Structural Analysis of Inositol Monophosphatase Complexes with Substrates†

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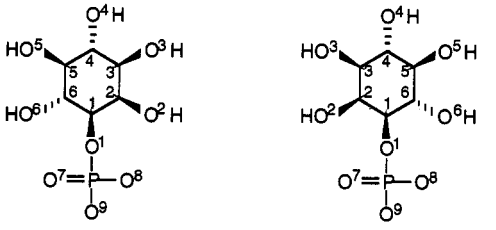
ABSTRACT: The structures of ternary complexes of human inositol monophosphatase with inhibitory Gd^{3+} and either D- or L-*myo*-inositol 1-phosphate have been determined to 2.2–2.3 Å resolution using X-ray crystallography. Substrate and metal are bound identically in each active site of the phosphatase dimer. The substrate is present at full occupancy, while the metal is present at only 35% occupancy, suggesting that Li^+ from the crystallization solvent partially replaces Gd^{3+} upon substrate binding. The phosphate groups of both substrates interact with the phosphatase in the same manner with one phosphate oxygen bound to the octahedrally coordinated active site metal and another oxygen forming hydrogen bonds with the amide groups of residues 94 and 95. The active site orientations of the inositol rings of D- and L-*myo*-inositol 1-phosphate differ by rotation of nearly 60° about the phosphate ester bond. Each substrate utilizes the same key residues (Asp 93, Ala 196, Glu 213, and Asp 220) to form the same number of hydrogen bonds with the enzyme. Mutagenesis experiments confirm the interaction of Glu 213 with the inositol ring and suggest that interactions with Ser 165 may develop during the transition state. The structural data suggest that the active site nucleophile is a metal-bound water that is activated by interaction with Glu 70 and Thr 95. Expulsion of the ester oxygen appears to be promoted by three aspartate residues acting together (90, 93, and 220), either to donate a proton to the leaving group or to form another metal binding site from which a second Mg^{2+} coordinates the leaving group during the transition state.

Inositol monophosphatase (EC 3.1.3.25) catalyzes the hydrolysis of inositol monophosphates produced both in the *de novo* biosynthesis of inositol from glucose 6-phosphate and in the recovery of inositol from inositol phosphates formed during phosphatidylinositol-mediated signal transduction (Berridge & Irvine, 1989; Berridge *et al.*, 1982, 1989; Sherman *et al.*, 1986; Hallcher & Sherman, 1980; Majerus *et al.*, 1988). On the basis of the role of the phosphatase in the phosphatidylinositol signaling pathway and the observation that the enzyme is inhibited uncompetitively by Li^+ (Hallcher & Sherman, 1980), it has been suggested as the molecular site of action of Li^+ therapy for manic-depressive illness (Berridge & Irvine, 1989). Although the enzyme does not catalyze the hydrolysis of inositol polyphosphates, it has broad specificity for phosphate monoesters with a hydroxyl substituent in an α position relative to the ester (Gee *et al.*, 1988; Ganzhorn & Chanal, 1990). In addition, the enzyme acts on both enantiomers of Ins(1)P[1] with the same catalytic efficiency, indicating that either stereoisomer of the α-hydroxyl group can be accommodated in the active site (Gee *et al.*, 1988; this work). Mg^{2+} is a required cofactor for the reaction (Gee *et al.*, 1988; McAllister *et al.*, 1992), and kinetic studies of bovine inositol monophosphatase have suggested that hydrolysis of phosphate esters is facilitated by acid–base catalysis (Ganzhorn & Chanal, 1990). However, these and other studies of the phosphatase (Shute *et al.*, 1988; Baker *et al.*, 1991; Leech et al., 1993) have not provided evidence to distinguish unequivocally between mechanisms involving either the direct attack of water or the attack of an enzyme nucleophile on the phosphate group of substrates.



D-*myo*-Inositol-1-Phosphate L-*myo*-Inositol-1-Phosphate

Recently, in an effort to aid in the design of novel inhibitors of the enzyme, the crystal structure of the human inositol monophosphatase complex with inhibitory Gd^{3+} and sulfate was determined to 2.1 Å resolution (Bone *et al.*, 1992). Gd^{3+} inhibits by displacing Mg^{2+} from the enzyme (Bone *et al.*, 1992), and sulfate competes with substrates for the active site (Hallcher & Sherman, 1980). On the basis of the metal and sulfate locations, it was proposed that the most likely nucleophile was a metal-bound water (Bone *et al.*, 1992). Using the phosphatase structure and the proposed mechanism, mutagenesis experiments have identified several residues that appear to play catalytic roles (Pollack *et al.*, 1993). However, the exact location of the inositol binding site and the mode of interaction of phosphate esters with the enzyme remained uncertain.

† The final refined coordinates have been deposited in the Protein Data Bank (entry names: 1IMA and 1IMB).

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[1] Abbreviations: D-Ins(1)P, D-*myo*-inositol 1-phosphate; L-Ins(1)P, L-*myo*-inositol 1-phosphate; Ins(1)P, *myo*-inositol 1-phosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylenebis(oxyethylenenitrilo)-tetraacetic acid; rms, root mean square.

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Efforts to use X-ray crystallography to determine where substrates bind and how metal cations activate inositol monophosphatase are hampered by the lack of both transition state analogs and nonhydrolyzable substrate analogs for the enzyme. For this reason, crystals of inositol monophosphatase grown from Li_2SO_4 in the presence of Gd^{3+} are ideal for studies of substrate binding: the crystals grow reproducibly and diffract to 2.1 Å resolution, ligands can be diffused into the active sites, and the presence of inhibitory Li^+ and Gd^{3+} prevents substrate hydrolysis.[2] In this work, we report determination of the structures of complexes of the Gd^{3+}-inhibited enzyme with D- and L-Ins(1)P. These structures identify the location of the substrate binding site and suggest that phosphate ester hydrolysis is accomplished by the direct attack of a metal-coordinated water on the phosphate group of substrates.

Because the structures of these substrate complexes were determined at high ionic strength in the presence of inhibitory cations, either the substrate or the enzyme may adopt a conformation which would not be observed in the presence of activating metals at low ionic strength. To address this concern, we have conducted mutagenesis experiments which, together with previous mutagenesis data (Pollack *et al.*, 1993, 1994), confirm the presence of several interactions between the enzyme and substrates. Furthermore, in the following paper, we report the structure of an inositol monophosphatase crystal form grown from low-ionic strength poly(ethylene glycol) solutions in the presence of activating Mn^{2+} cations, although the presence of the metal precludes the observation of enzyme-bound Ins(1)P (due to turnover). This Mn^{2+} crystal form is essentially isomorphous with the crystals grown from Li_2SO_4 in the presence of Gd^{3+}, and no significant enzyme conformational changes are observed as a result of crystal growth at low ionic strength or from the substitution of metal ions that activate the enzyme for inhibitory metals. The data presented in these two papers allow a composite picture of the mechanism of action of inositol monophosphate to be developed.

EXPERIMENTAL PROCEDURES

Oligonucleotide-directed mutagenesis was carried out by the method of Kunkel *et al.* (1987) using the pRSET vector containing the gene encoding human brain inositol monophosphatase (McAllister *et al.*, 1992). Oligonucleotides of 21 bases with either one or two mismatches in the central codon were synthesized on an Applied Biosystems 380 DNA synthesizer and used to introduce the appropriate mutation. Mutations were confirmed by DNA sequencing (Sanger *et al.*, 1977). Both mutant and wild-type inositol monophosphatases were expressed in *Escherichia coli* and purified as described by McAllister *et al.* (1992). Enzyme activity was monitored at 37 °C using either L-Ins(1)P (Billington *et al.*, 1987) and L-[U-^{14}C]Ins(1)P (Amersham) or D-Ins(1)P (Billington *et al.*, 1987) and D-[2-^{3}H]Ins(1)P (Amersham) and incubation times that gave less than 20% conversion to products (Ragan *et al.*, 1988). K_m, k_{cat}, and K_i values were calculated from initial velocity data using the RS1 software package (BBN Software Products, Cambridge, MA; Jackson *et al.*, 1989).

Cloned human inositol monophosphatase was crystallized from Li_2SO_4 in a complex with Gd^{3+} as previously described (Bone *et al.*, 1992). Ternary complexes of enzyme, Gd^{3+}, and Ins(1)P were formed by adding an equal volume of 70% saturated Li_2SO_4 containing D- (200 mM) or L- (100 mM) Ins(1)P (Billington *et al.*, 1987) to vapor diffusion drops containing large crystals of the enzyme–lanthanide complex. Crystals were undamaged by this procedure and mounted for data collection 4–5 h after the addition of substrates.

Table 1: Structure Determination Statistics for Inositol Monophosphatase Complexes with Substrates

	substrate complex	
parameter	D-*myo*-inositol 1-phosphate	L-*myo*-inositol 1-phosphate
space group	$P3_221$	$P3_221$
cell dimensions (Å)	$a = b = 86.9, c = 154.5$	$a = b = 86.9, c = 154.5$
resolution (Å)	8.0–2.3	8.0–2.2
R_{merge}[a]	0.079	0.075
% $I/\sigma_I > 2$ (%)	76	85
aveg I/σ_I	13.1	19.1
completeness (%)	93	84
refinement		
R_{cryst}[b]	0.181	0.167
rms bond dev (Å)	0.009	0.009
rms angle dev (degrees)	1.1	1.1

[a] $R_{merge} = (\Sigma\Sigma|I_j(h) - \langle I(h)\rangle|/(\Sigma\Sigma I_j(h))$; summations done over all reflections from a crystal. [b] $R_{cryst} = (\Sigma||F_{obs}| - |F_{calc}||)/(\Sigma|F_{obs}|)$.

Data for each ternary complex were collected from a single crystal using monochromatic Cu Kα X-rays (λ = 1.5418 Å) and a Siemens multiwire X-ray area detector and processed using version 2.0 of the Xengen software (Howard *et al.*, 1987). Cell dimensions and data reduction statistics for each of the two substrate complexes are shown in Table 1. Initial difference electron density maps were made using structure factors and phases calculated from the atomic model of the phosphatase complex with Gd^{3+} and sulfate (2.1 Å; Bone *et al.*, 1992) minus the sulfate and solvent molecules filling the active sites on each subunit. Although substrate positions were evident in these initial difference electron density maps, significant lack of isomorphism with the structure of the complex with Gd^{3+} and sulfate degraded the quality of the maps. Therefore, substrates and active site solvent molecules were placed into difference electron density only after preliminary rigid body and positional refinement using XPLOR (Brunger *et al.*, 1987) had improved the quality of the substrate electron density. The positions of substrate atoms were clear in these maps, and the program CHAIN (Sack, 1988) was used to place substrates and solvent into electron density maps and for additional adjustment of enzyme and substrate atomic positions. The structures were further refined using XPLOR (Brunger *et al.*, 1987), treating each subunit as an independent protein segment throughout refinement (see Table 1 for refinement statistics). Difference electron density is shown in Figure 1 for a map ($|F_o| - |F_c|$) with structure factors (F_c) and phases (ϕ_c) calculated from the final refined model of the inositol monophosphatase complex with Gd^{3+} and D-Ins(1)P minus the D-Ins(1)P. Residues that appeared to be disordered in the phosphatase complex with Gd^{3+} and sulfate also appeared to be disordered in the substrate complexes and were either left out of the model (N-terminal three residues and C-terminal residue) or assigned an occupancy value of 0.0 (disordered side chains).

RESULTS

Metal Binding. After initial rigid body and positional refinement, difference electron density maps clearly revealed

[2] Li^+ alone would not necessarily prevent substrate hydrolysis because it may act by binding to a complex of the enzyme with phosphate product (Leech *et al.*, 1993).

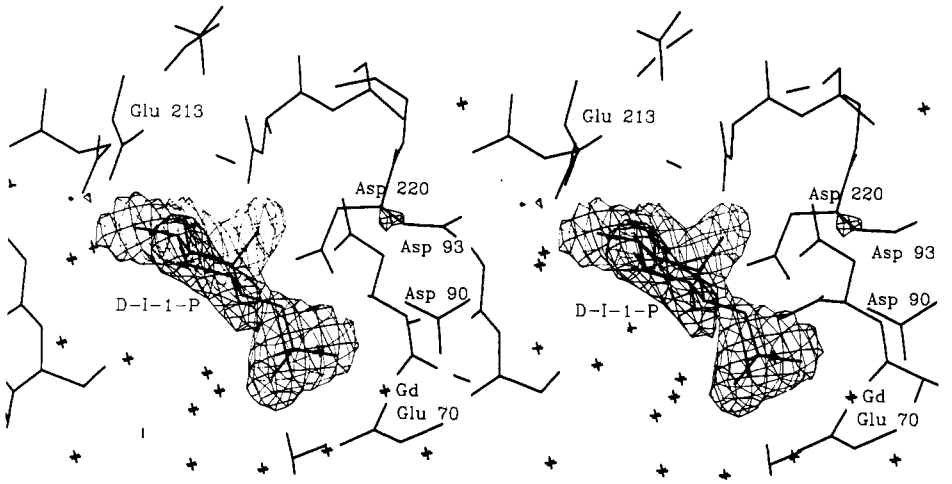

FIGURE 1: Stereodrawing of a difference electron density map ($|F_o| - |F_c|$) of the inositol monophosphatase complex with D-Ins(1)P and Gd^{3+}. Structure factors (F_c) and phases (ϕ_c) were calculated from the final refined coordinates of the complex minus the coordinates for D-Ins(1)P. Crosses represent water molecules and the metal (labeled Gd). Positive contours are displayed at 2.75 times the rms map value; no negative contours were observed in these sections at −2.75 times the rms map value.

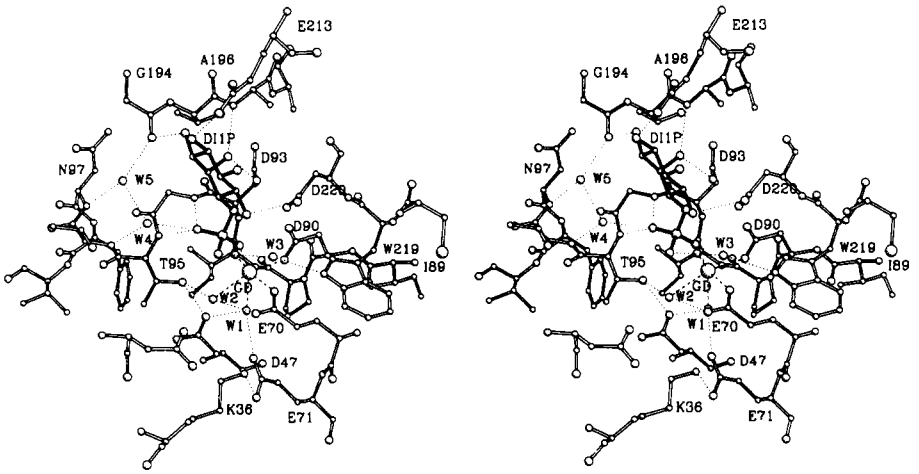

FIGURE 2: Stereodrawing of the active site of the structure of the complex of D-Ins(1)P with inositol monophosphatase and Gd^{3+}. Enzyme residues are shown in open bonds and D-Ins(1)P with filled bonds. Possible hydrogen bonds are shown with dotted lines, and coordinate bonds are shown with dashed lines.

the positions of the Ins(1)P binding sites on each subunit. Electron density was clear for the inositol ring and the phosphate for both substrates, though negative electron density was observed at the metal positions. Refinement using XPLOR (Brunger *et al.*, 1987) produced average temperature factors of 14.1 Å^2 for the substrates (13.4 for D-Ins(1)P and 14.8 for L-Ins(1)P and over 60 Å^2 for the metal cations, with negative electron density persisting around the metal coordinates. In comparison, the temperature factors for the sulfates and metals in the enzyme complex with sulfate and Gd^{3+} averaged 16.8 and 23.7 Å^2 (Bone *et al.*, 1992). From these observations, it was concluded that the inhibitory Gd^{3+} was not present at full occupancy in complexes with substrates. Alternating occupancy and temperature factor refinement for the metals, starting from occupancy and temperature factor values of 0.5 and 25 Å^2, led to temperature factors of 21.5 and 18.9 Å^2 and occupancy values of 0.37 and 0.35 in complexes with D-Ins(1)P and L-Ins(1)P, respectively. These occupancy values correspond to approximately 22 of the 61 Gd^{3+} electrons and would be clearly distinguishable from either full occupancy or no occupancy at the resolution of these studies (2.2–2.3 Å).

In contrast to the phosphatase complex with Gd^{3+} and sulfate in which nine possible metal ligands were observed (Bone *et al.*, 1992), the metal ions in these substrate complexes appeared to have just six ligands in a slightly distorted octahedral geometry (Figures 2 and 3, Table 2). The ligands, which appear the same on each subunit and in each substrate complex, include one carboxylate oxygen of Asp 90, the main chain carbonyl of Ile 92, one carboxylate oxygen of Glu 70, one phosphate oxygen, and two molecules of solvent. Of the nine possible ligands in the complex with Gd^{3+} and sulfate, only the six closest have been retained in the substrate complexes. The most notable changes are that Thr 95 is much too far from the metal to be considered a ligand (3.3 Å) and the phosphate group of the substrate contributes only one ligand to the metal while sulfate appeared to supply two. Despite evidence of partial metal occupancy, there was no evidence in difference electron density maps for alternate conformations of either the substrate or the active site residues. In addition, temperature factors for residues involved in metal binding were equivalent to values observed in the complex with Gd^{3+} and SO_4^{2-}.

Phosphate Binding. The phosphoryl groups of both D-Ins(1)P and L-Ins(1)P were bound by the enzyme in the same manner (Figures 2 and 3). In addition to the contribution of one ligand (O9) to the metal, another oxygen (O7) of the substrate phosphoryl group forms hydrogen bonds with the main chain amide groups of residues 94 and 95 (Table 2). Residues 93–95 form a kinked structure that wraps around the phosphate oxygen in a structure reminiscent of the oxyanion

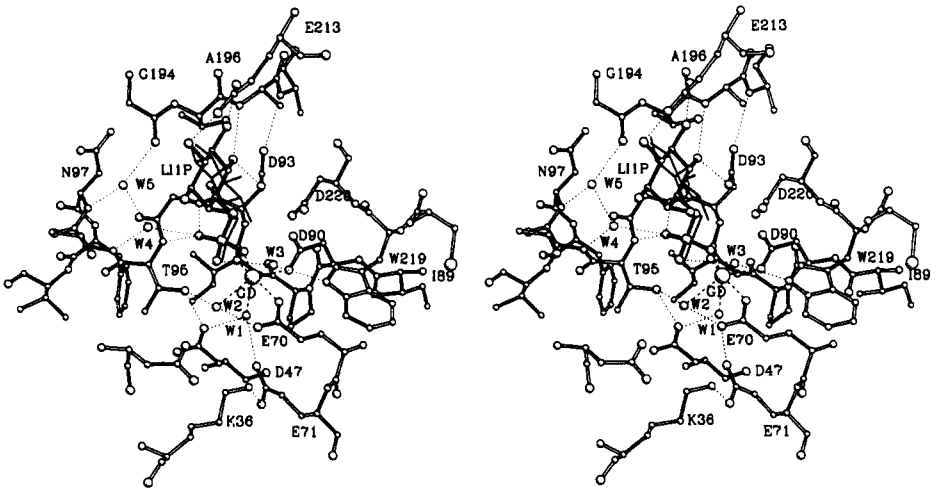

FIGURE 3: Stereodrawing of the active site of the structure of the complex of L-Ins(1)P with inositol monophosphatase and Gd^{3+}. Enzyme residues are shown in open bonds and D-Ins(1)P with filled bonds. Possible hydrogen bonds are shown with dotted lines, and coordinate bonds are shown with dashed lines. For comparison, a stick figure of D-Ins(1)P from the D-Ins(1)P complex with the phosphatase has been superimposed on the structure. The superposition was accomplished using the C_α atoms of subunit A of each structure.

Table 2: Interatomic Distances in Inositol Monophosphatase Complexes with Substrates and Gd^{3+}

	inositol monophosphatase complex[a]					
	distance (Å)					
	D-I-1-P		L-I-1-P		SO_4[b]	
atoms	A	B	A	B	A	B
Substrate Hydrogen Bonds						
Asp 220 OD2·I-1-P O1	2.85	3.16	3.09	3.33	3.00	3.07
Asp 93 OD2·I-1-P O1	2.82	2.87	3.00	3.11	2.97	2.81
Asp 93 OD2·I-1-P O2 (O6)[c]	2.71	2.87	2.66	2.68		
Ala 196 N·I-1-P O2 (O6)[c]	2.90	3.03	2.89	2.85		
Glu 213 OE2·I-1-P O5			3.07	3.08		
Glu 213 OE1·I-1-P O4	2.84	2.78	2.72	2.61		
Gly 194 O·I-1-P O3	2.87	2.87[d]				
Asp 220 OD1·I-1-P O6 (O2)[c]	2.78	2.75	3.66	3.75		
Gly 94 N·I-1-P O7	2.91	2.82	2.79	2.74	2.74	2.63
Thr 95 N·I-1-P O7	3.17	2.95	2.94	2.95	2.77	2.74
W4·I-1-P O7	2.75	2.63	2.82	2.68	2.62	2.63
W3·I-1-P O8	2.93	2.94	2.98	3.27	4.30	4.25[e]
W2·I-1-P P	3.61	3.66	3.48	3.62	3.55	3.61
Enzyme–Solvent Hydrogen Bonds						
Thr 95 OG1·W2	2.64	2.72	2.63	2.62	3.04	3.13
Glu 70 OE2·W2	2.95	2.96	2.89	2.99	2.52	2.57
Trp 219 NE2·W3	2.86	2.89	2.91	2.79	2.80	2.84
Thr 96 N·W4	3.01	3.17	2.94	3.06	2.89	3.02
W5·W4	2.85	2.75	2.79	2.79	2.95	2.63
Asn 97 N·W5	3.23	3.20	2.97	3.13	2.95	3.17
Gly 194 O·W5	2.80	2.68	2.87	2.69	2.79	2.70
Arg B191 NH1·W5	3.10	3.39	3.02	3.20	2.94	3.09
Enzyme Distances						
Asp 220 OD2–Asp 90 OD2	3.17	2.76	3.02	2.81	2.92	3.08
Asp 220 OD2–Asp 93 OD2	3.01	3.15	3.02	3.20	2.97	3.02
Asp 90 OD2–Asp 93 OD2	3.24	3.25	3.24	3.24	2.78	3.07
Metal Coordination						
W1–Gd	2.60	2.64	2.53	2.42	2.61	2.63
W2–Gd	2.81	2.66	2.72	2.62	2.59	2.62
I-1-P O9–Gd	2.05	2.10	2.06	2.25	2.71	2.86
Asp 90 OD1–Gd	2.31	2.36	2.30	2.30	2.34	2.42
Ile 92 O–Gd	2.03	2.19	2.10	2.21	2.56	2.32
Glu 70 OE1–Gd	2.28	2.25	2.39	2.26	2.43	2.57

[a] For each distance, the value for subunits A and B are given. [b] Values for the sulfate complex are from Bone *et al.* (1992). Distances are for the sulfate atoms equivalent to the atoms of the substrate indicated. The orientation of sulfate is rotated by approximately 60° about the S–O1 bond, and the molecule is translated by 0.55 Å relative to the phosphate group of the substrate. [c] Atoms listed are the D-I-1-P complex; the atoms in parentheses are for the complex with L-I-1-P. [d] This hydrogen bond is very bent; the C_{194}–O_{194}–$O3_{I-1-P}$ angle is 95°. [e] Because of the rotation of the orientation of the sulfate with respect to the phosphate groups of the substrates, this hydrogen bond is not made.

binding pocket in the serine proteases (Stroud *et al.*, 1974). Binding of the phosphate may be further stabilized by alignment of the dipole of a two-turn helical segment, which includes residues 95–100, with the phosphate oxygen (O7). The phosphate ester oxygen is within hydrogen-bond distance of the carboxylates of both Asp 220 and Asp 93, which are within hydrogen-bond distance of each other and Asp 90 (Figures 2 and 3, Table 2). The closest atoms of the three carboxylates and the ester oxygen appear to be organized in a tetrahedral geometry about a central point. The close proximity of these Asp residues to one another and to an electronegative atom of the substrate would be expected to be very destabilizing if all of the carboxylates are anionic.

Interactions between the phosphoryl oxygens of both substrates and the enzyme are also mediated by solvent molecules which form hydrogen bonds to both enzyme and substrate. Water W3 forms hydrogen bonds to both Trp 219 and one of the oxygens on the substrate phosphate group (Figures 2 and 3, Table 2). Water W4 is within hydrogen-bond distance of the amide group of residue 96, water W5, and one of the phosphoryl oxygens. Both of these water molecules form similar interactions with the enzyme and sulfate in the structure of the phosphatase complex with Gd^{3+} and sulfate (Bone *et al.*, 1992). It seems probable that these solvent binding sites are also filled in the unliganded enzyme and that these interactions between the substrate and ordered, active site solvent participate in the stabilization of the transition state for phosphoryl transfer.

Inositol Binding. Because inositol monophosphatase does not discriminate between the enantiomers of Ins(1)P (Table 3), it was of great interest to understand how the enzyme accommodates both substrates in the same active site. The binding modes of D-Ins(1)P and L-Ins(1)P differ in the orientation of the inositol rings and in the way each inositol ring interacts with the active site (Figure 3). The orientations of the inositol rings differ by rotation about the bond between the ester oxygen and the inositol ring (C2–C1–O1–P torsional angle), which has a torsional angle of −108° in the complex with D-Ins(1)P and −164° in the complex with L-Ins(1)P. Although the −108° C2–C1–O1–P torsional angle leaves all substituents on C1 staggered relative to phosphorus in the D-Ins(1)P complex, the hydrogen on C1 and the phosphorus are in a conformation which is nearly eclipsed in the L-Ins-(1)P complex.

Table 3: Kinetic Parameters for Inositol Monophosphatase Mutants

	D-*myo*-inositol 1-phosphate			L-*myo*-inositol 1-phosphate		
mutant	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (s^{-1} mM^{-1})
WT	16	42	380 000	23	62	370 000
E213Q	3.6	810	4400	4.9	600	8200
S165A	1.8	35	51 000	4.4	35	130 000
S165I	5.0	52	96 000	4.7	65	72 000

The inositol rings of both substrates interact with essentially the same active site residues and form four intermolecular hydrogen bonds with the enzyme in spite of the difference of approximately 60° in the orientations of the inositol rings (Table 2). In the monophosphatase complex with D-Ins(1)P, the axial 2-hydroxyl group forms hydrogen bonds with the carboxylate of Asp 93 and the main chain amide group of Ala 196 (Figure 2). In addition, the 4-hydroxyl group forms a hydrogen bond with the side chain of Glu 213 and the 6-hydroxyl group contributes a hydrogen bond to the side chain of Asp 220. The 3-hydroxyl group of D-Ins(1)P is within hydrogen-bond distance of the carbonyl group of Gly 194, but the hydrogen bond formed would be extremely bent (C–O–O3 angle = 95°) and probably does not contribute to complex stability. The 5-hydroxyl group of D-Ins(1)P does not form an intermolecular hydrogen bond with the enzyme, though it does form a hydrogen bond with an active site solvent molecule. In the phosphatase complex with L-Ins(1)P, the 4-hydroxyl group of inositol still forms a hydrogen bond with the side chain of Glu 213 (Figure 3). However, it is the equatorial 6-hydroxyl group rather than the axial 2-hydroxyl group of L-Ins(1)P that forms hydrogen bonds with the side chain of Asp 93 and the main chain amide group of Ala 196. The fourth hydrogen bond in the L-Ins(1)P complex is formed between the 5-hydroxyl group and the side chain of Glu 213, while the 2- and 3-hydroxyl groups interact with active site solvent. Although the 2-hydroxyl group of L-Ins(1)P is not close enough to Asp 220 to form a hydrogen bond, model building suggests that an interaction between Asp 220 and L-Ins(1)P may be possible during the transition state. For both substrates, solvation energy lost upon removal of the five inositol hydroxyl groups from water appears to be not fully compensated by productive interactions between each inositol hydroxyl group and the enzyme.

The inositol rings of both D-Ins(1)P and L-Ins(1)P displace the same six water molecules from the active site upon complex formation with the enzyme. The solvent molecules are bound in the enzyme complex with sulfate in positions filled in the substrate complexes by the 6-, 5-, 4-, and 2-hydroxyl groups of L-Ins(1)P or the 2-, 3-, 4-, and 6-hydroxyl groups of D-Ins-(1)P. These solvent molecules interact with the same enzyme groups as the inositol hydroxyl groups which displace them. In addition, several new molecules of solvent are observed in substrate complexes which appear to create a new water network that forms a bridge between the enzyme and substrate.

Mutagenesis of Inositol-Binding Residues. On the basis of the interactions observed in the structures of complexes of inositol monophosphatase with substrates, two of the residues that are involved in substrate selection are Glu 213 and Asp 93. Mutation of Glu 213, which forms hydrogen bonds to the 4-hydroxyl group of D-Ins(1)P and the 4- and 5-hydroxyl groups of L-Ins(1)P, to Gln decreases k_{cat}/K_m by factors of 86 and 45 for D-Ins(1)P and L-Ins(1)P, respectively (Table 3). The effects of the mutation were primarily on K_m values, which were increased approximately 15-fold, with smaller decreases in k_{cat}. These decreases in transition state stabilization result from changing a carboxylate group to an amide group and are in the range of values that would be expected to result from altering a hydrogen-bonding interaction from one involving an ionic partner to one involving only neutral partners (Fersht *et al.*, 1987). Similarly, mutation of Asp 93, which forms a hydrogen bond with the 2-hydroxyl group of D-Ins(1)P and the 6-hydroxyl group of L-Ins(1)P, to Asn increased the dissociation constant of Ins(1)P 14-fold (Pollack *et al.*, 1993).

Ser 165 is in close proximity to the 5-hydroxyl group of D-Ins(1)P and the 3-hydroxyl group of L-Ins(1)P. Although the Ser 165 hydroxyl group was not close enough to the substrate to form hydrogen bonds (O_γ–O5 distance = 3.5 Å), mutation of the residue to Ala and Ile decreased k_{cat}/K_m by factors of 3.0 and 5.2 for the hydrolysis of D-Ins(1)P and 7.0 and 3.9 for the hydrolysis of L-Ins(1)P, with the changes primarily due to reductions in k_{cat} rather than K_m (Table 3). Because mutation of Ser 165 to Ala or Ile affects values of k_{cat} rather than K_m, it appears that interactions between this residue and the inositol ring of the substrate may develop as the transition state is approached.

Substrate-Induced Conformational Changes. Despite some lack of isomorphism between the structures of the phosphatase complexes with sulfate, D-Ins(1)P, and L-Ins(1)P, the structure of the enzyme in the substrate complexes changes little relative to the structure in the sulfate complex (Bone *et al.*, 1992). The rms deviations between C_α positions in the Gd^{3+} and sulfate and the Gd^{3+} and substrate complexes are only 0.36 Å (D-Ins(1)P) and 0.32 Å (L-Ins(1)P) when phosphatase dimers are superimposed (0.24 and 0.21 Å when the A subunits are superimposed), and most of the conformational alterations appear to be in surface residues which have relatively high temperature factors. However, the structures differ significantly in the region of the metal binding site which appears to have expanded upon substrate binding (Figure 4). The C_α positions of residues 90 and 95, which are on opposite sides of a kink in the protein chain that wraps around the metal binding site, adjust away from each other by 1 Å when substrates are bound. This region can evidently act as a sort of spring, expanding or contracting depending on the type of stress put on it. In addition, other residues which interact either directly (Glu 70) or indirectly (Glu 71 and Asp 47) with the metal adjust away from the original Gd^{3+} location by approximately 0.5 Å.

Movement of individual residues away from the metal binding site is part of larger concerted adjustments of entire protein segments away from the binding site. For instance, adjustment of Asp 90 away from the original Gd^{3+} location is part of the movement of an entire β strand (residues 85–90) away from the metal binding site by 0.5 Å. Similar but smaller adjustments of entire segments of secondary structure are also observed in the region of Asp 47 and Glu 70. In contrast, adjustment of Thr 95 away from the metal appears to be absorbed into the two turns of α helix that follow it in the structure (Figure 4). The conformational transition is the same on each subunit and the same for each of the two substrates.

DISCUSSION

Specificity. The structures of complexes of inositol monophosphatase with substrates and inhibitory Gd^{3+} have been determined at resolutions of 2.2–2.3 Å. These structures show how the phosphoryl group of the substrate interacts with the enzyme and active site metal and where the inositol binding

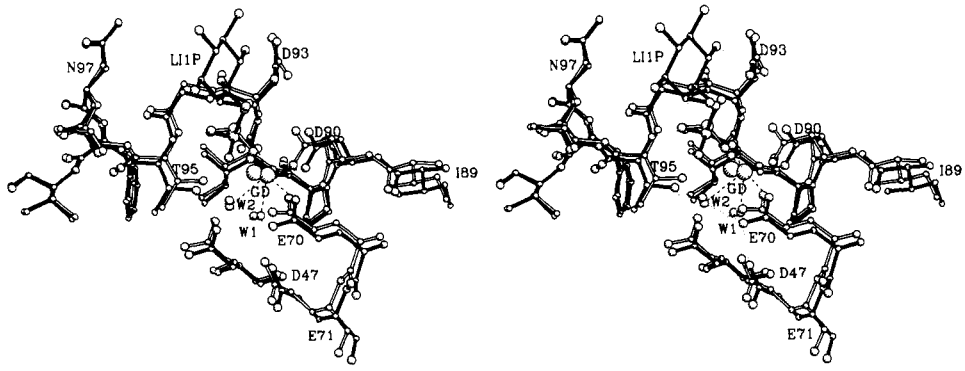

FIGURE 4: Stereodrawing of the superimposed structures (using all C_α atoms from the A subunits) of the complexes of inositol monophosphatase with sulfate and Gd^{3+} (open bonds; Bone *et al.*, 1992) and with L-Ins(1)P and Gd^{3+} (filled bonds). Possible hydrogen bonds are indicated by the dotted lines and metal coordination by the dashed lines for the complex with substrate.

site is located. The phosphate groups of the substrates interact identically with the enzyme in each complex and contribute one ligand to the active site metal. Ins(1)P enantiomers are bound by the enzyme with the inositol rings rotated by approximately 60° relative to one another such that the 2-, 4-, and 6-hydroxyl groups nearly superimpose, a result which had been predicted on the basis of earlier model building (Baker *et al.*, 1990). Each inositol group forms the same number of good hydrogen bonds with the enzyme (four), in accord with the observation that inositol monophosphatase does not distinguish between Ins(1)P enantiomers (Gee *et al.*, 1988; this work). The low intrinsic affinity of the enzyme for inositol (0.4 M; Leech *et al.*, 1993) is explained by the observation that only three of the five inositol hydroxyl groups form hydrogen bonds with the enzyme and only one hydroxyl group forms two hydrogen bonds.

The most important interaction between the inositol ring of the substrate and the enzyme appears to be the formation of two hydrogen bonds between a hydroxyl group adjacent to the phosphate ester and residues Asp 93 and Ala 196. The second most important specificity determinant appears to be the interaction of the 4-hydroxyl group of the substrate with Glu 213 which is observed in both substrate complexes. The importance of interactions between substrates and both Asp 93 and Glu 213 is confirmed by the effects of mutating either of these residues to the corresponding amide (Pollack *et al.*, 1993; this work). These as well as other enzyme–substrate interactions, such as hydrogen-bond formation between Ser 165 or Asp 220 and the inositol ring, may develop or improve as the transition state is approached.

In addition to catalyzing the hydrolysis of both D-Ins(1)P and L-Ins(1)P, inositol monophosphatase has activity toward other inositol monophosphates as well as other phosphate monoesters such as β-glycerophosphate, adenosine 2′-monophosphate, and *p*-nitrophenyl phosphate (Gee *et al.*, 1988; Ganzhorn & Chanal, 1990). The availability of alternate ring orientations to accommodate both axial and equatorial hydroxyl groups adjacent to the ester, the absence of strong interactions with all of the inositol hydroxyl groups, and the presence of a large solvent channel on one side of the inositol ring allow the rationalization of the broad specificity of the enzyme for phosphate monoesters. With the exception of *p*-nitrophenyl phosphate, a very poor substrate, each of the known substrates preserves the interaction of a hydroxyl group adjacent to the ester with the enzyme.

Gd^{3+} Binding Site. Surprisingly, the inhibitory lanthanide cation in these inositol monophosphatase complexes appears to be present at $^1/_3$ occupancy or less. Close proximity of six metal ligands and the absence of observed alternate conformations for residues involved in metal binding suggest strongly that some other cation is present at $^2/_3$ to full occupancy to shield the negatively charged or electronegative ligands from one another. If two different metal ions are partial occupants of the active site, then closely similar alternate conformations of the metal ligands might be present. However, these conformations could be undetectable at the resolution of these studies because the ionic radii of hexacoordinate Gd^{3+}, Li^+, and many other cations differ by only 0.1–0.3 Å (Huheey, 1978).

While it cannot be ruled out that the metal binding site is fully occupied by a contaminating metal ion with 20–30 electrons, this seems unlikely for the following reasons. The only inorganic cations present in crystallization solutions in excess of chelators (1 mM EGTA and 2 mM *o*-phenanthroline; Bone *et al.*, 1992) were Gd^{3+} (4–6 mM), Na^+ (3 mM), and Li^+ (3–4 M). In addition to being stored in the presence of chelating agents, the enzyme is purified in the presence of 10^{-3} M EGTA (McAllister *et al.*, 1992). In the absence of substrates, the presence of Gd^{3+} was proved by extremely strong anomalous scattering which was observed in anomalous difference Patterson maps (Bone *et al.*, 1992). Finally, to be observed at high occupancy, a metal ion contaminant would have to be present at relatively high concentration (0.3 mM or higher depending on the relative affinity of the chelators for Gd^{3+} and the contaminant). Because these observations suggest that the presence of a contaminating metal ion is unlikely, it appears that the Gd^{3+} may be partially replaced by Li^+, which is a known enzyme ligand and present at high concentration[3] (3–4 M; Bone *et al.*, 1992).

Several conclusions regarding the Gd^{3+} binding site can be drawn which do not depend on the identity of the metal ion(s) occupying the site. Residues 70, 90, and 92, the phosphate group of the substrate, and water molecules W1 and W2 coordinate to the metal ion(s) and appear to be present in only one conformation. The six metal ligands are organized in a roughly octahedral geometry, the coordination geometry expected when activating Mg^{2+} is present. Mutagenesis data presented in this and other work confirm the interaction of Glu 213, Asp 93, and Ser 165 with the inositol ring of the substrate and the involvement of several metal-binding residues in catalysis (Pollack *et al.*, 1993, 1994). In addition, the structures of inositol monophosphatase complexes with Mn^{2+} (Bone *et al.*, following paper in this issue) and Ca^{2+} (R. Bone,

[3] Because Li^+ is present at levels much higher than the K_i (K_i = 0.3 mM; McAllister *et al.*, 1992), the results do not necessarily indicate that Li^+ binds to the Michaelis complex at the Gd^{3+} binding site to produce the uncompetitive inhibition observed in kinetic experiments (Leech *et al.*, 1993).

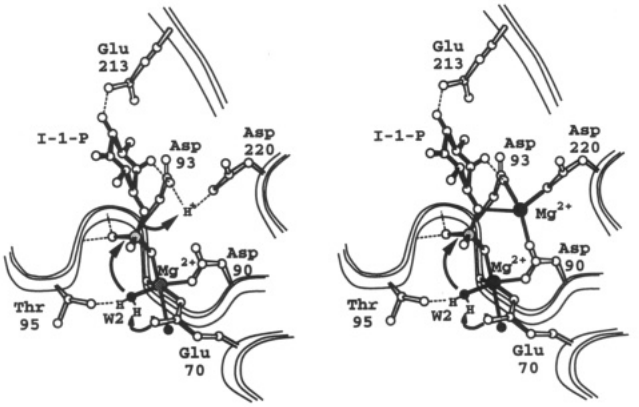

FIGURE 5: Proposed mechanisms of action of inositol monophosphatase. Glu 70 and Thr 95 activate a metal-bound water as a nucleophile, while Asp 93 and Asp 220 act together to donate a proton to the leaving group (inositol). The nucleophilic water and the leaving group are in line in the trigonal bipyramidal transition state. Alternately Asp 90, Asp 93, Asp 220, and the ester oxygen form a second Mg^{2+} binding site. Mg^{2+} coordination of the ester oxygen stabilizes the development of negative charge during the transition state and promotes inositol leaving.

unpublished observations) establish the binding of activating metal ions at the Gd^{3+} binding site and further confirm the nature of the metal ligands.

Mechanism. On the basis of the structures of these complexes between inositol monophosphatase, substrates, and Gd^{3+}, the mechanism of enzyme action appears to involve the direct attack of water on the substrate phosphoryl group (Figure 5). The most likely nucleophile is water W2, which is coordinated to the active site metal and positioned so as to be in line with the leaving group during the transition state. W2 is located 3.5–3.6 Å from the substrate phosphorus atom in both substrate complexes and appears to be activated as a nucleophile through the formation of hydrogen bonds to both Glu 70 and Thr 95. The trigonal bipyramidal transition state for phosphate ester hydrolysis would be stabilized by coordination of one of the phosphoryl oxygens to the metal, by the formation of hydrogen bonds between the amide groups of residues 94 and 95 and another of the phosphoryl oxygens, and by alignment of the dipole of the two-turn α helix spanning residues 95–100 with the phosphoryl group.

In support of the proposal that W2 is the active site nucleophile, mutation of Glu 70 to Gln decreased k_{cat} dramatically (7000-fold) while leaving metal binding unaffected (Pollack *et al.*, 1993). Gln 70 in the E70Q mutant would still be able to coordinate the metal and stabilize W2 as a ligand to the metal but would not function to activate W2 as a nucleophile. Similarly, mutation of Glu 70 to Asp reduced k_{cat} dramatically (400-fold) while leaving metal binding unaffected (Pollack *et al.*, 1993). Mutation of Thr 95 to Ala also decreased k_{cat} dramatically (14 000-fold) while increasing the K_m for Mg^{2+} 7-fold (Pollack *et al.*, 1993). As with E70Q, T95A would be poorer at activating W2 as a nucleophile.

Two mechanisms for promoting expulsion of the leaving group are consistent with the structural data. Asp 90, Asp 93, Asp 220, and the ester oxygen of the substrate are organized in an unstable arrangement in which each is in close proximity to the others (Table 2). The arrangement of aspartates could be stabilized if either Asp 220 or Asp 93 has an unusually high pK_a value and retains its proton at neutral pH. Either Asp 220 or Asp 93 would be in an ideal location to act as an acid catalyst by donating a proton to the ester oxygen to facilitate inositol leaving (Figure 5). An alternate possibility is that the arrangement of Asp residues is stabilized by formation of a complex with Li^+ from the crystallization solvent rather than by proton binding. Direct observation of Li^+ binding is not possible because Li^+ has just two electrons and is virtually invisible in these X-ray crystallography experiments. Li^+ placed at the center of the tetrahedral arrangement of oxygens would form coordinate bonds of 1.9–2.1 Å with the three Asp residues. The Li^+ would also be able to form coordinate bonds to both the ester oxygen and the nonbridging phosphate oxygen (O9) which is coordinated to the Gd^{3+}. An implication of this possibility is that under activating conditions a second Mg^{2+} might be bound between the three Asp residues and the ester oxygen and function by coordinating the ester oxygen to stabilize the development of negative charge on the leaving group during the transition state (Figure 5). To establish which of the two proposed mechanisms is used by the enzyme, we have obtained and determined the structure of crystals of inositol monophosphatase complexes with activating Mn^{2+} in the absence of inhibitory cations (Bone *et al.*, following paper in this issue). These studies provide direct evidence for the involvement of two metal ions in the catalytic mechanism of inositol monophosphatase.

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