The Temperature Dependence of the Inositol Monophosphatase K_m Correlates with Accumulation of Di-*myo*-inositol 1,1′-Phosphate in *Archaeoglobus fulgidus*†

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ABSTRACT: Di-*myo*-inositol 1,1′-phosphate (DIP) accumulates as a compatible solute in many hyperthermophilic archaea (e.g., *Archaeoglobus fulgidus*) when the cells are grown above 80 °C. Recent microarray analysis of *A. fulgidus* transcripts [Rohlin, L., et al. (2005) *J. Bacteriol. 187,* 6046] indicates that neither the *myo*-inositol-1-phosphate synthase, the first step in inositol biosynthesis, nor the inositol monophosphatase (IMPase), which generates *myo*-inositol, are significantly upregulated upon thermal stress. Although other factors could contribute to regulation of DIP synthesis in cells, there is an 8−10-fold decrease in the K_m of the IMPase for inositol phosphates between 75 and 85 °C (for L-I-1-P, the K_m decreased from 13.2 to 1.67 mM) that correlates with the observed accumulation of DIP in cells. Between 55 and 75 °C, K_m values decreased 2.3-fold at most. The enzyme also exhibits fructose bisphosphatase activity. However, the K_m for fructose 1,6-bisphosphate was low and the same (0.15 ± 0.01 mM) at 55 and 70 °C. This indicates that the unusual temperature dependence of K_m is specific for I-1-P substrates. ^{31}P NMR studies confirmed that the affinity of inositol 1-phosphate for the enzyme was indeed weak ($K_D \geq 5$ mM) below but increased significantly at 80 °C. In contrast, the IMPase from *Methanococcus jannaschii,* an organism in which DIP does not accumulate, had a low K_m for I-1-P over the entire temperature range. A structural comparison of the two archaeal IMPases identified a hydrogen bonding network present in the active site of the *A. fulgidus* enzyme and not in the *M. jannaschii* IMPase, the disruption (e.g., *A. fulgidus* IMPase S171A or T174L) of which prevented the drop in K_m at high temperatures. We suggest that the temperature-dependent synthesis and accumulation of DIP in *A. fulgidus* are regulated in part by the temperature dependence of the K_m of the IMPase activity in the cells.

Di-*myo*-inositol 1,1′-phosphate (DIP)[1] is a major intracellular solute in hyperthermophilic archaea, including *Pyrococcus woesei* (*1*), *Methanotorris igneus* (formerly *Methanococcus igneus*) (*2*), *Pyrococcus furiosus* (*3*), *Archaeoglobus fulgidus* (*4*), and several bacteria of the order Thermotogales (*5*). DIP functions as an osmolyte, since its intracellular concentration increases with an increasing external NaCl concentration in these cells. More intriguingly, DIP significantly accumulates only when hyperthermophilic Archaea and Thermotogales cells are grown above 75−80 °C (*2*, *3*, *5*). For example, in *A. fulgidus,* DIP levels are increased ∼30-fold (*4*). A biosynthetic pathway, deduced from isotopic labeling studies in *M. igneus,* proposed for DIP requires four activities: (i) *myo*-inositol-1-phosphate synthase (mIPS) to convert D-glucose 6-phosphate to L-inositol 1-phosphate (L-I-1-P), (ii) inositol monophosphatase (IMPase) to generate *myo*-inositol, (iii) CTP:L-I-1-P cytidylyltransferase to activate I-1-P by forming CDP-inositol, and (iv) DIP synthase which forms DIP from CDP-inositol and *myo*-inositol (*6*). Enzymes for the first two steps in the pathway have been identified, cloned, and overexpressed in *Escherichia coli* and characterized (*7*−*11*); genes for the cytidylyltransferase and DIP synthase have not been identified thus far in any of the organisms in which DIP accumulates.

mIPS, which catalyzes the first step in DIP production, might be expected as the point of regulation since it commits resources to make inositol compounds. Recent whole-genome microarray data (*12*) did not detect an increased level of transcript for mIPS. However, several hyperthermophilic archaea synthesize inositol-containing lipids with L-inositol units [e.g., *Aeropyrum pernix* (*13*, *14*)] that are presumably derived from L-I-1-P, the product of mIPS, making regulation of mIPS problematic. Modulation of the IMPase might be a better step for regulating DIP synthesis. The microarray data did detect a 1.4-fold increase in the level of the IMPase transcript (*12*). However, this would appear unlikely to lead to the 30-fold increase in the level of DIP accumulation in *A. fulgidus* incubated at high temperatures. If regulation of either of these two enzymes is critical to DIP accumulation, it does not occur at the level of transcription.

Functional inositol monophosphatase (IMPase) enzymes from several hyperthermophiles (including archaea) have

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[1] Abbreviations: IMPase, inositol monophosphatase; FBPase, fructose bisphosphatase; I-1-P, inositol 1-phosphate; FBP, fructose 1,6-bisphosphate; DIP, di-*myo*-inositol 1,1′-phosphate; mIPS, *myo*-inositol-1-phosphate synthase; QFF, Q-Sepharose fast flow.

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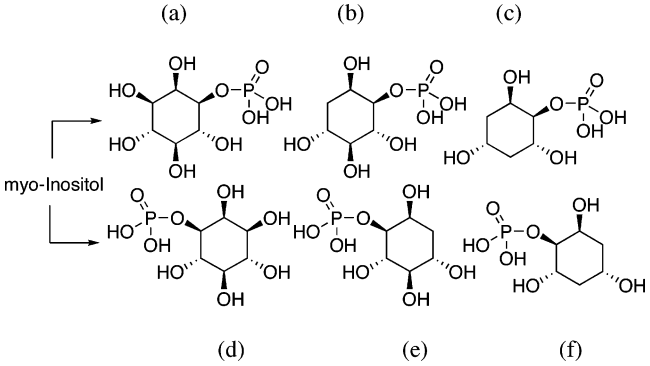

FIGURE 1: Synthetic I-1-P compounds and analogues examined in this study: (a) D-I-1-P, (b) 3-deoxy-D-I-1-P, (c) 3,5-dideoxy-D-I-1-P, (d) L-I-1-P, (e) 3-deoxy-L-I-1-P, and (f) 3,5-dideoxy-L-I-1-P.

been cloned, overexpressed, and characterized (*7*−*9*). To complicate matters, these IMPase homologues exhibit FBPase as well IMPase activity (*15*), although whether this FBPase activity is physiologically important is unclear (*16*). Previous studies of the structure of the *A. fulgidus* IMPase showed that two proximal cysteine residues form a disulfide upon oxidation that abolishes phosphatase activity (*17*). The proximity of two cysteine residues was not observed in the *Methanococcus jannaschii* IMPase structure (*18*). This could link IMPase in DIP biosynthesis to oxidative stress, but it is not obviously connected to temperature-dependent accumulation of the solute in *A. fulgidus*.

In this work, we look at kinetics of the IMPase enzyme from *A. fulgidus* and compare kinetic parameters for that IMPase (and its FBPase activity) to the same enzyme from *M. jannaschii*. The K_m for L-I-1-P, the substrate needed to make DIP in *A. fulgidus*, is very high at 75 °C and lower temperatures, but it drops substantially at 85 °C. The same trend for K_m is seen with D-I-1-P and the corresponding deoxyinositol phosphates. The FBP K_m does not exhibit the same trend but is low at all the temperatures that were examined. In contrast, *M. jannaschii* IMPase does not show a sudden drop in K_m at high temperatures. Rather, the K_m for I-1-P is low across the entire temperature range. ^{31}P line width studies of D-I-1-P confirm that the binding of the substrate becomes significantly tighter at high temperatures. In comparing structures of the two archaeal IMPases, we noticed a hydrogen bonding network present in the *A. fulgidus* enzyme active site that was not present in the *M. jannaschii* enzyme. The kinetics of the S171A mutant of the *A. fulgidus* enzyme, which should abolish this network, did not show a decrease in K_m above 75 °C, indicating that this network plays a part in the temperature-induced change in the structure that enhances substrate binding. These results suggest that modulation of IMPase substrate binding may be critical to the sharp temperature dependence of the intracellular accumulation of DIP in *A. fulgidus*.

MATERIALS AND METHODS

Chemicals. Chiral inositol phosphates [D-I-1-P, 3,5-dideoxy-D-I-1-P, L-I-1-P, and 3,5-dideoxy-L-I-1-P, bis(cyclohexylamine) salts] were synthesized using peptide-based catalysts for an enantiospecific phosphorylation methodology (*19*−*21*). The specific compounds used as substrates for IMPases are shown in Figure 1. Fructose bisphosphate, ammonium molybdate, and malachite green oxalate were purchased from Sigma. The QFF resin was obtained from Amersham Biosciences. Tryptone and yeast extract were purchased from DIFCO. SDS-PAGE molecular weight standards were purchased from Bio-Rad. The QuikChange site-directed mutagenesis kit was purchased from Stratagene.

Protein Expression, Purification, and Mutation. *A. fulgidus* IMPase (GenBank entry AF2372), *M. jannaschii* IMPase (GenBank entry MJ0109), and *Thermotoga maritima* IMPase proteins were prepared as described previously (*7*−*9*). The pET23a(+) plasmid containing the desired IMPase gene was transformed into BL21(DE3) competent cells for expression. The key steps in purification of these IMPase enzymes from hyperthermophiles included heating crude protein at 85 °C for 30 min, followed by chromatography on QFF resin. The S171A and T174L mutants of AF2372 were prepared using the QuikChange mutagenesis kit; altered genes were sequenced to confirm the specific mutations.

Phosphatase Assays. Phosphatase activity toward a variety of inositol phosphate substrates was measured by a colorimetric determination of the amount of released inorganic phosphate (*7*, *21*, *22*). Most assays for AF2372 were carried out with variable substrate concentrations in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 5 mM Mg^{2+}; the total assay volume was 20 μL. The assays for MJ0109 were carried out in 50 mM Tris-HCl (pH 8.0) with 1 mM EDTA and 10 mM Mg^2; the total assay volume was 200 μL. The amount of enzyme added was adjusted to give 15−20% conversion of substrate to inorganic phosphate (P_i) during the 2 min incubation. After incubation, 1 mL of ammonium molybdate and malachite green reagent was immediately added to the reaction mixtures. To monitor the FBPase K_m and V_{max} of purified AF2372 enzymes, a total volume of 200 μL was used; the reaction was carried out at 55 and 70 °C (for up to 5 min). A maximum temperature of 70 °C was used since nonenzymatic degradation of FBP becomes a severe problem at higher temperatures. A comparison of observed A_{660} changes to those for standard P_i samples was used to calculate the reaction rate. To determine k_{cat} and K_m at each temperature, at least five different substrate concentrations were used and each point was measured in duplicate.

Circular Dichroism Spectroscopy. The overall secondary content of S171A and T174L compared to wild-type recombinant *A. fulgidus* IMPase was checked by analyzing the molar ellipticity between 195 and 260 nm measured with an AVIV circular dichroism model 202 spectrometer with a 1 cm path length quartz cell. The concentrations of proteins were 0.05−0.07 mg/mL. To check the thermostability of proteins, the temperature-induced changes in ellipticity (measured in millidegrees) at 222 nm were measured as the cell temperature was increased from 37 to 100 °C in 1 °C intervals with a 1 min delay time between temperatures. The fraction of unfolded protein was estimated using the relationship $f_u = (\theta_{37°C} - \theta_T)/\theta_{37°C}$.

^{31}P NMR Spectroscopy. ^{31}P NMR spectra of D-I-1-P (2 mM) solubilized in 50 mM Tris-HCl (pH 8.0) with 5 mM EDTA in the absence and presence of recombinant *A. fulgidus* IMPase or S171A (3 mg/mL) were obtained at 202.3 MHz on a Varian INOVA 500 spectrometer. The parameters that were used included an 8000 Hz sweep width, a 90° pulse width, a 2.5 s delay tine between acquisitions, and 512 transients. The free induction decays were processed with a 1 Hz line broadening. The line width of D-I-1-P (measured

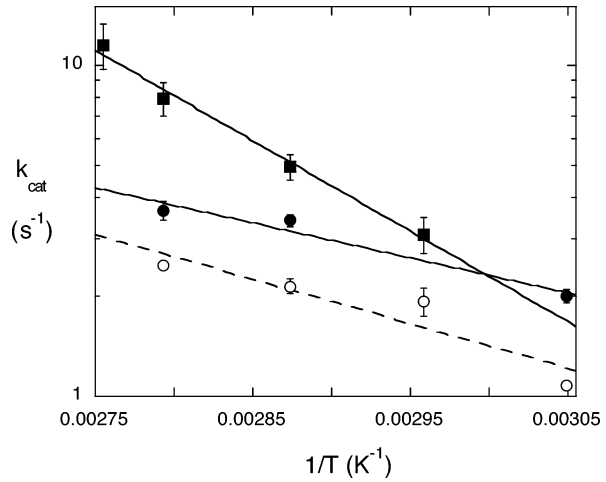

FIGURE 2: Arrhenius plot of k_{cat} vs inverse temperature for *A. fulgidus* IMPase hydrolysis of L-I-1-P (●) and dideoxy-D-I-1-P (○) and *M. jannaschii* hydrolysis of L-I-1-P (■).

Table 1: Activation Energies for Archaeal IMPase Hydrolysis of Different Inositol Phosphates

enzyme source	k_{cat} at 85 °C (s^{-1})	substrate	E_a (kJ/mol)
A. fulgidus	3.64 ± 0.25	L-I-1-P	19 ± 2
	5.78 ± 0.39	dideoxy-L-I-1-P	30 ± 4
	4.32 ± 0.27	D-I-1-P	20 ± 2
	2.49 ± 0.06	dideoxy-D-I-1-P	23 ± 2
M. jannaschii	7.92 ± 0.91	L-I-1-P	48 ± 4
	7.72 ± 0.41	D-I-1-P	58 ± 5
T. martitima	16.6[a]	L-I-1-P	36 ± 3
	104[a]	D-I-1-P	63 ± 5

[a] Extrapolated from an Arrhenius plot.

as the width at half-height with the applied line broadening subtracted) in the absence and presence of protein was examined at temperatures from 25 to 80 °C.

RESULTS

Archaeal IMPase Kinetics. Previously, we showed that at 85 °C, the growth temperature of the organism, the *A. fulgidus* enzyme exhibited a 10−20-fold higher K_m for the natural substrate L-I-1-P than for the D-I-1-P isomer (*21*), although k_{cat} values were similar. As a further investigation of this enzyme, we measured the activities of the recombinant *A. fulgidus* IMPase toward the synthetic inositol phosphates D-I-1-P, 3,5-dideoxy-D-I-1-P, L-I-1-P, and 3,5-dideoxy-L-I-1-P between 55 and 85 °C. Over this temperature range, k_{cat} values exhibited Arrhenius behavior (the temperature dependence for AF2372 hydrolysis of two of the substrates and for *M. jannaschii* MJ0109 is shown in Figure 2). Activation energies were similar for the substrates, although dideoxy-L-I-1-P exhibited a noticeably higher E_a than the other substrates for this enzyme (Table 1). In contrast to the behavior of k_{cat} with temperature, the K_m exhibited a very peculiar response to an increase in temperature for all four substrates (Figure 3). For most enzymes, K_m increases with an increase in temperature. However, for the *A. fulgidus* IMPase-catalyzed hydrolysis of all four substrates, K_m varied no more than 2.5-fold between 55 and 75 °C then decreased dramatically at 85 °C. The relative decrease was smallest for the 3,5-dideoxy-L-I-1-P, although it was still quite significant.

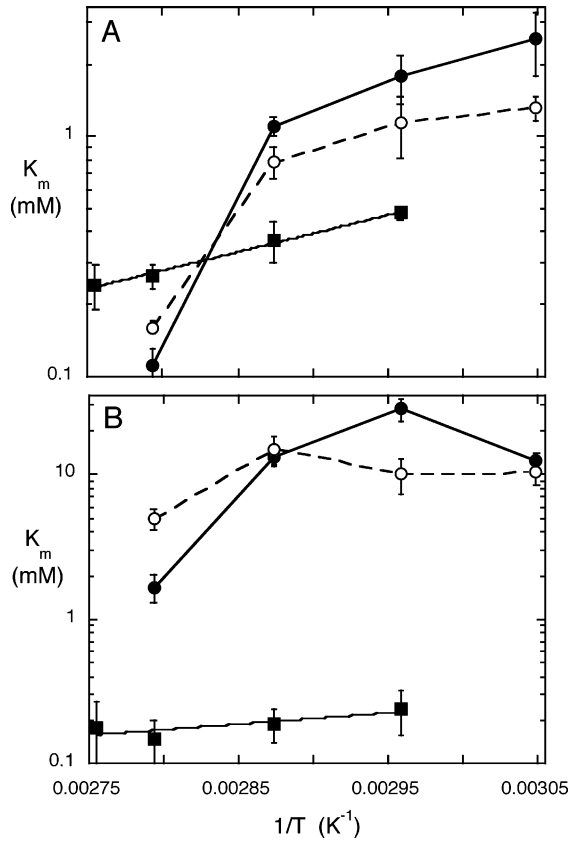

FIGURE 3: Temperature dependence of the substrate K_m for two archaeal inositol monophosphatases: (A) *A. fulgidus* IMPase toward (●) D-I-1-P or (○) 3,5-dideoxy-D-I-1-P and *M. jannaschii* IMPase toward D-I-1-P (■) and (B) *A. fulgidus* IMPase toward (●) L-I-1-P or (○) 3,5-dideoxy-L-I-1-P and *M. jannaschii* IMPase toward L-I-1-P (■).

Mg^{2+} is absolutely required for IMPase activity; the enzyme binds three metal ions in the active site (*9*). Mg^{2+} ions at higher concentrations will also interact with the substrate phosphate groups, so defining a K_D for Mg^{2+} in the presence of substrate is difficult. For the substrate temperature dependences shown here for a given enzyme, the assays all used the same concentration of $MgCl_2$ (5 or 10 mM). To see if the dramatic decline in K_m at high temperatures was caused by a pronounced temperature dependence in the requirement for Mg^{2+}, we examined AF2372-catalyzed hydrolysis rates for a fixed concentration of D-I-1-P (8 mM) and varied the Mg^{2+} concentration at 65, 75, and 85 °C. D-I-1-P was chosen as the substrate since the K_m values were well under 5 mM over this temperature range. Under these conditions, the apparent K_D for Mg^{2+} at these three temperatures varied less than 2-fold (Figure 4).

Since the enzyme also has FBPase activity, we examined the temperature dependence of K_m for AF2372 acting on that substrate (with 5 mM Mg^{2+}) as well. FBP is not very stable at high temperatures, so the K_m for this substrate was measured at 55 and 70 °C. AF2372 hydrolyzed the bisphosphate substrate (removing only the phosphate group esterified at C1) with a low K_m (0.15 mM) at both these temperatures (Figure 4). The second phosphate moiety on the inositol ring clearly enhanced binding of FBP to the enzyme at low temperatures.

For comparison to the results with the *A. fulgidus* IMPase, K_m and k_{cat} were measured at different temperatures for the

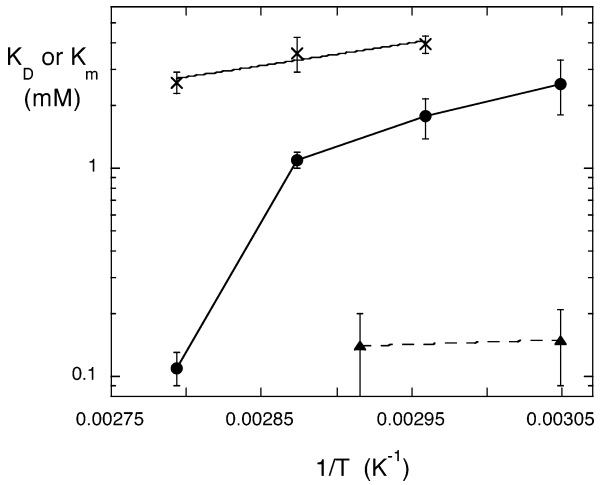

FIGURE 4: Temperature dependence of *A. fulgidus* IMPase K_D for Mg^{2+} (×) or K_m for D-I-1-P (●) or FBP (▲).

IMPase from *M. jannaschii* MJ0109 (*7*). Both the *A. fulgidus* and *M. jannnaschii* IMPase enzymes exhibit FBPase as well as IMPase activities. However, *M. jannaschii* does not have an identifiable mIPS gene and does not accumulate DIP, so its IMPase must have another role in the cells. As shown in Table 1 and Figure 2, MJ0109 had a significantly higher activation energy for hydrolysis of both L- and D-I-1-P substrates (Table 1 and Figure 1) than AF2372. For the *M. jannaschii* IMPase, K_m values were low (<0.5 mM) for both isomers with a relatively small decrease in K_m over a large temperature range (Figure 3).

T. maritima, a hyperthermophilic bacterium rather than archaeon, also accumulates DIP when grown at temperatures above 75 °C (*5*). The recombinant IMPase from this bacterium is a tetramer rather than a dimer and exhibits very high activity toward D-I-1-P (*8*). The activity of the enzyme toward the D-I-1-P is much higher than toward the L-isomer (Figure 5A). This IMPase displays a low K_m over the temperature range of 55−95 °C for both L- and D-I-1-P (Figure 5B), behavior similar to that of the *M. jannaschii* IMPase. This might suggest that the unusual kinetic behavior of the *A. fulgidus* IMPase may be a hallmark of archaeal proteins in organisms in which DIP accumulates.

Binding of Substrate to A. fulgidus IMPase. The drop in K_m between 75 and 85 °C exhibited by the *A. fulgidus* IMPase suggested that the enzyme must undergo a conformational change at high temperatures that facilitates productive substrate binding and hydrolysis. This was probed by ^{31}P NMR studies of 2 mM D-I-1-P in the absence or presence of enzyme (∼0.1 mM) as a function of temperature. EDTA (5 mM) was present to prevent any hydrolysis of substrate. Enzyme-bound D-I-1-P should have a much broader line width than free D-I-1-P (typically 1.5 Hz), and fast exchange between the two environments should result in broadening of the phosphorus resonance that will depend on the fraction of substrate bound to the enzyme. D-I-1-P was chosen since it had a lower K_m, and at least at high temperatures, a higher fraction of it compared to L-I-1-P would be bound to the enzyme. As shown in Figure 6, there was little line broadening of D-I-1-P at 25, 50, or 65 °C (<0.7 Hz increase). However, the line width increased by almost 4 Hz at 80 °C. The changes in line width were reversible, indicating that the changes reflect reversible binding of the substrate to the

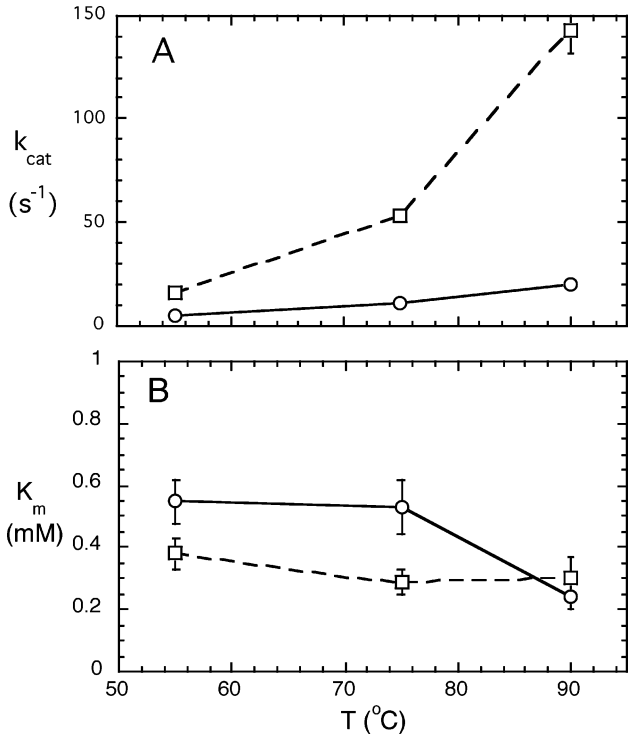

FIGURE 5: (A) Temperature dependence of the *T. maritima* IMPase k_{cat} for D-I-1-P (□) and L-I-1-P (○). (B) Temperature dependence of the *T. maritima* IMPase K_m for D-I-1-P (□) and L-I-1-P (○).

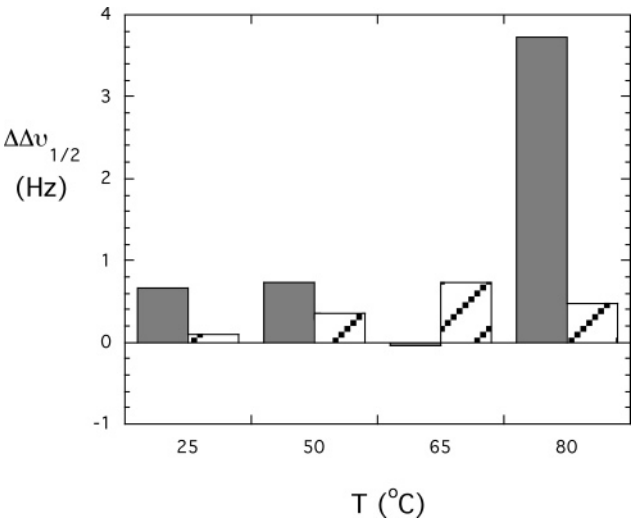

FIGURE 6: Increase in D-I-1-P (2 mM) ^{31}P line width ($\Delta\Delta\upsilon_{1/2}$) caused interactions with the *A. fulgidus* IMPase (3 mg/mL) at different temperatures: (gray) recombinant AF2372 and (hatched) S171A mutant.

AF2372 enzyme in the absence of Mg^{2+}. This indicates that, in the absence of Mg^{2+}, substrate D-I-1-P can bind tightly to enzyme at a temperature of only ≥80 °C, which is consistent with the dramatic decrease in the K_m at 85 °C. Under the assumption that the protein is saturated with D-I-1-P and that bound and free ligand are in fast exchange, the bound line width for D-I-1-P would be 70 Hz. Again, assuming that the bound line width at 80 °C represents a completely bound enzyme under these conditions, a smaller observed line width provides a rough estimate of the amount of enzyme with substrate bound. A maximum 1 Hz increase in line width at lower temperatures compared to an ∼4 Hz line width at 80 °C would be consistent with a K_D of ≥5 mM.

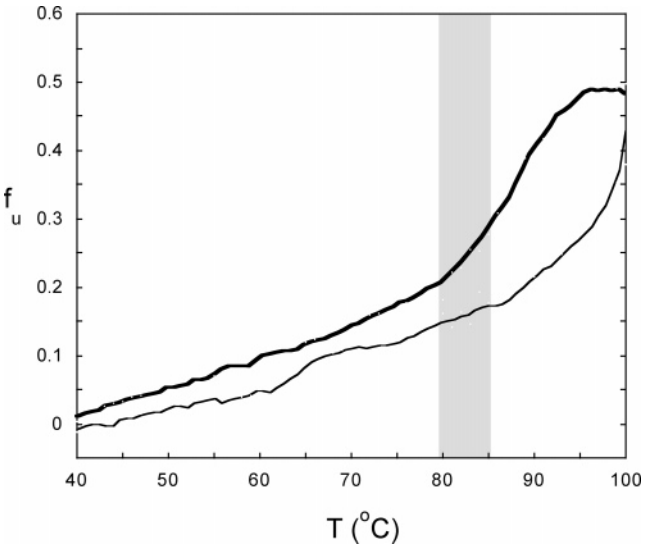

FIGURE 7: Effect of temperature on IMPase unfolding as monitored by the ellipticity at 222 nm for AF2372 (thick line) and MJ0109 (thin line). The fraction unfolded, f_u, at each temperature was calculated as $(\theta_{37°C} - \theta_T)/\theta_{37°C}$, where θ is the ellipticity in millidegrees at 222 nm. The gray band indicates the temperature range at which K_m for inositol phosphates decreases dramatically.

Thermostability of Archaeal IMPase Enzymes. The impaired substrate binding below 80 °C suggested that the *A. fulgidus* IMPase undergoes a structural and/or motional change around that temperature that is coupled to substrate binding. CD was used to examine structural changes in the protein at high temperatures. The ellipticity at 222 nm was monitored as a function of temperature for AF2372 at low concentrations in 0.01 mM borate (pH 8.0). As can be seen in Figure 7, there was a gradual loss of secondary structure (~17% of the secondary structure originally detected at 40 °C) with heating from 40 to 75 °C, presumably the result of an overall increase in the mobility of this thermozyme. However, above 80 °C, there was a larger and more cooperative loss of structure that leveled off at 50% residual structure near 100 °C. The midpoint of this partial unfolding transition was 88−89 °C. This larger loss of structure coincided with an irreversible loss of catalytic activity. Interestingly, the substantial decrease in the K_m for L-I-1-P occurs as the transition for this partial unfolding is approached. However, the CD studies used enzyme in borate buffer without any of the cofactors that might help it retain structure. For comparison, the unfolding of *M. jannaschii* MJ0109 and the loss of catalytic activity occurred at a much higher temperature (~95 °C as seen in Figure 7), yet MJ0109 exhibited a low K_m for I-1-P well below this loss of secondary structure. Therefore, tighter binding of substrate to an archaeal IMPase at 85 °C does not absolutely require the increased level of motion of the protein as it begins to thermally unfold. Something else must be responsible for the temperature dependence of the I-1-P K_m in the *A. fulgidus* enzyme.

Structural Differences in the Active Site of M. jannaschii versus A. fulgidus IMPase. The active site of AF2372 has a hydrogen bonding network of residues Tyr155, Glu175, Ser171, and Asp85 (Figure 8A). Of these four residues, Asp85 is a highly conserved and critical for catalysis. It binds not only metal 1 but also O2 of the inositol ring. The hydrogen bond between the hydroxyl group of Ser171 and

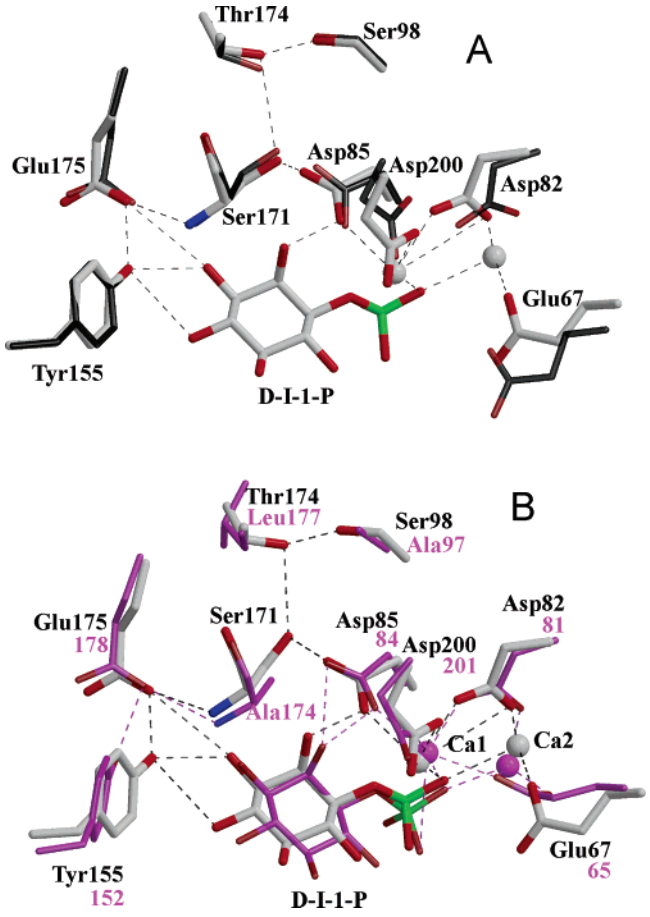

FIGURE 8: (A) Overlay of apo-AF2372 [PDB entry 1LBV (thin black line)] onto D-I-1-P-liganded AF2372 [PDB entry 1LBX (thick gray line)] showing the Tyr155−Glu175−Ser171−Asp85 network. (B) Overlay of I-1-P-liganded AF2372 [PDB entry 1LBX (thick gray lines)] with MJ0109 [PDB entry 1G0H (thin magenta line)] showing differences in coordination of I-1-P in the active site and disruption of the network seen in AF2372.

Asp85 is present in the active site in both the substrate-liganded form (thick gray lines) (PDB entry 1LBX) and the apo form of the AF2372 enzyme (black thin lines) (PDB entry 1LBV). In addition, in the D-I-1-P-liganded structure (PDB entry 1LBX), Tyr155, which is in the interfacial loop in AF2372, interacts with O3 and O4 of inositol. Tyr155 also interacts with Glu175, which in turn is 3.59 Å from O3 of the inositol ring. Glu175 is positioned to hydrogen bond with Ser171. This network is interrupted in the *M. jannaschii* protein (PDB entry 1G0H) since the amino acid that aligns with *A. fulgidus* Ser171 is Ala174. Ala174 cannot anchor or constrain the critical metal binding Asp84 in MJ0109 (Figure 8B). In addition, Tyr152 in MJ0109, which aligns with Tyr155 of AF2372, has a different orientation and does not coordinate inositol (Figure 8B). Since there is not yet a crystal structure of the *T. maritima* IMPase, a comparison with that protein cannot be made.

There are two other pronounced differences between the two archaeal IMPases. While these two enzymes share similar global secondary structure organizations, they have very different dimer interfaces. The contact in *M. jannaschii* IMPase is helical, while that for the *A. fulgidus* protein has strands and large loops (*9*). In AF2372, Tyr155 is adjacent to interfacial contacts, including Tyr156, a residue that has no counterpart in MJ0109 (Figure 9). The interface of

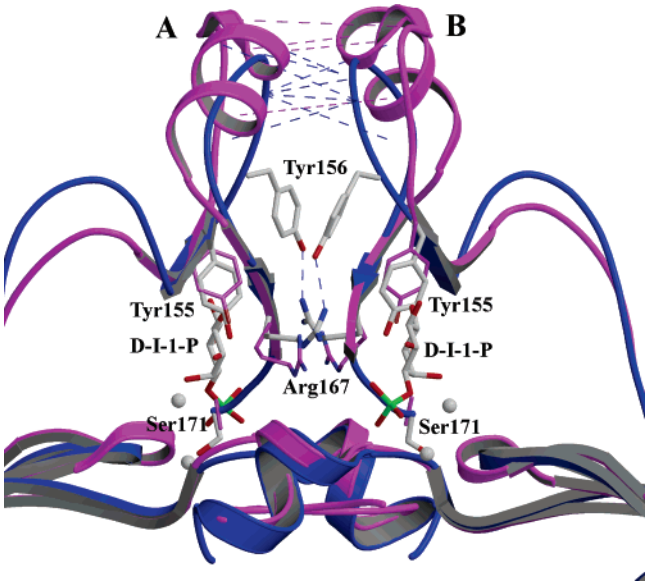

FIGURE 9: Dimer interface of AF2372 (blue, PDB entry 1LBX) overlaid with MJ0109 (magenta, PDB entry 1G0H). Each structure has D-I-1-P (phosphorus colored green) and two Ca^{2+} ions bound per monomer. Different interactions at the top of the dimer interfaces are emphasized with dashed lines (blue for AF2372 and magenta for MJ0109). Also note the interdimer interactions of Tyr156 and Arg167 in AF2372 whose behavior could influence active site residues.

AF2372 is also much more extensive (12 interactions at the dimer interface) than that of MJ0109 (six interfacial interactions). The dashed lines at the top of the dimer interface are an attempt to illustrate this schematically. In particular, the proximity of Tyr156 at the dimer interface to the Tyr155−Glu175−Ser171−Asp85 network suggests that these two features could be linked. The other difference in the crystal structures of the two enzymes with ligands bound (in this case, products) and activating Mn^{2+} metal ions (PDB entry 1G0I for MJ0109 and PDB entry 1LBY for AF2372) is in metal positioning in the active site with the largest change for the third and weakly bound metal ion (data not shown). This difference, however, is unlikely to be critical to the substrate binding reflected in the K_m since the ^{31}P NMR results showed that D-I-1-P binds to AF2372 at high temperatures (but not appreciably at low temperatures) in the absence of Mg^{2+}.

Substrate Binding and Temperature Stability of the AF2372 S171A Mutant. Of the structural differences between the two archaeal proteins, we chose to perturb the unique active site hydrogen-bonded network in AF2372, adjacent to the dimer interface. Mutation of Ser171 of AF2372 to alanine as in *M. jannaschii* was done to investigate if this single-amino acid substitution could account for differences in substrate binding at high temperatures. The S171A mutant enzyme exhibited the same secondary structure and stability as the wild-type protein when measured by CD (~50% total loss of secondary structure with a T_m of ~89 °C). The k_{cat} for S171A at 85 °C (3.5 s^{-1}) was ~80% of that for the wild-type recombinant protein. Mg^{2+} binding was impaired somewhat with apparent K_D values of 6.0 and 5.0 mM (with 8 mM D-I-1-P) at 75 and 85 °C, respectively. The S171A activity toward FBP was 54% of that of the wild type. However, the behavior of K_m with temperature was altered significantly (Figure 10). Instead of a decrease in the K_m at

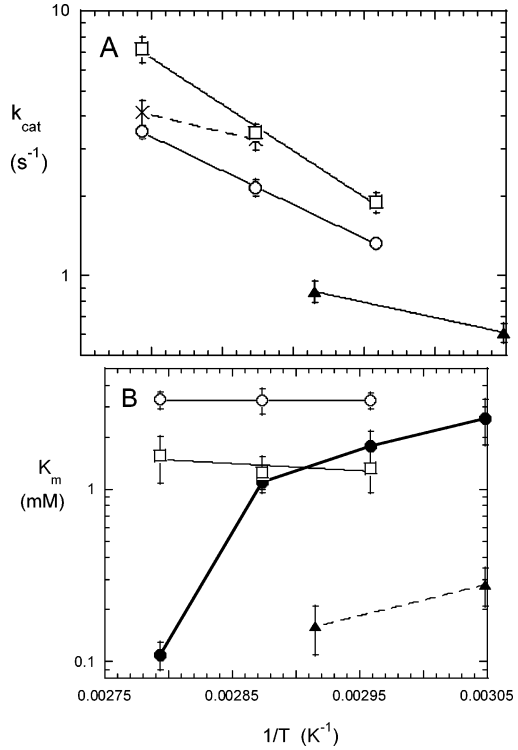

FIGURE 10: (A) Temperature dependence of k_{cat} for hydrolysis of D-I-1-P by S171A (○) and T174L (□) and k_{cat} for S171A hydrolysis of FBP (▲) with 5 mM Mg^{2+}; also shown is the k_{cat} for S171A extrapolated using 8 mM D-I-1-P and varying the Mg^{2+} concentration (×). (B) Temperature dependence of the K_m for D-I-1-P for S171A (○) and T174L (□) compared to the K_m for wild-type recombinant AF2372 (●); the K_m for S171A for FBP (▲) is also shown.

85 °C, the K_m for D-I-1-P was constant at 3.3 mM from 65 to 85 °C. However, the K_m for FBP was much lower at 55 °C (0.28 mM) and 70 °C (0.16 mM). That the binding of I-1-P was weak, even at high temperatures, was checked by examining the ^{31}P line width of D-I-1-P in the presence of S171A (3 mg/mL) and EDTA (Figure 6). Unlike the case for the wild-type recombinant enzyme, there was no increase in line width at 80 °C.

We also examined the temperature dependence of kinetic parameters for T174L acting on D-I-1-P. The Thr residue is also part of the more extended hydrogen bonding network at the *A. fulgidus* IMPase active site (Figure 8). Replacement of the Thr with Leu was done to introduce a residue comparable to that in the *M. jannaschii* protein. Like S171A, T174L exhibited a nearly temperature-invariant K_m from 65 to 85 °C (Figure 10). The 10-fold decrease in K_m observed for wild-type *A. fulgidus* IMPase upon going from 75 to 85 °C did not occur. Clearly, by disrupting this hydrogen bonding network at the active site of AF2372, we can trap the enzyme in a form with a high K_m for the phosphomonoester substrate and little change in k_{cat}.

DISCUSSION

Thermozymes, Activity, and Flexibility. Many enzymes from thermophiles ("thermozymes") are inactive at room temperature but exhibit high activity when the temperature is increased, often to near the growth temperature of the organism. It is thought that the major determinant of protein activity of thermozymes is a change in the flexibility of the

protein. Changes in flexibility can affect catalysis, substrate binding, or both steps. This hypothesis is supported by several studies. Studies on monofunctional hyperthermophilic indole-3-glycerol phosphate synthase showed that at higher temperatures the enzyme−substrate electrostatic interactions favor the correct binding of the substrate, whereas at lower temperatures, the substrate is bound in an unreactive and unproductive conformation (*23*). Zavodszky et al. (*24*) also provided evidence that the low activity of thermozymes at room temperature was caused by restricted conformational movements of the protein molecule. Hyperthermophilic enzymes have often demonstrated biphasic Arrhenius behavior, with an increased enthalpy of activation at temperatures below the break point [e.g., 6,7-dimethyl-8-ribityllumazine synthase (*25*) and the NADH:ubiquinone oxidoreductase (*26*) from the hyperthermophilic bacterium *Aquifex aeolicus* and archaeal ribulose 1,5-bisphosphate carboxylase/oxygenase (*27*)]. This behavior has been attributed to the increased protein rigidity at reduced temperatures (*28*).

The IMPase from *A. fulgidus* exhibits quite different kinetic behavior. For this particular thermozyme, k_{cat} clearly exhibits Arrhenius behavior with an activation energy that is moderately low (∼20 kJ/mol). However, substrate binding as reflected in K_m shows a pronounced break above 75 °C. The conformational change that is needed for substrate binding has little to do with the actual chemistry since the activation energies (from k_{cat}) are well-behaved. Furthermore, this behavior is associated with this particular archaeal IMPase and not with a related IMPase from *M. jannaschii*. CD studies clearly showed an overall increase in protein mobility above 75 °C that leads to an ∼50% loss of secondary structure with a midpoint for this transition of ∼88 °C. It is tempting to associate this increased protein mobility with the enhanced substrate binding. However, the loss of secondary structure for the *M. jannaschii* protein occurs at a much higher temperature (∼95 °C), yet it has a low K_m for the substrate over the same temperature range. The loosening of the structure above 75 °C has a specific effect on substrate binding interactions at the AF2372 active site.

The Tyr155−Glu175−Ser171−Asp85 Network Has an Effect on Substrate Binding. Ser171 in the *A. fulgidus* IMPase has no direct interactions with substrate, although it is part of a hydrogen bonding network of residues in the *A. fulgidus* IMPase active site where the other members form a number of interactions with substrate. When this residue was replaced with alanine, substrate binding was weak even at high temperatures, and the drop in K_m observed at 85 °C with the wild-type enzyme was not observed. By disrupting the Tyr155−Glu175−Ser171−Asp85 network, we have disabled the mechanism whereby increased flexibility of some part of the protein is transferred to the active site and converted to enhanced inositol phosphate binding. T174L, which disrupts interactions of Thr with Ser98 and Ser171 and so is an extended part of the network, also maintained a high K_m at 85 °C. Both these mutants suggest that this hydrogen bonding network is linked to the changes in the protein that enhance substrate binding at high temperatures.

How could this hydrogen bonding structural feature affect substrate binding? The Tyr155−Glu175−Ser171−Asp85 network is observed both in one of the apoenzyme crystal structures and in structures with substrates or products. The other apo-AF2372 structure, which crystallized in a different space group (*P*32), had 20 interfacial contacts and a weakened interaction of Ser171 with Asp85 (the distance from oxygen to oxygen is 3.89 Å, 1.5 Å longer than in the I-1-P-liganded structure). Which apo-AF2372 crystal structure approximates the solution structure at suboptimal temperatures is unclear. It is worth noting that for ligands to crystallize with the *A. fulgidus* IMPase, the protein had to be heated to 80 °C in the presence of metal ions and ligands before crystallization attempts (K. Stieglitz, unpublished results). Without this heat treatment, no ligands were observed in crystals. After heating and then cooling had been carried out, the ligands appeared to be kinetically trapped in the protein.

What is the mechanism for the temperature-induced increase in substrate affinity? It is possible that input of energy and/or heat is required to loosen the active site hydrogen bonding network so that substrate can bind. However, the results with S171A and T174L suggest that an intact network is important for the conformational changes with temperature that enhance substrate binding. Tyr155, at the active site, is part of a large interfacial loop (residues 153−165); it is also adjacent to Tyr156, which interacts with Arg167 of the adjacent monomer. This interaction is absent in MJ0109 (the residue that aligns with Tyr156 is a proline in the *M. jannaschii* IMPase). The increased mobility of the interfacial loop with temperature could alter the interactions of Tyr155 with the other participants in the hydrogen bonding network. In any event, by removing components of the network that are not in direct contact with the substrate or products, we have lost the tight binding of substrate at 85 °C.

Relevance of A. fulgidus IMPase K_m Behavior to DIP Accumulation. The unusual osmolyte DIP accumulates in hyperthermophiles only when they are grown above 75−80 °C (*2*−*4*). Since many of these organisms require inositol for their lipids, regulation of mIPS, the enzyme that converts D-glucose 6-phosphate to L-inositol 1-phosphate, is not an appropriate control point. Incorporation of inositol into lipids could involve a CDP−inositol intermediate, which is also an intermediate in DIP biosynthesis (*6*). If DIP but not lipid incorporation of inositol requires *myo*-inositol, control of DIP accumulation could occur at the level of the IMPase. As in many bacterial systems, the IMPase could be controlled at the level of protein synthesis. However, we have identified another way that directly links temperature to the control of *myo*-inositol generation and hence DIP synthesis. The 10-fold drop in the *A. fulgidus* IMPase K_m for inositol phosphates between 75 and 85 °C correlates with the sharp temperature dependence of DIP accumulation in vivo. With a K_m of 15 mM at 75 °C for L-I-1-P versus a value of 1.5 mM at 85 °C, little *myo*-inositol would be produced in that organism unless the intracellular concentration of I-1-P were quite high. Studies by Santos and co-workers (*5*) did not detect any I-1-P in *A. fulgidus*, so it is unlikely that such a high concentration exists. However, once the temperature crosses a threshold and the L-I-1-P K_m decreases substantially, some of this lower concentration of I-1-P can be converted to *myo*-inositol for DIP biosynthesis.

DIP does not accumulate in *M. jannaschii*, and its IMPase shows a low K_m over the entire temperature range. Its IMPase activity may be the serendipitous result of a more primitive active site with wide specificity. Thus, the in vivo function

of that enzyme is more likely to be as an FBPase in the central metabolism. The differences in the temperature dependence of substrate binding for the two archaeal IMPase activities suggest that the *A. fulgidus* enzyme evolved to have a secondary role in thermal stress. One might suggest that other archaeal homologues of the IMPase will fall into either those with a drop in K_m at high temperatures or those whose K_m shows little dependence on temperature depending on whether DIP accumulates in the organism.

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