The Structure of the 1L-\textit{myo}-inositol-1-phosphate Synthase-NAD\textsuperscript{+}-2-deoxy-\textit{d}-glucitol 6-(\textit{E})-Vinylhomophosphonate Complex Demands a Revision of the Enzyme Mechanism* \\

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1L-\textit{myo}-inositol 1-phosphate (MIP) synthase catalyzes the conversion of \textit{d}-glucose 6-phosphate to 1L-\textit{myo}-inositol 1-phosphate, the first and rate-limiting step in the biosynthesis of all inositol-containing compounds. It involves an oxidation, enolization, intramolecular aldol cyclization, and reduction. Here we present the structure of MIP synthase in complex with NAD\textsuperscript{+} and a high-affinity inhibitor, 2-deoxy-\textit{d}-glucitol 6-(\textit{E})-vinylhomophosphonate. This structure reveals interactions between the enzyme active site residues and the inhibitor that are significantly different from that proposed for 2-deoxy-\textit{d}-glucitol 6-phosphate in the previously published structure of MIP synthase-NAD\textsuperscript{+}-2-deoxy-\textit{d}-glucitol 6-phosphate. There are several other conformational changes in NAD\textsuperscript{+} and the enzyme active site as well. Based on the new structural data, we propose a new and completely different mechanism for MIP synthase.

Inositol-containing compounds play critical and diverse biological roles, including signal transduction, stress response, and cell wall biogenesis (1–4). Though large quantities of inositol are available from the diet, significant biosynthesis of inositol has been detected in organs where a significant blood barrier exists, such as the testes and brain (5–9). In fact, reduction of the brain inositol pool by inhibition of \textit{myo}-inositol (MI)\textsuperscript{+} monophosphatase has been suggested to be the mode of action for lithium in the treatment of bipolar disorder (10–13). Recent in vivo results in yeast (14) and dictyostelium (15) suggest that Valproate, a drug used in the treatment of depression, bipolar disorder, and seizure disorder, may act by inhibiting MIP synthase, thus lowering neuronal inositol pools similar to the action of lithium (14). Regulation of inositol biosynthesis itself may, therefore, play an important role in the regulation of second messenger signaling.

MIP synthase is remarkably conserved in eukaryotes, with better than 45% identity from yeast to humans (3, 16–25). In all cases, the enzyme displays modest catalytic activity, with turnover numbers ranging from 3 to 13 \(\mu\)mol/min/mg enzyme and with substrate \(K_m\) values in the 100 \(\mu\)M to 1 mM range (3, 17). The reaction path first proposed by Loewus et al. (shown in Fig. 1) remains the most likely (3, 26–29). A series of inhibitor studies indicates that the enzyme first binds the open acyclic tautomer of the substrate \textit{d}-glucose 6-phosphate, followed by oxidation to the C5 keto intermediate (30). Frost and coworkers propose that enolization is promoted by proton extraction by means of the substrate phosphate, which is consistent with the phosphate binding in a transoid conformation (30). They base this conclusion on the ability of 2-deoxy-\textit{d}-glucitol 6-(\textit{E})-vinylhomophosphonate, a substrate mimic of the enzyme that fixes the phosphate trans to C5, to strongly inhibit the enzyme, whereas the equivalent Z-mimic displays no affinity for the enzyme. 2-Deoxy-\textit{d}-glucitol 6-(\textit{E})-vinylhomophosphonate is, in fact, the most potent inhibitor of the enzyme known, having a \(K_i\) of 0.6 \(\mu\)M. Intramolecular aldol cyclization followed by reduction of the C5-ketone completes the formation of the product. None of the intermediates have been isolated or trapped, suggesting that all intermediates are tightly bound and not released until the final reduction to \textit{myo}-inositol 1-phosphate (31–33).

Crystal structures of several MIP synthase enzymes have been determined, including \textit{Saccharomyces cerevisiae} MIP synthase partially occupied with NAD\textsuperscript{+} and bound to fully occupied NAD\textsuperscript{+} and an inhibitor, 2-deoxy-\textit{d}-glucitol 6-phosphate (34). The inhibitor seemed to be bound in a relatively extended conformation, which is inconsistent with intramolecular aldol cyclization. Based on this data, a conformation that would be consistent with the cyclization was modeled, and a mechanism for the transformation was proposed. In addition, a possible location for an ammonium ion was identified, and it was proposed that the ammonium ion performed a similar function to that of a divalent cation in type II aldolases, stabilizing the developing negative charge on the enolate oxygen atom. This supposition was based on data showing that ammonium ions significantly activate \textit{S. cerevisiae} MIP synthase relative to other ions (17). The structure of \textit{Mycobacterium tuberculosis} MIP synthase bound to NAD\textsuperscript{+} also has recently been reported and a position for a Zn\textsuperscript{2+} proposed (35). However, the Zn\textsuperscript{2+} appears to be located between the amide of the nicotinamide and the nicotinamide phosphodiester on NAD\textsuperscript{+}. Though apparently not in a position to be directly involved in catalysis, this Zn\textsuperscript{2+} bridges the NAD\textsuperscript{+} and may help to define the nicotinamide position in the active site. Recently, we have determined the structure of \textit{S. cerevisiae} MIP synthase in the complete absence of NAD\textsuperscript{+} and in the presence of fully occupied NAD\textsuperscript{+} (36). We have also determined the structure of \textit{S. cerevisiae}
MIP synthase bound with NADH, phosphate, and glycerol (36). In both the apo and NAD\(^+\)-bound structures, several active site residues were disordered. When the enzyme was bound with NADH, several dramatic conformational changes were observed. All active site residues became ordered in this structure, and the conformation of NADH changed significantly with its nicotinamide ring moving more than 1 Å away from its position in the NAD\(^+\)-bound structure. A possible divalent cation was also observed in a position similar to that seen in the *M. tuberculosis* structure, between the nicotinamide phosphodiester and amide. Two small molecules were bound in the enzyme active site in this structure, and a phosphate and glycerol were modeled into these positions based on the shape and size of the electron density. However, the position of the phosphate was significantly different from that of the *S. cerevisiae* MIP synthase-NAD\(^+\)-2-deoxy-D-glucitol 6-phosphate complex structure (34), which is completely inconsistent with the position of the inhibitor in the active site and therefore calls into question the mechanism proposed based on the previous structure. Additionally, the putative ammonium ion proposed in this structure is one of the ligands for the putative metal ion, which is inconsistent with its identification as an ammonium ion.

These structural results call into question virtually the entire mechanism proposed for *S. cerevisiae* MIP synthase based on the 2-deoxyglucitol-6-phosphate-bound structure. Major issues include the location of the substrate phosphate, and indeed the entire substrate molecule during active catalysis, and the role of ammonium for stabilization of the enolate.

To better answer these and other questions regarding the mechanism of MIP synthase, we have determined the structure of *S. cerevisiae* MIP synthase in complex with NAD\(^+\) and a high affinity inhibitor 2-deoxy-D-glucitol 6-(E)-vinylhomophosphonate (\(K_i = 6.7 \times 10^{-6}\) M) at pH 5.5, a pH at which the enzyme still displays significant catalytic activity.

**EXPERIMENTAL PROCEDURES**

Crystallization, Data Collection, and Refinement—*S. cerevisiae* MIP synthase was purified as reported previously (34, 37). Protein was treated with activated charcoal for 30 min at 4 °C to remove cofactors. Crystals of apo *S. cerevisiae* MIP synthase were then grown by using the same condition used to grow the crystals of *S. cerevisiae* MIP synthase with partially occupied NAD\(^+\) and the *S. cerevisiae* MIP synthase-NAD\(^+\)-2-deoxy-D-glucitol 6-phosphate complex (37). Crystals of apo MIP synthase were then soaked in a stabilizer containing 5% polyethylene glycol 8000, 0.1 M NaAc, pH 5.5, 1 mM NAD\(^+\), 13.5 mM 2-deoxy-D-glucitol 6-(E)-vinylhomophosphonate for 24 h. For data collection, a single crystal was transferred to the cryo-protectant stabilizing solution (5% polyethylene glycol 8000, 0.1 M sodium acetate, pH 5.5, 30% glycerol) and flash-frozen. Data were collected at the synchrotron radiation source at the Industrial Macromolecular Crystallography Association Collaborative Access Team ID-17, Advanced Photon Source, Argonne National Laboratory. Diffraction data reduction and scaling were performed using HKL2000 (38). The structure was solved by using the *S. cerevisiae* MIP synthase-NADH-phosphate-glycerol complex structure (36) as an initial phasing model. The electron density maps were traced by using TURBO-FRODO, and multiple rounds of refine-
ment were conducted by using CNS (39). Data collection and final refinement statistics are tabulated in Table I. The final refinement model contained residues 9–533 in molecule A, residues 9–464 and 472–533 in molecule B in the asymmetric unit, and it also contained 400 water molecules. The inhibitor was present and ordered active site. The final R factor and R_{free} are 18.8 and 24.4%, respectively. Only 3 of 1041 residues are in the disallowed region in the Ramachandran plot (Asp-319 in molecule A, Asp-320 in molecules A and B) evaluated by PROCHECK (40).

Site-directed Mutagenesis and Enzyme Kinetic Assay—S. cerevisiae MIP synthase single mutants K369A, K412A, and K489A were constructed by using the QuikChange protocol (Stratagene). The primers used were: K369A, ATTTAGGCTCGAGAGATTTTCCAAA; K412A, GTGCGGGACTCAGATGGAA; and K489A, AGTTACTGCTTAGACGCCATTTA. Mutant enzymes were over-expressed and purified identically to that of wild-type enzyme. For the activity assay, the enzyme was incubated in the assay solution containing 50 mM Tris, 2 mM NH₄Cl, 0.2 mM diethylenetriothiophosphor, pH 7.7, with 1.5 mM NAD⁺ and various concentrations of the substrate 2-deoxy-D-glucose 6-phosphate. Reaction rates were monitored over a time period of 20 min and stopped by adding a 20% trichloroacetic acid solution to the reaction mixture. The reaction mixture was incubated with 0.2 mM NaNO₂ for one hour at 37 °C and quenched by the addition of 1.5 mM Na₂SO₃. Released inorganic phosphate was determined by the colorimetric method of Ames (41).

**Molecular Modeling**—All energy minimization calculations were performed by using the Insight II version 2000 software package (Molecular Simulations Inc., San Diego, CA). X-ray coordinates of the MIP synthase and 2-deoxy-D-glucose 6- (E)-vinylhomophosphonate were used to produce the starting coordinates of the substrate D-glucose 6-phosphate and the reaction intermediate myo-2-inosose 1-phosphate. The structures were parameterized by using the Discover/Insight II Extensible Systematic Force Field (ESFF) and incorporating the partial charges calculated for both substrates and the reaction intermediate by an electrostatic fitting procedure. Explicit hydrogens were added, and the amino acid residues were protonated so as to be consistent with the experimental pH value. Energy minimization was performed until the atomic root-mean-square deviation reached its minimum.

**RESULTS AND DISCUSSION**

**The Overall Structure of MIP Synthase**—The overall structure of the S. cerevisiae MIP synthase-NAD⁺-2-deoxy-D-glucose 6- (E)-vinylhomophosphonate complex is similar to that of the S. cerevisiae MIP synthase-NAD⁺-phosphate-glycerol complex (Fig. 2A; Ref. 36). The root-mean-square deviation between the two structures was only 0.49 Å. All of the active site residues are ordered in both molecules in the asymmetric unit, and the side chains of the active site residues overlap with those of the S. cerevisiae MIP synthase-NAD⁺-phosphate-glycerol complex structure very well. Cys-436 is the only exception, because its Ca and side chain sulfur atoms moved 2.4 Å and 3.5 Å, respectively, away from their positions in the S. cerevisiae MIP synthase-NAD⁺-phosphate-glycerol complex. In fact, the position of Cys-436 is identical to that of the apo, NAD⁺-bound, and the 2-deoxy-D-glucose 6-phosphate-bound structures (34, 36).

**The Conformation of NAD⁺**—The conformation of NAD⁺ is very similar to that of NADH in the S. cerevisiae MIP synthase-NAD⁺-phosphate-glycerol complex structure, differing significantly from that of the NAD⁺-bound and the NAD⁺-2-deoxy-D-glucose 6-phosphate-bound structures. However, the distance between the amide and phosphodiester is a bit closer than that of the MIP synthase-NAD⁺-phosphate-glycerol structure (3.2 Å versus 3.8 Å) (Fig. 2B). The putative divalent cation is present in this structure as well, but the fourth ligand seen in the
S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure (a water molecule) is not present. The fourth ligand is now Ser-439 O, identical to the coordination of the Zn$^{2+}$ in the M. tuberculosis MIP synthase structure. Interatomic distances between the putative divalent cation and its four ligands are 2.26, 2.58, 2.52, and 2.83 Å, respectively; bond angles are 95.0, 104.7, 92.01, and 112.8°. The conclusion to be drawn from these observations is that divalent cation binding is variable in S. cerevisiae MIP synthase, at least at low cation concentration. It also correlates with a well folded active site, as metal binding is only seen in structures of the S. cerevisiae MIP synthase when the active site is fully ordered. Therefore, this conformation of the cofactor probably represents the enzyme in its active state. Also, the conformational change of the cofactor is not solely due to the difference in the charge of the cofactor, as the present structure contains NAD$^+$, whereas NADH is bound in the previous structure.

The Structure of the Inhibitor 2-Deoxy-d-glucitol 6-(E)-Vinylhomophosphonate—The inhibitor 2-deoxy-d-glucitol 6-(E)-vinylhomophosphonate is bound in the enzyme active site in an extended conformation, as shown in the 1.8 e simulated annealing omit map shown in Fig. 3A. The phosphate group is in a transoid conformation relative to the inhibitor carbon backbone, fixed by the double bond between the phosphonate carbon and C6. The distance from the inhibitor C5 to nicotinamide C4 is 3.8 Å, a bit long for a direct hydride transfer. The inhibitor molecule is well nestled within the enzyme active site by hydrogen bond interactions with the active site residues (Fig. 3B).

The phosphate moiety is in an identical position to that of the phosphate in the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure (Fig. 2B) but is different from the position of the phosphate moiety of the inhibitor in the previously published S. cerevisiae MIP synthase-NAD$^+$-2-deoxy-d-glucitol 6-phosphate complex structure (34). In fact, the entire inhibitor molecule is rotated end to end, with the phosphate on the opposite side of the active site relative to the S. cerevisiae MIP synthase-NAD$^+$-2-deoxy-d-glucitol 6-phosphate complex structure (Fig. 4A). The phosphate makes hydrogen bonds with the main chain nitrogen atoms of Ser-323, Gly-324, Gln-325, and Thr-326. This motif is conserved among eukaryotes but not in prokaryotes (it is SQVGA in M. tuberculosis and TGEG in Archaeoglobus fulgidus). Conserved lysine residues Lys-412 and Lys-373 also make hydrogen bonds with the phosphate oxygens. All of the hydroxyl groups of the inhibitor except O1 make hydrogen bonds with side chains of conserved active site residues. There are hydrogen bonds between O3 and both Asp-356 OD1 and Lys-369 NZ; O4 and Asp-438 OD2; O5 and both Lys-369 NZ and Lys-489 NZ; O1P and Ser-323 N, Gly-324 N and Gln-325 N; O2P and Gly-324 N, Gln-325N and Lys-412 NZ; O3P and Thr-326 N, Thr-326 OG1 and Lys-373 NZ. All of these residues are absolutely conserved among eukaryotes from yeast to human. Residues Asp-356, Lys-369, Lys-373, Lys-412, Asp-438, Lys-489 are also conserved among MIP synthases from A. fulgidus and M. tuberculosis. Fig. 3B depicts all of the interactions between the inhibitor molecule and the active site residues. It is important to note that the putative divalent cation chelates the water molecule as part of hydrogen bond network that holds O3 and O4 of the inhibitor in their positions. However, the divalent cation position is quite far from O5, on the opposite side of the active site and seems not to be directly involved in the catalytic mechanism of the enzyme, ruling out a type II, metal-mediated aldol cyclization mechanism. The water molecule that is chelated to this metal is also on the opposite side of the active site but plays an important role in stabilizing residues in the active site (Asp-356 and Asp-438), both of which make hydrogen bonds to the hydroxyl groups of the inhibitor (Fig. 3B). Given the sequence similarity of the A. fulgidus MIP synthase to our S. cerevisiae MIP synthase enzyme, we conclude that this metal ion will likely be present in the A. fulgidus MIP synthase (17), though given the sensitivity of the Archaeabacteria enzyme toward EDTA and metal ions, the possibility of a second metal ion in or near the active site cannot be ruled out. The surprising result is the apparent lack of a requirement for the divalent in S. cerevisiae MIP synthase, because our structures together indicate that the nicotinamide is improperly positioned for catalysis in the absence of the divalent cation, as shown in our structures of the NAD$^+$-bound enzyme and the EDTA-treated NADH-bound enzymes (36). Clearly, more detailed analysis of the cationic requirements of S. cerevisiae MIP synthase is necessary to answer this question.

The constellation of new data from the S. cerevisiae MIP synthase-NAD$^+$ complex, the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex, and the S. cerevisiae MIP synthase-NAD$^+$-2-deoxy-d-glucitol 6-(E)-vinylhomophosphonate complex structures leads to the conclusion that the previous modeling of the inhibitor 2-deoxy-d-glucitol 6-phosphate is not consistent with substrate binding at neutral pH and that the mechanism proposed must, therefore, also be revised (34). (i) Asp-356 is in the same conformation in all of the above-mentioned structures, as well as M. tuberculosis MIP synthase and A. fulgidus MIP synthase, as opposed to the conformation in the previously reported S. cerevisiae MIP synthase-NAD$^+$-2-deoxy-d-glucitol 6-phosphate complex structure (Fig. 4A; Ref. 34), where it is flipped out of the active site. (ii) The phosphate moiety of 2-deoxy-d-glucitol 6-(E)-vinylhomophosphonate is in
S. cerevisiae synthase-NADH-phosphate-glycerol complex structure and the white positively charged for encapsulation of the substrate phosphate. In both the reaction of the substrate and the substrate-binding site of MIP synthase at physiological pH. There must be strong polar interactions between the substrate and the substrate-binding site of MIP synthase-NADH-phosphate-glycerol complex structure (Fig. 4A; Ref. 34). (iii) As shown in Fig. 4B, an analysis of surface charge distribution of the substrate-binding site indicates that the phosphate-binding pocket is positively charged for encapsulation of the substrate phosphate. an identical position to that of the phosphate in the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure (Fig. 2B), which is quite different from that of the inhibitor from the previously published S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure and the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure and the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure, the phosphate moieties are located in this positively charged region. However, in the previously published structure of the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure, the phosphate is located on the opposite side of the active site, where the surface is, in fact, negatively charged. (iv) The structure of the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex was determined at pH 5.5, closer to the optimal pH (7.2–7.7) than pH 4.5, at which the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure was determined. MIP synthase activity assays at different pH indicate that, at pH 4.5, the enzyme has very low activity, but at pH 5.5, the enzyme recovers about 50% of its optimum activity (Fig. 5). Finally, in the structure of the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex, the conformations of the substrate-interacting residues that are different from the previous S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure (34) agree well with that of the recently reported structure of M. tuberculosis MIP synthase (35), especially the region surrounding the phosphate-binding pocket. Specifically, Ser-323 is flipped into the active site in the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure (Fig. 4A), effectively blocking phosphate binding, whereas it is flipped out in the M. tuberculosis MIP synthase, S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex, and S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structures, allowing room for phosphate binding. This strongly indicates that Ser-323 belongs in the “flipped out” conformation when the enzyme is binding substrate, as we see in the S. cerevisiae MIP synthase-NADH-phosphate-glycerol complex structure.

**Modeling of the Substrate and Reaction Intermediates**—As described above, the inhibitor 2-deoxy-d-glucitol 6-(E)-vinylhomophosphonate is bound in the active site of the enzyme in an extended conformation and is incapable of cyclization (Fig. 3). When the substrate is modeled in the same conformation in the active site, a steric collision occurs between the 2-hydroxyl group and the residue Leu-360 in the active site (the distance from the 2-hydroxyl oxygen and Leu-360 carbon is 2 Å). This indicates that the substrate must bind differently in the active site than does the inhibitor in the vicinity of C1 and C2 (42). Based on the location and conformation of the inhibitor 2-deoxy-d-glucitol 6-(E)-vinylhomophosphonate, the substrate d-glucose 6-phosphate was modeled in a conformation consistent with cyclization. The phosphate portion was overlaid onto that of the inhibitor molecule with O6 at the position of the inhibitor C7, C6, C5, and C4 were overlaid onto the inhibitor C6, C5, and C4, respectively. Energy minimization using the program Insight II was performed to model the rest of the substrate in a minimum energy conformation. The result of modeling was such that none of the backbone atoms and the hydroxyl oxygen

![Image](https://i.imgur.com/1Q5Q5Q.png)
atoms would collide with the side chains of active site residues (Fig. 6A). The result of this modeling provided abundant information regarding potential interactions between the substrate in its pseudocyclic conformation and the enzyme active site. All but O2 and O3 of the substrate hydroxyl groups make hydrogen bonds with active site residue side chains. Fig. 6B depicts most of the interactions seen. The final reaction intermediate, myo-2-inosose 1-phosphate, was also modeled, and its energy minimized in the active site (Fig. 6C). This cyclic intermediate makes an additional hydrogen bond between O3 and Asp-438, which contributes to the stabilization of the cyclic conformation.

Proposed Mechanism of MIP Synthase—The structure of the S. cerevisiae MIP synthase-NAD$^+$-2-deoxy-D-glucitol 6-(E)-vinylhomophosphonate described above (34) is inconsistent with the mechanism proposed previously, which was based on the structure of the S. cerevisiae MIP synthase-NAD$^+$-2-deoxy-D-glucitol 6-phosphate complex. Based on the new structure of the S. cerevisiae MIP synthase-NAD$^+$-2-deoxy-D-glucitol 6-(E)-vinylhomophosphonate complex and modeling of the substrate and reaction intermediates, a new mechanism can be proposed (Fig. 7).

In the first step, the substrate is oxidized at C5 by NAD$^+$. This involves direct hydride transfer from C5 of D-glucose 6-phosphate to C4 of the nicotinamide moiety of NAD$^+$; this is consistent with the present crystal structure where the nicotinamide is located in a suitable orientation for hydride transfer to occur. In concert, a proton is lost from the C5 hydroxyl group of D-glucose 6-phosphate. This proton can be transferred to the Lys-369 terminal nitrogen atom, which is 2.8 Å away from the substrate O5. Asp-320, adjacent to Lys-369, could then accept this proton in a proton-shuffling system.

The second step is the enolization. During the enolization, the pro-R hydrogen of C6 is eliminated (43). From the crystal structure, either the phosphate monoester or Lys-489 may act as the base at the enolization step. The distance between C6 and the phosphate oxygen is 3 Å, and between Lys-489 NZ and C6 is 3.4 Å. The phosphate monoester acting as the base at the enolization step has precedent in the dehydroquinate synthase mechanism (44, 45). This hypothesis requires the phosphate to be in a transoid conformation relative to the carbon backbone of the substrate, which is consistent with the present structure (Fig. 3, A and B). From the modeling of the 5-keto-d-glucose 6-phosphate intermediate in the active site, Lys-489 is also in a suitable position to remove the pro-R hydrogen of C6. The developing negative charge on the enolate oxygen is stabilized by two lysines, Lys-369 and Lys-489.

In the aldol condensation step, the phosphate could transfer the proton abstracted from C6 to O1. Lys-412 could also transfer a proton to O1 in the aldol cyclization step; the developing negative charge on O1 would then be stabilized by Lys-373. From the crystal structure and the substrate modeling, a type I aldolase mechanism where Lys-369 or Lys-489 could form a Schiff base with C5 of the substrate also can not be ruled out. However, Schiff base formation would require significant conformational changes of either the enzyme main chain or the substrate, because neither Lys-369 nor Lys-489 can reach C5 in its present position in our structure.

The last step is the reduction by NADH. The hydride that was transferred in the first step to the nicotinamide C4 returns to the C5 of the intermediate myo-2-inosose 1-phosphate. Using the same proton-shuffling system, a proton could then be transferred to the C5 ketone oxygen from Asp-320, via Lys-369. It is important to realize that our mechanistic proposal is based largely on our inhibitor-bound structure and not our modeling, as the C5-O5 bond is already oriented properly for hydride transfer from NAD$^+$. On the other hand, our hypotheses re-
regarding activation of the O1 aldehyde by Lys-412 and Lys-373 are based on our modeling in the active site, though it is important to realize that both Lys-373 and Lys-412 are absolutely conserved in all MIP synthase sequences and that our mutagenesis results are consistent with this hypothesis in that the K412A mutation is completely inactive.

Mutagenesis—To verify the importance of residues Lys-369, Lys-412, Lys-489 in the mechanism, we have mutated these three lysine residues to alanine and investigated the activity of these mutants. All three mutations have resulted in complete loss of activity, as none of these mutants was able to turn the substrate over detectably. This result strongly suggests that these lysine residues indeed play very important mechanistic roles.

Though the mechanism described above is reasonable, it is by no means definitive. Verification of the mechanism proposed above requires further structural investigations of MIP synthase in complex with various structural analogues of reaction intermediates. At this point, there is still not enough data regarding whether the substrate binds in its cyclic form, followed by ring opening catalyzed by the enzyme, or binds in its acyclic form, which constitutes less than 0.4% of D-glucose 6-phosphate in solution. It is particularly important to produce a structure of a product-like inhibitor, which is already cyclized, to validate our modeling of the cyclic conformation in the active site.

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