'Active' Conformation of the Inositol Monophosphatase Substrates Adenosine 2'-Phosphate and Inositol Phosphate: Role of the Ribofuranosyl O-atom and Inositol O-atoms in Chelating a Second Magnesium Ion

Andrew G. Cole and David Gani*

School of Chemistry, The Purdie Building, The University, St. Andrews, Fife, UK KY16 9ST

A high energy conformation, in which the adenine moiety of adenosine 2'-phosphate occupies a C-1'-axial ribofuranosyl position, is stabilised through the chelation of a second (additional) Mg^{2+} ion by the 2'- and furanose ring O-atoms; with inositol 1-phosphate as the substrate, the 1- and 6-O-atoms chelate the second Mg^{2+} ion and for both substrates a different (buried) Mg^{2+} ion interacts directly with the phosphate moiety.

In mammalian brain cells, inositol monophosphatase is responsible for supplying free inositol for the biosynthesis of the secondary messenger precursor, phosphatidylinositol 4,5-bisphosphate.[1] The enzyme is able to process both enantiomers of Ins 1-P and Ins 4-P as well as 2'-AMP **1**.† Recent work from a number of laboratories has probed the structure and mechanism of the enzyme and several key features are now documented.[1-5] For example, it is established that the enzyme operates *via* a direct displacement ternary complex mechanism in which water is the attacking nucleophile,[2,3] rather than *via* the common substituted enzyme mechanism.[4] It is also evident that the phosphate dianion is the primary binding functionality for substrates and inhibitors and that the 1-O-atom and the 2- and 4-OH groups of Ins 1-P substrates (*e.g.* D-Ins 1-P **2**) are important in binding whilst the 6-OH group is important in catalysis (Fig. 1).[5,6] It is also known that Mg^{2+} is required for enzymic catalysis and is ligated by the phosphate group of substrates[3,7] and that Li^+ binds to enzyme–product complexes in place of Mg^{2+} in the inhibition of the enzyme.[3]

However, four important features of the system have not been explained. The first is the ability of the enzyme to process ribonucleoside 2'-phosphates (*e.g.* compound **1**) which lack the catalytically essential hydroxy group.[3,8] The second is that the position of the metal ion, Gd^{3+} (a competitive inhibitor for Mg^{2+}) in the X-ray crystal structure of a Gd^{3+}-sulfate complex of the enzyme[7] is completely inconsistent with the sequence of Mg^{2+} and substrate and product binding and debinding.[3] [The Gd^{3+} ion appears to be buried in such a way that it could not bind after the substrate (or debind before the product) contrary to expectations derived from kinetic studies.[3]] The third is that the optimum concentration of Mg^{2+} required for catalysis varies with the structure of the substrate[3,9] and that Mg^{2+} binding is cooperative for some substrates but not for others.[3] The fourth is that the mode of inhibition of the enzyme by Li^+ changes from uncompetitive to noncompetitive with increasing concentration[3] and that K_i for uncompetitive inhibition by Li^+ depends acutely on the structure of the substrate.[1] Here we provide a structural and mechanistic rationalisation for all of these properties.

2'-AMP **1** is a good substrate for the bovine brain monophosphatase[3] and is hydrolysed to give adenosine and inorganic phosphate (P_i). It is generally accepted that the 2'-, 3'- and 5'-oxygen atoms of the ribofuranosyl moiety in 2'-AMP (**1**; X = O) serve the same functions as the 1-, 2- and 4-oxygen atoms in D-Ins 1-P (**2**; X = O), respectively, in binding to the enzyme (Fig. 1). For Ins 1-P, the substrate binding groups are now well defined owing to elegant functional group deletion studies[5,6] which indicated that the 6-OH group of the substrate D-Ins 1-P is essential for catalysis and that its replacement by –OMe, –OR or by –H affords tight binding inhibitors.[10] Since 2'-AMP does not possess an equivalent catalytic group its ability to be processed by the enzyme was further examined.

The rate for the inositol monophosphatase‡ catalysed exchange of ^{18}O-label from ^{18}O-water into P_i was measured in the presence of adenosine (30 mmol dm^{-3}) and Mg^{2+} ions using assays similar to those used previously for measuring inositol-dependent ^{18}O-label incorporation.[2,3] Control experiments in which adenosine was replaced by inositol showed the expected rates of label incorporation[2,3] but those containing adenosine showed no incorporation whatsoever. Further-

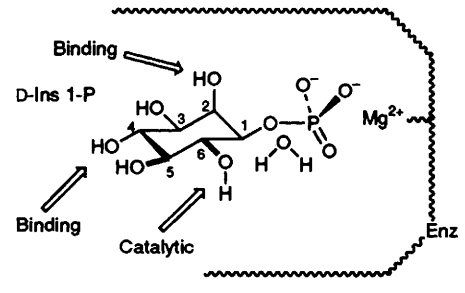
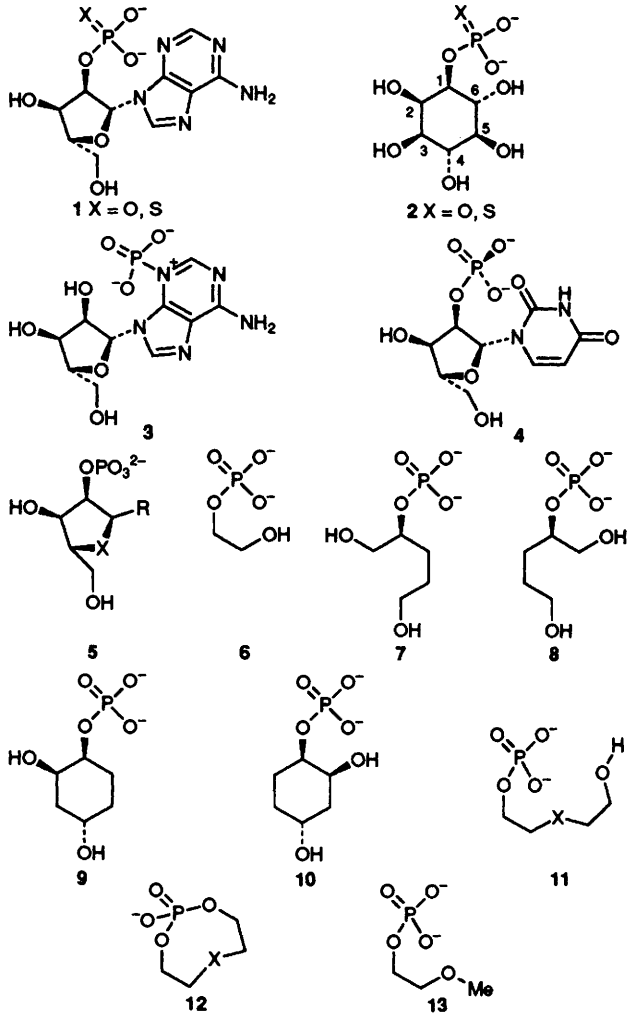


Fig. 1 Proposed roles of the flanking 2- and 6-hydroxy groups in binding to the enzyme and in facilitating catalysis. The active site nucleophile is a water molecule.



more, adenosine did not serve as an inhibitor for the hydrolysis reaction (K_i > 4 mol dm^{-3}) indicating that adenosine is not recognised as a product. Since adenosine-mediated ^{18}O-exchange into P_i is expected on the basis of microscopic reversibility, and since inositol-mediated exchange is facile, the results indicate that, for the adenosine system, the reverse reaction possesses at least one very slow step, *i.e.* a step in the hydrolysis reaction direction is essentially irreversible. Only two simple explanations can account for the differences in the kinetics of the two systems either 2′-AMP is hydrolysed by an alternative mechanism such that adenosine is not the true enzymic product, or the product binding site for adenosine on the enzyme requires an inaccessible high energy conformation.

Examination of Fig. 1 suggests that the catalytic 6-OH group of Ins 1-P might provide an anchor to orientate the nucleophilic water molecule to attack the phosphate P-atom *via* a seven-membered ring transition state.[1] While 2′-AMP lacks an equivalent hydroxy group the possibility that it could also form a seven-membered ring transition state leading to N^3-phosphoadenosine **3** *via* intramolecular trans-phosphorylation was discounted when it was shown that uridine 2′-phosphate **4** and the 5,6-dihydrouridine analogue (**5**; R = 5,6-dihydrouracil, X = O)§ underwent facile enzymic hydrolysis without forming C-2 phosphorylated uridine intermediates. In accord with these results, Leeson and coworkers[11] recently reported that ribofurans which lack the heterocyclic base (*e.g.* **5**; R = H, X = O) are substrates while carbocyclic ribofuran analogues (*e.g.* **5**; R = H, X = CH_2) are not.[11] Thus, it appears that enzyme-bound form of adenosine must exist in a high energy conformation and that the ribofuranosyl ring O-atom in 2′-AMP can serve as the surrogate catalytic site for the 6-OH group in Ins 1-P.

To probe further the enzyme's ability to recognise 2′-AMP but not adenosine, adenosine 2-phosphorothioate **1**, X = S, was prepared§ from adenosine and was tested as a substrate and as an inhibitor. It was already known that both L- and D-inositol phosphorothioate (**2**; X = S) were substrates for the enzyme (in the presence of Mg^{2+}) and possessed K_m and K_i values only tenfold higher than those for the corresponding phosphates.[12] Interestingly, the adenosine phosphorothioate **1**, X = S, did not serve as a substrate or as an inhibitor indicating that the enzyme could not recognise the molecule. However, when the compound was tested as a substrate in enzyme assays which contained thiophilic Mn^{2+} in place of the Mg^{2+}, the expected hydrolysis reaction occurred. These results clearly indicate that a strong binding interaction between an enzyme bound metal ion and the anionic group (either Mg^{2+} and phosphate, or Mn^{2+} and thiophosphate) is required to offset the energy required to reorganise the adenosine moiety into its active conformation.

In order to occupy the same position as the 6-OH group in Ins 1-P, relative to other functionally important groups, the furanosyl ring O-atom in 2′-AMP must move towards the 2′-O atom, so that their respective lone-pairs point towards each other. This conformation also suffers from an adverse 1,3-diaxial interaction between the adenine moiety and the 4′-hydroxymethyl group. Calculations predict that the structure is *ca.* 100 kJ mol^{-1} less stable than the unconstrained form (*K ca.* 10^{-17}) which would explain why adenosine is not recognised by the enzyme. By analogy to the Ins 1-P system (Fig. 1), the 2′-O atom and ribofuranosyl O-atom are key binding and catalytic functionalities respectively and must, therefore, interact with other species on the enzyme. Neither of the O-atoms possesses a hydrogen atom so that H-bonding cannot stabilise the system. However, chelation to a second Mg^{2+} ion to produce a five-membered metallocycle would substantially stabilise the system and also provide a possible site on the Mg^{2+} ion for hydroxide ion suitably disposed for attacking the P-atom [Fig. 2(*a*)].

The structure of the analogous catalytic complex for D-Ins 1-P [Fig. 2(*b*)] shows that only the 1- and 6- O-atoms of the inositol ring make contact with the second Mg^{2+} ion and only the phosphate moiety of the substrate makes contact with the first (buried) Mg^{2+} ion. Other interactions between the enzyme and the buried Mg^{2+} ion, as deduced from X-ray crystal data by others involve the β-carboxylate O-atoms of Asp-90, the amide O-atom of Ile-92, the β-OH group of Thr-95, two of the O-atoms of bound sulfate (*in lieu* of phosphate) and a γ-carboxylate O-atom of Glu-70 respectively.[7,13] With regard to a hydrolytic mechanism, the arrangement would suggest that the first Mg^{2+} ion should enhance the electrophilicity of the P-atom by coordinating to the phosphate O-atoms, while the second Mg^{2+} ion should stabilise the formation of hydroxide from a chelated water molecule, which would then be activated and disposed correctly to attack the P-atom.[14] The associative process may be aided through further stabilisation of the developing negative charge by the positive dipole of the Thr-195-Thr-205 α-helix which points directly at the sulfate anion in the published crystal structure,[7] and the Thr-95-His-100 α-helix which points towards the leaving 1-O-atom of inositol in modelled enzyme–substrate complexes. The second Mg^{2+} ion also requires a second counter-anion, in addition to hydroxide, and must interact with the enzyme. Modelling studies indicate that the carboxylate group of Asp 220 could fulfil this role. Thus, the minimum structure capable of acting as a substrate for the enzyme should be ethane-1,2-diol 1-phosphate **6**. Since this compound had not been used to probe the structure of the enzyme previously, it was prepared and tested and displayed a V_{max} value of 12% of the V_{max} for Ins 1-P, a K_m value of 0.7 mmol dm^{-3} and a K_i value for competitive inhibition of 1.0 mmol dm^{-3}. Note that the K_i value for P_i under these conditions is 0.5 mmol dm^{-3} and that the importance of the 2-OH was verified through its replacement by methyl, to give propyl phosphate, which possessed no inhibitory activity.

In order to probe the environment of the second Mg^{2+} ion, compound **6** was elaborated by incorporating extra oxygen functionalities into specific positions. Thus, (2*S*)- and (2*R*)-pentane-1,2,5-triol 2-phosphate (**7** and **8** respectively) were prepared as outlined in Scheme 1.

(2*S*)-Pentane-1,2,5-triol 2-phosphate **7** served as an inhibitor for the enzyme (K_i = 0.12 mmol dm^{-3}), as expected on the basis of the behaviour of the constrained cyclic analogue **9**.[6] Presumably, Mg^{2+} cannot bind in the absence of the 'catalytic' site [equivalent to the 6-OH group in Fig. 2(*b*)] and the interaction of the 1-OH and 5-OH groups of compound **7** with the enzyme account for the ninefold reduction in K_i over the diol phosphate **6** which can bind Mg^{2+}. On the other hand, by analogy with cyclic substrate **10**,[6] the (2*R*)-enantiomer of phosphate **8** might have been expected to serve as a substrate since it possesses all of the functional groups of the minimum substrate **6** as well as an OH group capable of binding to the

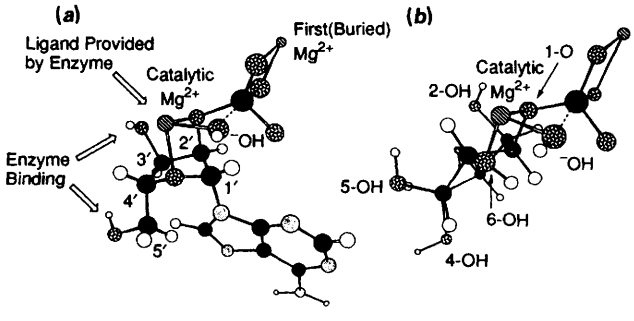
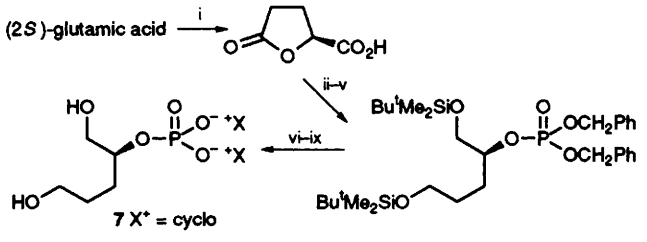


Fig. 2 (*a*) Model showing the role of the second (catalytic) Mg^{2+} ion in stabilising the conformation of the active form of 2′-AMP through chelation by the 2′- and ribofuranosyl O-atoms. (*b*) Showing the conformation of D-Ins 1-P and the important catalytic and binding interactions with the two Mg^{2+} ions.

site for the 4-OH group of Ins 1-P. However, compound **8** did not serve as a substrate and was a weak inhibitor, K_i = 3.8 mmol dm^{-3}. Indeed, phosphate **8** is the only small 1,2-diol phosphate that does not serve as a substrate. Thus, it appears that the flexible 5-OH group can provide a third ligand for the second Mg^{2+} ion which disrupts the geometry of the complex.

In another series of compounds, the 2-OH group of ethane-1,2-diol 1-phosphate **6** was elaborated to provide molecules that might displace the nucleophilic water molecule from its site on the second Mg^{2+} ion, see Fig. 2. Accordingly, phosphate esters (**11**, X = O or CH_2) and cyclic phosphate diesters (**12**, X = O or CH_2) were prepared. For comparison, 2-methoxyethanol 1-phosphate **13** was also prepared. Details of the syntheses will be reported elsewhere. Each of the compounds was tested as a potential substrate and inhibitor for the enzyme, and none was a substrate, as expected. However, their K_i values for competitive inhibition are informative and are consistent with structures depicted in Fig. 2. For example, the replacement of the 2-OH group in compound **6** by OMe to give **13**, led to a 25-fold reduction in binding affinity ($K_i \geqslant 25$ mmol dm^{-3}). Replacement of the methyl group by a 2-hydroxyethyl group, compound **11**; X = O, led to a eightfold increase in binding affinity (K_i = 3.5 mmol dm^{-3}). This increase in affinity is ascribed to the ability of the inhibitor to provide an extra ligand for the second Mg^{2+} ion by displacing the nucleophilic hydroxide ligand, see Fig. 2. The replacement of the ether O-atom to give compound **11**, X = CH_2, did not affect the K_i value, as would be expected if the ω-hydroxy group binds to the Mg^{2+} ion. The fact that neither compound served as a substrate excludes the possibility that the ω-hydroxy group binds in the site for the 5'-OH site for 2'-AMP [Fig. 2(*a*)]. The cyclic diester ether **12**, X = O, was a weak inhibitor (K_i = 8 mmol dm^{-3}). Nevertheless, this latter result is remarkable given that the compound is a monoanion and that K_i for sulfate is 20 mmol dm^{-3}.[8] As predicted from the properties of compounds **11**, X = O and CH_2, removal of the ether O-atom in the monoanion **12**, X = O, to give compound **12**, X = CH_2, had little effect on the value of K_i.

Thus, the properties of these new inhibitors are also completely consistent with the involvement of a second Mg^{2+} ion. Moreover, it can now be concluded that since substrate O-atoms provide two ligands for the second Mg^{2+} ion, its involvement accounts for the variance of the observed Mg^{2+} binding affinities for the enzyme with different substrates.[3,9] Most importantly the new findings explain the inconsistencies in the deduced binding sequences for species as derived from the crystal data for the Gd^{3+}–sulfate–enzyme complex which indicated that the metal was buried deeper in the active-site cleft than sulfate (phosphate), and, therefore, should bind



Scheme 1 *Reagents and conditions*: i, $NaNO_2$, HCl, H_2O, −10 °C, 76%; ii, $LiAlH_4$, THF, 45%; iii, Bu^tMe_2SiCl, imidazole, DMF, 68%; iv, $Pr^i_2NP(OCH_2Ph)_2$, 1*H*-tetrazole, MeCN; v, *m*CPBA, CH_2Cl_2 (78% for iv–v); vi, H_2 Pd/C; vii, Bu^tNF, THF; viii, Amberlite 118 (H^+); ix, cyclohexylamine, H_2O (69% for vi–ix)

first,[7] and our own kinetic studies which indicated that Mg^{2+} binds after the substrate.[3] Simply, the two approaches were each detecting one of two different metal ions. With regard to the mechanism of uncompetitive inhibition by Li^+, it is known that Li^+ binds in a site vacated by Mg^{2+} in an E–P_i product complex. With the emergence of the new (additional) Mg^{2+} ion site it is evident that, at low concentration, Li^+ must occupy the second site for Mg^{2+} to give an E–Mg^{2+}–P_i–Li^+ product complex. At higher concentration Li^+ is also able to bind into the first Mg^{2+} (buried) site on the free enzyme as is demonstrated by the almost unique change in the mode of inhibition by Li^+ from uncompetitive to noncompetitive with increasing Li^+ concentration.

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Footnotes

† *Abbreviations*: 2'-AMP, adenosine 2'-phosphate; P_i, inorganic phosphate; Ins 1-P, *myo*-inositol 1-phosphate; Ins 4-P, *myo*-inositol 4-phosphate.

‡ Inositol monophosphatase was purified and assayed as described in ref. 3.

§ All compounds and their intermediates were fully characterised and gave the expected spectral and analytic data. Full details will appear elsewhere.

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