A structural basis for lithium and substrate binding of an inositol phosphatase

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Edited by Wolfgang Peti

Inositol phosphate (IP) signaling is critically important to cellular communication networks. Among the numerous lipid and soluble IP messengers, inositol 1,4,5-trisphosphate (IP3) is crucial for mediating the agonist-induced release of intracellular calcium stores (1, 2). Termination of IP3 signaling occurs, in part, through the action of IP kinases and phosphatases, the latter of which also function to replenish stores of inositol—metabolism reviewed in (3, 4). Links of inositol signaling to the pharmacology of lithium emerged when it was first reported that treatment of rats with lithium resulted in decreased brain inositol levels and led to the accumulation of inositol monophosphates and polyphosphates (5). Two IP phosphatase activities, inositol monophosphate phosphatase (IMPA1, IMPA2) and inositol polyphosphate 1-phosphatase (INPP1) were reported to be potently inhibited by lithium (6–8). Cloning and characterization of IMPA1 and INPP1 gene products confirmed they encoded metal-dependent lithium-inhibited enzymes with minimal sequence homology aside from a short six amino acid motif (9, 10).

Structural studies of IMPA1 and INPP1 illuminated that the “DPIDxT” six-amino acid motif anchor metal binding sites likely involved catalysis (11, 12). Remarkably, superimposition of alpha-carbons of these six residues of IMPA1 and INPP1 aligned 13 secondary structural elements of a 280 amino acid common core fold. Sequence comparison based on this three-dimensional alignment indicates they are prototype members of a small family of structurally conserved phosphatases, whose activities were not only limited to inositol signaling but also include regulation of gluconeogenesis and nucleotide metabolism (13). Upon completion of the human and mouse genome sequences, we now know that the family comprised of seven genes including IMPA1 and its ortholog IMPA2; INPP1; FBPI and FBP2—fructose 1,6-bisphosphate phosphatas; BPNT1—adenosine 3’5’ biphosphate nucleotidase; and gPAPP—Golgi-resident 3’ phosphoadenosine 5’ phosphatase (14, 15). Of interest, all seven gene products are potently inhibited in vitro at or below therapeutic lithium concentrations (9, 10, 14–17).

Detailed comparison of the structures of four members of the family suggests a common metal-dependent catalytic mechanism (11, 12, 18, 19). Conserved secondary structural elements comprise juxtaposed metal binding pockets and define a shared sequence pattern of D9E10E11...E167 as shown for INPP1 (Fig. 1). Despite several reports that have defined the position of activating metal sites used for catalysis by this protein family (20–23), understanding the mechanism of uncompetitive lithium inhibition has been challenging. Nonetheless, studies for three family members, IMPA1, yeast 3’-nucleotidase (Hal2p), and FBPI, have provided insights into catalytic metal orientation and effects of lithium on these configurations (12, 18, 20, 24–26). Other data include fluorescence quenching experiments in IMPA1 (27) and inference from coordination geometries of Li+ modeled into the active site of IMPA1 (21). In opposition to those data, support for Li+ inhibition at metal site 3 (MG3) include structural studies of the archaeal IMPA1/FBPIs implicating conformational changes associated in a loop region responsible for forming metal site 3 (28, 29). These...
The activity of INPP1 is cooperative with Mg$^{2+}$ having a Hill co-efficient of 1.9 (7), supporting the idea that substrate binding alters Mg$^{2+}$ affinity at additional metal sites. We tested the effects of substrate addition by co-crystallization of INPP1 with a 10X molar excess of Ins(1,3,4)P$_3$ substrate and 2 mM inhibitory Gd$^{3+}$. Although we could not confidently model the substrate in the resultant structure, metal sites GD1 and GD3 became equivalently occupied (Fig. 2D). Difference Fourier analysis showed densities peaks of 20.5σ and 20.0σ in metal sites GD1 and GD3, respectively, giving a relative occupancy of 1.0. Consistent with the Fo-Fc differences, anomalous differences peaks of 15.4σ and 10.5σ were calculated, giving a relative occupancy of 2.4 (Fig. 2C). In the absence of substrate, both the Gd$^{3+}$ and Gd$^{3+}$/Li$^+$ structures had gadolinium atoms that were poorly ordered in metal site GD3, refined to only half occupancy and had B-factors more than two-fold higher than the global average B-factor. This indicates in the absence of substrate or product, the addition of Li$^+$ did not significantly affect the ratio of Gd$^{3+}$ at sites GD1 and GD3, with metal binding strongly preferred at site GD1.

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Lithium displaces metal in INPP1 at site GD3 in the presence of substrate

In the absence of substrate, the addition of lithium did not eliminate Gd$^{3+}$ metal binding in INPP1. These data are consistent with an uncompetitive pattern of Li$^+$ inhibition that predicts that substrate or product must be bound in order for Li$^+$ inhibition to occur. We therefore tested the effect lithium addition in the presence of substrate. INPP1 was co-crystallized in the presence of 10X molar excess of Ins(1,3,4)P$_3$ and 2 mM Gd$^{3+}$, but also 200 mM Li$^+$. As seen in Figure 2F, in the presence of substrate, no electron density peak was observed for Gd$^{3+}$ at metal site GD3 with little change in density observed at metal site GD1. A 15.4$\sigma$ difference density peak was found for metal site GD1, with no detectable peak for metal site GD3 indicating lithium displaces metal at site GD3 and that binding requires the presence of substrate. Interestingly, a 4.9$\sigma$ difference density peak was found for gadolinium binding at GD2, the equivalent of MG2, indicating poorly ordered Gd$^{3+}$ in that site (Fig. 2F). This provides further evidence Li$^+$ does not displace metals at either sites GD1 or GD2.

The active site metals of the lithium-inhibited phosphomonoesterase protein family

The native INPP1 structure was re-refined and agrees well with the original structure with an all-atom root mean square deviation of 0.25 Å. Importantly, the structural core and active site, including Mg$^{2+}$ in metals sites MG1 and MG2, are essentially identical. In conjunction with the four INPP1-Gd$^{3+}$ complexes presented here, at least three metal binding sites are seen in the active site of INPP1. Metal site MG1/GD1 has been observed in all structures, metal site MG2/GD2 has been observed in the Mg$^{2+}$/Li$^+$ and Gd$^{3+}$/Li$^+$/Ins(1,3,4)P$_3$ structures, and the inhibitory metal site GD3 has been observed in the Gd$^{3+}$/Gd$^{3+}$/Li$^+$, Gd$^{3+}$/Ins(1,3,4)P$_3$ structures.

The INPP1 GD3 metal site is mediated by two protein–metal contacts to residues D54 and E80. In other family members, there appears to be only one protein-mediated contact to the activating metal site MG3. The other five coordinating ligands of MG3 are water molecules giving this site a unique plasticity that could accommodate different coordination geometries (Fig. 3A) (21). The lithium sensitive GD3 binding site corresponds to one of the five waters that coordinate the MG3 metal site (Fig. 3A). The GD3/coordinating water site is also in close proximity to a lysine residue in IMPA1 that when mutated dramatically reduces lithium sensitivity and renders the enzyme resistant to Mg$^{2+}$ inhibition at high concentration (30), further suggesting a role for this site in lithium inhibition.

**Figure 2.** Difference Fourier analysis of INPP1 complexes with gadolinium and/or lithium in the presence and absence of substrate. Stereodiagrams of INPP1 active site metals are shown for four separate experimental conditions. The protein component of the INPP1 active site is depicted as a stick diagram. Active site gadolinium metals are shown as spheres with the following numbering: GD1 (orange), GD2 (purple), and GD3 (blue). For each experimental condition, Fo–Fc difference density (green mesh, panels A, B, D, F) was calculated using phases from the protein component of native INPP1. Anomalous Fo–Fc difference density (Magenta mesh, panels C and E) was calculated in a similar manner. The ratio of individual Fo–Fc peak heights for each metal site (GD3:GD2:GD1) is listed. Metal sites with no detectable peak are listed as ND. Structures of INPP1 complexes were solved under the following conditions: (A) Gd$^{3+}$/Li$^+$; (B–C) Gd$^{3+}$; (D–E) Gd$^{3+}$/Ins(1,3,4)P$_3$; and (F) Gd$^{3+}$/Ins(1,3,4)P$_3$/Li$^+$. GD1, first gadolinium site; INPP1, inositol polyphosphate 1-phosphatase; MG1, magnesium metal site 1.
A lithium binding motif

An overlay of the INPP-metal complexes with structurally related lithium-inhibited phosphomonoesterase proteins with one or more active site metals reveals that their active site metals cluster around defined sites (Fig. 3B) (11, 12, 18, 21–26, 28, 29, 32–36). The positions of metal sites corresponding to INPP1 MG1/GD1 and MG2/GD2 are well conserved with an average pairwise distance for 26 and 27 metal atoms of 0.8 Å and 0.7 Å, respectively, and represent two of three activating metal sites. In contrast, the lithium-sensitive and inhibitory gadolinium site (MG3) (Fig. 3B) has not yet been observed in INPP1. Members of this family conserve a third activating metal site (MG3) (Fig. 3B). Notably, this site is distinct from the Li/GD3 binding site observed in the INPP1 structures, and its position away from putative water nucleophile may explain the inhibitory effects these metals exert.

Lithium inhibition studies of INPP1 wild-type versus D54A mutant

Based on our crystallographic studies, we found that the lithium-sensitive, inhibitory metal binding site was coordinated by the carboxylates of two conserved residues, D54 and E80. Residue D54 was mutated to alanine, and we kinetically examined the effect of Li+ inhibition upon the wild-type and mutant enzymes. Mutant INPP1D54A exhibited a 4300-fold inactivation relative to the wild-type enzyme with a Vmax of 13.9 nmol/min/mg compared with 59.7 μmol/min/mg for the wild-type enzyme (Fig. 4A) (31). While a large change was observed for Vmax values, the substrate affinity appeared to be relatively unaltered with Km values of 11 μM to 31 μM for the D54A mutant and wild-type enzymes (Fig. 4A) (31). Although the D54A mutation does not significantly alter Km, there was a large effect on Kt. Figure 4, B–C show linear plots of 1/v versus [Li+] for the hydrolysis of Ins(1,3,4)P3 by the D54A mutant and wild-type enzyme. As seen in Figure 4B, there is a 100-fold decrease in Li+ sensitivity for the D54A mutant compared with the wild-type INPP1 (Fig. 4C). The results from the kinetic studies corroborate our structural studies that the site of Li+ inhibition in INPP1 is located at metal site GD3.

Figure 3. Comparison of active sites from metal-dependent, lithium-inhibited phosphomonoesterases. A, stereo-diagram of the superimposed active sites of a high-resolution IMPA1-Mg2+ complex (PDB ID 2BJI) and INPP1-Gd3+/Ins(1,3,4)P3 complex. The protein component of the INPP1 (gray) and IMPA1 (green, numbering in parenthesis) complexes are shown as sticks. Metals atoms are shown as large spheres for the MG1/GD1 (orange), MG2/GD2 (purple), MG3 (cyan), and GD3 (blue) sites. Water molecules, shown as small red spheres, are taken from the IMPA1 structure. Red dashed lines depict the coordination of the activating Mg2+ metals of IMPA1. The small yellow sphere represents a water atom that coordinates metal site MG3 in the IMPA1 structure. This water also corresponds to lithium binding site (GD3) in the INPP1 complexes. A sulfate ion from the INPP1 complex (1G0H, 1G0I, 1FPI, 1FPJ, 1FKK, 1CNQ, 1EYI, 1EYJ, 1EYK), FBP-IMP: archael hybrid fructose and inositol phosphatase (PDBs = 1LBX,1DK4), IMP: inositol monophosphatase (PDBs =P2BJ, 28, 29, 32).

Figure 4. Lithium inhibition of the INPP1 and INPP1D54A lithium binding site mutant. A, specific activity for the INPP1D54A catalyzed hydrolysis of Ins(1,3,4)P3. Enzyme velocity (nmol/min/mg) is plotted as a function of substrate concentration ([Ins(1,3,4)P3]). The Vmax and Km of the reaction are indicated. B–C, dixon plots of 1/v versus [Li+] for the hydrolysis of Ins(1,3,4)P3 by the INPP1D54A mutant and INPP1 wild-type enzyme. Respective Kfi values are indicated. INPP1, inositol monophosphatase 1-phosphatase; INPP1, inositol polyphosphate 1-phosphatase; MG1, magnesium metal site 1.
Active site sulfate/phosphates found in INPP1

Two sulfate and/or phosphate ions were found in the active sites of the INPP1 structures. In the Gd$^{3+}$ and Gd$^{3+}$/Li$^+$ structures, the sulfates originated presumably as the Gd$^{3+}$ counter-ion from the Gd$_2$(SO$_4$)$_3$ used in the crystallization conditions. In the Gd$^{3+}$/Ins(1,3,4)P$_3$ and Gd$^{3+}$/Li$^+$/Ins(1,3,4)P$_3$ structures, the ion could either be a phosphate from the Ins(1,3,4)P$_3$ substrate molecule or a sulfate from the Gd$_2$(SO$_4$)$_3$ in the crystallization solutions. We were unable to distinguish these possibilities. However, as seen in Figure 3A, a sulfate (or phosphate) in the INPP1 structures correlates well with the 1-position phosphate of Ins(1)P$_1$ observed in the IMPA1/Ins(1)P$_1$ complex (25), and the inositol ring from this structure is omitted for clarity. The second sulfate is located near the 4-position of the inositol ring from Ins(1)P$_1$ and was therefore labeled 4'SO$_4$. While we did not observe the Ins(1,3,4)P$_3$ substrate, we hypothesize the sulfates and/or phosphates seen in the INPP1 structures represent the 1- and 4-PO$_4$ positions that would be found with ordered substrate or reaction product.

Structure determination INPP1$^{D54A}$ metal and substrate complexes

We next attempted to directly examine substrate binding by INPP1. As mutation of D54 results in loss of INPP1 activity without significantly changing the substrate affinity, we hypothesize that the D54A mutant can be utilized to trap the substrate in INPP1. To rule out the possibility of unanticipated conformational changes, we first determined the structure of INPP1$^{D54A}$ in the presence of calcium. One calcium ion at metal site 1 is found in the INPP1$^{D54A}$/Ca$^{2+}$ structure, alongside two sulfate ions similar to those found in Gd$^{3+}$ structures. The INPP1$^{D54A}$/Ca$^{2+}$ structure reveals that D54A mutation does not cause obvious conformational changes, exhibiting an root mean square deviation of 0.6 Å between main-chains of the mutant and the wild-type protein.

We next soaked the INPP1$^{D54A}$/Ca$^{2+}$ crystals with the Ins(1,4)P$_2$ substrate. Indeed, the D54A mutant led to structure determination of the substrate bound INPP1$^{D54A}$/Ca$^{2+}$/Ins(1,4)P$_2$ complex. Two calcium ions (CA1 and CA2) are found at metal sites 1 and 2. We did not observe any metal binding at MG3/GD3 in the D54A mutant. The mFo-DFc omit map of the Ins(1,4)P$_2$ substrate exhibits continuous electron density that agrees with the shape of Ins(1,4)P$_2$ in the active site of the enzyme (Fig. 5). Substrate binding is largely contributed by multiple main-chain amide groups involving E269, G290, and A291. Furthermore, binding of Ins(1,4)P$_2$ is reinforced by interactions between the inositol ring and surrounding residues. Together, the INPP1$^{D54A}$/Ca$^{2+}$/Ins(1,4)P$_2$ complex structure reveals the molecular basis for substrate recognition by INPP1.

Discussion

In this report, we provide evidence for how lithium binds to and inhibits the inositide phosphatase INPP1, a prototype member of a family of structurally conserved family of enzymes whose activities are potently inhibited by lithium. Using X-ray crystallographic analysis of several INPP1 complexes in the presence and absence of substrate and/or lithium, we define structural bases for (1) the role of a conserved sequence motif responsible for lithium’s effect; (2) lithium’s uncompetitive pattern of inhibition; and (3) Ins(1,4)P$_2$ substrate binding. Our systematic approach provides snapshots of how substrate and/or product influence metal binding as well as outlining critical residues that mediate the effects. Overall, we summarize our model using the schematic (Fig. 6A). INPP1 catalyzes the metal-dependent hydrolysis of the phosphomonoester bond from the 1 position phosphate of Ins(1,4)P$_2$ or Ins(1,3,4)P$_3$ to yield inositol 4-phosphate and inositol 3,4-bisphosphate, respectively (6, 7, 37, 38). Consistent with other family members for which a detailed kinetic mechanism has been described (21–23), we postulate a nucleophilic water coordinated by Mg$^{2+}$ in metal sites 2 and 3 is activated for an ionic attack on the labile phosphate by a conserved threonine residue, T158. Metal site 2 is coordinated by three protein-mediated contacts to residues E79, D153, and I155. Metal site 3 is coordinated by two protein-mediated contacts, D54 and E80. Three conserved aspartic acids, D153, D156, and D317, compose metal site 1, from which Mg$^{2+}$ coordinates the ester oxygen and helps to stabilize the developing negative charge, as the phosphate ester is cleaved.

We specifically chose to use Gd$^{3+}$, an electron dense lanthanide, as a proxy for catalytically required Mg$^{2+}$. Despite gadolinium’s coordination ligand number of 6 to 9, as compared with 4 to 6 for magnesium, we observed that the
A lithium binding motif

![Diagram of INPP1 active site with metal binding](Image)

**Figure 6. Mechanistic model for the metal-dependent hydrolysis of phosphomonoester bonds by INPP1.** A, three metal sites in the active site of INPP1 are shown as hollow spheres labeled 1, 2, and 3—derived from various Mg^{2+}, Ca^{2+}, and Gd^{3+} structural data. Mg^{2+} in metal sites 2 and 3 coordinate a nucleophilic water that we postulate is activated by threonine residue 158 for an inline attack on 1'PO₄ of Ins(1,4)P₂. Metal site 2 is coordinated by three protein-mediated contacts to residues E79, D153, and carbonyl oxygen of I155. Metal site 3 is coordinated by two protein-mediated contacts, D54 and E80. Three conserved aspartic acids, D153, D156 (omitted for clarity), and D317, compose metal site 1, from which Mg^{2+} coordinates the ester oxygen and helps to stabilize the developing negative charge, as the phosphate ester is cleaved. B, uncompetitive lithium inhibition of the INPP1 reaction may occur through its binding at metal site 3, possibly interfering with nucleophilic attack by water or through altering substrate reloading/binding. INPP1, inositol polyphosphate 1-phosphatase.

positioning of three Gd^{3+} sites identified are similar to those observed or proposed for Mg^{2+}. In the absence of substrate, co-crystallization of INPP1 with Gd^{3+}, a competitive inhibitor of Mg^{2+}, resulted in preferred metal binding to site GD1 to GD3. Addition of Li^{+} in the absence of substrate did not significantly affect the ratio of Gd^{3+} at these sites. Co-crystallization with Gd^{3+} but in the presence of substrate (I(1,3,4)P₃) resulted in equivalent occupancies of metal sites GD1 and GD3 consistent with the hypothesis that substrate binding alters affinity at metal site GD3 by contributing an additional coordinating ligand through phosphate oxygen. The observation occupancy at a second/third site is also consistent with previous kinetic studies which demonstrated metal binding in INPP1 is cooperative with a Hill co-efficient of 1.9 which predicts if metal site 1 is predominantly bound in the absence of substrate, that an additional two metals are bound cooperatively upon substrate binding. It is noteworthy that the addition of substrate resulted in a significant change in the ratio and intensity of electron density peaks such that metal sites equivalent occupancies of sites GD1 and GD3, each having a tremendous signal exceeding 20 sigma. Anomalous signals confirm this is because of metal binding.

An important mechanistic insight gleaned from our studies relates to the constitutive occupancy of metal at site 1. Regardless of experimental condition, the INPP1 metal site MG1/GD1 is found fully occupied and is accompanied by a strong, relatively unchanged, electron density peak. This is in stark contrast to what is observed at metal site GD3, which undergoes dramatic changes in the electron density signal depending on the addition of substrate and lithium. Furthermore, mechanistically, MG1/GD1 metal binding does not appear cooperative with respect to substrate binding nor is susceptible to displacement by lithium under any of the conditions examined. Our conclusions of a three-metal–assisted catalytic mechanism for INPP1 are consistent with proposed models for several other members of the Mg^{2+}-dependent/Li+-sensitive phosphoric monoester hydrolase family, including IMPA1 (20, 21), FBP1 (23), and FBP1/IMPA1 (22). Our work supports an evolutionary conservation of the enzymology of this important family.

The uncompetitive pattern inhibition of INPP1 by Li^{+} predicts that substrate or product must be bound in order for Li^{+} inhibition to occur. As discussed earlier, addition of Li^{+} alone had minimal observable effect on the electron density observed for Gd^{3+} at any of the three sites. In dramatic contrast, co-crystallization of INPP1 with Gd^{3+}, I(1,3,4)P₃ and Li^{+} resulted in a stunning complete loss of electron density at metal site GD3 (no observable density even at below 1 sigma) with little change in density at site GD1 (remained above 20 sigma). Because we did not observe significant changes in the side-chain geometry for residues that coordinate the metal binding sites, we infer the parsimonious explanation is lithium replaces gadolinium at metal site 3. A partially ordered Gd^{3+} was found in metal site GD2 in the substrate-bound, lithium-treated structure and importantly excludes this metal site as the lithium-binding site. This hypothesis is supported by our kinetic analysis of the INPP1D54A mutant protein which demonstrated a 100-fold reduction in the potency of lithium inhibition (K₈ went from 1 mM to over 100 mM with no significant change in Kₘ).

Given the systematic structural approach utilized, our work helps to unambiguously define a basis for lithium’s uncompetitive pattern of inhibition through its effects at metal site 3 (Fig. 6B). It is important to note, studies of IMPA1 indicate potential differences for the precise site at which lithium acts among family members (21). It is possible that given the high concentrations of metals used in different studies may account for subtle differences, for example, the inhibitory metals gadolinium and lithium bound in an INPP1 site that corresponds to a water molecule used for the coordination of MG3 (Fig. 3B) (21). Therefore, while some support exists for lithium
inhibition at MG1 (12, 18, 21, 24, 27), our data are most consistent with lithium binding disrupting the coordination of metal site MG3 (GD3). The determination that lithium binds to a site responsible for the coordination of MG3 is corroborated by several previous studies. Crystal structures of *Archaeal IMPA1/FBP1s* show that the conformation and length of a loop region containing residues that help form metal sites GD3 and MG3 directly correlates to the sensitivity to Li⁺ inhibition (28, 29). Further, mutagenesis of a conserved IMPA1 lysine residue in close proximity to this site results in reduced sensitivity to lithium inhibition and resistance to inhibition at high Mg²⁺ concentrations (30). Because these observations support our findings and given the remarkable conservation of their active sites, the results presented here may extend to other family members.

**Experimental procedures**

**Protein expression, purification, and crystallization**

Recombinant INPP1 was prepared as previously described (31). Briefly, cDNA encoding bovine INPP1 was transfected into Sf9 insect cells via Baculovirus infection and grown until enzyme levels were >25 mg/l of medium. Over-expressed protein was concentrated, dialyzed, and sequentially passed over *diethylaminoethyl cellulose* and phenyl HPLC columns and was purified to homogeneity as judged by SDS-PAGE analysis. The INPP1D54A mutant used for the kinetic and structural studies was over-expressed and purified to homogeneity as described for wild type.

INPP1 tetragonal-shaped crystals amenable to diffraction studies were grown by the hanging drop vapor diffusion method on silanized glass coverslips at 20 °C and grew with dimensions routinely exceeding 0.1 × 0.1 × 0.5 mm. Gd³⁺, a competitive inhibitor of Mg²⁺, has a useful anomalous absorption edge at the CuKa wavelength and therefore was used in the co-crystallization or soaking conditions. Four separate conditions were used to produce crystals in the course of these studies. In each case, purified recombinant bovine INPP1 from pooled Phenyl HPLC fractions was concentrated in a *Centricon-30* concentrator, then exchanged with buffer 50 mM Tris HCl (pH 6.5) before the use of Rfree test data sets (43). We therefore sought to re-examine the INPP1 model in context of the current studies by taking advantage of a Rfree test set and modern structure refinement software. The original reflection file for the native INPP1 (11) was converted to mtz format and assigned a conserved Rfree set using the program f2mtz (41).

INPP1D54A and substrate complex diffraction data were collected on an R-Axis IIc image plate detector using monochromatic Cu Kα X-rays (λ = 1.5418 Å) at 4 °C to prevent crystal decay and were processed using DENZO and SCALEPACK (42).

**Re-refinement of the native INPP1 structure**

The native INPP1 structure (11NP) was solved and refined before the use of Rfree test data sets (43). We therefore sought to re-examine the INPP1 model in context of the current studies by taking advantage of a Rfree test set and modern structure refinement software. The original reflection file for the native INPP1 (11) was converted to mtz format and assigned a conserved Rfree set for re-refinement. The Rfree set was taken an INPP1–IP2 complex that crystallized in the same space group and unit cell dimensions. The native INPP1 data with conserved Rfree set were solved by molecular replacement with Phaser (44) using the protein component of the INPP1–IP2 complex. Model building was completed with iterative rounds of building in Coot (45) using difference Fourier maps.
and automated refinement with an MLF target in Refmac5 (46), including isotropic B-factor and TLS refinement (47).

**Structure determination and refinement of INPP1 and INPP1D54A complexes**

The structures of INPP1 co-crystallized or soaked in the presence of Gd\(^{3+}\), Gd\(^{3+}/Li^+\), Gd\(^{3+}/Ins(1,3,4)P_3\), and Gd\(^{3+}/Li^+\)/Ins(1,3,4)P\(_3\) were solved by direct Fourier transform in Refmac (46) using phases derived from the protein component of the re-refined native INPP1 structure. Each structure refined independently against data collected to 3.00 Å, 2.50 Å, 2.80 Å, and 3.20 Å, respectively. Model building was completed with iterative rounds of building in Coot (45) using difference Fourier maps and automated refinement with an MLF target with Refmac5 (46), including isotropic B-factor refinement for the Gd\(^{3+}/Li^+\) data and overall B-factor refinement for the Gd\(^{3+}\), Gd\(^{3+}/Ins(1,3,4)P_3\), and Gd\(^{3+}/Li^+\)/Ins(1,3,4)P\(_3\) data. Anomalous difference maps for the Gd\(^{3+}\) and Gd\(^{3+}/Ins(1,3,4)P_3\) data were calculated in fast fourier transform (41) using phases derived from the INPP1D54A/Ca\(^{2+}\) structure. Structure validation for all structures was performed using MolProbity (48). Molecular graphics and structural alignments were created with PyMol (http://www.pymol.org), Data collection and refinement statistics are given in Table 1.

The structures of INPP1D54A/Ca\(^{2+}\) and INPP1D54A/Ca\(^{2+}\)/Ins(1,4)P\(_2\) were solved by direct Fourier transform in Refmac (46) using phases derived from the protein component of the re-refined native INPP1 structure. Difference Fourier map with (|Fo|−|Fc|)\(_{\text{calc}}\) coefficients was then calculated to check the electron density around the side-chain of carboxyl group of D54. The negative density around the carboxyl group of D54 confirmed the nature of the mutation. For the INPP1D54A/Ca\(^{2+}\)/Ins(1,4)P\(_2\) structure, programs from the CCP4 suite (41) were used to calculate sigma-weighted difference Fourier maps with phases derived from the INPP1D54A/Ca\(^{2+}\) structure. Substrate Ins(1,4)P\(_2\) was initially modeled to the positive density using Turbo/Frodo program. Model building was completed with iterative rounds of building in Coot (45). Refinement of INPP1D54A/Ca\(^{2+}\) and INPP1D54A/Ca\(^{2+}\)/Ins(1,4)P\(_2\) was carried out in both reciprocal space and real space using Phenix (49) with secondary structure restraints. Data collection and refinement statistics are given in Table 2.

**Lithium inhibition of the INPP1-PDP1-D54A mutant**

Li\(^+\) inhibition of the D54A mutant INPP1 was determined as follows: 25 μl reactions containing 2.4 μg of enzyme, 40 μM (4 × Km) Ins(1,3,4)P\(_3\), ~2000 cpm of \(^3\)H-Ins(1,3,4)P\(_3\), 50 mM HEPES, pH 7.5, 1 mM EGTA, 3 mM MgCl\(_2\), and 0 to 100 mM LiCl (to a total of 100 mM with KCl, to keep ionic strength constant) were incubated at 37 °C for 15 min, then diluted with 0.5 ml of 0.35 M NH\(_4\)COOH, 0.01 M COOH (IP3 formate) to stop the reactions. Stopped reaction mixtures were divided into two fractions and passed over 200 μl AG 1-X8 (200–400 mesh, formate form) anion exchange columns (Bio-Rad). The columns were each washed with 3 ml IP3 fluid (Tru-Lab). Vials were counted in a Packard 1600 TR liquid scintillation analyzer. Percent hydrolysis of the substrate was 20 to 40% for each of the reactions. Nonradioactive Ins(1,3,4)P\(_3\) was purchased from Matreya, and \(^3\)H-Ins(1,3,4)P\(_3\) was prepared by digesting 1 μCi of \(^3\)H-Ins(1,3,4,5)P\(_4\) with ~100 ng of type I 5-phosphatase for 1 h.
at 37 °C, stopping the reaction at 100 °C for 10 min, then centrifuging the mixture to clear the 5-phosphatase. An aliquot of the supernatant was run on an anion exchange HPLC column to confirm complete digestion of the substrate. The V_max and K_m values for the D54A INPP1 mutant were determined by similar kinetic studies using 0 to 50 μM Ins(1,3,4)P_3 in the absence of Li^+.

All kinetic data were evaluated using the program Prism (GraphPad Software, Inc). V_max and K_m values for the D54A mutant were determined by fitting kinetic data by nonlinear regression to the one site binding hyperbola equation Y = V_max×X/(K_M + X) with an r^2 value = 0.99 and the kinetic parameters determined. Data for Li^+ inhibition for the D54A mutant and wild-type enzyme were fit by linear regression with r^2 values of 0.95 and 0.99, respectively, to a plot of 1/v versus [Li^+], for the hydrolysis of Ins-1,3,4-P_3. The K_i value was determined according to the following equation: X int = -K_i (1 + K_M/[Ins-1,3,4-P_3]). All enzyme activities are the averages of three independent measurements.

### Data availability

Most data are provided in the manuscript; however, diffusion data and coordinates have been deposited in the Protein Data Bank for following INPP1 complexes: INPP1/Mg^2+/Li^+ (11INP), INPP1/Gd^{3+} (6WRO), INPP1/Gd^{3+}/Li^+ (6WRR), INPP1/Gd^{3+}/Ins(1,3,4)P_3 (6WRY), and INPP1/Gd^{3+}/Li^+/Ins(1,3,4)P_3 (6X25); INPP1^{D54A}/Ca^{2+} (7KIO); INPP1^{D54A}/Ca^{2+}/Ins(1,4)P_3 (7KIR).

#### Acknowledgments

We thank members of the York lab for helpful discussions. John York personally wishes to dedicate this manuscript to our teacher, mentor and friend, Dr Philip W. Majerus, who passed away June 2016.

#### References

1. Michell, R. H. (1992) Inositol lipids in cellular signalling mechanisms. *Trends Biochem. Sci.* 17, 274–276
2. Berridge, M. J., and Irvine, R. F. (1989) Inositol phosphates and cell signalling. *Nature* 341, 197–205
3. Irvine, R. F., and Schell, M. J. (2001) Back in the water: the return of the inositol phosphates. *Nat. Rev. Mol. Cell Biol.* 2, 327–338
4. Majerus, P. W. (1992) Inositol phosphatase biochemistry. *Annu. Rev. Biochem.* 61, 225–250
5. Allison, J. C., and Majerus, P. W. (1990) Isolation and heterologous expression of a cDNA encoding bovine inositol polyphosphate 1-phosphatase. *J. Biol. Chem.* 265, 14559–14565
6. Inhorn, R. C., and Majerus, P. W. (1988) Properties of inositol polyphosphate 1-phosphatase. *J. Biol. Chem.* 263, 233, 267–268
7. Inhorn, R. C., and Majerus, P. W. (1987) Inositol polyphosphate 1-phosphatase from calf brain. *J. Biol. Chem.* 262, 15946–15952
8. Hallacher, L. M., and Sherman, W. R. (1980) The effects of lithium and other agents on the activity of myo-inositol-1-phosphatase from bovine brain. *J. Biol. Chem.* 255, 10896–10901
9. York, J. D., and Majerus, P. W. (1990) Isolation and heterologous expression of a cDNA encoding bovine inositol polyphosphate 1-phosphatase. *Proc. Natl. Acad. Sci. U. S. A.* 87, 9548–9552
10. Diehl, R. E., Whitin, P., Potter, J., Gee, N., Ragan, C. I., Linemeyer, D., Schoepfer, R., Bennett, C., and Dixon, R. A. (1990) Cloning and expression of bovine brain inositol monophosphate phosphohydrolase. *J. Biol. Chem.* 265, 5946–5949
11. York, J. D., Ponder, J. W., Chen, Z. W., Mathews, F. S., and Majerus, P. W. (1994) Crystal structure of inositol polyphosphate 1-phosphatase at 2.3-A resolution. *Biochemistry* 33, 13164–13171
12. Bone, R., Springer, J. P., and Atack, J. R. (1992) Structure of inositol monophosphatase, the putative target of lithium therapy. *Proc. Natl. Acad. Sci. U. S. A.* 89, 10031–10035
13. York, J. D., Ponder, J. W., and Majerus, P. W. (1995) Definition of a metal-dependent/Li(+)–inhibited phosphomonoesterase protein family based upon a conserved three-dimensional core structure. *Proc. Natl. Acad. Sci. U. S. A.* 92, 5149–5153
14. Frederick, J. P., Tafari, A. T., Wu, S. M., Megosh, L. C., Chiou, S. T., Irving, R. P., and York, J. D. (2008) A role for a lithium-inhibited Golgi nucleotidase in skeletal development and sulfation. *Proc. Natl. Acad. Sci. U. S. A.* 105, 11605–11612
15. Spiegelberg, B. D., dela Cruz, J., Law, T. H., and York, J. D. (2005) Alteration of lithium pharmacology through manipulation of phospho-adenosine phosphate metabolism. *J. Biol. Chem.* 280, 5400–5405
16. Hudson, B. H., and York, J. D. (2012) Roles for nucleotide phosphatases in sulfate assimilation and skeletal disease. *Adv. Biol. Regul.* 52, 229–238
17. Spiegelberg, B. D., Xiong, J. P., Smith, J. I., Gu, R. F., and York, J. D. (1999) Cloning and characterization of a mammalian lithium-sensitive bisphosphate 3’-nucleotidase inhibited by inositol 1,4-bisphosphate. *J. Biol. Chem.* 274, 13619–13628

#### Table 2

| Data collection and refinement statistics for INPP1^{D54A} structures |
|-----------------------------|-----------------------------|
| **Diffraction data** | INPP1^{D54A}; Ca^{2+} | INPP1^{D54A}; Ca^{2+}/Ins(1,4)P_3 |
| PDB code | 7KIO | 7KIR |
| Space group | P4_1 | P4_1 |
| a (Å) | 51.45 | 51.44 |
| c (Å) | 142.24 | 143.12 |
| Resolution (Å) | 28.9 | 28.9 |
| Last Shell (Å) | 2.49 | 2.49 |
| Unique Reflections (last shell) | 14,344 (1311) | 10,601 (929) |
| Completeness (%) (last shell) | 96.8 (90.3) | 92.8 (80.3) |
| Average I/σ | 15.4 | 15.4 |
| Rmerge (%) | 9.0 | 9.0 |
| Crystallographic refinement | | |
| Resolution range (Å) | 28.9–2.40 | 28.9–2.40 |
| Reflections | 143,388 | 143,388 |
| Rms deviation from ideality | 10,595 | 10,595 |
| Bond lengths (Å) | 0.005 | 0.005 |
| Bond angles (°) | 0.76 | 0.76 |
| Rotamer outliers | 0.0 | 0.0 |
| Ramachandran Outliers | 0.0 | 0.0 |
| Allowed | 100% | 100% |
| Favorable | 98.7% | 98.7% |
| R value (%) | 19.1 | 19.1 |
| R_free (%) | 23.9 | 23.9 |

**Abbreviations**—The abbreviations used are: GD1, first gadolinium site; INPP1, inositol polyphosphate 1-phosphatase; IP, inositol phosphate; IP3, inositol 1,4,5-trisphosphate; MG1, magnesium metal site 1.
A lithium binding motif

18. Albert, A., Yenush, L., Gil-Mascarell, M. R., Rodriguez, P. L., Patel, S., Martinez-Ripoll, M., Blundell, T. L., and Serrano, R. (2000) X-ray structure of yeast Hal2p, a major target of lithium and sodium toxicity, and identification of framework interactions determining cation sensitivity. *J. Mol. Biol.* 295, 927–938

19. Ke, H., Thorpe, C. M., Seaton, B. A., Marcus, F., and Lipscomb, W. N. (1989) Molecular structure of fructose-1,6-bisphosphatase at 2.8 Å resolution. *Proc. Natl. Acad. Sci. U. S. A.* 86, 1475–1479

20. Dutta, A., Bhattacharyya, S., Dutta, D., and Das, A. K. (2014) Structural elucidation of the binding site and mode of inhibition of Li(+) and Mg(2+) in inositol monophosphatase. *FEBS J.* 281, 5309–5324

21. Gill, R., Mohammed, F., Badyal, R., Coates, L., Erskine, P., Thompson, D., Cooper, J., Gore, M., and Wood, S. (2005) High-resolution structure of inositol monophosphatase, the putative target of lithium therapy. *Acta Crystallogr.* D Struct. Biol. 61, 545–555

22. Johnson, K. A., Chen, L., Yang, H., Roberts, M. F., and Stieglitz, K. A., Johnson, K. A., Yang, H., Roberts, M. F., Seaton, B. A., Stieglitz, K. A., Albert, A., Yenush, L., Gil-Mascarell, M. R., Rodriguez, P. L., Patel, S., and Albert, A. (2002) Structural enzymology of Li(+)-sensitive/Mg(2+)-dependent phosphatases. *J. Mol. Biol.* 320, 1087–1094

23. Patel, S., Yenush, L., Rodriguez, P. L., Serrano, R., and Blundell, T. L. (2002) Crystal structure of an enzyme displaying both inositol-polyphosphate-1-phosphatase and 3’-phosphoadenosine-5’-phosphate phosphatase activities: a novel target of lithium therapy. *J. Mol. Biol.* 315, 677–685

24. Villeret, V., Huang, S., Fromm, H. J., and Lipscomb, W. N. (1995) Crystallographic evidence for the action of potassium, thallium, and lithium ions on fructose-1,6-bisphosphatase. *Biochemistry* 34, 9468–9476

25. Barnaby, R. J., Ragan, C. I., and Baker, R., Fletcher, S. R., Iversen, L. L., and Broughton, H. B. (1994) Inositol monophosphatase: evidence for two-metal ion catalysis. *Biochemistry* 33, 9469–9476

26. Pollack, S. J., Atack, J. R., Knowles, M. R., McAllister, G., Ragan, C. I., Broughton, H. B., Baker, R., and Fletcher, S. R. (1994) Structural analysis of inositol monophosphatase complexes with substrates. *Biochemistry* 33, 9460–9467

27. Pollack, S. J., Atack, J. R., Knowles, M. R., McAllister, G., Ragan, C. I., Baker, R., Fletcher, S. R., Iversen, L. L., and Broughton, H. B. (1994) Mechanism of inositol monophosphatase, the putative target of lithium therapy. *Proc. Natl. Acad. Sci. U. S. A.* 91, 5766–5770

28. Stieglitz, K. A., Johnson, K. A., Yang, H., Roberts, M. F., Seaton, B. A., Head, J. F., and Stieglitz, K. A. (2002) Crystal structure of a dual activity IMpase/FBpase (AF2372) from Archaeoglobus fulgidus. The story of a mobile loop. *J. Biol. Chem.* 277, 22863–22874

29. Ganzhorn, A. J., Legape, P., Pelton, P. D., Strasser, F., Vincendon, P., and Rondeau, J. M. (1996) The contribution of lysine-36 to catalysis by human myo-inositol monophosphatase. *Biochemistry* 35, 10957–10966

30. York, J. D., Chen, Z. W., Ponder, J. W., Chauhan, A. K., Mathews, F. S., and Majerus, P. W. (1994) Crystallization and initial X-ray crystallographic characterization of recombinant bovine inositol polyphosphate 1-phosphatase produced in Spodopter frugiperda cells. *J. Mol. Biol.* 236, 584–589

31. Choe, J. Y., Poland, B. W., Fromm, H. J., and Honzatko, R. B. (2000) Crystal structures of fructose 1,6-bisphosphatase: mechanism of catalysis and allosteric inhibition revealed in published complex structures. *Biochemistry* 39, 8565–8574

32. Patel, S., Martinez-Ripoll, M., Blundell, T. L., and Albert, A. (2002) Structural enzymology of Li(+)–sensitive/Mg(2+)–dependent phosphatases. *J. Mol. Biol.* 320, 1087–1094

33. Patel, S., Yenush, L., Rodriguez, P. L., Serrano, R., and Blundell, T. L. (2002) Crystal structure of an enzyme displaying both inositol-polyphosphate-1-phosphatase and 3’-phosphoadenosine-5’-phosphate phosphatase activities: a novel target of lithium therapy. *J. Mol. Biol.* 315, 677–685

34. Stieglitz, K. A., Roberts, M. F., Li, W., and Stec, B. (2007) Crystal structure of the tetrmeric inositol 1-phosphate phosphatase (TM1415) from the hyperthermophile, Thermotoga maritima. *FEBS J.* 274, 2461–2469

35. Hamlin, R. (1985) Multiwire area X-ray diffractometers. *Methods Enzymol.* 114, 416–452

36. Howard, A. J., Nielsen, C., and Xuong, N. H. (1985) Software for a diffractometer with multiwire area detector. *Methods Enzymol.* 114, 452–472

37. Albert, A., Yenush, L., Gil-Mascarell, M. R., Rodriguez, P. L., Patel, S., Martinez-Ripoll, M., Blundell, T. L., and Serrano, R. (2000) X-ray structure of yeast Hal2p, a major target of lithium and sodium toxicity, and identification of framework interactions determining cation sensitivity. *J. Mol. Biol.* 295, 927–938

38. Ke, H., Thorpe, C. M., Seaton, B. A., Marcus, F., and Lipscomb, W. N. (1989) Molecular structure of fructose-1,6-bisphosphatase at 2.8 Å resolution. *Proc. Natl. Acad. Sci. U. S. A.* 86, 1475–1479

39. Gill, R., Mohammed, F., Badyal, R., Coates, L., Erskine, P., Thompson, D., Cooper, J., Gore, M., and Wood, S. (2005) High-resolution structure of myo-inositol monophosphatase, the putative target of lithium therapy. *Acta Crystallogr.* D Struct. Biol. 61, 545–555

40. Johnson, K. A., Chen, L., Yang, H., Roberts, M. F., and Stieglitz, K. A., Johnson, K. A., Yang, H., Roberts, M. F., Seaton, B. A., Stieglitz, K. A., Albert, A., Yenush, L., Gil-Mascarell, M. R., Rodriguez, P. L., Patel, S., and Albert, A. (2002) Structural enzymology of Li(+)-sensitive/Mg(2+)-dependent phosphatases. *J. Mol. Biol.* 320, 1087–1094