Probing the Mechanism of the *Archaeoglobus fulgidus* Inositol-1-phosphate Synthase*

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myo-Inositol-1-phosphate synthase (mIPS) catalyzes the conversion of glucose-6-phosphate (G-6-P) to inositol-1-phosphate. In the sulfate-reducing archaeon *Archaeoglobus fulgidus* it is a metal-dependent therozyme that catalyzes the first step in the biosynthetic pathway of the unusual osmolyte di-myoinositol-1,1'-phosphate. Several site-specific mutants of the archaeal mIPS were prepared and characterized to probe the details of the catalytic mechanism that was suggested by the recently solved crystal structure and by the comparison to the yeast mIPS. Six charged residues in the active site (Asp225, Lys274, Lys278, Lys306, Asp332, and Lys367) and two noncharged residues (Asn255 and Leu257) have been changed to alanine. The charged residues are located at the active site and were proposed to play binding and/or direct catalytic roles, whereas noncharged residues are likely to be involved in proper binding of the substrate. Kinetic studies showed that only N255A retains any measurable activity, whereas two other mutants, K306A and D332A, can carry out the initial oxidation of G-6-P and reduction of NAD⁺ to NADH. The rest of the mutant enzymes show major changes in binding of G-6-P (monitored by the 31P line width of inorganic phosphate when G-6-P is added in the presence of EDTA) or NAD⁺ (detected via changes in the protein intrinsic fluorescence). Characterization of these mutants provides new twists on the catalytic mechanism previously proposed for this enzyme.

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Asp$^{32}$ that might aid in binding of substrate, cofactor, or divalent metal ion to the enzyme. The placement of these residues in the active site of *A. fulgidus* mIPS is shown in Fig. 1. Characterization of enzyme activity, the ability to carry out the initial oxidation of G-6-P, and ligand binding for each of these mutants provide definitive roles for several of these residues but also suggest a modified catalytic mechanism for the archaeal mIPS.

**MATERIALS AND METHODS**

**Construction of Mutants—** *A. fulgidus* mIPS mutants were constructed using a QuikChange site-directed mutagenesis kit from Stratagene. Overexpression and purification of the mutants were carried out according to the procedures for the wild type mIPS described previously (12). SDS-PAGE on 12% polyacrylamide gel was used to assess the purity of the mutants, and absorbance at 280 nm was used to determine the concentration of the mutants (the extinction coefficient at 280 nm is 50210 M$^{-1}$ cm$^{-1}$ as calculated from the sequence). All of the mutants were stored at 4 °C in 50 mM Tris acetate buffer, pH 7.5.

**mIPS Specific Activity Assay—** Conversion of G-6-P to I-1-P was monitored via $^{31}$P (202.3 MHz) NMR spectroscopy (12) using a Varian INOVA 500 system with a broadband probe. Assay mixtures (5 mM G-6-P, 1 mM NAD$^+$, 1 mM MgCl$_2$ in 50 mM Tris-HCl, pH 7.5, with 20% D$_2$O) were incubated with mIPS (typically 0.5 mM enzyme in 50 mM Tris, pH 7.5, or H$_2$O, pH 7) was monitored by the CD spectra in the far UV region. The CD spectra of all proteins in 10 mM potassium phosphate at pH 7 were acquired using an AVIV 202 circular dichroism spectrometer. Enzyme samples (~1 μM) in both 10 mM KH$_2$PO$_4$, pH 7, or H$_2$O, pH 7 were analyzed. Wavelength scans were taken from 300 to 190 nm at room temperature with a bandwidth of 1.0 nm and an averaging time of 1 s.

**RESULTS**

**Mutant Expression and Stabilization by P$_i$—** The eight mutants expressed were purified to >90% homogeneity as monitored by SDS-PAGE. The secondary structure of these proteins was monitored by the CD spectra in the far UV region. The CD spectra of all proteins in 10 mM potassium phosphate at pH 7 were comparable (12.5% α-helix, 40.2% β-sheet, 22.4% β-turn, and 29.4% random coil as obtained by the CD spectra deconvolution software; Neural Networks, version 2.0d). That fact suggested the overall secondary structure content of the proteins under these conditions is not altered by the different mutations. Given the unstructured regions around the active site in the yeast crystal structure in the absence of substrate (14), it is likely that the P$_i$ binding promotes structural stability and correct folding of the *A. fulgidus* IPS active site region. Older preparations of wild type mIPS often lose activity that correlates with precipitation of the protein. As little as 5 mM potassium phosphate added to precipitated enzyme is able to resolubilize the protein and restore activity. Thus, P$_i$ may aid in limited refolding of the active site regions as well as stabilize a specific folded conformation of the archaeal mIPS.

**Intrinsic Fluorescence Assay for NAD$^+$ Binding—** A Jobin y Van Halogenorax 3 spectrophotofluorimeter was used to monitor the binding of NAD$^+$ to IPS and IPS mutants (1–μM in 10 mM EDTA with 10 mM KH$_2$PO$_4$, pH 7). Steady state fluorescence measurements were taken at room temperature with an excitation wavelength of 290 nm and 2-mm excitation and emission slit widths. The mIPS emission was scanned from 300 to 400 nm in the absence and presence of 1–20 μM NAD$^+$. Controls, run in parallel, of mIPS where only buffer was added were used to account for small volume changes associated with dilution of the mIPS when the NAD$^+$ was added. The addition of NAD$^+$ caused a decrease in the mIPS intrinsic fluorescence, which was plotted as Δ$F$ = $F_b$ – $F$ (in arbitrary units). The concentration of NAD$^+$ for 50% of the maximum fluorescence change was used to characterize the apparent $K_D$ for NAD$^+$.

**CD Analysis of Secondary Structure—** CD spectra in the far UV were acquired using an AVIV 202 circular dichroism spectrometer. Enzyme samples (~1 μM) in both 10 mM KH$_2$PO$_4$, pH 7, or H$_2$O, pH 7 were analyzed. Wavelength scans were taken from 300 to 190 nm at room temperature with a bandwidth of 1.0 nm and an averaging time of 1 s.

**DISCUSSION**
Activity of Mutants—$^{31}$P NMR spectroscopy provides an excellent way to monitor mIPS activity (12). In the $^{31}$P spectrum, the $\alpha$ and $\beta$ isomers of G-6-P (substrate) at $-4.4$ ppm are well separated from the I-1-P (product) resonance at 4.0 ppm. The NAD$^+$ cofactor at 1 mM, which is well in excess of what is needed to saturate the wild type enzyme, is characterized by an AB quartet at $-9.9$ ppm. The increase in I-1-P resonance intensity compared with G-6-P (at a concentration of 5 mM, well above the $K_m$) was used to estimate the mIPS specific activity. Mg$^{2+}$ (1 mM) present in the assay mixtures was also considerably in excess of the $K_m$ of 24 $\mu$M established for the recombinant enzyme (12). Under these conditions, mIPS exhibited a specific activity of 13.2 $\mu$mol min$^{-1}$ mg$^{-1}$ at 90°C. The only mutant that exhibited any conversion of G-6-P to I-1-P was N255A, although the reaction was significantly reduced. The estimated specific activity for N255A was 0.012 $\mu$mol min$^{-1}$ mg$^{-1}$, 0.09% that of recombinant wild type mIPS. All of the other mutants had specific activities much less than 0.001 $\mu$mol min$^{-1}$ mg$^{-1}$ because no product was detected over long periods of time and with an increased amount of protein added to assay mixtures.

Generation of NADH by mIPS Mutants—Although most of the mutants did not produce I-1-P, it is possible that they could still carry out the initial oxidation of G-6-P and even the cyclization step to form the inosose compound. The addition of EDTA to the assay mix inhibits the A. fulgidus mIPS cyclization of 5-keto-G-6-P to myo-2-inosose-1-phosphate (12), because divalent metal cations are absolutely required for the aldol condensation reaction. As shown in Fig. 2, with wild type protein, stoichiometric amounts of NADH were produced after incubation of protein with excess G-6-P and NAD$^+$ in the presence of EDTA at 90°C. Although depletion of divalent cations dramatically slows down this first step of the reaction (12), this assay can be used to see whether any of the mutants are able to carry out the first step (of the many steps and two intermediates) in 1-P-P production. Of the eight mutants, NADH was produced in stoichiometric amounts to protein in only two of the mutants, K306A and D332A. Most of the mutants (e.g. L257A in Fig. 2) could not form NADH under these incubation conditions. The only mutant that exhibited very low catalytic activity, N255A, did not exhibit stoichiometric NADH production, perhaps suggesting that one or both of the ligands were weakly bound under these assay conditions. The ability to form NADH indicates that the involvement of Lys$^{306}$ and Asp$^{332}$ in catalysis must occur after the oxidation step. For the other residues, the lack of NADH formation suggests that they must be involved in substrate and cofactor binding in the first part of the reaction, stabilization of linearized G-6-P in the active site, deprotonation of the C-5 hydroxyl group, and/or oxidation of C-5.

Detection of G-6-P Substrate and Substrate Analog Binding—The five mutants that were unable to form NADH might, alternatively, be unable to bind substrates (NAD$^+$ and G-6-P).

Because P$_i$ bound to mIPS could be detected in the $^{31}$P spectrum of the isolated protein, this method could be applicable for detecting G-6-P and other (containing phosphate group) potential ligands binding to the enzyme. However, the $^{31}$P spectrum of ~70 $\mu$M wt mIPS mixed with 1 mM G-6-P in the presence of EDTA showed no broadening of the G-6-P phosphorus line width (which was <2 Hz in the buffer alone under these conditions). If the $K_m$ for G-6-P is comparable with the $K_m$ for this substrate, most of the enzyme would be saturated with G-6-P, leading to a significant fraction of enzyme-bound G-6-P. This observation has three possible explanations: (i) G-6-P does not bind without divalent cations bound to the protein, (ii) the bound G-6-P is not in fast exchange with free G-6-P (a possibility that must be considered because during the reaction the intermediates are tightly bound and not released into solution), and (iii) with residual NAD$^+$ present, 5-keto-G-6-P might be formed and tightly bound to the enzyme so that it is not released into solution.

In contrast to what was observed with G-6-P, excess P$_i$ added to the protein with EDTA present was significantly broadened, indicating that both free and bound P$_i$ were in exchange. The P$_i$ bound to the enzyme (~180 kDa) would have a long correlation time because of the large size of the complex. The 13 Hz line width observed for P$_i$ bound to the purified protein is consistent with P$_i$ bound to a large complex. When additional P$_i$ was titrated into the mIPS (68 $\mu$M) solution, the P$_i$ line width increased and then narrowed as the P$_i$ concentration increased further (Fig. 3). A fast exchange analysis ($\Delta$,$\Delta_0$) was the difference in line width for free P$_i$, at a given concentration and P$_i$ when enzyme was present, $E_0$ was the total enzyme concentration, and $L_0$ was the total P$_i$ concentration) was used to estimate the enzyme bound line width, $\Delta_b$, and $K_p$ for P$_i$ binding to recombinant mIPS. Values of 19 ± 5 Hz and 0.05 ± 0.02 mM for $\Delta_b$ and $K_p$ were obtained. That $\Delta_b$ was larger than the 13 Hz line width measured for P$_i$ bound to mIPS in the absence of any added P$_i$ might suggest that the ligand binding is in an intermediate exchange regime.

With a concentration of P$_i$ and mIPS that leads to substantial line broadening, we can monitor whether, in the presence of EDTA (and at room temperature), substrate can displace P$_i$ from the mIPS active site. Any solute added that binds to the active site and displaces the P$_i$ should narrow the P$_i$ resonance because the bound solute would reduce the population of enzyme-bound P$_i$ that is averaged into the observed line width. When G-6-P (1 and 2 mM) was added to mIPS (56 $\mu$M) and 0.5 mM P$_i$, the observed P$_i$ line width ($\Delta_0$,$\Delta_1$) narrowed significantly, from an initial value of 7 ± 1 to 3 ± 0.5 Hz with 2 mM G-6-P (Fig. 4). This indicates that G-6-P and P$_i$ bind to the same location in the active site. Thus, P$_i$ binding can be used indirectly to assess whether other substrate-like molecules bind to the mIPS protein as well as to mIPS mutants.

Previously, we examined 2-deoxy-glucose-6-phosphate, glucose-6-sulfate, mannose-6-phosphate, glucose, and glycerol 3-phosphate as potential substrate analogs for the wt A. fulgidus mIPS. Although none of these molecules were substrates (12), they could still bind to the protein as inhibitors. In the presence of A. fulgidus mIPS, the P$_i$ line width decreased significantly only when increasing concentrations (up to 2 mM) of glycerol-3-phosphate were added to the solution (Fig. 4). Hence, this small organophosphate must bind to the mIPS active site to displace P$_i$. The other phosphate- or sulfate-containing compounds, where the cyclic form is in equilibrium with a linear form, cannot bind tightly to the mIPS, because they do not lead to a narrowing of the P$_i$ resonance. The addition of glucose also failed to decrease the P$_i$ line width.
However, this last result must be viewed with some caution because although it could indicate that this moiety does not bind to mIPS, it might also mean that both glucose and Pi can occupy the active site.

The line width of Pi in the presence of mIPS could be used to see whether, for mutant mIPS enzymes, (i) Pi can bind to the enzyme and (ii) G-6-P can displace the bound Pi. In the eight mutants generated, binding of Pi (as monitored by an increased line width in the presence of protein) was observed for all but L257A (Table I). The lack of Pi line-broadening in the presence of L257A suggests that its active site is sufficiently perturbed so that it no longer binds Pi tightly (and by inference G-6-P); if the $K_D$ increased from 0.05 mM (wild type enzyme) to 2 mM, we would be unlikely to detect any line width changes under the conditions used. As shown in Fig. 5 for several of the mutants, observed Pi line widths reflecting Pi binding to mIPS varied depending on the mutant. This could reflect an altered mobility in the Pi-mIPS complex or an altered $K_D$ that affects the off-rate of the complex. For example, the much larger Pi line width in the presence of D332A suggests that removal of this aspartate has increased the affinity of Pi for the active site (possibly by relieving like charge repulsion) where exchange between enzyme-bound and free Pi occurs on an intermediate time scale. In contrast, the smaller line width for Pi in the presence of a comparable concentration of K274A could indicate that Pi does not bind as tightly to this mutant.

The increase in Pi line width in the presence of mIPS protein was used to check for G-6-P binding to the mutant proteins. Using 0.5 mM Pi and 50–70 mM mIPS to generate a Pi line width broadened by exchange (with $\Delta u_{obs}$ ranging from 3.6 to 15.6 Hz compared with free Pi of 1 Hz; Table I and Fig. 5), we then added 5 mM G-6-P to see whether the mIPS substrate could bind to the mutant and displace Pi. As shown in Table I, for most of the mutants, G-6-P could displace bound Pi as evidenced by the decreased line width observed for Pi. As shown in Table I, for most of the mutants, G-6-P could displace bound Pi, as evidenced by the decreased line width observed for Pi. D225A, K274A, K278A, K306A, and D332A all exhibited narrower Pi line widths when G-6-P was added. However, N255A and K367 showed little change in the line width of the bound Pi, when 5 mM G-6-P was added. This indicates that these two mutations have impaired G-6-P binding that is much weaker than Pi binding to the wild type protein.

Binding of NAD$^+$—A. fulgidus mIPS activity was shown to
be significantly increased when exogenous NAD$^+$ was added to the protein (12). Although some of the subunits could have NAD$^+$ bound already, adding NAD$^+$ saturates all the active sites with this cofactor. NAD$^+$ binding to vacant sites decreases the intrinsic fluorescence intensity but not the wavelength of maximum emission of the archaeal mIPS protein. This change in fluorescence intensity can be used to quantify NAD$^+$ binding to the proteins by measuring the intrinsic fluorescence intensity at 334 nm compared with that for the protein where only buffer was added over an NAD$^+$ concentration range of 1–20 μM. There are two tryptophans near the active site of A. fulgidus mIPS (13) that could have altered characteristics upon NAD$^+$ binding. From the change in fluorescence intensity as a function of added NAD$^+$, we used the concentration for 50% of the maximum change to define an apparent $K_d$ for NAD$^+$ binding (see Fig. 6 for fluorescence changes for several of the mutants). Although the wild type, N255A, L257A, K306A, and K367A were characterized by $K_d$ values $<10$ μM (Table II), D225A, K274A, and D332A showed considerably weaker binding of the cofactor. NAD$^+$ did not induce any changes in the intrinsic fluorescence of K274A in the cofactor concentration range studied. This could indicate a dramatically reduced affinity of the mutant protein for NAD$^+$. However, if mIPS

### Table I

| mIPS          | $\Delta v_{obs}$ | $v_{obs} - v_{free}$ |
|---------------|-----------------|----------------------|
| Wild type     | 6.2             | -4.9                 |
| D225A         | 6.5             | -5.9                 |
| N255A         | 3.6             | -0.5                 |
| L257A         | 1.2             | -1.8                 |
| K274A         | 4.8             | -4.1                 |
| K278A         | 6.3             | -3.2                 |
| K306A         | 5.3             | -4.5                 |
| D332A         | 15.6            | -11                  |
| K367A         | 4.1             | -0.2                 |

$^a$ Line width observed for 0.5 mM Pi, with 0.07–0.08 mM mIPS. 
$^b$ Decrease in P$i$ line width upon the addition of 5 mM G-6-P. The initial P$i$ line width in the presence of mIPS under these conditions was typically in the range of 4–7 Hz, except for D332A, where the initial line width under these conditions was ~16 Hz, and L257A, which only exhibited a very narrow P$i$ resonance (and in essence serves as a control for free P$i$).

### Table II

| mIPS          | $K_d$ (μM) |
|---------------|------------|
| Wild type     | 1.0 ± 0.2  |
| D225A         | 44 ± 16    |
| N255A         | 2.8 ± 1.2  |
| L257A         | 6.5 ± 3.4  |
| K274A         | 10 ± 2     |
| K278A         | 70 ± 21    |
| K306A         | 4.4 ± 1.8  |
| D332A         | 16 ± 8     |
| K367A         | 1.7 ± 0.6  |

$^c$ The $K_d$ value for wild type protein is very similar to the kinetic $K_m$ for this substrate (12). 
$^d$ There was no change in the fluorescence intensity of this mutant when NAD$^+$ was added to the protein.

### Discussion

Understanding the function of key residues responsible for substrate binding and reaction intermediate formation is critical toward formulating a catalytic mechanism of an enzyme. In a complex enzyme such as mIPS with two ligands required for catalysis to commence and two intermediates, characterization of mutants solely by loss of catalytic activity is inadequate. Additional evidence for significant conformational changes along the pathway and sequestration of the intermediates from dissociation into solution are needed to draw more detailed conclusions about the role of a given residue in catalysis. Indeed, several active site mutants of yeast and M. tuberculosis mIPS have been generated based on the crystal structures (17, 18) and shown to be inactive. Exactly at what point in the mechanism the activity has been impaired was difficult to deduce from those experiments. At best the mutagenesis indicated that a particular residue is critical for catalysis, but whether or not it is involved directly in catalysis or indirectly (ligand binding or structural role) cannot be gleaned from loss-of-function studies only.

Our prior studies have shown that A. fulgidus mIPS requires
divalent cations for the aldol cyclization step, so the catalysis can be halted after the first step (oxidation at C-5) by chelating the cations with EDTA (12). This enables us to identify residues important for at least the first step of the reaction by monitoring the production of 5-keto-G-6-P and NADH. The addition of assays for substrate binding in mutants that cannot carry out the initial redox step provides a better test of specific defects in archaeal mIPS mutants. A summary of the A. fulgidus mIPS mutants generated, and their responses in the divergent assays and initial roles proposed based on the crystal structure, is presented in Table III. The residues mutated were chosen based on the A. fulgidus mIPS crystal structure with P<sub>i</sub>, and NAD<sup>+</sup> present and its structural homologies to the yeast and M. tuberculosis mIPS enzymes (13).

N255A was the only mutant to show any formation of I-1-P. The specific activity was less than 0.1% that of the wt enzyme. Because this mutant can convert G-6-P to I-1-P, it must form NADH. However, in the presence of EDTA and excess substrates very little NADH was detected (certainly not stoichiometrically). This suggests that binding of one or both ligands to the mutant enzyme has been impaired. NAD<sup>+</sup> binding to the protein, as assessed by changes in the intrinsic fluorescence of the protein, exhibited a KD similar to wild type protein, whereas NAD<sup>+</sup> binding was weakened. The replacement of Asn<sub>255</sub> by Ala significantly weakens G-6-P binding to the protein.

K274A was essentially inactive (no I-1-P was detected under the assay conditions) leading to an upper limit for NADH produced. This means that that residue plays a role in the fluorescence change upon cofactor binding. For D225A, P<sub>i</sub> and G-6-P binding are similar to wild type, whereas NAD<sup>+</sup> binding is much more weakly than wild type protein, which might suggest that the second ion is critical for correct orientation of the cofactor.

The other five mutant mIPS were blocked before the first redox step. In the crystal structure, Leu<sup>257</sup> is thought to be coplanar with the closed ring of the product and could help to stabilize binding of glucose moieties. Clearly, L257A does not bind G-6-P or even P<sub>i</sub> well, suggesting a significant reorganization of active site residues. Thus, Leu<sup>257</sup> has a primary role in substrate binding possibly by aiding the organization of the active site. A plausible hypothesis is that it participates in folding of a short helical fragment of residues 259–264 that is critical for active site structure. Lys<sup>367</sup> was proposed to abstract a proton from the hydroxyl group of G-6-P C-5 (13). The various assays are consistent with that role if one assumes that the complete lack of K274A quenching with added NAD<sup>+</sup> means that that residue plays a role in the fluorescence change upon cofactor binding. For D225A, P<sub>i</sub> and G-6-P binding are similar to wild type protein, whereas NAD<sup>+</sup> binding is much weaker (an apparent KD of 44 μM compared with 1 μM for recombinant mIPS). It is likely that the main role of this residue must be in stabilization of NAD<sup>+</sup> binding.

K367A can bind P<sub>i</sub> but it has lost or at least significantly reduced its affinity for G-6-P, which would explain its inability to produce NADH. Lys<sup>367</sup> was initially proposed as the base in the enolization step that occurs after formation of 5-keto-G-6-P (13). Because the first step does not occur with this mutant, this residue must have a primary role in an earlier event, likely substrate binding. The remaining mutation, K278A, generated protein that could bind G-6-P but had greatly reduced affinity for NAD<sup>+</sup>. The crystal structure of A. fulgidus mIPS shows that Lys<sup>278</sup> is not close to the active site of the nicotinamide ring. Nonetheless, the ability to bind NAD<sup>+</sup> productively and generate NADH has been lost.

Several of these results are surprising. For example, one of the residues whose removal seemed to have the largest effect...
on NAD\(^+\) binding (e.g., K278A) is not close enough to directly participate in stabilization of bound NAD\(^+\). Therefore, the effect of Lys\(^{278}\) on NAD\(^+\) binding must work through some secondary effects such as electrostatic or Van der Waals' stabilization of the active site. The impaired substrate (but not Pi) binding to K367A and the lack of conversion to NADH with substrate and EDTA present indicate that the primary defect for Lys\(^{367}\) must occur prior to the first oxidation step. This is in opposition to what the crystal structure suggested. Clearly, the mutagenesis results suggest this lysine plays an initial role in binding the acyclic G-6-P. However, Lys\(^{278}\) is also proposed to be critical for binding the substrate via interactions with the phosphate, and although substrate binding may be weaker in K278A, it still is observed (albeit indirectly in the \(^{31}\)P NMR experiment where bound Pi is displaced). The interesting question is how to interpret the lack of G-6-P binding by K367A.

There is a major problem in all the structural work with mIPS. The yeast enzyme is the only one with a substrate analog bound, and it is oriented in an extended conformation that does not have substrate C-1 or C-6 near one another (17). Inhibitors of the enzyme that are analogs of acyclic G-6-P bind quite tightly and are potent inhibitors of the yeast mIPS, whereas cyclic compounds are very poor inhibitors (19). However, to generate the inosose ring, C-1 and C-6 need to be near one another. For this, one needs an enzyme-bound pseudocyclic form of G-6-P, undoubtedly a high energy conformation. The small amount of acyclic G-6-P in solution will not exist in a pseudo-cyclic conformation but will adapt extended confor-

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**FIG. 7.** Proposed mechanism for *A. fulgidus* mIPS based on the characterization of mIPS mutants along with the crystal structure of this archaeal enzyme.
mations. When it binds to the enzyme it would have to interact with the correct combination of Lys and Asp groups that line the active site. Modeling studies with the A. fulgidus mIPS end up generating an extended acyclic G-6-P orientation that cannot be what exists on the enzyme along the catalytic pathway. Indeed, cyclic G-6-P cannot be modeled into the active site of the archaeal enzyme; a similar result was observed with the yeast enzyme. Nonetheless, binding a cyclic molecule must be possible because inosose-1-P is formed and held tightly in the active site prior to reduction to I-1-P and release into solution.

There must be a sizeable conformational change that brings C-1 and C-6 near one another for C-C bond formation. This conformational change sequesters the active site from solution so that bound G-6-P is not in exchange with free G-6-P. Assuming that the \( K_d \) for substrate binding is comparable with the \( K_m \) (0.1 mM) and the likely \(^{31}P\) chemical shift difference is small (\(^{31}P\) chemical shifts are dominated by the ionization state of the phosphate and the shift for \( P_i \) suggests it has a net charge of \(-2\) equivalent to what it is in solution at pH 7.5–8), the exchange between bound and free G-6-P forms should not be in the slow exchange regime if the two forms are in equilibrium. Because no line broadening of G-6-P was observed in the presence of mIPS, whereas \( P_i \), was effectively broadened by the protein, free and bound G-6-P must not be in equilibrium, at least on the NMR time scale. The slow NMR step could be interconversion of cyclic and acyclic G-6-P bound to the enzyme. A large conformational change in the protein could further retard the exchange of free and bound solute. With a large change involving the active site and its accessibility to solution, it may be difficult to predict what ligand/protein interactions really exist in the active site. In support of a large conformational change, we have noted that protein solubility decreases from at least 10 mg/ml to <2 mg/ml when G-6-P is incubated with the protein, excess \( P_i \), and EDTA and heated to 85 °C to generate bound NADH and 5-keto-G-6-P.2

So how do the mutagenesis results fit into the proposed mechanism for mIPS? Most are consistent with what one would predict from the crystal structure. The two real anomalies are K367A and N255A. There is the complete loss of activity for K367A and the nearly complete loss of activity of N255A. Although one could explain decreased activity for both these mutants, what is more surprising is the dramatically reduced binding of G-6-P to each enzyme, whereas \( P_i \) still binds to both. By analogy to the yeast enzyme (14, 16, 17), \( \text{Asn}^{255} \) could interact with the 2-OH of linearized G-6-P (although it is further away in the A. fulgidus enzyme), and \( \text{Lys}^{367} \) would be necessary for orienting the 5-hydroxy of G-6-P (or the keto group in 5-keto-G-6-P). There are several other residues in the active site that could hydrogen bond to acyclic G-6-P. It may not be oriented quite right, but one would expect linearized G-6-P to still bind to K367A. Our indirect assay of G-6-P binding uses a large excess of G-6-P (5 mM) added to displace \( P_i \) (total concentration 0.5 mM) from the active site of mIPS. For a \(<0.2\)-Hz decrease in line width, the G-6-P would have to bind much more weakly to the protein. However, there is another potential explanation. The 5-OH is not available in the cyclic G-6-P form because that oxygen is the ring oxygen; this might strongly suggest that \( \text{Lys}^{367} \) aids in promoting and stabilizing the acyclic form and generating the bound pseudo-cyclic G-6-P by protonating the ring G-6-P ring oxygen. \( \text{Lys}^{367} \) may also be involved in proton transfer reactions involving the C-5 oxygen at later steps in the reaction, but its removal halts catalysis at a much earlier step. \( \text{Asn}^{255} \) could either directly interact with the C-1 hydroxyl or aid in proton transfer to an acidic side chain. Deprotonation of the C-1 oxygen would stabilize the aldehyde. If both of these side chains work concurrently, one might imagine generating a pseudo-cyclic conformation of G-6-P bound to the enzyme that is poised for formation of the C-C bond in the second chemical step of the reaction. The absence of \( \text{Lys}^{367} \) in this model would preclude stabilizing G-6-P in a pseudo-cyclic orientation and G-6-P would not bind well, although \( P_i \) should have no difficulty binding to the mIPS mutant.

The mechanism suggested for this mIPS is presented in Fig. 7. The first step is the stabilization of a bound acyclic form of G-6-P, which we propose is carried out in large part by \( \text{Lys}^{367} \) and \( \text{Asn}^{255} \). \( \text{Asn}^{255} \) is shown as having a direct interaction with the substrate C-1 oxygen, but it could be part of a network that interacts with this part of the substrate. The first chemical step would become the simultaneous deprotonation of the C-5 hydroxyl by \( \text{Lys}^{274} \) (or \( \text{Lys}^{367} \)) and oxidation of C5 with direct hydride transfer to \( P_i \). Subsequently, the withdrawal of the pro-R proton from the C-6 position (by the phosphate group or one of the many lysine residues that has become deprotonated) promotes enolization of the C-5-C-6 bond. The developing negative charge is stabilized by \( \text{Lys}^{274} \) and/or \( \text{Lys}^{367} \). At the other end of the active site \( \text{Lys}^{306} \) in concert with \( \text{Asp}^{232} \) protonates the C1 oxygen in concert with the aldol condensation. The C-C bond formation is only completed in the presence of the second metal ion. Therefore the second metal ion must bind close or directly coordinate one of the neighboring OH at positions C-1 or C-2. Finally, the reduction of the oxygen atom at C-5 is achieved by hydride transfer and one of the nearby lysine residues protonates the oxygen to generate I-1-P.

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