**Bacillus cereus** Phosphopentomutase Is an Alkaline Phosphatase Family Member That Exhibits an Altered Entry Point into the Catalytic Cycle*

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Bacterial phosphopentomutases (PPMs) are alkaline phosphatase superfamily members that interconvert α-D-ribose 5-phosphate (ribose 5-phosphate) and α-D-ribose 1-phosphate (ribose 1-phosphate). We investigated the reaction mechanism of *Bacillus cereus* PPM using a combination of structural and biochemical studies. Four high resolution crystal structures of *B. cereus* PPM revealed the active site architecture, identified binding sites for the substrate ribose 5-phosphate and the activator α-D-glucose 1,6-bisphosphate (glucose 1,6-bisphosphate), and demonstrated that glucose 1,6-bisphosphate increased phosphorylation of the active site residue Thr-85. The phosphorylation of Thr-85 was confirmed by Western and mass spectroscopic analyses. Biochemical assays identified Mn2+-dependent enzyme turnover and demonstrated that glucose 1,6-bisphosphate treatment increases enzyme activity. These results suggest that protein phosphorylation activates the enzyme, which supports an intermolecular transferase mechanism. We confirmed intermolecular phosphoryl transfer using an isotope relay assay in which PPM reactions containing mixtures of ribose 5-[18O3]phosphate and [U-13C5]ribose 5-phosphate were analyzed by mass spectrometry. This intermolecular phosphoryl transfer is seemingly counter to what is anticipated from phosphomutases employing a general alkaline phosphatase reaction mechanism, which are reported to catalyze intramolecular phosphoryl transfer. However, the two mechanisms may be reconciled if substrate encounters the enzyme at a different point in the catalytic cycle.

Enzyme-catalyzed phosphoryl transfer forms the basis for many biological, bioenergetic, and regulatory processes and is one of the most common cellular reactions (1). Numerous enzyme families have evolved mechanistically distinct solutions for phosphoryl transfer (2). Phosphomutases are phospho-transfer enzymes that rearrange the position of phosphate within a substrate molecule through either intramolecular (i.e. the phosphate is transferred to a different position on the same molecule) or intermolecular phosphoryl transfer (i.e. the phosphate is transferred from one substrate molecule to another).

Bacterial phosphopentomutases (PPMs) (EC 5.4.2.7) interconvert ribose 1-phosphate and ribose 5-phosphate, which bridges glucose metabolism and RNA biosynthesis (3). The importance of this reaction has recently been underscored by the observation that targeted deletion of the gene encoding PPM in the pathogen *Francisella tularensis* (deoB) results in markedly decreased virulence (4). PPMs appear to be biochemically and structurally distinct from their human congeners (5, 6), making them potential targets for antibiotic development.

Sequence clustering classifies prokaryotic PPMs within the alkaline phosphatase superfamily of metalloenzymes, which includes a range of functionally diverse enzymes such as cofactor-independent phosphoglycerate mutase, phosphodiesterase, and estrone and aryl sulfatases (7). The majority of alkaline phosphatase superfamilies catalyze a hydrolase reaction; however, both PPM (5) and the cofactor-independent phosphoglycerate mutase catalyze phosphomutase reactions (8, 9).

All previously characterized alkaline phosphatase superfamily members follow a unified general reaction mechanism (Fig. 1) (10). In alkaline phosphatase itself (11, 12), the catalytically competent enzyme has an unphosphorylated phosphoenzyme intermediate (state 1). Turnover is initiated when the metallocenter activates a phosphoester donor substrate (R$_2$-OPO$_3$H$^-$) (Fig. 1, state 2) to transfer the phosphoryl group to the hydroxyl of Ser-120 (Fig. 1, state 3). This results in a covalent phosphoenzyme intermediate (E-OPO$_3$H$^-$) (Fig. 1, state 4). A second phosphoryl transfer from the enzyme to the acceptor water molecule (Fig. 1, states 5 and 6) completes the reaction cycle. This general reaction mechanism has also been verified.

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The atomic coordinates and structure factors (codes 3MBW, 3MY8, 3MBZ, and 3OT9) 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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3 The abbreviations used are: PPM, phosphopentomutase; ribose 5-phosphate, α-D-ribose 5-phosphate; glucose 1,6-bisphosphate, α-D-glucose 1,6-bisphosphate; BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl) methane; PDB, Protein Data Bank.
for cofactor-independent phosphoglycerate mutase (8, 9), a phosphomutase in the alkaline phosphatase family. In this case, the incoming substrate is also the acceptor molecule, thus defining it as an intramolecular phosphomutase.

By comparison, characterization of bacterial PPM has been limited. Biochemical analysis of the *Escherichia coli* enzyme revealed that *E. coli* PPM catalyzes Mn$^{2+}$- or Co$^{2+}$-dependent interconversion of ribose 5-phosphate and ribose 1-phosphate (5) and that glucose 1,6-bisphosphate stimulates activity. Given the wealth of information available for alkaline phosphatase superfamly members, one might reasonably expect to infer the mechanism of bacterial PPM based on its classification within that family. This would predict that, like cofactor-independent phosphoglycerate mutase, PPM acts as an intramolecular phosphomutase. However, to our knowledge, no study has directly investigated the catalytic cycle of PPM.

This work investigates the biochemical mechanism of *Bacillus cereus* PPM in the context of the alkaline phosphatase superfamly fold. Surprisingly, our data demonstrate that PPM acts as an intermolecular transferase, which would require an adaptation of the alkaline phosphatase reaction mechanism. In one possible modification, substrate could be acted upon by phosphorylated enzyme (Fig. 1, state 4) and could proceed through the catalytic cycle from this altered entry point. These findings expand upon the mechanistic repertoire for the broadly conserved alkaline phosphatase scaffold.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were of the highest commercially available grade and were used without further purification. Competent expression cells were obtained from Stratagene Inc. (Materials—Protein Expression and Purification).

**Protein Expression and Purification—*B. cereus* (ATCC 14579) PPM was heterologously overexpressed and purified from *E. coli* BL21-Gold (DE3) freshly transformed with pET28a-PPM, as described previously (13), resulting in protein appended with Gly-Ser–His at the N terminus. The T85A variant was generated from pET28a-PPM using the Stratagene QuikChange Lightning site-directed mutagenesis kit with oligonucleotide primers (5′-CTGGTAAAGATGCAATGACACAG-GTCACCTGG-3′ and 5′-CCATGCCCTGTACCTGATCTTTACCAG-3′). This variant was purified using the protocol developed for the wild-type enzyme. Purified PPM was concentrated and stored in aliquots at −80 °C in 25 mM Tris–HCl, 1 mM MnCl$_2$, pH 8.0. Xanthine oxidase was prepared from raw milk using the standard procedure (14), and human purine nucleoside phosphorylase was purified as previously reported (15).

**Crystallization and Structure Determination**—The structure of PPM was determined alone, co-crystallized with ribose 5-phosphate, co-crystallized with glucose 1,6-bisphosphate, and following activation with glucose 1,6-bisphosphate. Crystals of PPM (12 mg/ml buffered in 25 mM Tris–HCl, pH 7.4, 1 mM MnCl$_2$) were grown at 18 °C as described previously (13) using the hanging drop vapor diffusion method. Protein solution (1 μl) and 1 μl of crystallization reservoir solution were equilibrated over a reservoir containing 100 mM BisTris, pH 5.5, 50 mM MnCl$_2$, 14% polyethylene glycol 3350, and 75 mM NH$_4$CH$_3$COO. Co-crystals of PPM with ribose 5-phosphate were prepared by soaking fully formed crystals in a solution containing all of the crystallization components and 10 mM ribose 5-phosphate for 1 h at 18 °C. Co-crystals of PPM with glucose 1,6-bisphosphate were prepared with the same procedure but were soaked with 5 mM glucose 1,6-bisphosphate.

Crystals of activated PPM were grown from purified protein preincubated with 5 mM glucose 1,6-bisphosphate for 30 min at 22 °C before crystallization over a reservoir solution containing 100 mM BisTris, pH 5.5, 50 mM MnCl$_2$, 14% polyethylene glycol 3350, and 50 mM CH$_3$CO$_2$NH$_4$. Prior to data collection, crystals were cryoprotected in a solution containing the crystallization components and 30% glycerol and then flash-cooled in liquid nitrogen. Crystal quality was assessed by diffraction-based feedback at both the Advanced Photon Source beamline 21-ID-G and the Stanford Synchrotron Radiation Lightsource beamline 9-2. All x-ray diffraction data sets were collected at Advanced Photon Source 21-ID-G using a temperature of −173 °C, a wavelength of 0.979 Å, and a MAR 225 CCD detector. Data were processed using the HKL2000 (16) and CCP4 (17) program suites. Crystals formed in the monoclinic space group P2$_1$ with unit cell dimensions listed in Table 1. The structure of native PPM was determined by molecular replacement with the program PHASER (18) using the unpublished crystal structure of a putative PPM from *Streptococcus mutans* (PDB code 2109; New York SGX Research Center for Structural Genomics) as the search model. Residues 133–145 and 199–208 were absent from the search model and were built into 2|F$_{o}$| − |F$_{c}$| omit maps using COOT (19). A careful procedure of omit mapping during refinement was used to avoid model bias.

Model refinement was performed in the programs CNS (20) and REFMAC5 (21) with TLS (translation/libration/screw) (22) groups determined in the TLS Motion Determination server (23). Superposition of residues 2–393 of the final model of *B. cereus* PPM with the equivalent residues (positions 3–402) of the putative PPM from *S. mutans* resulted in an overall root mean square (r.m.s.) deviation of 1.0 Å for the core domain (residues 2–99 and 219–393) and an r.m.s. deviation of 0.7 Å for the cap domain (residues 102–216).
Because all crystals of the *B. cereus* PPM were isomorphous, the refined model of native PPM was subjected to rigid body refinement in CNS (20) and used as a starting model for the remaining structures. Restrayment libraries for ribose 5-phosphate and Thr(P) were generated using the PRODRG server (24). The *R*\(_{free}\) test set of data were composed of 5847 (5\%) reflections randomly selected in CNS (20). The reflections of the *R*\(_{free}\) test set were selected to be identical in the remaining data sets with additional reflections randomly selected in the higher resolution structure of PPM in complex with glucose 1,6-bisphosphate (Table 1). Refinement statistics and PDB accession codes are shown in Fig. 2, and the Ramachandran analysis is shown in Table 1. PDB accession codes are shown in Table 1. Additional reflections were used in the higher resolution structure of PPM in complex with glucose 1,6-bisphosphate (Table 1). Refinement statistics and PDB accession codes are shown in Fig. 2. The electrostatic surface calculation shown in Fig. 2E was prepared using the PDB2PQR (27) server set to use the CHARMm force field (25). Surface maps were calculated using the APBS tools (29) plug-in for PyMOL (26).

**Phosphothreonine Western Analysis**—Either wild type or the T85A variant of PPM (at a concentration of 2 \(\mu\)M and buffered in 50 mM Tris-HCl, pH 8.0, and 0.1 mM MnCl\(_2\)) were incubated with 0, 1, 2.5, 5, 7.5, 10, 25, 50, 75, 100, and 1000 \(\mu\)M glucose 1,6-bisphosphate (wild type) or 0 and 1000 \(\mu\)M glucose 1,6-bisphosphate (T85A) at 23 °C for 30 min. SDS sample buffer was added, and each reaction was separated by SDS-PAGE and transferred to a 0.45-\(\mu\)m nitrocellulose membrane. Total protein was estimated with Ponceau S staining, which was subsequently removed by washing in water. The membrane was then blocked with Odyssey blocking buffer (LI-COR Biosciences) overnight and incubated with an anti-phosphothreonine antibody (Cell Signaling) diluted 1:1000 in TBS containing 0.5% BSA and 0.1% Tween 20 (BSA-TTBS) for 4 h at room temperature. Binding of the primary antibody was detected by incubation with an AlexaFluor680-conjugated goat anti-rabbit antibody (Invitrogen) diluted 1:10,000 in BSA-TTBS for 1 h at room temperature and visualized using an Odyssey infrared imaging system. Both total protein and anti-phosphothreonine band intensities were determined by plotting lane intensities and then measuring the area of the peak corresponding to the band at 44 kDa in the program ImageJ (30).

**Identification of Phosphorylation by LC/MS/MS**—Purified PPM (10 \(\mu\)g) was subjected to proteolytic digestion by trypsin, elastase, and subtilisin in parallel using a modification of a published procedure (31). Data from each digest of the sample were acquired with three separate 90-min LC/MS/MS data acquisitions using a Thermo-Fisher LTQ-orbitrap equipped with a nanospray source coupled to an Eksigent 1D nano-LC pump. Peptide tandem mass spectra were extracted from the instrument files (Scansifter) and searched against a data base containing *B. cereus* proteins using SEQUEST (32), considering possible phosphorylation events on serine, threonine, and tyrosine. Search results were collated and filtered using IDPicker (33).

**Hypoxanthine Consumption Assay**—Enzyme turnover was calculated by coupling the PPM-catalyzed formation of ribose 1-phosphate with human purine nucleoside phosphorylase in the presence of hypoxanthine to form inosine. Inosine formation at end points was determined via correlating to hypoxanthine to form inosine. Search results were collated and filtered using IDPicker (33).
which has a maximal absorbance at 546 nm. The amount of human purine nucleoside phosphorylase required for catalytic excess was determined empirically for reactions via a concentration series. In assays containing 50 nM PPM and 750 μM hypoxanthine, 1.1 μM human purine nucleoside phosphorylase was determined to be the concentration above which no greater rate of product formation was observed. A 3-fold excess of human purine nucleoside phosphorylase (3.3 μM) was used in all experiments to ensure catalytic excess. At specified time points, corresponding to <15% ribose 5-phosphate consumption, reactions were quenched by either heat denaturation or acidification, as described below. Hypoxanthine concentrations were determined by the addition of Developer Mix and comparison of ΔA_{546} with a standard curve run in parallel to biochemical assays. Developer Mix contained 25 mM Tris-HCl, pH 8.0, 0.356% Triton X-100, 7.13 mM iodonitrotetrazolium chloride, and 2.4 mg/ml xanthine oxidase. Details of individual assay formats are provided below in each study. This assay meets the requirements for a valid tandem assay, which are as follows: 1) the first reaction must be zero-order with respect to substrate, and 2) the second stage reaction must not be rate-limiting (35).

Metal Dependence—Endogenous metal ions were removed from affinity-purified PPM by treatment of 100 μM stock solutions with 1 mM EDTA for 1 h at 4°C. The protein was then exchanged into 25 mM Tris-HCl, pH 8.0, using a HiTrap desalting column. To determine metal dependence of PPM, chelated preparations were incubated at 23 °C with 1 mM MnCl₂, ZnCl₂, NiCl₂, MgCl₂, or CoCl₂ for 3 min prior to the biochemical assay. Relative turnover assays (100 μl) contained 1 μM glucose 1,6-bisphosphate, 10 nM reconstituted PPM, 25 mM Tris-HCl, pH 8.0, 750 μM hypoxanthine, and 3.3 μM human purine nucleoside phosphorylase and were initiated by the addition of 500 μM ribose 5-phosphate. PPM and purine nucleoside phosphorylase activities were quenched after an 8-min reaction by heat denaturation at 95 °C for 5 min. Product formation was measured via the hypoxanthine consumption assay, performed by adding 20 μl of Developer Mix to 75 μl of the turnover reaction. The absorbance at 546 nm was monitored at 15-s intervals until no further absorption change was observed, which typically occurred within 10 min.

Glucose 1,6-Bisphosphate Dependence—The initial velocity of PPM was measured in the presence of increasing concentrations of glucose 1,6-bisphosphate in microtiter plates. PPM (10 μl of a 200 nM stock solution) was activated by incubating with 80 μl of a solution containing 0, 0.1, 0.25, 0.5, and 1 μM glucose 1,6-bisphosphate in 25 mM Tris-HCl, pH 8.0, 111 μM MnCl₂, 937.5 μM hypoxanthine, and 4.1 μM human purine nucleoside phosphorylase. Turnover was initiated by the addition of 10 μl of 5 mM ribose 5-phosphate, resulting in a final concentration of 0.5 mM. Reactions were quenched after 4 min by acid denaturation with 5 μl of a solution containing 1 M HCl and 1 M CaCl₂. Reactions were neutralized by the addition of 5 μl of 1 M NaOH immediately prior to determination of the hypoxanthine concentration. Hypoxanthine consumption was measured by removing a 75-μl aliquot of quenched/neutralized assay mixture and combining with 20 μl of Developer Mix. The absorbance at 546 nm was monitored at 15-s intervals until no further absorption change was observed, which usually occurred within 10 min.

Steady State Kinetics—Biochemical data for determination of kinetic parameters was determined in 96-well microtiter plates. Enzyme Mix was freshly prepared by preactivating 200 nM PPM in 25 mM Tris-HCl, pH 8.0, with 5 μM glucose 1,6-bisphosphate and 100 μM MnCl₂ at 23 °C for 10 min and maintained at 4 °C until assayed. In the 96-well plate assay, 100-μl reactions contained 10 μl of Enzyme Mix and 80 μl of Assay Mix comprising 25 mM Tris-HCl, pH 8.0, and 111 μM MnCl₂, 937.5 μM hypoxanthine, and 4.1 μM human purine nucleoside phosphorylase. Reactions were initiated by the addition of 10 μl of the appropriate 10× substrate stock solutions and quenched after 4 min by the addition of 5 μl of a solution containing 1 M HCl and 1 M CaCl₂. Reactions were neutralized by the addition of 5 μl of 1 M NaOH immediately prior to determination of the hypoxanthine concentration. Correspondingly, initial velocities of the activated enzyme were measured in a final concentration of 25 mM Tris-HCl, pH 8.0, 100 μM MnCl₂, 0.5 μM glucose 1,6-bisphosphate, 20 nM activated PPM, and final substrate concentrations of 50, 75, 100, 170, 220, 270, 350, 500, and 700 μM ribose 5-phosphate. Hypoxanthine consumption was measured by combining 75 μl of quenched assay mixture with 20 μl of Developer Mix as described for the preceding glucose 1,6-bisphosphate dependence assay. Hypoxanthine concentrations were determined by comparison of ΔA_{546} with a standard curve run in parallel, and initial velocities were fit to the Michaelis-Menten equation using non-linear regression analyses in the GraphPad Prism software package version 5.01 for Windows.

Phosphatase Assays—Phosphatase activity of PPM was measured during ribose 5-phosphate turnover using the phosphate sensor-based assay (Invitrogen), which monitors the change in fluorescence of a fluorophore-labeled E. coli phosphate-binding protein upon phosphate binding. The reaction mixture contained 0.1 μM PPM, 0.1 mM MnCl₂, 25 mM Tris-HCl, pH 8.0, and 0.5 μM phosphate sensor. The reaction was initiated with 5 μM ribose 5-phosphate and 5 μM glucose 1,6-bisphosphate, and the fluorescence was monitored for 10 min at room temperature in a Cary Eclipse spectrophotometer with λ_{ex} = 430 nm and λ_{em} = 470 nm. The concentration of phosphate formed by PPM was determined by comparison with a standard curve of potassium phosphate in 0.1 μM PPM, 0.1 mM MnCl₂, and 25 mM Tris-HCl.

Synthesis of Ribose 5-[^18O]₄[Phosphate, [¹³C]₅Ribose 5-Phosphate, and [U-¹³C₆]Ribose 5-[^18O]₄[Phosphate—Isotopically labeled ribose 5-phosphates were synthesized using ribokinase with ribose or [U-¹³C₆]ribose and ATP or [γ-¹⁸O]₄ATP (Cambridge Isotope Laboratories). Ribokinase was expressed and purified as previously described (36) and stored at −80 °C in 25 mM Tris-HCl, pH 8.0, and 5 mM MgCl₂. Preparative reactions contained a final concentration of 1 mM ribose (ribose or [U-¹³C₆]ribose), 1 mM ATP ([γ-¹⁸O]₄ATP or ATP), 25 mM Tris-HCl, pH 8, 30 mM KCl, 0.5 mM MnCl₂, 0.5 mM MgCl₂, and 0.5 μM ribokinase. Reactions were incubated at 25 °C and quenched after 5 min by heat denaturation of ribokinase at 94 °C for 5 min. After centrifugation for 2 min at 15,000 × g at 4 °C, the enzymatically synthesized ribose 5-phosphate species were stored at 4 °C until used.
**RESULTS**

**Overall Structure of the B. cereus PPM**—B. cereus PPM folds into two distinct domains: a core domain (residues 2–99 and 219–393) and a cap domain (residues 102–216) (Fig. 2A). The core domain is organized around an alkaline phosphatase fold (Fig. 2, B and C), and superposition with the E. coli alkaline phosphatase (PDB entry 1ALK) (12) results in an r.m.s. deviation of C$_\alpha$ atoms of 2.9 Å. A homology search using the EMBL DaliLite server (37) established that the fold of the cap domain is unique to prokaryotic PPMs and contains three helices surrounding a 5-stranded mixed β-sheet (Fig. 2, A and D).

**Active Site Architecture**—An electropositive cleft at the interface between the core and cap domains (Fig. 2E) houses the active site (Fig. 3A) and contains electron density consistent with two metals ions. Collection of diffraction data at the manganese edge (λ = 1.89 Å) revealed an anomalous signal for both metals at this wavelength (not shown). To be consistent with the nomenclature adopted for the alkaline phosphatase superfamily, we refer to these as Mn-1 and Mn-2. Comparison of the PPM with structurally characterized members of the alkaline phosphatase superfamily revealed that the coordinating ligands to this dimetallo center are structurally conserved with the exception of an additional ligand to Mn-1 that appears to be unique to prokaryotic PPMs (Asp-156 O of the catalytic cycle (Fig. 1, E) nucleophile, Ser-102, in the active site with E. coli alkaline phosphatase (Fig. 3B) and other structurally characterized members of the alkaline phosphatase superfamily revealed that the coordinating ligands to this dimetallo center are structurally conserved with the exception of an additional ligand to Mn-1 that appears to be unique to prokaryotic PPMs (Asp-156 O61).

Among the coordinating ligands of the dimetallo center is Thr-85, which is structurally homologous to the catalytic nucleophile, Ser-102, in the E. coli alkaline phosphatase (Fig. 3B). In accordance with the alkaline phosphatase reaction mechanism (Fig. 1), Ser-102 is not phosphorylated at the start of the catalytic cycle (Fig. 1, step 1) but becomes transiently phosphorylated as the catalytic intermediate (Fig. 1, states 3–5). This transient phosphorylation was challenging to trap for crystallographic studies of the E. coli alkaline phosphatase (38). As a
result, observation of electron density consistent with phosphorylation of Thr-85 in the *B. cereus* PPM was unexpected (Fig. 3C). The electron density at Thr-85 was weaker than expected for a phosphate modification but could not be explained by a water molecule. This suggested that the crystals formed from a pool of protein containing a mixture of both phosphorylated and unphosphorylated enzyme. Crystals grown from protein preincubated with the reported activator glucose 1,6-bisphosphate (5) appeared to have stronger electron density for the phosphate modification of Thr-85 (Fig. 3D), suggesting that the activator transfers a phosphate to Thr-85 in the unphosphorylated enzyme.

**Structure of PPM in Complex with Ribose 5-Phosphate**—Soaking preformed crystals of *B. cereus* PPM with the substrate, ribose 5-phosphate, resulted in the appearance of new electron density within the active site (Fig. 4C) that was not observed in either native crystals or crystals grown from protein preincubated with the activator but without ribose 5-phosphate (Fig. 4, A and B). This electron density (Fig. 4C) is not fully explained by the modeling of a single ribose 5-phosphate molecule. Because substrate reorientation is a likely event during catalysis, multiple binding positions are anticipated. Indeed, the most reasonable explanation of this electron density is that it reflects a mixed state, with ribose 5-phosphate in two mutually exclusive positions.

In the first binding position of ribose 5-phosphate (Fig. 4D), Thr-85 is unphosphorylated, and the 5-phosphate of the substrate bridges Mn-1 and Mn-2. As a result, we term this the coordinating position. In the coordinating position, the substrate is additionally stabilized by two hydrogen bonds between Arg-193 Nq and the 1-OH of the furanose ring and between Asp-286 Oy and the 3-OH of the furanose ring and by two water-mediated interactions with the protein. A similar binding position has previously been observed in structures of alkaline phosphatase family members crystallized in complex with products.

The second binding position of ribose 5-phosphate has markedly clearer electron density (Fig. 4E). Although substrate bound in this position is oriented with the 1-OH of the furanose ring facing toward the phosphate of Thr(P)-85, the distance between Thr(P)-85 and the 1-OH is 8.5 Å, and no part of the molecule directly interacts with the dimetallo catalytic center. As a result, we term this the distal position. In the distal position, ribose 5-phosphate is stabilized by seven putative hydrogen bonds to the cap domain of the protein and four water-mediated interactions. Four of the hydrogen bonds and two of the water-mediated interactions are to the 5-phosphate, which binds within a well-defined pocket. The remaining interactions are between the Nq1 and Nq2 atoms of Arg-193 and the 1-OH and 2-OH of the furanose ring, between Ser-154 Oy and the 3-OH of the furanose ring, and two water-mediated interactions with the 1-OH of the furanose ring. A similar binding position has not previously been observed in structures of alkaline phosphatase superfamily members.

**Structure of PPM in Complex with Glucose 1,6-Bisphosphate**—Soaking of preformed crystals of *B. cereus* PPM with glucose 1,6-bisphosphate resulted in the appearance of new electron density consistent with a phosphate modification of Thr-85 (Fig. 4F). This electron density is not fully explained by the modeling of a single glucose 1,6-bisphosphate molecule. The 1-OH and 2-OH of the furanose ring, between Ser-154 Oy and the 3-OH of the furanose ring, and two water-mediated interactions with the 1-OH of the furanose ring. A similar binding position has not previously been observed in structures of alkaline phosphatase superfamily members.
1,6-bisphosphate resulted in the appearance of clear electron density within the active site consistent with a single binding site for glucose 1,6-bisphosphate (Fig. 4F). In this binding position, the pyranose ring and the 6-phosphate superimpose closely with the furanose ring and 5-phosphate of ribose 5-phosphate in the distal position. The 1-phosphate binds within a second phosphoester binding pocket at the interface of the core and cap domains and includes contacts to the N\textsubscript{9256}/H\textsubscript{9256} atom of Lys-240 and the N\textsubscript{9257}/H\textsubscript{9257}1 and N\textsubscript{9257}2 atoms of Arg-212. This position of glucose 1,6-bisphosphate is stabilized by seven direct contacts to the protein, only one of which is to the pyranose ring (between Ser-154 O\textsubscript{9253} and the 3-OH of the pyranose ring), and three water-mediated contacts. Interestingly, the hydrogen-bonding contacts to the 6-phosphate of glucose 1,6-bisphosphate slightly differ from those to the 5-phosphate of ribose 5-phosphate, with one hydrogen bond shifting from a direct contact to a water-mediated contact. Steric differences between the phosphate orientations on a five- versus six-membered sugar ring may underlie this alteration in phosphate binding.

Verification of Thr-85 Phosphorylation by Western and Mass Spectral Analysis—Because the phosphorylation of the catalytic nucleophile, Thr-85, was unanticipated, and post-translational modifications cannot be unambiguously identified with even the best crystallographic data, we confirmed the phosphorylation of Thr-85 with both mass spectral and Western analyses.

LTQ-orbitrap analysis of B. cereus PPM digested with trypsin, elastase, or subtilisin resulted in 82% sequence coverage. Of the peptides analyzed, eight unique peptides contained Thr-85, one of which (residues 80–95, STGKD7MTGHWEMGL, mass of 1876.76 Da) deviated from its predicted m/z ratio in a manner consistent with phosphorylation. Tandem mass spectrometry confirmed both the presence and location of the site of phosphorylation in this peptide (Fig. 5A). This spectrum shows not only the loss of an m/z of 98 (H\textsubscript{3}PO\textsubscript{4}) from the parent ion but also from several of the fragment ions (e.g. b6, b10, and b12). Localization of the phosphate modification to Thr-85 is evidenced specifically with the b5, b6, y10, and y11 fragments flanking the site of phosphorylation. The identification of a mixture of phosphorylated and unphosphorylated peptides is
consistent with the crystal structure of PPM having only partial occupancy of the phosphate modification (Fig. 3C).

To further verify both the phosphorylation of Thr-85, and an increase in phosphorylation upon the addition of glucose 1,6-bisphosphate, we used Western analysis. Both wild-type and T85A PPM were analyzed using a phosphothreonine-specific antibody after incubation with increasing amounts of glucose 1,6-bisphosphate (Fig. 5, B and C). This antibody only recognized the wild-type enzyme, consistent with Thr-85 being the only site of phosphorylation. The phosphorylation of the wild-type enzyme increased upon the addition of the activator, glucose 1,6-bisphosphate, consistent with the observation that preincubation of PPM with glucose 1,6-bisphosphate appeared to increase the percentage of protein with phosphorylated Thr-85 in the crystal structure (Fig. 3D).

Activity of PPM—The crystallographic, Western, and mass spectrometric analyses demonstrated that the addition of glucose 1,6-bisphosphate increased phosphorylation of Thr-85 in the wild-type enzyme. To assess the effect of increased phosphorylation on activity, we preincubated B. cereus PPM with increasing concentrations of glucose 1,6-bisphosphate and assessed catalytic turnover (Fig. 6A). We observed a concentration-dependent enhancement of PPM activity between 0.1 and 2.5 μM glucose 1,6-bisphosphate, at which point maximal activity was reached. We confirmed the metal dependence of B. cereus PPM by measuring the activity of chelated protein reconstituted with Mn²⁺, Zn²⁺, Ni²⁺, Mg²⁺, and Co²⁺. Only the Mn²⁺-incorporated enzyme showed significant enzyme activity (Fig. 6B). In these optimized conditions, we determined that the $V_{max}$, $k_{cat}$, and $K_m$ are 12.3 ± 0.7 μmol min⁻¹, 10.2 ± 0.6 s⁻¹, and 263 ± 34 μM, respectively (Fig. 6C).

Having established basic kinetic parameters for the conversion of ribose 5-phosphate to ribose 1-phosphate, we sought to determine whether the enzyme had intrinsic phosphatase activity, in which the phosphoryl group from Thr-85 is hydrolyzed. Although statistically significant phosphatase activity was measured, the rate of hydrolysis was near the detection limit of the assay and appeared to be ~10,000-fold lower than the rate of ribose 1-phosphate and ribose 5-phosphate interconversion (data not shown).

Reaction of PPM with Ribose 5-[¹⁸O₃]Phosphate and [U-¹³C₅]Ribose 5-Phosphate—Phosphomutases that are unphosphorylated when active commonly catalyze intramolecular phosphoryl transfer (Fig. 7A), whereas those that are phosphorylated commonly catalyze intermolecular phosphoryl transfer (Fig. 7B) with respect to substrate (39). Accordingly, the observed correlation between enzyme phosphorylation and activity in PPM suggests that it catalyzes intermolecular phosphoryl transfer; however, this is counter to what is anticipated from the alkaline phosphatase general mechanism. In order to unambiguously demonstrate intermolecular transfer, we performed an isotope relay assay. In this assay, we incubated a 1:1 stoichiometric mixture of ribose 5-[¹⁸O₃]phosphate and [U-¹³C₅]ribose 5-phosphate with PPM under standard reaction conditions (Fig. 7C). These substrates are labeled with heavy atom isotopes in the phosphoryl group (+6 Da) and the ribose ring (+5 Da), respectively, allowing all possible reaction products to be distinguished by mass spectrometric analysis. In the
event of intramolecular transfer (mutase mechanism), the isotopic distribution of the molecular ions for the two compounds should remain unchanged. Conversely, in the event of intermolecular transfer (transferase mechanism), in which the transferred phosphoryl group is derived from an antecedent substrate, the isotopic distribution for the molecular ions should scramble into four species of unique masses. It is unambiguous from these results (Fig. 7D) that intermolecular phosphoryl group transfer is occurring under the PPM reaction conditions used in this study, with four isotopologues represented at roughly equal total ion current intensities. Moreover, when human purine nucleoside phosphorylase and hypoxanthine were added to this reaction, consumption of the ribose phosphates was apparent. Notably, newly formed isotopologues were consumed more rapidly than isotopologue masses corresponding to precursors, suggesting that the new isotopologues corresponded to predominantly ribose 1-phosphate.

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Reaction of PPM with [U-13C5]Ribose 5-[18O3]Phosphate and Ribose 5-Phosphate—To support the isotope relay results observed in the preceding study, we used identical conditions with a 1:1 mixture of [U-13C5]ribose 5-[18O3]phosphate and ribose 5-phosphate. As in the prior case, the isotopologue distribution of this mixture should remain unchanged in the event of an intramolecular reaction, whereas an intermolecular reaction should engender isotope relay of [18O3]phosphate to unlabeled ribose and relay of unlabeled phosphate to [U-13C5]ribose (Fig. 7E). Again, the incubation of substrates with PPM led to the generation of isotopologues in equal ratios, and the addition of human nucleoside phosphorylase to reactions resulted in the disappearance of these newly formed isotopologues (Fig. 7F).

To determine if ribose could be a discrete intermediate or reversible shunt product in the PPM-catalyzed reaction, we performed the PPM reaction as described above, with the addition of 1 mM [U-13C5]ribose. In these reactions, the initial isotopologue distribution of the reaction mixture remained unchanged (data not shown), ruling out ribose as a diffusible intermediate.

DISCUSSION

Implications for the Catalytic Mechanism—Extrapolation of the general alkaline phosphatase reaction mechanism to PPM predicts that the enzyme should be active when unphosphorylated (Fig. 1, state 1) and that catalysis should proceed via an intramolecular mutase mechanism. In such a mechanism, the reaction would proceed through an intermediate in which the enzyme is phosphorylated, but the substrate is dephosphorylated (Fig. 1, state 4). Indeed, this is exactly what was observed for cofactor-independent phosphoglycerate mutase (8, 9), which is the only other characterized phosphomutase in the alkaline phosphatase family.

Surprisingly, our results are inconsistent with this precedent. We demonstrated that glucose 1,6-bisphosphate both increased enzyme activity (Fig. 6A) and resulted in increased phosphorylation of Thr-85 (Figs. 3 (C and D) and 5 (B and C)), suggesting that glucose 1,6-bisphosphate activates PPM by phosphorylating the catalytic nucleophile. These data are therefore consistent with substrates being acted upon by the phosphorylated enzyme and generally proscribe an intramolecular sequence analogous to phosphoglycerate mutase.

To unambiguously demonstrate that PPM proceeds via intramolecular or intermolecular transfer, we performed a series of isotope relay experiments. Under all assayed reaction conditions, the phosphoryl group of the product is indeed derived from an antecedent substrate (Fig. 7, D and F). Notably, analogous isotopic labeling experiments performed for phosphoglycerate mutase of the alkaline phosphatase superfamily revealed no relay of labeled phosphoryl groups (or glyceryl groups), demonstrating the opposite result (8, 9).

Having ruled out a purely intramolecular process, there are several general scenarios that may result in the intermolecular transfer of phosphoryl groups (a full range of scenarios has been outlined by Britton et al. (39) for phosphoglycerate mutase). As shown in Fig. 7B, one limiting case assumes a primed (phosphorylated) enzyme. In this scenario, the phosphoenzyme would transfer its phosphoryl group to the 1-position of ribose
5-phosphate, resulting in intermediary ribose 1,5-bisphosphate. A phosphoryl group would be subsequently transferred from the 5-position of this intermediate to the enzyme nucleophile, concomitantly generating the product ribose 1-phosphate and priming the enzyme for the next reaction cycle.

Although there are alternatives to this reaction mechanism, a dephosphorylated starting enzyme must be invoked, which is inconsistent with our data. Briefly, to catalyze intermolecular transfer with a dephosphorylated enzyme, the dephosphorylation of ribose 5-phosphate by PPM must be followed by diffusion of the ribose intermediate at a rate competitive with phosphoryl transfer over the course of the assay. Despite our evidence that the active enzyme is phosphorylated, we assayed for this "leaky" mutase possibility by performing the PPM reaction with [18O]-labeled and unlabeled ribose 5-phosphates synthesized from [U-13C5]ribose or unenriched ribose and γ-[18O]ATP or ATP were incubated with PPM for 30 min, and the masses of the products were measured by LC-MS. C, theoretical masses for starting materials and the predicted products of either intramolecular or intermolecular phosphoryl transfer. D, mass spectra of the experiment outlined in C. In the absence of enzyme (black line) only the m/z 229 and 240 peaks corresponding to the starting materials are observed. Following incubation with PPM, peaks at m/z 229 and 240 appear (green line). The addition of human purine nucleoside phosphorylase to this reaction, which removes the product ribose 1-phosphate, decreases the intensity of these new peaks (gray line). E, theoretical masses for starting materials and the predicted products of either intramolecular or intermolecular phosphoryl transfer. F, mass spectra of the experiment outlined in E. In the absence of enzyme, only the m/z 229 and 240 peaks corresponding to the starting materials are present (black line). Incubation of the starting materials with PPM resulted in the appearance of peaks at m/z 234 and 235 (green line). These new peaks decreased in intensity following incubation with human purine nucleoside phosphorylase (gray line), which removes ribose 1-phosphate.
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most parsimonious interpretation supports the mechanism depicted in Fig. 7B.

To reconcile this intermolecular transferase mechanism with the general alkaline phosphatase mechanism (Figs. 1 and 8), we suggest that bacterial PPMs have adapted to favor substrate entry in the catalytic cycle at an alternate point. In this scenario, phosphorylated PPM (Fig. 8, state 4) binds the substrate ribose 5-phosphate (Fig. 8, state 5) and then transfers a phosphoryl group from Thr(P)-85 to ribose 5-phosphate, forming a ribose 1,5-bisphosphate intermediate (Fig. 8, state 6) and a dephosphorylated enzyme (Fig. 1, state 1). The reaction cycle is completed with transfer of the 5-phosphate of ribose 1,5-bisphosphate to Thr-85 (Fig. 8, states 2 and 3), priming the enzyme for the next turnover cycle (Fig. 8, state 4).

The distinct entry point into the catalytic cycle requires that PPM differ from characterized enzymes within the alkaline phosphatase superfamily with respect to the stability of the phosphorylated catalytic nucleophile. In alkaline phosphatase, the nucleophile, Ser-102, is only transiently phosphorylated during the catalytic cycle, and efforts to stabilize this reaction intermediate proved challenging. The eventual crystal structure of phosphorylated alkaline phosphatase required both that the Zn$^{2+}$ ions of the dimetallo center be replaced with catalytically inactive Cd$^{2+}$ and that Ser-102 be mutated to threonine (38). In peptides, phosphoserine has been shown to be more labile than phosphothreonine under alkaline conditions (40). This cursory implies that the use of a threonine as the active site nucleophile in PPM contributes to the improved longevity of the phosphoenzyme. However, the folded protein has a profound influence on the stability of phosphate modifications, and phosphoserines in phosphoryl transfer enzymes have measured $t_{1/2}$ values on the order of years (41). Although the origins of the improved stability of the phosphorylated nucleophile in PPM remain unclear, the substrate nevertheless probably encounters phosphorylated enzyme under physiological conditions.

Comparison of the Structures of the B. cereus and S. mutans PPMs—The structure of B. cereus PPM was determined by molecular replacement using the unpublished coordinates for a putative PPM from S. mutans (PDB entry 2I09; New York SGX Research Center for Structural Genomics) as a search model. Although the backbone accuracy was sufficient for use as a molecular replacement search model, the S. mutans structure contains numerous errors. For example, four substantial errors at the active site would prevent the development of a structure-based reaction mechanism using the S. mutans structure as a guide. First, a short frameshift (residues 218–220) places incorrect residues at the active site; second, no metals are included; third, the side chains of three active site residues, including the catalytic threonine, are misoriented; fourth, the post-translational phosphorylation of the active site threonine is modeled as a water molecule. We can verify that these are model building errors and not differences between PPM from these two organisms because both the coordinates and the structure factors were deposited for the S. mutans PPM. After the submission of this manuscript, the coordinates for the S. mutans PPM were superseded with a newly refined model (PDB entry 3MV7). Although this revised included metal ions and corrected some of the errors, incorrectly positioned side chains in the active site would still preclude the development of a structure-based mechanism from these updated coordinates. We used a careful procedure of omit mapping to prevent these errors from propagating into the present study.

Comparison with Phosphomutases in the $\alpha$-Phosphohexomutase and Haloacid Dehalogenase Superfamilies—Phosphomutases have long been grouped into cofactor-independent enzymes that catalyze intramolecular transfer and bisphosphate-dependent enzymes that catalyze intermolecular transfer. The intermolecular transferase mechanism of PPM is shared with several well studied cofactor-dependent phosphomutases within the $\alpha$-phosphohexomutase and haloacid dehalogenase superfamilies (42–45). Perhaps unsurprisingly, numerous parallels exist between PPM and phosphomutase enzymes within both of these superfamilies. For example, PPM and the phosphomutases within the $\alpha$-phosphohexomutase and haloacid dehalogenase superfamilies are multidomain enzymes with an active site located in a positively charged cleft between domains (43, 46–48). Activity is dependent upon divalent cations, the reactions proceed through bisphosphate intermediates (42, 49), and the enzymes require the continued presence of bisphosphate activators (44, 45) to counter the slow inactivation that occurs when the reaction intermediate prematurely dissociates from the enzyme or when the phosphoryl group is transferred to water (1). Taken together, these broad mechanistic parallels suggest that the details of catalysis iden-
tified for phosphomutases within the α-phosphohexomutase and haloacid dehalogenase superfamilies may guide future studies on PPM.

Conclusions—The results presented here include the first published crystal structure and structure-based functional study of a prokaryotic PPM. Although prokaryotic PPMs belong to the alkaline phosphatase superfamily, the data presented here provide evidence that the catalytic cycles of these enzymes are distinct. This study serves as a reminder that caution should be used when extrapolating reaction mechanisms, even in closely related systems.

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