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Solution structure and DNA-binding properties of the phosphoesterase domain of DNA ligase D

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ABSTRACT

The phosphoesterase (PE) domain of the bacterial DNA repair enzyme LigD possesses distinctive manganese-dependent 3'-phosphomonoesterase and 3'-phosphodiesterase activities. PE exemplifies a new family of DNA end-healing enzymes found in all phylogenetic domains. Here, we determined the structure of the PE domain of *Pseudomonas aeruginosa* LigD (*PaePE*) using solution NMR methodology. *PaePE* has a disordered N-terminus and a well-folded core that differs in instructive ways from the crystal structure of a *PaePE*•Mn²⁺•sulfate complex, especially at the active site that is found to be conformationally dynamic. Chemical shift perturbations in the presence of primer-template duplexes with 3'-deoxynucleotide, 3'-deoxynucleotide 3'-phosphate, or 3' ribonucleotide termini reveal the surface used by *PaePE* to bind substrate DNA and suggest a more efficient engagement in the presence of a 3'-ribonucleotide. Spectral perturbations measured in the presence of weakly catalytic (Cd²⁺) and inhibitory (Zn²⁺) metals provide evidence for significant conformational changes at and near the active site, compared to the relatively modest changes elicited by Mn²⁺.

INTRODUCTION

Many bacteria have two independent mechanisms to repair DNA double-strand breaks (DSBs)—homologous recombination and non-homologous end joining (NHEJ) (1,2). DNA ligase D (LigD) is the central catalyst of bacterial NHEJ *in vivo* (3). LigD is composed of three

autonomous enzymatic modules: an ATP-dependent ligase (LIG) domain that joins 3'-OH and 5'-phosphate ends (4,5); a polymerase (POL) domain that adds ribonucleotides or deoxyribonucleotides to DSB ends and gaps (6–11); and a 3'-phosphoesterase (PE) domain (12) that hydrolyzes 3'-monophosphate termini to 3'-OH ends that can either be extended by POL or sealed by LIG. The PE domain also has a unique 3'-phosphodiesterase activity that resects short 3' ribonucleotide tracts created at DSB ends by the POL domain, to leave a 3'-OH monoribonucleotide end that is the preferred substrate for sealing by bacterial NHEJ ligases (12–16). Mutational studies of the PE domain of *Pseudomonas aeruginosa* LigD (*PaePE*) have identified residues essential for activity (13). Whereas His42, His48, Asp50, Arg52, His84 and Tyr88 are important for both the monoesterase and diesterase activities, Arg14, Asp15, Glu21 and Glu82 are implicated specifically in the monoesterase activity.

In order to understand the structural basis for catalysis by the PE domain, we pursued crystallographic and solution NMR studies of *PaePE* in parallel. The 1.9-Å crystal structure of the active phosphodiesterase core of *PaePE* revealed a novel fold in which an 8-strand β -barrel with a hydrophobic interior supports a distinctive crescent-shaped hydrophilic active site on its outer surface, wherein six of the essential side chains coordinate manganese and a sulfate anion (17). Subsequent crystal structures of two archaeal PE proteins at high resolution—1.1 Å for *Candidatus Korarchaeum cryptofilum* PE (*KcoPE*) and 2.1 Å for *Methanosarcina barkeri* PE (*MbaPE*)—highlighted conservation of the *PaePE* β -barrel fold, active site constituents and metal-binding site (18). Thus, *PaePE* exemplifies a novel family of 3'-end healing enzymes found in diverse taxa of the bacterial and archaeal domains of life. Putative PE homologs were also detected in several eukaryal proteomes (17).

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Missing from the *PaePE* crystal structure was an N-terminal peptide segment, deletion of which selectively diminished the 3'-phosphomonoesterase activity of *PaePE* without affecting the 3'-phosphodiesterase activity (16). The *MbaPE* protein has the homologous N-terminal peptide, but electron density corresponding to this segment was missing in the *MbaPE* crystal structure, suggesting that it is disordered (18). *CkoPE* naturally lacks an equivalent of the N-peptide, and the associated 3'-phosphomonoesterase activity. It was notable that the crystallization of *PaePE*, *CkoPE* and *MbaPE* could only be achieved in the presence of Mn^{2+} and an oxyanion (either sulfate or phosphate), both of which were present as a Mn^{2+} •oxyanion complex in the respective PE active sites (18). The phosphate and sulfate anions in the crystal structures were taken to be mimetics of the scissile 3'-phosphate of the PE nucleic acid substrate.

Our aim in the present study was to obtain a solution structure of *PaePE* in order to determine its conformation in the absence of metals or substrate mimetics and, if possible, to visualize the N-terminal peptide, in the absence of any perturbations including external non-physiological additives and crystal-packing forces. Initial NMR studies were performed with *PaePE*-(1–187), which produced spectra that were suitable for obtaining backbone assignments, but were not of high enough quality to yield a sufficient number of sidechain 1H assignments to allow structure determination. Serial C-terminal truncations identified *PaePE*-(1–177) as a catalytically active 3'-monoesterase/3'-diesterase that produced spectra of considerably higher quality and yielded sufficient number of 1H assignments (19) suitable for calculating an NMR structure of this highly dynamic enzymatic module. Here, we present the solution structure of *PaePE*-(1–177) determined using standard NOE-based approaches. By investigating spectral changes in the presence of each of three different oligonucleotide primer–template duplexes and a series of bivalent metal ions, we provide insight to the DNA binding and metal ion recognition properties of *PaePE*.

MATERIALS AND METHODS

Protein expression and purification

PaePE-(1–177) (referred to hereafter as *PaePE*) was produced in *E. coli* as an N-terminal His₁₀-Smt3 fusion; isotope labeling and purification of the tag-free *PaePE* protein are described in detail elsewhere (19). *PaePE* samples for NMR experiments were prepared in a buffer containing 50 mM Bis-Tris (pH 6.5), 200 mM NaCl and 5 mM DTT (referred to as the NMR buffer from hereon forward) and typically contained ~300 μ M protein. The lack of chemical shift changes in the ^{15}N , 1H TROSY spectra acquired with and without a 10-fold excess of EDTA argued against the retention or acquisition of enzyme-bound bivalent metal ions during the purification process. Thus, all subsequent spectra were acquired in the absence of EDTA.

Resonance assignment

Backbone 1H , ^{15}N , $^{13}C\alpha$, $^{13}C'$ and sidechain $^{13}C\beta$ assignments were obtained via TROSY-based HNCO, HN(CA)CO, HNCACB and HN(CO)CACB experiments (20) utilizing uniformly 2H , ^{13}C , ^{15}N -labeled *PaePE*. Sidechain ^{13}C and 1H assignments were obtained via (H)C(CO)NH and H(CCO)NH experiments utilizing uniformly ^{13}C , ^{15}N -labeled *PaePE* (21). Experimental details have been described at length elsewhere (19).

Distance and angular constraints

Backbone $^{13}C\alpha$, $^{13}C\beta$, $^{13}C'$ and ^{15}N chemical shifts were converted into dihedral ϕ and ψ angular restraints using the TALOS+ software suite (22). NOE-derived distance constraints to the backbone amide 1H nuclei were obtained using a ^{15}N -edited NOESY-HSQC (utilizing a uniformly ^{15}N -labeled sample) with a 150-ms mixing time at a field strength of 800 MHz. Distance restraints involving sidechain protons were obtained from 3-dimensional ^{13}C -edited NOESY experiments in D₂O (150 ms mixing time, 800 MHz), separately optimized for aliphatic and aromatic regions of the spectrum. Uniformly ^{15}N , ^{13}C -labeled samples were utilized in both cases. NOESY crosspeak intensities were obtained using NMRView (23) and converted into distance restraints using the ambiguous distance restraints (ADR) protocol as implemented in the ARIA (24) software suite. Manual NOE assignments were supplemented with those assigned automatically using the ARIA scheme as described previously (24).

Structure calculations

To facilitate structure calculation, the ordered regions of *PaePE* were determined using an improved pulse sequence to measure the ^{15}N - $\{^1H\}$ steady-state NOE at 600 MHz (25). Spectra were acquired with several repetitions of a τ - π - τ (τ = 11 ms) symmetric 1H saturation scheme for a total of 3 s. An additional delay of 2 s was used to allow lock stabilization. The reference experiment included a 5 s pre-delay. Spectra were acquired using sweep-widths of 14 ppm (512 complex points) and 32 ppm (128 complex points) for the 1H and ^{15}N dimensions respectively, using 64 transients per t_1 point. Based on the ^{15}N - $\{^1H\}$ steady-state NOE values, structure calculations were initially performed excluding the flexible N- and C-termini of *PaePE* and including only residues Thr32–Gly163. After the ordered 'core' had been suitably refined, the N-terminal 31 residues were included in the structure calculations to obtain the final structural ensemble. Few resonance assignments were available for the disordered C-terminal region (164–177) that is dispensable for catalytic activity. Consequently, this polypeptide fragment was not included in the structure calculations.

Manually assigned NOEs were combined with dihedral and hydrogen-bonding constraints into a standard assignment/structure-calculation protocol using the ARIA 2.3/CNS 1.1 (24) software suite. Pro65 and Pro72 were constrained to be in the *cis* conformation using the *cis*-proline patch during the structure calculation. Additional

assignments that were obtained automatically during a run were inspected for correctness and incorporated into subsequent runs. Structure calculations were performed using a Cartesian dynamics simulated annealing protocol that consisted of: (i) high temperature dynamics at 2000 K (10 000 steps); (ii) 4000 steps of refinement; (iii) a Cartesian dynamics cooling stage from 2000 to 1000 K (6000 steps); and (iv) a second Cartesian dynamics cooling stage from 1000 to 50 K (5000 steps). Force constants of 5, 25 and 200 kCal mol⁻¹ Å⁻² for the dihedral constraints and 10, 10 and 50 kCal mol⁻¹ Å⁻² for the distance constraints (ambiguous, unambiguous and hydrogen bond) were used during the three temperature stages of the Cartesian dynamics protocol. Prochiral H β and H γ from long-chain amino acids and methyl groups from Val and Leu, were treated using the floating chirality method (24). For the final refinement step, a subset of the lowest energy structures were solvated in a 7.5-Å box of explicit solvent and refined using the full Lenard-Jones non-bonded potential and electrostatic interactions from the OPLS force-field using a protocol described in detail previously (26). In the final run, 2048 structures were calculated and the resultant 128 lowest energy structures were used in the water-refinement protocol. Fifteen of the lowest energy water-refined structures with the least number of violations of experimental constraints were chosen to represent the final structural ensemble of *PaePE* (note that only residues 1–163 were included in the final round of structure calculations). The quality of the resulting NMR structures was evaluated with PROCHECK (27). Regions of secondary structure were defined using Promotif 3.0 (28,29).

Preparation of oligonucleotides

HPLC purified DNA oligos D16F1R1 (5'-TTTTTTGGGACCGGTC(C_{2'}F)(rC); wherein the penultimate nucleoside is 2'-fluorocytidine and the 3' terminal nucleoside is ribocytidine), D18 (5'-TTTTTTGGGACCGGTCCC) and D18p (5'-TTTTTTGGGACCGGTCCCp: where p is a 3'-monophosphate) were purchased from Integrated DNA Technologies Incorporated. Stock solutions containing 100 μ l of aqueous D16F1R1 (9.2 mM) or 50 μ l of aqueous D18 (11.6 mM) or 50 μ l of aqueous D18p (6 mM) were annealed in an Eppendorf thermocycler at 95°C for 3 min, at 60°C for 2 min, at 40°C for 10 min and finally at 25°C for 10 min, to generate duplex primer-templates each containing a 6-nt single-stranded tail. The concentrations of the annealed primer-template duplexes were estimated from absorption measurements at wavelengths of 260 and 280 nm (see Figure 4a for details of the annealed duplexes).

NMR measurements of protein-oligonucleotide interactions

Samples of uniformly ²H, ¹⁵N-labeled *PaePE* (100 μ M) in NMR buffer were used for NMR titrations. For all the primer-templates, ¹⁵N, ¹H TROSY spectra were acquired for *PaePE*:oligo ratios of 1:0.05, 1:0.12, 1:0.2, 1:0.3, 1:0.5, 1:1, 1:2 and 1:3 for D18; 1:0.05, 1:0.1, 1:0.2, 1:0.3, 1:0.5, 1:1, 1:2 and 1:3 for D18p. Data were acquired at 600 MHz at 25°C using sweep-widths of 15 ppm

(512 complex points) and 36.2 ppm (128 complex points) in the direct and indirect dimensions, respectively, using a recycle delay of 1.5 s and 48 transients per t_1 point. For the D16F1R1 primer-template, the following *PaePE*:oligo ratios were used 1:0.05, 1:0.1, 1:0.2, 1:0.3, 1:0.5, 1:1, 1:2 and 1:3 and ¹⁵N, ¹H TROSY spectra were acquired at 800 MHz at 25°C using sweep-widths of 14 ppm (512 complex points) and 32 ppm (128 complex points) in the direct and indirect dimensions respectively using a recycle delay of 1.5 s and 64 transients per t_1 point.

Scaled chemical shift differences were calculated using the following equation:

$$\Delta\delta(i) = \sqrt{(\delta_{o,H} - \delta_{i,H})^2 + 0.11(\delta_{o,N} - \delta_{i,N})^2} \quad (1)$$

where $\delta_{o,H}$ and $\delta_{o,N}$ are the ¹H and ¹⁵N chemical shifts for a given residue in apo *PaePE*, respectively, while $\delta_{i,H}$ and $\delta_{i,N}$ are the corresponding shifts at the i th titration point.

NMR measurements of divalent cation binding

Samples of uniformly ¹⁵N, ²H labeled *PaePE* (100 μ M) in the NMR buffer (excluding DTT; removal of DTT produced no spectral perturbations) were used for the metal ion titrations (Mn²⁺, Cd²⁺ and Zn²⁺). Metal ions were added as dilutions from 7.5 mM stock solutions of MnCl₂, CdCl₂ or ZnCl₂. A series of ¹⁵N, ¹H HSQC datasets were obtained at 700 MHz (25°C) with sweep-widths of 14 ppm (512 complex points) and 32 ppm (128 complex points) in the direct and indirect dimensions respectively, using recycle delays of 1.5 s (5.5 s for Mn²⁺; to allow a more accurate comparison between the peak intensities in the reference state and those in the presence of the paramagnetic metal ion resulting in increased R_1 rates and enhanced recovery toward equilibrium between scans) and 32 transients per t_1 point. The following protein-to-metal ratios were used: Cd²⁺—1:0.25, 1:0.5, 1:1 and 1:2; Zn²⁺—1:0.5, 1:1; Mn²⁺—1:0.25, 1:0.5 and 1:1. In addition, the effects of phosphate were analyzed using a 10-fold excess of phosphate in buffer containing equimolar ratios of *PaePE* and each of the three divalent cations. An additional dataset was collected at 600 MHz (25°C) for Zn²⁺ in the presence of DTT using the following ratios of *PaePE* and Zn²⁺—1:0.2, 1:0.4, 1:0.6, 1:0.8, 1:1, 1:1.5 and 1:2. These data were fully consistent with those collected in the absence of DTT. The reversibility of the spectral perturbations induced by metal ion binding in all cases was tested by addition of an excess of EDTA.

Fluorescence measurements of protein-DNA interactions

A 1 μ M sample of *PaePE* in the NMR buffer was used for the fluorescence measurements using a 3-ml cuvette that allowed continuous mixing. All measurements were performed on a Spex Fluorolog-3 (Horiba Jobin Yvon) at 25°C. Tryptophan emission was monitored following excitation at 280 nm. Emission spectra were recorded from 300 to 450 nm in 1 nm increments using a 0.5-s integration time and averaged over four scans. For the D16F1R1 primer-template, the following *PaePE*:oligo ratios were used—1:0, 1:0.05, 1:0.1, 1:0.2, 1:0.3, 1:0.5, 1:0.75, 1:1,

1:1.5, 1:2, 1:3, 1:5, 1:8, 1:12, 1:18, 1:25 and 1:40. Experiments were performed first in the absence of divalent metal ions and then in the presence of 100 μ M Mn^{2+} . Apparent K_d values were obtained by fitting the change in fluorescence intensity at the emission maximum in the absence of oligos (331 nm) with increasing D16F1R1 concentration to a quadratic binding isotherm [Equation (2)] using in-house software that utilized the ODRPACK libraries (30).

$$I_0 - I = I_\infty \frac{K_d + [D] + [P]_0 - \sqrt{(K_d + [D] + [P]_0)^2 - 4[D][P]_0}}{2[P]_0} \quad (2)$$

where I is the intensity at 331 nm at a dsDNA concentration of $[D]$, I_0 is the corresponding intensity in the absence of DNA, I_∞ is the intensity difference at saturation, $[P]_0$ is the protein concentration (held constant at 1 μ M). Additionally, fluorescence titrations of 1 μ M *PaePE* with Mn^{2+} were performed in the absence of oligonucleotide using the following *PaePE*:metal ion ratios—1:0, 1:0.05, 1:0.1, 1:0.2, 1:0.5, 1:1, 1:2, 1:5, 1:10, 1:20, 1:40 and 1:80.

RESULTS AND DISCUSSION

Solution structure of *PaePE*

Inspection of the final NMR ensemble of 15 structures (see Table 1 for statistics) reveals a disordered N-terminus (1–31). This fact is also borne out by the extremely low ^{15}N - $\{^1H\}$ NOE values (0.08 ± 0.21 ; see Figure 1) for this region. However, some degree of local ordering is seen encompassing residues Asp15–Thr19 (^{15}N - $\{^1H\}$ NOE: 0.18 ± 0.01). This region contains Asp15 and is flanked by Arg14 and Glu21; these three sidechains are important for the 3'-monoesterase activity of *PaePE* (16). The distribution of ϕ/ψ angles for the Asp15–Thr19 segment indicates an overall extended structure (Supplementary Figure S1). A hydrogen bond between the carbonyl oxygen of Thr19 and the amide of Glu21 is found in all 15 structures of the final ensemble.

The core region (Gly33–Gly163) of *PaePE* forms a β -barrel consisting of eight antiparallel β -stands (Figure 2) as follows: Leu35–His42 (β 1), His48–Leu55 (β 2), Thr58–Ala63 (β 3), Arg76–His84 (β 4), Val105–Pro115 (β 5), His127–Gly134 (β 6), Ser138–Arg145 (β 7) and Trp155–Lys159 (β 8). A single α -helix (Pro119–Lys125) bridges the β 5 and β 6 strands. The locations of the secondary structural elements are consistent with those predicted from chemical shift data (19) and those seen in the crystal structure (in complex with Mn^{2+} and sulfate) (17). A ninth β -strand, which packs against the N-terminus of β 5 and includes residues Gly93 and Ser94, is seen in the majority of the structures in the solution ensemble; this strand extends to residues Phe91 and Ile95 in several of the structures in the ensemble. The crystal structure also shows a strand between residues Gly93 and Ile95. However, the low ^{15}N - $\{^1H\}$ NOE values for this region (0.38 ± 0.15 for residues 91–95; Figure 1) and the elevated local

Table 1. NMR restraints and structure statistics

Restraints and statistics	
Total number of restraints	2380
NOE restraints	2080
Unambiguous	1676
Intraresidue	563
Sequential ($ i-j = 1$)	488
Short-range ($ i-j = 2,3$)	153
Medium-range ($ i-j = 4,5$)	53
Long-range ($ i-j > 5$)	419
Ambiguous	404
Dihedral angle restraints	212
Hydrogen bond restraints ^a	88
Constraint violations ^b	
NOE violations > 0.5 Å	0
Dihedral violations $> 5^\circ$	4
Deviation from idealized geometry	
Bond lengths (Å)	$5.55 \times 10^{-3} \pm 0.12 \times 10^{-3}$
Bond angles ($^\circ$)	0.74 ± 0.02
Impropers ($^\circ$)	2.05 ± 0.10
Energies (kcal mol ⁻¹)	
Total	-4334.8 ± 106.7
Van der Waals	-303.8 ± 23.9
Distance restraints	321.5 ± 17.3
RMSD from average structure ^c	
All residues (236–298)	
Backbone (N, C α , C) (Å)	0.46 ± 0.07
Heavy atoms (Å)	1.29 ± 0.19
Ramachandran statistics ^d	
Most favored region (%)	65.0 (82.1)
Additionally allowed (%)	28.0 (17.2)
Generously allowed (%)	5.5 (0.6)
Disallowed (%)	1.5 ^e (0.1)

^aHydrogen bond restraints were H^N-O distance of 1.8–2.3 Å and an N–O distance of 2.8–3.3 Å.

^bTotal number of violated constraints for the final ensemble of 15 water-refined structures including residues 1–163.

^cRMSD (from the mean) for residues in regions of definite secondary structure 35–42, 48–55, 58–63, 76–84, 105–115, 119–125, 127–134, 138–144, 155–159.

^dRamachandran statistics for residues 33–163 for the 15 structures in the final water refined ensemble. The numbers in the parenthesis indicate the statistics for residues in regions of definite secondary structure: 35–42, 48–55, 58–63, 76–84, 105–115, 119–125, 127–134, 138–144, 155–159.

^eA majority of the residues found in the disallowed regions of the Ramachandran plot belong to Asp71 that flanks the *cis* Pro72. The remainder of the disallowed residues preceded or followed those for which no mainchain 1H , ^{15}N assignments were available.

displacement in the NMR ensemble (Supplementary Figure S2) suggest its disorder in solution. This disordered strand (β 9, Gly93–Ser94) is colored purple in Figure 2b. In addition, there are three instances of 3_{10} helices, comprising residues Tyr88–Asp90 (in 40% of the structures of the final ensemble), Gln97–His99 (20% of structures) and Thr146–Leu148 (20% of the structures). Only the first of these 3_{10} helices is seen in the crystal structure (17), albeit between residues Leu86–Asp90. There is no electron density in the crystal structure corresponding to the second 3_{10} helix, indicating significant disorder of this segment, as confirmed by the elevated spin–spin relaxation rates (R_2 at 900 MHz; data not shown) suggesting μ s–ms timescale dynamics.

Analysis of the final NMR ensemble reveals high local displacement (Supplementary Figure S2) in the β -hairpin

Figure 1. $^{15}\text{N}\{-^1\text{H}\}$ Steady-state NOE for *PaePE*. Data was acquired at 600 MHz. NOE values for the β -strands and the α -helix are shown in red and blue respectively.

between $\beta 1$ and $\beta 2$ (Asp43–Tyr49, $0.69 \pm 0.18 \text{ \AA}$, including the N-terminus of $\beta 2$) and in the loop between $\beta 4$ and $\beta 5$ (Phe91–Asp104, $0.40 \pm 0.13 \text{ \AA}$) that includes a dynamic 3_{10} helix as discussed above. Some disorder is also seen in the loop between $\beta 7$ and $\beta 8$ ($0.30 \pm 0.09 \text{ \AA}$). Regions of definite secondary structure are generally well defined ($0.10 \pm 0.08 \text{ \AA}$) except the extreme N-terminus of $\beta 2$. The regions of high disorder, i.e. elevated mean local displacement values in the loop regions, also display low $^{15}\text{N}\{-^1\text{H}\}$ NOE values (Figure 1) indicating significant subnanosecond timescale dynamics.

Differences between the NMR and crystal structures of *PaePE*

Whereas the overall fold in solution is very similar to the crystal structure (17), significant differences can be seen, with the largest deviations being near the active site. The crystal structure (colored red in Figure 3) adopts a more closed conformation with a concave active site, compared to a more open conformation seen in the NMR ensemble in solution (the member of the NMR ensemble closest to the mean is colored cyan in Figure 3). The open–closed conformational switch is indicated by the dashed line with arrowheads in Figure 3. The largest distortions occur at the face of the β -barrel comprising strands $\beta 1$, $\beta 2$ and $\beta 5$. Significantly smaller deviations are seen on the opposite face comprising strands $\beta 6$, $\beta 7$ and $\beta 8$. In the crystal structure, the catalytic Mn^{2+} ion is coordinated with octahedral geometry by the sidechains of His42 ($\beta 1$), His48 ($\beta 2$) and Asp50 ($\beta 2$), the sulfate anion and two waters. This network might favor a relatively rigid closed structure, whereas a more open structure, on average, forms in the absence of the metal ion and crystal packing forces. The C-terminal tail (177–187) in the crystal structure, missing from our *PaePE* NMR construct, tucks in under the $\beta 1$ – $\beta 2$ – $\beta 5$ sheet and thus might also stabilize the closed conformation seen *in crystallo*. Note that the NMR ensemble (Figure 2a) includes both open as well as closed structures, though the former constitute a larger fraction of the ensemble. The archaeal PE crystal structures

Figure 2. (A) NMR ensemble of 15 water-refined structures of *PaePE* (shown in cross-eyed stereo view) showing only residues Ser31–Gly163. The β -strands and the α -helix are shown in red and blue, respectively. The disordered segment that lies between Phe91 and Asp104 is shown in yellow (this includes a disordered 9th β -strand between Gly93 and Ser94). The $\beta 3$ – $\beta 4$ loop containing the two proline residues in *cis* conformation (Pro65 and Pro72) is shown in green. A 3_{10} helix (indicated in cyan) forms in $\sim 40\%$ of structures in the final NMR ensemble. (B) Ribbon diagram for a representative structure (closest to the mean) of the NMR ensemble. A ninth β -strand that forms in majority of the structures between Gly93 and Ser94 is colored purple.

(Supplementary Figure S3a) illustrate varying degrees of closure around the active site. In the present study, main-chain ^1H , ^{15}N assignments could not be made for *PaePE* residues His42, Ala44, Ser45, Leu47, His48, Tyr49 and Tyr88 (though mainchain $^{13}\text{C}\alpha$, $^1\text{H}\alpha$ assignments could be obtained for all of these residues except His48). $^{15}\text{N}\{-^1\text{H}\}$ NOE data for residues in this region where ^1H , ^{15}N resonance assignments are available, suggest overall rigidity on the ps–ns timescale. However, substantial line broadening (confirmed by elevated R_2 values, data collected at 900 MHz; data not shown) associated with assigned resonances in this region indicate significant conformational dynamics on the μs –ms timescale. The conformational flexibility seen at the active site in the NMR ensemble is not evident in the crystal structure of the *PaePE*• Mn^{2+} •sulfate complex. It therefore appears that the catalytic metal ion and the phosphomimetic sulfate are necessary to induce order at the active site and orient the important sidechains into their catalytically competent conformations. The extensive dynamics is also the likely

Figure 3. Differences between the NMR and X-Ray structures of *PaePE*. The X-Ray structure (red) was solved in the presence of a catalytic Mn^{2+} ion and a phosphomimetic sulfate anion. The key residues that lie in the co-ordination sphere of the cation and anion are shown in green and blue, respectively. The regions where the differences are the largest are indicated—the $\beta 1$ – $\beta 2$ – $\beta 5$ face near the catalytic site (dashed arrow), the $\beta 3$ – $\beta 4$ loop (blue dashed oval) that contains the two *cis* prolines (*trans* in the X-Ray structure) and the $\beta 7$ – $\beta 8$ loop (red dashed oval). Only the core region is shown in both cases.

reason that *PaePE* (or any other PE domain) could not be crystallized in the absence of metal ions.

Notable differences between the crystal and NMR structures are also seen in the long loop between $\beta 3$ and $\beta 4$ (circled in blue in Figure 3, right panel). Two proline residues (Pro65 and Pro72) in this segment were predicted to be in the *cis* conformation (albeit in a *trans* configuration in the crystal structure), based on the differences between their $^{13}\text{C}\beta$ and $^{13}\text{C}\gamma$ chemical shifts (19). Whereas the *cis* conformation for Pro65 could be confirmed by the presence of $\text{H}\alpha^{i-1}$ – $\text{H}\alpha^i$ NOE cross-peaks (Supplementary Figure S4), extensive spectral overlap prevented the unambiguous assignment of either $\text{H}\alpha^{i-1}$ – $\text{H}\alpha^i$ (characteristic of *cis* proline) or $\text{H}\alpha^{i-1}$ – $\text{H}\delta^i$ (characteristic of *trans* proline) crosspeaks corresponding to Pro72. Nevertheless, the two prolines residues were locked in *cis* conformation for the structure calculations. The presence of *cis* prolines in the $\beta 3$ – $\beta 4$ loop does not lead to significant deviations between the crystal and NMR structures for the strands $\beta 3$ and $\beta 4$ themselves, except for some minor differences at the extreme C- and N-terminal ends of $\beta 3$ and $\beta 4$, respectively. The biological role, if any, of the *cis* prolines is not immediately obvious though this loop contains two to four prolines in bacterial and archaeal PE domains (Supplementary Figure S5).

The loop between $\beta 7$ and $\beta 8$ also displays differences between the crystal structure and the solution structure (circled in red in Figure 3). In the NMR ensemble, this loop folds back at an angle of $\sim 90^\circ$ about residues Asn147 and Ser153. This region is also quite dynamic on the ps–ns timescale with moderately low ^{15}N – $\{^1\text{H}\}$ NOE values (0.44 ± 0.09) compared with the flanking regions, a fact that is reflected by the relatively high mean local displacement values in the NMR ensemble (Supplementary Figure S2). Different degrees of closure for this loop are also seen in the case of *MbaPE* and *CkoPE* (Supplementary Figure S3a).

Comparison of the observed $^{13}\text{C}\alpha$ chemical shifts and those calculated from the crystal structures of *PaePE*

using the program SHIFTX2 (31) averaged over three residues (Supplementary Figure S3b) reveals that largest differences occur around the regions that also display the largest structural deviations between the crystal and NMR structures. Most notable among these include the $\beta 1$ – $\beta 2$ hairpin and the $\beta 4$ – $\beta 5$ loop. The largest residue-specific deviations occur for Leu86 (calculated to be shifted 3.4 ppm downfield from that observed in solution) and Asp87 (3.13 ppm downfield) suggesting a higher degree of helicity *in crystallo* compared to that in solution, in agreement with that seen comparing the NMR and crystal structures (Figure 3).

The sidechains of the catalytic residues in the NMR ensemble sample orientations similar to those seen in the crystal structure (Supplementary Figure S6). It is likely that the catalytically optimal orientations are locked in by the presence of the metal ion as discussed earlier. However, Glu82 (implicated in the monoesterase activity) samples sidechain orientations that are quite different from those seen in the crystal structure. The reasons for this are not immediately apparent. Orientations for His48, for which no resonance assignments are available, were not calculated.

In order to further probe the differences between the NMR and crystal structures of *PaePE*, we tested several alignment media (32) in an attempt to obtain a set of residual dipolar couplings. However, in spite of our best efforts, all these attempts failed because *PaePE* was unstable under all conditions tested.

NMR analysis of the interactions of *PaePE* with DNA

Positively charged residues distributed over the surface of *PaePE* (Supplementary Figure S7) could potentially act as loci for DNA binding. In order to determine the DNA-binding surface of *PaePE*, we analyzed spectral perturbations induced in ^{15}N , ^1H TROSY spectra of uniformly ^{15}N , ^2H -labeled protein by increasing concentrations of the 18-mer primer–template duplex, D18 (Figure 4a). Many spectral perturbations were seen in the presence of

Figure 4. (A) The three primer-template constructs used to measure spectral perturbations in *PaePE*: D18p contains a 3'-phosphate; D16F1R1, contains a 2'-fluorocytidine (to closely mimic a ribose) in the penultimate position and a 3'-terminal ribocytidine. (B) Results of the NMR titration of *PaePE* with D18. ^{15}N , ^1H TROSY spectra acquired at 600 MHz in the absence (red) and the presence of equimolar amounts of D18 (blue). The inset shows the trajectory of the resonance corresponding to Leu157 that lies on $\beta 8$ during the course of the titration.

D18 (Figure 4b) reflecting the alteration of the chemical environment of the main chain nuclei due the combined effects of direct binding and induced conformational changes. Many residues were broadened beyond the threshold of detection early in the titration course (Supplementary Table S1). This extensive line broadening indicates exchange on the intermediate timescale (33). The broadened resonances correspond to residues situated near the active site, on the $\beta 3$ – $\beta 4$ loop, on strands $\beta 4$, $\beta 5$ and $\beta 7$, and on the $\beta 7$ – $\beta 8$ loop. The positively charged residues His127, Lys136 and Arg140 were also completely broadened out early during the titration. From this group of perturbed residues, those on $\beta 5$ (Asp109 and Gly111 flank the positively charged Arg110), and the set of positively charged residues that show localized perturbations (Lys136, Arg140) are quite distant from the catalytic site, all lying on the opposite face of the protein (Figure 5c). If one or more of these residues were to take part in a productive binding event, a substantial distortion would be required of the duplex bound to this patch to access the catalytic site. A conformational change in this region in response to DNA binding at the opposite face, while also possible, is difficult to

rationalize without further evidence. It is therefore likely that the spectral perturbations seen in the presence of the D18 primer–template result from both productive and non-productive binding events. In addition to these extensive line-broadening effects, significant chemical shift changes (Supplementary Figure S8) were also seen for several residues in and around strands $\beta 4$ and $\beta 8$.

Given the ability of *PaePE* to catalyze the hydrolysis of the 3'-PO₄ of an all-DNA primer–template, we tested whether the presence of a phosphate group at the 3'-end (primer–template D18p) resulted in any alteration in the spectral perturbations. The overall distribution of the perturbations (Supplementary Figure S8 and Supplementary Table S1) was quite similar to those seen for D18. Thus, the presence of a 3'-PO₄ group did not seem to have a substantial effect on the nature of spectral perturbations, at least in the absence of the catalytic Mn^{2+} ions.

The phosphodiesterase activity of *PaePE* requires a 2'-OH in the penultimate nucleobase (12). Thus, successive base removal occurs until a single ribonucleotide remains at the 3'-end. Given this scenario, we tested the influence of a primer–template containing a 3'-terminal ribonucleotide on the spectral perturbations in *PaePE*. A primer–template construct (D16F1R1, Figure 4a) that was also 2'-fluoro modified at the penultimate base (to mimic closely an RNA base) was used in the NMR titrations. In the presence of D16F1R1, substantial changes in the spectral perturbations were seen compared to D18 and D18p. The perturbations for residues comprising $\beta 5$ and the isolated perturbations (seen in the cases of the D18 and D18p constructs) near the positively charged residues His127, Lys136 and Arg140 were no longer seen (highlighted in Supplementary Table S1). While Lys151 on the catalytic face was no longer significantly perturbed, the perturbation in Gly150 persisted, albeit at a much higher concentration of DNA (Supplementary Table S1). Line broadening effects were limited to residues near the active site (Tyr37, Lys41 and Val105), the $\beta 3$ – $\beta 4$ loop (Val64, Leu70), $\beta 4$ (Arg76, Leu77, Ala78, Val79, Val81, Glu82 and His84) and on the $\beta 7$ – $\beta 8$ loop (Thr146, Asn147 and Gly150). In addition, large chemical shift changes (>0.11 ppm at an equimolar ratio with D16F1R1, Figure 5d and Supplementary Figure S8) were seen near the catalytic site (Glu40: 0.12 ppm), the $\beta 3$ – $\beta 4$ loop (Val74: 0.15 ppm; Lys75: 0.25 ppm), $\beta 4$ (Gln80: 0.11 ppm), $\beta 7$ (Ile144: 0.16 ppm), the $\beta 7$ – $\beta 8$ loop (Gln152: 0.22 ppm; Ser153: 0.12 ppm and Gln154: 0.33 ppm) and $\beta 8$ (Trp155: 0.37 ppm; Phe156: 0.13 ppm; Leu157: 0.2 ppm and Lys159: 0.15 ppm). Based on these observations, it appears that the binding site for DNA that is productive for catalysis involves a surface composed of portions of strands $\beta 3$, $\beta 4$, $\beta 7$, $\beta 8$ and intervening loops (Figure 5b and d). This region shows a high degree of conservation in PE domains found across the bacterial and archaeal taxa (Figure 5e). In the absence of a preferred ribose base at the 3'-end, detectable oligonucleotide binding seems to occur at additional putatively non-productive sites at the opposite face of the protein. The inability to fit the chemical shift or intensity changes with increasing oligonucleotide concentration to simple quadratic binding also suggests a more complex interaction dynamics than

Figure 5. (A) Residues important for the catalytic activity of *PaePE*. Gln40 and Glu82 (along with Arg14 and Glu15 on the disordered N-terminus, not shown) important for the 3'-phosphomonoesterase activity are colored magenta; Lys66 and Arg76 that influence the 3'-phosphodiesterase activity are colored blue; His42, His48, Asp50, His84 and Trp88 influence both activities, are colored cyan. (B) Schematic representation of *PaePE* showing the secondary structural elements in the same orientation (looking down into the catalytic cavity) as in (a) and the left panels of (C–E). The location of the catalytic residues as in (a) is indicated by the dotted oval. The strands comprising the $\beta 3$ – $\beta 4$ – $\beta 7$ – $\beta 8$ face that shows the largest perturbations in the presence of oligonucleotide is indicated by the red dots. (c) Influence of D18 on *PaePE*. Residues that are broadened out beyond detection during the titration course are colored blue and residues that display chemical shift changes [$\Delta\delta$, Equation (1)] >0.11 ppm at an equimolar protein:oligonucleotide ratio, are colored red. The circled patches represent perturbations involving residues near pockets of positive charges on the face opposite to that bearing the catalytic residues. These perturbations are likely due to non-productive binding events. Residues for which data were not analyzed due to missing assignments, spectral overlap or weak peaks in the reference state, are colored green. Residues that show no significant spectral perturbations in the presence of oligonucleotide are shown in gray. The effects of D18p are quite similar to those of D18. (d) While the overall spectral perturbations on the catalytic face, in the presence of D16F1R1, are similar as in D18 and D18p, the perturbations on the face opposite to that bearing the catalytic residues, are not seen. (e) Sequence conservation in bacterial and archaeal PE domains are depicted on the *PaePE* surface using a cyan (least conserved) to maroon (most conserved) gradient. The catalytic residues and a large part of the putative oligonucleotide binding surface are well conserved.

a single-site binding event (also suggested by the fluorescence-based assays described below). It is tempting to speculate that the extensive line broadening effects seen could in part be due to exchange between the productive and non-productive sites. The presence of the ribose enables a higher degree of selectivity in the occupation of the productive sites.

No significant changes in the chemical shifts were seen for the N-terminal segment with any of the primer-templates used in this study. No spectral perturbations were seen in the Gly98-Gly101 segment (missing from the crystal structure); it was conjectured that this segment might grip the primer-template and position it at the catalytic site (18).

No perturbations were detected for the N-terminal tail in a complex consisting of *PaePE*, D16F1R1 and Mn^{2+} in a 1:0.5:0.5 ratio in a buffer containing 2 mM phosphate (to mimic a state immediately following the 3'-monoesterase activity in the presence of the product inorganic phosphate). Sub-stoichiometric amounts of the paramagnetic Mn^{2+} and D16F1R1 were used to minimize the line-broadening effects due to the former and allow the visualization of a maximal number of resonances (Supplementary Table S1). Based on the lack of observed perturbations at the N-terminus, it is conceivable that an intact phosphorylated 3'-end is necessary to observe the conformational changes, if any, required for the monoesterase activity. It is also possible that the conformational changes are quite fast or transient on the chemical shift timescale and thus do not give rise to any observable chemical shift changes or line broadening effects.

Fluorescence studies of the interactions of *PaePE* with DNA

In addition to the NMR titration experiments discussed above, we measured native tryptophan fluorescence for *PaePE* to probe its interactions with D16F1R1. The protein contains five tryptophan residues (Trp62, Trp108, Trp113, Trp141 and Trp155), all of which are buried in the structure, as confirmed by a fluorescence emission maximum around 331 nm (34) following excitation at 280 nm. The change in fluorescence emission seen in the presence of D16F1R1 (Figure 6) was fit to a simple one-site binding model [Equation (2)] yielding an apparent K_d value of $5.6 \pm 0.6 \mu\text{M}$. However, the quality of the fit to the single-site binding model is quite poor (Figure 6; black symbols), as evidenced by the nature of the residuals, clearly suggesting that this simple model is not suitable for *PaePE*/DNA interactions. Note, it is unlikely that this change in fluorescence emission is due to direct quenching by DNA, but rather due to a conformational change in the protein. Surface exposure of the tryptophan residues to allow contact with DNA would result in a red shift of the fluorescence maximum to around 340 nm (34). Based on the NMR titrations, the most perturbed tryptophan residues are likely to be Trp62, Trp155 and Trp158.

Better fits to a quadratic isotherm were obtained in the presence of an excess of Mn^{2+} (see Figure 6, red symbols)

Figure 6. Change in *PaePE* tryptophan fluorescence (measured at 331 nm) in the presence of D16F1R1 with (red) and without (black) $100 \mu\text{M}$ Mn^{2+} . Experimental data are shown as filled circles and fits to a quadratic isotherm [Equation (2)] by solid lines. Almost no change in tryptophan fluorescence is seen with an excess of Mn^{2+} ions alone (blue circles). The inset depicts the residuals for the fits in the absence (black dashed line) or the presence (red dashed line) of Mn^{2+} . Note the reduced deviation from a simple quadratic binding isotherm and the improvement in the quality of the fits seen in the presence of Mn^{2+} .

with greatly improved residuals and a concomitant decrease in the χ^2 value per degree of freedom from 2509 in the absence of Mn^{2+} to 995 in its presence, albeit with a similar apparent K_d value of $3.0 \pm 0.1 \mu\text{M}$. This suggests that the presence of the catalytic Mn^{2+} ions in addition to a ribose base at the 3'-end further reduces binding at the non-productive site and helps localize the oligonucleotide substrate more efficiently near the active site at the productive binding surface. No significant change in fluorescence emission was seen with Mn^{2+} ions in the absence of DNA (see Figure 6; blue symbols). As stated earlier, *PaePE* requires Mn^{2+} for catalytic activity but our results indicate that Mn^{2+} alone does not induce conformational changes that are detectable by tryptophan fluorescence. A comparison of the NMR ensemble with the crystal structure of *PaePE*• Mn^{2+} •sulfate complex indicates that the orientations of the tryptophan residues are quite similar. Therefore it is not surprising that the metal induced conformational changes are not reflected in our fluorescence data. However, as will be demonstrated in the following section, the overall conformational changes induced by the catalytic Mn^{2+} are far more modest compared to those induced by Cd^{2+} that partially supports catalysis and Zn^{2+} that is highly inhibitory (12).

Interactions with metal ions

As mentioned previously, *PaePE* depends on Mn^{2+} for its phosphomonoesterase and phosphodiesterase activities. Cd^{2+} weakly supports the phosphodiesterase activity but not the subsequent hydrolysis of the 3'- PO_4 (12). In order to test whether Cd^{2+} also engaged the residues that constitute the Mn^{2+} binding site revealed by the crystal structure, we analyzed spectral perturbations in *PaePE* in the presence of increasing amounts of Cd^{2+} (Supplementary Figure S9). Resonances corresponding to almost all

residues (for which data could be analyzed) that lie on sheets $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 5$ together with the intervening loops (see Figure 7b and Supplementary Figure S10) were progressively attenuated during the course of the titration and finally broadened beyond the threshold of detection at an equimolar ratio of protein to Cd^{2+} ions. Minimal further changes were seen upon increasing the metal ion concentration, suggesting a single ion-binding site on *PaePE*. The Cd^{2+} -induced spectral perturbations could be reversed by addition of EDTA. Based on the nature of the spectral perturbations seen (no significant chemical shift changes or the appearance of new resonances), it appears that the *PaePE*- Cd^{2+} interaction

occurs on the intermediate exchange regime on the chemical shift timescale.

Next, we tested the ability of *PaePE* to bind Zn^{2+} that has been shown to be inhibitory in the presence of Mn^{2+} (12). As in the case of Cd^{2+} , strong spectral perturbations (Supplementary Figure S9), that reached their maximal value around a 1:1 stoichiometry, reversible by EDTA, were noted on the $\beta 1$ - $\beta 2$ - $\beta 3$ - $\beta 5$ face of the protein (Figure 7c). Additionally, some smaller but significant perturbations, not present in the case of Cd^{2+} ions (indicated by the black oval in Figure 7), were seen on the α -helix. More importantly, unlike in the case of Cd^{2+} , a new set of resonances appeared during the course of the titration

Figure 7. (A) Schematic representation of *PaePE* showing the secondary structural elements in the same orientation as in the left panels of (B–D). The largest conformational changes in the presence of metal ions involve the face formed by the strands $\beta 1$, $\beta 2$, $\beta 3$ and $\beta 5$ (indicated by the red dots). Some weak binding is also seen in the α -helical segment [indicated by an asterisk, also circled in panels (b–d)]. Spectral perturbations in the presence of (b) Cd^{2+} , (c) Zn^{2+} and (d) Mn^{2+} ions are mapped onto the *PaePE* surface. Reduction in peak intensity (I/I_0) in ^{15}N , ^1H HSQC spectra of *PaePE* in the presence of an equimolar ratio of metal ions is depicted using a red (maximum attenuation in the presence of metal with respect to the reference state) to blue (minimum attenuation) scale (also see Supplementary Figure S10). Residues for which perturbations could not be analyzed due to missing assignments, spectral overlap or weak peaks in the reference state are shown in gray. Note that the signal attenuation in the presence of Mn^{2+} is due to a combination of conformational changes and paramagnetic relaxation enhancement effects.

(reaching their maximum intensity around an equimolar protein-to-metal ratio) suggesting an interaction in the slow exchange regime on the chemical shift timescale (see Supplementary Figure S11 for a representative example). Given the extensive nature of the spectral perturbations seen in the case of both Cd^{2+} and Zn^{2+} , it appears they are the likely result of a conformational change involving the $\beta 1$ – $\beta 2$ – $\beta 3$ – $\beta 5$ face of the protein. This is also the region where the largest differences were seen between the NMR and crystal structures (Figure 3).

Finally, we analyzed the spectral changes for *PaePE* in the presence of Mn^{2+} (Supplementary Figure S9). Modest perturbations (Supplementary Figure S10), a combination of the effects of paramagnetic relaxation enhancement and conformational changes, were seen. The location of the line-broadening effects (see Figure 7d), though smaller in magnitude, were consistent with those seen in the cases of Cd^{2+} and Zn^{2+} , suggesting, as expected, a common binding site and similar conformational changes in the presence of Mn^{2+} . As in the case of Zn^{2+} , additional line-broadening effects were also seen (Figure 7d, indicated by the black oval) localized on the α -helical segment. It is therefore likely that the presence of several charged residues on this helix facilitate a weaker, non-specific interaction with bivalent metal ions. This binding is observable with Zn^{2+} that appears to have the highest overall affinity toward *PaePE* and with Mn^{2+} where the spectral perturbations due to binding events, even weak or transient ones, are greatly exaggerated due to relaxation enhancement induced by the paramagnetic nature of Mn^{2+} . No significant differences in spectral perturbations were seen at an equimolar protein-to-metal ratio in the presence or absence of a 10-fold excess of phosphate for any of the metal ions except perhaps in the case of Mn^{2+} where some additional attenuation involving Lys41 and Asp43 (that flank the catalytic His42) was noted at the active site (Supplementary Figure S10). It is plausible that a 3'- PO_4 facilitates the proper orientation of the catalytic Mn^{2+} at the active site. Overall, our data suggest that whereas *PaePE* relies on Mn^{2+} for catalysis, Mn^{2+} induces only modest conformational changes compared to Cd^{2+} or Zn^{2+} .

A crystal structure of *CkoPE* bound to Zn^{2+} and phosphate has recently been determined (35). The authors found that the structure of *CkoPE*• Zn^{2+} •phosphate complex is essentially identical to that of the previously determined *CkoPE*• Mn^{2+} •sulfate complex. However, the main difference lies in the coordination geometry of the metal ions: Zn^{2+} and Mn^{2+} were found to have tetrahedral and octahedral coordination geometries, respectively. This is in contrast to the large-scale spectral perturbations seen in our NMR data for *PaePE* in the presence of Zn^{2+} (and to a certain extent Cd^{2+}) but not for Mn^{2+} when compared to the metal-free enzyme. These differences cannot be explained simply by an alteration of the co-ordination sphere of the metal, rather points to a significant conformational change involving a large part of the catalytic face. While it is possible that the conformational changes induced by Zn^{2+} are different for the *PaePE* and *CkoPE* domains [Zn^{2+} acts as inhibitor for both proteins (12,35)],

it is more likely that the conformers that display these changes are unstable under crystal packing forces.

CONCLUSIONS

We have obtained the solution structure of the PE domain of *Pseudomonas aeruginosa* LigD using solution NMR techniques. PE in solution displays the 8-stranded β -barrel fold observed in the crystal structures of *PaePE* and two archaeal PEs. However significant differences between the solution and crystal structures are seen near the catalytic site. This involves strands $\beta 1$, $\beta 2$ and $\beta 5$ that form a dynamic structure that samples multiple conformations on a μs – ms timescale leading to an open conformation, on average, compared to a more closed conformation seen in the crystal structure in the presence of Mn^{2+} and sulfate. Additional differences are seen in the proline-rich $\beta 3$ – $\beta 4$ loop where the two flanking proline residues are found to be in the *cis* conformation while *trans* conformations are seen in the crystal structure.

Measurement of the spectral perturbations in the presence of three different primer–template duplexes reveal the location of the DNA binding surface of *PaePE* to be at a large positively charged patch on the face that hosts the catalytic residues. In addition what appears to be non-productive binding of DNA is also seen around positively charged residues in two of the all-DNA primer–template duplexes. However this mode of binding is greatly reduced in the presence of a terminal ribonucleotide base that is the preferred substrate for LigD, as evident from the NMR spectral perturbations, and further decreased in the presence of Mn^{2+} ions, as revealed by fluorescence measurements. It therefore appears that a ribose 3'-end and the catalytic metal ion help localize the oligonucleotide efficiently near the catalytic site. In contrast, no DNA-induced perturbations are seen for the disordered N-terminus that has been implicated in the monoesterase activity of LigD, whether in the absence or the presence of substoichiometric amounts of Mn^{2+} . Thus, the nature of the contribution of this segment to the 3'-monoesterase activity of LigD remains a mystery.

Whereas Mn^{2+} is essential for the catalytic activity of *PaePE*, we have found evidence for significant conformational changes in the presence of both Cd^{2+} and Zn^{2+} ions, with spectral features in the latter confirming the existence of an alternate conformation(s) in the presence of metal ions, this is in contrast to the minimal conformational changes seen by comparing the crystal structures of the *CkoPE*• Mn^{2+} •sulfate and *CkoPE*• Zn^{2+} •phosphate complexes, beyond the co-ordination sphere of the metal cation. Our spectral perturbation data opens up an intriguing possibility—it is plausible that within the conformational ensemble sampled by *PaePE* in solution, there exists a dynamic equilibrium between conformers that are catalytically competent and others that are not. Zn^{2+} ions help stabilize states compromised in their catalytic ability and greatly reduce the rate of exchange with the catalytically competent states (or equivalently, shifts the equilibrium toward the catalytically incompetent states) to an extent that this process is far slower than the catalytic

timescale leading to inhibition even in the presence of Mn^{2+} ions. This would imply that the new resonances that appear in the presence of Zn^{2+} correspond to an ensemble that is dominated by conformers unsuitable to induce catalysis. This shift in equilibrium is not as drastic in the case of Cd^{2+} ions where the exchange between the conformers appears to be in the intermediate regime on the chemical shift timescale and is still fast enough to support some level of catalytic activity (12).

ACCESSION NUMBERS

The coordinates for the core residues for the 15 structures of the final ensemble have been deposited in the PDB with accession code 2LJ6.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–11 and Supplementary Table 1.

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REFERENCES

- Gupta, R., Barkan, D., Redelman-Sidi, G., Shuman, S. and Glickman, M.S. (2011) Mycobacteria exploit three genetically distinct DNA double-strand break repair pathways. *Mol. Microbiol.*, **79**, 316–330.
- Shuman, S. and Glickman, M.S. (2007) Bacterial DNA repair by non-homologous end joining. *Nat. Rev. Microbiol.*, **5**, 852–861.
- Aniukwu, J., Glickman, M.S. and Shuman, S. (2008) The pathways and outcomes of mycobacterial NHEJ depend on the structure of the broken DNA ends. *Genes Dev.*, **22**, 512–527.
- Akey, D., Martins, A., Aniukwu, J., Glickman, M.S., Shuman, S. and Berger, J.M. (2006) Crystal structure and nonhomologous end-joining function of the ligase component of Mycobacterium DNA ligase D. *J. Biol. Chem.*, **281**, 13412–13423.
- Gong, C., Martins, A., Bongiorno, P., Glickman, M. and Shuman, S. (2004) Biochemical and genetic analysis of the four DNA ligases of mycobacteria. *J. Biol. Chem.*, **279**, 20594–20606.
- Zhu, H., Nandakumar, J., Aniukwu, J., Wang, L.K., Glickman, M.S., Lima, C.D. and Shuman, S. (2006) Atomic structure and nonhomologous end-joining function of the polymerase component of bacterial DNA ligase D. *Proc. Natl Acad. Sci. USA*, **103**, 1711–1716.
- Zhu, H. and Shuman, S. (2005) A primer-dependent polymerase function of pseudomonas aeruginosa ATP-dependent DNA ligase (LigD). *J. Biol. Chem.*, **280**, 418–427.
- Zhu, H. and Shuman, S. (2010) Gap filling activities of Pseudomonas DNA ligase D (LigD) polymerase and functional interactions of LigD with the DNA end-binding Ku protein. *J. Biol. Chem.*, **285**, 4815–4825.
- Brissett, N.C., Pitcher, R.S., Juarez, R., Picher, A.J., Green, A.J., Dafforn, T.R., Fox, G.C., Blanco, L. and Doherty, A.J. (2007) Structure of a NHEJ polymerase-mediated DNA synaptic complex. *Science*, **318**, 456–459.
- Pitcher, R.S., Brissett, N.C., Picher, A.J., Andrade, P., Juarez, R., Thompson, D., Fox, G.C., Blanco, L. and Doherty, A.J. (2007) Structure and function of a mycobacterial NHEJ DNA repair polymerase. *J. Mol. Biol.*, **366**, 391–405.
- Brissett, N.C., Martin, M.J., Pitcher, R.S., Bianchi, J., Juarez, R., Green, A.J., Fox, G.C., Blanco, L. and Doherty, A.J. (2011) Structure of a preternary complex involving a prokaryotic NHEJ DNA polymerase. *Mol. Cell*, **41**, 221–231.
- Zhu, H. and Shuman, S. (2005) Novel 3'-ribonuclease and 3'-phosphatase activities of the bacterial non-homologous end-joining protein, DNA ligase D. *J. Biol. Chem.*, **280**, 25973–25981.
- Zhu, H. and Shuman, S. (2006) Substrate specificity and structure-function analysis of the 3'-phosphoesterase component of the bacterial NHEJ protein, DNA ligase D. *J. Biol. Chem.*, **281**, 13873–13881.
- Zhu, H. and Shuman, S. (2007) Characterization of Agrobacterium tumefaciens DNA ligases C and D. *Nucleic Acids Res.*, **35**, 3631–3645.
- Zhu, H. and Shuman, S. (2008) Bacterial nonhomologous end joining ligases preferentially seal breaks with a 3'-OH monoribonucleotide. *J. Biol. Chem.*, **283**, 8331–8339.
- Zhu, H., Wang, L.K. and Shuman, S. (2005) Essential constituents of the 3'-phosphoesterase domain of bacterial DNA ligase D, a nonhomologous end-joining enzyme. *J. Biol. Chem.*, **280**, 33707–33715.
- Nair, P.A., Smith, P. and Shuman, S. (2010) Structure of bacterial LigD 3'-phosphoesterase unveils a DNA repair superfamily. *Proc. Natl Acad. Sci. USA*, **107**, 12822–12827.
- Smith, P., Nair, P.A., Das, U., Zhu, H. and Shuman, S. (2011) Structures and activities of archaeal members of the LigD 3'-phosphoesterase DNA repair enzyme superfamily. *Nucleic Acids Res.*, **39**, 3310–3320.
- Dutta, K., Natarajan, A., Nair, P.A., Shuman, S. and Ghose, R. (2011) Sequence-specific 1H , ^{13}C and ^{15}N assignments of the phosphoesterase (PE) domain of *Pseudomonas aeruginosa* DNA ligase D (LigD). *Biomol. NMR Assign.*, **5**, 151–155.
- Salzmann, M., Pervushin, K., Wider, G., Senn, H. and Wuthrich, K. (1998) TROSY in triple-resonance experiments: new perspectives for sequential NMR assignment of large proteins. *Proc. Natl Acad. Sci. USA*, **95**, 13585–13590.
- Cavanagh, J., Fairbrother, W.J., Palmer, A.G. III, Rance, M. and Skelton, N.J. (2007) *Protein NMR Spectroscopy*, 2nd edn. Academic Press, San Diego.
- Shen, Y., Delaglio, F., Cornilescu, G. and Bax, A. (2009) TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J. Biomol. NMR*, **44**, 213–223.
- Johnson, B.A. (2004) Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol. Biol.*, **278**, 313–352.
- Habeck, M., Rieping, W., Linge, J.P. and Nilges, M. (2004) NOE assignment with ARIA 2.0: the nuts and bolts. *Methods Mol. Biol.*, **278**, 379–402.
- Ferrage, F., Cowburn, D. and Ghose, R. (2009) Accurate sampling of high-frequency motions in proteins by steady-state ^{15}N - $\{^1H\}$ nuclear Overhauser effect measurements in the presence of cross-correlated relaxation. *J. Am. Chem. Soc.*, **131**, 6048–6049.
- Linge, J.P., Williams, M.A., Spronk, C.A., Bonvin, A.M. and Nilges, M. (2003) Refinement of protein structures in explicit solvent. *Proteins*, **50**, 496–506.
- Laskowski, R.A., Rullmann, J.A., MacArthur, M.W., Kaptein, R. and Thornton, J.M. (1996) AQUA and PROCHECK-NMR: programs for checking the quality of protein structures solved by NMR. *J. Biomol. NMR*, **8**, 477–486.
- Hutchinson, E.G. and Thornton, J.M. (1996) PROMOTIF - a program to identify and analyze structural motifs in proteins. *Protein Sci.*, **5**, 212–220.
- Laskowski, R.A. (2001) PDBsum: summaries and analyses of PDB structures. *Nucleic Acids Res.*, **29**, 221–222.

30. Boggs, P.T., Donaldson, J.R., Byrd, R.H. and Schnabel, R.B. (1989) ODRPACK software for weighted orthogonal distance regression. *ACM Trans. Math. Software*, **15**, 348–364.
31. Han, B., Liu, Y., Ginzinger, S.W. and Wishart, D.S. (2011) SHIFTX2: significantly improved protein chemical shift prediction. *J. Biomol. NMR*, **50**, 43–57.
32. Prestegard, J.H., Bougault, C.M. and Kishore, A.I. (2004) Residual dipolar couplings in structure determination of biomolecules. *Chem. Rev.*, **104**, 3519–3540.
33. Zuiderweg, E.R.P. (2002) Mapping Protein-Protein Interactions in Solution by NMR Spectroscopy. *Biochemistry*, **41**, 1–7.
34. Burstein, E.A., Vedenkina, N.S. and Ivkova, M.N. (1973) Fluorescence and the location of tryptophan residues in protein molecules. *Photochem. Photobiol.*, **18**, 263–279.
35. Das, U., Smith, P. and Shuman, S. (2011) Structural insights to the metal specificity of an archaeal member of the LigD 3'-phosphoesterase DNA repair enzyme family. *Nucleic Acids Res.* (in press).