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Molecular differences between a mutase and a phosphatase: Investigations of the activation step in *Bacillus cereus* phosphopentomutase[‡]

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Abstract

Prokaryotic phosphopentomutases (PPMs) are di-Mn²⁺ enzymes that catalyze the interconversion of α -D-ribose 5-phosphate and α -D-ribose 1-phosphate at an active site located between two independently-folded domains. These prokaryotic PPMs belong to the alkaline phosphatase superfamily, but previous studies on *Bacillus cereus* PPM suggested adaptations of the conserved alkaline phosphatase catalytic cycle. Notably, *B. cereus* PPM engages substrate when the active site nucleophile, Thr-85, is phosphorylated. Further, the phosphoenzyme is stable throughout purification and crystallization. In contrast, alkaline phosphatase engages substrates when the active site nucleophile is dephosphorylated, and the phosphoenzyme reaction intermediate is only stably trapped in catalytically compromised enzyme. Studies were undertaken to understand the divergence of these mechanisms. Crystallographic and biochemical investigations on the PPM^{T85E} phosphomimetic variant and the neutral corollary PPM^{T85Q} identified that the side chain of Lys-240 changed conformation in response to active site charge, which modestly influenced affinity for the small molecule activator α -D-glucose 1,6-bisphosphate. More strikingly, the structure of unphosphorylated *B. cereus* PPM revealed a dramatic change in interdomain angle and a new hydrogen-bonding interaction between the side chain of Asp-156 and the active site nucleophile, Thr-85. This hydrogen-bonding interaction is predicted to align and activate Thr-85 for nucleophilic addition to α -D-glucose 1,6-bisphosphate, favoring the observed equilibrium phosphorylated state. Indeed, phosphorylation of Thr-85 is severely impaired in the PPM^{D156A} variant even under stringent activation conditions. These results permit a proposal for activation of PPM, and explain some of the essential features that distinguish between the catalytic cycles of PPM and alkaline phosphatase.

Keywords

Phosphopentomutase; alkaline phosphatase superfamily; ribose 5-phosphate; ribose 1-phosphate; phosphoenzyme; catalytic nucleophile; metalloproteins; metalloenzymes

[‡]The atomic coordinates and structure factors for all structures have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>) with accession codes 3TWZ, 3TX0, 3UN2, 3UN3, 3UN5, 3UNY, and 3UO0.

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Phosphopentomutases (PPMs) interconvert α -D-ribose 5-phosphate (ribose 5-phosphate) and α -D-ribose 1-phosphate (ribose 1-phosphate). Physiologically, this enables products of RNA breakdown to be used in glycolysis or purine and pyrimidine synthesis (1). PPM activity has been observed in both prokaryotes and eukaryotes (2, 3); however, sequence and structural comparisons indicate that prokaryotic and eukaryotic PPMs are unrelated (4, 5).

Bacterial PPMs belong to the alkaline phosphatase superfamily, a large and diverse family of dimetallo enzymes that catalyze phosphoryl or sulfonyl transfer via a conserved reaction cycle (4). Indeed, the structure of *B. cereus* PPM is predominated by a core domain with a fold related to alkaline phosphatase; however, it also contains a cap domain that is unique to phosphopentomutases (Figure 1) (6). The active site is located at the interface between these two domains. Previous structure-based enzymology of *B. cereus* PPM (6) revealed numerous architectural and mechanistic parallels between PPM and alkaline phosphatase. The active sites of PPM and alkaline phosphatase exhibit virtually identical geometry with both containing a catalytic nucleophile (Thr-85 in *B. cereus* PPM and Ser-102 in *Escherichia coli* alkaline phosphatase (7)), and a dimetallo center (di-Mn²⁺ in *B. cereus* PPM and di-Zn²⁺ in *E. coli* alkaline phosphatase (8)). The coordinating residues to the dimetallo center are absolutely conserved in both sequence and structure with the exception of an additional ligand within the *B. cereus* active site, Asp-156, that is located on the unique, cap domain (6, 9). Accordingly, the alkaline phosphatase reaction cycle was used as a starting point for developing a reaction mechanism specific for PPM (6).

Interestingly, *B. cereus* PPM appears to have several mechanistic adaptations as compared to the remainder of the alkaline phosphatase superfamily (Figure 2A,B). The most surprising difference between PPM and alkaline phosphatase is that the active site phosphorylation status of the catalytic form of the enzymes differs. In PPM, substrate is acted upon by enzyme in which the catalytic nucleophile, Thr-85 in *B. cereus* PPM, is phosphorylated (p-Thr-85). The catalytic cycle proceeds through an intermediate with unphosphorylated enzyme and doubly phosphorylated substrate (6). Conversely, in alkaline phosphatases, the substrates encounter an enzyme in which the catalytic nucleophile, Ser-102 in the *E. coli* alkaline phosphatase, is dephosphorylated. The catalytic cycle proceeds through a transient intermediate in which the enzyme is phosphorylated and the substrate is dephosphorylated (9, 10). This key difference requires that PPM use an altered entry point to the catalytic cycle in comparison to other characterized members of the alkaline phosphatase superfamily, but overall the catalytic cycle itself is conserved (Figure 2A,B).

Phosphorylation of PPM requires a dedicated chemical activation step, which is achieved *in vitro* using the small molecule activator α -D-glucose 1,6-bisphosphate (glucose 1,6-bisphosphate). Since this activation is one requirement for the mechanistic divergence between PPM and alkaline phosphatase, studies were undertaken to identify the mechanisms of the activation process. Structures of a phosphomimetic variant of PPM (PPM^{T85E}), the neutral corollary (PPM^{T85Q}), and a fully unphosphorylated form of PPM suggest a mechanism favoring activation that is unique to phosphopentomutases within the alkaline phosphatase superfamily.

EXPERIMENTAL PROCEDURES

Materials

Unless otherwise stated, all materials were of the highest grade commercially available and were used without further purification.

Cloning, Expression, and Purification

N-terminally hexahistidine tagged *B. cereus* PPM was cloned, expressed, and purified as described (11). Site directed substitutions were prepared from the gene contained in plasmid pET28+ (11) using the QuikChange mutagenesis kit (Stratagene). Primers for the PPM^{T85E} variant were

GCAAGAGAAATCTACTGGTAAAGATGAAATGACAGGTCCTGGGAAATCATGG
GCC and

GGCCCATGATTTCCCAGTGACCTGTCATTTTCATCTTTACCAGTAGATTCTCTTG
C, for the PPM^{T85Q} variant were

GCAAGAGAAATCTACTGGTAAAGATCAAATGACAGGTCCTGGGAAATCATGG
GCC and

GGCCCATGATTTCCCAGTGACCTGTCATTTGATCTTTACCAGTAGATTCTCTTG
C, for the PPM^{K240A} variant were

CGATGTAATTGCTATTGGTGCAATCTCTGATATTTATGATGG and

CCATCATAAATATCAGAGATTGCACCAATAGCAATTACATCG, and for the

PPM^{D156A} variant were

CAGGCTCTTTAATCGTTTATACTTCTGCTGCCAGCGTATTGCAAATTGCAGCACA
CGAAG and

CTTCGTGTGCTGCAATTTGCAATACGCTGGCAGCAGAAGTATAAACGATTAAAG
AGCCTG.

Wild type and variant PPMs were expressed and purified using a previously reported protocol (11). Briefly, plasmids were transformed into *E. coli* BL21 (*DE3*). The cells were grown in LB medium at 37 °C and induced with 0.3 mM IPTG after the OD₆₀₀ reached 0.4–0.6. Cells were disrupted by sonication and the protein was purified from clarified lysate using two chromatographic steps. The first step used a HisTrap (GE Healthcare) Ni-affinity column, in which the protein was bound to the column in 50 mM Tris-HCl, 200 mM NaCl, 1 mM MnCl₂, and 10 mM imidazole, pH 7.5, and eluted from the column in 50 mM Tris-HCl, 200 mM NaCl, 1 mM MnCl₂, and 300 mM imidazole, pH 7.5. The hexahistidine affinity tag was then removed by overnight thrombin cleavage at 22°C in buffer containing 50 mM Tris-HCl, 200 mM NaCl, 1 mM MnCl₂, pH 7.5. The protein was further purified using size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated with 25 mM Tris-HCl, 1 mM MnCl₂, pH 7.5.

Crystallization, data collection, and structure determination of wild-type and variant PPM in the P2₁ crystal form

Crystals of PPM variants were grown using the hanging drop vapor diffusion method using a modification of crystallization conditions described previously (11). All proteins were at a concentration of 10 mg ml⁻¹ buffered in 25 mM Tris-HCl, 1 mM MnCl₂, pH 7.5. Crystals of the PPM^{T85E} variant grew over a reservoir solution containing 100 mM Bis-Tris pH 5.5, 50 mM MnCl₂, 17% polyethylene glycol 3350, and 75 mM NH₄CH₃COO. Crystals of the PPM^{T85Q} variant crystallized over a reservoir solution containing 100 mM Bis-Tris pH 5.5, 50 mM MnCl₂, 13% polyethylene glycol 3350, and 75 mM NH₄CH₃COO. Co-crystals of each variant with glucose 1,6-bisphosphate were prepared by soaking fully-formed crystals in 5 mM glucose 1,6-bisphosphate. For the co-structure of activated wild-type PPM bound to glucose 1,6-bisphosphate, PPM was incubated with 5 mM glucose 1,6-bisphosphate in 25 mM Tris-HCl pH 7.4 and 1 mM MnCl₂ at room temperature for 30 minutes prior to setting drops. Crystals grew over a reservoir solution containing 100 mM Bis-Tris pH 5.5, 50 mM MnCl₂, 14% polyethylene glycol 3350, and 50 mM NH₄CH₃COO. Fully formed crystals of the activated enzyme were then soaked in a solution containing all of the crystallization components and 5 mM glucose 1,6-bisphosphate for 1 hour at 18 °C. All crystals were

cryoprotected in a solution that was 70% v/v reservoir solution and 30% v/v glycerol prior to cryo cooling in liquid nitrogen.

Crystal quality was assessed at the Stanford Synchrotron Radiation Lightsource beamline 9–2 and the Advanced Photon Source (APS) beamline 21-ID-G; all data sets were collected on the APS beamline 21-ID-G. Data were processed using the HKL2000 (12) and CCP4 (13) suites of programs (Table 1). Crystals grown from these conditions formed in the primitive monoclinic space group $P2_1$. The unit cell dimensions of wild-type and the PPM^{T85Q} variant were similar to those previously reported (6). Accordingly, initial phases were determined by rigid body refinement of the structure of *B. cereus* PPM (PDBID 3M8W (6)) using CNS (14). Crystals of the PPM^{T85E} variant had unit cell dimensions significantly different from those previously reported (6). The structure of the PPM^{T85E} variant was determined in the program PHASER (15), using the structure of wild-type *B. cereus* PPM (PDBID 3M8W (6)) as the search model. Models were improved with iterative rounds of model building in COOT (16) and refinement in CNS (14) and REFMAC (17, 18). For the latter, Translation/Libration/Screw (TLS) (19) groups were assigned using the TLS Motion Determination server (20).

Crystallization, data collection, and structure determination of unphosphorylated PPM in the $P2_12_12_1$ crystal form

Crystal packing interactions in the $P2_1$ crystal form restrict the interdomain orientation (see Supporting Information, Fig. S1). Given the possibility of interdomain rotation during catalysis, we sought to identify a new crystal form for the unphosphorylated enzyme. Accordingly, we identified protein of low phosphorylation level and performed sparse-matrix screening. Both previous enzyme activity assays and Western analyses suggested that different protein preparations of PPM varied in phosphorylation level of Thr-85 (unpublished observations). Some of the variables influencing the degree of phosphorylation may include the length of time taken during the overall purification process, the age of the protein preparation, and the storage temperature. Sparse matrix screening was performed with enzyme preparations comparable to those where Western analyses did not detect measurable p-Thr and are thus expected to have low levels of phosphorylation (<5%) in sitting drops with a Mosquito drop setter (TTP LabTech) by mixing 200 nL protein and 200 nL reservoir solution and equilibrating over 100 μ L reservoir solution. The crystals used for data collection were grown using the hanging drop vapor diffusion method by equilibrating either 1 μ L 10 mg ml⁻¹ unphosphorylated *B. cereus* PPM (buffered in 25 mM Tris-HCl, 1 mM MnCl₂, pH 7.5, 5 mM Na-orthovanadate, and 5 mM ribose 5-phosphate) and 1 μ L reservoir solution (125 mM MnCl₂ and 1.8 M Ammonium Citrate, pH 7.2) over 1 mL reservoir solution or 1 μ L 10 mg ml⁻¹ phosphorylated *B. cereus* PPM (buffered in 25 mM Tris-HCl, 1 mM MnCl₂) over the same reservoir solution. Crystals were cryo protected using a 1:1 mixture of paratone-N and mineral oil prior to flash cooling in liquid nitrogen.

Diffraction data were collected at 100 K at the LS-CAT beamline 21-ID-G using a wavelength of 0.979 Å and a MAR 225 CCD detector. Data were processed and scaled using the HKL2000 suite of programs (12), which demonstrated that these crystals formed in the primitive orthorhombic space group $P2_12_12_1$ (Table 2). The unit cell dimensions were distinct from those observed in the previously-reported $P2_1$ space group.

Structure determination and refinement of phosphorylated and unphosphorylated PPM in the $P2_12_12_1$ space group

Structures of both phosphorylated and unphosphorylated *B. cereus* PPM in the $P2_12_12_1$ space group were determined by molecular replacement in the program PHASER (15) using the structure of *B. cereus* PPM in the $P2_1$ space group (PDB entry 3M8W; (11)) as the

search model. The structure of the phosphorylated protein used the entire protein as the search model, while the structure of the unphosphorylated protein could only be determined when the isolated alkaline phosphatase-like core domain (residues 2–99 and 216–393) was used as the search model. The models were improved using iterative rounds of model building in COOT (16) and refinement in CNS (14) and REFMAC (18). TLS groups for REFMAC were determined using the TLS Motion Determination server (20). Refinement statistics for the final models are given in Table 2. Subsequent analysis of the crystal packing interactions in the P2₁2₁2₁ crystal form confirmed that this crystal form did not restrict the orientation between domains in PPM (see Supporting Information, Fig. S1).

Structural analysis and molecular visualization

Changes in the interdomain angle were calculated using DynDom (21). Structural comparisons were performed in O (22). Figures 1, 3, 5, 6, and 7 were prepared using Molscript (23), Bobscript (24), and Raster3D (25). The supplementary video was made using PyMol (26).

Hypoxanthine Consumption Assay for PPM activity

The activity of wild-type PPM and the PPM^{T85Q}, PPM^{T85E}, PPM^{K240A}, and PPM^{D156A} variants was measured in a tandem assay with human purine nucleoside phosphorylase and measured by hypoxanthine consumption by xanthine oxidase; both of these enzymes were expressed and purified using previously reported protocols (27, 28). In this assay, ribose 1-phosphate formed by PPM was consumed by a catalytic excess of purine nucleoside phosphorylase in the presence of hypoxanthine to form inosine. Inosine formation was determined at endpoints by measuring hypoxanthine consumption using a typical hypoxanthine detection assay (29) containing xanthine oxidase and iodinitrotetrazolium chloride, which produces a colored formazan, quantified at 546 nm.

All reactions were performed in 100 μ L volumes in 96-well plates. Wild-type or variant PPM buffered in 25 mM Tris-HCl, pH 8, 0.1 mM MnCl₂ and at a concentration 10-fold higher than that used in the assay was pre-activated for 10 minutes at room temperature with 5 μ M glucose 1,6-bisphosphate, and then held at 4°C until assayed. Final assay conditions were 20 nM wild-type PPM, and 50, 100, 250 or 500 nM of each of the PPM variants buffered in 0.1 mM MnCl₂, 25 mM Tris-HCl, pH 8, 5 μ M human purine nucleoside phosphorylase, 600 μ M hypoxanthine, and 1 mM ribose 5-phosphate. Reactions were initiated by the addition of the substrate, ribose 5-phosphate, were incubated for 10 minutes at room temperature, and then were quenched by addition of 5 μ L 2 M NaOH. After 20 minutes at room temperature, the reactions were neutralized by the addition of 5 μ L of 2 M HCl. 75 μ L of the quenched assay was combined with 20 μ L of 0.2% Triton X-100, 7.5 mM iodinitrotetrazolium chloride and xanthine oxidase in 25 mM Tris-HCl, pH 8. Hypoxanthine consumption was determined based on the absorbance at 546 nm in comparison to a standard curve run in parallel.

Measurement of binding affinity for glucose 1,6-bisphosphate by intrinsic fluorescence

Intrinsic fluorescence of protein samples was measured at 20 °C in a Cary Eclipse Spectrophotometer with excitation and emission slits set to 20 nm and the detector voltage set to 675 mV. For each measurement, 114 μ L of 0.5 M wild-type or variant PPM (in buffer containing 50 mM Tris-HCl pH 8.0 0.1 mM MnCl₂) was mixed with 6 μ L of the appropriate concentration of glucose 1,6-bisphosphate immediately prior to placing the sample into the instrument. After one minute, the sample was excited at 280 nm and the emission spectrum read from 300 to 400 nm. Each individual sample was measured three times in immediate succession, and each data point is the average of three samples for a total of 9 measurements. The concentration dependent change in fluorescence following the addition

of glucose 1,6-bisphosphate was significant for all samples tested. The K_d was calculated by plotting the change in fluorescence intensity at 350 nm against the concentration of glucose 1,6-bisphosphate and fitting to a single site binding site model with GraphPad Prism version 5.01.

Phosphothreonine Western analysis

Either wild-type or variant PPM (2 μ M) was incubated in 25 mM Tris-HCl, pH 8.0, 0.1 mM $MnCl_2$ and a varied concentration of glucose 1,6-bisphosphate at room temperature for 30 minutes. Samples were quenched by addition of SDS sample buffer and heating at 95° C for 5 minutes before separation by SDS-PAGE and subsequent transfer to a 0.45 μ m nitrocellulose membrane. Total protein was observed by Ponceau S staining, which was removed by washing with water after imaging. The membrane was blocked using Odyssey blocking buffer (LICORP Biosciences) overnight and incubated with an anti-phosphothreonine antibody (Cell Signaling) diluted 1:1000 in BSA-TTBS (TBS containing 0.5% BSA and 0.1% Tween 20) for 2 hours at 22°C. The membrane was then incubated for 1 hour at 22°C with an AlexaFluor680-conjugated goat anti-rabbit antibody (Invitrogen) diluted 1:20000 in BSA-TTBS, and was visualized using an Odyssey Infrared Imaging System.

Phosphate Release Assay

The phosphatase activity of wild-type and variant PPM was measured using molybdate-based methods as previously described (30) with 20 nM wild-type PPM, either 1 μ M or 5 μ M of the PPM^{D156A} variant enzyme, 1 mM ribose 5-phosphate or 1 mM glucose 1,6-bisphosphate, and 0.1 mM $MnCl_2$, 25 mM Tris-HCl, pH 8. Background absorbance values from substrate and enzyme reaction mix were subtracted from the reaction measurement and phosphate turnover was determined by comparison to a standard curve.

RESULTS

Structures of p-Thr-85 phosphomimetic mutants and the conformation of the Lys-240 side chain

Previous studies of the *B. cereus* PPM demonstrated that activity required phosphorylation of the catalytic nucleophile, Thr-85 (6). As a starting point to understanding how the phosphothreonine was stabilized, we sought to identify if there were structural consequences of phosphorylation. Accordingly, we engineered site-directed substitutions of the catalytic nucleophile, Thr-85, changing this residue to the phosphomimetics glutamate (PPM^{T85E}; activated mimetic), or the neutral corollary glutamine (PPM^{T85Q}; unactivated mimetic). As anticipated, neither of these variants displayed detectable phosphomutase activity (data not shown) and both displayed a monodisperse elution profile in size exclusion chromatography with an elution volume similar to that of the wild-type enzyme.

The structures of the PPM^{T85Q} and PPM^{T85E} variants (Figure 3A, B) showed that these proteins were properly folded, and that neither exhibited significant backbone conformational changes when compared to the wild-type enzyme. Interestingly, the side chain of Lys-240 in the PPM^{T85Q} variant was oriented such that it contributed to the formation of a glucose 1,6-bisphosphate binding pocket (Figure 3A), a conformation that resembled that observed in the structure of unactivated PPM (Figure 3C; PDB entry 3M8W, (6)). Conversely, the orientation of the side chain of Lys-240 in the PPM^{T85E} variant formed an interaction with the side chain of Glu-85 (Figure 3B), which resembles that observed in the structure of activated PPM (Figure 3D; PDB entry 3M8Y, (6)). Taken together, these structures suggest that net charge at the active site could influence the conformation of the Lys-240 side chain.

Influence of the conformation of Lys-240 on affinity for the activator glucose 1,6-bisphosphate

To verify that the location of the glucose 1,6-bisphosphate binding site is not altered upon enzyme mutation, we next determined the crystal structures of the complexes between activated wild-type PPM, the PPM^{T85Q} variant, and the PPM^{T85E} variant after soaking with 5 mM glucose 1,6-bisphosphate (Figure 3E–H). In all cases, electron density for the activator (if apparent) was observed in the same location as was previously identified (6), however the quality of the electron density suggested different binding occupancy in each case. Clear electron density for glucose 1,6-bisphosphate was observed in the co-structure of the activator with the unactivated mimetic PPM^{T85Q} (Figure 3E), and the intensity of this electron density was similar to that observed in the structure determined from crystals of unactivated PPM soaked with glucose 1,6-bisphosphate (Figure 3G; PDB entry 3OT9 (6)). Conversely, virtually no electron density corresponding to the activator was observed in the co-structure of glucose 1,6-bisphosphate with the PPM^{T85E} activated mimetic (Figure 3F), while only poor electron density was observed in the co-structure of activated PPM soaked with glucose 1,6-bisphosphate (Figure 3H). In the former structure, coordinates for glucose 1,6-bisphosphate were not included in the final model.

Since the PPM^{T85Q} variant mimics the active site charge of the unactivated state of the enzyme, and the position of the side chain of Lys-240 may be predicted to contribute to the formation of a binding site for the activator in the absence of phosphorylation, we quantified whether this conformational change altered the binding affinity of the enzyme for the activator, glucose 1,6-bisphosphate. To assess the affinity, we took advantage of the proximity of Trp-90 to the active site and developed a fluorescence assay to monitor ligand binding. With this assay, we measured the affinity of glucose 1,6-bisphosphate in the unactivated wild-type enzyme, the PPM^{T85Q} variant (unactivated mimetic), and the PPM^{T85E} variant (activated mimetic) (Figure 4). Measurements of affinity on the activated wild-type enzyme were precluded since activation requires the continued presence of glucose 1,6-bisphosphate, and therefore fully-activated PPM could not be isolated from glucose 1,6-bisphosphate. Consistent with the hypothesis that the conformational change in Lys-240 alters enzyme affinity for the activator, both unactivated wild-type enzyme and the PPM^{T85Q} variant exhibited similar affinities for glucose 1,6-bisphosphate of 0.47 ± 0.04 μ M, and 0.23 ± 0.02 μ M, respectively, while the PPM^{T85E} variant had a 4.5-fold lower affinity of 2.05 ± 0.39 μ M.

Structural changes in unphosphorylated *B. cereus* PPM

Previous structures of unactivated PPM were likely determined from a mixed population of enzyme, with some of the protein phosphorylated and some unphosphorylated. In these structures, the occupancy of the Thr-85 phosphate was estimated as 30% (6). Determination of the structure of fully unphosphorylated *B. cereus* PPM required the identification of a new crystal form. Both phosphorylated and unphosphorylated *B. cereus* PPM could be crystallized in this new crystal form, and comparison of the two structures allowed conformational changes associated with crystal packing to be differentiated from conformational changes associated with the loss of active site phosphate.

At a global level, the structure of *B. cereus* PPM in the P2₁2₁2₁ space group (Figure 1) appears similar to the structure determined in the previously-reported P2₁ space group. Superposition of the individual domains of PPM determined from protein crystallized in the P2₁2₁2₁ space group with the structures of the equivalent domains determined from the previously-published P2₁ space group (PDB entry 3M8W; (6)) resulted in an RMS deviation of C $_{\alpha}$ atoms of the core domain of 0.5 Å, and an RMS deviation of C $_{\alpha}$ atoms of the cap domain of 0.3 Å.

Although the folds of the individual domains of both phosphorylated and unphosphorylated PPM are statistically identical, an interdomain angle change accompanied the loss of active site phosphorylation (Figure 5). The angles of interdomain reorganization in *B. cereus* PPM are restricted to two directions, twist and closure, by the main chain backbone that acts as the hinge (Figure 5A). Comparisons between unphosphorylated PPM and phosphorylated PPM show that the unphosphorylated enzyme is associated with an 18° twist and a 4° closure of the cap domain relative to the core domain (Figure 5B, Supplementary Video 1). Conversely, a comparison of the phosphorylated structure of *B. cereus* PPM determined in the P₂₁2₁2₁ space group to the previously-reported phosphorylated PPM in the P₂₁ space group (PDB entries 3M8W, 3M8Y, 3M8Z, and 3OTY; (6)) showed only a 2° twist and a 1° closure of the cap domain relative to the core domain.

In *B. cereus* PPM, the interdomain contacts (Figure 6A) appear to be dictated by three types of molecular interactions: hydrophobic interactions, hydrogen-bonding interactions, and metal coordination. The most extensive interaction mediating the interdomain interface is a hydrophobic cluster containing side chains from both the core and cap domains (Figure 6B, Table 3). These hydrophobic interactions are not significantly altered upon domain rotation. Hydrogen-bonding interactions further contribute to the interdomain orientation. Here, a difference is observed upon the change in domain angle. Two interdomain hydrogen-bonding interactions are observed in phosphorylated PPM, while four interdomain hydrogen-bonding interactions are observed in the crystal structure of unphosphorylated PPM (Figure 6C, D, Table 3).

Surprisingly, the interdomain contacts through the active site Mn²⁺ also differ between phosphorylated and unphosphorylated PPM. First, it is noted that comparison between the active site of phosphorylated PPM in the P₂₁2₁2₁ space group with that of phosphorylated PPM in the P₂₁ space group (6) shows no significant differences in metal coordination upon the change in space group (Figure 7A, B). Interestingly, unphosphorylated PPM shows a change in coordination number and geometry of the Mn-1 ion (Figure 7C). In the phosphorylated enzyme, the O1P atom of p-Thr-85 contributes to the coordination of Mn-1. This ligand is lost in the unphosphorylated enzyme, and a compensatory change in rotamer of Asp-156 is observed. In the new conformation, the side chain Oδ2 remains a ligand to Mn-1, and Oδ1 now forms a hydrogen-bonding interaction to the Oγ of unphosphorylated Thr-85. This change in side chain conformation converts Mn-1 from a 5-coordinate distorted square pyramidal to a 4-coordinate distorted tetrahedral geometry. Outside of the change in rotamer of Asp-156, the active site side chains of unphosphorylated *B. cereus* PPM do not show any significant changes in conformation.

Roles for Lys-240 and Asp-156 in activation

The studies on the PPM^{T85E} and PPM^{T85Q} variants demonstrated that a change in the conformation of Lys-240 influences affinity for the activator glucose 1,6-bisphosphate (Figures 3, 4), and suggest a role for this side chain in the activation process. The role of the Asp-156 side chain in activation and catalysis is less immediately clear. The new orientation of the Asp-156 side chain in the unphosphorylated enzyme partially restricts access to Mn-1 in the active site (compare the location of the Asp-156 side chain in Figure 7C to the location of the phosphate in Figure 7B), but also forms a tight hydrogen-bonding interaction with the Oγ of Thr-85, which is expected to enhance the nucleophilicity of the Oγ and modify its orientation with respect to the glucose 1,6-bisphosphate phosphate donor. This leads to two opposing hypotheses. Asp-156 could be a key residue for enzyme activation by favoring nucleophilic displacement of the glucose 1,6-bisphosphate donor and thereby boosting the steady state phosphorylation of Thr-85. Conversely, by sterically restricting access to Mn-1, Asp-156 could be acting to self-inhibit the enzyme and prevent off-pathway phosphatase activity.

To test these hypotheses, we created the PPM^{D156A} and PPM^{K240A} variants, with the former possibly influencing activation by influencing chemical reactivity, and the latter possibly influencing activation by reducing the affinity of PPM for the small molecule activator glucose 1,6-bisphosphate (Figures 3, 4). Both variants expressed to levels similar to the wild-type enzyme and were folded properly, as assessed by comparing the size exclusion chromatography profiles of the wild-type enzyme, PPM^{K240A}, and PPM^{D156A} variant enzymes (data not shown). Neither variant enzyme exhibited detectable phosphopentomutase activity under the conditions tested (data not shown). We used Western analysis to assess the ability of these variants to be phosphorylated. Excitingly, the PPM^{D156A} variant displays dramatically reduced phosphorylation of the active site Thr-85, while both the wild-type enzyme and the PPM^{K240A} variant can be phosphorylated by the small molecule activator glucose 1,6-bisphosphate (Figure 8). This suggests that the hydrogen-bonding interaction between Asp-156 and Thr-85 in the unphosphorylated enzyme is important for activation by glucose 1,6-bisphosphate, and that the influence of the Lys-240 side chain is of lesser importance in the activation process. Interestingly, the PPM^{K240A} variant appears to be phosphorylated more readily than the wild-type enzyme. That the PPM^{K240A} variant retains the ability to be robustly phosphorylated (Figure 8) yet lacks catalytic activity suggests that this side chain plays an additional mechanistic role in the catalytic cycle that is presently being investigated.

We next tested whether the Asp-156 side chain influenced the reaction outcome (Figure 2) by restricting access to Mn-1 when PPM is unphosphorylated (Figure 7C). If this were true, removal of this restriction could be sufficient to convert PPM to a phosphatase. Accordingly, we assessed the rate of phosphate hydrolysis of ribose 5-phosphate and glucose 1,6-bisphosphate by both wild-type PPM and the PPM^{D156A} variant. We did not detect an increase in phosphatase activity in the PPM^{D156A} variant as compared to the wild-type enzyme (data not shown). That the PPM^{D156A} variant did not acquire phosphatase activity suggests that the Asp-156 side chain is unlikely to sterically prevent phosphatase activity in unactivated PPM. Instead, additional differences between PPM and alkaline phosphatase likely tune these related enzymes to perform their respective reactions.

DISCUSSION

Mechanisms of activation of *B. cereus* PPM

In general, enzymes classified within a superfamily have similar structures and catalytic mechanisms. For example, in the catalytic cycle of the cofactor-independent phosphoglycerate mutase, which belongs to the alkaline phosphatase family, it has been concluded that the enzyme follows the same catalytic cycle as alkaline phosphatase (Figure 2B) despite having an overall reaction more similar to PPM. The reaction cycle of cofactor-independent phosphoglycerate mutase results in the transfer of a phosphoryl group from one position on the substrate molecule to a second position in the same molecule (31) via an intermediate with the enzyme transiently phosphorylated and the substrate transiently unphosphorylated. In this case, the incoming substrate, rather than a water molecule, acts as the phosphoryl acceptor ((32); Figure 2B, steps 5 & 6). This defines cofactor-independent phosphoglycerate mutase as an intramolecular mutase.

Since sequence and structural analyses place PPM within the alkaline phosphatase superfamily, one of the most intriguing surprises was the observation of a mechanistic deviation between PPM and other biochemically-characterized alkaline phosphatase family members, including other characterized mutases within the alkaline phosphatase family. Alkaline phosphatase is catalytically active when the active site nucleophile is unphosphorylated, and the reaction proceeds through a transiently-phosphorylated catalytic intermediate (Figure 2B). Conversely, PPM is catalytically active when the active site

nucleophile is phosphorylated, and the reaction proceeds through a transiently-unphosphorylated intermediate ((6); Figure 2A), making it an intermolecular mutase. *B. cereus* PPM is the only characterized alkaline phosphatase family member where this difference in the catalytic cycle has been demonstrated. In order to diverge from the alkaline phosphatase catalytic cycle, one requirement is that PPM must have a mechanism for the phosphorylation of the active site nucleophile. These studies investigate that mechanism.

The active site ligands to the di-metallo center are absolutely conserved between PPM and alkaline phosphatase with the exception of Asp-156, which is unique to PPM (6). Since no previously-characterized alkaline phosphatase superfamily member has a side chain equivalent to Asp-156 ligating the metallo-center, the presence of this additional ligand is unexpected. In our previous studies, we identified that binding of the substrate, ribose 5-phosphate, (PDB entry 3M8Z; (6)) resulted in the side chain of Asp-156 adopting an alternative conformation where its carboxylate was 4.5 Å from Mn-1, which is too far to act as a ligand. This strongly suggests that the predominant role of Asp-156 is not in metal binding. The structure of unphosphorylated PPM revealed that the side chain of Asp-156 forms a tight hydrogen-bonding interaction with the O γ of the unphosphorylated active site nucleophile, Thr-85 (Figure 7C). This is predicted to both enhance the reactivity of the Thr-85 O γ , which is more sterically hindered than its serine congener in the alkaline phosphatases, and aid in positioning this residue for the catalytic requirement of an in-line attack of glucose 1,6-bisphosphate, which would allow it to become phosphorylated more readily. Indeed, mutation of Asp-156 dramatically reduces enzyme activation by glucose 1,6-bisphosphate (Figure 8). It therefore seems possible that the crystal structure of unphosphorylated enzyme reflects the conformation of the enzyme after protein synthesis and prior to the first activation.

The poisoning of Asp-156 in an orientation that activates Thr-85 is associated with a large interdomain rotation. Hints at a possible mechanism for stabilizing the interdomain angle change come from careful examination of the phosphorylated and unphosphorylated PPM structures, which have been determined at resolutions sufficient to model alternate side chain conformations confidently. Importantly, Met-86, Asp-156, and Arg-193, all residues that mediate the interdomain contacts, have been observed in alternate conformations in crystal structures presented here (Figure 6) and previously (6). A combination of the changes in conformation of these side chains and the rotation of the domains results in the unphosphorylated form of *B. cereus* PPM containing an increased number of interdomain hydrogen-bonds when compared to phosphorylated enzyme (Figure 6B, C, Table 3).

Interestingly, the structure of the PPM^{T85Q} variant did not show a similar change in interdomain angle, but instead only showed a change in the conformation of the Lys-240 side chain (Figure 3). There are multiple possibilities for why this could be so. First, the glutamine chain may sterically prevent the interdomain rotation as these are each similar in size to p-Thr. In addition, the structure of the PPM^{T85Q} variant was determined in a crystal form that constrained the orientation of the domains with respect to each other. By comparison, the structure of the fully unphosphorylated enzyme was determined from a crystal form where packing did not constrain the interdomain orientation. It is therefore possible that the PPM^{T85Q} variant could be in conformational equilibrium in solution, with some enzymes having the domain orientation observed in the crystal structure reported here and some having rotated domains, but that the crystallization conditions selected for those members of the population with their domains similar to the activated enzyme.

Perhaps the most interesting possibility for a mechanism favoring Thr-85 phosphorylation incorporates both structural observations. During one type of unproductive turnover in PPM, a bisphosphorylated product could diffuse from the active site and leave unphosphorylated

enzyme (Figure 2A – diffusion would occur between steps 3 & 5). A fast conformational change of the Lys-240 side chain is associated with a modest increase in affinity for the activator glucose 1,6-bisphosphate (Figures 3, 4) and could increase the likelihood that the PPM could be rephosphorylated quickly. In the absence of activator, the enzyme could then undergo a larger, slower conformational change that more substantially increases the likelihood of rephosphorylation upon the next encounter with glucose 1,6-bisphosphate. Combined, these two mechanisms for improving activation of PPM enhance the affinity for small molecule activator and increase the reactivity of the Thr-85 nucleophile. Whether these are the only factors that favor increased Thr-85 phosphorylation is presently unknown.

Possible additional roles for interdomain angle changes

Enzymes with a global architecture similar to PPM, with an active site at an interdomain interface, commonly have angle changes between the domains that can be important for function. Interdomain closure is frequently proposed to accompany catalysis (see, for example (33–38)). For example, β -phosphoglucomutase is a member of the haloacid dehalogenase superfamily with mechanistic similarities to PPM despite a lack of sequence identity. In β -phosphoglucomutase, comparison of crystal structures determined in the presence (39) or absence (40) of a transition state analog altered the orientation of domains relative to each other. The interdomain angle change of unphosphorylated *B. cereus* PPM is predominantly a twist of the cap domain. However, both the $P2_12_12_1$ crystal form presented here, and molecules of the previously-reported $P2_1$ crystal form have small angle changes ($<2^\circ$) along the axis of closure. This direction of angle change hints at the possibility that interdomain closure could accompany catalysis, which is the subject of ongoing study.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Structure of phosphorylated *B. cereus* PPM. The structure of *B. cereus* PPM is colored by secondary structural elements, with α -helices in green and β -strands in cyan. The active site contains two Mn²⁺ ions, colored burgundy. The phosphorylated catalytic nucleophile, p-Thr-85, is shown as a stick model with carbon colored grey, oxygen colored red, and phosphorous colored magenta.

Figure 2.

Proposed catalytic cycles of PPM and alkaline phosphatase. **A.** Proposed catalytic cycle for the interconversion of ribose 5-phosphate and ribose 1-phosphate by *B. cereus* PPM. This 6-step mechanism is extrapolated from the alkaline phosphatase mechanism (see **Figure 2B**; (6)) and is discussed from steps 1 through 6, although all steps are reversible. In **step 1**, the enzyme is phosphorylated and active. In **step 2**, the substrate, ribose 5-phosphate encounters active enzyme. In **step 3**, a phosphoryl group is transferred from the enzyme to the 1-OH of ribose 5-phosphate, resulting in a ribose 1,5-bisphosphate chemical intermediate and an unphosphorylated enzyme intermediate. In **step 4**, the ribose 1,5-bisphosphate is reoriented in the enzyme active site. In **step 5**, the reoriented ribose 1,5-bisphosphate encounters unphosphorylated enzyme. In **step 6**, a phosphoryl group is transferred from ribose 1,5-bisphosphate back to the enzyme. The end result is a ribose 1-phosphate chemical product and a phosphorylated, active enzyme. **B.** Proposed mechanism for alkaline phosphatase. Alkaline phosphatase is a promiscuous enzyme that acts upon a variety of phosphorylated substrates and can either use water or a second organic molecule as a phosphate acceptor; a representative phosphatase reaction, where water is the phosphate acceptor, is shown. **Step 1** of this 6-step mechanism starts with active, dephosphorylated enzyme (compare to **step 4** of the PPM mechanism). In **step 2**, phosphorylated substrate enters the active site (compare to **step 5** of the PPM mechanism). In **step 3**, a phosphoryl group is transferred from substrate to the enzyme, resulting in a dephosphorylated substrate and a phosphorylated enzyme intermediate (compare to **step 6** of the PPM mechanism). In **step 4**, the dephosphorylated chemical substrate disassociates from the enzyme (compare to **step 1** of the PPM mechanism). In **step 5**, a water molecule enters the active site and encounters phosphorylated enzyme (compare to **step 2** of the PPM mechanism). In **step 6**, the phosphoryl group is transferred from the enzyme to the water molecule to release phosphate (compare to **step 3** of the PPM mechanism). The end result is a dephosphorylated substrate, a free phosphate, and a dephosphorylated, active enzyme.

Figure 3.

Comparison of the position of the Lys-240 side chain and the binding of glucose 1,6-bisphosphate in the PPM^{T85E} and PPM^{T85Q} variant enzymes and in the unactivated and activated wild-type enzymes. Carbons are colored grey, nitrogens blue, oxygens red, and the Mn²⁺ ions are shown as cyan spheres. Metal ligation (distances <2.2 Å) are shown as solid black lines, and hydrogen-bonding interactions are shown as dashed grey lines. All figure panels show the final model superimposed with $|F_o| - |F_c|$ simulated annealing electron density (green mesh) calculated after the omission of both the Lys-240 side chain (panels A–H) and glucose 1,6-bisphosphate (panels E–H) and displayed at 3 σ . The side chain of Asp-286, a metal ligand to Mn-2, has been omitted from all figure panels for clarity. **A.** The structure of PPM^{T85Q} variant crystallized without glucose 1,6-bisphosphate. **B.** The structure of the PPM^{T85E} variant crystallized without glucose 1,6-bisphosphate. **C.** The structure of unactivated PPM (low phosphorylation level; PDB entry 3M8W (6)) crystallized without glucose 1,6-bisphosphate. Thr-85 is in a mixed state in this structure with an estimated occupancy of the phosphate of 30%. The position of the phosphate is omitted for clarity. **D.** The structure of activated PPM (high phosphorylation level; PDB entry 3M8Y (6)) crystallized without glucose 1,6-bisphosphate. **E.** The structure of the PPM^{T85Q} variant crystallized with glucose 1,6-bisphosphate. The estimated occupancy of the glucose 1,6-bisphosphate is 75%. **F.** The structure of the PPM^{T85E} variant crystallized with glucose 1,6-bisphosphate. The electron density was not sufficient to include glucose 1,6-bisphosphate in the model, even at low occupancy. **G.** The structure of unactivated PPM (low phosphorylation level; PDB entry 3OTY (6)) crystallized with glucose 1,6-bisphosphate. The estimated occupancy of the phosphate is 30%, and the estimated occupancy of glucose 1,6-bisphosphate is 75%. The position of the phosphate is omitted for clarity. **H.** The structure activated PPM crystallized with glucose 1,6-bisphosphate. The estimated occupancy of the phosphate is 100% and the estimated occupancy of the glucose 1,6-bisphosphate is 50%.

Figure 4.

Changes in the intrinsic fluorescence spectra of wild-type and variant PPM following the addition of glucose 1,6-bisphosphate. **A – C.** Representative spectra of **A.** wild-type, unphosphorylated PPM, **B.** the PPM^{T85E} variant, and **C.** the PPM^{T85Q} variant collected in the absence (solid line) and presence (dashed line) of 10 μM glucose 1,6-bisphosphate. **D – F.** Plot of the change in fluorescence intensity at 350 nm following the addition of glucose 1,6-bisphosphate for **D.** wild-type, unphosphorylated PPM, **E.** the PPM^{T85E} variant, and **F.** the PPM^{T85Q} variant. Points are the average of three separately-prepared samples each measured three times, and error bars are the standard deviation of the mean.

Figure 5.

Phosphorylation dependent interdomain orientation in *B. cereus* PPM. **A.** Diagram of the twist and closure axes in relation to the core and cap domains of the structure of PPM. The backbone is represented as a cyan trace and the Mn²⁺ ions are shown as burgundy spheres. **B.** Stereoview of the overlay of the core domains of the phosphorylated PPM (cyan) with that of unphosphorylated PPM (grey) demonstrates the relative difference in the orientation of the cap domain in the two states of the enzyme. When divided along the two axes of rotation, 18° of the angle change is around the twist axis, while only 4° is around the closure axis.

Figure 6.

Contacts stabilizing the interdomain orientation in phosphorylated and unphosphorylated *B. cereus* PPM. All panels are colored with the core domain in grey and the cap domain in magenta. **A.** A global view of *B. cereus* PPM with Mn^{2+} ions highlighted in cyan and p-Thr-85 depicted as a stick model. The region highlighted in panels **(B)** – **(D)** is delineated in the box. **B.** Highlight of the hydrophobic cluster of residues stabilizing the interdomain contacts. Residues emanating from the core domain are colored green and residues emanating from the cap domain are colored orange. **C.** Interdomain hydrogen-bonding interactions in phosphorylated *B. cereus* PPM. The structure of PPM determined from the $\text{P2}_12_12_1$ crystal form is shown, but the hydrogen-bonding interactions are unchanged between the $\text{P2}_12_12_1$ crystal form and the P2_1 crystal form. **D.** Interdomain hydrogen-bonding interactions in the unphosphorylated *B. cereus* PPM.

Figure 7.

Active site of phosphorylated and unphosphorylated PPM. The active site of PPM is colored with both backbone and carbons emanating from the core domain in grey, and with backbone and carbons emanating from the cap domain in magenta. Mn^{2+} ions are shown as cyan spheres. Metal ligation ($<2.2 \text{ \AA}$) is shown as solid black lines, and hydrogen-bonds are shown as dashed grey lines. The side chain of Asp-286, a metal ligand to Mn-2, has been omitted from all figure panels for clarity. **A.** The active site of phosphorylated *B. cereus* PPM in the $P2_1$ crystal form (6). **B.** The active site of phosphorylated *B. cereus* PPM in the $P2_12_12_1$ crystal form. **C.** The active site of unphosphorylated *B. cereus* PPM in the $P2_12_12_1$ crystal form. Note the movement of the Asp-156 ligand and the newly-formed hydrogen-bonding interaction to Thr-85.

Figure 8.

Western analysis of phosphothreonine levels of wild-type and variant *B. cereus* PPM after incubation with glucose 1,6-bisphosphate. The upper panel (labeled **Total**) shows the Ponceau S stained nitrocellulose membrane, which confirms equal protein loading between lanes. The lower panel (labeled **p-Thr**) shows the Odyssey image of the same nitrocellulose membrane following incubation with a phosphothreonine-specific primary antibody and an Alexafluor680-labeled secondary antibody. The concentration of glucose 1,6-bisphosphate (in μM) and enzyme identity are indicated above the lanes.

Table 1
Data collection and refinement statistics for wild-type and PPM variants crystallized in the P2₁ space group

PDB entry	3UN5	3UNY	3UN2	3UN3	3UO0
<i>Data Collection:</i>					
Resolution (Å) ^a	20–1.8 (1.86–1.8)	20–1.95 (2.01–1.95)	20–1.8 (1.86–1.8)	20 – 1.8 (1.86–1.8)	20–2.3 (2.38–2.3)
Unit-cell parameters (Å)	a = 106.4	a = 106.6	a = 91.9	a=91.6	a=91.8
	b = 76.8	b=76.8	b = 76.7	b=76.8	b=77.1
	c = 182.8	c = 182.3	c = 107.2	c = 106.8	c=108.3
	β = 106.0°	β = 106.1	β = 108.7°	β = 108.6°	β=108.9°
Total reflections	896,177	686,614	461,139	424,067	231,036
Unique reflections	254,487	206,406	130,700	125,668	63,622
1/σ	22.3 (2.5)	13.9 (2.6)	22.5 (2.6)	19.6 (2.2)	15.8 (3.2)
Completeness (%)	97.4 (84.8)	99.2 (95.9)	97.5 (81.4)	96.3 (77.4)	99.7 (97.8)
R _{sym} ^b	0.06 (0.34)	0.08 (0.34)	0.06 (0.36)	0.07 (0.39)	0.08 (0.36)
<i>Model Refinement:</i>					
Reflections in test set	12,792	10,161	6,535	5,997	4,517
R _{cry} ^c	0.179	0.167	0.180	0.165	0.173
R _{free}	0.207	0.199	0.208	0.191	0.225

^aValues in parentheses are for the highest resolution shell

$$bR_{sym} = \sum |I_{obs} - I_{avg}| / I_{avg}$$

$$cR_{crys} = \left[\sum ||F_{obs}| - |F_{calc}|| \right] / \sum |F_{obs}|$$

^dR_{free} is calculated using the same equation as R_{crys} using a subset of reflections omitted from refinement and reserved in the test set of data.

Table 2

Data collection and refinement statistics for phosphorylated and unphosphorylated PPM in the P2₁2₁2₁ space group

	Phosphorylated PPM	Unphosphorylated PPM
PDB entry	3TX0	3TWZ
<i>Data collection:</i>		
Resolution (Å)	50–1.75 (1.80–1.75) ^a	50–2.26 (2.32–2.26)
Unit-cell parameters (Å)	a = 68.7	a = 61.0
	b = 76.6	b = 75.7
	c = 107.2	c = 95.6
Resolution (Å)	50–1.75 (1.81–1.75)	50–2.25 (2.33–2.25)
Total reflections	265,458	73,732
Unique reflections	47,942	19,421
I/σ	17.5 (3.3)	24.3 (3.5)
Completeness (%)	91.6 (63.4)	92.1 (64.3)
R _{sym} ^b	0.08 (0.33)	0.07 (0.26)
<i>Model Refinement:</i>		
Number of R _{free} reflections	2428	989
R _{cryst} ^c	0.177	0.189
R _{free} ^d	0.196	0.246

^aValues in parentheses are for the highest resolution shell.

^b $R_{\text{sym}} = \sum [I_i - \langle I \rangle] / \sum [\langle I \rangle]$ where i is ith measurement and $\langle I \rangle$ is the weighted mean of I.

^c $R_{\text{cryst}} = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$

^dR_{free} is calculated using the same equation as R_{cryst} using a subset of reflections omitted from refinement and reserved in the test set of the data.

Table 3
Interdomain interactions in phosphorylated and unphosphorylated *B. cereus* PPM

Phosphorylated		Unphosphorylated	
Hydrophobic cluster [#]			
Core domain	Cap domain	Core domain	Cap domain
Met-86	Phe-101	Met-86	Phe-101
Trp-90	Leu-185	Trp-90	Leu-185
Ile-97	Met-190	Ile-97	Met-190
Leu-217		Leu-217	
Phe-220		Phe-220	
Hydrogen-bonding interactions ^{\$}			
Core domain	Cap domain	Distance	Cap domain
Asp-243 O	Arg-193 Nη1	2.6 Å	Arg-193 Nη2
Asp-246 Oδ1	Lys-218Nζ	~ 29 Å	Lys-218Nζ
			~ 29 Å
			29 Å
			2.9 Å
			2.4 Å
Interdomain metal ligation			
Core domain	Cap domain	Distance	Cap domain
Mn-1	Asp-156 Oδ2	2.1 Å *	Asp-156 Oδ2
			2.1 Å *

[#] Residues in the hydrophobic cluster are listed in numerical order, and the listing of the residues on the same line does not imply specific interactions between the side chains. The indicated side chains form a compact cluster with contributions from all residues.

^{\$} Hydrogen-bonding interactions were identified and measured using the program O (22).

* distance is consistent with metal ligation