Structure of PhnP, a Phosphodiesterase of the Carbon-Phosphorus Lyase Pathway for Phosphonate Degradation*

Received for publication, November 3, 2008, and in revised form, March 12, 2009. Published, JBC Papers in Press, April 14, 2009, DOI 10.1074/jbc.M808392200

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Carbon-phosphorus lyase is a multienzyme system encoded by the phn operon that enables bacteria to metabolize organophosphonates when the preferred nutrient, inorganic phosphate, is scarce. One of the enzymes encoded by this operon, PhnP, is predicted by sequence homology to be a metal-dependent hydrolase of the β-lactamase superfamily. Screening with a wide array of hydrolytically sensitive substrates indicated that PhnP is an enzyme with phosphodiesterase activity, having the greatest specificity toward bis(p-nitrophophenyl)phosphate and 2',3'-cyclic nucleotides. No activity was observed toward RNA. The metal ion dependence of PhnP with bis(p-nitrophosphophenyl)phosphate as substrate revealed a distinct preference for Mn2+ and Ni2+ for catalysis, whereas Zn2+ afforded poor activity. The three-dimensional structure of PhnP was solved by x-ray crystallography to 1.4 Å resolution. The overall fold of PhnP is very similar to that of the tRNase Z endonucleases but lacks the long exosite module used by these enzymes to bind their tRNA substrates. The active site of PhnP contains what are probably two Mn2+ ions surrounded by an array of active site residues that are identical to those observed in the tRNase Z enzymes. A second, remote Zn2+ binding site is also observed, composed of a set of cysteine and histidine residues that are strictly conserved in the PhnP family. This second metal ion site appears to stabilize a structural motif.

In many environments inorganic phosphate, an essential nutrient, can fall to extremely low concentrations, forcing microorganisms to utilize other forms of phosphorus to survive. In such cases, organophosphonates can comprise a major fraction of the total phosphorus available to biological systems (e.g. 2-aminoethylphosphonate is a widespread natural product). However, cleavage of the highly stable carbon-phosphorus (CP) bond to release inorganic phosphate requires specialized enzymes. One such enzyme activity found widely in bacteria is CP-lyase (1). Cleavage of the CP bond of organophosphonates by CP-lyase yields inorganic phosphate and, remarkably, a hydrocarbon. CP-lyase is actually a multienzyme system, encoded by the phn operon (phnCDEFGHIJKLMNOP), which is induced by low concentrations of phosphate as part of the pho regulon. Gene deletions studies in Escherichia coli have shown that phnGHIJKLMNOP are essential for catalysis of CP bond cleavage, whereas the remaining genes probably encode transport, regulatory, or accessory functions (2). Only a handful of the proteins encoded by the phn operon have been characterized to date. PhnD was shown to be a periplasmic binding protein with high affinity for organophosphonates (3); the three-dimensional structure of PhnH, one of the proteins essential for CP-lyase catalysis, was recently solved, but a function has yet to be determined (4); PhnN was shown to be an ATP-dependent kinase that provides a redundant pathway to 5-phospho-d-riboburanosyl-α-1-diphosphate (5); and PhnO was demonstrated to be an acetyl-CoA-dependent N-acyltransferase with activity toward a wide range of aminoalkylphosphonates (6). Although the phnP gene is not essential for CP bond cleavage by cells in liquid culture (2), cell growth on solid media supplemented with methylphosphonate or phosphite as the sole phosphorus source is prevented by phnP mutations (7), suggesting a critical regulatory or accessory role for PhnP. Accordingly, phnP appears frequently in the phn operon in various species of bacteria, typically following the phnN gene (8). PhnP is predicted based on its sequence to be a member of the β-lactamase family of metal-dependent hydrolases with greatest homology to enzymes from the tRNase Z (ProDom family PD352433) and ElaC families (9), the latter erroneously annotated as composed of arylsulfatases but later determined to also belong to the tRNase Z family (10, 11). The tRNase Z enzymes are endonucleases used by prokaryotes and eukaryotes to cleave a specific

* This work was supported in part by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) and the Canadian Institute for Health Research. The atomic coordinates and structure factors (code 3GIP) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
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5 The abbreviations used are: CP, carbon-phosphorus; CHES, 2-(cyclohexylamino)ethanesulfonic acid; bpNP, bis(p-nitrophosphophenyl) phosphate; ICP, inductively coupled plasma; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; ppGpp, guanosine 3',5'-bisphosphophosphate; ppGpp, guanosine 3'-diphosphate-5'-triphosphate; RFU, relative fluorescence units; WT, wild type enzyme; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; pPG2, 3'-p; pNP-TMP, thymidine-5'-monophosphate p-nitrophosphophenyl ester.
phosphodiester bond near the 3′-end of pre-tRNA, yielding a 3′-end that can be coupled to an amino acid. These enzymes typically use two active site bound Zn$^{2+}$ ions to simultaneously lower the pK$_a$ of a nucleophilic water molecule and stabilize negative charge development on the phosphodiester linkage undergoing nucleophilic attack (12). Since it is not clear how a tRNase activity would support cell growth with an organophosphonate as a sole phosphorus source, we set out to characterize the substrate specificity and three-dimensional structure of PhnP to learn more about this critical CP-lyase enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—All buffers, substrates, and chemicals, unless otherwise noted, were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Canada). DNA oligonucleotides were synthesized by Sigma-Genosys Canada (Oakville, Canada). Microbiology media were purchased from Thermo-Fisher Scientific Canada Ltd. (Ottawa, Canada). DNA sequencing was performed at the Robarts Research Institute (London, Canada). Calf intestinal alkaline phophatase was obtained from New England Biolabs. RNase A was from Ambion. J. T. Baker, Inc. brand polyethyleneimine-cellulose TLC plates were obtained from Mallinckrodt Baker.

Expression, Purification, and Size Exclusion Chromatography of PhnP and Mutants—The cloning and expression of phnP as well as the purification and crystallization of wild type PhnP were described previously (13). Briefly, wild type PhnP with a C-terminal His$_6$ tag was expressed from the plasmid pH520-phin in BL21(DE3) cells (Novagen) co-transformed with pLacI (Novagen). The plasmid pH520-phin is a derivative of pUHE 23–2 which contains T7 promoter and lac operator sites (14). Cells were grown in LB medium supplemented with ampicillin and chloramphenicol at 30 °C and induced at A$_{600}$ ~ 0.6 with 0.5 mM isopropyl-$\beta$-d-1-thiogalactopyranoside. The cells were then grown at 15 °C for 24 h and collected by centrifugation. After cell lysis and centrifugation, PhnP was purified by Ni$^{2+}$-nitritrolactiate acid affinity and size exclusion chromatography. MALDI-MS confirmed the predicted molecular mass of His$_6$-tagged PhnP as 28,670 Da (data not shown). PhnP mutants were created using the QuikChange$^\text{TM}$ protocol (Stratagene). For the D80A mutant, the primer 5′-ACGCATTATCATATGCTCAGTCCAGGGGCTCCGCCAG-3′ and its complementary sequence were used. For the C21S/C23S/C26S triple mutant, the primer 5′-CGGGCATGGGGTCAGTCTGCGGCCTCCGCCAG-AAGCCCG-3′ and its complimentary sequence were used. Mutagenic codons are underlined. The mutant genes were verified by sequencing both DNA strands. The mutant alleles were transferred into the Robarts Research Institute (London, Canada).

Substrate Screening—PhnP activity was assayed against a panel of naturally occurring phosphodiesterases and phosphonohydrides (all obtained from Sigma), as described previously (15). Each reaction (80 µl), containing 50 mM Tricine hydrochloride (pH 8.5), 5 mM MgCl$_2$, 0.5 mM each MnCl$_2$, NiCl$_2$, and CoCl$_2$, 0.25 mM substrate, and 2 µg of PhnP, was incubated at 37 °C for 20 min and then stopped by the addition of 80 µl of 0.2 M CHES, 10 mM MgCl$_2$, pH 9.0. The reactions were then incubated with 1 unit of shrimp alkaline phosphatase for 10 min at 37 °C, followed by the addition of 40 µl of malachite green reagent (16) and measurement of the absorbance at 630 nm to determine production of orthophosphate.

Steady-state Kinetic Analysis—Reactions of PhnP with bis(p-nitrophenyl)phosphate (bpNPP), thymidine-5′-monophosphate-p-nitrophenyl ester (pNP-TMP), or 2′,3′-cyclic nucleotides were performed in 50 mM Tris-HCl (pH 7.2), 150 mM NaCl, divalent metal (0.1 mM ZnCl$_2$, 1 mM MnCl$_2$, or 1 mM NiCl$_2$) and 1 mg/ml bovine serum albumin at 25 °C. Release of p-nitrophenolate was monitored at 405 nm (Δε$_{405}$ = 11,500 M$^{-1}$ cm$^{-1}$) on a Cary Bio-300 spectrophotometer (Varian). Phosphodiesterase activity toward 2′,3′-cyclic nucleotides was measured as follows. A 400-µl solution of substrate, 1 mM MnCl$_2$, 50 mM Tris-HCl (pH 7.2), and 150 mM NaCl was reacted with 1.5 µM PhnP for 20 min and then stopped with 400 µl of 0.2 M Tris-HCl, 10 mM MgCl$_2$, pH 9.0. One unit of calf intestinal alkaline phophatase was added, and the reaction was incubated at 37 °C for 10 min. Finally, 200 µl of malachite green reagent (16) was added, and free phosphate was determined colorimetrically at 630 nm using an extinction coefficient of Δε$_{630}$ = 90,000 M$^{-1}$ cm$^{-1}$. Duplicate reactions containing no PhnP were performed to measure residual inorganic phosphate. The kinetic parameters $k_{cat}$ and $K_m$ were determined by fitting the dependence of the initial reaction velocities on the substrate concentration with the Michaelis-Menten or Hill (17) equations using GraFit 6 (Erithacus Software Ltd.).

RNase Activity Assay—The activity of PhnP and the D80A mutant was assayed with the RNaseAlert Lab Test Kit from Ambion (catalogue number 1964). RNase-free pipette tips, plasticware, and water were used for the assay. A 50-µl reaction containing 10 ng of PhnP, 20 mM HEPEs (pH 7.5), 150 mM NaCl, 10 µM MnCl$_2$, and RNA substrate was incubated at 37 °C for 1 h. Control reactions contained 0.2 µg of RNase A or buffer only. Cleavage of the RNA oligonucleotide was monitored by fluorescence at λ$_{ex}$ = 490 nm and λ$_{em}$ = 520 nm using a SpectraMax Gemini XS spectrofluorimeter (Molecular Devices, Sunnyvale, CA).

ICP-MS Analysis—The metal content of wild type PhnP was analyzed using a Varian ICP-MS (Ultra Mass). PhnP was dissolved into 10 mM HEPEs, pH 7.2, and diluted to 10 µM for analysis. Metal content was determined by comparison with standard curves of Mn$^{2+}$ and Zn$^{2+}$ ranging from 0 to 1000 ppm. Measurements were performed in triplicate and corrected for residual metal content by analyzing buffer alone.

Metal Dependence of PhnP Activity—Metal-free “apo-PhnP” was obtained by incubation with 10 mM EDTA for 1 h at 4 °C, followed by dialysis into 50 mM Tris-HCl, 150 mM NaCl, pH 7.2. Apo-PhnP was then incubated with different metal ions by the addition of 0.2 mM ZnCl$_2$, MnCl$_2$, NiCl$_2$, CoCl$_2$, CaCl$_2$, FeCl$_2$, FeCl$_3$, or MgCl$_2$. After 1 h of incubation at 4 °C, an aliquot of each PhnP sample was added to a reaction containing 2 mM bpNPP in 50 mM Tris-HCl, 150 mM NaCl, 1 mg/ml bovine serum albumin, pH 7.2, equilibrated at 25 °C (the final concentration of PhnP was 2 µM). Initial rates were measured by mon-
itoring p-nitrophenolate production as before. PhnP reconstituted with Zn²⁺ was “rescued” by incubation with 10 mM EDTA a second time followed by dialysis. After incubation with 0.2 mM Mn²⁺ as before, PhnP (2 μM) was assayed with bpNPP. The apparent Km values (Km(apply) for Mn²⁺ and Ni²⁺ were obtained by an assay of wild type apo-PhnP or the D80A mutant with 1 mM bpNPP and various concentrations of the metal ion. The resulting curve of initial rates versus metal ion concentration was fit to the Michaelis-Menten equation using GraFit 6.0.

**Crystall Structure of PhnP**

A reaction containing 700 μl of 50 mM Tris (pH 7.2), 150 mM NaCl, 2 mM MnCl₂, 10 mM 2',3'-cAMP, and 26 μM wild type PhnP was incubated for 7 h at 21°C. A control reaction lacking PhnP was incubated under the same conditions. Aliquots (5 μl) of these reactions along with standards of 3'-AMP and 2'-AMP (10 mM each in the above reaction buffer lacking PhnP) were spotted onto a polyethyleneimine-cellulose TLC plate (5 × 20 cm, polyester-backed, with a fluorescent indicator). Once the aliquots had dried, the TLC plate was washed in MeOH, dried, and then developed in saturated ammonium sulfate (pH 3.5), as described by Bochner (18). After washing the plate again in MeOH, the dried plate was visualized under UV light (254 nm) and photographed with a standard digital camera.

**PhnP Structure Solution and Refinement**—Diffraction data were collected as previously described (19). Data were indexed, integrated, and scaled using DENZO and SCALEPACK (20). Initial phases were determined by single wavelength anomalous dispersion at the selenium peak energy using the program autoSHARP (21), and 10 of the 12 expected selenium atoms in the asymmetric unit were located. The initial model was built automatically using autoSHARP and completed manually in XFIT/XTALVIEW (22). Structure refinement was performed using REFMACS (23). The final model contained a dimer in the asymmetric unit, 882 water molecules, four manganese ions, two zinc ions, and two (S)-malate molecules.

**RESULTS**

**Substrate Specificity and Product Analysis**—The amino acid sequence of *E. coli* PhnP shows significant homology to metal ion-dependent enzymes that comprise the β-lactamase superfamily (Fig. 1). Best homology is found with enzymes that hydrolyze phosphodiester bonds, particularly the tRNase Z endonucleases (21 and 24% sequence identity, respectively, with *E. coli* ZipD and *B. subtilis* tRNaseZ), where all residues involved in active site metal ion binding are strictly conserved (Fig. 1). Accordingly, *E. coli* PhnP showed high activity against the generic phosphodiesterase substrate bpNPP. The pH optimum for this reaction was 7.2 (data not shown). PhnP was inactive against the corresponding monoester, p-nitrophenyl phosphate. To identify a potential *in vivo* substrate, PhnP was screened for hydrolytic activity against a wide array of naturally occurring phosphodiesters and phosphoanhydrides in the presence of a mixture of metal ions (Mg²⁺, Mn²⁺, and Ni²⁺) (Fig. 2). Highest activity was observed with 2',3’-cyclic nucleotides, whereas virtually no activity was observed with the corresponding 3',5’-cyclic nucleotides. The reaction with 2',3’-nucleotides is regiospecific, with only the 3’-nucleotide product being observed by TLC (Fig. 3). The low apparent activity observed in the screen with FAD, 3',5'-cIMP, and UDP-GlcA could not be detected by direct initial rate kinetics and is probably due to free phosphate in the substrate preparations. PhnP also did not display activity against single-stranded RNA beyond background hydrolysis (specific activity of 2.8 × 10³ RFU min⁻¹ mg⁻¹). This was confirmed when the D80A mutant of PhnP, which is virtually inactive against bpNPP (see Table 3), afforded essentially the same low specific activity against the RNA substrate (4.2 × 10³ RFU min⁻¹ mg⁻¹) as the wild type enzyme. This is consistent with the background hydrolysis arising from another enzyme, probably a constant, trace level of RNase in our PhnP preparations. In contrast, a positive control with RNase A showed specific activity of 2.2 × 10¹³ RFU min⁻¹ mg⁻¹ toward this single-stranded RNA substrate.

**Metal Ion Dependence of PhnP—ICP-MS analysis of PhnP revealed nearly stoichiometric amounts of Zn²⁺ (1.38/monomer) and low levels of Mn²⁺ (0.13/monomer; Table 1). To determine which of these metals was essential for catalysis, PhnP was treated with EDTA and then dialyzed against metal free buffer. Although it was possible to reduce the Mn²⁺ content to background levels (as measured by ICP-MS), nearly stoichiometric amounts of Zn²⁺ (0.78/monomer) remained (Table 1), suggesting the presence of a high affinity Zn²⁺ binding site on the enzyme. The Mn²⁺-free form of PhnP was essentially inactive against bpNPP (Table 2), whereas incubation of PhnP with 200 μM Mn²⁺ or Ni²⁺ restored substantial activity (5000–13,000-fold). Zinc ion restored considerably less activity (~200-fold) and in fact appeared to be inhibitory, since equimolar mixtures of Zn²⁺ and Mn²⁺ or Ni²⁺ likewise only restored ~140–190-fold activity. However, substantial activity (1500-fold greater than apo-PhnP) could be restored by treating the Zn²⁺-reconstituted PhnP with EDTA and then reincubating with Mn²⁺ (Table 2). The reaction rate of apo-PhnP with bpNPP as a function of metal ion concentration affords an apparent Km value (Km(apply)) of 0.13 ± 0.02 mM for Mn²⁺ and 0.090 ± 0.02 mM for Ni²⁺ (Table 3), indicating that at least one metal ion occupies a site of relatively low affinity. Further evidence for Mn²⁺ bound in the active site was obtained by mutating Asp⁸⁰, one of the conserved metal ion ligands. The PhnP D80A mutant, in addition to being largely inactive, experienced a 100-fold increase in Km(apply) for Mn²⁺ (13 ± 2 mM; Table 3). Consistent with this dramatically reduced affinity for the metal ion, the D80A mutant no longer co-purified with Mn²⁺, as indicated by ICP-MS (Table 1), whereas the amount of Zn²⁺ bound did not change significantly. More detailed kinetic analysis of PhnP using bpNPP as substrate and saturating metal ion concentrations confirmed an apparent Km value (Km(apply)) of 0.13 ± 0.02 mM for Mn²⁺ and 0.090 ± 0.02 mM for Ni²⁺ (Table 3), indicating that at least one metal ion occupies a site of relatively low affinity. Further evidence for Mn²⁺ bound in the active site was obtained by mutating Asp⁸⁰, one of the conserved metal ion ligands. The PhnP D80A mutant, in addition to being largely inactive, experienced a 100-fold increase in Km(apply) for Mn²⁺ (13 ± 2 mM; Table 3). Consistent with this dramatically reduced affinity for the metal ion, the D80A mutant no longer co-purified with Mn²⁺, as indicated by ICP-MS (Table 1), whereas the amount of Zn²⁺ bound did not change significantly. More detailed kinetic analysis of PhnP using bpNPP as substrate and saturating metal ion concentrations confirmed that considerably greater kcat and kcat/Km values are achieved with Ni²⁺ and Mn²⁺ than with Zn²⁺ (Table 3). Overall, these data suggest that the binuclear active site of PhnP has greater activity with Mn²⁺ and Ni²⁺ ions and that a single Zn²⁺ ion is bound at a separate, high affinity site.

**Kinetic Analysis of Wild Type PhnP**—The initial substrate screening results were confirmed by more detailed kinetic analysis (Table 3). In the presence of saturating Mn²⁺ (1 mM), PhnP has greatest kcat/Km values with 2',3'-cAMP, 2',3'-cCMP and 2',3'-cGMP followed by bpNPP. Analysis of the activity at low
FIGURE 1. Multiple sequence alignment of PhnP homologues. Amino acid sequences are for E. coli K-12 PhnP (gi: 536936), Pseudomonas stutzeri PhnP (gi: 40804950), Pyrococcus horikoshii OT3 PhnP (gi: 14591382), Marinobacter aquaeolei VT8 PhnP (gi: 120555210), Pseudomonas putida PqqB (gi: 56967240; Protein Data Bank code 1xto), E. coli ZipD (gi: 90109091; Protein Data Bank code 2cbn), and B. subtilis tRNaseZ (gi: 60594108; Protein Data Bank code 1y44). The percentage of sequence identity to E. coli PhnP is shown in parentheses. α-Helices and β-strands observed in the E. coli PhnP structure are indicated as cylinders and block arrows, respectively. Residues involved in binding the two active site metal ions are highlighted in red. The putative general acid catalyst (GAC) is highlighted in turquoise. Residues involved in binding the structural zinc ion are highlighted in yellow. The exosite of the tRNase Z enzymes involved in binding tRNA is underlined. Strictly, highly and moderately conserved residues are indicated by asterisks, colons, and periods, respectively. The sequence alignment was performed using ClustalW (53) and then edited manually.
FIGURE 2. Screening PhnP hydrolytic activity against a series of phosphat diesters and phosphoanhydrides. PhnP (2 μg) was incubated with each substrate (0.25 mM) in the presence of a mixture of Mg2+/Mn2+ (0.25 mM each) or Mg2+/Ni2+ (0.25 mM each) in pH 8.5 buffer for 20 min at 37°C. After treatment with alkaline phosphatase to hydrolyze any monooester product generated by PhnP, the liberated orthophosphate was quantified by absorbance at 630 nm with a malachite green assay. Assay conditions are described under “Experimental Procedures.”

Metal ion dependence of PhnP

Table 2

| Sample*  | \( V_{o}/[E]_p \) | Relative rate |
|----------|------------------|---------------|
| Apo-PhnP | 0.004            | 1             |
| Mn2⁺     | 24.3             | 5600          |
| Ni2⁺     | 58.7             | 13,500        |
| Mn2⁺/Ni2⁺| 46.1             | 10,600        |
| Zn2⁺     | 0.891            | 205           |
| Zn2⁺/Mn2⁺| 0.600            | 138           |
| Zn2⁺/Ni2⁺| 0.817            | 188           |
| 1) Zn2⁺ | 6.57             | 1510          |
| 2) EDTA | 0.557            | 128           |
| 3) Mn2⁺ | 0.561            | 129           |
| Co2⁺     | 0.261            | 60            |
| Cu2⁺     | 0.013            | 30            |
| Mg2⁺     | 0.022            | 5.0           |
| Ca2⁺     | 0.026            | 6.0           |

* Metal-free apo-PhnP was obtained by incubation with EDTA followed by dialysis. Apo-PhnP was incubated with a 0.2 mM concentration of the metal ions listed above then assayed with bpNPP, as described under “Experimental Procedures.” Specific activities of bpNPP hydrolysis for metal-reconstituted PhnP and 2 mM bpNPP at 25°C. \( V_{o} \) = initial rate; \( [E]_p \) = total enzyme concentration.

* Apo-PhnP incubated with Zn2⁺ was stripped with EDTA, dialyzed, incubated with Mn2⁺, and then assayed with bpNPP.

FIGURE 3. TLC (polyethyleneimine-cellulose) analysis of the reaction product of PhnP with 2′,3′-cAMP. Lanes 1 and 2, standards of 2′-AMP and 3′-AMP, respectively (5 μl each from 10 μM solutions in reaction buffer). Lane 3, an aliquot (5 μl) of the PhnP reaction with 2′,3′-cAMP (26 μM PhnP, 10 mM 2′,3′-cAMP in reaction buffer, incubated at 21°C for 7 h). Lane 4, an aliquot (5 μl) of 2′,3′-cAMP (10 mM in reaction buffer) incubated under the same conditions as the PhnP reaction. Reaction conditions and TLC development are described under “Experimental Procedures.”

TABLE 1

| PhnP sample     | [Mn2⁺][μM] | [Zn2⁺][μM] | Mn2⁺/PhnP monomer | Zn2⁺/PhnP monomer |
|-----------------|------------|------------|-------------------|-------------------|
| WT as purified  | 1.3 ± 0.1² | 13.8 ± 1.1 | 0.13              | 1.38              |
| WT EDTA-treated | 0.06 ± 0.01| 7.8 ± 0.03 | 0.01              | 0.78              |
| D80A            | ND*        | 10.5 ± 0.2 | 0                 | 1.05              |

* S.D. values based on the average of three samples.

* Not detectable; value indistinguishable from that obtained with buffer alone.

bpNPP concentrations followed by fitting of the data to the Hill equation revealed modest cooperativity (nH = 1.55 ± 0.04), similar to that observed with E. coli ZipD (nH = 1.6) (10). This cooperativity was not observed with the 2′,3′-cyclic nucleotides. Very low activity was also observed with pNP-TMP (kcat/Km = 5.3 M⁻¹ s⁻¹), another general phosphodiesterase substrate (15). The greater specificity of PhnP toward the 2′,3′-cyclic nucleotides is manifested almost entirely by a drop in the value of Km (10–310 μM) relative to bpNPP (Km = 2.9 ± 0.5 mM), suggesting greater recognition for these substrates in the ground state (in the absence of kinetically significant enzyme-substrate intermediates).

The Crystal Structure of PhnP—The three-dimensional structure of PhnP from E. coli K12 was determined to 1.4 Å resolution using a single-wavelength anomalous dispersion method. The asymmetric unit of the PhnP crystal contained a dimer (see Fig. 5A). This is probably representative of the oligomeric state of PhnP in solution, as opposed to crystal packing. The PhnP monomer has a predicted molecular mass of 28.67 kDa (Fig. 4), suggesting the formation of a compact dimer in solution. Of 258 residues, molecule A contains residues 2–250, including all of the side chains, whereas molecule B contains residues 3–250. Since only the peptide backbone density was visible for residues 2 and 251 in molecule B, they were refined as alanine and glycine, respectively. There was no clear density present for the C-terminal hexahistidine tag in either of the subunits (residues 253–258). The model was refined to an R-factor of 18.6% and an Rfree value of 21.0%, with 882 molecules of water (Table 4). The model possesses excellent geometry, where 87.2% of the residues fall in the allowed regions, 1.9% in generously allowed regions, and 88.2% in restraints.

The PhnP fold belongs to the a/β class of proteins and falls into the metallo-hydrolase superfamily. The monomer core consists of two mixed β-sheets that are sandwiched between two layers of α-helices. The smaller sheet contains six strands, the first three parallel (β5–β3) and the next three antiparallel (β2, β1, and β3). The larger sheet contains seven strands, the first four of which are parallel (β12–β9); the remaining three sheets are antiparallel (β8–β6). Strands 6 and 7 are joined by a two-residue Type I’ β-hairpin, whereas strands 2 and 3 are con-
The dimerization of PhnP results in formation of a deep cleft on the surface of each monomer close to the dimerization interface. The two His\(^{58}\) side chains from loop 5 of each monomer are in the off-centered parallel orientation, creating a disallowed region. The two His\(^{58}\) side chains from loop 5 of each monomer extend far into the hydrophobic pocket of the other monomer. The two His\(^{58}\) side chains from loop 5 of each monomer are in the off-centered parallel orientation, creating a disallowed region. The two His\(^{58}\) side chains from loop 5 of each monomer extend far into the hydrophobic pocket of the other monomer.

Structural Homology to tRNase Z Endonucleases—A search of the Protein Data Bank using DALI revealed that PhnP has high structural homology to metal-dependent hydrolases of the \(\beta\)-lactamase superfamily, particularly the tRNase Z endonucleases (Fig. 6A). Using root mean square deviation values based on the least squares superimposition of the structurally equivalent C-\(\alpha\) atoms, the nearest tRNase Z homologue is the B. subtilis enzyme (Protein Data Bank code 1y44) with a root mean square deviation of 2.7 Å (Z score 23). The tRNases are also homodimers, and the active site residues used for coordination of two active site metal ions are strictly conserved with PhnP (Fig. 6B). However, several profound structural differences are also observed. The characteristic long exosite used for pre-tRNA binding by E. coli ZipD and B. subtilis tRNase Z is absent in PhnP (Fig. 6A). PhnP, like E. coli ZipD, possesses fully metal-loaded active sites in both monomers, whereas only one monomer is metal-loaded in the B. subtilis enzyme. This is due to...
to a dramatic conformational change between two monomers in the *B. subtilis* tRNase Z, where one monomer has a distorted active site, whereas the other one lacks a resolved exosite but retains a functional active site (24). His140 and His247 in *B. subtilis* tRNase Z (His143 and His200 in PhnP, respectively) move far out of position in the “inactive” monomer, which prevents metal binding. It has been suggested that tRNA binding to the inactivated monomer causes a conformational change and subsequent activation of the second active site, which would result in cooperative behavior. Another feature that distinguishes PhnP from the tRNase Z hydrolases is the presence of an additional α-helix containing the second metal ion binding site (see below).

The Zn\(^{2+}\) Binding Site—Unlike other members of the β-lactamase family, the PhnP subfamily possesses three strictly conserved cysteines near the N terminus (Fig. 1) (25). Such sulfur-rich binding sites typically bind Zn\(^{2+}\) ions, and this is clearly observed in the structure of PhnP (Fig. 6C). This metal-binding site is located at the edge of the monomer next to the dimerization interface. A tetrahedral coordination sphere for single Zn\(^{2+}\) ion is formed by Cys19, Cys21, Cys23, and His225 residues. The cysteine residues are contributed by loop 1 and α-helix 1, whereas His225 is contributed by α-helix 8. Helices 1 and 2 are flanked by loop 3 and a long extended region of loop 1. This stretch of secondary structure forms a lobe that is tethered to the main body of the protein through hydrophobic interactions with helix 8 and is further stabilized by two intraprotein salt bridges and several hydrogen bonds. Loops 1 and 3 also provide the residues for two of three interprotein salt bridges as well as residues that form the majority of the interprotein hydrogen bonds. As mentioned previously, loop 1 also contains Trp19, which forms extensive hydrophobic interactions with the deep pocket created by residues of helices 3 and 4 and loops 6 and 8 of the other monomer. Therefore, the area around the Zn\(^{2+}\) site is responsible for providing the majority of protein–protein interaction, and its integrity is crucial for the overall stability of the dimer. The structural role of this site is supported by the observation that simultaneous alteration of all three cysteines to serines (C21S/C23S/C26S) produced an insoluble protein.

**Structural Homology to PqqB**—Intriguingly, one of the closest structural homologues to PhnP, based on a DALI search of the Protein Data Bank, is PqqB (Fig. 6A; Protein Data Bank code 1xt0), an enzyme that appears to be involved in the transport of an intermediate in the pyrroloquinoline quinone biosynthetic pathway (26, 27). Despite the low sequence identity of 22%, PhnP and PqqB monomer structures align with a root mean square deviation of 2.7 Å and Z-score of 24.7. PhnP and PqqB share the Zn\(^{2+}\) binding site and structural motif, which is also strictly conserved in the PqqB family of enzymes (Fig. 1). The arrangement of the scaffold and the three coordinating cysteine residues is virtually identical between the two proteins, whereas the fourth residue is Asn272 in PqqB rather than histidine as in PhnP (Fig. 6C). In contrast, there is only moderate sequence and structural conservation between PhnP and PqqB active sites, and the latter does not have metal ions bound (Fig. 1).

**The PhnP Active Site**—The active site in PhnP is located at the loop aggregation area at the edge of the β-sandwich at the dimerization interface (Fig. 5B). Density was observed for two metal ions in the active site, surrounded by residues that are conserved among PhnP homologues and known to bind metal ions (Fig. 1). At a resolution of 1.4 Å, the difference density for the metal ions at this site was noticeably smaller than the one found for Zn\(^{2+}\) at the cysteine site described above, suggesting the presence of a lighter metal ion. Although 0.13 Mn\(^{2+}\)/monomer co-purified with PhnP (Table 1), this does not necessarily represent the metal ion occupancy in the crystal, since a metal-enriched form of the enzyme may have been selectively crystalized. Combining this observation with the co-purification of Mn\(^{2+}\) with wild type PhnP, the reduced affinity for Mn\(^{2+}\) in the active site mutant D80A (Tables 1 and 3), and the distinct pref-
ference for Mn\textsuperscript{2+} over Zn\textsuperscript{2+} for activity (Tables 2 and 3), the active site metals were assigned as two Mn\textsuperscript{2+} ions. Distances between metal ions and coordinating residues are summarized in Table 5. The metal ions are 3.5 Å apart, and are located about 19 Å away from the Zn\textsuperscript{2+} ion of the same monomer. Surprisingly, a molecule of (S)-malate was stereoselectively seques-
tered from 0.1M racemic malate present in the crystallization buffer (MMT buffer; Qiagen). The (S)-malate molecule binds in a bidendate fashion to the more solvent-exposed MnA ion (Fig. 7A). Metal ions are labeled as described by Vogel et al. (17). The MnA ion has octahedral coordination geometry, with axial bonds provided by the (S)-malate α-carboxyl and His\textsuperscript{76}, whereas equatorial bonds are provided by His\textsuperscript{78}, His\textsuperscript{143}, Asp\textsuperscript{164}, and the (S)-malate hydroxyl. The coordination geometry of less solvent-exposed MnB ion is dis-
torted octahedral. The equatorial bonds are provided by Asp\textsuperscript{80}, Asp\textsuperscript{164}, His\textsuperscript{222}, and the (S)-malate hydroxyl, whereas the axial bonds are formed with His\textsuperscript{81} and a water molecule, which is located 1.6 Å above the MnB ion. A significant B-factor increase was observed for MnB ion compared with the MnA ion in both monomers, indicating higher mobility of the former. This probably reflects differing affinities for the two metal ions, which have been observed by ITC for E. coli ZipD and related β-lactamase fam-
ily enzymes (28). The hydroxyl group of (S)-malate is not equidis-
 tant from each Mn\textsuperscript{2+} due to the bidentate interaction with the MnA ion; the distance to the more solvent-exposed MnA ion is 2.2 Å, whereas the distance to the more buried MnB ion is 3.1 Å (Table 5). The malate hydroxyl makes an intriguing interaction with Asp\textsuperscript{80}, a conserved residue critical for PhnP catalysis (Table 3). This hydroxyl remains protonated, despite its Lewis acidic environment and thus is able to form a short 2.6-Å hydrogen bond to OD1 of Asp\textsuperscript{80}, which in turn maintains a weak ligand inter-
action with MnA that is 3.1 Å away from OD2. The ionized α-carboxyl group of (S)-malate also forms an ionic hydrogen bond (2.8 Å) with NE2 of a protonated His\textsuperscript{200} (Fig. 7B). An ionized Asp\textsuperscript{187} in turn stabilizes His\textsuperscript{200} through an ionic hydrogen bond (2.6 Å). His\textsuperscript{200} is strictly con-
served in tRNase Z endonucleases (His\textsuperscript{247} of B. subtilis tRNase Z and His\textsuperscript{248} of E. coli ZipD) and has been observed to interact with inorganic phosphate bound in the active site of the B. subtilis enzyme (29) analogous to the (S)-malate interaction observed with PhnP. Notably, (S)-malate bound in one monomer makes additional contacts with Arg\textsuperscript{80}, Asp\textsuperscript{108}, and Asp\textsuperscript{109} of the other monomer using its second car-
boxyl group (Fig. 7B; distances of 3.9, 4.7, and 3.7 Å, respec-
tively). Although probably not a physiological substrate, the interaction of (S)-malate with the residues of the second mon-
omer might be similar to the one provided by the actual sub-
strate, where substrate binding to one monomer may affect a conformational change in the dimer that confers higher affinity for the substrate in the second active site. These interactions could account for the modest cooperativity PhnP showed toward bpNPP.
**DISCUSSION**

The hallmark of the β-lactamase family of hydrolases is the use of a pair of active site metal ions as Lewis acid catalysts. The metal ions are thought to simultaneously polarize the P=O or C=O bonds of their respective substrates and lower the Pκa of the attacking water molecule, which is typically sandwiched between the two metals. A range of metal ions are utilized in the β-lactamase family, most commonly Zn²⁺, Fe³⁺, and Mn²⁺, with some individual enzymes displaying activity with all three of these metal ions (30), and mixed metal pairs (31). PhnP is notable in that it has a distinct preference for Ni²⁺ and Mn²⁺ ions for hydrolysis of bpNPP, whereas Zn²⁺ affords considerably lower activity. In contrast, tRNase Z endonucleases do not show a marked change in activity against bpNPP with Mn²⁺ (a minor 3-fold increase was reported for one enzyme (32)). Rather, despite sharing the same active site residues as PhnP, Zn²⁺ appears to be the active metal ion for the tRNase Z enzymes (17, 24, 32) (Fig. 6B). Oddly, Mn²⁺ does dramatically enhance activity of these enzymes against more complex tRNA substrates (32–34). In this case, it is thought that Mn²⁺ mediates RNA folding into a hydrolytically sensitive conformation or mediates binding to the enzyme itself. However, in the case of PhnP, a “chaperone” role between Mn²⁺ and a small substrate like bpNPP is an unlikely reason for enhanced activity with this metal ion. Likewise, the distinct electron density difference observed between the Zn²⁺ site on PhnP (a convenient internal control) and the active site metals argues for a lighter metal, such as Mn²⁺, bound in the active site and supporting catalysis. The possibility of “second shell” side chains that modulate the hardness of the metal binding site has been put forward to account for the metal binding preference of *Salmonella typhimurium* glyoxylase II (30), but here too there is considerable conservation between PhnP and the Zn²⁺-dependent tRNase Z enzymes. Evidently there is subtle plasticity that dictates metal ion specificity in the β-lactamase family.

It is certainly possible that *in vivo* Zn²⁺ may serve as the active metal ion in PhnP, since cytoplasmic Zn²⁺ concentrations are maintained at 45 μM in *E. coli* (35), which would afford a low level of activity (Table 3) sufficient for cell growth. However, *E. coli* also has a dedicated Mn²⁺ transport system (36) and can achieve cytoplasmic levels of this metal ion well into the 10⁻⁴ M range (37, 38). This would match or exceed the apparent *Kₘ* for this metal with PhnP (Table 3) and would stimulate much greater activity. Interestingly, Mn²⁺ levels are typically highest in stationary, slowly growing bacteria that are nutrient-deprived (38), which would be the case when expression of the *phn* operon is increased in response to low phosphate levels. This may provide another level of control of PhnP activity in phosphate-starved cells.

The high resolution structure of PhnP fortuitously complexed with (S)-malate provides excellent insight into the catalytic features of the active site, particularly into the roles of Asp⁸⁰ and His²⁰⁰. (S)-Malate binds to the more solvent-exposed manganese (MnA) in a bidentate fashion, forcing the hydroxyl closer to MnB than MnB (Fig. 7A and Table 5). Remarkably, the OD2 oxygen of Asp⁸⁰ appears to “follow” this hydroxyl in order to maintain a short hydrogen bond (2.6 Å), seemingly in preference to OD1 forming a close ligand interaction to MnB, which is 3.1 Å distant (for comparison, the metal-bridging OD2 of Asp¹⁶⁴ is 2.0 and 2.3 Å from MnB and MnA, respectively). The malate hydroxyl appears to mimic the attacking water molecule (or hydroxide) that distinguishes the β-lactamase family of hydrolases (39). The close interaction of Asp⁸⁰ with this hydroxyl, even in the presence of a Lewis acidic metal ion (MnB), illustrates its potential to participate in general base catalysis or positioning of a nucleophilic water (or hydroxide). The importance of Asp⁸⁰ to catalysis is highlighted by its strict conservation in the β-lactamase family. Studies with β-lactamases have suggested primarily a metal ion binding role for this residue, possibly combined with positioning or general base catalysis (40–42). Likewise, PhnP D80A shows reduced Mn²⁺ affinity and a great loss of activity (Tables 1 and 3). One would expect a close interaction of Asp⁸⁰ with the MnB ion to lower its pKₐ and impair its suitability as a general base or

### TABLE 5

Distances between PhnP active site residues, metal ions, and (S)-malate

| Atom 1   | Atom 2       | Distance Å |
|----------|--------------|------------|
| MnA      | MnB          | 3.5        |
| MnA      | His²⁷ (ND1)  | 2.2        |
| MnA      | His⁴⁴ (NE2)  | 2.2        |
| MnA      | Asp¹⁶⁴ (OD2)  | 2.3        |
| MnA      | Malate hydroxyl | 2.2 |
| MnA      | Malate α-carboxyl | 2.2 |
| MnB      | Asp⁸⁰ (OD2)  | 3.1        |
| MnB      | His²²² (NE2) | 2.5        |
| MnB      | His⁴³ (NE2)  | 2.3        |
| MnB      | Asp¹⁶⁴ (OD2)  | 2.0        |
| MnB      | Malate hydroxyl | 3.1 |
| MnB      | Water        | 1.6        |
| Malate α-carboxyl | His²⁰⁰ (NE2) | 2.8 |
| Asp¹⁸⁷ (OD2) | His²⁰⁰ (ND1) | 2.6 |
| Malate hydroxyl | Asp⁸⁰ (OD1) | 2.6 |
| Malate δ-carboxyl | R⁹⁹ (NH₂) | 3.9 |
| Malate δ-carboxyl | Asp¹⁰⁹ (OD2) | 3.7 |
| Malate δ-carboxyl | Asp¹¹⁸ (OD2) | 4.7 |

**FIGURE 7. Active site of PhnP.** A, interaction of (S)-malate with Mn²⁺ ions and Asp⁸⁰. A bound water molecule is shown in blue. The difference density for Mn²⁺ ions at the 5 σ level is shown as red mesh. B, interactions of (S)-malate with His²⁰⁰ and adjacent monomer (residues are shown in gray). The difference density is contoured at the 5 σ level. Distances between interacting groups are given in Table 5.
hydrogen bond acceptor. That a preferential hydrogen bond is formed by Asp\textsuperscript{80} in the presence of a competing metal ion suggests that the Lewis acidity of MnB is either dampened by its other ligands, such as a closer interaction by Asp\textsuperscript{164}, or that Asp\textsuperscript{80} is physically constrained from approaching MnB more closely.

The (S)-malate complex also reveals a potential role for His\textsuperscript{200} in stabilizing negative charge on a phosphodiester substrate. The large decreases in $K_{\text{cat}}$ upon mutation of this residue in these enzymes (17, 34, 43) indicate that most of this interaction with a phosphodiester substrate takes place in the transition state. This might arise from stabilization of increasing negative charge development on the nonbridging oxygens in a phosphorane transition state or general acid-catalyzed proton transfer to the leaving group oxygen.

Although PhnP shares close overall structural and active site homology with tRNase Z endonucleases, there are a number of notable differences. In addition to its metal ion preferences, PhnP lacks the distinctive tRNA binding exosite as well as activity against RNA. However, PhnP does exhibit regiospecific activity against 2',3'-cyclic nucleotides. The \textit{E. coli} tRNase ZipD is not active against 2',3'- or 3',5'-cyclic nucleotides (10) but will cleave short sequences of unstructured RNA (44). The noncatalytic Zn\textsuperscript{2+} binding site of PhnP is one of the striking features of this structure, which, intriguingly, is shared by another "accessory" protein, PqqB, of the pyrroloquinoline quinone biosynthetic pathway. PqqB does not appear to play a direct catalytic role in the synthesis of pyrroloquinoline quinone (26, 45). Deletion of the \textit{pqxB} gene in this pathway does not prevent synthesis of pyrroloquinoline quinone but instead leads to accumulation of a biosynthetic intermediate (27). For this reason, PqqB is suggested to facilitate transport of the final product pyrroloquinoline quinone or an intermediate across the cytosolic membrane to the periplasm and thereby alleviate product inhibition of PqqC (26). This echoes observations of the importance of PhnP in organophosphonate degradation. Although disruption of the \textit{phnP} gene in \textit{E. coli} does not prevent CP bond cleavage by cells in liquid culture (2), cell growth on solid media supplemented with methylphosphonate or phosphite as a sole phosphorus source is prevented (7). Interestingly, simultaneous disruption of \textit{phnN} and \textit{phnP} allows weak growth on solid media, suggesting that PhnP is only essential when active PhnN is present (7). The product of the PhnN-catalyzed reaction is 5-phospho-D-ribofuranosyl-\alpha-1-diphosphate, a glycosyl donor used in the biosynthesis of purine, pyrimidine, and pyridine nucleotides (46).

It is not clear how the phosphodiesterase activity of PhnP relates to the PhnN-catalyzed reaction. Nevertheless, the degradation of cyclic nucleotides appears to be a highly conserved activity in the CP-lyase pathway. A survey in the SEED data base (available on the World Wide Web) (47) of 54 bacterial \textit{phn} operons containing \textit{phnM} (an essential gene for CP bond cleavage) revealed 27 occurrences of \textit{phnP}. Intriguingly, in 16 operons where \textit{phnP} was absent, the gene \textit{rcsf} was present in its stead (the remaining 11 operons contained neither \textit{phnP} nor \textit{rcsf}). The \textit{rcsf} gene product (DUF1045, pfam06299) belongs to the 2H-phosphodiesterase superfamily and is uniquely associated with \textit{phn} operons (48). This family of phosphodiesterases hydrolyze 2',3'-cyclic nucleotides or ribosyl-1',2'-cyclic phosphates as part of tRNA splicing reactions and signal transduction. However, unlike PhnP, these enzymes do not employ active site metals and instead use two histidines as general acid-base catalysts to cleave phosphodiester bonds. It is also noteworthy that the \textit{phnP} and \textit{rcsf} genes almost always occur together with \textit{phnN} (only three \textit{phn} operons of the 54 examined above contained a \textit{phnN} gene without \textit{phnP} or \textit{rcsf}). Analogous to PqqB, PhnP (or RcsF) may be involved in transport or processing of an intermediate of organophosphonate metabolism that contains a cyclic phosphate diester or hydrolysis of a 2',3'-cyclic nucleotide as part of a signaling pathway. The latter is a distinct possibility, since phosphate starvation in \textit{E. coli} (and other bacteria) leads to the production of the "alarmones" guanosine 3',5'-bis(diphosphate) (ppGpp) and ppGpp in a SpoT ((pp)ppGpp phosphohydrolase)-dependent (but RelA ((p)ppGpp synthase)-independent) fashion as part of the bacterial "stringent response" (49). These alarmones are believed to induce expression of genes of the \textit{pha} regulon (induction of \textit{phoA} and \textit{ptsS} have been directly observed) (50), of which the \textit{phn} operon is a member. Intriguingly, guanosine 5'-diphosphate 2',3'-cyclic monophosphate (ppG2',3'p) was observed in crystal structures of SpoT (51) and adenylosuccinate synthetase (52). In both cases, ppG2',3'p is observed to bind to these enzymes in an inhibitory fashion. Since adenylosuccinate synthetase is an essential enzyme for the synthesis of AMP, its inhibition results in reduced cell growth, probably as a mechanism for bacterial cells to conserve resources under nutrient-limiting conditions. Likewise, inhibition of SpoT by ppG2',3'p would prevent the hydrolyase activity of this enzyme from degrading ppGpp and halting the stringent response. However, once the expression of the \textit{phn} operon is induced and local organophosphonates are degraded at a rate sufficient to meet the phosphate demands of the cell, it will become necessary to degrade ppG2',3'p. The 2',3'-cyclic phosphodiesterase activity of PhnP may provide this mechanism.

\textbf{Acknowledgment—}We thank Prof. Diane Beauchemin (Department of Chemistry, Queen's University) for generously performing the ICP-MS analyses.

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\textit{Crystal Structure of PhnP}
