Substrate Binding in Protein-tyrosine Phosphatase-like Inositol Polyphosphatases*

Received for publication, October 19, 2011, and in revised form, November 30, 2011
Published, JBC Papers in Press, December 2, 2011, DOI 10.1074/jbc.M111.309872

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Background: PTP-like inositol polyphosphatases hydrolyze myo-inositol hexakisphosphate via an ordered pathway.

Results: X-ray structures in complex with substrates and fluorescence binding reveal novel features of the kinetic mechanism.

Conclusion: PTP-like inositol polyphosphatases have a two-step binding mechanism that facilitates specificity and catalysis.

Significance: Structural and binding studies are essential for understanding complex kinetic mechanisms.

Protein-tyrosine phosphatase-like inositol polyphosphatases are microbial enzymes that catalyze the stepwise removal of one or more phosphates from high-phosphorylated myo-inositolos via a relatively ordered pathway. To understand the substrate specificity and kinetic mechanism of these enzymes we have determined high resolution, single crystal, x-ray crystallographic structures of inactive Selenomonas ruminantium PhyA in complex with myo-inositol hexa- and pentakisphosphate. These structures provide the first glimpse of a myo-inositol polyphosphatase-ligand complex consistent with its known specificity and reveal novel features of the kinetic mechanism. To complement the structural studies, fluorescent binding assays have been developed and demonstrate that the $K_d$ for this enzyme is several orders of magnitude lower than the $K_m$. Together with rapid kinetics data, these results suggest that the protein tyrosine phosphatase-like inositol polyphosphatases have a two-step, substrate-binding mechanism that facilitates catalysis.

myo-Inositol polyphosphates (IPPs) are ubiquitous in nature and are involved in a number of important cellular signaling events (1). myo-Inositol 1,2,3,4,5,6-hexakisphosphate (phytic acid, InsP$_6$) is the most abundant cellular IPP and plays a central role in numerous essential cellular processes, including DNA repair, RNA processing, mRNA export, plant development, apoptosis, and bacterial pathogenicity (2–7). Inositol polyphosphates (IPPases) that degrade IPPs are found throughout nature and have been identified in prokaryotes, protists, fungi, animals, and plants (8–10). IPPases that are able to hydrolyze InsP$_6$ are generally referred to as phytases. To date, four classes of IPPases have been described that are structurally and mechanistically diverse, including histidine acid phosphatases, β-propeller phytases, purple acid phosphatases, and protein-tyrosine phosphatase-like phytases (PTPLPs) (11–13). Each of the IPPase classes catalyze the stepwise removal of one or more phosphates from IPPs. The products generated during the breakdown of a specific IPP are released from the enzyme and serve as substrate for subsequent cycles of hydrolysis. Although the biological function of some of these enzymes is unclear, they have been found in a wide range of bacteria, including plant and human pathogens (14, 15).

PTPLPs are particularly interesting because the stepwise removal of phosphate from IPP substrates occurs in a relatively specific order. They have a protein-tyrosine phosphatase (PTP) fold, including the P-loop or PTP active-site signature sequence (CXR$_2$R/S/T) and utilize a classical PTP reaction mechanism (12–13). Interestingly, these enzymes display no catalytic activity against classical PTP substrates due to several unique structural features that confer specificity for IPPs (12–13, 15–17). The structural basis of IPP binding to a representative PTPLP (PhyAsr; Selenomonas ruminantium PhyA) has been investigated by x-ray crystallography using the competitive inhibitor, myo-inositol hexasulfate (MIHS) (13). As expected, the inhibitor binds in a deep electropositive cleft where it makes extensive contacts with both the active-site signature sequence (P-loop) and structural determinants that are not present in PTPs. Notably, MIHS binds with the C5 sulfate adjacent to the nucleophilic thiol in contrast to the known specificity of the enzyme for the C3 phosphate of InsP$_6$ suggesting that the binding modes for substrate and inhibitor are distinct.

In this work, we describe high resolution x-ray crystallographic structures of inactive PhyAsr mutants in complex with InsP$_6$ and Ins(1,2,3,5,6)P$_5$. The PhyAsrC252S/InsP$_6$ complex structure is the first IPPase-InsP$_6$ structure that is consistent with the known substrate specificity of the enzyme. The PhyAsrC252A/Ins(1,2,3,5,6)P$_5$ complex structure and previous inhibitor structures (13) suggest that IPP substrates have multiple, overlapping binding sites within the binding pocket of the enzyme. To complement the structural studies, fluorescent binding assays were developed and demonstrate that the $K_d$ for InsP$_6$ is several orders of magnitude lower than the apparent $K_m$. 

* This work was supported by National Science and Engineering Research Council of Canada (NSERC) discovery grants (to H.-J. W., L. B. S., and S. C. M.) and NSERC studentships (to R. J. G., S. D., and L. M. B.).

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‡ This article contains supplemental Fig. S1.

1 The abbreviations used are: IPP, myo-inositol polyphosphate; S-1AF, S-iodoacetamidofluorescein; Ins(1,2,3,5,6)P$_5$, myo-inositol pentakisphosphate; InsP$_6$, myo-inositol hexakisphosphate; IPPase, inositol polyphosphatase; PhyAsr, S. ruminantium IPPase (PhyA); MIHS, myo-inositol hexasulfate; PDB, Protein Data Bank; PTP, protein-tyrosine phosphatase; PTPLP, PTP-like phytase.

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determined from steady-state kinetic studies. These data suggest that the earliest steps of the PhyAsr reaction mechanism involve an initial substrate binding event followed by a slower catalytic step that may require substrate reorientation. This interpretation is supported by preliminary results from a rapid kinetic study of substrate binding which suggest a multiphasic binding involving, at minimum, a rapid initial phase and another slower phase.

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis—The pET28b expression construct (13) containing the full-length *S. ruminantium* ORF (minus the putative signal peptide) was used as the template for all cloning and mutagenesis procedures. The signal peptide sequence was determined using SignalP 3.0 (19). PhyAsr numbering begins with 1 at the N terminus of the protein sequence found in GenBank (AAQ13669) including the predicted 27-residue signal peptide. All mutant proteins (C252S, C252A, H188C, and H188C/C252S) were prepared by site-directed mutagenesis using PCR amplification of the expression plasmid as described previously (20). To verify the identity of the construct and the presence of the desired mutations, all PCR products were sequenced at the University of Calgary Core DNA and Protein services facilities. Sequence data were analyzed using the programs SEQUENCHER 4.0 (Gene Codes Corp.) and MacDNAAsis 3.2 (Hitachi Software Engineering Co.). All of the primers used in this study were purchased from Integrated DNA Technologies.

Protein Production and Purification—Protein expression was carried out in *Escherichia coli* BL21(DE3) cells (Novagen) for 18 h at 310 K. Cells were grown to an *A*600 nm of 0.6–0.8, and protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Induced cells were harvested and resuspended in lysis buffer (20 mM KH₂PO₄ (pH 7.0), 300 mM NaCl, 1 mM β-mercaptoethanol), (15 mM imidazole (pH 8.0)). Cells were lysed by sonication, and cell debris was removed by centrifugation at 20,000 *g*. PhyAsr was purified to homogeneity by metal chelating affinity chromatography (nickel-nitrilotriacetic acid-agarose; Qiagen). Bound protein was washed with lysis buffer and eluted with lysis buffer containing 400 mM imidazole (pH 8.0). PhyAsr was further purified by cation exchange (Macro-Prep High S) and size exclusion chromatography (GE Healthcare, S200). The homogeneity of the purified protein was confirmed by SDS-PAGE (21) and Coomassie Brilliant Blue R-250 staining. Protein (containing an N-terminal His tag) was either used immediately or dialyzed into 20 mM ammonium bicarbonate (pH 8.0) and lyophilized for long term storage.

Crystallization and Ligand Soaking—Crystallization experiments were carried out at room temperature using sitting-drop vapor diffusion with a drop ratio of 2 μl of 20 mg/ml protein solution (in water) to 2 μl of reservoir solution. Crystals were grown in 8–10% PEG 8000, 200–300 mM NaCl, 50 mM potassium acetate (pH 4.8). Ligand solutions were prepared by dissolving Ins₆P₉ (Sigma-Aldrich) in mother liquor to a final concentration of 2 mM. Ligand solutions were added to drops containing crystals and incubated for approximately 1 h prior to flash freezing in a solution containing the crystallization reagents, ligand, and 25% glycerol.

Data Collection, Image Processing, and Structure Refinement—Diffraction data (λ = 1.1159 Å) for both complexes were collected at 100 K on beamline 8.3.1 at the Advanced Light Source. Diffraction images were processed with MOSFLM and scaled with SCALA within the CCP4 program suite. Phases derived from the isomorphous D223N structure (Protein Data Bank (PDB) code 2B4P) (13) were used to solve the structures of the PhyAsr ligand complexes. CNS 1.2 (22) was used to refine the models iteratively using simulated annealing and positional and B-factor refinement followed by manual model building in XFIT (23). The N-terminal His tag and residues 27–32 lack corresponding electron density and were not included in the final model of either structure. Unless indicated otherwise, figures were prepared with PyMOL (24). Initial topology and parameter files for the ligands were generated using the structure of Ins₆P₉ bound to the *E. coli* phytase (PDB code 1DKQ) as a model. For the refinement of the Ins₁,2,3,5,6,P₉ complex, these files were edited to reflect the structures of the ligands. PROCHECK was used throughout refinement to assess the stereochemistry of the model (25). Statistics for the data collection and refinement are presented in Table 1.

Fluorescent Labeling—150 nmol of PhyAsrH188C or PhyAsrH188C/C252S was batch-bound to nickel-nitrilotriacetic acid resin (Qiagen) in binding buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM β-mercaptoethanol), and buffer was exchanged with 4 column volumes of labeling buffer (20

### Table 1

| Parameter | PhyAsrC252S-Ins₆P₉ | PhyAsrC252A-Ins₆P₉ |
|-----------|-------------------|-------------------|
| Space group | P₂₁ | P₂₁ |
| a, b, c (Å) | 46.1, 136.9, 79.9 | 46.0, 137.3, 80.0 |
| β (°) | 102.9 | 103.0 |
| Wavelength (Å) | 1.1159 | 1.1159 |
| Resolution (Å) | 51.1-1.60 | 37.5-1.55 |
| Observed reflections | 560,803 | 456,682 |
| Unique reflections | 120,565 | 126,093 |
| Completeness (%) | 92.9 (77.0) | 89.9 (56.8) |
| Redundancy | 4.6 (4.2) | 3.6 (2.8) |
| Rmerge | 0.083 (0.76) | 0.074 (0.58) |
| | 10.7 (1.5) | 15.5 (2.8) |

### Refinement statistics

- Resolution: 50-1.60 Å, 50-1.55 Å
- No. reflections: 13,394, 11,967
- No. reflections test set: 6,015, 6,346
- Rmerge/Repr (%): 17.0/18.5, 16.4/16.9
- Asymmetric unit: Dimer, Dimer
- Protein atoms: 5,103, 5,304
- Solvent atoms: 932, 886
- Ligand atoms: 80, 72
- Small molecule atoms*: 47, 47
- Wilson B (Å²): 17.9, 17.9
- Average B protein (Å²): 14.6, 14.6
- Average B solvent (Å²): 29.3, 29.3
- Average B ligand (Å²): 23.2, 23.2
- R.m.s.d. bond (Å): 0.013, 0.011
- R.m.s.d. angle (°): 1.66, 1.65

### Additional

- Ramachandran distribution:
  - Most favored (%): 93.1, 92.7
  - Additionally allowed (%): 6.6, 6.9
  - Generously allowed (%): 0.4, 0.4

*: Each structure contains 1 chloride, 4 acetate, and 5 glycerol molecules.

| Parameter | Ins₆P₉ PhyAsrC252A-Ins₆P₉ |
|-----------|---------------------------|
| Rwork (%) | 17.0/18.5, 16.4/16.9 |
| Rfree (%) | 17.0/18.5, 16.4/16.9 |
| Rmerge/Repr (%): 17.0/18.5, 16.4/16.9 |
| Asymmetric unit: Dimer, Dimer |
| Protein atoms: 5,103, 5,304 |
| Solvent atoms: 932, 886 |
| Ligand atoms: 80, 72 |
| Small molecule atoms*: 47, 47 |
| Wilson B (Å²): 17.9, 17.9 |
| Average B protein (Å²): 14.6, 14.6 |
| Average B solvent (Å²): 29.3, 29.3 |
| Average B ligand (Å²): 23.2, 23.2 |
| R.m.s.d. bond (Å): 0.013, 0.011 |
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### Additional

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#### Notes

* Each structure contains 1 chloride, 4 acetate, and 5 glycerol molecules.

* R.m.s.d., root mean square deviation.
mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol). A 20-fold molar excess of 5-iodoacetamidofluorescein (5-IAF) was added to 2 ml of labeling buffer and incubated with the bound protein for 48 h at 310 K with gentle agitation. The bound protein was washed with labeling buffer until the flow-through was colorless, eluted with elution buffer (20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 2.5 M imidazole (pH 8.0)), and subsequently buffer-exchanged into storage buffer (20 mM sodium acetate (pH 5.0), 300 mM NaCl, 20% glycerol). Labeling efficiencies were /H1101170%/H11011.

Binding Studies—Dissociation constants (Kd) were determined by titrating a fixed amount of 5-IAF labeled PhyAsrH188C/C252S with InsP6. Fluorescence measurements were made using a Cary Eclipse fluorometer (Varian). The 5-IAF fluorophore was excited at 480 nm, and the resulting emission between 490 nm and 550 nm was measured in 0.5-nm increments at a rate of 300 nm/min after a 2-min equilibration. Runs were performed in quadruplicate at 293 K after a 2-min equilibration period following injection of titrant. Blank titrations were performed by titrating 5-IAF-labeled PhyAsrH188C/C252S with buffer. Fluorescent data points were obtained by averaging data from fluorescent emissions across 20 nm, centered on the fluorescence emission maxima (517 nm). The change in fluorescence was obtained by subtracting the blank titrations from the InsP6 titrations. The resulting difference data were evaluated using the following equation,

$$\Delta F = 0.5\left(\frac{B_{\text{max}}}{[P]_t} + [L]_t - \frac{K_d + [P]_t + [L]_t - 4[P]_t[L]_t}{1/2}\right)$$

(Eq. 1)

where B_{max} is amplitude, [P]_t is the total concentration of PhyAsrH188C/C252S, [L]_t is the total concentration of InsP6/MIHS, and K_d is the dissociation constant (26).

Phosphate Inhibition—PhyAsr (1 μM) was incubated with 2–50 mM InsP6 in the presence and absence of 1.0 and 10.0 mM inorganic phosphate for 5 min, and the products were separated using a CarboPax-100 column (Dionex) following standard protocols (27).

RESULTS

Structure of PhyAsrH188C/C252S-InsP6 Complex—To examine the interactions between PhyAsr and its substrate, InsP6, we have determined the high resolution structure (1.6 Å) of the inactive C252S mutant (active-site nucleophile) in complex with InsP6 (PDB code 3MMJ). Analysis of 2Fo - Fc and difference electron density maps clearly identify density for InsP6 bound in the active site. MIHS binds with the C2 phosphate in an equatorial position and with the C5 sulfate in the active site. C, divergent stereo view of the interactions between InsP6 and PhyAsrH188C. Residues involved in InsP6 binding are shown as sticks, and dashed lines represent specific hydrogen bonding or electrostatic interactions. The P-loop (yellow) accounts for all backbone contacts with InsP6, whereas the GA loop (cyan) and six additional residues (green) make direct contacts through their side chains.
atoms in the ligand with the exception of the C3 phosphate. To account for this density, we modeled PhyAsrC252S as binding either InsP6 or inorganic phosphate (an impurity in commercial InsP6) with occupancies of 0.75 and 0.25, respectively. Subsequent hydrolysis assays in the presence and absence of inorganic phosphate confirm product inhibition occurs (supplemental Fig. S1) under conditions similar to those used for crystallization and likely accounts for the observed phosphate binding.

In the PhyAsrC252S-InsP6 complex structure, the ligand conformation and orientation are significantly different from previous complex structures with the competitive inhibitor MIHS (13). InsP6 binds to PhyAsrC252S in a chair conformation with the C1 and C3–C6 phosphates in equatorial positions, whereas the MIHS inhibitor was modeled in a chair conformation with only C2 adopting an equatorial position (Fig. 1B). As a consequence of this conformational difference, the InsP6 leaving group oxygen of the scissile phosphate is 0.60 Å closer to the general acid (Asp-223) than in the inhibitor complex (12). This was previously predicted from substrate docking studies that suggested an InsP6 conformation similar to that observed in our complex has the lowest conformational energy and the lowest binding energy and positions the leaving group oxygen for protonation by the general acid (13). Next, InsP6 binds to PhyAsrC252S with the C3 phosphate in the active site (Fig. 1C) in agreement with the known PhyAsr degradation pathway that shows InsP6 dephosphorylation is initiated at and highly specific for the C3 phosphate (13). Finally, we note that InsP6 binding induces strain in the ligand with the inositol ring adopting a partially twisted chair conformation and increasing the C-O-P angle for the active-site phosphate by 27 degrees relative to the other phosphates. The twisted conformation of the ligand maximizes the agreement with the electron density for the ligand and relieves steric interactions between the general acid loop (Asp-223, His-224) and the C2 phosphate. This conformation is stabilized by the extensive contacts made to both the C2 phosphate and the scissile phosphate.

**InsP6 Contacts with PhyAsrC252S**—The binding pocket of PhyAsr is an electropositive cleft that is large enough to accommodate the highly negative InsP6 molecule (Fig. 2A). The C2 phosphate is bound adjacent to C252S and positioned for nucleophilic attack. There are extensive contacts between the C2, C3, and C4 phosphates and the protein at the base of the
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binding pocket and relatively few contacts involving the solvent-exposed C1, C5, and C6 phosphates (Fig. 2). Main-chain contacts between PhyAsrC252S and InsP6 are limited to the scissile C3 phosphate (five contacts) and the adjacent C4 phosphate (one contact). To facilitate the discussion of ligand binding to various IPPs, we will refer to the PhyAsr phosphate binding sites as Ps (scissile phosphate) Pa/Pb/Ph11032 (adjacent to scissile phosphate), Pb/Pb/Ph11032, and Pc (Fig. 2c). Residues that form hydrogen bonds and ion pairs with the substrate are listed in Table 2 and shown schematically in Fig. 2b. Ordered solvent molecules are observed coordinating the phosphates in the Pa, Pb, and Pc sites. Interestingly, most of the residues involved in binding InsPα are also involved in binding MIHS (12) despite the significant conformational differences between ligands. Because there are no significant changes in the main-chain conformation of PhyAsr residues contacting the ligands in the two structures (0.2 Å root mean square deviation for 15 residues), it appears that there are several modes or ways in which the binding pocket can accommodate ligands.

Two notable differences in the binding of InsPα and MIHS involve residues Arg-68 and Gly-257. In the absence of substrate and in the MIHS complex structure, the Arg-68 side chain is directed away from the binding pocket and into the solvent. In the InsPα complex structure, the Arg-68 side chain undergoes a large, induced-fit movement that brings it into contact with the C1 phosphate. This movement is a result of a large rotation about χ1 and χ2 that shifts the Nη1 and Nη2 atoms of the guanidinium group by 8.3 and 9.8 Å, respectively. This movement results in the formation of a 3.1 Å contact with the C1 phosphate and may be important for productive substrate binding. Unlike Arg-68, Gly-257 does not change its conformation in the absence of substrate or in the presence of either ligand. Instead, differences in the ligand binding modes give rise to an additional hydrogen bond between the amine of Gly-257 and the bridging oxygen of the C4 phosphate (Pa site) in the InsP6 complex structure. This hydrogen bond is absent in the inhibitor complex structure and may have a role fixing the orientation of InsP6 relative to the nucleophile.

Structure of PhyAsrC252A Bound to Ins(1,2,3,5,6)P5—In an attempt to obtain a structure of the PhyAsrC252A mutant in complex with InsP6 we unexpectedly observed the binding of Ins(1,2,3,5,6)P5 and inorganic phosphate (Fig. 3A, PDB code 3MOZ). This inositol polyphosphate is a contaminant in commercial InsP6 purified from plant sources and has previously been observed in the structure of a pleckstrin homology domain soaked with InsP6 (28). The smaller alanine side chain enables the inorganic phosphate to bind 0.8 Å deeper within the active site than the scissile phosphate of the InsP6 complex. The binding of inorganic phosphate sterically prevents InsP6 binding while allowing the binding of Ins(1,2,3,5,6)P5. The inorganic phosphate in the active site makes a 2.6-Å hydrogen bond with the C4-hydroxyl of Ins(1,2,3,5,6)P5. The Ins(1,2,3,5,6)P5 ring

FIGURE 3. Binding of Ins(1,2,3,5,6)P5 and inorganic phosphate to PhyAsrC252A. A, divergent stereo view of the interactions between Ins(1,2,3,5,6)P5, inorganic phosphate, and PhyAsrC252A. Residues contacting the substrate are shown as sticks and colored as in Fig. 1c. Electrostatic interactions and H-bonds are shown as dashed lines. B, divergent stereo view of the least squares superposition of the PhyAsrC252A-InsPα (gray) and PhyAsrC252A-Ins(1,2,3,5,6)P5 (red) including the inorganic phosphate bound within the active site. The InsP6 is rotated and translated away from the P-loop to accommodate the inorganic phosphate. C, schematic, two-dimensional representation of the contacts (dashed lines) between PhyAsrC252A and each of the Ins(1,2,3,5,6)P5 phosphates.
adopts a chair conformation similar to the InsP₆ ring without the slight twist. However, the ring of Ins(1,2,3,5,6)P₅ is shifted away from the base of the active site by roughly 1 Å and rotated relative to InsP₆ (Fig. 3B).

PhyAsrC252A contacts with Ins(1,2,3,5,6)P₅ are closely similar to those observed in both the InsP₆ and MIHS complex structures despite the differences in ligand orientation within the active site (Table 2). The C3, C5, C6, and C2 phosphates of Ins(1,2,3,5,6)P₅ contact residues that form the Pₐ, Pₐ’, Pₜ, and Pₜ’ sites, respectively, in the PhyAsrC252S-InsP₆ complex (Fig. 3, B and C). The solvent exposed C1 phosphate fails to make direct contacts with PhyAsrC252A whereas the anion makes 10 contacts at the base of the active site, involving main-chain amines of the P-loop and the side chains of Arg-258 and Thr-259.

Based on the shift in the inorganic phosphate position and the smaller size of the alanine side chain, we investigated the possibility that the inorganic phosphate was positioned similarly to the cysteinyl-phosphate intermediate in PTP1B (29). Superposition of the PhyAsrC252A-Ins(1,2,3,5,6)P₅ complex with the PTP1B phosphoenzyme intermediate (PDB code 1A5Y) reveals only small differences in the position of the phosphates that are likely due to the noncovalent nature of the bound inorganic phosphate in our structure (Fig. 4). The similarities suggest the hypothesis is reasonable and the observed contacts between the phosphate anion and PhyAsrC252A will be analogous to those present in a PhyAsr cysteinyl-phosphate intermediate.

**InsP₆ Binding Studies**—To date, the reported binding affinities of phytases for InsP₆ or any other inositol phosphates have been based on the Michaelis-Menten kinetic parameter \( K_m \). To examine the \( K_m \) of PhyAsr for InsP₆, we developed a fluorescent based assay to measure substrate binding. The binding curves obtained for InsP₆ are characteristic of a tight binding interaction and indicate that the \( K_m \) for InsP₆ binding is comparable with, or lower than, the concentration of protein (1 \( \mu \)M) used in the assay (Fig. 5A) (30). The sensitivity of our current fluorescent based assay precludes us from significantly decreasing the protein concentration. Although we cannot currently obtain an accurate \( K_m \) value, the curve is consistent with PhyAsrC252S having a submicromolar binding affinity for InsP₆. In contrast, \( K_m \) values for InsP₆ are dependent on ionic strength and vary from 1290 ± 240 \( \mu \)M (100 mM ionic strength) to 150 ± 10 \( \mu \)M (500 mM ionic strength) (31). At all ionic strengths, the \( K_m \) for InsP₆ determined from the fluorescence binding assay is 2 or more orders of magnitude less than the \( K_m \). These finding suggest that PhyAsr does not utilize a classical Michaelis-Menten reaction mechanism as assumed in previous works.

**DISCUSSION**

**Substrate Conformation**—This first structure of a PTPLP in complex with InsP₆ has allowed us to identify novel features not observed in the MIHS inhibitor complex. In particular, our PhyAsrC252S-InsP₆ structure is consistent with the known InsP₆ degradation pathway, positions the leaving group oxygen for protonation by Asp-223 (12), and the InsP₆ adopts the energetically preferred ring conformation present under physiolog-
In the PhyAsrC252A·Ins(1,2,3,5,6)P₅ complex structure, the observed phosphate anion contacts resemble those of a cysteinyl-phosphate intermediate, whereas the ligand adopts the same energetically preferred ring conformation observed in the PhyAsrC252S·InsP₆ complex. However, the Ins(1,2,3,5,6)P₅ is shifted away from the active site. This accommodates the bound phosphate anion and relieves the steric and electrostatic interactions that induce strain in InsP₆ (Fig. 2). These observations are consistent with the PhyAsrC252S·InsP₆ complex structure representing interactions prior to catalysis and the PhyAsrC252A·Ins(1,2,3,5,6)P₅ structure representing a postcatalytic complex (Fig. 4). Notably, the bound phosphate anion cannot be released from the active site in the observed structure without prior dissociation of the Ins(1,2,3,5,6)P₅ ligand. This agrees with previous findings that clearly indicate that PhyAsr is nonprocessive and releases all products prior to subsequent catalytic cycles (12).

Phosphoryl Group Binding Sites—We have identified several conserved phosphoryl group binding sites (Pₛ,Pₐ, and Pₐ/H₁₁₀₃₂) near the base of the active site, and several variable, solvent-accessible binding sites (P₄,P₉, and P₉). The Pₛ site is the catalytic center and as such is directly involved in catalysis. Notably, this site is primarily composed of P-loop residues and accounts for virtually all the main-chain interactions with the Ins(1,2,3,5,6)P₅, InsP₆, and MIHS ligands. The Pₐ and Pₐ/H sites (Arg-57, Lys-189, Asp-223, His-224, and Lys-312) are located adjacent to the catalytic site and appear to function as anchor points that ensure the scissile phosphate is correctly oriented for nucleophilic attack. The functions of the variable sites are less clear, but they may be important for binding and discriminating between the lower phosphorylated IPPs that are generated during the degradation of InsP₆.

The catalytic properties of several PTPLPs have recently been described (12, 16–18), and these enzymes can be separated into high activity and low activity groups. Interestingly, all of the binding residues identified in PhyAsr are conserved in all other high activity PTPLPs whereas the low activity PTPLPs have conservative and nonconservative mutations or deletions at several of these sites. The conservation of these binding residues also appears to play a role in defining substrate specificity because two of the enzymes with mutations to binding residue have significantly altered substrate specificity (16–17, 35).

In each complex structure, residues that form the conserved Pₛ,Pₐ, and Pₐ/H subsites (Arg-57, Lys-189, Asp-223, His-224, and Lys-312, and the P-loop) account for the majority of the contacts to the ligand. In contrast, residues that compose the variable sub-sites (P₄,P₉, and P₉) make relatively few contacts with solvent-exposed phosphoryl groups. Further, there are differences in both the number and nature of the contacts. With the exception of the remote standby site identified by Chu et al. (13) the same residues are involved in binding the distinctive Ins(1,2,3,5,6)P₅, InsP₆, and MIHS inhibitor conformations. The Pₛ,Pₐ, and variable sub-sites are almost exclusively composed of side chain atoms. Consequently, differences of several angstroms in phosphoryl group position can be accommodated by the movement of side-chain atoms without significant main-chain movements.
Arg-68 adopts multiple conformations in the InsP$_6$ complex structure and apparently undergoes a large induced fit movement upon substrate binding and makes a specific contact with the C1 phosphate of InsP$_6$. While this residue is not interacting with Ins(1,2,3,5,6)P$_5$ substrate it is observed in a closed conformation. In contrast, this residue adopts an open conformation in all PhyAsr structures lacking substrate. Although Arg-68 is not strictly conserved in known PTPLPs, it may participate in substrate binding prior to catalysis, either guiding or orienting the ligand within the PhyAsr active site.

Mechanistic Insight into PhyAsr Catalysis—The $K_d$ values for InsP$_6$ binding (this study) are at least 2 orders of magnitude lower than previously reported $K_m$ values under similar conditions (12, 31). There are several potential explanations for the large, reported $K_m$ values. Based on our structural analysis, binding studies, and the findings of Puhl et al. (12) we suggest that a two-step binding mechanism accounts for the discrepancy. Preliminary rapid kinetics data (Fig. 5, B and C) suggest that binding involves a rapid initial binding of the substrate followed by a slower step. Structural studies clearly indicate that PhyAsr binds substrate or inhibitor in at least three distinct conformations within the electropositive binding pocket. Further, only one conformation (InsP$_6$ complex) is consistent with catalytic competence. We speculate that the rapid initial binding phase represents substrate association with the electropositive binding pocket in one of several conformations followed by a slower phase that results in the substrate adopting a catalytically competent conformation.

To our knowledge, there have been no studies examining the binding of InsP$_6$ to phytases; however, the $K_d$ for the binding of the competitive inhibitor MIHS ($K_i$) has been determined for both PhyA and PhyB from *Aspergillus fumigatus* (36) and PhyA from *Aspergillus niger* (37). Interestingly, for each of these enzymes their $K_i$ is at least 1 order of magnitude smaller than their $K_m$ for InsP$_6$. These phytases belong to the histidine acid phosphatase family of phytase and are structurally similar to the *E. coli* phytase. It is not known whether the $K_d$ for the substrate, InsP$_6$, is significantly lower than the $K_m$ for these enzymes. Interestingly, the only other phytate-degrading enzyme that has been structurally characterized in complex with InsP$_6$ is the *E. coli* phytase (PDB code 1DKQ) (14). This enzyme hydrolyzes the C6 phosphate preferentially but was observed to bind the C3 phosphate within the catalytic site. This result suggests that *E. coli* phytase can bind InsP$_6$ in multiple conformations as observed for the PTPLP, PhyAsr. Similar to PhyAsr, the binding pocket of this enzyme is a highly basic cleft that makes extensive contacts with the phosphoryl groups near the catalytic site at the base of pocket.

The discrepancy between the binding affinity of MIHS and the $K_m$ of InsP$_6$ in histidine acid phosphatases, the similarities in substrate binding by PhyAsr and the *E. coli* phytase, and the finding that the *E. coli* phytase can bind InsP$_6$ in multiple conformations, all suggest that histidine acid phosphatases and PTPLPs may both utilize a similar binding mechanism. This mechanism likely involves a rapid initial substrate binding followed by a slower reorientation and may be a general feature of all IPPases.

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