Activity and structure of *Pseudomonas putida* MPE, a manganese-dependent single-strand DNA endonuclease encoded in a nucleic acid repair gene cluster

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A recently identified and widely prevalent prokaryal gene cluster encodes a suite of enzymes with imputed roles in nucleic acid repair. The enzymes are: MPE, a DNA endonuclease; Lhr-Core, a 3'-5' DNA helicase; LIG, an ATP-dependent DNA ligase; and Exo, a metallo-β-lactamase-family nuclease. Bacterial and archaeal MPE proteins belong to the binuclear metallophosphoesterase superfamily that includes the well-studied DNA repair nucleases Mre11 and SbcD. Here we report that the *Pseudomonas putida* MPE protein is a manganese-dependent DNA endonuclease that incises either linear single strands or the single-strand loops of stem-loop DNA structures. MPE has feeble activity on duplex DNA. A crystal structure of MPE at 2.2 Å resolution revealed that the active site includes two octahedrally coordinated manganese ions. Seven signature amino acids of the binuclear metallophosphoesterase superfamily serve as the enzymic metal ligands in MPE: Asp33, His35, Asp78, Asn112, His124, His146, and His158. A swath of positive surface potential on either side of the active site pocket suggests a binding site for the single-strand DNA substrate. The structure of MPE differs significantly from Mre11 and SbcD in several key respects: (i) MPE is a monomer, whereas Mre11 and SbcD are homodimers; (ii) MPE lacks the capping domain present in Mre11 and SbcD; and (iii) the topology of the β sandwich that comprises the core of the metallophosphoesterase fold differs in MPE vis-à-vis Mre11 and SbcD. We surmise that MPE exemplifies a novel clade of DNA endonuclease within the binuclear metallophosphoesterase superfamily.

Nucleases, helicases, and ligases are indispensable agents of DNA repair found in all taxa. These enzymes come in various flavors that have distinctive structures and biochemical specificities. It is often the case that multiple nucleases, helicases and ligases coexist in the same organism, whereby the enzyme paralogs or homologs specialize in the repair of particular types of DNA damage. In prokaryons, the clustering of genes encoding nucleases, helicases, or ligases in operons can provide strong clues to the existence of a coherent repair pathway. In this vein, we recently identified a cluster of four genes encoding predicted nucleases, helicase, and ligase enzymes that is widely prevalent in diverse bacteria from distinct phyla (1). The four enzymes are: (i) Lhr-Core, a 3'-5' DNA helicase; (ii) MPE, a DNA endonuclease of the binuclear metallophosphoesterase family; (iii) Exo, a putative exonuclease of the metallo-β-lactamase family; and (iv) an ATP-dependent DNA ligase. Many other bacteria and archaea have a two-gene cluster encoding Lhr-Core and MPE (1).

To understand whether and how these enzymes might contribute to nucleic acid repair, we undertook previously to purify and characterize the Lhr-Core and MPE proteins specified by the Exo•Lig•Lhr-Core•MPE gene cluster of *Pseudomonas putida* (1). The 816-amino acid *P. putida* Lhr-Core polypeptide is homologous to the core ATPase/helicase domain of the 1507-amino acid *M. smegmatis* Lhr protein (2,3). We reported that *P. putida* Lhr-Core is a single-strand DNA-dependent ATPase/dATPase (Km 0.37 mM ATP; kcat 3.3 s⁻¹), an ATP-dependent 3'- to-5' single-strand DNA translocase, and an ATP-dependent 3'- to-5' helicase. Lhr-Core unwinds 3'-tailed duplexes in which the loading/tracking strand is DNA and the displaced strand is either DNA or RNA (1).

Nucleases, helicases, and ligases are indispensable agents of DNA repair found in all taxa. These enzymes come in various flavors that have distinctive structures and biochemical specificities. It is often the case that multiple nucleases, helicases and ligases coexist in the same organism, whereby the enzyme paralogs or homologs specialize in the repair of particular types of DNA damage. In prokaryons, the clustering of genes encoding nucleases, helicases, or ligases in operons can provide strong clues to the existence of a coherent repair pathway. In this vein, we recently identified a cluster of four genes encoding predicted nucleases, helicase, and ligase enzymes that is widely prevalent in diverse bacteria from distinct phyla (1). The four enzymes are: (i) Lhr-Core, a 3'-5' DNA helicase; (ii) MPE, a DNA endonuclease of the binuclear metallophosphoesterase family; (iii) Exo, a putative exonuclease of the metallo-β-lactamase family; and (iv) an ATP-dependent DNA ligase. Many other bacteria and archaea have a two-gene cluster encoding Lhr-Core and MPE (1).

The 216-amino acid *P. putida* MPE protein is a member of the metallophosphoesterase superfamily of enzymes that utilize a binuclear transition-metal ion center to catalyze phosphonomonoester or phosphodiesters hydrolysis (4). We reported that *P. putida* MPE is a manganese-dependent phosphodiesterase that released p-nitrophenol from bis-p-nitrophenyl phosphate (kcat 212 s⁻¹) and p-nitrophenyl-5'-thymidylate (kcat 34 s⁻¹) but displayed no detectable phosphomonoesterase activity against p-nitrophenyl phosphate. MPE is also a manganese-dependent DNA nuclease that sequentially converted a closed circle plasmid DNA to nicked circle...
and linear forms prior to converting the linear DNA to smaller fragments (1). This initial characterization of MPE suggested that it might be a new bacterial homolog of the archaeal and eukaryal DNA nuclease Mre11 and the homologous bacterial DNA nuclease SbcD, which are also binuclear metallophosphoesterases (5-12).

In the present study, we examine in greater detail the nuclease activity of *P. putida* MPE and find it to be a manganese-dependent single-strand DNA endonuclease that incises either linear single strands or the single-stranded loops of DNA stem-loop structures. MPE has feeble activity on duplex DNA. We determined crystal structures of MPE in two different space groups at 2.0 to 2.2 Å resolution. The MPE active site includes two octahedrally coordinated manganese ions. The defining amino acids of the metallophosphoesterase superfAMILY serve as the enzymic metal ligands. We discuss how the structure of MPE differs significantly from Mre11 and SbcD.

**RESULTS and DISCUSSION**

*P. putida* MPE incises single-strand DNA. A 30-min reaction of 1 pmol (0.1 µM) 5'-32P-labeled 50-mer single-strand DNA oligonucleotide with 1 pmol MPE in the presence of 1 mM manganese resulted in incision of a fraction of the input 50-mer DNA to form a ladder of smaller 5'-32P-labeled DNAs that were resolved by urea–PAGE (Fig. 1). One cleavage product was especially prominent at limiting enzyme (denoted by ▲ in Fig. 1). Increasing the input MPE to 2.5, 5 and 10 pmol resulted in progressive depletion of the 50-mer substrate, which was complete at 10 pmol of MPE (Fig. 1). By contrast, when 10 pmol MPE was reacted with 1 pmol 5'-32P-labeled 50-mer duplex DNA the majority of the labeled strand was intact and there was only scant formation of shorter cleavage products (Fig. 1).

To gauge the effect of DNA strand length on endonuclease activity, we reacted 2.5 and 10 pmol MPE with 1 pmol of 5'-32P-labeled 50-mer, 40-mer, 30-mer, 20-mer, and 10-mer single-strand DNA oligonucleotides of identical 5'-terminal nucleobase sequence (Fig. 2). The trend seen was that shortening the DNA resulted in less effective endonuclease activity, as reflected in the level of residual uncleaved substrate at 2.5 pmol of MPE. The transition from 20-mer to 10-mer elicited a sharp decrement in activity, such that there was on only scant cleavage of the 10-mer at 10 pmol of input MPE (Fig. 2). From the cleavage ladders in Fig. 2, we could identify the prominent cleavage product generated by reaction of 2.5 pmol of MPE with the 50-mer DNA as a 15-nucleotide fragment resulting from incision after a dG nucleotide at the tip of a palindromic sequence 5'-GGTACCGGAGGATCC-3' that has the potential to form an intramolecular hairpin.

**MPE incises the single-strand loop of DNA stem-loop structures.** In the experiment in Fig. 3, MPE (10 pmol) was reacted in the presence of manganese with 1 pmol of a 56-nucleotide 5'-32P-labeled DNA stem-loop substrate consisting of a self-complementary 18-bp stem and a 20-nucleotide single-strand loop. In a parallel reaction, the 56-mer stem-loop substrate was incubated with *Mycobacterium smegmatis* 5'-flap endonuclease/5'-exonuclease FenA, which cleaves the DNA principally at the first duplex phosphodiester adjacent to the 5'-flap (to yield a 5'-32P-labeled 39-mer product) and at the 5'-terminal phosphodiester (to release a mononucleotide product) (13). The MPE and FenA reaction products were analyzed by urea-PAGE alongside an 5'-32P-labeled 18-mer corresponding the 5'-terminal 18-nucleotide segment of the stem-loop DNA. We found that 10 pmol MPE incised the stem-loop within the single-strand loop to yield a cluster of about twenty 5'-32P-labeled products migrating between the FenA flap cleavage product and the 18-mer marker (Fig. 3). 5'-32P-labeled products migrating faster than the 18-mer were also present.

We proceeded to test two other 5'-32P-labeled stem-loop structures, of 46 and 41 nucleotides respectively, composed of the same 18-bp duplex stem segment but with shorter 10-nucleotide or 5-nucleotide single-strand loops (Fig. 3). MPE cleaved the 46-mer DNA primarily with the loop to form a cluster of about ten 5'-32P-labeled products migrating between the FenA flap cleavage product (in this case a doublet) and the 18-mer marker. MPE cleaved the 41-mer DNA within the loop to generate a cluster of five 5'-32P-labeled products extending upward from the 18-mer marker. (Note that FenA was unable to cleave the 5'-flap when the loop was shortened to 5 nucleotides.) Products shorter than 18 nucleotides were also formed during the MPE reaction with the 46-mer and 41-mer stem-loop DNAs.

**Characterization of the MPE endonuclease.** Further studies of MPE activity were performed with the 56-nucleotide 5'-32P-labeled stem-loop DNA substrate. An enzyme titration experiment in Fig. 4A showed that at limiting levels of MPE (0.5 and 1 pmol), at which not all of the input substrate was consumed, the cleavage events were confined to, and relatively evenly
distributed among, the inter-nucleotide linkages in the 20-nucleotide loop segment. As the enzyme was increased, and all substrate was cleaved at least once, the distribution of cleavage events within the loop shifted to yield shorter 5′ radiolabeled products and there also appeared cleavages within the 5′-duplex segment to form labeled products of less than 18 nucleotides. A similar pattern was observed when we followed the temporal profile of 56-mer stem-loop cleavage by 10 pmol of MPE (Fig. 4B), whereby at early times when a fraction of the input DNA was consumed, the enzyme incised at all sites within the loop segment and at later times, when all substrate was cleaved at least once, the loop cleavage product distribution shifted to smaller size and cleavages within the 18-nucleotide 5′ duplex segment became evident. These results affirm MPE as an endonuclease that preferentially cleaves single-strand DNA. The absence of cleavages within the 3′ 18-nucleotide stem duplex at early reaction times, and the paucity of cleavages near the 5′ terminus of the 56-mer DNA at saturating enzyme or at late times, indicate that MPE is not an exonuclease that sequentially cleaves single nucleotides from the termini.

The divalent cation specificity of the MPE endonuclease was examined in the experiment in Fig. 4C, in which reactions were performed either in the absence of added metal, or in the presence of 1 mM calcium, cadmium, cobalt, copper, magnesium, manganese, nickel, or zinc. No loop incision was detected in the absence of metal and the metal requirement was satisfied optimally by manganese. Nickel was less active, as gauged by the extent of consumption of the 56-mer DNA. Cadmium and cobalt supported trace levels of loop cleavage. Calcium, copper, magnesium and zinc were inactive (Fig. 4C).

Additional insights to the metal specificity of MPE were provided by mixing experiments, in which reactions containing 1 mM manganese were supplemented with 1 mM of another divalent cation (Fig. 5). The mixture of nickel and manganese reduced endonuclease activity to the level of a reaction containing nickel only. Mixing manganese with cadmium or cobalt resulted in trace activity comparable to that of cadmium or cobalt alone. Copper and zinc abolished nuclease activity in the presence of manganese. These results suggest that each of these four “soft” metals might out-compete manganese for one or both metal-binding sites on the enzyme, wherein engaged they are either unable to support phosphodiesterase reaction chemistry (copper and zinc) or support a lower level of activity vis-à-vis manganese (nickel, cadmium, and cobalt). By contrast, magnesium and calcium had no such deleterious effect in combination with manganese, implying that these “hard” metals do not bind effectively to the MPE active site.

**Crystal structure of *P. putida* MPE.** Crystals grown from a solution of 0.5 mM MPE and 5 mM MnCl₂ had two distinct morphologies: a hexagonal form (crystal form 1) that diffracted to 2.0 Å resolution in space group P6₁22 and a rod-shaped form (crystal form 2) that diffracted to 2.2 Å resolution in space group P2₁2₁2 (Table 1). The structure of MPE in the form 1 crystal was solved by SIRAS phasing on manganese (Rwork/Rfree = 0.192/0.243). The structure of MPE in the form 2 crystal was then solved by molecular replacement using the structure of the crystal form 1 as a search model (Rwork/Rfree = 0.204/0.264). Both crystal forms contained a single MPE protomer and two manganese ions in the asymmetric unit. MPE was monomorphic in each crystal. The MPE folds in the two crystal forms superimposed with a Z score of 33.4 and an rmsd of 1.2 Å at 194 °C positions, as determined by pairwise comparison in DALI (14). One of the manganese ions in the form 1 crystal was situated on a crystallographic two-fold axis in a position that we construed to be off-pathway (to be discussed later). Because both manganese ions in the form 2 crystal were located in the enzyme active site, we will focus henceforth on the form 2 structure of MPE.

The MPE tertiary structure, shown in stereo view in Fig. 6A, consists of sixteen β strands, three α helices, and three 3₁₀ helices. The secondary structure elements are displayed above the MPE amino acid sequence in Fig. 6B. The strands are organized into an extended β sandwich. The topologies of the two β sheets of the sandwich are β₁↓•β₂↑•β₃↓•β₄↑•β₅↑•β₆↑•β₇↑ and β₈↓•β₉↑•β₁₀↑•β₁₁↑•β₁₂↓•β₁₃↑•β₁₄↑. An additional three-strand β sheet (β₁₂↓•β₁₁↑•β₁₄↑) emanates from the top surface of the second sheet of the β sandwich (Fig. 6A). The active site containing the binuclear manganese cluster (green spheres) is formed by constituents of the inter-strand loops on the forward surface of MPE (Fig. 6A). The seven amino acids that bind the metals – Asp33, His35, Asp78, Asn112, His142, His156, and His158 – are depicted as stick models (Fig. 6A) and denoted by red dots below the amino acid sequence (Fig. 6B).
MPE active site and surface electrostatics. A stereo view of the active site highlighting the binuclear metal complex is shown in Fig. 7A. The metal-binding mode is characteristic of the enzyme superfamily. The Mn1 atom is octahedrally coordinated to Asp33-Od, His35-Ne, Asp78-Od, His158-Ne, and two waters. The octahedral Mn2 coordination complex includes Asp78-Od, Asn112-Od, His142-Ne, His156-Ne, and two waters. The Mn1 and Mn2 atoms are 3.6 Å apart and are bridged by Asp78 and one of the waters. We showed previously that mutation of Asp78 to alanine squelches MPE phosphodiesterase activity with the generic substrate bis-\(p\)-nitrophenylphosphate and its endonuclease activity on supercoiled plasmid DNA (1). The metal-bridging water may correspond to the nucleophile in the phosphodiesterase reaction, insofar as its proximity to the two metals will significantly lower the pKa of the water and activate it for attack on the DNA backbone. In the same vein, we speculate that the other two waters of the M1 and M2 coordination spheres occupy the positions of two of the phosphate oxygens of the scissile phosphodiester. The His113 side chain located above the metal complex (Fig. 7A) is conserved in many phosphodiesterase/nuclease enzymes of the metallophosphoesterase superfamily; this histidine serves as a general acid catalyst of DNA cleavage, by donating a proton to the 3'-OH leaving group.

A surface electrostatic model of MPE highlights a swath of positive potential on either side of the negatively charged metal-binding pocket (Fig. 7B). The positive surface suggests a binding site for the negatively charged backbone of the single-strand DNA substrate of the MPE endonuclease reaction. MPE amino acids that contribute to the positive surface include (proceeding from top to bottom in the view in Fig. 7B) Arg86, Arg83, His81, Lys38, Arg115, Arg172, Arg170, and Arg164. Of these, Arg115, His81, and Lys38 are located near the active site (Fig. 7A).

MPE crystal form 1. The structure of MPE in space group P6\(_2\)2\(_2\) had several notable features from the crystallographic standpoint. First, whereas it clearly contained two manganese ions in the asymmetric unit, as affirmed by the anomalous difference peaks over the metal atoms (Fig. 8A and B), one of the metals was located on a two-fold crystallographic symmetry axis (the z-axis looking down on the images in Fig. 8) and was coordinated by ligands in neighboring MPE protomers on either side of the axis. The axial manganese (labeled Mn2 in Fig. 8B) was octahedrally coordinated to two His156 side chains (via Ne, rather than by Nd that engages Mn2 in the form 2 structure), two Glu143 side chains (which is not a metal ligand in the form 1 structure), two waters bridged to Glu143, and two waters on the symmetry axis (Fig. 8B). Asn112 and His142, which are Mn2 ligands in the form 2 structure, do not interact with a metal in crystal form 1. The other manganese, equivalent to Mn1 in the form 2 MPE structure described above, was located in the active site in an octahedral coordination complex with Asp33-Od, His35-Ne, Asp78-Od, His158-Ne, and two waters. We surmise that an off-pathway complex of the Mn2 ion aide in formation of the hexagonal crystal lattice.

Second, the main difference in the MPE fold in the two crystal forms entailed a shift of the 85ARAP88 loop segment immediately preceding the \(\beta\)5 strand (by 5.9 Å at the Pro88 Ca atom, 6.9 Å at the Ala87 Ca atom, 7.4 Å at the Arg86 Ca atom, and 6.2 Å at the Ala85 Ca atom). Third, Pro120 in the loop following the \(\alpha\)3 helix adopts a \(\alpha\)8 conformation in the form 2 crystal versus a \(\alpha\)9 conformation in the form 1 crystal.

Comparison to other binuclear metallophosphoesterases and nucleases. A DALI search (14) of the Protein Database with the \(P.\ putida\) MPE structure identified many members of the binuclear metallophosphoesterase enzyme superfamily as structural homologs. The "top hits" were *Pseudomonas aeruginosa* UDP-diacylglicosamine pyrophosphohydrolase LpxH (pdb 5B4C: Z score 11.1; 2.5 Å rmsd at 133 Ca positions; 22% amino acids identity) (15) and human Mre11 (pdb 3T1I: Z score 10.5; 2.8 Å rmsd at 145 Ca positions; 19% amino acid identity) (10). Other notable hits (besides Mre11 homologs from other taxa) were: *E. coli* YfcE (pdb 1SU1: Z score 10.0; 2.7 Å rmsd at 131 Ca positions; 21% identity) (16); *E. coli* SbcD (pdb 4LU9: Z score 9.3; 3.0 Å rmsd at 141 Ca positions; 20% identity) (12); and *Methanococcus jannaschii* MJ0396 (pdb 1S3M: Z score 9.2; 2.6 Å rmsd at 123 Ca positions; 18% identity) (17). As one might expect, the structural conservation among MPE and these other family members centers on the amino acid constituents and spatial arrangements of their respective binuclear metal-binding sites. Superposition of the di-manganese sites of *P. putida* MPE, human Mre11, and *E. coli* SbcD is shown in Fig. 9.

The relationship of MPE to Mre11 and SbcD is pertinent because the latter two are manganese-
dependent DNA nucleases. Mre11 is the nuclease subunit of a DNA end-processing complex, which also includes an SMC-like ATPase subunit Rad50, that functions in cellular pathways of double-strand break repair, e.g., homologous recombination (HR) and non-homologous end-joining (NHEJ) (reviewed in 18). SbcD and its SMC-like ATPase partner SbcC form an SbcCD complex (19), a homolog of Mre11•Rad50, that triggers replication-dependent double-strand breaks at palindromic sequences in the bacterial chromosome (20). The Mre11•Rad50 and SbcCD complexes have ATP-dependent double-strand DNA exonuclease activity and ATP-independent single-strand endonuclease activity. Like MPE, SbcC is adept at cleaving the single-strand loops of DNA hairpin structures (21).

A number of structural features distinguish MPE from Mre11 and/or SbcD. These are highlighted in Fig. 10, which shows a side-by-side superposition of the structures of P. putida MPE, human Mre11 (10), and E. coli SbcD (12), aligned with respect to their metal-binding sites, with the structural elements in Mre11 and SbcD that are not present in MPE shaded gray. First, the 216-aa MPE protein is smaller than the human Mre11 catalytic core (416-aa) or E. coli SbcD (400-aa). MPE lacks the so-called capping domain, comprising a β sheet and two α-helices, that is appended to the C-terminus of the metallophosphoesterase fold of Mre11 orthologs and SbcD. The capping domain is implicated in interaction of Pyrococcus Mre11 with duplex DNA (6) and of Thermotoga Mre11 with Rad50 (11). P. putida MPE and its homologs are not linked genetically with an SMC-like ATPase protein and thus may have no need for a capping domain.

Second, the monomeric quaternary structure of MPE in solution (as gauged by gel filtration [1]) and in both crystal forms reported here (as analyzed in PISA) contrasts with the homodimeric quaternary structures of Mre11 and SbcD (5-12). The homodimerization interface of SbcD and archaeal and fungal Mre11 proteins consists of a four-helix bundle formed by the α2 and α3 helices from each protomer (as indicated in Fig. 10, right panel). Whereas MPE has a counterpart of the α2 helix (albeit with no amino acid sequence identity to the SbcD α2 helix), it lacks an equivalent of the α3 helix. Human Mre11 has a distinctive homodimer interface (10) involving the α3 helix and several loops (Fig. 10, middle panel), none of which are present in MPE. Third, Mre11 and SbcD have additional α helices and loops below the central β sandwich that are not present in MPE (Fig. 10). Finally, the topology of the β sheets that form the central sandwich differs in MPE versus Mre11 and SbcD. For example, Mre11 and SbcD have no equivalent of the β1 strand of MPE. The topology of the rest of this β sheet (which is β2↑•β3↓•β4↑•β5↑•β6↑•β7↑ in MPE) is different in human Mre11, where the strand order is β14↑•β13↓•β1↑•β2↑•β3↑•β4↑ (10). Mre11 and SbcD also have no equivalent of the MPE β15 and β16 strands within the other sheet of the β sandwich, or of the β11 and β14 strands. The topology of the rest of the second sheet in the sandwich (which is β8↓•β9↑•β10↑•β13↑•β12↓ in MPE) is different in human Mre11, where the strand order and direction is β7↑•β9↑•β10↑•β12↑•β11↓ (10).

Conclusions and speculations. We surmise from the results presented above that P. putida MPE exemplifies a novel clade of bacterial DNA endonucleases within the binuclear metallophosphoesterase enzyme superfamily. MPE’s activity in cleaving single-strand DNA and single-strand regions in duplex DNA is compatible with a variety of potential DNA repair functions, especially considering its signature genetic functions, especially considering its signature genetic

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EXPERIMENTAL PROCEDURES

Purification and crystallization of P. putida MPE. Recombinant MPE was produced in E. coli as a His_{6}-Smt3 fusion and purified from a soluble extract by serial nickel-affinity, tag removal, and gel filtration steps as described previously (1). Protein concentration was determined with the BioRad dye reagent using BSA as the standard. MPE crystals were grown at 22°C by sitting drop vapor diffusion. The MPE protein preparation (12.5 mg/ml in 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM DTT, 10% glycerol) was adjusted to 5 mM MnCl₂ and incubated for 20 min. Aliquots of the protein solution (1 µl) were then mixed with an equal volume of precipitant solution containing either 0.1 mM Tris-HCl, pH 7.4, 16% PEG-MME-2000 (Crystal form 1) or 0.1 mM Tris-HCl, pH 7.2, 16% PEG-MME-2000 (Crystal form 2). Crystals grew to their full size in 1 or 2 days. Single crystals were harvested and transferred briefly to precipitant solution containing 25% glycerol prior to flash-freezing in liquid nitrogen.

Diffraction data collection and structure determination. X-ray diffraction data were collected from single crystals at the Advanced Photon Source beamline 24-ID-C at wavelength of 1.7712 Å near the manganese X-ray absorption edge. Form 1 and form 2 crystals diffracted to 2.0 Å and 2.2 Å resolution, respectively. Indexing and merging of the diffraction data were performed in HKL2000 (22). The form 1 crystal was in space group P6₁2₂. The phases for solving the form 1 structure were obtained by single isomorphous replacement with anomalous scattering (SIRAS) as implemented in SHELX (23). Two strong manganese anomalous scattering peaks were identified per asymmetric unit containing one MPE protomer. One of the manganese atoms in the form 1 crystal was situated on a crystallographic two-fold symmetry axis. Iterative model building was performed in O (24). Refinement was accomplished with PHENIX (25). The model of the MPE protomer in the form 1 crystal, refined to 2.0 Å resolution (R_{work}/R_{free} = 0.192/0.243), consisted of three polypeptide segments – Asn2 to Tyr42, Gly52 to Gly165, Arg170 to Gly209, and Arg212 to Leu216 – separated by disordered surface loops. Data collection and refinement statistics for both crystal forms are compiled in Table 1.

Accession numbers. Structure coordinates have been deposited in the Protein Data Bank database under accession codes 6NVO and 6NVP.

Nuclease substrates. The 5' 3²P-labeled single-strand DNA and stem-loop DNA substrates were prepared by reaction of synthetic oligonucleotides with T4 polynucleotide kinase (Pnk) and [γ³²P]ATP. The kinase reaction mixture was heated to 95°C to inactivate T4 Pnk. The DNA was separated from free ATP by electrophoresis through a nondenaturing 18% polyacrylamide gel and then eluted from an excised gel slice by overnight incubation at 4°C in 200 µl of 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. To form the 50-mer DNA duplex, the radiolabeled pDNA strand and template DNA were annealed at 1:1.5 molar ratio in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA. The reactions were quenched at the times specified by adding 10 µl of 90% formamide, 50 mM EDTA. The samples were heated at 95°C for 5 min and then analyzed by electrophoresis through a 40-cm 18% polyacrylamide gel containing 7.5 M urea in 44.5 mM Tris-borate, pH 8.3, 1 mM EDTA. The products were visualized by autoradiography.

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Table 1
Crystallographic data and refinement statistics

|                          | Crystal form 1                        | Crystal form 2                        |
|--------------------------|---------------------------------------|---------------------------------------|
| **Data collection**       |                                       |                                       |
| Beamline                 | APS 24-ID-C                           | APS 24-ID-C                           |
| Space group              | P6\(_2\)22                            | P2\(_1\)2\(_1\)2                       |
| **Cell dimensions**       |                                       |                                       |
| a, b, c (Å)               | 45.39, 45.39, 317.40                  | 58.22, 73.00, 41.75                   |
| α, β, γ (°)               | 90, 90, 120                           | 90, 90, 90                            |
| Resolution (Å)            | 40–2.0 (2.07–2.0)                     | 40–2.2 (2.24–2.2)                     |
| Wavelength (Å)            | 1.7712                                | 1.7712                                |
| R\(_{\text{sym}}\)        | 0.031 (0.345)                         | 0.034 (0.194)                         |
| CC(1/2)                  | 0.995 (0.794)                         | 0.993 (0.925)                         |
| <I>/<of>                 | 28.1 (2.0)                            | 27.8 (4.4)                            |
| Completeness (%)          | 98.3 (91.5)                           | 93.9 (72.3)                           |
| Redundancy               | 4.3 (2.4)                             | 4.6 (3.3)                             |
| Unique reflections        | 14173                                 | 8980                                  |

**Phasing**

|                          |                                       |                                       |
|                          | Heavy atom sites                     | 2                                     |
|                          | Figure of merit                      | 0.53                                  |

**Refinement**

|                          |                                       |                                       |
|                          | R\(_{\text{work}}\) / R\(_{\text{free}}\) | 0.192 / 0.243                        |
|                          | B-factors (Å\(^2\))                  |                                       |
| Average / Wilson         | protein                               | 51.3 / 36.8                           |
|                          | ligands                               | 34.3                                  |
|                          | solvent                               | 51.9                                  |
| RMS deviations           | bond lengths (Å)                      | 0.007                                 |
|                          | bond angles (°)                       | 0.823                                 |
| Ramachandran plot        | % favored                             | 94.2                                  |
|                          | % allowed                             | 5.8                                   |
|                          | outliers                              | 0                                     |

**Model contents**

|                          |                                       |                                       |
|                          | Protomers / ASU                       | 1                                     |
|                          | Protein residues                      | 197                                   |
|                          | Ions                                  | 2                                     |
|                          | Water                                 | 105                                   |
| **PDB ID**               |                                        | 6NVP                                   |

Values in parentheses refer to the highest resolution shell.

R\(_{\text{free}}\) set consists of 10% of data chosen randomly against which structures were not refined.
FIGURE LEGENDS

Figure 1. MPE cleaves single-stranded DNA. Reaction mixtures (10 µl) containing 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 mM MnCl₂, 1 mM DTT, 1 pmol (0.1 µM) 5'-32P-labeled 50-mer ssDNA or dsDNA substrates (shown at bottom), and MPE as specified were incubated for 30 min at 37°C. The reaction mixtures were analyzed by urea-PAGE and the labeled DNAs were visualized by autoradiography. A cleavage product that was especially prominent at limiting enzyme is denoted by ▶ on the left.

Figure 2. Effect of ssDNA length on MPE cleavage. Reaction mixtures (10 µl) containing 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 mM MnCl₂, 1 mM DTT, 1 pmol (0.1 µM) 5'-32P-labeled 50-mer, 40-mer, 30-mer, 20-mer, or 10-mer ssDNA substrates (shown at bottom), and either 2.5 or 10 pmol MPE as specified were incubated for 30 min at 37°C. The products were analyzed by urea-PAGE and visualized by autoradiography.

Figure 3. MPE incises the single-strand loop of DNA stem-loop structures. Reaction mixtures (10 µl) containing 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 mM MnCl₂, 1 mM DTT, 1 pmol (0.1 µM) 5'-32P-labeled stem-loop DNAs (shown at bottom), and either 10 pmol (1 µM) MPE or 1 pmol (0.1 µM) M. smegmatis FenA (where indicated by +) were incubated for 30 min at 37°C. The reaction mixtures were analyzed by urea-PAGE (in parallel with a 5'-32P-labeled 18-nucleotide oligonucleotide size marker corresponding to the 5' end of the 18-bp stem) and the labeled DNAs were visualized by autoradiography. The predominant site of flap cleavage of the 20-nt loop substrates by FenA is indicated by an arrow.

Figure 4. Characterization of MPE cleavage of stem-loop DNA. (A) MPE titration. Reaction mixtures (10 µl) containing 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 mM MnCl₂, 1 mM DTT, 1 pmol (0.1 µM) 5'-32P-labeled 20-nt stem-loop DNA, and 0, 0.5, 1, 2.5, 5, 10, or 20 pmol MPE or 1 pmol FenA were incubated for 30 min at 37°C. (B) Time course. The reaction mixture (110 µl) contained 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 mM MnCl₂, 1 mM DTT, and 1 pmol (0.1 µM) 5'-32P-labeled 20-nt stem-loop DNA. The time 0 sample (10 µl) was removed prior to the addition of 100 pmol MPE and incubation at 37°C. Aliquots (10 µl) were withdrawn at the times specified and quenched immediately with 10 µl of 90% formamide, 50 mM EDTA. (C) Divalent cation specificity. Reaction mixtures (10 µl) containing 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 pmol (0.1 µM) 5'-32P-labeled 20-nt stem-loop DNA, 10 pmol (1 µM) MPE, and either no added metal (–) or 1 mM Ca, Cd, Co, Cu, Mg, Mn, Ni, or Zn (as chloride salts) were incubated for 30 min at 37°C. The products in panels A–C were analyzed by urea-PAGE (in parallel with a 5'-32P-labeled 18-nucleotide oligonucleotide marker) and the labeled DNAs were visualized by autoradiography.

Figure 5. Effect of other divalent metals on manganese-dependent endonuclease activity. Reaction mixtures (10 µl) containing 20 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 mM MnCl₂, plus 1 mM of the indicated divalent metals (Ca, Cd, Co, Cu, Mg, Mn, Ni, or Zn, as chloride salts) were incubated for 30 min at 37°C. Control reactions lacked enzyme (–MPE) or added metal (–Mn). The products were analyzed by urea-PAGE and visualized by autoradiography.

Figure 6. Overview of MPE crystal structure. (A) Stereo view of the MPE tertiary structure in crystal form 2, depicted as a cartoon model with magenta β strands, cyan α helices, and blue 3₁₀ helices. The secondary structure elements are labeled. Manganese ions are shown as green spheres. Amino acids that bind the metal ions are depicted as stick models. (B) The secondary structure elements of MPE are displayed above the amino acid sequence. The seven metal-binding amino acids are indicated by red dots.

Figure 7. MPE active site and surface electrostatics. (A) Stereo view of the active site and metal-coordination complexes. MPE amino acids are depicted as stick models with beige carbons. Mn₁ and Mn₂ are rendered as green spheres. Waters are depicted as red spheres. Atomic contacts of the manganese ions are denoted by black dashed lines. (B) Surface electrostatic model of the MPE protein generated in Pymol. Manganese ions in the active site pocket are depicted as green spheres. The view highlights areas of positive
surface potential (blue) flanking the pocket as a likely binding site for the phosphodiester backbone of the single-strand DNA substrate. The asterisk denotes amino acid Tyr42, which demarcates the start of a 9-aa disordered surface loop $^{[43]RALHPVPR^{51}}$ between $\beta 4$ and $\alpha 1$.

Figure 8. MPE crystal form 1. (A) The tertiary structure of the MPE protomer in crystal form 1 (space group P6$_1$22) is shown in green; a symmetry-related MPE protomer is colored cyan. The amino terminus of the polypeptide is indicated by $N$. Manganese ions are magenta spheres. Each MPE protomer contains a manganese ion in its active site (equivalent to Mn1 in Fig. 6A). A third manganese ion is situated at the crystallographic symmetry axis. (B) Closeup view of the active sites and metal contacts in the symmetry-related MPE protomers (colored as in panel A). Waters are depicted as red spheres. Anomalous difference density for the manganese atoms, contoured at 5$\sigma$, is shown in pink mesh.

Figure 9. Superposition of the metal-binding sites of $P$. putida MPE, human Mre1, and $E$. coli SbcD. A stereo view of the superimposed di-manganese sites and metal-binding amino acids of $P$. putida MPE (amino acids shown as stick models with beige carbons and metals as orange spheres), human Mre11 (green amino acid carbons and metal spheres), and $E$. coli SbcD (cyan amino acid carbons and metal spheres).

Figure 10. Comparison of the tertiary structures of $P$. putida MPE, human Mre1, and $E$. coli SbcD. The $P$. putida MPE, human Mre11, and $E$. coli SbcD structures were superimposed with respect to their di-manganese binding sites and then offset horizontally. MPE is colored by secondary structure (as in Fig. 6A) with Mn atoms as green spheres. The core metallophosphoesterase folds of human Mre11 and $E$. coli SbcD that are conserved in MPE are colored green and cyan, respectively, with Mn atoms as magenta spheres. The capping domains and other structural elements in human Mre11 and $E$. coli SbcD that are not conserved in MPE are colored gray. The elements that comprise the human Mre11 and $E$. coli SbcD homodimer interfaces are indicated.
Figure 1
| 50-mer | 40-mer | 30-mer | 20-mer | 10-mer |
|--------|--------|--------|--------|--------|
| 0  2.5 10 | 0  2.5 10 | 0  2.5 10 | 0  2.5 10 | 0  2.5 10 |

\[ p_{CGAGCTCGGTACCCGGGATCCTCTAGAGTCGACCTGCAAGC } \] 50-mer

\[ p_{CGAGCTCGGTACCCGGGATCCTCTAGAGTCGACCTGCAG } \] 40-mer

\[ p_{CGAGCTCGGTACCCGGGATCCTCTAGAGT} \] 30-mer

\[ p_{CGAGCTCGGTACCGGGA} \] 20-mer

\[ p_{CGAGCTCGGT} \] 10-mer

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 9
Figure 10
Activity and structure of *Pseudomonas putida* MPE, a manganese-dependent single-strand DNA endonuclease encoded in a nucleic acid repair gene cluster
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