure 1). Purified ZiPD migrates to about 36 kDa on SDS-PAGE which corresponds well to the calculated molecular mass for the monomer of 36.04 kDa. The main fraction of the
ElaC encodes a novel binuclear zinc phosphodiesterase recombinant protein eluted from the gel filtration column at a volume corresponding to a dimer of about 70 kDa. The profile of the phosphodiesterase activity coincided with the protein profile. After several weeks of storage at 4°C these dimers tend to form multimers and aggregates which elute with the void volume of the gel filtration column (Figure 2). The ratio of activity to protein absorbance shows that the phosphodiesterase activity of these higher molecular weight forms was significantly lower than of the dimer. These data demonstrate that the active species of ZiPD in solution is a dimer.

**ZiPD binds two metal atoms**

The metallo-β-lactamase proteins currently known have been shown to bind up to two zinc or iron atoms. Metal analysis by PIXE using the known protein bound sulfur content as an internal standard showed that a. i.-ZiPD contained 0.07 iron and 0.3 zinc atoms per protein molecule (Table I). The nickel content (0.16) is probably due to the purification protocol used. The content of other transition metals such as copper and manganese was negligible. Most of the zinc could be extracted with EDTA. After incubation of the apo-ZiPD with either an excess of zinc or iron ions followed by dialysis of the unbound metals, the protein was shown to bind approximately two metal ions (Table I). The stoichiometry of zinc binding was also confirmed by ICP-MS analysis from which 1.8 ± 0.1 equivalents of zinc for Zn-ZiPD were determined. In order to determine the fraction of protein bound zinc, the ICP-MS was coupled on-line to a size-exclusion chromatograph. No free zinc could be detected. Instead all zinc co-eluted with the protein, confirming that it was completely bound by ZiPD (data not shown). ZiPD was isolated as a His-tag fusion protein. The His-tag could not be cleaved by TEV protease, indicating a conformationally blocked TEV cleavage site at the N-terminus. In order to test the influence of the His-tag on zinc binding, we used another member of the metallo-β-lactamase domain protein family (cytosolic oxygen reductase from *E. coli*), which was expressed using the same modified pET-vector. Incubation of this protein with TEV
ElaC encodes a novel binuclear zinc phosphodiesterase protease removed the His-tag. PIXE analysis of the zinc incubated His-tagged and TEV cleaved protein showed no significant difference in zinc stoichiometry (unpublished results). This identifies ZiPD as a metalloprotein with two metal binding sites capable of binding either zinc or iron.

**Active site model derived from EXAFS analysis**

EXAFS analysis allowed the first structural characterization of the Zn binding sites of ZiPD. The Zn K-edge EXAFS spectrum (Figure 3) is modeled with 2.0 histidine residues, 1.0 carboxylate group, and 1.5 oxygen atoms at an average distance of 1.99 \(\pm 0.02\) Å with an R-factor\(^2\) of 32.3 %. In some metallo-\(\beta\)-lactamases, one Zn atom is coordinated to a cystein-sulfur atom (1, 23). The presence of a sulfur ligand in ZiPD was tested by inclusion of a sulfur atom with half occupancy into the model (2.0 His, 1.0 carboxylate, 1.0 oxygen, 0.5 sulfur). A poorer fit was obtained as indicated by a significantly increased R-factor (35.1 % compared to 32.3 %). Based on the EXAFS analysis, we argue against a sulfur ligand for ZiPD. Metallo-\(\beta\)-lactamase domain proteins are known to have binuclear metal sites and metal analysis of ZiPD revealed 1.7 equivalents of zinc, therefore, we investigated the presence of a second neighboring zinc atom in ZiPD. An active site model with a refined zinc-zinc distance of 3.32 \(\pm 0.03\) Å resulted in a fit with a slightly decreased R-factor of 31.9 % (Table II). It has been demonstrated that the contribution of a neighboring metal in proteins can be hidden by the backscattering contributions of carbon atoms, especially from imidazole ligands (24). This could explain the low significance of the contribution from the second zinc atom. With the present data, EXAFS does not unambiguously confirm a binuclear metal site.

**ZiPD is a phosphodiesterase**

Based on the sequence homology to an arylsulfatase, we probed ZiPD first for arylsulfatase activity using the standard substrates \(p\)-nitrocatechol sulfate and \(p\)-nitrophenylsulfate. In
ElaC encodes a novel binuclear zinc phosphodiesterase addition, the related glycosulfatase activity was tested with the substrate glucose-6-sulfate. No activity towards these substrates with any of the different ZiPD preparations listed in Table I could be detected at pH 7.4 or 5.0 (data not shown). In addition, reactions catalyzed by other β-lactamase-domain enzymes were tested including β-lactamase- and glyoxalase II substrates. Again, none of these substrates were hydrolyzed by ZiPD.

Finally, a commercially available substrate screening plate utilizing 188 different substrates was applied: 95 different substrates for peptidases, 17 substrates for diverse reactions, and 76 substrates for glycolytic enzymes, phosphatases, and esterases. None of these substrates were hydrolyzed by Fe-ZiPD (data not shown). For Zn-ZiPD, however, two different substrates were identified: the phosphodiesters thymidine-5’-p-nitrophenylphosphate (TpNPP) and bis(p-nitrophenyl)phosphate (bpNPP) (Figure 4). The substrate bpNPP is cleaved into p-nitrophenol and p-nitrophenylphosphate. We could not detect any activity for Zn-ZiPD towards the latter product. This enzyme is therefore a phosphodiesterase and does not act on phosphomonoesters.

The enzymatic activity on TpNPP could indicate a function as a nuclease, but no hydrolytic activity for different types of nucleic acids was found (Table III). Furthermore, neither phospholipase C or D activity, nor cleavage of various phosphoric acid anhydrides was detected after incubation with Zn-ZiPD (Table III).

The dependence of the velocity on the substrate concentration for bpNPP and TpNPP was measured for Zn-ZiPD. The curve for bpNPP did not show hyperbolic behavior, which would be expected for Michaelis-Menten kinetics, but could be analyzed according to the Hill equation. The sigmoidal curve for bpNPP indicated positive cooperativity with a Hill coefficient $n_H = 1.6$, a value consistent with a homodimeric enzyme (Figure 5). Thus, in the case of bpNPP the binding of the first substrate molecule increases the affinity for the second molecule. This behavior changes for the substrate TpNPP. Here, the Hill coefficient equals
ElaC encodes a novel binuclear zinc phosphodiesterase, and the enzyme follows Michaelis-Menten kinetics (Figure 5). The turnover number $k_{\text{cat}}$ for bpNPP ($59 \text{ s}^{-1}$) is about 6.5 times higher than for TpNPP ($9 \text{ s}^{-1}$) (Table IV) and the $K'$ value was found to be in the millimolar range (4 and 3 mM, respectively) for both compounds.

**Phosphodiesterase is dependent on zinc**

Determination of the specific activity towards bpNPP for the different preparations of ZiPD demonstrates that activity is strongly dependent on zinc. The isolated enzyme with 0.3 equivalents of zinc displays an activity of 5.8 U/mg and is inactivated by the loss of zinc upon EDTA incubation (Table I). An activity of 62 U/mg was achieved for the dizinc form, whereas the diiron form exhibits an activity of only 0.17 U/mg.

Determination of the phosphodiesterase activity with increasing zinc concentration showed an optimal activation with 0.1 to 0.2 mM Zn$^{2+}$ (Figure 6 A). Higher Zn$^{2+}$ concentrations lead to inactivation of the enzyme, which was accompanied with a precipitation of the protein. It is therefore more likely that the inactivation is due to a denaturation of ZiPD rather than to an enzymatic inhibition by zinc.

Addition of different relevant physiological divalent metals to the isolated enzyme confirms this strong and selective zinc dependence. None of these metals, except zinc, could significantly activate the enzyme (Figure 6 B), although a slight activation by ferrous and nickel ions was observed. Copper at 0.2 mM concentration, in contrast, inactivated ZiPD. The metal analysis together with these activity tests identifies ZiPD as a binuclear zinc enzyme.

**DISCUSSION**

Multinuclear zinc enzymes are frequently found to hydrolyze peptide, thioester, and phosphoester bonds (25, 26). The previously characterized phosphodiesterases within this group utilize three zinc atoms, as shown for phospholipase C (27), nuclease P1 (28), and
ElaC encodes a novel binuclear zinc phosphodiesterase (29). Two of the three zinc ions form a binuclear cluster and are bridged by water and carboxylate groups, whereas the third metal ion is about 5 Å away (25). This group of multinuclear phosphodiesterases utilizing zinc can now be extended by the enzyme characterized in this study.

Sequence analysis assigned ZiPD to the metallo-β-lactamase family (8, 10), which suggests a binuclear metal binding site. The metal coordination sites of the structurally characterized members of this family are identified in part by residues from the conserved sequence motif HxHxDH (7). Additional weakly conserved amino acid residues and water molecules complement the 4-6 fold coordination. A coordinating cysteine residue has been found so far only in the β-lactamases from Bacteroides fragilis and Bacillus cereus (1, 23). Other structurally characterized members, which are glyoxalase II, ROO, and β-lactamase from Xanthomonas maltophilia (2, 3, 30), contain a pure N/O donor set. EXAFS analysis of Zn-ZiPD provided the first structural characterization of the average zinc coordination environment. A 4.5-fold coordination was found with 2.0 histidine residues, 1.0 carboxylate group, and 1.5 oxygen atoms, which could be present as water ligands. A coordinating sulfur atom was excluded based on the EXAFS analysis. The metal-ligand distance of 1.99 Å is in agreement with Zn-ligand distances found in proteins and model complexes with a 4- or 5-fold coordination (31). This model is most similar to the zinc coordination found in β-lactamase from X. maltophilia (30). However, the model is also consistent with the binuclear cluster found in phospholipase C, nuclease P1, and endonuclease IV (27-29). Thus, a low coordination number and an N/O donor set seems to be required for effective phosphodiester cleavage at a binuclear zinc site.

We demonstrated that the protein encoded by the E. coli ElaC gene hydrolyzes the phosphodiester bpNPP with high efficiency. The k_{cat}/K value of Zn-ZiPD for bpNPP is orders of magnitude higher than for the nucleases BfiI and Nuc (Table IV). The phosphonate
ElaC encodes a novel binuclear zinc phosphodiesterase from *Burkholderia caryophilli* hydrolyzes bpNPP with a $k_{cat}/K_m$ of 12 s\(^{-1}\) mM\(^{-1}\) (32) which is very similar to the corresponding value for ZiPD (15 s\(^{-1}\) mM\(^{-1}\)). Furthermore, both enzymes hydrolyze TpNPP at a significantly lower rate than bpNPP, which could indicate that they have similar physiological substrates. In fact, it was proposed that PEH could act *in vivo* as a phosphodiesterase (32) in addition to its ability to utilize the phoshonate monoester glyceryl glyphosate as a sole phosphorous source. A potential physiological phosphodiester substrate for PEH, however, has not yet been found. An interesting fact is that other binuclear zinc enzymes with an established physiological function, such as aminopeptidase (33), phosphotriesterase (34), and alkaline phosphatase (phosphoric acid monoester hydrolase) (35) also display activity to artificial phosphodiesters (Table IV). Although the activity is orders of magnitude lower compared to that of ZiPD, this shows that a binuclear zinc active site is preferred for phosphodiester cleavage. The high $k_{cat}/K'$ value for ZiPD towards bpNPP makes it very likely that the physiological substrate for this enzyme is a phosphodiester.

The main classes of naturally occurring phosphodiesters are nucleic acids and phospholipids. None of the various types of nucleic acids tested here were hydrolyzed by ZiPD, excluding the function of ZiPD as a nuclease (Table III). The assays also included cyclic nucleotides which act as important second messengers. We analyzed the hydrolysis of phosphatidylcholine as a prototype of a phospholipid. Neither phospholipase C nor D activity could be detected with this substrate. Furthermore, no activity towards the chromophoric substrate *p*-nitrophenylphosphorylcholine, a phospholipase C substrate, was found. Finally, it was also shown that phosphoric acid anhydrides like pyrophosphate, ATP, and ADP do not serve as substrates for ZiPD. Although no natural substrate has been found in this study, the substrate profile indicates specificity. Since naturally occurring phosphodiesterases show a high degree of variety, especially in the case of phospholipids, the number of possible substrates is...
ElaC encodes a novel binuclear zinc phosphodiesterase far too high to test them individually. Nevertheless, the results in this study present a more rational way to search for ZiPD’s natural substrate and to investigate its physiological function. This is especially interesting in the case of the human ElaC genes which have been associated with cancer (11, 12). It is expected that these proteins have similar enzymatic characteristics.

The functional assignment of the ElaC gene product demonstrates that the annotation of ElaC as an arylsulfatase, which was already adopted in literature and led to the definition of the arylsulfatase/ElaC family (8, 10), was wrong. This underlines the importance of substantiating data from pure sequence analysis by experimental data. The combination of rational and random substrate screenings performed here proved to be a successful way for the detection of an enzymatic function for a newly purified protein. Since many proteins are waiting for functional characterization, the usage of substrate screening plates might be a useful general strategy for characterizing proteins of unknown function.

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FOOTNOTES

1 Abbreviations used: ROO, rubredoxin:oxygen oxidoreductase; EXAFS, extended X-ray absorption fine structure; ZiPD, zinc phosphodiesterase; PIXE, proton induced X-ray emission; ICP-MS, inductively coupled plasma mass spectrometry; TEV, tobacco etch virus; bpNPP, bis(p-nitrophenyl)phosphate; TpNPP, thymidine-5’-p-nitrophenylphosphate; PEH, phosphonate monoester hydrolase

2 The R-factor is used as a measure of the goodness of the fit. Smaller values indicate better fits. \[ R = \frac{1}{N} \sum_{i}^{N} \left( \frac{1}{\sigma_i} \sum_{k} \left| k \exp(k) - k \text{model}(k) \right| \right) \times 100\% \text{ with } \frac{1}{\sigma_i} = \sum_{k} \frac{k(j)^n}{k(j)^n |\text{Exp}(j)|} \]
LEGENDS TO FIGURES

Figure 1 Purification of ZiPD

*E. coli* ZiPD was overexpressed in *E. coli* BL21(DE3) harboring pETM-ZiPD. Protein samples of subsequent purification steps were analyzed by 10 % NuPAGE gels (Novex) followed by coomassie blue staining. *Lane 1*, total cell extract; *Lane 2*, soluble fraction; *Lane 3*, eluted fraction from Ni-chelate column; *Lane 4*, eluted fraction from gel filtration column.

Figure 2 Active ZiPD consists as a dimer in solution

Gel filtration analysis of purified ZiPD after 4 weeks of storage at 4ºC using Superdex 200 30/10 (Amersham Pharmacia Biotech). The solid line represents the absorption at 280 nm and the circles phosphodiesterase activity towards bpNPP in units/ml. The elution volume of gelfiltration standards (Biorad) used for calibration is depicted at the top x-axis. The peak of main activity corresponds to 70 kDa, equivalent to a ZiPD dimer.

Figure 3 EXAFS spectrum for Zn-ZiPD

Data were recorded at the Zn K-edge as described in the experimental section. A) EXAFS spectrum, B) Fourier transform. Thin lines represent the experimental data and thick lines the calculated spectrum based on the model given in Table II. $\chi(k)$ is the EXAFS amplitude; $r'$ is the metal-ligand distance corrected for first shell phase shifts; a.u., arbitrary units.

Figure 4 ZiPD is a phosphodiesterase

Substrate screen using 188 different hydrolytic substrates on Taxa Profile E plate (Merlin). The plate contains two identical tests (top and bottom separated by line) which were carried out with Zn-ZiPD (0.004 mg/ml) and buffer (150 mM NaCl), respectively. Positive reactions for ZiPD, which were not seen in the buffer control are circled. These are: 1 and 2, thymidine-
ElaC encodes a novel binuclear zinc phosphodiesterase

5′-p-nitrophenylphosphate (TpNPP) sodium and ammonium salt, respectively; 3, bis(p-nitrophenyl)phosphate (bpNPP).

**Figure 5**  Cooperativity of Zn-ZiPD

The reaction velocity vs. substrate concentration (cS) dependence was determined with freshly prepared Zn-ZiPD for the substrates bpNPP and TpNPP. Measurements were performed in triplicate. The lines represent the best fit of the data using the Hill equation. The Hill coefficients obtained were 1.6 for bpNPP and 1 for TpNPP.

**Figure 6**  ZiPD is a zinc-dependent enzyme

a.i.-ZiPD was incubated with divalent cations for three hours at room temperature and the phosphodiesterase activity was determined as described in the experimental section. The activity of a.i.-ZiPD remained constant during the incubation period. Measurements were done in triplicate. A) Dependence of the ZiPD activity on the zinc concentration. B) a.i.-ZiPD (—) was incubated with 0.2 mM of the indicated divalent metal ions before measuring the phosphodiesterase activity with bpNPP.
### TABLES

**Table I** Metal content of ZiPD samples related to phosphodiesterase activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Metal Content per Monomer</th>
<th>Specific Activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fe</td>
<td>Zn</td>
</tr>
<tr>
<td>a. i.-ZiPD</td>
<td>0.07 (2)</td>
<td>0.32 (8)</td>
</tr>
<tr>
<td>apo-ZiPD(^c)</td>
<td>0.09 (2)</td>
<td>0.07 (2)</td>
</tr>
<tr>
<td>Zn-ZiPD</td>
<td>0.17 (4)</td>
<td>1.7 (3)</td>
</tr>
<tr>
<td>Fe-ZiPD</td>
<td>2.3 (5)</td>
<td>0.09 (2)</td>
</tr>
</tbody>
</table>

\(^a\)Metal content determined by PIXE analysis using the protein bound sulfur as internal standard calculated from Cys and Met content in ZiPD. Values in brackets represent the uncertainties of the last digit.

\(^b\)Determined using bpNPP (7.5 mM) in 20 mM Tris/HCl pH 7.4, 150 mM NaCl.

\(^c\)Obtained after EDTA incubation and subsequent dialysis as described under experimental procedures.
ElaC encodes a novel binuclear zinc phosphodiesterase

Table II  Mixed zinc coordination of Zn-ZiPD as derived by EXAFS data analysis\textsuperscript{a}

<table>
<thead>
<tr>
<th>ligand\textsuperscript{b}</th>
<th>atom\textsuperscript{c}</th>
<th>N</th>
<th>r  (Å)</th>
<th>2(\sigma^2) (Å(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>His</td>
<td>N\textsubscript{1}</td>
<td>2.0</td>
<td>1.99</td>
<td>0.014 (1)</td>
</tr>
<tr>
<td></td>
<td>C\textsubscript{2}</td>
<td></td>
<td>3.00</td>
<td>0.011 (3)</td>
</tr>
<tr>
<td></td>
<td>C\textsubscript{2}</td>
<td></td>
<td>3.06</td>
<td>0.011 (3)</td>
</tr>
<tr>
<td></td>
<td>C\textsubscript{3}</td>
<td></td>
<td>4.13</td>
<td>0.008 (1)</td>
</tr>
<tr>
<td></td>
<td>N\textsubscript{3}</td>
<td></td>
<td>4.18</td>
<td>0.008 (1)</td>
</tr>
<tr>
<td>Asp/Glu</td>
<td>O\textsubscript{1}</td>
<td>1.0</td>
<td>1.99</td>
<td>0.014 (1)</td>
</tr>
<tr>
<td></td>
<td>C\textsubscript{2}</td>
<td></td>
<td>2.92</td>
<td>0.011 (3)</td>
</tr>
<tr>
<td></td>
<td>O\textsubscript{2}</td>
<td></td>
<td>3.17</td>
<td>0.011 (3)</td>
</tr>
<tr>
<td></td>
<td>C\textsubscript{3}</td>
<td></td>
<td>4.30</td>
<td>0.008 (1)</td>
</tr>
<tr>
<td></td>
<td>O\textsubscript{1}</td>
<td>1.5</td>
<td>1.99</td>
<td>0.014 (1)</td>
</tr>
<tr>
<td>Zn</td>
<td>Zn</td>
<td>1.0</td>
<td>3.32</td>
<td>0.02 (1)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} N is the coordination number, r is the mean interatomic distance and 2\(\sigma^2\) the Debye-Waller factor. The R-factor of this model is 31.9 % and the Fermi Energy offset -6.47 eV. The error of N is estimated to 0.5. The error of r is 0.02 Å for the first shell and 0.03 Å for the zinc atom. Numbers in brackets represent the uncertainties of the last digit.

\textsuperscript{b} Histidine residues are represented by an imidazole and aspartate/glutamate residues by a carboxylate group. These structures were taken from EXCURV98 and subjected to rigid body refinement.

\textsuperscript{c} Atoms with similar distances were grouped together for collective refinement of the Debye-Waller factors and the distance to the metal center in the case of the first shell. These groups are marked by the same subscript.
Table III  
**Substrate profile of Zn-ZiPD towards phosphodiesters**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>rel. $V_{max}$ (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>bpNPP</td>
<td>100</td>
</tr>
<tr>
<td>TpNPP</td>
<td>15</td>
</tr>
<tr>
<td>3’ TpNPP</td>
<td>n.d.</td>
</tr>
<tr>
<td>dsDNA</td>
<td>n.d.</td>
</tr>
<tr>
<td>ssDNA</td>
<td>n.d.</td>
</tr>
<tr>
<td>oligonucleotides</td>
<td>n.d.</td>
</tr>
<tr>
<td>RNA</td>
<td>n.d.</td>
</tr>
<tr>
<td>5’,3’-cAMP</td>
<td>n.d.</td>
</tr>
<tr>
<td>2’,3’-cAMP</td>
<td>n.d.</td>
</tr>
<tr>
<td>ATP</td>
<td>n.d.</td>
</tr>
<tr>
<td>ADP</td>
<td>n.d.</td>
</tr>
<tr>
<td>AMP</td>
<td>n.d.</td>
</tr>
<tr>
<td>pyrophosphate</td>
<td>n.d.</td>
</tr>
<tr>
<td>phosphatidylcholine$^b$</td>
<td>n.d.</td>
</tr>
<tr>
<td>$p$-nitrophenylphosphorylcholine</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^a$Activity for bpNPP set to 100 %; n.d., not detected

$^b$phospholipase C and D activity was tested
Table IV  Kinetic constants of phosphodiesterases

<table>
<thead>
<tr>
<th>enzyme</th>
<th>substrate</th>
<th>$k_{cat}$ ($s^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ s$^{-1}$)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZiPD$^{a,b}$</td>
<td>bpNPP</td>
<td>59</td>
<td>4</td>
<td>15</td>
<td>this work</td>
</tr>
<tr>
<td>ZiPD$^a$</td>
<td>TpNPP</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>this work</td>
</tr>
<tr>
<td>restriction enzyme BfiI</td>
<td>bpNPP</td>
<td>—</td>
<td>—</td>
<td>0.0042</td>
<td>(36)</td>
</tr>
<tr>
<td>endonuclease Nuc</td>
<td>bpNPP</td>
<td>0.12</td>
<td>8.5</td>
<td>0.014</td>
<td>(37)</td>
</tr>
<tr>
<td>PEH$^c$</td>
<td>bpNPP</td>
<td>10.3</td>
<td>0.9</td>
<td>12</td>
<td>(32)</td>
</tr>
<tr>
<td>PEH$^c$</td>
<td>TpNPP</td>
<td>1.5</td>
<td>4.7</td>
<td>0.4</td>
<td>(32)</td>
</tr>
<tr>
<td>aminopeptidase</td>
<td>bpNPP</td>
<td>0.45</td>
<td>4.5</td>
<td>0.1</td>
<td>(33)</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>bpNPP</td>
<td>—</td>
<td>—</td>
<td>0.05 $\times 10^{-3}$</td>
<td>(35)</td>
</tr>
<tr>
<td>phosphotriesterase</td>
<td>ethyl-pNPP</td>
<td>0.06</td>
<td>—</td>
<td>0.0016</td>
<td>(34)</td>
</tr>
</tbody>
</table>

—, not reported

$^a$values obtained from three independent Zn-ZiPD preparations which were measured each in triplicate. The error is estimated to 20 %.

$^b$K’ instead of $K_m$ obtained from fit to Hill equation with a Hill coefficient of 1.6 ± 0.2

$^c$PEH, phosphonate monoester hydrolase
Figure 1, Vogel et al.
Figure 2, Vogel et al.
Figure 3, Vogel et al.
Figure 4, Vogel et al.
Figure 5, Vogel et al.
Figure 6, Vogel et al.
ElaC encodes a novel binuclear zinc phosphodiesterase
Andreas Vogel, Oliver Schilling, Manfred Niecke, Joerg Bettmer and Wolfram
Meyer-Klaucke

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