A Third Metal Is Required for Catalytic Activity of the Signal-transducing Protein Phosphatase M tPphA

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Protein phosphatase M (PPM) regulates key signaling pathways in prokaryotes and eukaryotes. Novel structures of bacterial PPM members revealed three divalent metal ions in their catalytic centers. The function of metal 3 (M3) remained unclear. To reveal its function, we created variants of tPphA from Thermosynechococcus elongatus in all-metal-coordinating residues, and multiple variants were created for the M3 coordinating Asp-119 residue. The structures of variants D119A and D193A were resolved, showing loss of M3 binding but unaffected binding of M1 and M2 in the catalytic center of D119A, with the nucleophilic water molecule in the correct place. The catalytic activity of this variant was highly impaired. This and further structure-function analyses showed that M3 is required for catalysis by providing a water molecule as a proton donor during catalysis. Mutation of the homologue Asp residue in human PP2Cα also caused loss of function, suggesting a general requirement of M3 in PPM-catalyzed reactions.

Serine/threonine phosphatases of the PPM family are widely present in eukaryotes and prokaryotes and regulate key signaling pathways involved in cell proliferation, stress responses, or metabolic control (1). PPM phosphatases are metalloenzymes requiring Mg2+ or Mn2+ ions, which are coordinated by a universally conserved core of aspartate residues.

The human PPM member PP2Cα has been the defining representative of this family. As shown by structural analysis and site-directed mutagenesis studies, a binuclear metal center (with metals M1 and M2) activates a catalytic water molecule for nucleophilic attack of the phosphate group (2, 3). The importance of the M1-M2 core was also demonstrated for other PPM members, such as BA-Stp1, a bacterial PPM member from Bacillus anthracis (4). Recently, the structure of several bacterial members (tPphA from Thermosynechococcus elongatus (5), MtPstP from Mycobacterium tuberculosis (6), MspP from Mycobacterium smegmatis (7), STP from Streptococcus agalactiae (8), as well as the structure of Fab1 from Arabidopsis thaliana in co-crystal with its inhibitor, abscisic acid receptor Pyl2 (9)) has been solved. A third metal ion (M3) in proximity of the M1-M2 core could be revealed in several crystal forms of these proteins; however, the function of the third metal was controversially discussed. It was proposed to directly take part in catalysis in the case of SaSTP (8) or to have a regulatory role in the conformation of the flexible FLAP subdomain, which may be important for substrate recognition (10).

The PPM tPphA from the thermophilic cyanobacterium T. elongatus is ideally suited to elucidate the role of M3. Because of its thermophilic origin, it has a robust structure, and the M3 coordination was almost invariant in various crystal forms (Fig. 1) (5), whereas M3 coordination was variable in other PPM members (7, 8). Furthermore, tPphA reacts readily with the artificial substrate pNPP, and moreover, its natural substrate, the phosphorylated Pii signal transduction protein (Pii-P), is available (11). In this study, mutant tPphA variants affected in M3 coordination were generated and analyzed with respect to enzymatic properties, metal binding, and three-dimensional structures, demonstrating a catalytic role of M3 in the dephosphorylation reaction.

EXPERIMENTAL PROCEDURES

Detailed protocols are available in the supplemental material.

Cloning, Overexpression, and Purification of tPphA and Its Variants—The tPphA gene was cloned into the His tag vector pET15b (Novagen) according to standard procedures. Site-directed mutagenesis of tPphA was carried out with the QuickChange XL site-directed mutagenesis kit (Stratagene). Artificial genes for human PP2C fragment (residues 1–297) and the corresponding PP2C-variant D146A were synthesized and cloned into His tag pET15b vector by GENEART (Regensburg, Germany). Overexpression and purification of His-tagged proteins were performed as described in the supplemental material.

His tag-free tPphA variants D119A and D193A for crystallization were constructed from the plasmids pET15b + D119A and pET15b + D193A by standard PCR methods. Protein overproduction and purification were carried out as described previously for wild-type tPphA (5).

Crystallization, Data Collection, and Structure Determination—Crystals of the tPphA mutants D119A and D193A were obtained by mixing 0.8 μl of protein solution (36 mg/ml)
and 0.8 μl of reservoir at 293 K after 1 day. The reservoir consists of 30% PEG3350, 0.2 mM CaCl₂, and 0.1 mM Tris, pH 8.0, for the D119A mutant and 0.1 mM Hepes, pH 7.4, for the D193A mutant. Before x-ray measurement, the crystals were transferred to a cryosolution containing the reservoir solution and 15% 2,3-butanediol as cryoprotectant. Data sets were collected on a VP-ITC titration calorimeter (MicroCal, GE Healthcare). To remove any divalent cations from the purified proteins, all buffers were treated with Chelex 100 (Sigma), and the proteins were dialyzed four times at 4 °C in dialyzed metal-free buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl) in the presence of excess Chelex 100 in the dialysis reservoir buffer. ITC experiments were performed at 25 °C. Protein samples (50 μM) were titrated in the calorimeter cell (1.43 ml) by 50 successive injections (4 μl) of 500 μM MnCl₂ solution containing 250 μM DTT. The data were evaluated using the ORIGIN 7.0 software, provided by the manufacturer. Human PP2C ITC procedures were same as for tPphA.

Elemental Analysis by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES)—ICP-OES (Optima 5300DV, PerkinElmer Life Sciences) was used to quantify the amount of Mn²⁺ in tPphA variants. The sensitivity of ICP-OES to measure Mn²⁺ in liquid condition is 1 μg/liter. Chelex 100 was used to remove any divalent cations in the protein samples and buffers (same ITC procedure as above). After removing divalent cations, tPphA variants were incubated with 2 mM MnCl₂ and 1 mM DTT on ice for 30 min. To remove unbound Mn²⁺, the samples were loaded onto CentriSpin columns (from Princeton Separations) equilibrated by the final dialysis buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl). The columns were centrifuged at 750 × g for 2 min. Only Mn²⁺ tightly bound to tPphA should pass through the column. The average protein recovery was 70%. Identical concentrations of the protein variants were adjusted using the final dialysis buffer before ICP-OES analysis. As a background control for ICP-OES calibration,
50 μl of the final dialysis buffer was supplemented with 2 mM MnCl₂ and 1 mM DTT, and the mixture was subjected to the same desalting procedure as the protein samples. The CentriSpin column eluate, collected in parallel with the protein samples, was used as background control for unspecific carry-over of Mn²⁺. The concentration of Mn²⁺ was calculated according to a Mn²⁺ standard (0–1000 μM/liter) prepared in the final dialysis buffer.

RESULTS AND DISCUSSION

**Initial Characterization of tPphA Variants**—To study the catalytic core of tPphA (Fig. 1), the highly conserved aspartate residues (Asp-18, Asp-34, Asp-119, Asp-193, and Asp-231) coordinating the three metal ions in the trinuclear metal center were site-directed mutated. Asp-18, Asp-34, Asp-193, and Asp-231 were changed to alanine. Asp-119 coordinating M3 was changed to alanine, threonine, glutamate, or asparagine. The recombinant proteins were purified to apparent homogeneity and characterized biochemically. First, the effect of the mutations on catalytic activity was investigated with pNPP as substrate. pNPP is a commonly used artificial substrate to characterize the catalytic activity of protein phosphatases (20). Previously, we showed that pNPP hydrolysis by tPphA requires Mn²⁺ as a divalent metal ion, as is the case for many other PP2C members (6, 7, 21, 22). Under standard assay conditions, all aspartate variants except D119N lost any detectable activity toward pNPP. Only after increasing the enzyme concentration 40-fold compared with standard assay conditions could a marginal activity be detected in variants D119A, D119E, D119T, and D231A; variants D18A, D34A, and D193A remained completely inactive (see Table 2). To confirm that the latter mutants were completely inactive, the Mn²⁺ concentration was further increased 5-fold, and the assays were incubated for prolonged periods. However, under no conditions could pNPP hydrolysis be detected. Therefore, it is safe to assume that D18A, D34A, and D193A have completely lost the ability to dephosphorylate pNPP. Variants D119A, D119E, D119T, and D231A lost about 97.6, 99.9, 99.9, and 99.7% catalytic efficiency compared with the wild type in pNPP assay (as derived from the $K_{cat}/K_{m}$ values in Table 2). The complete loss of activity of variants impaired in M1 or M2 coordination confirms the pivotal role of the M1-M2 core in PPM catalysis (3, 4). The very low activities of Asp-119 variants could either indicate a role of M3 in catalysis or may be explained by an indirect effect on the coordination of the M1-M2 center. To distinguish between these possibilities, the crystal structures of two representative tPphA variants, D119A and D193A, were solved.

**Structure of tPphA Variants D119A and D193A**—The crystallization conditions for variants D119A and D193A are nearly identical for wild-type tPphA (5). The overall structure of these variants is very similar to wild-type tPphA (Fig. 2A). The centrally buried β-sheets and the two anti-parallel α-helices of the variants fully merged with wild-type tPphA. In contrast to the wild type, only two metal ions (M1 and M2) are resolved in

| Enzyme variant | $K_{m}$ (pNPP) $s^{-1}$ | $K_{cat}$ (pNPP) $s^{-1} M^{-1}$ | $K_{cat}/K_{m}$ $s^{-1} M^{-1}$ |
|----------------|------------------------|-------------------------------|-------------------------------|
| tPphA WT      | 0.47 ± 0.07            | 0.85 ± 0.055                  | 1809 ± 117                    |
| D18A          | –                      | –                             | –                             |
| D34A          | –                      | –                             | –                             |
| D119A         | 0.27 ± 0.07            | 0.012 ± 0.001                 | 44 ± 4                        |
| D119E         | 1.67 ± 0.77            | 0.032 ± 0.01                  | 19 ± 6                        |
| D119N         | 1.22 ± 0.21            | 0.15 ± 0.015                  | 123 ± 12                      |
| D119T         | 0.8 ± 0.03             | 0.027 ± 0.001                 | 33 ± 0.001                    |
| D193A         | –                      | –                             | –                             |
| D231A         | 2.90 ± 0.59            | 0.018 ± 0.002                 | 6 ± 1                         |
| hPP2Ca WT     | 0.57 ± 0.03            | 2.69 ± 0.06                   | 5725 ± 105                    |
| hPP2Ca D146A  | –                      | –                             | –                             |

$^a$—means not determined.
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the catalytic center of D119A and D193A (Fig. 2, B and C). Furthermore, the variants differed from the wild type in the structure of the lower part of the FLAP subdomain near the M3 site. This indicates that the occupation of the M3 site could affect the structure of the FLAP subdomain proximal to the catalytic cavity, as suggested previously (10).

Compared with the wild type, the catalytic core of the D119A variant displayed identical positions of M1 and M2 and all residues, except for the lack of the aspartyl side chain and the lack of M3, revealing that the Asp-119 mutation specifically affected the coordination of M3. By contrast, the Asp-193 mutation causes a shift in the position of M1, probably due to the lack of coordination by the Asp-193 carboxyl group (Fig. 2C). The complete loss of activity of the D193A variant can therefore be explained by the destruction of the bimetallic metal core that activates the nucleophilic water. However, in case of the D119A variant, this metal core was entirely preserved, but nevertheless activity was drastically reduced. Activity was lost due to a highly reduced $K_{\text{cat}}$, whereas the $K_m$ value for $pNPP$ was almost not affected. Similarly, variants D119T and D119E also displayed greatly reduced $K_{\text{cat}}$ values but only slightly different $K_m$ values, indicating that substrate turnover rather than substrate binding was affected.

Mutation of the Asp-119 homologue residue in PstP to asparagine has previously revealed only a minor effect on catalytic activity at high Mn$^{2+}$ concentrations (6), which was taken as evidence against a catalytic role of M3. However, the carboxyl oxygen of asparagine could still coordinate M3, although less efficiently, explaining the high Mn$^{2+}$ requirement of this variant. To reveal if such an explanation could also apply for the tPphA D119N variant, the $K_m$ value of Mn$^{2+}$ was determined (Table 3). Indeed, a 10-fold higher $K_m$ value for Mn$^{2+}$ compared with the wild type was measured, whereas the $K_{\text{cat}}$ of the reaction at saturating Mn$^{2+}$ was only moderately (about 2-fold) lowered. This result is in perfect agreement with the above assumption of a decreased efficiency in M3 binding.

Reactivity of tPphA Variants toward Phosphoserine and Phosphopeptides—Next, the reactivity of the various tPphA variants was determined toward phosphoserine and five different phosphopeptides. Two peptides were commonly used artificial protein phosphatase substrates as follows: the hexapeptides containing either a phosphothreonyl residue (RRAR(pT)VA subsequently termed pT peptide) or a phosphosereryl residue (RRAR(pS)VA, subsequently termed pS peptide). The other three peptides were derived from the sequence surrounding the phosphorylated seryl residue in the natural substrate, the T-loop of the PII protein (G(pS)E, RG(pS)EY, and CRYRG(pS)EYTV) (Table 4). Phosphoserine alone was not used as a substrate by any tPphA variant. Wild-type tPphA turns over the pT peptide (7.73 ± 1.10 nmol/min/μg) more rapidly than any of the other phosphoseryl-containing peptides. The preference for pT over the pS peptides is frequently found in PP2C-like phosphatases (19, 23, 24). Activity correlates with the length of the peptides as follows: toward longer peptides the activity is higher than toward smaller substrates; the three-residue peptide (G(pS)E) is apparently too short to be recognized as a substrate (Table 4). As for $pNPP$ dephosphorylation, variants D18A, D34A, and D193A had no detectable activity toward any of the peptides, even when the incubation time was prolonged up to 2 h, and D119A, D119T, and D231A variants had some marginal activity (Table 4). The D119E variant showed no residual activity toward any phosphopeptide, highlighting the severe defect of this variant. The D119N variant displayed variable defects; only a low activity (5–10%) was measured against “foreign” peptides; however, more than 50% of wild-type activity was recorded toward the 10-amino acid T-loop peptide, suggesting that the sequence surrounding the phosphoseryl residue can partially compensate the D119N mutation.

Reactivity of tPphA Variants toward Phosphorylated PII Protein as Substrate—The various mutants were also tested with phosphorylated PII protein (P$_{\text{II}}$-P) as substrate. Because the recombinant P$_{\text{II}}$ protein cannot be phosphorylated in vitro until now, P$_{\text{II}}$-P extracted from the cyanobacterium $S$. elongatus was used as substrate. The phosphorylation status of P$_{\text{II}}$ was detected by a well proven procedure, in which unphospho-

### TABLE 3

Kinetic parameters of tPphA and its mutants towards Mn$^{2+}$ (pNPP used as substrate)

| Variant  | $K_m$ (Mn$^{2+}$) | $K_{\text{cat}}$(Mn$^{2+}$) |
|----------|------------------|--------------------------|
| WT       | 0.58 ± 0.088     | 1.16 ± 0.048             |
| D119N    | 5.25 ± 0.78      | 0.47 ± 0.037             |
| Human PP2Ca | 0.24 ± 0.013   | 2.51 ± 0.034             |

### TABLE 4

The activity of tPphA and its mutants toward three phosphopeptides

| Variant  | RRA(pT)VA | RRA(pS)VA | Phosphoserine | G(pS)E | RG(pS)EY | CRYRG(pS)EYTV |
|----------|-----------|-----------|---------------|--------|----------|---------------|
| WT       | 7.73 ± 1.10 | 3.06 ± 0.21 | –             | –      | –        | –             |
| D18A     | –         | –         | –             | –      | –        | –             |
| D34A     | –         | –         | –             | –      | –        | –             |
| D119A    | –         | –         | –             | –      | –        | –             |
| D119E    | –         | –         | –             | –      | –        | –             |
| D119N    | 0.72 ± 0.02 | 0.16 ± 0.01 | –             | –      | 0.17 ± 0.01 | 2.36 ± 0.46 |
| D119T    | –         | –         | –             | –      | –        | –             |
| D193A    | –         | –         | –             | –      | –        | –             |
| D231A    | 0.04 ± 0.02 | 0.10 ± 0.01 | –             | –      | –        | –             |
| hPP2Ca   | 3.732 ± 0.184 | 1.82 ± 0.12 | –             | –      | 0.23 ± 0.03 | 1.89 ± 0.39 |
| hPP2Ca D146A | –       | –         | –             | –      | –        | –             |

– means the activity is below 0.01 nmol/min/μg.
labeled and the three phosphorylated forms (each subunit of the trimeric PII may be phosphorylated on Ser-49) are separated by non-denaturing PAGE and detected by immunoblot analysis (11). PII-P extract was incubated with the various tPphA variants, and at different time points, aliquots were removed, and the phosphorylation status of PII was analyzed. The resulting electrophoretic pattern, after incubating PII-P with the phosphatase variants, is shown in supplemental Fig. 1. From this pattern, the degree of PII dephosphorylation was determined densitometrically (19) and plotted against the reaction time (Fig. 3). In Mn²⁺-supplemented buffer, wild-type tPphA dephosphorylated PII-P already after 5 min (Fig. 3). Under these assay conditions, the D119N variant was fairly active, and a two-step sequential binding model (Fig. 6). The calorimetric signals could not be unambiguously fitted to a unique binding model; therefore, only a qualitative evaluation can be safely performed, because fitting to different models yields different thermodynamic constants (Table 5). The decreased binding isotherms indicate reduced but nevertheless clearly detectable metal binding. The observed residual binding signals in these variants probably represent binding of either M2 (in case of D193A and D231A mutants) or M1 (in case of D18A mutant) to the enzyme. The amount of firmly bound Mn²⁺ to tPphA variants was determined by ICP-OES, a very sensitive method for quantitative elemental analysis. Before ICP-OES analysis, the protein samples were passed through a CentriSpin desalting column, equilibrated with a Mn²⁺-free buffer, to remove unbound or loosely bound Mn²⁺ ions from the protein. Only Mn²⁺, which was tightly bound, can pass through the column. From the Mn²⁺ concentration measured by ICP-OES and the protein concentration, the stoichiometry of tightly bound Mn²⁺ to tPphA was calculated (Table 6). Wild-type tPphA and variants D119E and D119N had Mn²⁺:protein stoichiometries close to 2:1, whereas that of the D34A variant was 0.3:1. The latter result agrees with the ITC measurement, which showed that D34A was almost completely impaired in metal binding. This low value indicates that the experimental procedure only reveals tightly protein-bound Mn²⁺. Asp-34 is placed in the middle of the catalytic metal center, and the oxygen atoms of its carbonyl group coordinates both M1 and M2 (see Fig. 1), explaining the strongly reduced metal binding. Variants D18A, D193A, and D231A have stoichiometries near 1:1, indicating that they can

**TABLE 5**
The affinity and thermodynamic parameters of WT and the mutants from ITC assay

| Fitting model | WT | D119E | D119N |
|---------------|----|-------|-------|
| Three-step sequential binding model | 18 | 61 | 85 |
| | 18 | 85 | 178 |
| | 82 | 50 | 48 |
| | -26 | -24 | -24 |
| | 283 | -282 | -27 |
| | 161 | -33 | |
| Two-step sequential binding model | D119E | D119N | D119T |
| | 1.9:1 | 0.7:1 | 0.3:1 |
| | 0.8:1 | 1.5:1 | 2.2:1 |
| | 1.4:1 | 1.2:1 | 1.1:1 |

**TABLE 6**
The stoichiometry of Mn²⁺ and proteins calculated from ICP-OES measurement

| Mn²⁺:WT | 1.9:1 |
| Mn²⁺:D18A | 0.7:1 |
| Mn²⁺:D34A | 0.3:1 |
| Mn²⁺:D119A | 0.8:1 |
| Mn²⁺:D119E | 1.5:1 |
| Mn²⁺:D119N | 2.2:1 |
| Mn²⁺:D119T | 1.4:1 |
| Mn²⁺:D193A | 1.2:1 |
| Mn²⁺:D231A | 1.1:1 |

The Asp-34 mutant shows the strongest impairment in Mn²⁺ binding, with almost no heat release upon MnCl₂ addition. The Mn²⁺-binding isotherms of the mutants D18A (close to M2), D193A (close to M1 and M3), and D231A (close to M1) show that these mutants were still able to bind Mn²⁺; however, binding was clearly impaired and released less heat (calorimetric signals decreasing by 70, 50, or 60% for D18A, D193A, or D231A mutants, respectively) compared with wild-type enzyme. The calorimetric signals could not be unambiguously fitted to a unique binding model; therefore, only a qualitative evaluation can be safely performed, because fitting to different models yields different thermodynamic constants (Table 5). The decreased binding isotherms indicate reduced but nevertheless clearly detectable metal binding. The observed residual binding signals in these variants probably represent binding of either M2 (in case of D193A and D231A mutants) or M1 (in case of D18A mutant) to the enzyme.
only tightly bind one metal, either M1 or M2, depending on their mutation. What happens to M3? We suggest an inherent weak binding of M3 to explain the 2:1 stoichiometry found in wild-type tPphA. In agreement with the rather weak and superficial coordination of M3 by the enzyme, it probably binds loosely to the catalytic core and is therefore never observed by this experimental procedure, which involves a gel filtration step. Under conditions of protein crystallization, however, the M3 site is always occupied in wild-type tPphA, due to high surrounding divalent metal ion concentration.

Comparison with Human PP2C—The results of this study strongly argue in favor of a catalytic role of M3, most likely in activating a water molecule as proton donor (see below). The absolute conservation of the M3-coordinating residue Asp-119 in all other PPM phosphatases, including human PP2Cα (hPP2Cα), raises the question of a general mechanism in this class of enzymes. We addressed this question by producing recombinant hPP2Cα and a variant, in which the homologue residue Asp-146 was changed to Ala, and characterized the phenotype of these variants as described above for tPphA. The enzymatic constants of hPP2Cα toward pNPP were similar to tPphA; the $K_m$ value for pNPP was almost the same, and the $K_{cat}$ was $\sim$3-fold higher. Strikingly, the hPP2Cα variant D146A was completely inactive toward pNPP (Table 2). When using the various phosphopeptides as substrate, again the wild-type variant of hPP2Cα turned out to be similar to wild-type tPphA, whereas the D146A variant had no obvious activity toward any phosphopeptide. Intriguingly, hPP2Cα wild type was completely inactive toward P11-P, either in the presence of Mn$^{2+}$ or in the presence of Mg$^{2+}$ (supplemental Fig. 2). This result illustrates a high degree of specificity toward the substrate phosphoprotein, which deserves further investigation. When assaying the binding of Mn$^{2+}$ to hPP2Cα by ITC, the calorimetric signals of the wild-type variant were very similar to the D146A variant (Fig. 4), and both could be fitted according to a two-step sequential binding model. This result indicates that only two metals, presumably M1 and M2, bound to hPP2Cα in such a manner that they yielded a calorimetric signal. Nevertheless, Asp-146 is essential for catalysis. As shown by the tPphA D119A structure, this mutation did not cause a distortion of the M1-M2 core. Taking into consideration the high degree of structural conservation in the catalytic site of PP2C homologues, including tPphA, it is conceivable that Asp-146 coordinates a loosely bound divalent metal to the M3 site. The assumption of a loosely bound M3 would explain the stoichiometry of Mn$^{2+}$ binding to tPphA; the short passage through a desalting column is obviously sufficient to loosen M3, which therefore seems to be a “volatile” metal in PPM. The degree of volatility could differ between the different enzymes, in agreement with the variable occupation of M3 in various PPM crystals (STP and MtPstP). The M3 site is always occupied in the tPphA crystal, indicating that M3 in tPphA may be less volatile than in other PP2C homologues, possibly because this enzyme is derived from a thermophilic organism and is therefore more rigid.

Conclusions—This study has provided clear evidence that the correct coordination of M3 in tPphA is decisive for catalytic activity. As shown by the structure-function study of the D119A variant, the M1-M2 metal core is not sufficient for catalysis. The absence of M3 causes a highly reduced substrate turnover, implying a catalytic role for M3. The requirement for the M3-coordinating residue Asp-119 was not only confined to tPphA but a similar phenotype was obtained with the defining member of this family, hPP2Cα. One contradicting report concerns the M. tuberculosis PPM member MtPstP, where the homologue D118A
mutation resulted in no significant alteration in pNPP hydrolysis activity (10). However, the activity of this enzyme toward pNPP ($K_{cat}/K_{m}$, 76 ± 9 M$^{-1}$ s$^{-1}$) was almost as low as the activity of the tPphA variant D119A, suggesting that pNPP may not be a suitable substrate for MtPstP. The reported activity could therefore correspond to the slow background reaction that is observed in the tPphA variant D119A, which occurs in the absence of M3.

Structures at the atomic resolution of the PPM member MspP (from M. smegmatis) in various complexes with phosphate, sulfate, or the phosphate analogue cacodylate gave insight into potential reaction intermediates (7). According to these structures, the oxygens of the substrate phosphate group can directly interact with M1 and M2 by bidentate coordination, rather than indirectly through water-mediated interactions (2). This configuration places the catalytic water between M1 and M2 in a very favorable position for nucleophilic attack of the phosphorus atom. The MspP-cacodylate complex was suggested to mimic the competent enzyme-substrate complex (7). Modeling the cacodylate molecule from the MspP structure into the tPphA structure in such a way that the distance between cacodylate and M1 and M2 remained constant shows that the cacodylate oxygen atoms would replace water molecules coordinated by M1 and M2 in tPphA (Fig. 5, A and B). If a phospho-substrate binds to this position, water molecule 2067 coordinated by M3 (colored in blue in Fig. 5, A and B) is now in a very favorable distance (3.17 Å) to function as a proton donor for the phosphate leaving group. This general acid for catalysis has previously been assumed to be a histidine residue in hPP2Ca. However, this residue is not conserved in bacterial PPM members, and it has been suggested that a water molecule or a functional group from the protein substrate could function as a general acid (7). This work strongly argues in favor of the water molecule 2067 coordinated by M3 to act as proton donor; M3 can act as Lewis acid to facilitate the proton transfer from water 2067 to the phosphate leaving group, as depicted in Fig. 5C. The phenotype of the hPP2Ca variant D146A suggests that
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the proposed mode of reaction may be general for PPM phosphatases, because the M3 coordinating aspartate residue is universally conserved, and mutation to alanine causes a loss of function phenotype, similar to that in tPphA.

The proposed mechanism explains all observed phenotypes of the Asp-119 variants; the variants with Ala or Thr in place of Asp display a very slow substrate turnover. When the M3 is vacant and consequently no Mn$^{2+}$-dependent activation of water molecule 2067 occurs, a water molecule from the solvent could occasionally act as a proton donor, explaining the back-ground reaction with a highly reduced $K_{cat}$ of the D119A variant. When Asp is replaced by Glu, metal analysis suggests that M3 could still bind, however, shifted by the length of a $-\text{CH}_2$ unit (1.54 Å), thus placing the metal in improper reaction distance and resulting in loss of function. However, when Asp is replaced by Asn, M3 could still bind at the same position as in the wild type, albeit with reduced affinity (implied by the elevated $K_m$ for Mn$^{2+}$). When at high Mn$^{2+}$ concentrations the site becomes occupied, the reaction proceeds almost as efficiently as in the wild type. According to the structure of the two tPphA variants D119A and D193A and as shown for the structure of the bacterial PPM member SaSTP (8), binding of M3 affects the conformation of part of the FLAP domain, which is close to the catalytic site. Conversely, conformational changes of this part of the FLAP domain could modulate the binding of M3. If the conformation of the FLAP domain is influenced by substrate binding, the connection between FLAP conformation and M3 binding could provide a functional link between substrate binding and catalytic activity and could contribute to the mechanistic basis of substrate specificity of PPM-catalyzed dephosphorylation reactions.

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