3-Dimensional interactions between inositol monophosphatase and its substrates, inhibitors and metal ion cofactors

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Experiment has shown that bovine inositol monophosphatase requires the presence of two Mg^{2+} cations for activity, the second of which interacts either directly or indirectly with peripheral O-atoms on the substrate. Here we describe the 3-D structure of the active enzyme di-magnesium ion complex and the modelled binding modes for five known and dissimilar substrates, D-inositol 1-phosphate, L-inositol 1-phosphate, inositol 4-phosphate, 2′-adenosine monophosphate and ethane-1,2-diol 1-phosphate The expected location and functional role of the second metal ion within the protein in the presence of each of these substrates is very similar. The predicted binding modes and interactions with the metal ions and the protein are also presented for a range of known inhibitors. These structures highlight the small differences that exist between substrates and inhibitors in the mode of coordination for the first Mg^{2+} ion within the enzyme and the large differences that exist in the mode of binding for the second Mg^{2+} ion. This analysis indicates that the hydrolytic water molecule is located on the second Mg^{2+} ion and that hydrolysis proceeds *via* a non-inline, direct displacement. The so called 'catalytic' oxygen functionality of substrates serves as a hydrogen-bond acceptor for the hydrolytic water molecule and holds it on the second Mg^{2+} ion, but does not appear to bind to the metal ion directly. Some of the predictions of this refined new mechanism have been tested by experiment and the binding conformations of new potential inhibitors which may be able to block the site for the nucleophile on the second Mg^{2+} ion have been examined. A comparison of the proposed pseudorotation mechanism with an alternative in-line displacement mechanism is presented.

Introduction

Inositol monophosphatase (EC 3.1.3.25) catalyses the hydrolysis of a wide range of inositol monophosphates and ribonucleoside 2′-monophosphates *via* a mechanism involving the direct displacement of the alcohol leaving group by a nucleophilic water molecule. The phosphatase shows an absolute requirement for two divalent metal cations,[1] such as Mg^{2+} or Mn^{2+}, and it is known that one metal ion binds to the enzyme before the substrate and that one metal ion binds after the substrate.[2] The enzyme is inhibited at high Mg^{2+} ion concentrations and is also inhibited by Li^+ ion. Inhibition by Li^+ ion is complex and is uncompetitive with respect to substrate at low Li^+ concentration[1] and noncompetitive at high concentration.[2] Uncompetitive inhibition by Li^+ and Mg^{2+} is mutually exclusive, which indicates that both metal ions bind in the same site. On the basis of results of pre-steady-state and steady-state kinetic studies it is evident that this site is the site for the second catalytic Mg^{2+} ion ($Mg^{2+}2$) in the enzyme-phosphate product complex. Thus, occupation of the second Mg^{2+} site prevents the product, P_i, from debinding.

Considerable effort has also gone into defining the structural requirements for substrate activity and into establishing the determinants for inhibitor-binding propensity for the inositol monophosphatase system. It has been shown that neither of the 3- or 5-hydroxy groups [with respect to D-inositol 1-phosphate D-Ins 1-*P*)] have any effect on binding or catalysis[3] and that the removal of the 6-hydroxy group abolishes catalytic activity. However, both the 2- and 4-hydroxy groups are involved in binding to the enzyme but are not essential in conferring activity as a substrate.[4] The smallest molecule capable of acting as a substrate has been shown to be ethane-1,2-diol 1-phosphate[5,6] but, interestingly, not all 1,2-diol monophosphates can serve as substrates, even those which are known to be small enough to fit into the active-site cleft and which can act as competitive inhibitors.[7]

There is good evidence from structure–activity studies to show that both O-atoms of the monophosphodiol moiety of substrates interact directly or indirectly with $Mg^{2+}2$ in the active quaternary enzyme complex.[5,6] In order to account for the ability of some 1,2-diol monophosphates but not others to serve as substrates it has been suggested, therefore, that the active-site nucleophile, a water molecule, is coordinated to the second Mg^{2+} ion.[6] Hence, the position of the nucleophile would be critically dependent upon the accessible dihedral angles in the diol moiety which positions $Mg^{2+}2$ and its attached nucleophile, Fig. 1.

Here we examine the conformational structures of several substrates and several inhibitors for the enzyme within the confines of the protein using the X-ray coordinates for a Gd^{3+} sulfate form of the enzyme[8] and kinetic data[2,6,9] as a starting point. We also provide a structure for the Li^+ ion-inhibited enzyme–phosphate product complex. This complex may be the inactive species that is formed during the treatment of manic depression.[10] Furthermore, evidence is presented which suggests that $Mg^{2+}1$ does not provide the site for the nucleophilic water molecule,[11–13] but rather that $Mg^{2+}2$ activates the nucleophile. The analysis indicates that inositol monophosphatase should operate *via* a direct adjacent displacement mechanism in which the stereochemical configuration of chiral phosphate in the substrate would be preserved in the product. We also provide evidence to suggest that the 'catalytic' oxygen functionality does not directly chelate to $Mg^{2+}2$ but rather serves as an H-bond acceptor for the nucleophilic water molecule that is attached to, and activated by, $Mg^{2+}2$. In the analysis and interpretation of our findings, a detailed comparison is made with the recent findings of the Merck, Sharp and Dohme group.[11–13] In broad terms there is much agreement, although there are significant differences in opinion regarding the exact hydrolytic mechanism. These are high-lighted at the appropriate place throughout the text.

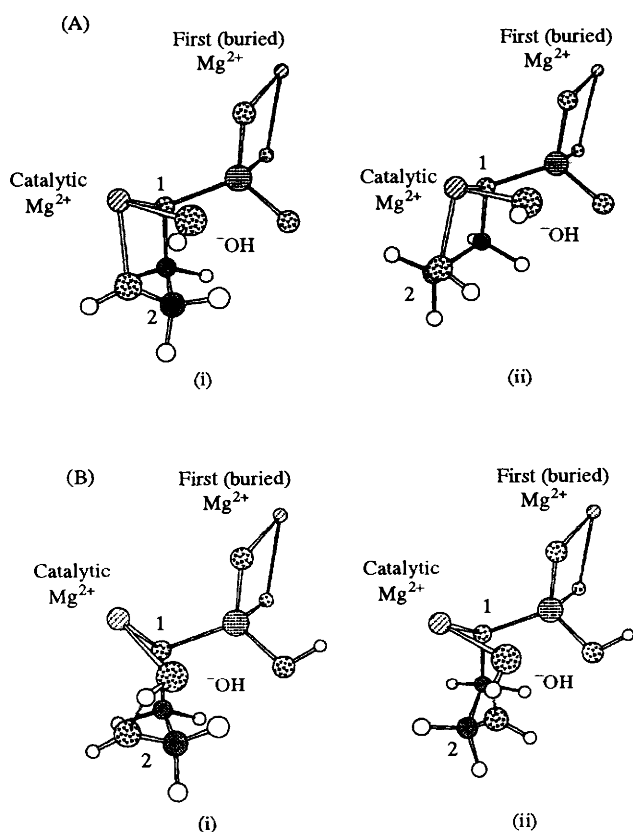
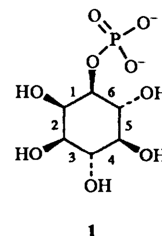


Fig. 1 Proposed role of metal ions according to Cole *et al.* [5,6,9] The catalytic Mg^{2+} ion interacts directly with the bridging phosphate ester O-atom and positions the nucleophilic water molecule for attack on the P-atom. The 2-OH group could also interact directly with metal ion through chelation (A; i and ii), or indirectly by H-bonding to the water molecule (B; i and ii).

Results

Comparison of tertiary structures of human and bovine enzyme

Most of the reported kinetic data for inositol monophosphatase were obtained using the enzyme from bovine brain.[2–7,9,10,14–18] The published X-ray crystal coordinates for inositol monophosphatase, however, were obtained from diffraction data collected for a Gd^{3+}–sulfate form of the cloned human brain enzyme[8] and more recent X-ray data were also obtained for complexes of the human enzyme.[11–13] Both proteins are homodimers of ~ 30 000 Da subunits and the bovine enzyme comprises 277 residues per subunit and differs from the human enzyme in thirty-two residues (~ 11.5%). Of these, twenty two can be described as highly conservative mutations. Here there is little change in the type of functional group in the side-chain (*e.g.*, Leu to Val, Lys to Arg). Moreover, only four residues from each subunit lie within 10 Å of either of the two active sites. Specifically Val-40, Phe-183, Ser-192 and Val-198 are replaced by Ala, Leu, Gly and Leu, respectively, in the bovine enzyme. Of these four variable residues, Val-(Leu)-198 resides the closest to the substrate binding cleft at 6.0 Å. None of the mutations would be expected to perturb the substrate-binding conformations or affect the enzyme mechanism and this was confirmed by optimising the structures of the bovine and human enzyme using the X-ray structure derived from the human enzyme; see Experimental section. Overlaying of the two optimised structures revealed that there were no differences between the coordinates for the key residues in the substrate-bound forms of human and bovine inositol monophosphatase. Thus, it is reasonable to assume that arguments regarding the mechanism of the enzyme will be equally relevant to both forms.



Binding of D-inositol 1-phosphate and Mg^{2+} to inositol monophosphatase

The optimised complex for D-Ins 1-*P* **1** is shown in Fig. 2, and was generated from the published 2.3 Å resolution X-ray crystal structure for a Gd^{3+}–sulfate form of the human enzyme.[8] Gd^{3+} and sulfate are competitive inhibitors for Mg^{2+} and phosphate, respectively, and, therefore, it is reasonable to expect that the inhibitory ions bind in the same sites as Mg^{2+} 1 and phosphate in the enzyme product complex.[8,15]

In order to determine the structure of the active complex, the Gd^{3+} ion in the crystal structure was replaced by Mg^{2+} and the phosphate group of the substrate was placed so that the P-atom occupied the position occupied by the S-atom of the sulfate dianion in the binding cleft in the published structure.[8] The dihedral angles of the substrate were then adjusted in accord with available data on the kinetic properties of substrates and substrate analogues for IMPase and a second Mg^{2+} ion was placed in a site created by the phosphate bridging O-atom of the substrate and three aspartate residues from the protein. This position corresponded closely to that required from the analysis of structure–activity relationships, as described in the preceding article.[6] The positions of both metal ions, the substrate and the active-site residues were then optimised following the protocols described in the Experimental section. As noted above, the structures generated for the human and bovine enzyme were virtually identical in the vicinity of the active site.

The position of one of the metal ions (Mg^{2+} 1, site 1) in the D-Ins 1-*P* complex closely corresponds to that for the crystallographic Gd^{3+} ion in a recently published X-ray structure for the Gd^{3+}–D-Ins 1-*P* enzyme–substrate complex.[11,12] Mg^{2+} 1 coordinates to the side-chain carboxylate groups of Glu-70 and Asp-90, the hydroxy group of Thr-95 and to the carbonyl O-atom of Ile-92 as well as to two of the non-bridging phosphate O-atoms, Fig. 3. One coordination site on Mg^{2+} 1 is vacant and lies on the opposite face from that to which phosphate is attached. In the published Gd^{3+}–sulfate structure[8] this site is occupied by a protein-enclosed water molecule and is hydrogen bonded to Asp-47. This water molecule (which was subsequently added to complete the coordination sphere of Mg^{2+} 1; see Fig. 3) appears to be unable to interact with the phosphate group of the substrate and cannot reach the bulk solvent. Another water molecule which is attached to Mg^{2+} 1 and H-bonds to Thr 95 in the Gd^{3+}–D-Ins 1-*P* X-ray structure[12] may be displaced by one of the inorganic phosphate O-atoms in the product complex. Indeed, in a recently published X-ray crystal structure for a di-Mn^{2+} ion complex with phosphate, one of the phosphate O-atoms occupies this position.[13] The site for Mg^{2+} 1 lies at the bottom of the active-site cleft and, after the first coordination sphere, the entire site for the Mg^{2+} ion is encapsulated by the protein. A long α-helix (residues 44–61) runs along the bottom of the protein and prevents large movements in the ligands for Mg^{2+} 1. Access of solvent to the site, other than from the top of the active-site cleft, would require large movements in the protein backbone. It appears that Mg^{2+} 1 would enter the active site

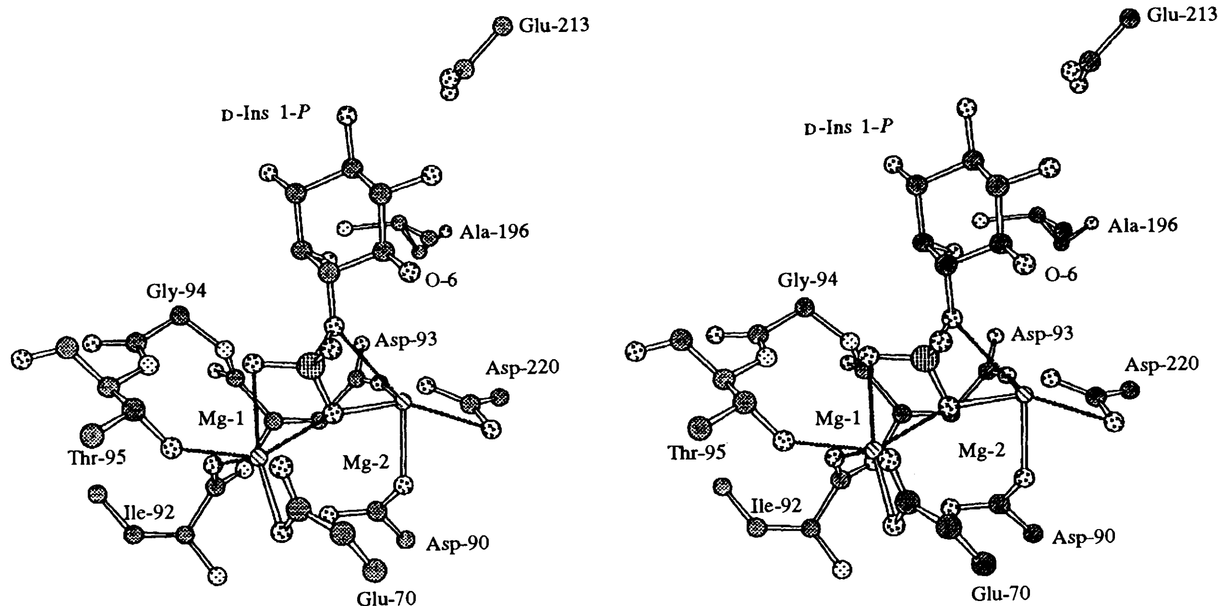


Fig. 2 Stereo view of the optimised active-site structure for the bound Mg^{2+}–D-Ins 1-*P* complex showing the interactions with key amino acid residues (see text for details)

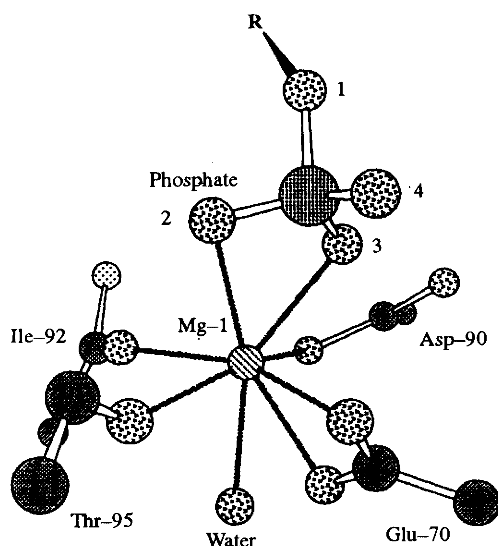


Fig. 3 The primary coordination sphere of $Mg^{2+}1$ in the optimised Mg^{2+}–enzyme–substrate complex

through the top of the active-site cleft, before any of the other species, and would not be able to escape between individual catalytic events at saturating concentrations of substrates. This sequence of events is entirely consistent with the results of kinetic studies (Scheme 1)[2] which, in the context of a two-Mg^{2+} ion mechanism, show that the binding of the substrate to the enzyme occurs after $Mg^{2+}1$ and before $Mg^{2+}2$.[6]

A second metal site for Mg^{2+} is formed in the enzyme–D-Ins 1-*P* substrate complex comprising ligands from both the enzyme and the substrate; see Table 1. $Mg^{2+}2$ coordinates to the enzyme through the carboxylate groups of Asp-90, Asp-93 and Asp-220, and to substrate through one of the three equivalent phosphate O-atoms and to the bridging ester O-atom, Fig. 4. Again one face of the metal is free, but in this case it is accessible to bulk solvent. The inositol ring binds in the active-site cleft through its hydroxy groups by forming several hydrogen bonds, one from the 4-OH group to the carboxylate group of Glu-213, one from the 2-OH group to the side-chain of Asp-93 and one from the backbone NH of Ala-196 to the 2-O-

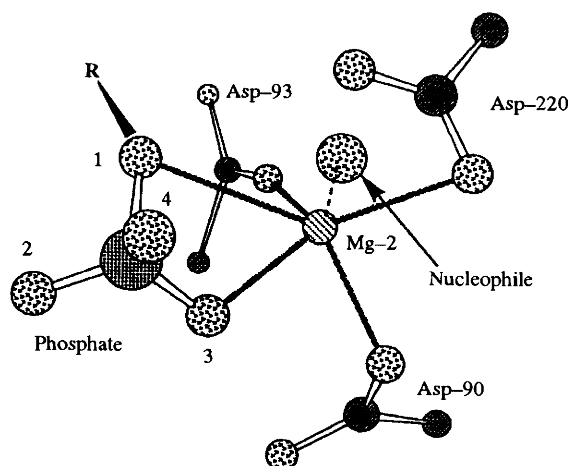
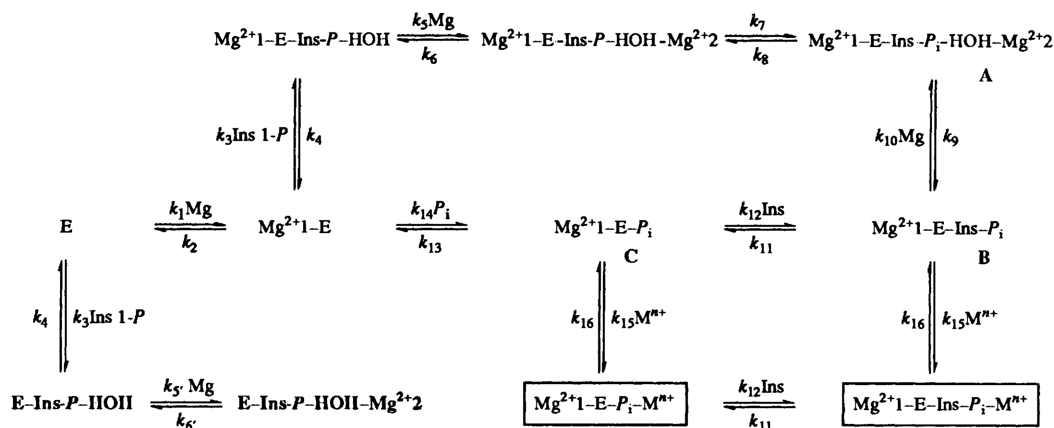


Fig. 4 The primary coordination sphere of $Mg^{2+}2$ in the optimised Mg^{2+}–enzyme–substrate complex

atom. The residue Asp-220, in addition to providing a ligand for $Mg^{2+}2$, also accepts a hydrogen bond from the hydroxy group at C-6. These interactions are in accord with the findings of recent crystallographic and modelling studies.[11,12]

The phosphate ester binds to both metals and to the backbone NH moieties of Thr-95 and Gly-94; see Table 1. The 6-OH group of D-Ins 1-*P*, described on the basis of kinetic data as the 'catalytic' OH group,[10] does not form any direct interaction with the $Mg^{2+}2$ ion and lies some 4.02 Å away (Fig. 4). This result was initially somewhat surprising given the volume of information describing its importance[9] and the results of the Merck group[11] which showed a direct 6-O-$M^{2+}2$ interaction in modelling studies of a di-Mn^{2+}–Ins 1-*P*–enzyme complex (see below), but is completely consistent with the kinetic behaviour of several substrates and substrate analogues.[6] The catalytic hydroxy group is positioned to coordinate to a water molecule and that, in turn, completes the coordination shell of $Mg^{2+}2$ to give approximate octahedral geometry. This water molecule is well placed to function as the nucleophile in the hydrolytic reaction, Fig. 4. The water molecule is also within hydrogen-bonding distance of the carboxylate group of Asp-220 which may act as a base (Fig. 5).



Scheme 1 Full kinetic scheme for catalysis and for inhibition (where M^{n+} = Li^+ or Mg^{2+}). The concentration of the bold complexes become significant for mutant enzymes which possess a low binding affinity for Mg^{2+} at site 1. These dead-end complexes are inactive and reduce the apparent value of k_{cat} although the actual magnitude of the rate constant for the hydrolytic step (k_7) could be identical in the wild-type and mutant enzymes. The fact that the steady-state concentrations of the complexes **B** and **C** [which Li^+ (and Mg^{2+}) is able to trap to give uncompetitive inhibition (boxed complexes)] are similar in the wild-type and Gln-70 and Asp-70 mutants indicate that Glu-70 in the native enzyme only plays a role in binding to Mg^{2+}1. Note that, in the absence of inositol, the concentration of complex **B** is very low; steps 7 and 13 are each partially rate-limiting. Also note that inositol can debind from complex **A** but this parallel pathway is not shown for the sake of simplicity. See text for further details.

Table 1 Interactions in the ground-state complexes of inositol monophosphatase with its substrates, both metal ions and water molecules; see Figs. 2, 3, 4 and 18B, and 10 and 11

Substrate interactions	D-Ins 1-*P*	L-Ins 1-*P*	D-Ins 4-*P*	2′-AMP
Asp-93 (O-4)	3.35 (O-2)	3.14 (O-6)	4.11 (O-3)	3.29 (O-3′)
Gly-94 (NH)	3.06 (O2-P)	2.99 (O2-P)	2.92 (O2-P)	3.10 (O2-P)
Thr-95 (NH)	2.84 (O2-P)	2.77 (O2-P)	2.83 (O2-P)	2.79 (O2-P)
Ala-196 (NH)	3.01 (O-2)	3.00 (O-6)	4.05 (O-3)	3.25 (O-3′)
Glu-213 (O-5)	3.11 (O-4)	3.07 (O-4)	2.80 (O-1)	2.57 (O-5′)
Asp-220 (O-4)	3.07 (O-6)	3.42 (O-2)	4.16 (O-5)	2.78 (OW2)
Mg^{2+}1	2.23 (O3-P)	2.18 (O3-P)	2.31 (O3-P)	2.19 (O3-P)
Mg^{2+}1	2.46 (O2-P)	2.57 (O2-P)	2.30 (O2-P)	2.34 (O2-P)
Mg^{2+}2	2.11 (O3-P)	2.04 (O3-P)	2.12 (O3-P)	2.13 (O3-P)
Mg^{2+}2	2.73 (O1-P)	2.77 (O1-P)	2.41 (O1-P)	2.70 (O1-P)
Mg^{2+}2	4.02 (O-6)	4.31 (O-2)	4.49 (O-5)	4.98 (OW2)
Mg^{2+}2	2.09 (nucleophile)	2.25 (nucleophile)	3.20 (nucleophile)	2.29 (nucleophile)
Metal–protein interactions				
Mg^{2+}1	2.19 (Glu-70)	2.21 (Glu-70)	2.19 (Glu-70)	2.19 (Glu-70)
Mg^{2+}1	3.09 (Asp-90)	3.09 (Asp-90)	3.18 (Asp-90)	3.28 (Asp-90)
Mg^{2+}1	2.24 (Ile-92)	2.24 (Ile-92)	2.24 (Ile-92)	2.24 (Ile-92)
Mg^{2+}1	2.32 (O-3 Thr-95)	2.35 (O-3 Thr-95)	2.31 (O-3 Thr-95)	2.27 (O-3 Thr-95)
Mg^{2+}2	2.15 (Asp-90)	2.16 (Asp-90)	2.12 (Asp-90)	2.14 (Asp-90)
Mg^{2+}2	2.19 (Asp-93)	2.20 (Asp-93)	2.17 (Asp-93)	2.20 (Asp-93)
Mg^{2+}2	2.21 (Asp-220)	2.21 (Asp-220)	2.22 (Asp-220)	2.22 (Asp-220)

In the modelling work performed by the Merck group[11] and referred to above, four different models of the enzyme–Ins 1-*P* complex containing two different metal ion occupancies in sites 1 and 2, respectively, were examined; a Gd^{3+}/Li^+ model (with and without Li^+ bound in a third site) and an Mn^{2+}/Mn^{2+} model (with and without Mn^{2+} bound in a third site). In one of the models, the di-Mn^{2+}–Ins 1-*P*–enzyme complex, there was a stable interaction during molecular dynamics between the 6-O-atom of the substrate and Mn^{2+}2 but at the expense (weakening or removal) of the interaction of the 2-hydroxy group with Asp-93. In the Gd^{3+}/Li^+ models these contacts were reversed and similar to our own findings for the di-Mg^{2+} complex, Fig. 4. Thus, there is a consensus that the interaction of the 2-hydroxy group with Asp-93 and the *direct* interaction of the 6-O-atom of the substrate with M^{2+}2 cannot be maintained simultaneously. However, in our model both sets of interactions are maintained, the latter indirectly, through a hydrogen bond between the 6-OH group and the Mg^{2+}2-chelated hydrolytic water molecule.

Also relevant to the mechanism of the hydrolytic reaction, the N-terminal (positive pole)[19,20] of an α-helix spanning residues 195–205 is directed at the phosphate ester group, which could aid in binding, while the N-terminal of an α-helix spanning residues 95–100 is directed at the bridging phosphate ester O-atom and at the nucleophilic water molecule. The former O-atom develops negative charge during the reaction and becomes the alcoholate leaving group while the latter is expected to attack the P-atom by ionising to become hydroxide ion. Thus, these interactions with the helix[19,20] would be expected to stabilise the transition state.

The di-Mg^{2+}–D-Ins 1-*P*–complex containing the nucleophilic water molecule was subjected to 7 ps of molecular dynamics at 300 K and the final geometry was reoptimised. It was found that both the substrate and the proposed nucleophile retained their approximate positions, and that all of the interactions described above were retained throughout the simulation. It is worthy of note that the nucleophilic water molecule has an unimpeded pathway into the bulk solvent of the active site. Table 1 lists the key interactions.

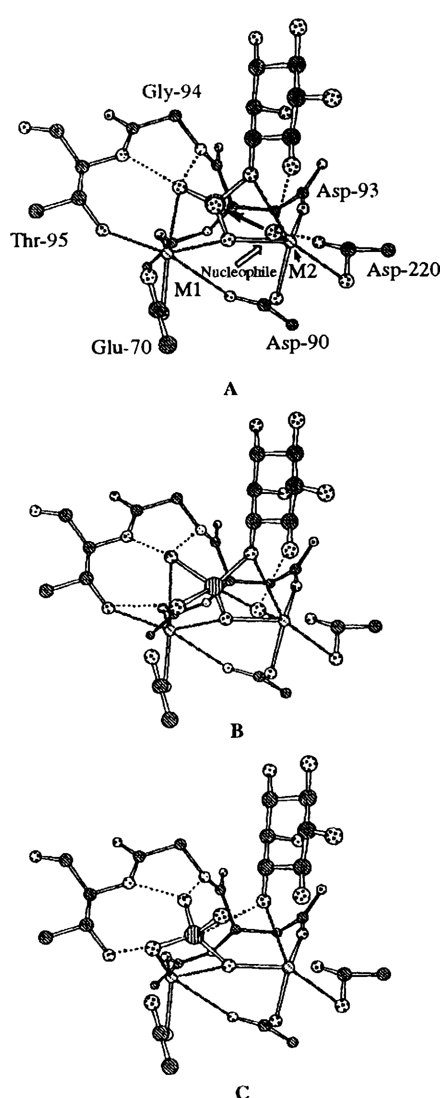


Fig. 5 Possible adjacent displacement (with pseudorotation) and in-line displacement mechanisms for the inositol monophosphatase reaction with D-Ins 1-*P* as the substrate. For both mechanisms the positions of the Mg^{2+} ions in sites 1 and 2 and the positions of the inositol-derived atoms are similar but there are small differences in the positions of the side-chain of Thr-95 and the position of the phosphate group in the ground states **A**. The position of the nucleophile, indicated by an open arrow, differs considerably for the two mechanisms and forms the sixth ligand for Mg^{2+}2 in Fig. 5 and the sixth ligand for Mg^{2+} 1 in Fig. 6 (qv.). Note that one water ligand for Mg^{2+}1 which resides at lowest site of the approximate octahedron is omitted in both schemes for the sake of clarity.

In Fig. 5 the nucleophile is H-bonded to the 6-OH of the substrate and is activated and positioned by the metal ion and Asp-220. Attack on phosphorus occurs from opposite the O-atom that is H-bonded to the backbone NH moieties of Gly-94 and Thr-95. The P–O bond lengths increase as the nucleophile attacks and the unbound phosphate O-atom moves towards Mg^{2+}1 such that its position is stabilised by interactions between the side-chain hydroxy group of Thr-95 and Mg^{2+}1 in the stable intermediate **B**. The O–1–P bond then begins to lengthen further and the other phosphate group P–O bonds shorten. The O-atom of the nucleophile moves out of the coordination sphere of Mg^{2+}2 as O–1 of the substrate, stabilised as alkoxide, becomes protonated by (the now acidic) Asp-220, possibly *via* the intermediacy of the 6-OH group, to give the product complex **C**. The interaction of Mg^{2+}1 and the side-chain of Thr-95 with the pseudorotated phosphate O-atom then resembles those observed in an X-ray crystal structure for the di-Mn^{2+}–phosphate product complex.[18] The inositol product and Mg^{2+}2 then depart from the active site in a random order, followed by the phosphate group.

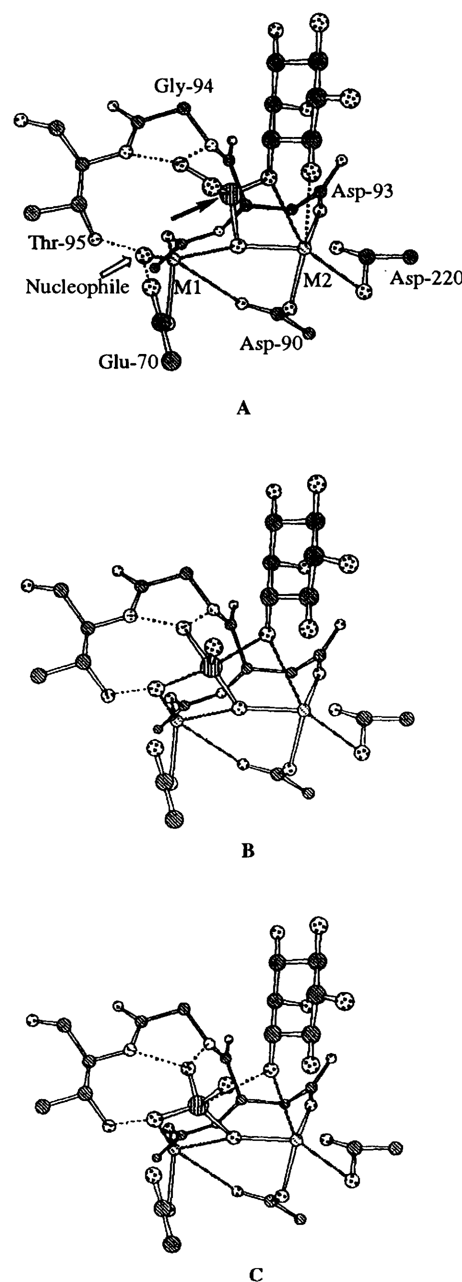


Fig. 6 As proposed by the Merck group, the nucleophile is H-bonded to the 3-OH group of Thr-95 and is activated and positioned by Mg^{2+}1 and Glu-70. Attack on phosphorus occurs from opposite the 1-O-atom of the substrate to give a stable, trigonal bipyramidal intermediate or transition state, **B**. The 1-O-atom of the substrate, stabilised as alkoxide, then moves away from the P-atom to give the product ground state, **C**, containing an inverted phosphate group. This structure appears to be very similar for both mechanisms. The 1-O-atom of the substrate is subsequently protonated, possibly by bulk solvent in the active site, and the products are released as for Fig. 5.

Overall, this analysis of the binding arrangement for D-Ins 1-*P* correlates closely with the crystal structure for the inactive enzyme–Gd^{3+}–D-Ins 1-*P* complex recently described by Pollack and co-workers[11,12] and also fits the known kinetic data, substrate-binding data and the established structural requirements for catalysis. However, Pollack, Bone and co-workers[11–13] suggest a different hydrolytic mechanism (Fig. 6) in which Mg^{2+}1 chelates the attacking nucleophile; see below.

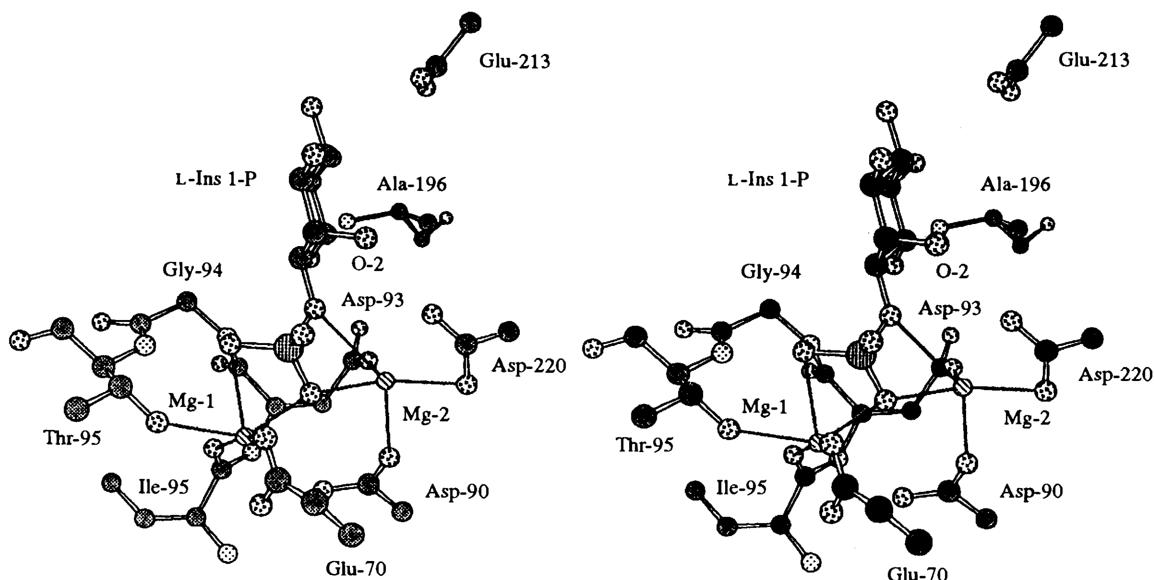
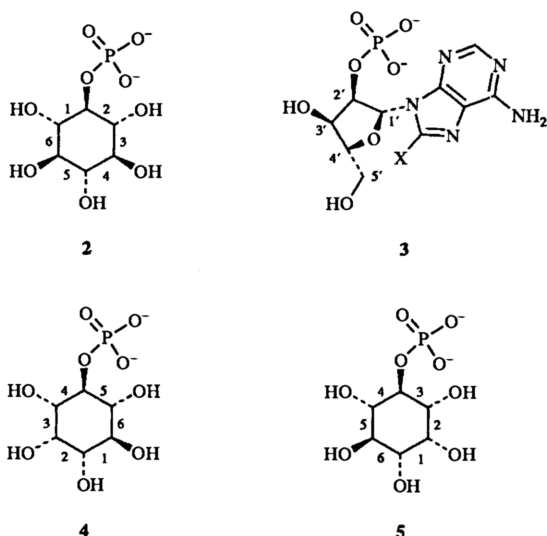


Fig. 7 Stereo view of the optimised active site structure of the Mg^{2+}–L-Ins 1-*P* complex showing key enzyme–substrate interactions



Binding of alternative substrates

The modes of binding of the alternative substrates L-inositol 1-phosphate (L-Ins 1-*P*) **2** and adenosine 2′-phosphate (2′-AMP) **3** (X = H) and both enantiomers of *myo*-inositol 4-phosphate (Ins 4-*P*) (**4** and **5**) to inositol monophosphatase (IMPase) were also examined. The enzyme displays similar binding affinities and reaction rates for D-Ins 1-*P* **1** and L-Ins 1-*P* **2**,[12] and, therefore, the two molecules might be expected to bind in a similar manner, despite the apparent differences in their structures, as indicated in the preceding article.[6] 2′-AMP **3** (X = H), on the other hand, is known to bind 10-fold less tightly than Ins 1-*P*, as a substrate and as an inhibitor, and on the basis of our earlier analyses[9] was expected to exist in a high-energy enzyme-bound arrangement.

To optimise the binding interactions for both L-Ins 1-*P* and 2′-AMP, as before, the phosphate ester moiety was centred on the crystallographic SO_4^{2-} position using the X-ray coordinates of the published Gd^{3+} structure as a starting point.[8] The remainder of each molecule was then rotated to give a binding mode consistent with known kinetic and thermodynamic data acquired from analysis of substrate and inhibitor structure–activity relationships.[3,4,6,9] The binding interactions with both metal ions and the enzyme were then optimised for each system using the procedures detailed in the Experimental section. The optimised structures are shown in Figs. 7 and 8.

For both substrates, the positions of the phosphate ester moiety and each of the metal ions deviate only slightly from their respective positions in the D-Ins 1-*P* complex, Figs. 7 and 8. The 4-OH group of L-Ins 1-*P* (Fig. 7) interacts with the carboxylate group of Glu-213 in accord with expectations. Note that the mutation of Glu-213 to a glutamine residue increased the value of K_m for both D-Ins 1-*P* and L-Ins 1-*P* by a factor of 20- and 10-fold, respectively.[12] The corresponding 5′-OH group of 2′-AMP (Fig. 8) also formed a hydrogen bond with Glu-213. Despite the change in stereochemistry, the 6-OH group of L-Ins 1-*P* was able to hydrogen bond to the carboxylate group of Asp-93 and, relative to the structure for D-Ins 1-*P* (Fig. 7), the entire inositol ring of the L-enantiomer was rotated by 55° about the C(1)–C(4) axis. This arrangement is qualitatively similar to the results of preliminary modelling studies performed by the Merck group[3a] and more recent in-depth studies.[11]

The analogous 3′-OH group of 2′-AMP likewise appears to form a hydrogen bond to the carboxylate group of Asp-93, but is also sufficiently close to coordinate to Mg^{2+}, and displaces the non-bridging phosphate O-atom from the coordination sphere of the metal ion. The 'catalytic' 2-OH group of L-Ins 1-*P* serves the same function as the 6-OH group of its D-antipode, that is as a hydrogen-bond donor/acceptor to the catalytic water molecule chelated to $Mg^{2+}2$. Thus, the structure for the complex of L-Ins 1-*P* is very similar to that for the D-antipode except that the inositol ring rotates by 55° about the C(1)–C(4) axis to optimise the number of H-bonds it can form with the enzyme (see Table 1). These findings are again very similar to those reported recently for the crystal structure of an inactive Gd^{3+}–L-Ins 1-*P* enzyme complex.[12]

The situation for 2′-AMP, however, is rather different. As originally proposed by Cole and Gani[5,9] in rationalising the substrate specificity of the enzyme, the ribofuranosyl ring O-atom can fulfil the same catalytic role as the 6-OH group of D-Ins 1-*P* through the ribose ring adopting an otherwise high-energy conformation in the presence of a metal ion; see Fig. 9 and Table 2. The enzyme-bound complex (Fig. 8) shows the

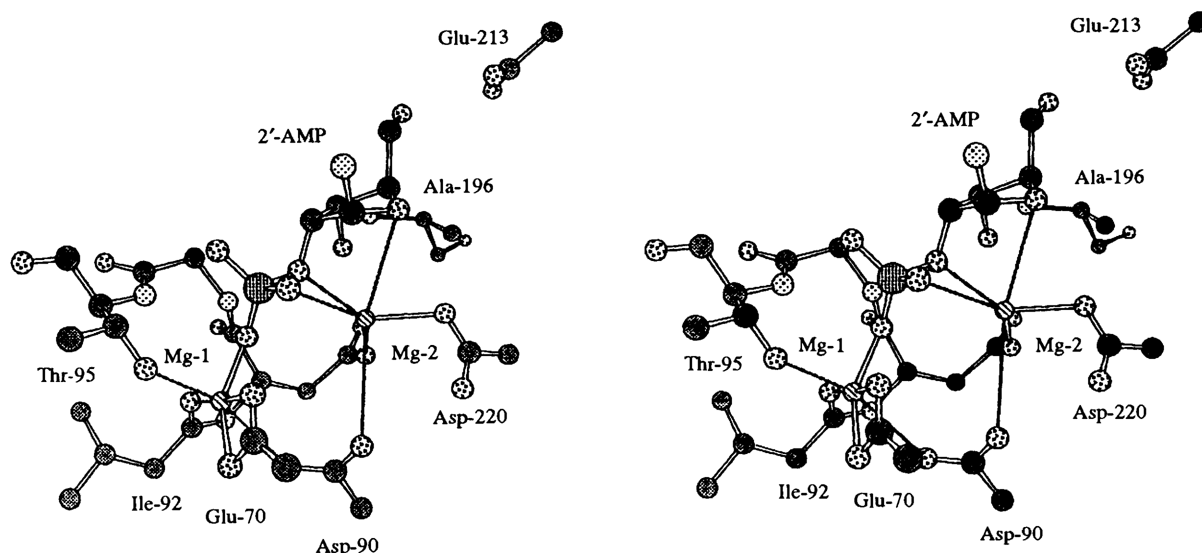


Fig. 8 Stereo view of the optimised active site structure of the Mg^{2+}–2'-AMP complex showing key enzyme–substrate interactions. This structure does not contain added water molecules; see Experimental section for details.

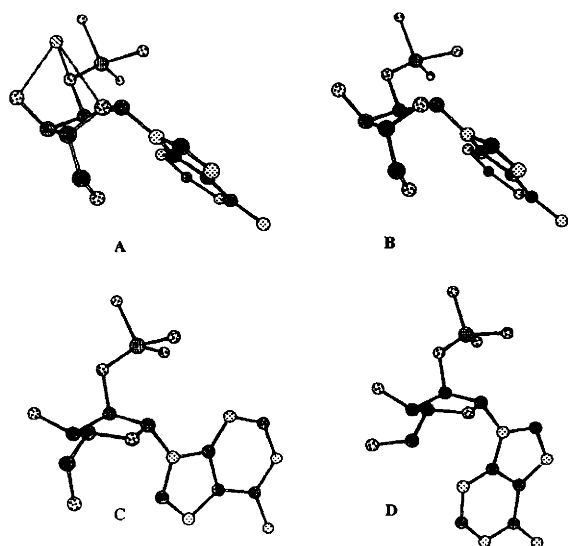


Fig. 9 Conformations and energies of 2'-AMP; A, M^{2+}-stabilised conformation; B, 'high-energy' form in absence of the metal ion (− 19.77 kJ mol^{-1}); C, ground-state conformation (−44.94 kJ mol^{-1}); and D, enzyme-bound conformation. Similar calculations were performed for adenosine, see Table 2 and Experimental section.

ribose ring in a similar 'high-energy' conformation, stabilised by an interaction with Mg^{2+}2, but the Mg–O distance is rather long at 3.2 Å. By comparison, the 6-OH and 2·OH groups respectively of D-Ins 1-*P* and L-Ins 1-*P* do not coordinate directly to Mg^{2+}2, but rather to a single water molecule in the coordination shell of Mg^{2+}2 (Figs. 2 and 7) The long Mg–O distance needed some further analysis and this was performed by considering the known properties of other substrates, and the binding conformations for D- and L-Ins 1-*P* (Figs. 2 and 7).

In our original analysis (in the absence of the protein) we had believed that the 1'-O-atom might interact directly with Mg^{2+}2, in order to stabilise the unfavourable conformation of the ribose ring, and form part of metal ion's binding site.[5,9] Within the protein environment, however, it becomes clear that such an interaction disturbs several others including the interaction between Mg^{2+}2 and the 2'-O-atom. Moreover, because Mg^{2+}2 forms four strong interactions with Asp-90, Asp-93, Asp-220 and one non-bridging phosphate O-atom, the approach of the 3'-OH and 1'-O-ether groups of the substrate fills its coordination sphere and leaves no room for the nucleophile. An alternative suggestion that was also consistent with the available kinetic data was that the ribofuranosyl ring O-atom might interact with Mg^{2+}2 indirectly through an Mg^{2+}2-chelated water molecule.[9] Such a situation could still fulfil the kinetic requirement that the bound species 2'-AMPS and, probably, 2'-AMP should exist in high-energy arrangements (see below) but, in this latter situation the internal conformation energy of the substrates would be reduced at the expense of decreasing the entropy of the bound water molecule.

Accordingly, the structure for the enzyme complex for 2'-AMP was modified by placing a catalytic water molecule on Mg^{2+}2, in the same position as it is in D- and L-Ins 1-*P* complexes. The energy calculations were then re-performed. The resulting structure changed slightly from that shown in Fig. 8. However, the energy of the entire system was reduced further, and interactions between the enzyme and the substrate were improved further, by including another water molecule (OW2) between the nucleophilic water molecule (OW1) and the 1'-O-atom of the ribofuranosyl ring. This minimum-energy structure was subjected to molecular dynamics simulations and was reasonably stable over a short time period (4 ps). In particular, both water molecules retained their positions and their hydrogen-bonding network despite being accessible to the bulk solvent in the active site. The optimised structure (Fig. 10) was still very similar to that shown in Fig. 8 and retained all of the key H-bonding interactions between the peripheral hydroxy groups of the ribofuranosyl ring and the enzyme although the H-bond length for the interaction of the 5'-OH group within Glu 213 shortened to 2.57 Å. The most notable differences were that the 3'- and 1'-O-atoms of the substrate moved out of the coordination sphere of Mg^{2+}2 and the bridging 2'-O-atom of the phosphate ester moved back in, see Fig. 10 and Table 1. The position of the phosphate ester matched that for D- and L-Ins 1-*P* exactly with the nucleophilic water (OW1) properly aligned for attack on the P-atom. Indeed, the immediate environments of the Mg^{2+}2 ion in each of the three structures (Figs. 2, 7 and 10) were almost indistinguishable. The ribofuranosyl ring retained some of its puckered character but the 1,3-diaxial arrangement of the adenine and the 5'-hydroxymethyl group (Figs. 7 and 9) relaxed so that both groups moved towards equatorial positions. This is in line with the predicted outcome

Table 2 Calculated energies for various bound and unbound conformations of 2'-AMP and adenosine

	Metal-bound (E/kJ mol^{-1})	M^{2+}-free (E/kJ mol^{-1})	Ground state (E/kJ mol^{-1})	Difference[a] (E/kJ mol^{-1})	System	Force-field/ Dielectric
1	−316.89	50.79	−40.92	91.7	2'-AMP	CVFF, 1.0
2	106.72	408.68	53.67	355.0	adenosine	CVFF, 1.0
3	−2030.14	−711.27	−960.86	249.6	2'-AMP	AMBER, 1.0
4	−776.35	−247.54	−317.76	70.2	adenosine	AMBER, 1.0
5	−200.35	−19.77	−44.94	25.2	2'-AMP	AMBER, 4.0
6	−75.16	22.45	13.21	9.2	adenosine	AMBER, 4.0
7	n/a	−40.76	−60.9	20.1	2'-AMP/2H_2O	AMBER, 4.0
8	n/a	40.88	15.26	25.6	adenosine/2H_2O	AMBER, 4.0

[a] Energy of the M^{2+}-free bound form minus energy of the ground-state form. Entries 7 and 8 refer to the binding conformations of 2'-AMP and adenosine within the active site of the enzyme as deduced from modelling studies described above.

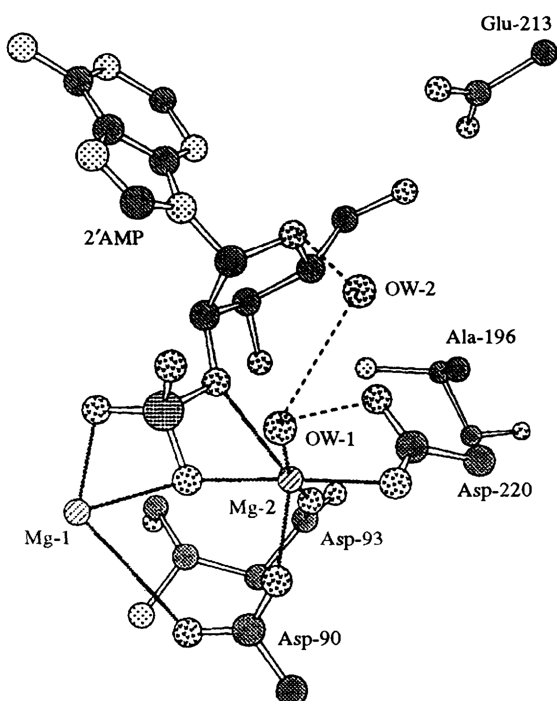


Fig. 10 Optimised active-site structure for the Mg^{2+}-2'-AMP reactant complex with both the nucleophilic and intermediate water molecules. The adenine moiety has been deleted for clarity.

for the removal of the 1'-O-atom from the coordination sphere of the metal ion;[5,9] also see Table 2 and Experimental section. The position of the second water (OW2) in the optimum structure for 2'-AMP (Fig. 10) was coincident with the 6-OH of D-Ins 1-*P* and the 2-OH of L-Ins 1-*P* and was stabilised by H-bonding between the 1'-O-atom and the nucleophilic water. Thus, the 'catalytic O-atom' for 2'-AMP and, indeed, for other cyclic-ether-containing phosphates, see below, appears to be the O-atom of a water molecule that is H-bonded to the substrate.

The structure of the bound 2'-AMP molecule depicted in Fig. 10, when removed from the active site, does not occupy a high-energy conformation and is only 20.1 kJ mol^{-1} less stable than the minimum-energy conformer; see Fig. 9 and Table 2. However, within the protein environment, two water molecules appear to be required to stabilise this conformation and thus the arrangement could still occupy an elevated energy state due to the loss of entropy associated with fixing of the positions of the water molecules. Recall that several lines of evidence point to the likelihood that 2'-AMPS and 2'-AMP exist in elevated-energy arrangements[9] compared with inositol 1-phosphates and 4-phosphates and that it is necessary (ultimately) for the model to be able to account for all of the differences.

First, the ternary enzyme-Mg^{2+}1-2'-AMP complex shows a low binding affinity for Mg^{2+}2 compared with complexes derived from *myo*-inositol 1-phosphate (Ins 1-*P*) and Ins 4-*P*.[2] In this paper[2] the differences in the K_i-values for Mg^{2+}1 and Mg^{2+}2 were detected as cooperativity effects where double-reciprocal plots of initial rate *versus* Mg^{2+} ion concentration show marked curvature. These differences are consistent with the models proposed here. Second, K_m- and K_i-values for 2'-AMP and other nucleoside 2'-phosphates[9] and several other tetrahydrofuran polyol phosphates that do not contain a nucleoside base[7] fall in a narrow range (0.9 to 4.0 mmol dm^{-3}) and are 10-fold or more higher than for Ins 1·*P*, although the values of V_{max} for the substrates are very similar and fall in the narrow range of 60–200% of V_{max} for Ins 1-*P*. These results indicate that the bound form of 2'-AMP (and other tetrahydrofuran phosphates) is as well set up for reaction as the bound form of Ins 1-*P* and, therefore, that the substrates differ in only their equilibria between the bound and unbound forms. Third, inositol phosphorothioates are good substrates for the di-Mg^{2+}-enzyme but 2'-AMPS is not and also fails to act as an inhibitor.[5,9] This result indicates that the energy gained from the interaction of the phosphorothioate group in 2'-AMPS with its binding site is not sufficient to overcome the energy penalty associated with binding, in contrast to the case for 2'-AMP. Fourth, both 2'-AMP and 2'-AMPS are good substrates for the di-Mn^{2+}-enzyme.[5,9] Finally, inositol serves as a product inhibitor and mediates the exchange of ^{18}O-label from [^{18}O] water into inorganic phosphate.[2] Adenosine shows neither of these properties and is not recognised by the enzyme.[9]

Although the first four points highlighted above are in accord with the analysis presented here, so far we have not been able to account for the fact that adenosine is not recognised by the enzyme as a product inhibitor.[9] This latter property can be no longer ascribed to a possible high-energy conformation in which the adenine moiety and the 5'-hydroxymethyl group are pseudoaxial on the ribofuranosyl ring[5,9] since it is emerging that such a conformation is probably not populated in the bound form of the substrate, 2'-AMP. However, the organisation of the species in the product complex immediately after the hydrolysis of 2'-AMP is likely to be very different to that in the analogous Ins 1-*P* system because the catalytic OH group in the adenosine system (OW2 in Fig. 10 which occupies the same position as the 6-OH group of D-Ins 1-*P*) is not actually covalently attached to the product alcohol.

Kinetic studies show that inositol and Li^+ are independent inhibitors[2] which indicates that the species can bind randomly to the Mg^{2+}1-E-P_i complex (see Scheme 1). Since Li^+ binds in the site occupied by Mg^{2+}2 in the Mg^{2+}1-E-P_i-Mg^{2+}2 complex it is reasonable to believe that inositol on the one hand, and adenosine and water on the other, can debind randomly with Mg^{2+}2 from their respective product complexes, Mg^{2+}1-E-P_i-Mg^{2+}2-Ins and Mg^{2+}1-E-P_i-Mg^{2+}2-Aden-H_2O (not shown explicitly in Scheme 1). If this is the case then to generate

(from the products) the kinetically competent enzyme product complexes that are capable of sustaining ^{18}O-label exchange between the solvent and P_i, it would be necessary to assemble five discrete molecular species in the case of the inositol system (Scheme 1) and six in the case of the adenosine system. Given that the steady-state concentration of E–Mg^{2+}1–P_i would be high in the presence of 2 mmol dm^{-3} Mg^{2+} and 20 mmol dm^{-3} inorganic phosphate (the conditions used for ^{18}O-label exchange experiments)[9] and that the concentration of E–Mg^{2+}1–P_i–Mg^{2+}2 would be very significant, the problem of assembling the kinetically competent product complexes would reduce to simply binding inositol or binding, simultaneously, both adenosine and water. As in the product, adenosine, there is no phosphate group to pay back the energy associated with organising the additional water molecule (equivalent to OW2 in Fig. 10), the formation of the full product complex would be expected to be unfavourable. Thus, the model provides a reasonable explanation for the differential activities of inositol and adenosine in facilitating ^{18}O-label exchange. Note that the H-bonds from the furanose 1′-O-atom to Asp-220 in the substrate complex are provided indirectly by OW2 and by OW1 and that the latter becomes one of the phosphate O-atoms in the product complex, according to our proposed mechanism (Fig. 5). The new phosphate O-atom (derived from OW1) may be able to H-bond with OW2 in the putative pentacoordinate intermediate but, following pseudorotation and expulsion of the 2′-O alkoxide leaving group, the 2′-O-atom would be a better H-bonding partner. Asp-220, which it is expected would activate the nucleophile for attack by removing a proton, could then reprotonate the 2′-O-atom *via* a proton relay involving OW2; see Discussion section below.

Several ribofuranosyl 2′-phosphates including the large 8-bromoadenosine 2′-phosphate **3** (X = Br), are substrates for the enzyme[5,7,9] and it was of interest to consider the structures of these within the active site. The conformation of the adenine moiety in Fig. 10 is such that the purine 8-hydrogen approaches two of the three equivalent phosphate O-atoms quite closely. However, there remains sufficient space to accommodate the extra bulk of an 8-bromo group and to allow rotation of the purine moiety. There is also sufficient space to accommodate the pyrimidine moieties of uracil and dihydrouracil when the ribofuranosyl 2′-phosphate group is bound to the enzyme as shown in Fig. 10. Hence, the binding conformation of the ribofuranosyl 2′-phosphate moiety accounts for the known substrate activity of the 2′-phosphates of 8-bromoadenosine, 5,6-dihydrouridine and uridine,[9] and, indeed, also guanosine 2′-phosphate (2′-GMP).[1]

The binding conformation in the Michaelis complex for each enantiomer of the other natural substrate, Ins 4-*P* (**4** and **5**), was determined using similar protocols to those described above, starting from the optimised structures for both D-Ins 1-*P* (Fig. 2) and L-Ins 1-*P* (Fig. 7). The *myo*-inositol 4-phosphates differ from both 1-phosphates in that, with respect to the 4-phosphate ester group, both of the flanking adjacent hydroxy groups (3-OH and 5-OH) are equatorial. Unlike the situation for both Ins 1-*P* enantiomers (see above), the adjacent 'binding' hydroxy group of Ins 4-*P* is not able to form strong hydrogen bonds to Asp-93 and Ala-196 while simultaneously maintaining the correct position for the catalytic hydroxy group (Fig. 11A). This is due to the difference in the distance between the flanking hydroxy groups in the two types of substrate. For Ins 4-*P*, the O(3)·O(5) distance is 4.9 Å while the equivalent O(2)·O(6) distance for Ins 1-*P* is 4.2 Å. Nevertheless, Glu-213 can form an H-bond to the distal 1-OH group (see Table 4). There is also an alternative possible binding mode for Ins 4-*P* in which the hydrogen bonds between the adjacent 'binding' hydroxy group and Asp-93 and Ala-196 are retained as for the inositol 1-phosphates. However, here it appears that the position of the 'catalytic' hydroxy group is altered such that the hydroxy group can no longer interact either directly with the Mg^{2+}2-bound nucleophile (Fig. 11B) or, indirectly *via* an intermediate water molecule, as is the case for 2′-AMP, due to obstruction by the phosphate ester group. Thus, only the first binding mode for Ins 4-*P* would be expected to lead to catalytic hydrolysis and by comparison with the inositol 1-phosphates, the 4-phosphates would be expected to display reduced affinity for the enzyme. These predictions are consistent with the observed 3.5-fold increase in K_m for Ins 4-*P* over Ins 1-*P*.[2] Note that the two enantiomers of the 4-phosphate compounds (**4** and **5**) bind to the enzyme in an almost identical conformation because the only stereochemical differences

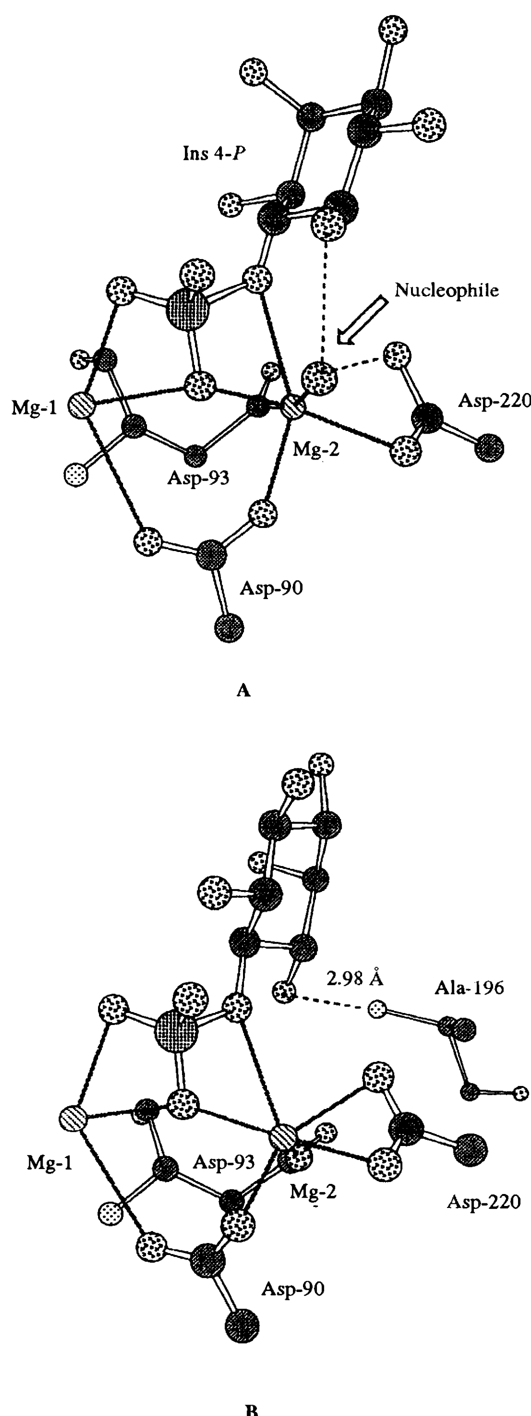


Fig. 11 Optimised active-site structures for two different binding modes of Ins 4-*P* showing key enzyme–substrate interactions

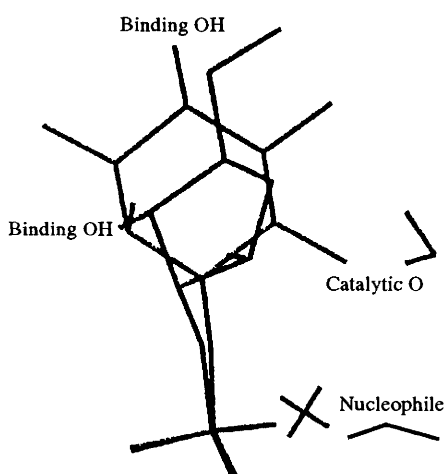


Fig. 12 Overlay of D-Ins 1-*P* and 2'-AMP, including both nucleophilic and intermediate water molecule, showing the congruence of 'catalytic' and 'binding' O-atoms. The adenine moiety has been removed for clarity.

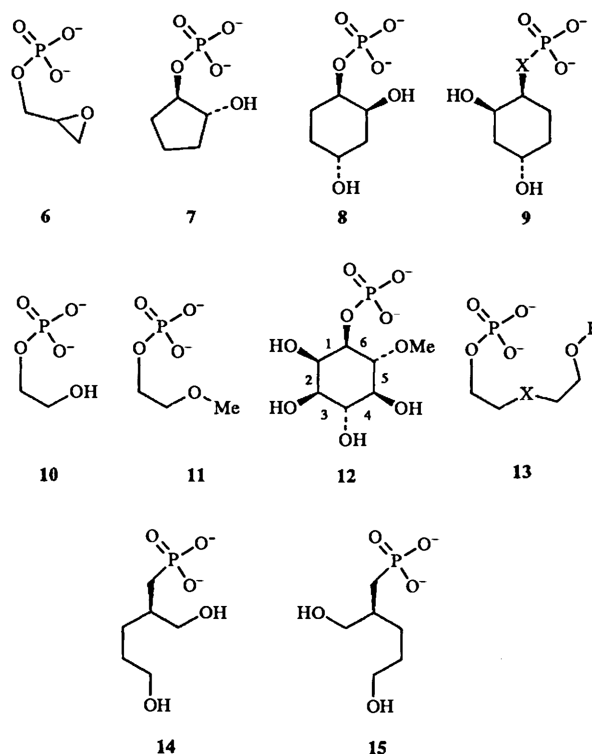
between the two antipodes occur in positions 2- and 6- of the ring. These positions are equivalent to positions 3- and 5- in D-Ins 1-*P* **1** and do not form specific interactions with the enzyme.[3,4]

Collectively, it appears, the optimised structures for each of the substrates highlight the requirements of the 'catalytic' group on the substrate. In each of the four structures, a lone pair on the catalytic O-atom of the substrate [which is an H-bonded water molecule (OW2) for 2'-AMP] provides an anchor for the nucleophilic water molecule that is chelated to $Mg^{2+}2$ (see overlay in Fig. 12). This analysis refines our earlier examination, which was performed in the absence of the protein environment, and suggests that it is the accessible conformations of seven-membered H-bonded trioxametallocycles rather than five-membered dioxametallocycles (Figs. 1A and 1B) that determines substrate activity. That this analysis is useful is demonstrated by its predictive ability. In addition to rationalising several of the known properties of substrates and inhibitors for the enzyme (see below), the refined model mechanism correctly predicted the activities of the potential substrates glycidol phosphate **6**, which contains no hydroxy groups, and *trans*-cyclopentane-1,2-diol phosphate **7**, and various inhibitors; see below. Full reports on these studies will appear elsewhere.[21]

Binding of other substrate analogues

Some of the small substrate analogues used in the experiments to determine the important substrate and inhibitor characteristics[3,9] were also docked into the active site of the enzyme. Not surprisingly, the (*R*)- and (*S*)-cyclohexanetriol monophosphate analogues **8** and **9** (X = O)[3] bound in the same fashion as the parent inositol phosphates. [Ref. 11 indicates that an X-ray structure for an enzyme–compound **9** (X = O) complex may be available soon and that preliminary work is in accord with our expectations.] The smallest functional substrate, ethane-1,2-diol 1-phosphate **10**,[6] it appears, can bind in two different low-energy orientations. In one of these (Fig. 13 A) the 2-OH group is positioned in the site for the 'catalytic' 6-OH group of D-Ins 1-*P* such that compound **10** would act as a substrate. In the other orientation (Fig. 13 B), the 2-OH group is positioned in the 'binding' site for the 2-OH of D-Ins 1-*P* (see Fig. 2) such that the compound would act as an inhibitor. It is not possible accurately to compare the energies of these two binding orientations as each possesses more than one stable conformation. The energies for the most stable conformations in each orientation are similar and, hence, it is likely that both orientations are populated.

The methyl ether derivative **11** is a very weak inhibitor ($K_i \geqslant 25$ mmol dm^{-3}) and binds to the enzyme 25 times less efficiently than does the diol phosphate **10**.[6] Although it appears to be able to bind in the same positions and orientations as the diol phosphate **10** neither of the binding modes is stabilised through interaction with Asp-93 or Asp-220 because the molecule lacks an acidic hydrogen and cannot serve as an H-bond donor. There are two H-bond donors that a lone pair on the ether oxygen could bond with, the backbone NH group of Ala-196 (inhibitory conformation) and the $Mg^{2+}2$-chelated water molecule [substrate conformation (compare with Fig. 13 A)]. In this latter conformation, however, the ether O-atom would need to be fixed and in the absence of an additional H-bonding interaction with Asp-220 (see Fig. 2) and a torsional constraint on ether-O-atom motion, the formation of one H-bond would not be expected to compensate for the loss of entropy.



A similar rationale may apply to the inhibitor D-6-*O*-methylinositol 1-*P* **12**, a known inhibitor of the enzyme.[7] Here, the peripheral inositol ring-binding interactions with the enzyme are very similar to those for D-Ins 1-*P* (Fig. 2). In the modelled structure the 6-OH group of the substrate D-Ins 1-*P* is within H-bonding distance of Asp-220 but, for inhibitor **12**, because the methoxy group is a substituent on a constrained cyclic system, there are only two conformations in which the lone pair of the methoxy group could H-bond to the nucleophilic water molecule, one for each lone pair. Each of these causes severe destabilisation or disruption to the catalytically competent conformation. In one conformation the methyl group has a 1,3-diaxial interaction with 5-OH group, and in the other the methyl group occupies the space normally utilised by the substrate, D-Ins 1-*P*, in forming a hydrogen bond with Asp-220. Thus, at best, Asp-220 would be expected to be pushed away so that the binding site for $Mg^{2+}2$ would be disrupted or distorted.

Replacing the methyl group in compound **11** with a 2-hydroxyethyl group gives an eight-fold better inhibitor (K_i =

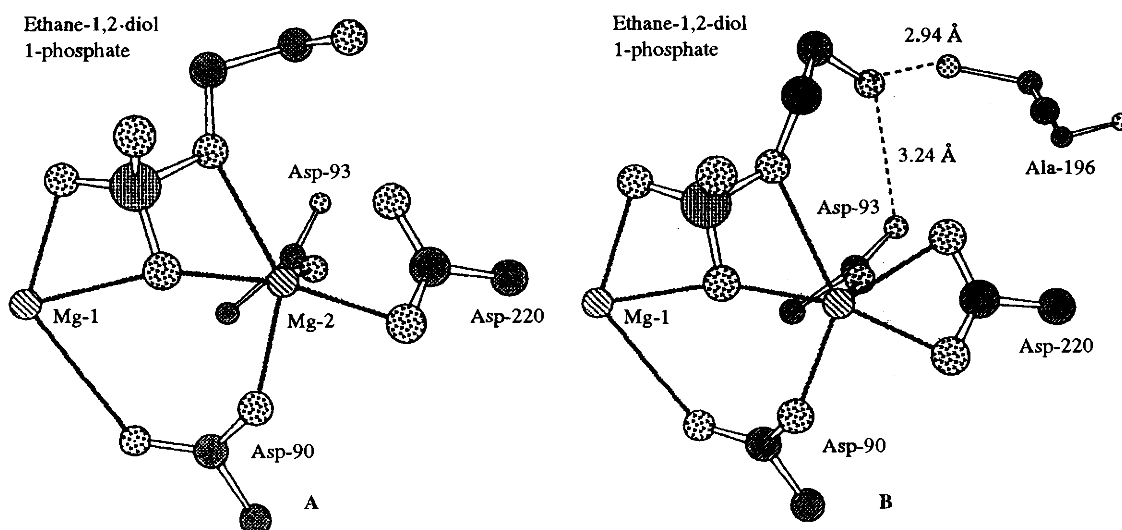


Fig. 13 Optimised active-site structures for ethane-1,2-diol 1-phosphate; **A**, bound as a substrate; **B**, bound as an inhibitor

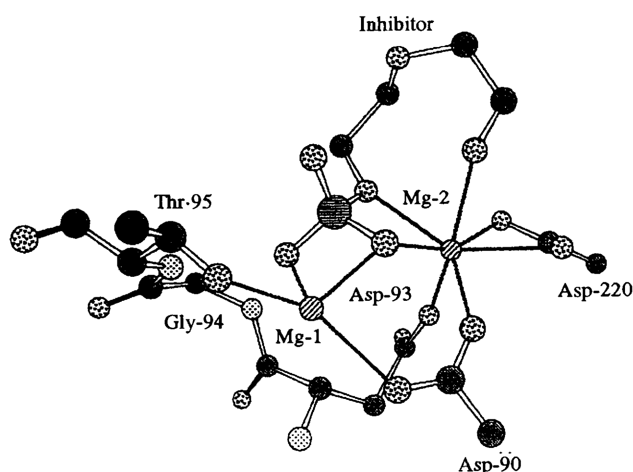


Fig. 14 Optimised active-site structure for the E–Mg^{2+}–compound **13** (X = O) complex

3.5 mmol dm^{-3}), compound **13** (X = O). The modelled interaction of the compound with the enzyme (Fig. 14) shows that the pendant hydroxy group could coordinate to $Mg^{2+}2$ and form a hydrogen bond with Asp-220. Binding in this manner would prevent access for the nucleophile. In this conformation, the ether O-atom would not be able to coordinate to the metal ion or accept a new H-bond from the nucleophilic water molecule (because the water molecule has been displaced) and this might account for the lack of any change in the value of K_i when the ether O-atom is replaced by a methylene group, compound **13** (X = CH_2).[6] It should be noted that the pendant hydroxy group can also form a hydrogen bond with Glu-213 and that conformations which mimic the conformation of the ribofuranosyl ring in 2′-AMP with the ether O-atom positioned similarly to the 1′-O-atom of 2′-AMP are accessible. Thus, compound **13** (X = O) might be expected to serve as a substrate. However, as has been discussed above, 2′-AMP appears to require an extra water molecule to function as a substrate (Fig. 10) and the negative entropic demands for the analogous situation with the acyclic analogue **13** (X = O) are probably too large to meet.

The most interesting experimental result came from the (2*R*)-enantiomer of pentane-1,2,5-triol 2-phosphate **14**, an acyclic analogue of D-Ins 1-*P* **1** and compound **8**. The compound possesses all of the structural features of the minimal substrate ethane-1,2-diol phosphate **10**, and a 5-OH group which can bind in the site for the 4-OH group of D-Ins 1-*P*, yet it functions as an inhibitor (K_i = 3.8 mmol dm^{-3}) rather than as a substrate.[6] The finding that compound **14** is an inhibitor is even more curious in the light of the fact that the (2*S*)-enantiomer **15** which contains an extra binding group at C-1 but no catalytic hydroxy group, behaves as expected[3] and is a good inhibitor (K_i = 0.12 mmol dm^{-3}).[6]

The greater flexibility afforded by the open chain allows the (2*R*)-enantiomer **14** to bind in several different conformations. When it binds in a manner expected from inspection of D-Ins 1-*P* (Fig. 2), where the 1-OH group donates a hydrogen bond to Asp-220 and accepts a hydrogen bond from the nucleophilic water, and the 5-OH group hydrogen-bonds to Glu-213, the hydrophobic chain at C-3 and C-4 is exposed to the solvent and the hydrophilic carboxylate group of Asp-93 is forced to desolvate, raising the energy penalty for binding. Note that compound **14** cannot achieve exactly the same conformation as similar cyclic compounds due to the unfavourable interactions that would result between the terminal methylene groups (which would also raise the energy penalty for binding). An alternative conformation, in which 5-OH group displaces the nucleophile to form a strong interaction with $Mg^{2+}2$ also allows the solvation of the hydrophilic enzyme residues whilst shielding some of the hydrophobic surface of the compound from the solvent, Fig. 15. In this more stable binding conformation the compound would be unable to act as a substrate.

Binding of 6-substituted inositol monophosphate ethers

The catalytic mechanism proposed by Cole and Gani,[5,6,9] and elaborated by the modelling studies described here, involves the nucleophilic attack on phosphorus by water coordinated to $Mg^{2+}2$ (Fig. 5). This mechanism suggests that an inositol derivative capable of displacing the nucleophile should act as an efficient, tight-binding inhibitor of the enzyme. With a view to designing such inhibitors, the binding of two different ether derivatives has been studied. The first, compound **16**, is a cyclic ester and thus possesses a reduced negative charge on the phosphate moiety, while the other, a D-6-*O*-(2-hydroxyethyl)-inositol 1-*P* analogue **17** possesses an hydroxyethyl ether side arm and the same charge on the phosphate moiety as substrates.

The modelled enzyme-bound conformation of the cyclic ester **16** indicates that it should bind in the same manner as D-Ins 1-*P*

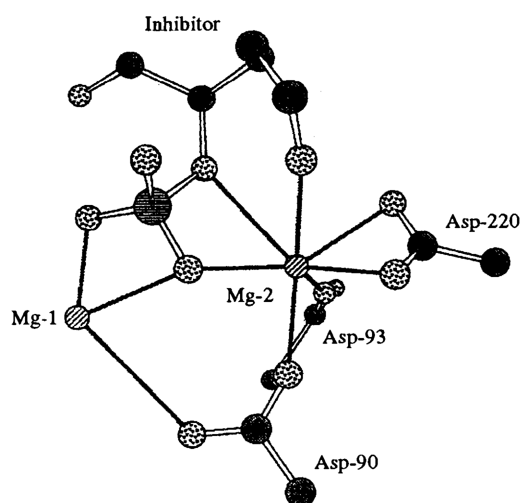


Fig. 15 Optimised conformation of the acyclic Ins 1-*P* analogue **14** binding as an inhibitor, showing the orientation relative to key catalytic enzyme residues

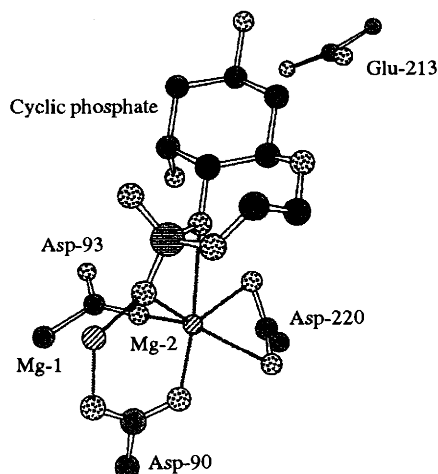
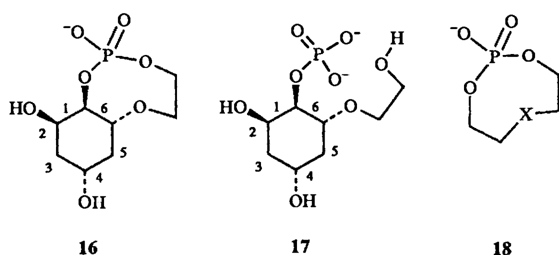


Fig. 16 Optimised active-site structure with the cyclic phosphate diester **16**



from which it is derived, interacting with the same amino acids, but now blocking access of solvent to the vacant face of $Mg^{2+}2$, *i.e.* to the position normally accessed by the nucleophilic water molecule (Fig. 16). This arrangement suggests that compound **16** should be an inhibitor for the enzyme and the nature of its activity should give insight into the catalytic mechanism. If the nucleophilic water is, indeed, activated by coordination to $Mg^{2+}2$, compound **16** is expected to be a good competitive inhibitor. Alternatively, if the attacking nucleophile comes from $Mg^{2+}1$, then, since the modification has no effect on access to this site, compound **16** might function as a substrate for the enzyme, leading to ring opening. Note that although such cyclic diesters possess a reduced negative charge and can only exist as monoanions, the only two experimentally studied examples **18** (X = O or CH_2) show remarkably low K_i-values (~8 mmol dm^{-3})[6] given that several tetrahedral dianions show much lower affinity (⩾20 mmol dm^{-3}), for example, the dianions of compound **11**[6] and sulfate.[18] On this issue it is important to note the sulphate and phosphate do not appear to bind to the enzyme in a similar manner as judged by comparison of the three published crystal structures for inositol monophosphatase.[8,12,13] Indeed, the position of sulfate resembles that of the phosphate moiety of substrates[12] (alkyl phosphates) while phosphate appears to bind as the dianion in an inverted conformation[13] where one O-atom is protonated and occupies a similar position to that occupied by W2 in the Gd^{3+}–sulphate structure.[8] This arrangement for inorganic phosphate which allows additional interactions with Thr-95 and Glu-70 would explain why the species[2] is bound so much more tightly than sulfate.[15]

The 6-O-(hydroxyethyl ether) analogue **17** (numbered according to *myo*-inositol ring) should form a dianion at neutral pH and so should not suffer from poor affinity for the enzyme on the grounds of reduced negative charge. As the pendant arm is the same as that embodied in the cyclic ester **16** it is also capable of coordinating to the second, catalytic Mg^{2+} ion so preventing access of the nucleophile to its activator site. Optimisation of compound **17** in the active site of the enzyme (Fig. 17A) shows this possible binding mode. In this structure the phosphate moiety and hydroxy groups at positions 2- and 4- bind in the same manner as for D-Ins 1-*P* while the hydroxy group of the hydroxyethyl pendant arm fills the last co-ordination site on $Mg^{2+}2$. The pendant arm is rather flexible, however, and is also able to reach across the active site to form a hydrogen bond to the side-chain of Ser-165 and also, *via* a water molecule, to the carbonyl O-atom of Val-40 and backbone NH moiety of Leu-42, Fig. 17B. In this second orientation, the nucleophile is able to access both the proposed activating $Mg^{2+}2$ ion and the phosphate group, allowing compound **17** to act as a substrate.

In order to evaluate the importance of the two conformations, both structures were subjected to dynamics calculations. The simulation for the inhibitory complex was run for 19 ps while the active complex was examined for 7 ps. Whilst the hydroxyethyl side-arm remained coordinated to $Mg^{2+}2$ (as in Fig. 17A) throughout the first simulation, the interaction of the hydroxyethyl group with Ser-165, Val-40 and Leu-42 in the second simulation was purely transient at 300 K. This result suggests that the conformation in which compound **17** binds as an inhibitor is the more stable of the two possibilities. It could be argued that 19 ps is too short for a dynamics calculation of this type, but significantly longer calculations are impractical (a 1 ps simulation requires 27 h of dedicated R4000 CPU time) and the difference in stability of the two binding modes is striking even over this short time period. [Indeed, recent work in our own laboratory has provided each of the enantiomers of the 6-O-(2-hydroxyethyl) inositol phosphate analogue **17** and these compounds show the expected biological activities. Full details on the syntheses and the possible substrate, inhibitory and transphosphorylation activities for these compounds and for compounds **6** and **7** will be reported elsewhere.][21]

Lithium-inhibited complex

There has been, for some time, good evidence to show that Li^+ acts as an uncompetitive inhibitor for the enzyme, with respect to the substrate, by blocking the release of the hydrolysis product, inorganic phosphate, from the active site,[22] step 13 in Scheme 1. More recently it has been shown that high concentrations of Mg^{2+} also inhibit the enzyme in an uncompetitive mode[16] and that the two metal ions achieve inhibition

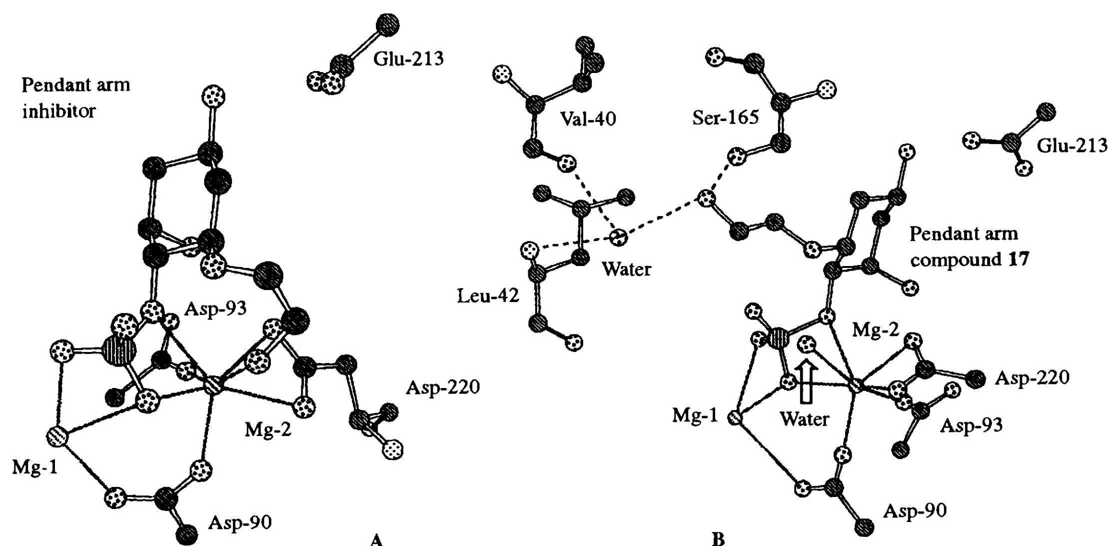


Fig. 17 Binding modes for the 2-hydroxyethyl pendant-arm compound **17**; **A**, bound as an inhibitor; **B**, bound as a potential substrate

by binding in the same site in the enzyme product complex.[2] It has also been shown that at high Li^+ concentration (> 5 mmol dm^{-3}), Li^+ can act as a *competitive* inhibitor for $Mg^{2+}1$ but it is now known that at the therapeutic levels of Li^+ used in the treatment of manic depression (~ 1 mmol dm^{-3}),[10] Li^+ binds only in the site for $Mg^{2+}2$.[6] The *apparent* K_i values for uncompetitive inhibition by both Li^+ and Mg^{2+} depend acutely on the structure of the substrate[2,16] (or the enzyme, in the case of mutants[23]) and this phenomenon can be explained by the fact that the metal ions trap the same species, the product complexes (B and C in Scheme 1) comprising enzyme, $Mg^{2+}1$ and P_i which exist at different steady-state concentrations depending on the substrate. Note that inhibitory metal ions do not prevent inositol from debinding, so that the complex C in Scheme 1 is the most important. There are two partially rate-limiting steps for the inositol monophosphatase reaction, the chemical step, phosphate ester hydrolysis (step 7 in Scheme 1),[16] and P_i release from $Mg^{2+}1$-E-P_i (step 13 in Scheme 1). Release of P_i occurs at the same rate for all substrates, since P_i is known to debind last,[2] and, therefore, the affinity of the inhibitory metal ions would be expected to increase with an increase in the steady-state concentration of the $Mg^{2+}1$-E-P_i complex.[2,6] This, of course, would be highest for the fastest substrates for the enzyme, and, indeed, there is an excellent correlation between V_{max} and the observed affinities of both Li^+ and Mg^{2+} in serving as uncompetitive inhibitors for a wide range of given substrates.[10] However, in each case, the affinity for Li^+ is significantly higher than for Mg^{2+}, *i.e.* the apparent K_i-values for inhibition by Li^+ are significantly lower than those for inhibition by Mg^{2+}.[16]

Prior to obtaining details on the environment of the protein around the binding site for the second catalytic metal ion, $Mg^{2+}2$, in the active enzyme–substrate complex, it was not possible to comment on why Li^+ should bind better than Mg^{2+} to the $Mg^{2+}1$-E-P_i complex, or, indeed, why Mg^{2+} should bind better to the $Mg^{2+}1$-E-S complex than does Li^+.[2] However, following the departure of inositol from the active site, regardless of the exact chemical mechanism for the hydrolytic step [the direct in-line displacement as proposed by Pollack and Bone and co-workers,[11-13] or the adjacent displacement followed by pseudorotation as proposed by us[5,6] (Figs. 5 and 6)], there would be a reduction in the number of ligands available to coordinate to $Mg^{2+}2$. By our own analysis the closest four ligands for $Mg^{2+}2$ in these product complexes would be the carboxylate O-atoms of Asp-90, Asp-93 and Asp-220 and one of the phosphate O-atoms of the product. These are approximately tetrahedrally arranged in space and broadly in keeping with their positions in a recently reported di-Mn^{2+}–P_i X-ray crystal structure.[13]

The replacement of the $Mg^{2+}2$ ion by a Li^+ ion gave a stable complex which optimised with the phosphate dianion slightly closer to the $Mg^{2+}1$ ion (Fig. 18) than in the corresponding complex containing two Mg^{2+} ions. Lithium ion is known to prefer tetrahedral coordination geometry[24] while octahedral geometry is favoured by Mg^{2+} ions and these properties appear to account for the experimentally observed inhibitory propensities of the two metal ions.

Discussion

The calculated binding modes for the known substrates **1–5** show clearly the location of the second metal site, predicted from kinetic and inhibition studies, and fit well with the known structural requirements for substrates.[1,4,8-10,14-16] The predicted second magnesium binding site correlates well with the metal binding site of the related enzyme fructose bisphosphatase.[25] The principal source of binding energy comes from the interaction of the phosphate group of Ins 1-*P* substrates with the enzyme and metal ions, while 2-OH and 4-OH groups contribute to specificity through the formation of hydrogen bonds to Glu-213, Asp-93 and Ala-196. This model suggests that the 'catalytic' oxygen of both enantiomers of Ins 1-*P* and Ins 4-*P* functions by helping to bind the nucleophilic water to $Mg^{2+}2$ and by ensuring its correct orientation relative to the phosphate group. Very recently published crystallographic work[11,12] is broadly in agreement with the binding interactions for D- and L-Ins 1-*P* presented here. The hydrogen bonds between the inositol ring and Glu-213, Asp-93 and Ala-196 are all found in the X-ray structures of a Gd^{3+}–Ins 1-*P* complex,[11] while both metal sites have been shown to be occupied by Mn^{2+} in an Mn^{2+}–phosphate enzyme complex.[12] These crystallographic studies, however, differ in the interpretation of the enzyme mechanism. Bone *et al.*[12,13] propose a more conventional in-line substitution mechanism in which the nucleophilic water is attached to $M^{2+}1$ and is activated by Glu-70 and Thr-95. This in-line displacement mechanism is contrasted with the pseudorotation mechanism below (see Figs. 5 and 6).

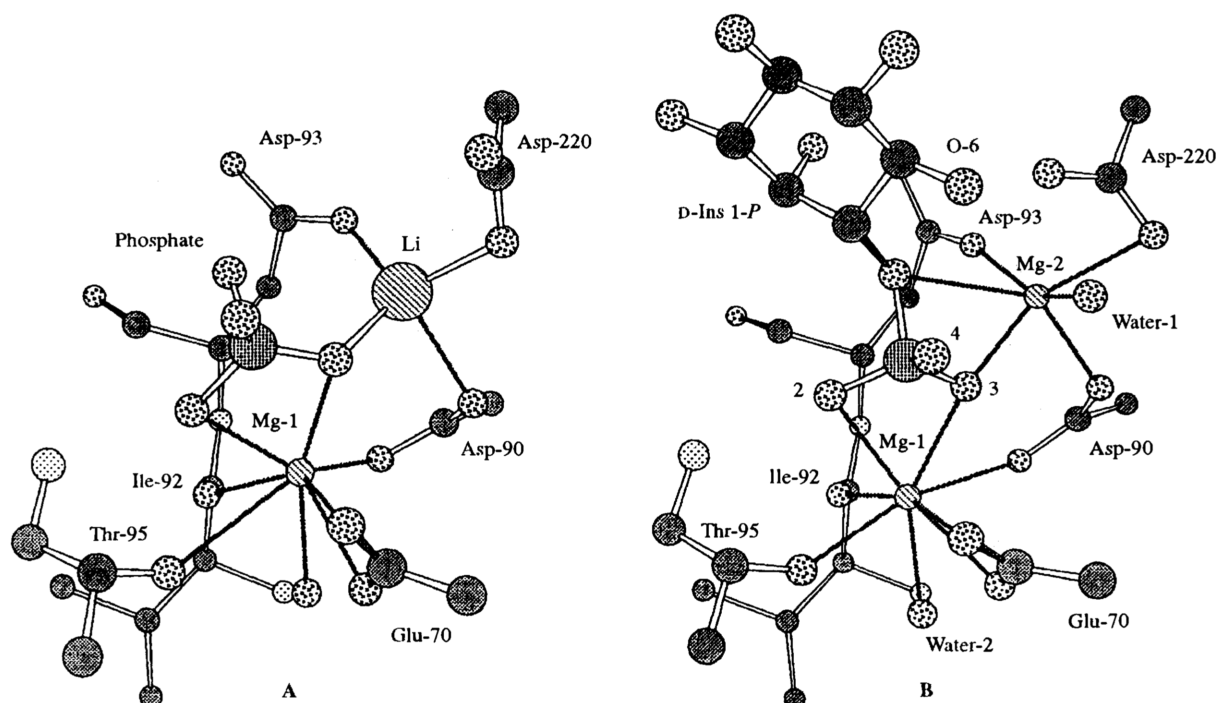


Fig. 18 Optimised active-site structures for; **A** the lithium-inhibited complex, E–Mg^{2+}1–HPO_4^{2-}–Li^+, and, for comparison, **B** the E–Mg^{2+}1–D–Ins 1-*P*–Mg^{2+}2 complex. The structures are displayed in identical orientations.

Comparison of the in-line and pseudorotation mechanisms

The in-line displacement mechanism in which the nucleophilic water is attached to M^{2+}1 and is activated by Glu-70 and Thr-95 is supported by the observation of a rather long $Gd^{3+}\cdots$O (Thr-95) distance of 3.3 Å[16] (as opposed to 2.32 Å in the calculated, Mg^{2+}, structure reported here). This finding indicates there is no interaction between the metal ion and Thr-95 and suggests that there is room for the hydrolytic water molecule (W2) approximately 3.6 Å away from the phosphate P-atom. It should be emphasised that such an arrangement is not uniquely consistent with an in-line displacement at the P-atom since such a water molecule could be displaced from the metal ion by one of the phosphate O-atoms in the pentacoordinate intermediate that would be formed *via* the pseudorotation mechanism. Indeed, it would expected that one of the phosphate O-atoms would be diametrically opposite the leaving ester O-atom in the pentacoordinate intermediate and that the inorganic phosphate group would be inverted compared with the substrate (see Figs. 5 and 6 and the discussion below). Furthermore, we should be mindful of the fact that the crystal structure was solved to 2.2–2.3 Å resolution, that only one metal ion was present in the active site, and that this possessed a higher formal charge of +3 rather than of +2, the charge required for activating metal ions (cofactors) of the correct size. There is no experimental evidence that we know of to indicate that there is a water molecule equivalent to W2 located on M^{2+}1 in any enzyme–inhibitor–divalent metal ion complex although detailed modelling studies suggest that such a water molecule could be stably bound in this position by Mn^{2+}1, Thr-95 and Glu-70 and activated by Glu-70 in a di-Mn^{2+}–Ins 1-*P* complex.[11] There is clearly one inorganic phosphate O-atom bound near the site for W2 in a crystal structure of a di-Mn^{2+}–phosphate–enzyme complex, which also shows that the phosphate group is inverted[13] compared with its position in the crystal structure of a Gd^{3+}–Ins 1-*P*–enzyme complex.[12] It is, therefore, very reasonable to argue that this phosphate O-atom would be derived from the in-line attack of a water molecule (W2) on the phosphate P-atom. However, if W2 does exist in kinetically competent ground-state complexes and is bound too tightly, it may not be able to serve as a nucleophile or exchange rapidly in [^{18}O]water phosphate ligand-exchange experiments[9] as was discussed in the previous article.[6]

Another argument presented in favour of the in-line displacement mechanism in which a water molecule on Mg^{2+}1 serves as the nucleophile was developed from the results of site-specific mutagenesis experiments.[11,12] It was proposed that Glu-70 would activate the nucleophile.[11,12] When Glu-70 was altered to glutamine or aspartate, k_{cat} was reduced drastically, by 7000-fold and 400-fold respectively.[23] However, K_m, K_i for inhibition by Li^+, the value of IC_{50} for inhibition by Mg^{2+} and the affinity for Mg^{2+} as a cofactor were found to be the same as for the wild-type enzyme. While, superficially, the data seem to support a direct catalytic role for Glu-70, the interpretation is not consistent with the known dependencies of the K_i-values for inhibition by Li^+ and Mg^{2+} on the steady-state concentration of the E–Mg^{2+}–P_i complex (complex C in Scheme 1); see above. This concentration would be reduced drastically if the hydrolytic step were decreased by the same magnitude as k_{cat}. Much more probably, the reduced values of k_{cat} reflect the fact that dead-end complexes form which lack Mg^{2+}1 (*e.g.*, E–Ins-*P*–Mg^{2+}2, see Scheme 1) due to the drastically reduced affinity of the enzyme for Mg^{2+}1 (recall that the species bind in the order Mg^{2+}1, substrate, then Mg^{2+}2). In the small remaining number of enzyme molecules which do bind an Mg^{2+} ion before the substrate binds, the hydrolytic rate seems to be very similar to that for the wild-type enzyme as evidenced by the unaltered values of the inhibition constants for Li^+ and Mg^{2+} ion.[23] Clearly the mutants display the same affinity for the second cofactor ion which is, it appears, what is being measured in the kinetic experiments since only the enzyme molecules which contain an Mg^{2+}1 ion will actually act as catalysts and provide kinetic data, Scheme 1. That the affinities of each of the mutant enzymes for the first metal ion are much lower than those for the wild-type enzyme is more reliably interpreted from the fact that the alteration of Glu-70 caused a decrease in the binding affinities for Tb^{3+} and Gd^{3+}

of greater than 30-fold. In these experiments direct fluorescence-quenching methods as well as kinetic methods were used.[23]

The mutation of Thr-95 to Ser and Ala gave enzymes which did display kinetic properties consistent with a mechanistic role for Thr-95. Both mutants showed reduced values of k_{cat} and inhibitory Li^+- and Mg^{2+}-binding properties consistent with a significantly reduced steady-state concentration of the E–Mg^{2+}–P_i complex (complex C in Scheme 1), see above. Unfortunately, this result is consistent with both proposed mechanisms (Figs. 5 and 6) since both generate pentacoordinate phosphorus intermediates where one phosphate O-atom could H-bond to Thr-95. This analysis is in accord with the X-ray structure for the di-Mn^{2+}–phosphate–enzyme complex which shows an 'inverted' phosphate group.[13] Other mutations which tested the importance of the Asp-90, Asp-93 and Asp-220 ligands for the second metal ion[11] gave enzymes with low k_{cat}-values and these data are also consistent with both proposed mechanisms. This appraisal for the role of Glu-70 highlights the problems associated with interpreting kinetic data from site-specific mutation studies for systems which require that the species bind in a given order[2] and, in the light of our own interpretation, it would seem that the only role for Glu-70 is in binding $Mg^{2+}1$. This analysis is not consistent with the proposed in-line displacement mechanism.[11-13]

At this time we cannot rule out the operation of an in-line displacement mechanism and it must remain a possibility. However, there are several experimental findings, in addition to those highlighted above, that are difficult to rationalise if the nucleophile is activated by the buried metal ion, $Mg^{2+}1$, and Glu-70. These include the high rates of inositol-mediated ^{18}O-exchange from labelled water into P_i, the fast rates for the hydrolysis of phosphorothioate substrates, and the inhibitory behaviour of certain potential substrates that do not hydrolyse even though they contain all of the functional groups required for an in-line displacement.

Most of these factors have been considered previously,[6] but, the latter issue deserves further consideration. If the second metal ion is only required to stabilise the leaving group why do compounds such as D-6-*O*-methylinositol 1-phosphate **12**[4] and 6-deoxyinositol 1-phosphate[4] fail to act as substrates? The former, compound **12**, may be able to prevent the second Mg^{2+} ion from binding (although this is highly improbable, see discussion above concerning this compound and the arguments below) but the latter should not. 4-Nitrophenyl phosphate dianion is a substrate and requires both metal ions to be present.[16] The E–$Mg^{2+}1$–substrate complex does show a low comparative affinity for $Mg^{2+}2$ (7-fold lower than Ins 1-*P*),[16] presumably because a water molecule is required to fulfil the role of the catalytic hydroxy group of other substrates, but $Mg^{2+}2$ does bind. Even when the enzyme is saturated with Mg^{2+} the substrate is processed very slowly (V_{max} = 5% of V_{max} for Ins 1-*P*), which could be interpreted to indicate that the second metal ion does more than just serve as a Lewis acid. A similar explanation could account for the absent (or very low) substrate activity of 6-deoxyinositol 1-phosphate.[4] $Mg^{2+}2$ should be able to bind, and reasonably should be able to coordinate to the bridging phosphate ester 1-O-atom, yet reaction does not occur. Again, it appears that the second metal ion may need to do more than just serve as a Lewis acid. Furthermore, why are such compounds such effective inhibitors? The replacement of the 6-OH group of D-Ins 1-*P* **1** by OMe gave a good competitive inhibitor, compound **12** [K_i = 0.035 mmol dm^{-3} (the racemate gave a K_i-value of 0.070 mmol dm^{-3})][4] and, likewise, the deletion of the 6-OH group to give the 6-deoxyIns 1-*P* resulted in a good inhibitor [0.035 mmol dm^{-3} (the racemate gave a K_i-value of 0.070 mmol dm^{-3}].[4] Both values of K_i are substantially lower than the value of K_i for Ins 1-*P* which is 0.1 mmol dm^{-3} and equal to K_m at pH 8.0 as determined in our laboratory,[2] or 0.16 mmol dm^{-3} (value for K_m as cited by the Merck group).[3b]

Equally striking is the comparison of the binding affinities of the analogous compounds in the D-3,5-dideoxyinositol 1-*P* series. Merck demonstrated very elegantly that the 3- and 5-hydroxy groups are not important in binding or catalysis[4] and that the parent compound, D-3,5-dideoxy Ins 1-*P* was a good substrate (K_m = 0.025 mmol dm^{-3}, V_{max} = 61% of V_{max} for Ins 1-*P*).[3b] Here, the further removal of the 6-OH group to give compound **9** (X = O) resulted in a highly potent competitive inhibitor (K_i = 0.003 mmol dm^{-3})[3a] while the replacement of the 6-OH group by an MeO group gave an equally efficacious inhibitory compound [K_i = 0.002 mmol dm^{-3} (the racemate gave a K_i-value of 0.004 mmol dm^{-3})].[21] Note that for the racemic compounds lacking the 6-OH group, only the enantiomers derived from D-Ins 1-*P* are expected to be inhibitors.[3,4,10]

When the findings reported for the corresponding phosphonates (where the 1-O-atoms in Ins 1-*P* and in D-3,5-dideoxyinositol 1-*P* are replaced by a methylene group)[26] are included in this analysis, there seems to be no other explanation except that $Mg^{2+}2$ can bind to the 1-O-atom of the substrates and inhibitors cited above. These phosphonates were reported to be completely inactive as inhibitors[26] which we interpret to mean that the K_i-values were greater that 2 mmol dm^{-3}. This corresponds to an approximate 500-fold reduction in binding affinity upon replacing the 1-O-atom in, for example, compound **9** (X = O) by a methylene group. Furthermore, there appears to be no other potential binding group in the vicinity of the 1-O-atom at the active site of the enzyme, either by our own analysis or by that of the Merck group.[11-13] So if the 1-O-atom of, for example, 6-O-MeIns 1-*P*, 6-deoxyIns 1-*P* and compound **9** (X = O) can bind to $Mg^{2+}2$ in the respective enzyme complexes, which appears to be the case, and $Mg^{2+}1$ provides the nucleophile, why do the compounds not serve as substrates instead of inhibitors?

The similarity of the trends for the properties of these two series of compounds is striking and is indicative of a common explanation. It is difficult to argue that removing a hydroxy group and replacing it with both a smaller group, a hydrogen, and a larger group, a methoxy group, should lead to an enthalpic energy decrease for the enzyme-bound forms, if the hydroxy group forms stabilising interactions with the holoenzyme as appears to be the case. Recall that X-ray data,[12] the modelling studies of the Merck group,[11] and our own modelling studies (Fig. 2) indicate that the 6-OH group of D-Ins 1-*P* interacts with Asp-220. However, it is quite reasonable to propose that the disruption caused by replacing the 6-OH group in the substrate (which is *confirmed* to result in the abolition of substrate activity in each case)[3,4,21] might result in relaxing a ground-state destabilising interaction (possibly entropic) that is associated with catalysis. This analysis would fit quite well with the pseudorotation mechanism (Fig. 5) in which $Mg^{2+}2$ must not only bind, but must be properly positioned and chelated in order to activate and orientate the nucleophile. The alternative in-line mechanism (Fig. 6) could accommodate these results if the modified substrates were able to prevent $Mg^{2+}2$ from chelating to the bridging ester O-atom. As alluded to above, from the properties of the 4-nitrophenyl phosphate system[16] and the phosphonates,[26] this would seem unlikely.

The proposed pseudorotation mechanism does seem able to accommodate the observed properties of the enzyme and has been successful in predicting the activities of previously untested compounds (see above).[21] The proposed mechanism has also been successful in designing highly potent non-lipophilic mechanism-based inhibitors in which the space for

the nucleophile on $Mg^{2+}2$ is expected to be occupied by the ω-hydroxy group of a pendant arm (*e.g.*, as in compound **17**, see Fig. 17A); full details will be reported elsewhere.[21] Albeit the final test for the validity of the hypothesis will come when the stereochemical course of the reaction with respect to the phosphorus atom is determined. Such an investigation is underway.

Comparison with fructose bisphosphatase

It may be tempting to subscribe to the view that fructose bisphosphatase and inositol monophosphatase operate in an identical manner because they both require two divalent metal ions to catalyse a hydrolysis involving the direct displacement of the leaving group by a nucleophilic water and share conserved amino acid residues for the metal-ion-binding sites. However, while there is a conserved heptapeptide forming part of the $Mg^{2+}1$ site[27] and the phosphate-binding site of both enzymes, -Asp-Pro-Ile-Asp-Gly-Thr-Thr-, starting at Asp-90 for inositol monophosphatase, and -Asp-Pro-Leu-Asp-Gly-Ser-Ser-, starting at Asp-118 for fructose bisphosphatase, there are few other structural similarities. To start with, the relative Mg^{2+}-ion-binding affinities at the two metal-binding sites is reversed for the two enzymes.[27,28] Thus, the equivalent of the $Mg^{2+}2$ site in inositol monophosphatase is filled first in fructose bisphosphatase and is, therefore, the $Mg^{2+}1$ site. This site contains an extra carboxylate ligand compared with inositol monophosphatase and this is derived from Glu-97. Glu-98 in fructose bisphosphatase is equivalent to Glu-70 in inositol monophosphatase, but there is no residue equivalent to Glu-97. The residue which occupies position 69 in inositol monophosphatase is a Gly residue and this is highly significant because it is believed that Glu-97 of fructose bisphosphatase also activates the hydrolytic water molecule for an in-line attack which proceeds with inversion of configuration at P.[29] For fructose bisphosphatase this water molecule is attached to $Mg^{2+}2$ (*i.e.*, the second ion to bind) and, in stark contrast to the situation for inositol monophosphatase, this site is extremely close to the surface of the enzyme, is not shielded by protein, and is completely accessible to the solvent. Thus, for fructose bisphosphatase, as we believe may be the case for inositol monophosphatase (Fig. 5), it is the second metal ion to bind and the one that is most accessible to the solvent which serves to activate the nucleophilic water molecule.

Experimental

D-Inositol 1-phosphate 1

Optimisations. The conformations and binding modes of all substrates and inhibitors (ligands) for inositol monophosphatase described here were determined using the AMBER[30,31] all-atom molecular mechanics force-field and the InsightII[32] program. In all cases, multiple starting points were derived from the published Gd^{3+}–SO_4^{2-}–IMPase X-ray structure[8] (2HHM in the Brookhaven database[33]) by following the procedure described below. AMBER default charges were used for the solvent and protein, while InsightII-determined charges were used for the ligands as they are compatible with the AMBER force-field. Magnesium ions were given a double positive charge as this leads to appropriate metal–oxygen distances for small test systems. The enzyme–ligand complexes were solvated, using the soak option in InsightII[32] to give a double solvent layer around the whole enzyme and a sphere of radius 10 Å around the ligand, giving a complex of some 11 000 atoms. This ensures that the active site is filled, that all charged surface residues are fully solvated, and allows the use of a dielectric constant of 4.0. The optimisations themselves were carried out in three stages; first, the solvent alone was allowed to relax; second, the ligand was also allowed to relax into the site, with the protein frozen; and finally the positions of all protein residues and solvent within 10 Å of the ligand, along with the ligand itself, were optimised. Convergence for the initial two steps was accepted once all gradients had fallen below 42 kJ $Å^{-1}$ mol^{-1} (10 kcal $Å^{-1}$ mol^{-1}), but for the final optimisation this was tightened to 4.2 kJ $Å^{-1}$ mol^{-1} (1 kcal $Å^{-1}$ mol^{-1}). By restricting the final optimisation to a 10 Å radius sphere, there is no need to apply a non-bonded cut-off and so a more complete description of the ligand binding energies is obtained. The resulting structures were visualised using InsightII.[32] Printed structures contained in this paper (*e.g.*, Fig. 2) were generated by editing full structures and then the atom coordinates were transferred to CSC Chem 3D (a chemical structure modelling display package).[34]

Initially, no water molecules were placed in possible or presumed locations for a nucleophile, as any water molecule close to the metal would become tightly bound and force the system to adopt a conformation broadly similar to the starting arrangement. Rather, when an accessible vacancy was found in the coordination sphere of $Mg^{2+}2$ it was filled by a water molecule and the structure was reoptimised. In this work no such vacancy was found for the $Mg^{2+}1$ site corresponding to the space occupied by water molecule, W2, in the published structures for Gd^{3+}–Ins 1-*P*–enzyme complexes,[17] although there was a vacancy corresponding to the position occupied by W1; see Fig. 3.

Starting points. In all cases, the starting points for the optimisations were derived from the published Gd^{3+}–SO_4^{2-}–IMPase X-ray structure.[8] Initially, the Gd^{3+} ion was replaced by Mg^{2+} and the SO_4^{2-} dianion was replaced by PO_4^{3-} trianion and optimised according to the above protocol with the crystallographic waters being removed on completion. In order to determine the positions of the bound ligands, the phosphate groups of various substrates and inhibitors were superimposed on the optimised PO_4^{3-} trianion position (note: there are two possibilities for this as two of the PO_4^{3-} oxygens are accessible from the inositol cleft) and all rotatable bonds of the ligand were allowed to rotate while the enzyme remained fixed. All conformations so generated that fitted with known experimental data were retained and were used as starting points for the optimisations.

Dynamics. Key optimised structures were subjected to a short period of dynamics to test the stability of binding. These calculations were run at 300 K, with the same solvation, dielectric constant, charges and constraints as the preceding optimisation, using a time step of 1 fs. As even these restricted calculations take a considerable time on Silicon Graphics workstations (1 ps in 27 h on an R4000 processor, longer on an R4600), it is not practicable to run these calculations for more than a few ps. Since this is not long enough to allow gross changes in domain orientations to occur, it is reasonable to fix the coordinates of all residues in which all of the atoms are more than 10 Å away from any part of the bound molecule. Dynamics calculations were run for between 4 and 19 ps; for specific details, see below. Note that the determining factor for molecular dynamics calculations was the program stability over such lengthy real-times, 4.5–20 days per calculation. Such short simulation times can only give a qualitative assessment of the stability of binding and can provide only limited conformational space sampling. Multiple reoptimisations of structures obtained during the dynamics run gave some assurance against being caught in an inappropriate high-energy conformation. A more complete sampling of nearby local minima was obtained by using several different starting points for the optimisation of the bound molecules; see the above procedure. Note that for the D-Ins 1-*P* system the reoptimised structure (following 7 ps dynamics) gave coordinates for the substrate, proposed nucleophile, and for the enzyme of less than 1 Å root mean

square (RMS) deviation from the conformation of the originally optimised structure. Interactions between the substrate and the enzyme and metal ions are given in Table 1.

L-Inositol 1-phosphate 2

Starting points were generated and optimised using the method described for D-Ins 1-*P* above. The calculated binding modes (Fig. 7) were very similar to the D-enantiomer and as the D-Ins 1-*P* structure was stable and optimisation of the final dynamics structure occupied the same well as the initial structure, further dynamics were not performed. Interactions between the substrate and the enzyme and metal ions are given in Table 1.

2′-AMP 3

Starting points were generated as for D-Ins 1-*P* above and were optimised as before. This resulted in a structure with poor contacts between the furanosyl O-atom–$Mg^{2+}2$ and between the 5′-OH group and Glu-213 (Fig. 8). Further optimisations were carried out by placing one water molecule (not shown) or two water molecules (Fig. 10) between the $Mg^{2+}2$ ion and the furanosyl O-atom. On optimisation, these waters positioned themselves so as to occupy the proposed nucleophilic binding site and the position of the 6-OH of D-Ins 1-*P*, determined above. The structure with only a single added water molecule did not optimise to give good contacts between the added water molecule and both the $Mg^{2+}2$ ion and the furanosyl O-atom. As a result, it was not considered further. The 2′-AMP–2 H_2O–IMPase complex was subjected to 4 ps dynamics (see above). Interactions between the substrate and the enzyme and metal ions are given in Table 1.

Inositol 4-phosphate 4 and 5

Starting structures for both enantiomers were generated and optimised as described for D-Ins 1-*P*. The protocol gave two different binding modes for each enantiomer. Fig. 11 shows these binding modes for D-Ins 4-*P*, which differ only in the interactions of the 3-OH and 5-OH with the enzyme. Note that the enantiomers of Ins 4-*P* possess the same arrangement of binding and catalytic hydroxy groups and, therefore, bind in a fashion identical with each other. As the 3-OH ··· 5-OH distance of Ins 4-*P* differs from the comparable 2-OH ··· 6-OH distance of D-Ins 1-*P*, each of these bound conformations (Figs. 11A and 11B) were subjected to molecular dynamics simulations at 300 K. The substrate-bound conformation (Fig. 11A) remained stable throughout a 4 ps simulation and the alternative conformation (Fig. 11B) remained stable throughout a 6 ps simulation. Interactions between the substrate and the enzyme and metal ions are given in Table 1.

Glycidol phosphate 6 and *trans*-cyclopentane-1,2-diol phosphate 7

The bound conformations of these potential substrates were determined using the approach described for D-Ins 1-*P* starting from conformations generated from the Gd^{3+}–SO_4^{2-}–IMPase crystal structure in the manner described above. Both compounds appeared to be able to bind in a manner that would support catalysis. Thus, if the compounds can bind to the enzyme it would be expected that they would serve as substrates and not inhibitors. However, the compounds contain minimal functionality and no binding interactions with the enzyme (other than through the phosphate moiety) are possible.

(1*R*)-Cyclohexane-1,2,4-triol 1-phosphate 8 and (1*S*)-cyclohexane-1,2,4-triol 1-phosphate 9 (X = O)

The bound conformations were determined using the approach described for D-Ins 1-*P* starting from conformations generated from the Gd^{3+}–SO_4^{2-}–IMPase crystal structure in the manner described above.

Ethane-1,2-diol 1-phosphate 10 (Fig. 13) and 2-methoxyethyl phosphate 11

The bound conformations were determined using the approach described for D-Ins 1-*P*, starting from conformations generated from the Gd^{3+}–SO_4^{2-}–IMPase crystal structure, in the manner described above. In neither case did any of the identified minima have the 2-O-atom located in a similar position to that of the furanosyl O-atom of 2′-AMP in its optimised, bound form; see calculations performed for 2′-AMP to determine its likely binding modes.

D-6-*O*-Methylinositol 1-phosphate 12

The bound conformation was determined using the approach described for D-Ins 1-*P* and starting from conformations generated from the Gd^{3+}–SO_4^{2-}–IMPase crystal structure in the manner described above.

Diethylene glycol monophosphate 13 (X = O) (Fig. 14), pentane-1,5-diol 1-phosphate 13 (X = CH_2), (2*R*)-pentane-1,2,5-triol 2-phosphate 14 (Fig. 15) and (2*S*)-pentane-1,2,5-triol 2-phosphate 15

The bound conformations were determined using the approach described for D-Ins 1-*P* starting from conformations generated from the Gd^{3+}–SO_4^{2-}–IMPase crystal structure in the manner described above. This procedure generated and optimised a number of binding modes, with either the ω-hydroxy group hydrogen bonding to Glu-213 or chelating to $Mg^{2+}2$.

L-3-*O*-(2-Hydroxyethyl)-1,2/3,5-cyclohexanetetraol cyclic 2-phosphate 16

The bound conformation was determined (Fig. 16) using the approach described for D-Ins 1-*P* starting from conformations generated from the Gd^{3+}–SO_4^{2-}–IMPase crystal structure in the manner described above.

L-3-*O*-(2-Hydroxyethyl)-1,2/3,5-cyclohexanetetraol 2-phosphate 17

Both the inhibitor binding mode and substrate binding mode (Fig. 17) were determined using the method described above. The stability of each bound mode was tested by molecular dynamics and while the inhibitory mode remained stable over 19 ps, the active mode was stable for less than 6 ps.

Pentane-1,5-diol 1,5-cyclic phosphate 18 (X = CH_2) and diethylene glycol cyclic phosphate 18 (X = O)

For both molecules, the bound conformation was calculated using the procedure described above. In each case there was only one possible orientation of the molecule in the active site, so the starting geometry could be generated by inspection.

Mg^{2+}–HPO_4^{3-}–Li^+–IMPase complex

The starting point for this calculation was generated from the optimised D-Ins 1-*P*–2 Mg^{2+}–IMPase complex, replacing the $Mg^{2+}2$ ion with an Li^+ ion and the substrate with HPO_4^{2-}. The resulting complex (Fig. 18) was then optimised in the manner described above.

Analysis of the relative energies of conformations of 2′-AMP and adenosine

Optimised structures for 2′-AMP and adenosine were obtained in both the presence and absence of a divalent metal cation (Mg^{2+} or Ca^{2+}) positioned so as to interact with both the 1-*O*′-furanosyl O-atom and the 2′-O-atom (Fig. 9). These possible interactions with an additional metal ion ($Mg^{2+}2$) at the active site of the phosphate were implicated by the consideration of

kinetic and binding data for 2′-AMP, and some analogues, and adenosine. The purpose of this analysis was to determine the preferred ring conformations for bound and unbound species and to estimate the differences in the internal energies of the species in each conformation.

To determine the internal energy penalty required for the furanosyl ring of 2′-AMP and adenosine to adopt a 'high-energy' conformation with the adenine and 5′-hydroxymethyl groups pseudo-axial (Fig. 9 and Table 2), the minimum-energy conformation of the metal-stabilised form was determined. The interactions of the molecule with the metal ion were then removed, by removing the metal ions, but keeping the exact same conformations. The energy of the structure was then recalculated allowing no relaxation of the structure. This internal energy (an estimate of the internal energy for the molecule in the bound state) was then compared with the calculated energy of the conformation of the free unbound system with the lowest energy. The difference was used as a guide to the amount of binding energy that would be required to 'dock' the molecule into the active site of the enzyme in that specific conformation.

The initial calculations[5,9] utilised the CVFF molecular mechanics parameter set[35] (default in InsightII[32]) and the default-optimisation procedure (dielectric constant = 1.0, optimisation terminated with all gradients < 0.004 kJ Å^{-1}, using a modified Newton–Raphson algorithm), with Ca^{2+} as the cation (Mg^{2+} parameters unavailable in this set). This model system showed two binding minima for the metal ion, one with interactions involving the 1′-O, 2′-O and 3′-O atoms (shown in Fig. 9) and a more stable arrangement with an interaction involving only the phosphate group (not shown), corresponding to potential interactions with the metal ions at sites 2 and 1, respectively.

For compatibility with the substrate and inhibitor-binding calculations described in this paper, the structures of 2′-AMP and adenosine were reoptimised in both the presence and absence of divalent metal cation, using the same conditions as for the enzyme-binding calculations namely AMBER parameters, dielectric constant of 4.0, Mg^{2+} cations and optimisation until all of the gradients were < 0.04 kJ Å^{-1} using conjugate gradients. In this case, no minima could be found in which the furanosyl O-atom formed part of the metal-ion-coordination sphere (due to the change in the dielectric constant and the differences in the method for representing electrostatic interactions between the two parameter sets), so a half-harmonic distance constraint (optimum distance 2.0 Å, force constant 4.2 kJ Å^{-2}) was added between the furanosyl O-atom and the metal ion. Typically, this forcing potential added ~ 2 kJ mol^{-1} to the conformational energy, which was subtracted before any energy comparisons. The energy of the 2′-AMP conformation from the 2′-AMP–$2H_2O$–enzyme complex was compared with that of the free optimised conformation using the AMBER force-field and dielectric of 4.0; see Table 2.

Initial calculations[5,9] suggested that there was a very large internal energy penalty (100 kJ mol^{-1}) required to achieve the pseudo-axial conformation of 2′-AMP needed for binding to the enzyme, this energy being supplied by direct interaction of a divalent metal cation with the furanosyl O-atom (entry 1, Table 2). However, subsequent calculations (entries 3 and 5, Table 2) have shown that the deduced magnitude of the interaction is highly dependent upon the local dielectric constant. Increasing the dielectric constant from 1.0 to 4.0 abolished the local energy minimum in which the furanosyl O-atom formed part of the metal-coordination sphere, and, even when forced to so bind, reduced the degree of distortion of the ring pucker such that the energy was only 25 kJ mol^{-1} above the ground state (entry 5). Similarly, the conformation of 2′-AMP in the 2′-AMP–2 H_2O–IMPase complex (entry 7) was found to be only 20 kJ mol^{-1} above the ground state. Comparable energies were obtained for adenosine bound first to a single Mg^{2+} ion (9 kJ mol^{-1} above the ground state, entry 6), and then in the same relative orientation as found in the 2′-AMP–$2H_2O$–IMPase complex (internal energy penalty of 25 kJ mol^{-1}, entry 8).

According to the proposed model (see Discussion section), organising the reactant complex for either hydrolysis (2′-AMP, $2H_2O$) or ^{18}O-label exchange (P_i, adenosine, $2H_2O$) requires a considerable decrease in entropy, especially for binding of water. In the case of hydrolysis, this can be compensated for by the binding interaction of the phosphate moiety of the substrate in addition to contacts involving the 3′-OH and 5′-OH which could allow the furanosyl O-atom and the Mg^{2+}2 ion to provide sufficient binding energy to hold the two water molecules. For the ^{18}O-label exchange, and also for the reverse reaction, one 'substrate', adenosine, appears to form only the weak interactions with the enzyme, at the 3′-OH and 5′-OH groups. These interactions may not be sufficient to overcome the requisite increase in entropy such that the high free-energy complex cannot form. The energetic advantage for the substrate binding (in the presence of 2 mmol dm^{-3} Mg^{2+} ion) is likely to be no more than 21 kJ mol^{-1} over and above that for the product, adenosine. This is calculated from the value of the product-inhibition constant for P_i of 0.3 mmol dm^{-3} in the presence of 2 mmol dm^{-3} Mg^{2+} ion. Note that at higher concentrations of Mg^{2+} ion the energetic advantage is greater for the substrate as it is trapped on the active site (it must bind before the second Mg^{2+} ion)[2] whereas the product (in this case adenosine) can debind in a random fashion and is therefore not trapped on the enzyme by Mg^{2+}; see Scheme 1 and Discussion section.

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