Mobile loop mutations in an archaeal inositol monophosphatase: Modulating three-metal ion assisted catalysis and lithium inhibition

Zheng Li,¹ Kimberly A. Stieglitz,² Anthony L. Shrout,³ Yang Wei,¹ Robert M. Weis,³ Boguslaw Stec,⁴ and Mary F. Roberts^{1*}

¹Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02467

²Science, Technology, Engineering, and Mathematics, Roxbury Community College, Boston, Massachusetts 02120

³Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003

⁴The Burnham Institute for Medical Research, La Jolla, California 92037

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Abstract: The inositol monophosphatase (IMPase) enzyme from the hyperthermophilic archaeon *Methanocaldococcus jannaschii* requires Mg^{2+} for activity and binds three to four ions tightly in the absence of ligands: $K_D = 0.8 \mu\text{M}$ for one ion with a K_D of $38 \mu\text{M}$ for the other Mg^{2+} ions. However, the enzyme requires 5–10 mM Mg^{2+} for optimum catalysis, suggesting substrate alters the metal ion affinity. In crystal structures of this archaeal IMPase with products, one of the three metal ions is coordinated by only one protein contact, Asp38. The importance of this and three other acidic residues in a mobile loop that approaches the active site was probed with mutational studies. Only D38A exhibited an increased kinetic K_D for Mg^{2+} ; D26A, E39A, and E41A showed no significant change in the Mg^{2+} requirement for optimal activity. D38A also showed an increased K_m , but little effect on k_{cat} . This behavior is consistent with this side chain coordinating the third metal ion in the substrate complex, but with sufficient flexibility in the loop such that other acidic residues could position the Mg^{2+} in the active site in the absence of Asp38. While lithium ion inhibition of the archaeal IMPase is very poor ($\text{IC}_{50} \sim 250 \text{ mM}$), the D38A enzyme has a dramatically enhanced sensitivity to Li^+ with an IC_{50} of 12 mM. These results constitute additional evidence for three metal ion assisted catalysis with substrate and product binding reducing affinity of the third necessary metal ion. They also suggest a specific mode of action for lithium inhibition in the IMPase superfamily.

Keywords: inositol monophosphatase; metal-assisted catalysis; magnesium binding; mutagenesis; mobile loop; lithium inhibition

Introduction

Inositol monophosphatase (IMPase) enzymes are critical for the generation of *myo*-inositol from *myo*-inositol-1-phosphate (I-1-P). In eukaryotes and some

bacteria, *myo*-inositol is a necessary intermediate in the synthesis of phosphatidylinositol.^{1,2} However, in some hyperthermophilic microorganisms, this enzyme is critical for the synthesis of di-*myo*-inositol-1,1'-phosphate, a solute accumulated in response to salt and temperature stress.^{3–6} The enzymes from hyperthermophiles have also been shown to catalyze the specific cleavage of the C-1 phosphate of fructose-1,6-bisphosphate (FBP) as well as the NADP(H) phosphate.^{7–9} These IMPase enzymes, part of a larger IMPase superfamily,¹⁰ are usually symmetric homodimers (although the enzyme from *T. maritima*

Anthony Shrout's current address is P.A. Technologies, LLC, Amherst, Massachusetts.

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*Correspondence to: Mary F. Roberts, Department of Chemistry, Boston College, Chestnut Hill, MA 02467.
E-mail: mary.roberts@bc.edu

exists as a tetramer¹¹) with each subunit organized in a five-layered $\alpha\beta\alpha\beta\alpha$ sandwich.^{8,12–14} A mobile loop serves as a gateway to substrate binding at the active site.¹⁴ The divalent cations Mg^{2+} or Mn^{2+} are needed for catalytic activity (other divalent cations such as Zn^{2+} and Ca^{2+} are inhibitory). A hallmark of many members of the IMPase superfamily is that they are inhibited by millimolar lithium ions.^{15,16} However, the archaeal homologues require much higher concentrations ($\text{Li}^+ > 100 \text{ mM}$), a difference attributed to alterations in the mobile loop conformation.^{14,17} There is evidence that Li^+ binds to a single site on the protein¹⁸ and that it competes with Mg^{2+} for binding to the protein.¹⁹

The mechanism for I-1-P hydrolysis by the human IMPase was initially proposed to use two-metal ions as, although three metal ions were sometimes seen in crystal structures, two ions were always present.^{12,13,15,20} More recently, with the structures of archaeal homologues^{7,8,14} as well as the bovine IMPase²¹ it was proposed that these enzymes use three metal ions to facilitate I-1-P hydrolysis, as structures of the enzyme can have three metal ions bound.

The IMPase from the hyperthermophile *Methanocaldococcus jannaschii* (MJ0109) has been crystallized and characterized with metal ions and bound substrate or analogs.^{7,8} This archaeal IMPase is extremely stable and active over a wide temperature range.^{17,22} Like other homologues from hyperthermophiles, it has a range of phosphatase activities.^{7,9} In MJ0109, metal 1 is coordinated by Asp84, Asp201, and two phosphate oxygens from substrate or product in a tetrahedral conformation; metal 2 is coordinated by Asp84, Asp81, Glu65, a phosphate oxygen, and a water molecule. A third metal ion was found in the crystals with Mn^{2+} (PDB 1G0H) or Zn^{2+} and products cocrystallized (but not in the structure with substrate I-1-P and inhibitory Ca^{2+} in place of the cofactor divalent cations). This third metal ion is $\sim 3 \text{ \AA}$ distant from Asp38, a residue in the middle of the active site mobile loop (sequence ²²FGRKDKSYVVGTSPPSGDTEI⁴²). Metal 3 was postulated to aid in establishing the correct orientation of the substrate and nucleophilic water.⁸ Asp38 is the only protein residue in contact with this third metal ion in the crystal structure, but along with Asp38, there are three other acidic residues in this mobile loop (Asp26, Glu39, and Glu41). These other negatively charged side chains also have the potential to stabilize a third Mg^{2+} bound in the active site. Another nearby acidic residue, Asp44, is close to the end of this mobile active site loop and also in close proximity to the I-1-P substrate in the crystal where it is likely to contribute to the positioning of substrate. The relative orientation and close proximity between Asp26, Asp38, Glu39, Glu41, and Asp44, the three metal ions, and the products

Figure 1. Ribbon diagram of mobile loop and active site elements showing acidic residues mutated, the three activating metal ions (indicated by Met1, Met2, and Met3), and product inorganic phosphate. Note that Asp38 is a direct ligand of the third metal ion.

inositol and inorganic phosphate (Pi) are shown in Figure 1.

Two approaches were taken to test if the third Mg^{2+} was critical for IMPase activity and to see the importance of mobile loop acidic residues for binding of this ion: (i) isothermal titration calorimetry (ITC) measurement of the K_D for ions binding to the recombinant *M. jannaschii* protein in the absence of substrate, and (ii) kinetic determination of the apparent K_D for Mg^{2+} (as well as other kinetic parameters) in recombinant enzyme and several mobile loop mutant enzymes. The results clearly show that three to four Mg^{2+} bind tightly to the archaeal protein in the absence of substrate as measured by ITC ($K_D < 0.05 \text{ mM}$). For comparison, the kinetic apparent K_D for Mg^{2+} was in the high mM range. That Asp38 is likely a key ligand of this third metal ion was confirmed by examining kinetics of the mutants of this and the other acidic groups in the mobile loop. D38A exhibited a significantly increased kinetic K_D for Mg^{2+} , while other mobile loop mutant enzymes showed either no change or a decrease in the apparent K_D for Mg^{2+} . An unexpected but particularly interesting kinetic effect in D38A was a very dramatically increased sensitivity to inhibition by Li^+ . For MJ0109, the K_i for this monovalent cation was previously measured to be 250 mM .¹⁷ However, D38A exhibited a K_i of 12 mM . This result is consistent with a proposed mechanism for Li^+ inhibition where monovalent cation binding to the ternary complex of IMPase, one or two Mg^{2+} , and substrate prevents occupation and alignment, via the mobile loop, of the third Mg^{2+} at the active site.

Results

Stoichiometry of Mg^{2+} binding to *M. jannaschii* IMPase and effect on secondary structure

ITC can be used to measure ligand-binding parameters by monitoring the heat release or input when ligands bind to a macromolecule.^{23,24} Calorimetric measurement at 25°C of the *M. jannaschii* IMPase titrated with Mg^{2+} detected two types of Mg^{2+} sites per monomer (Fig. 2 and Table I) – a tight site for one Mg^{2+} and two or three weaker sites that could not be distinguished. The estimate for the number of low affinity sites is less certain than the tight site because the low affinity sites were not fully saturated at the end of the titrations. The K_D for the tight metal-binding site was $0.83 \pm 0.07 \mu M$, while for the weaker metal-binding sites the binding was ~ 50 -fold weaker ($K_D = 38 \pm 13 \mu M$). Even though the estimate of K_D for the weaker sites has considerable error, all Mg^{2+} sites had K_D values less than 0.06 mM, much tighter than the K_D values reported for the human IMPase (K_D of 0.3 mM for a tight site and 3 mM for a weaker site²⁵). Binding of Mg^{2+} in the presence of products was too complex for analysis by this method, likely due to complexation between inorganic phosphate and the divalent cations, so the stoichiometry of Mg^{2+} bound to the protein could not be determined under those conditions.

Figure 2. (A) Heat released ($\mu cal/s$) upon *M. jannaschii* IMPase binding aliquots of Mg^{2+} ; (B) Enthalpy ($kcal/mol$) of binding each aliquot of Mg^{2+} as a function of ratio of Mg^{2+} to IMPase monomer.

Table I. Thermodynamic Parameters for Mg^{2+} Binding to *M. jannaschii* IMPase Determined from ITC^a

Parameter ^b	Site 1	Site 2
N	0.89 ± 0.09	2.90 ± 0.40
K_A (M^{-1})	$1.2 \pm 0.1 \times 10^6$	$2.6 \pm 0.8 \times 10^4$
K_D (μM)	0.83 ± 0.07	38 ± 13
ΔH (cal/mol)	-2980 ± 380	-1320 ± 40
ΔS ($cal/K\cdot mol$)	17.8 ± 1.0	15.8 ± 0.5

^a Parameter estimates and uncertainties are averages and standard deviations, respectively, calculated from duplicate experiments.

^b N is the number of Mg^{2+} ions bound per IMPase monomer.

Binding of Mg^{2+} ions to both types of sites in the archaeal IMPase was exothermic, although the ΔH for the first site was larger in magnitude (2.2-fold) than for the second weaker sites; entropy terms for binding were positive and comparable for the two types of sites. All else being equal (no major conformational change in the protein), the three sites should be occupied in the absence of substrate at the higher assay temperature for this enzyme from a hyperthermophile. If the mobile loop were to exhibit a pronounced and increased flexibility with increasing temperature in response to a conformational change, it is possible that one of the ions would bind less tightly.

The tight binding of Mg^{2+} to the protein affects the secondary structure of the protein as assessed by examining the far-UV CD spectrum (Fig. 3). The addition of the Mg^{2+} at 10 μM , a concentration where the tight site should be filled with partial (but low) occupancy of the other two sites, caused an increase in β -sheet formation. Up to 50 mM Mg^{2+} had no further effect on secondary structure. In the crystal structure of MJ0109 (PDB id 1DK4), Asp84, which coordinates metal 1, is in a turn region

Figure 3. Far-UV CD spectrum for the *M. jannaschii* IMPase in the absence of Mg^{2+} (thick solid line) and with 10 μM (—) and 100 μM (---) Mg^{2+} added. The inset shows the difference spectrum showing the increase in β -sheet after adding 10 μM Mg^{2+} .

adjacent to a sheet region, while Glu65 and Asp81, which coordinate metal 2, are in a turn region and in sheet region, respectively. These could be the regions whose β -sheets are stabilized when Mg^{2+} binds.

With 100 μM Mg^{2+} , the MJ0109 CD spectrum was extremely stable to increasing temperature showing little change or loss of secondary structure at 85°C, the assay temperature of the enzyme. These results are in contrast to mammalian IMPase where the far-UV CD spectrum was sensitive to mM Mg^{2+} and showed a significant loss of secondary structure with a ' K_D ' of 4 mM.²⁶ The latter could reflect structural changes related to the kinetic inhibition at higher Mg^{2+} that is seen with the mammalian IMPase but not with archaeal IMPase enzymes.¹⁷

Mutations of mobile loop residues

The ITC results indicate that three Mg^{2+} ions can bind well to the archaeal protein in the absence of substrate, much tighter than Mg^{2+} binding to mammalian IMPase at comparable temperatures. However, the Mg^{2+} concentrations required for recombinant archaeal IMPase catalysis are in the mM range (>5 mM) with the exact value depending on pH, temperature, and substrate concentrations (at high concentrations the substrate I-I-P can chelate Mg^{2+} and the apparent K_D increases). In the crystal structure of MJ0109 with substrate D-I-I-P bound, only two Ca^{2+} ions were bound to the protein.⁸ Crystal structures with products (and Mn^{2+} or Zn^{2+}) showed three ions bound. The third ion had at most a single contact with the protein, Asp38, a residue in the mobile loop at the mouth of the active site. The mobile loop also has several other acidic residues, Asp26, Glu39, and Glu41, that could interact with Mg^{2+} . To test the importance of the loop acidic residues, we constructed four mutant enzymes where an aspartate or glutamate residue in the mobile loop was changed to alanine. All four mutant proteins expressed well. Their secondary structure content and thermostability ($T_m > 95^\circ\text{C}$) were similar to recombinant *M. jannaschii* IMPase as monitored by far-UV CD spectroscopy.

Assays at 85°C using 2 mM D-I-I-P (a concentration significantly above the substrate K_m for wild type recombinant enzyme) and varying the Mg^{2+} concentration allow us to compare an apparent K_D for Mg^{2+} for recombinant IMPase and the different mutant loop enzymes. The kinetically determined ' K_D ' is the concentration of Mg^{2+} that is needed for half the optimum activity. For a combination of tight and weak sites, this parameter will reflect the weakest affinity sites that need to be filled to make the enzyme a competent catalyst. For recombinant IMPase under these conditions, the kinetically determined K_D for Mg^{2+} was found to be 7.6 ± 1.6 mM. Three of the four loop mutants, D26A, E39A, and

E31A, had K_D values for Mg^{2+} comparable or lower than recombinant enzyme (6.8 ± 1.7 , 2.3 ± 0.7 , and 1.7 ± 0.3 mM, respectively). However, when Asp38 was replaced by alanine (D38A), a much higher K_D for Mg^{2+} , 30.7 ± 4.8 mM was obtained [Fig. 4(A)]. The only other mutation of an acidic residue leading to an increased K_D for Mg^{2+} was D44A, a residue outside the mobile loop but part of the active site. Thus, of the mutant enzymes examined, only two exhibited weaker affinity for Mg^{2+} – one where the acidic residue is in the active site (Asp44) and the other where the acidic group, Asp38, was observed to chelate the third metal ion in a crystal structure of the enzyme with products and Mn^{2+} .

The substrate (D-I-I-P) K_m and k_{cat} values for these mutant enzymes were determined and compared to recombinant IMPase. Concentrations of Mg^{2+} ion were chosen as 10 mM for wildtype, D26A, E39A, and E41A enzymes, and 50 mM for D38A and D44A. These concentrations of Mg^{2+} would not generate the maximum activity for the two Mg^{2+} -binding impaired mutant enzymes, but should allow us to at least compare K_m (or k_{cat}) among the mutants. As shown in Figure 4(B), all mobile loop mutants exhibited K_m values from 0.11 to 0.59 mM (the wildtype IMPase K_m was 0.26 mM under these conditions). D26A kinetic behavior was unusually variable from batch to batch of protein so that the error in the substrate K_m value was larger than for the other mutants, but still within the range of what was determined for wildtype enzyme. Interestingly, this variability affected K_m but had a smaller effect on k_{cat} . With the exception of D44A, the k_{cat} values of these mutant enzymes were 30–70% that of the recombinant enzyme [Fig. 4(C)]. D26A exhibited a significantly lower k_{cat} than the other loop mutant enzymes. This negatively charged residue is flanked by Arg24, Lys25, and Lys27. In the absence of Asp26, this very cationic section of the mobile loop might adopt an altered conformation that weakens or slightly misaligns the I-I-P at the active site. However, as the Mg^{2+} requirement sensed by the kinetic K_D is unaltered for D26A, the anionic segment of the mobile loop near the mouth of the active site is unlikely to be affected in this mutation.

For all mutant enzymes but D44A, the observation of a k_{cat} near that of wild type enzyme indicates that the individual acidic residues in the mobile loop that approaches the active site are not absolutely essential for catalysis. For D38A, the only one suggested by crystal structures to bind the third Mg^{2+} , a true k_{cat} would be higher as the concentration of Mg^{2+} used in these assays was ~ 1.5 times the K_D determined for Mg^{2+} . Clearly, Mg^{2+} can still bind in the absence of Asp38, albeit more weakly, presumably by interacting with other nearby acidic groups (either directly or via a negative potential generated by several of these residues) in the mobile loop.

Figure 4. Kinetic parameters for recombinant *M. jannaschii* IMPase (WT) and mobile loop mutants: (A) K_D (mM) for cofactor Mg^{2+} evaluated by varying Mg^{2+} concentration at 2 mM D-I-1-P (a concentration chosen to be significantly above K_m for that substrate); (B) K_m (mM) for D-I-1-P substrate evaluated at fixed Mg^{2+} (10 mM for WT IMPase and 50 mM for Mg^{2+} -impaired mutants); (C) k_{cat} (s^{-1}) extrapolated from V_{max} ; (D) k_{cat}/K_m ($M^{-1}s^{-1}$). Bars indicate errors on each extracted kinetic parameter.

Enzyme efficiency is shown in Figure 4(D). Aside from D44A, which is involved in substrate binding at the active site and might be expected to have a low k_{cat} , D26A, and D38A are less efficient but only D38A has impaired Mg^{2+} binding.

Li⁺ inhibition of D38A

The inhibition of IMPases by Li^+ has been suggested to occur by competition of the monovalent ion with one of the bound Mg^{2+} ions,¹⁹ although it was also noted that the mobile loop conformation was distinctly different in crystal structures of Li^+ -sensitive versus insensitive (archaeal) IMPases.¹⁴ Given the higher K_D for Mg^{2+} of the mobile loop mutant enzyme D38A, we decided to examine the sensitivity of this mutant enzyme to Li^+ . For these experiments, FBP rather than I-1-P was used as the substrate, although the trends are the same with both substrates. Assay conditions included 5 mM FBP (saturating substrate) with 10 mM Mg^{2+} , a concentration that is not saturating but slightly above the K_D for wildtype enzyme but well below the K_D for D38A. As shown in Figure 5(A), wildtype MJ0109 was not affected by up to 100 mM Li^+ added to the assay mix. However, the D38A enzyme was dramatically inhibited by the monovalent cation with an apparent IC_{50} of 12 mM.

The Mg^{2+} concentration used in these assays was well below the D38A K_D . The sensitivity to Li^+ could, in fact, be related to the weaker affinity for Mg^{2+} . To assess this possibility, we checked the activity of wildtype MJ0109 with lower Mg^{2+} (2 mM) and lower substrate (0.5 mM). The lower substrate was chosen to avoid FBP interactions with Mg^{2+} competing with protein binding of the Mg^{2+} . As shown in Figure 5(B), at the lower substrate concentration and 10 mM Mg^{2+} there was no inhibition of the IMPase phosphatase reaction. For comparison, the effects of a comparable concentration of KCl were examined. At high Mg^{2+} the added KCl was not inhibitory, but rather slightly increased the enzyme specific activity. Such behavior was previously seen with I-1-P hydrolysis and added KCl.¹⁷ However, when the Mg^{2+} concentration was decreased to 2 mM (now significantly below the kinetic K_D of wildtype enzyme for this cation), the enzyme was significantly inhibited by Li^+ . Under these conditions, the IC_{50} for Li^+ was 0.1 M, measurable but still well above the value observed for D38A (0.012 M). The lower substrate and Mg^{2+} concentrations also allow K^+ to be somewhat inhibitory, although the IC_{50} for K^+ is still quite high.

The increased sensitivity of D38A to Li^+ is unique among reported mutations of any IMPase family enzymes. Most mutations reduced Li^+

Figure 5. Monovalent cation inhibition of MJ0109 FBPase activity, (A) Effect of LiCl on MJ0109 D38A (filled circle) and wildtype (open square) activity towards 2 mM FBP with 10 mM MgCl₂; (B) Effect of LiCl (circles) and KCl (squares) on wildtype MJ0109 FBPase activity towards 0.5 mM FBP in the presence of 2 mM (filled symbols) or 10 mM (open symbols) MgCl₂.

sensitivity (e.g., H217Q for mammalian IMPase,²⁵ modification of Val170 and Trp293 in Hal2,²⁷ and L171A in *Mycobacterium tuberculosis*²⁸). A previous mutation of MJ0109, A199C, only decreased the IC₅₀ a factor of two.¹⁷ *M. jannaschii* D38A IMPase is unique with its substantial decrease in the IC₅₀ for Li⁺.

To establish whether the mutant enzyme had any significant structural variations that could be linked to the increased sensitivity to Li⁺, we have crystallized the mutant enzyme in two crystal forms. The overall structure showed very little divergence from the wildtype protein previously solved. How-

ever, out of the four independent observations of the mobile loop in two dimers in two crystal structures, only one is relatively well-folded because it is stabilized by the crystal contact. It varies slightly from the canonical conformational state but is relatively close and within the variation shown before [Fig. 6(A)]. This means that the mutation of Asp to Ala did not influence the potential local structure in any major way. However, the observation of a single loop in four possible subunits indicates that the mobility of the loop significantly increased. The loss of that negatively charged residue could, on average, displace the loop slightly from the Mg²⁺ site – a result

Figure 6. (A) Electron density map for MJ0109 D38A mobile loop and active site shown in green with the model for the protein backbone as green ribbon. In blue is shown the canonical MJ0109 loop structure for wild type protein. Note the shift in the mobile loop away from the active site; (B) Model for D38A with Li⁺ bound at the active site in the place of the third Mg²⁺ ion. The loop is proposed to orient towards this ion via a water molecule rather than the direct contact seen in the wild type structure.

that would be expected to weaken the affinity of the IMPase for the most weakly bound Mg^{2+} .

Discussion

The results from ITC and mobile loop mutant enzymes strongly support a three metal ion assisted mechanism for this IMPase. They suggest that at high temperatures where this enzyme is active, and in the absence of substrate, three or four Mg^{2+} are likely bound to the protein unless either (i) the mobile loop has very high mobility at 80–85°C that significantly reduces the affinity of the enzyme for the weaker Mg^{2+} , or (ii) the binding of the anionic substrate weakens the binding of one of these Mg^{2+} . In the absence of substrate, the very tight binding of Mg^{2+} is not expected to weaken three orders of magnitude to reflect the kinetic K_D for this ion. However, adding the substrate to the active site could alter Mg^{2+} affinity substantially. The net result is that a much higher concentration of Mg^{2+} is needed to provide the critical Mg^{2+} that helps to orient the nucleophilic water for attack on the I-1-P (or other phosphate monoester substrate) as well as aid in inorganic phosphate removal.¹⁹ Of the multiple acidic residues in the mobile loop in MJ0109 that closes over the active site, Asp38 contributes the most to the binding of this Mg^{2+} as sensed in the kinetic experiments with mobile loop mutant enzymes. However, Asp38 is not absolutely necessary, suggesting that the negative charges of the other residues can compensate (electrostatically if not by direct coordination) in positioning the weakly bound Mg^{2+} for its role in catalysis. The observation that D38A exhibited a k_{cat} like wildtype enzyme, suggests that Asp38 contributes to the binding of the third Mg^{2+} , which is important for polarizing the attacking water molecule⁸ or aiding in product release,¹⁹ but not to substrate binding and catalysis as the substrate K_m and k_{cat} were essentially unaltered.

One of the most interesting insights these experiments have provided is that Li^+ inhibition is inversely related to affinity for the third Mg^{2+} when substrate is bound. The enhanced Li^+ inhibition of D38A suggests that the protein conformation of this mutant is the preferred target for the monovalent cation. Given the high K_D for Mg^{2+} of D38A, the complex of protein/2 Mg^{2+} /I-1-P (or FBP) is likely the major enzyme form in the assay solution. We suggest Li^+ binds to this complex preventing the last Mg^{2+} from binding. Li^+ in this complex would impair hydrolysis and/or potentially lock in the product complex as has been suggested by others.¹⁹ Other acidic residues in this mobile loop can help orient this third Mg^{2+} as at enough high divalent cation, the activity of D38A is comparable to wildtype enzyme. However, the more weakly bound third Mg^{2+} does not compete as well with Li^+ . In support of this, wildtype enzyme is significantly more sensi-

tive to Li^+ when the Mg^{2+} concentration is lowered. The lower Mg^{2+} favors a ternary complex with substrate and one or two (but not three) Mg^{2+} bound. As the IC_{50} for the wildtype enzyme is still much higher than that of D38A, the Asp38 side chain cannot contribute to Li^+ binding. Rather, its removal allows enhanced binding of Li^+ compared to Mg^{2+} binding by removing the best protein ligand for the third Mg^{2+} ion.

So what is the correlation of the mobile loop with lithium sensitivity? If the target of Li^+ is the 2 Mg^{2+} /substrate complex, then the ability of the mobile loop to promote binding of the third Mg^{2+} will determine, which enzymes are susceptible to Li^+ . The lithium insensitive IMPases have smaller, more compact mobile loops poised to aid in binding of the third Mg^{2+} as suggested by the much tighter binding of Mg^{2+} to the protein in the absence of substrate compared to the lithium sensitive mammalian IMPases. This active site loop appears to be a critical factor in the development of this family of enzymes. The relatively weak conservation of the individual residues as well as the changeable length of the mobile loop through the IMPase superfamily suggest that loop mobility and variability allows different enzymes to tailor their individual sequences to the catalytic need of the particular environment.

Clearly, the removal of the anionic Asp38 side chain weakens Mg^{2+} binding to this IMPase. One of the results of this mutation is that the active site mobile loop became more disordered in the crystal structure. Enhanced mobility associated with this disorder should allow other acidic residues in the loop to move and create the positive environment for binding of a third cation, which could be Mg^{2+} or Li^+ . Our proposal for why Mg^{2+} binding is preferentially weakened compared to Li^+ ion is that the removal of Asp38 would introduce a water molecule between the loop and the third bound Mg^{2+} , as has been observed in structures of Li^+ -sensitive IMPases. In the MJ0109 crystal structure with products and three bound Mg^{2+} , no such water mediates the Mg^{2+} -Asp38 interaction. Li^+ still can bind in this site in D38A [Fig. 6(B)] because the general electrostatic character of the site does not change, as it is controlled by the pair of Glu residues not in the mobile loop (residues 65–66). However, the hydration sphere might favor the slightly smaller Li^+ binding at the modified site compared to Mg^{2+} .

Materials and Methods

Chemicals

D-inositol-1-phosphate (D-I-1-P), initially purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), was also synthesized using a peptide enantiospecific phosphorylation catalyst.²⁹ Fructose-1,6-bisphosphate, ammonium molybdate, and malachite green

oxalate were also purchased from Sigma-Aldrich. The Q-sepharose fast flow (QFF) resin was obtained from Amersham Biosciences, Piscataway, NJ). Tryp-
tone and yeast extract were purchased from DIFCO. SDS-PAGE molecular weight standards were purchased from Bio-Rad.

MJ0109 expression and purification

The recombinant MJ0109-pET23a(+) plasmid was transformed into BL21(DE3)pLysS for overexpression as described previously.¹⁷ After lysis of cell pellets and heating to 80°C for 25 min, the IMPase was purified from the supernatant by chromatography on a Q-sepharose fast flow column.¹⁷ The *M. jannaschii* IMPase (>95% pure as judged by SDS-PAGE) was concentrated to about 10 mg/mL and stored at 4°C.

The QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used for preparation of mutated MJ0109 genes. Two primers (obtained from Operon Technology, Huntsville, AL) complementary to one of the opposite strands of the target plasmid, were designed with the mutated nucleotides in the middle of them and amplified by PCR. The amplification product was digested with the *Dpn I* to remove parental strands, then transformed into Novablue supercompetent cells for plasmid propagation. The QIAprep Miniprep kit (Qiagen, Valencia, CA) was used for purification of the plasmid DNA. Double strand DNA sequencing was carried out by Sequegen (Worcester, MA) to confirm the positive mutated clones. The mutants were overexpressed and purified as described for recombinant MJ0109. All proteins were overexpressed in BL21(DE3)pLysS *E. coli* cells. After purification, the final yield was comparable to the wild type protein (1–5 mg/L media); the proteins were >90% pure as judged by SDS-PAGE.

Kinetics

IMPase specific activity was measured by colorimetric phosphate assay with an ammonium molybdate Malachite Green reagent.¹⁷ The reaction (typically in 20 μ L) was carried out at 85°C for 1 min, and stopped by cooling down the samples to room temperature and immediately adding 1 mL colorimetric assay dye (1:3 mixture of 4.2% ammonium molybdate and 0.05% malachite green oxalate). The specific activity was calculated from the absorbance of samples compared to a calibration curve (2–20 μ M) using KH_2PO_4 as the standard. As the D44A mutant was much less active than the wild type recombinant protein or other mutants, a total assay volume of 200 μ L and 92 μ g D44A were used to give a reasonable A_{660} . To determine the K_m for D-I-1-P substrate, a range of substrate concentrations from 25 μ M to 5 mM was used; Mg^{2+} was fixed at either 10 mM or 50 mM (D38A and D44A). To determine the

kinetic K_D for Mg^{2+} , the D-I-1-P was fixed at 2 mM (above the K_m for all these mutants) and Mg^{2+} varied from 0.1 to 130 mM, the exact range depending on the mutant.

For Li^+ inhibition experiments, both wildtype and D38A IMPase activities were measured using FBP (5 or 0.5 mM) as the substrate and 2 or 10 mM Mg^{2+} in 50 mM Tris HCl, pH 8.0. With wildtype recombinant IMPase, the amount of enzyme added was adjusted to give 15–20% conversion of substrate to inorganic phosphate (Pi) during the 2 min incubation at 85°C and the assay volume used was 50 μ L. The effect of KCl was used as a control to judge the specificity of the Li^+ inhibition.

Isothermal titration calorimetry

MJ0109 IMPase was stored at 10°C before the titration experiments until use (within 2 weeks). Titrations were conducted according to published procedures²³ in a Microcal MCS ITC (Northampton, MA) thermostated at 25°C with a buffer pH of 7.5. Magnesium solutions were prepared by dissolving solid MgCl_2 into the protein dialysis buffer generated during the last step of protein purification. The pH of the protein and metal solutions were matched after a 10 min degassing period. The IMPase solution (0.23 mM monomer) in the calorimeter cell (1.353 mL) was titrated with a Mg^{2+} solution (4.0–4.3 mM) delivered in a schedule of 30 injections comprised of 9 or 10 μ L injection volumes. The raw data were integrated and normalized according to published procedures²³ and the integrated titration curve was shifted along the heat (Y) axis before fitting to account for nonspecific heats, e.g. heats of dilution and other unaccounted buffer mismatches, in a way that reduced their influence on the fit model. The data were fit to the two independent sets of sites model (provided by Microcal in the Origin analysis software). The thermodynamic parameters reported in "Results" are the averages of two experiments and the uncertainties are standard deviations.

Circular dichroism

Secondary structure content of mutant proteins, assessed from far-UV CD spectra, were measured with an AVIV 202 CD spectrophotometer (Lakewood, NJ) using 0.05 mg/mL protein in 10 mM sodium borate or phosphate buffer, pH 7, in a 1 cm cuvette. For assessing the effect of Mg^{2+} on MJ0109 secondary structure, the wildtype protein was dialyzed extensively to remove any contaminating divalent metal ions, placed in a 1 cm cuvette incubated at 25°C, and the ellipticity was measured from 250 down to 190 nm after each addition of MgCl_2 . Protein stability was monitored by setting the wavelength at 222 nm and heating the solution from 25 to 98°C at a rate of 1°C/min.

Table II. Data Collection and Refinement Summary
(form 1: P2₁2₁2₁; Form 2: P1)

Crystal form	1	2
Space group	P2 ₁ 2 ₁ 2 ₁	P1
$d_{\min}$ (Å)	3.2	2.7
Reflections unique	8243	24288
$\langle I/\sigma(I) \rangle^a$	14.1 (1.1)	15.1 (1.3)
Completeness (%) ^a	81.3 (94.5)	80.0 (56.5)
Average redundancy ^a	3.5 (2.2)	2.4 (2.3)
Unit cell (Å)	$a = 67.83$ $b = 94.32$ $c = 96.30$	$a = 47.65$ $b = 62.29$ $c = 99.83$
(deg)		$\alpha = 90.06$, $\beta = 76.12$, $\gamma = 67.53$
R_{merge}	0.11 (0.61)	0.12 (0.65)
R -factor	0.24 (0.28)	0.24 (0.29)
Free R -factor (5%)	0.32 (0.48)	0.35 (0.42)
RMS Deviations		
Bond lengths (Å)	0.022	0.021
Bond angles (Å)	2.34	2.35
Ramachandran plot (%)		
Most preferred	95	93
Allowed	3	3
Generously allowed	1	1
Disallowed	0	0

^a Last shell data in parenthesis.

Crystallography

The initial crystallization trials were carried out using the grid encompassing the previously reported conditions for MJ0109⁸ of 11% w/v PEG 3500 in Tris HCl, pH 7.5. The crystallization succeeded when the concentration of metal ion additive MgCl₂ was raised to 50 mM. This crystallization condition produced a novel crystal form in the P2₁2₁2₁ space group with a single dimer in an asymmetric part of the unit cell (form 1). We also initiated a search for other crystallization conditions with other metal ions. A second crystal form in the P1 space group was obtained with addition of CaCl₂. This crystal form has a single molecule in an asymmetric unit (form 2).

Data were collected on a Rigaku Cu rotating anode X-ray source with imaging plate detector (Rigaku RaxisIV, Tokyo, Japan) and processed with HKL2000.³⁰ Molecular replacement and refinement were done with MolRep,³¹ and refinement conducted with CNS,³² and RefMac,³³ and model building using Coot.³⁴ The models were refined at the 3.2 and 2.7 Å resolution. The summary of the data collection and the refinement statistics can be found in Table II.

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