Inositol-1-phosphate Synthase from *Archaeoglobus fulgidus* Is a Class II Aldolase†

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Received June 30, 2000

ABSTRACT: A gene putatively identified as the *Archaeoglobus fulgidus* inositol-1-phosphate synthase (IPS) gene was overexpressed to high level (about 30−40% of total soluble cellular proteins) in *Escherichia coli*. The recombinant protein was purified to homogeneity by heat treatment followed by two column chromatographic steps. The native enzyme was a tetramer of 168 ± 4 kDa (subunit molecular mass of 44 kDa). At 90 °C the K_m values for glucose-6-phosphate and NAD^+ were estimated as 0.12 ± 0.04 mM and 5.1 ± 0.9 μM, respectively. Use of (D)-[5-^{13}C]glucose-6-phosphate as a substrate confirmed that the stereochemistry of the product of the IPS reaction was L-*myo*-inositol-1-phosphate. This archaeal enzyme, with the highest activity at its optimum growth temperature among all IPS reported (k_{cat} = 9.6 ± 0.4 s^{-1} with an estimated activation energy of 69 kJ/mol), was extremely heat stable. However, the most unique feature of *A. fulgidus* IPS was that it absolutely required divalent metal ions for activity. Zn^{2+} and Mn^{2+} were the best activators with $K_D \approx 1$ μM, while NH_4^+ (a critical activator for all the other characterized IPS enzymes) had no effect on the enzyme. These properties suggested that this archaeal IPS was a class II aldolase. In support of this, stoichiometric reduction of NAD^+ to NADH could be followed spectrophotometrically when EDTA was present along with glucose-6-phosphate.

Inositol-1-phosphate synthase (EC 5.5.1.4) catalyzes the irreversible conversion of (D)-glucose-6-phosphate to (L)-*myo*-inositol-1-phosphate (*1*−*6*). The enzyme is essential to many organisms as the sole supplier of the inositol ring, a six-carbon cyclitol. Inositol containing compounds play important roles in signal transduction (*7*−*11*), stress response (*12*−*16*), and cell wall biogenesis (*17*, *18*). IPS[1] enzymes from various organisms including animals (*19*, *20*), plants (*21*), fungi (*22*), bacteria (*23*, *24*), and yeast (*25*) have been characterized. The eukaryotic enzymes are >550 residues, whereas the bacterial IPS homologues [identified by sequence alignment of a 150-residue conserved domain in the C-terminus of all IPS (*26*)] appear to be smaller with 350−420 residues. The catalytic mechanism, first proposed by Loewus and Kelly (*1*) and supported by others (*1*, *4*−*6*), involves oxidation of substrate glucose-6-phosphate to 5-keto-(D)-glucose-6-phosphate, cyclization to *myo*-inosose-2 1-phosphate [which is also a competitive inhibitor of IPS (*27*)], and reduction to (L)-*myo*-inositol-1-phosphate (Scheme 1). Since the two keto intermediates have not been detected directly in studies of known IPS enzymes, it has been assumed that they are tightly bound to the enzyme. IPS has an absolute requirement for NAD^+ as a cofactor, but unlike other NAD^+-requiring enzymes, IPS uses this cofactor as an active-site catalytic residue in a tightly coupled oxidation and reduction. Studies of these synthases from animals, plants, and yeast show that in carrying out the cyclization step they are neither Schiff base forming enzymes (class I aldolase) nor divalent metal requiring ones (class II aldolase). In fact, they can be stimulated only by monocations with NH_4^+ the most effective activator. The best-studied IPS is the enzyme from yeast; it is the product of the *INO1* gene and extensively regulated at the mRNA level (*28*−*33*). The transcriptional regulation and expression are sensitive to various precursor molecules of phospholipid biosynthesis, such as inositol and choline (*28*, *33*).

Our interest in IPS stems from the observation that it is the first enzyme involved in the biosynthesis of di-*myo*-inositol-1,1′-phosphate (DIP) (*34*), an osmolyte found in hyperthermophilic archaea including *Pyrococcus woesei* (*13*), *P. furiosus* (*15*), *Methanococcus igneus* (*14*), *Archaeoglobus fulgidus* (*35*), *Thermococcus spp*. (*16*), and the hyperthermophilic bacterium *Thermotoga maritima* (*36*, *37*). Annotation of the complete genome of *A. fulgidus* (*38*) identified AF1794 as an IPS based on a 53.1% similarity to the *INO1* gene, although the predicted subunit (44 kDa) was much smaller than eukaryotic IPS subunits (∼60 kDa). We have cloned AF1794 into the bacterial expression vector pET23a(+) and overexpressed it in *Escherichia coli*. The recombinant enzyme has IPS activity and is extremely heat stable. The stereochemistry of the product was demonstrated to be (L)-I-1-P by using ^{13}C-labeled substrate [(D)-[5-^{13}C]G-6-P)]. The most unique aspect of this archaeal IPS, unlike all other previously characterized IPS, is that it requires divalent metal ions for activity. Exploitation of this has allowed us to observe the stoichiometric reduction of NAD^+ to NADH in the presence of G-6-P and EDTA. This indicates that the enzyme is a class II aldolase.

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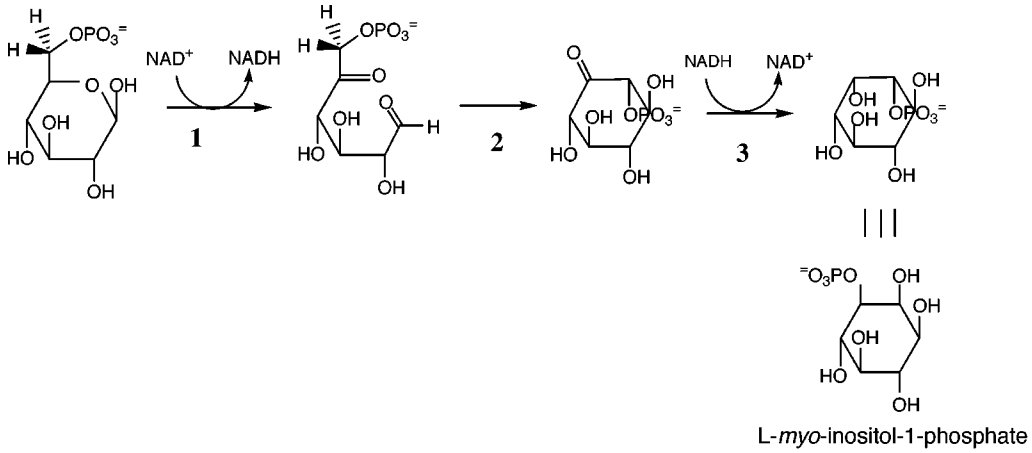
† This work has been supported by DE-FG02-91ER20025 from the Department of Energy Biosciences Division and GER-9023617 from the National Science Foundation.

[1] Abbreviations: IPS, inositol-1-phosphate synthase; G-6-P, glucose-6-phosphate; I-1-P, inositol-1-phosphate; DIP, di-myo-inositol-1,1′-phosphate; OD, optical density; I-1-Pase, inositol-1-phosphate phosphatase; ORF, open reading frame.

10.1021/bi001517q CCC: $19.00
Published on Web 09/15/2000

Scheme 1



MATERIALS AND METHODS

Chemicals. The PCR Gene-Amp kit was purchased from Perkin-Elmer; *pfu* DNA polymerase was obtained from Stratagene. The T4 DNA ligation kit, pET23a(+) vector and *E. coli* strains [Novablue and BL21(DE3)pLysS] were purchased from Novagen. The gene cleaning kit was obtained from Bio-101. Restriction enzymes were obtained from New England BioLab. Oligonucleotide primers were synthesized by Operon Technologies. G-6-P, glucose-6-sulfate (G-6-S), 2-deoxy-G-6-P, NAD^+, SDS−PAGE molecular weight markers, gel filtration molecular weight markers, Coomassie brilliant blue 250, and hexokinase were obtained from Sigma. Q-sepharose fast flow and phenyl-sepharose resins were from Pharmacia; AG1-X8 resin and Bio-gel A 0.5 m were purchased from BioRad. (D)-[5-^{13}C]Glucose and deuterated Tris-d_{11} were obtained from Cambridge Isotope Laboratories.

Recombinant Plasmid Construction. Genomic DNA from *A. fulgidus* was provided by Dr. Harold Schreier, University of Maryland. On the basis of the flanking region of AF1794 (*38*), oligonucleotide primers, 5′-ggaggtgatgcCATATGaaggtctg-3′ (containing an *Nde*I site) and 5′-tttgacgatgagAAGCTTggggaaagta-3′ (containing a *Hin*dIII site), were used to amplify this fragment. High fidelity *pfu* DNA polymerase was used in PCR to reduce possible random mutations. The 25-cycle PCR products were cut with *Nde*I−*Hin*dIII and inserted between the *Nde*I and *Hin*dIII sites of the bacterial expression vector pET23a(+) to form plasmid pBC/IPS1. The recombinant plasmid was transformed into Novablue competent cells for plasmid preparation. pBC/IPS1 was confirmed to contain the AF1794 fragment by restriction mapping.

Overexpression of IPS in E. coli. The recombinant vectors were transformed into BL21(DE3)pLysS cells for expression of protein. A single colony of BL21(DE3)pLysS containing the recombinant pBC/IPS1 was grown in 5 mL of LB medium with 100 μg/mL ampicillin and 34 μg/mL chloramphenicol until the OD at 560 nm reached 0.6. Cell pellets from 4 mL of culture were used to inoculate 2 L fresh LB medium containing the ampicillin and chloramphenicol. These cultures were grown at 37 °C to $OD_{560} \approx 0.7$. Production of recombinant protein was induced by the addition of IPTG to a final concentration of 0.4 mM and continued growth for another 4 h. Cells were harvested by centrifugation and stored at −70 °C until needed. The expression of protein was monitored by SDS−PAGE (IPS corresponds to the band at 44 kDa). Crude cell extract had high IPS activity, whereas the BL21(DE3)pLysS/pET23a-(+) cell extract, as a control, did not have the corresponding overexpressed band on SDS−PAGE and no detectable IPS activity (no IPS gene has been identified in the complete sequence of the *E. coli* genome).

Purification of IPS. Frozen cells (4 g from 2 L culture) were thawed and resuspended in 20 mL buffer A (50 mM Tris HCl, pH 7.5), then lysed by sonication for 10 × 30 s on ice. The supernatant was separated from cell debris by centrifugation (12400*g* for 10 min). DNase (0.8 mg) was added to the supernatant, and the resultant solution was dialyzed against 4 L of buffer A. The dialyzed crude extracts were then heated at 80 °C for 5 min. After centrifugation to remove precipitated *E. coli* host proteins, the supernatant was loaded onto a 2.5 × 12 cm Q-sepharose fast flow column and eluted with a linear gradient of 0 to 0.5 M NH_4Cl in buffer A (400 mL total). Elution of IPS was detected by activity; the purity of each fraction was monitored by SDS−PAGE. Fractions with >85% pure IPS were pooled, and $(NH_4)_2SO_4$ was added to a final concentration of 30%. This sample was loaded onto a 1.6 × 20 cm phenyl-sepharose column preequilibrated with buffer B [30% $(NH_4)_2SO_4$ in 50 mM Tris HCl, pH 7.5]. The column was washed with buffer B and eluted with 200 mL of a linear gradient of 0 to 100% buffer A. IPS fractions (99% pure as judged by SDS−PAGE) were pooled and dialyzed against 2 × 2 L buffer A. The dialyzed protein solution was concentrated to 10 mg/mL and stored at 4 °C.

Gel Filtration. A column (1.6 × 70 cm) of Bio-gel A 0.5 m equilibrated with buffer A was used to determine the molecular mass of recombinant *A. fulgidus* IPS. The void volume was determined with Blue Dextran, and the column was calibrated with apoferritin (442 kDa), yeast IPS (240 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Samples (1 mL of $OD_{280} \approx 2-3$) were applied to the column and eluted at a flow rate of 0.33 mL/min. Fractions of 2 mL were collected.

Native Gel Electrophoresis. Native PAGE (*39*) was also used to determine the molecular mass of *A. fulgidus* IPS. Recombinant IPS and standards were run at different gel concentrations (7, 8, 10, and 12% polyacrylamide gels). The

relative mobility (R_m) of a protein is a logarithmic function of gel concentration. The slope of ln(R_m) versus gel concentration is proportional to molecular mass, hence the molecular mass of a protein can be extracted from a standard curve. Yeast IPS (240 kDa), β-amylase (200 kDa), *E. coli* aspartate transcarbamoylase catalytic subunit (99 kDa, provided by Dr. Evan Kantrowitz, Boston College), bovine serum albumin (66 kDa), *M. jannaschii* I-1-Pase [56 kDa, purified as described previously (*40*)], and carbonic anhydrase (29 kDa) were used as molecular mass standards.

IPS Assays. ^{1}H coupled ^{31}P NMR (202.7 MHz) spectroscopy was first used to confirm and characterize the conversion of G-6-P to I-1-P as described previously (*34*) but with slight modifications (*41*). The typical assay mixture (0.5 mL) contained 5 mM G-6-P, 0.02−1 mM NAD^+, 0.02−1 mM divalent metal ion, 50 mM Tris acetate, pH 7.5, and 20% D_2O (for the spectrometer lock). *A. fulgidus* IPS is heat stable, and all assays were carried out at 90 °C and were fixed time point assays unless otherwise noted. The assay mixture was first heated to 90 °C, then enzyme (10−20 μg) was added to initiate the reaction. After incubation for 5 min, the IPS reaction was stopped by the addition of EDTA. The resultant solution was stored on ice until a ^{31}P spectrum could be acquired. The specific activity was obtained from the ratio of I-1-P to G-6-P resonances; protein concentration was determined by the Bradford (*42*) assay method with bovine serum albumin as standard. For experiments to measure the K_m for G-6-P, the reaction was run in a larger volume and the sample concentrated prior to NMR analysis. Reaction mixtures used to measure the pH dependence of IPS included 5 mM G-6-P, 500 μM NAD^+, 50 μM Zn^{2+} and 50 mM Mes (pH 6 and 6.5), Tes (pH 7 and 7.5), or Tris (pH 8 and 8.5). Samples (containing 20% D_2O for the spectrometer lock) were analyzed at room temperature by ^{31}P NMR spectroscopy after 3 min incubations at 85 °C.

Absorption and Fluorescence Spectra of IPS. Absorption spectra of IPS (20 μM) protein in 50 mM Tris, pH 7.5, with 300 μM NAD^+, and 400 μM EDTA were obtained at room temperature with a Beckman DU 640 spectrometer using 1 cm cuvettes. The protein solution was scanned from 300 to 400 nm before the addition of 5 mM G-6-P, then after adding the substrate and heating the sample for 30 min at 85 °C. To monitor the time course for NADH generation, the IPS sample was heated for various times (1−30 min) at 85 °C, then cooled to room temperature to obtain the UV spectrum. The NADH extinction coefficient used to measure the amount of NADH produced was $\epsilon_{mM}(340\ nm) = 6.2$; protein concentration was measured by Bradford assay. Steady-state fluorescence measurements were performed with a Shimadzu RF 5000 V spectrofluorimeter (with a Xenon light source) at 23 °C. The excitation wavelength was either 280 or 340 nm, with both excitation and emission slit widths set at 1.5 nm. The emission was scanned from 300 to 600 nm in the case of excitation at 280 nm and from 360 to 600 nm for excitation at 340 nm.

Enzymatic Synthesis and Purification of [5-^{13}C]G-6-P. (D)-[5-^{13}C]Glucose was converted to (D)-[5-^{13}C]G-6-P with hexokinase and ATP as described for the preparation of [5-^{3}H]G-6-P (*43*). (D)-[5-^{13}C]Glucose (0.05 g) and 0.23 g of ATP were dissolved in 5 mL of 100 mM Tris acetate, pH 7.5, containing 5 mM Mg^{2+}. Bakers yeast hexokinase (10 units) was added and the reaction mixture was incubated at 37 °C overnight. ^{31}P or ^{13}C NMR spectra were used to monitor the reaction and the purification process. (D)-[5-^{13}C]G-6-P was purified from the reaction mixture using an AG1-X8 anion exchange column (formate form). The reaction mixture was applied to the column and eluted with 0.2−0.8 M formic acid. Fractions containing (D)-[5-^{13}C]G-6-P (identified by ^{31}P spectra) were pooled, adjusted to pH 7 with NH_4OH, and then lyophilized to remove ammonium formate.

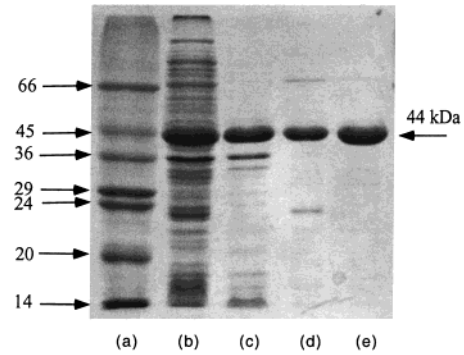


FIGURE 1: Optimized *A. fulgidus* IPS expression and purification analyzed by 12% SDS−PAGE stained with Coomassie brilliant blue: lane (a) molecular mass standards (sizes in kilodaltons indicated to the left of the gel), (b) crude supernatant, (c) heat treated supernatant, (d) after QFF column, and (e) after phenyl sepharose column.

^{1}H NMR Spectroscopy. ^{1}H NMR 500 MHz spectra were obtained with a Varian Unity 500 spectrometer using an indirect probe. 2D spectra were acquired in a phase-sensitive mode using hypercomplex data sets. The ^{1}H carrier was set on the residual HDO resonance, and transmitter presaturation pulse sequences were used. A TOCSY experiment with 60 ms mixing time was used to confirm the identity of the final product as I-1-P.

RESULTS

Expression and Purification of IPS. The expression of cloned yeast *INO1* gene in *E. coli* was very sensitive to the stage of induction and growth temperature. Reasonable overexpression of protein only occurred with induction at $OD_{560} \approx 0.4$ and growth at 28 °C (*41*). In marked contrast to the yeast gene, the expression of AF1794 was very straightforward. Under the induction conditions used (IPTG added at $OD_{560} = 0.7$ and incubation at 37 °C for 4 h), overexpressed AF1794 gene product represented 30−40% of total soluble cellular proteins as estimated by SDS−PAGE (Figure 1).

Since *A. fulgidus* is a hyperthermophile that grows at temperatures between 64 and 92 °C, the first step in the purification process was heat treatment. Most of the *E. coli* proteins were denatured after heating the crude cell protein supernatant at 80 °C for 5 min (Figure 1, compare lane b and lane c). The two chromatographic steps generated very pure IPS (lane e). With this protocol, 18 mg of pure (~99%) IPS was obtained from 2 L of culture with 37% recovery and 7.5-fold purification (Table 1). Purified IPS showed a single band in SDS−PAGE with a subunit size of 44 kDa in agreement with the 44.1 kDa predicted from the DNA sequence. The native molecular mass of the protein was measured by gel filtration a and native PAGE (Figure 2). An average of 168 kDa was obtained indicating that native

Table 1: Purification of Recombinant *A. fulgidus* IPS

	total protein (mg)	specific activity[a]	total units[b]	yield (%)	purification fold
crude extract	381	1.56	594	100	1
heat treatment	71.3	7.43	530	89	4.8
QFF column	37.6	9.15	344	57	5.9
phenyl sepharose column	18.8	11.8	220	37	7.5

[a] Specific activity (measured at 90 °C) units are μmol min^{-1} mg^{-1}. [b] The total units in the sample are defined by the specific activity times the total protein.

Table 2: Kinetic Parameters for Recombinant *A. fulgidus* IPS (assayed at 90 °C)

substrate/cofactor	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
G-6-P	124 ± 39	13.1 ± 0.6
NAD^+	5.08 ± 0.87	13.0 ± 0.4
Zn^{2+}	1.11 ± 0.28	13.4 ± 0.7
Mn^{2+}	0.77 ± 0.22	12.7 ± 0.7
Mg^{2+}	24.2 ± 5.1	12.2 ± 0.5

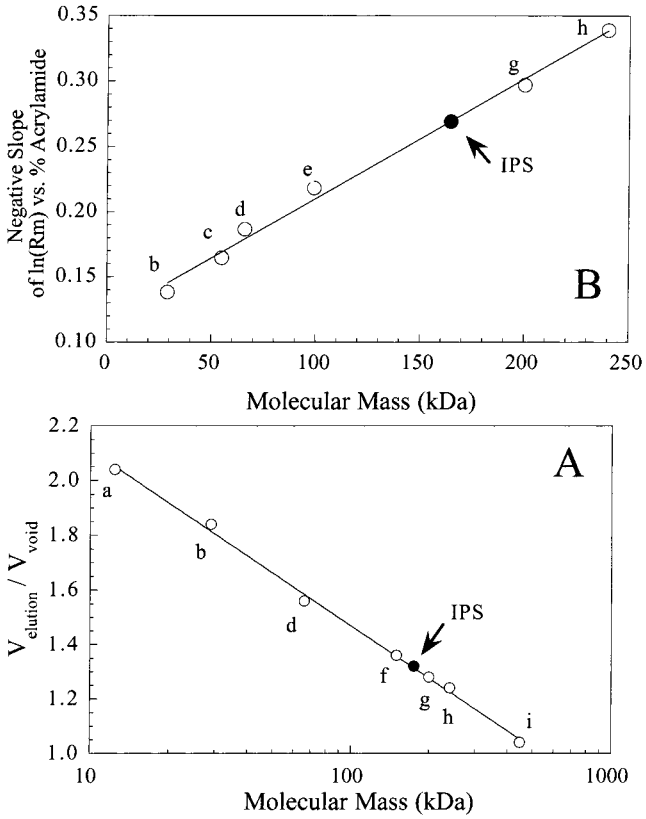


FIGURE 2: Native molecular mass determination for recombinant *A. fulgidus* IPS (●) and protein standards (○): (A) gel filtration on Biogel A 0.5 m resin; (B) negative slopes, obtained from plots of relative mobilities (R_m) of standards and *A. fulgidus* IPS versus gel concentration (7−12%). The standards labeled in each panel include (a) cytochrome *c* (12.4 kDa), (b) carbonic anhydrase (29 kDa), (c) *M. jannaschii* I-1-Pase (56 kDa), (d) bovine serum albumin (66 kDa), (e) *E. coli* ATCase catalytic subunit (99 kDa), (f) alcohol dehydrogenase (150 kDa), (g) β-amylase (200 kDa), (h) yeast IPS (240 kDa), and (i) apoferritin (442 kDa).

A. fulgidus IPS is a tetramer. All other known and characterized IPS enzymes are tetramers or trimers although their subunits are mostly around 60 kDa.

Identification of Archaeal IPS Product as L*-myo-I-1-P.* That the recombinant enzyme catalyzed the conversion of

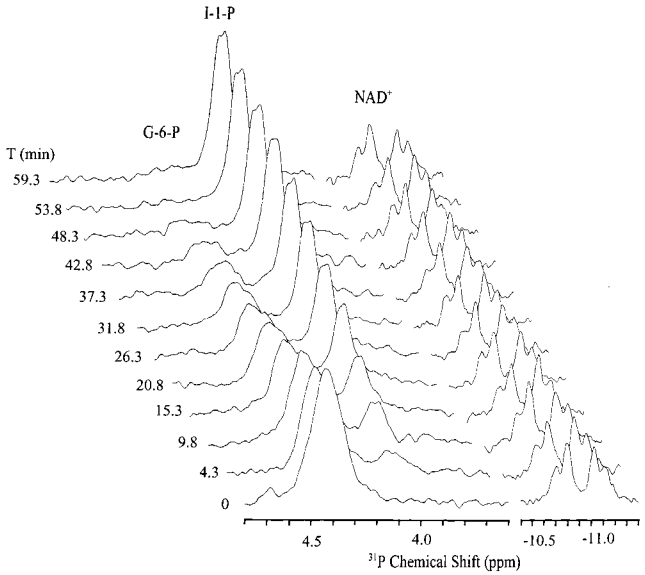


FIGURE 3: ^{1}H-coupled ^{31}P NMR (202.7 MHz) spectra as a function of incubation time (shown to the left of each spectrum) at 70 °C for an IPS assay mixture containing 5 mM G-6-P, 2 mM NAD^+, 50 mM Tris acetate, pH 7.5, and 20 μg of purified IPS. The final spectrum shows 100% conversion of G-6-P to I-1-P.

(D)-G-6-P to I-1-P was confirmed by ^{31}P and ^{1}H NMR spectra. ^{1}H coupled ^{31}P NMR spectroscopy was used to follow the IPS reaction at 70 °C (Figure 3). The conversion of (D)-G-6-P ($\delta_P \approx 4.4$ ppm, resonances for both α and β anomers are in an intermediate to fast exchange regime at this temperature that gives rise to a broad ^{31}P resonance) to I-1-P (~4.0 ppm, a doublet with $J_{H-P} = 8$ Hz) was essentially complete. Resonances for the G-6-P (substrate), I-1-P (product), and NAD^+ (cofactor) were the only phosphorus-containing species observed during the reaction. A ^{1}H-^{1}H TOCSY experiment with this solution clearly showed the six proton spin system characteristic of the inositol ring (Figure 4). The chemical shifts of the six resonances [ppm 3.9 (H-1), 4.1 (H-2), 3.5 (H-3), 3.6 (H-4), 3.3 (H-5), 3.7 (H-6)] were identical to those determined for authentic I-1-P under the same conditions [ppm 3.8 (H-1), 4.22 (H-2), 3.51 (H-3), 3.59 (H-4), 3.27 (H-5), 3.71 (H-6)]. (D)-[5-^{13}C]G-6-P was used to determine the stereochemistry of the product. If the product was (L)-I-1-P, ^{13}C-5 in (D)-G-6-P would be converted to (L)-I-1-P with the ^{13}C incorporated into C-2; the C-2- proton would exhibit a large splitting of about 150 Hz. In contrast, if the product was (D)-I-1-P, ^{13}C-5 in (D)-G-6-P would become ^{13}C-6 in I-1-P and the C-6 proton would be split by the attached ^{13}C. The ^{1}H spectrum of the IPS reaction product is shown in Figure 5. The H-2 resonance was a doublet with J_{H-C}=152 Hz, indicating that *A. fulgidus* IPS, like other characterized IPS, stereospecifically forms (L)-I-1-P.

Kinetic Parameters. At 90 °C and pH 7.5, the K_m for G-6-P was found to be 0.12 ± 0.04 mM, a value 10-fold lower than the K_m for yeast IPS (*41*). A V_{max} of 13.1 ± 0.5 μmol min^{-1} mg^{-1} ($k_{cat} = 9.6 \pm 0.4$ s^{-1}) at this temperature is the highest activity reported among IPS (*33*). Interestingly, the archaeal IPS could be activated by divalent metal ions (Figure 6A). The basal activity (without added divalent metal ion) was about 20−30% of the maximally activated enzyme. Both Zn^{2+} and Mn^{2+} were the best activators, followed by Co^{2+} and Mg^{2+}; Fe^{2+}, Ni^{2+}, Cu^{2+}, Ba^{2+}, and Ca^{2+} had no effect.

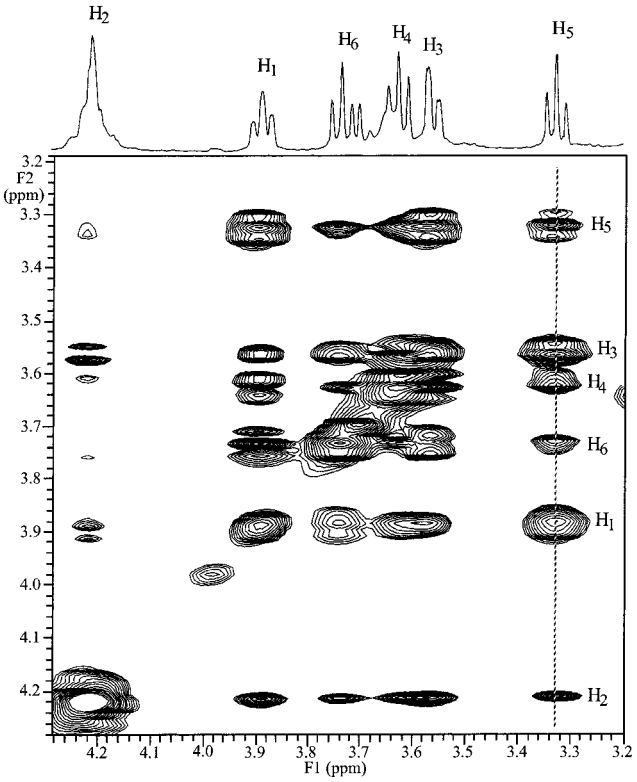


FIGURE 4: ^{1}H-^{1}H TOCSY spectrum of the enzymatically prepared I-1-P in 20 mM Tris-d_{11} in D_2O. The vertical dotted line identifies all six inositol protons in the ring spin system.

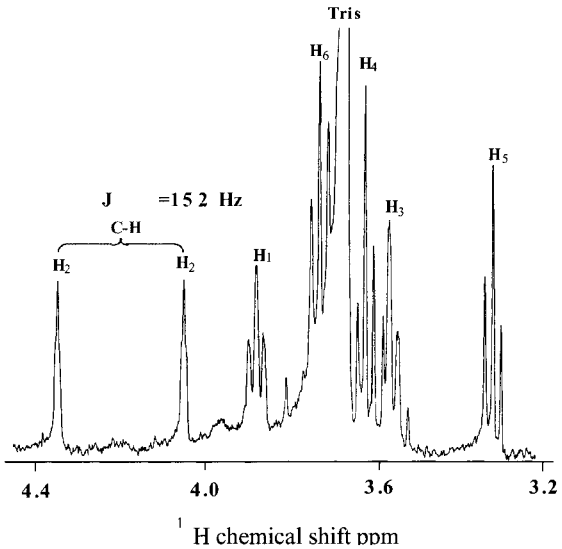


FIGURE 5: ^{1}H NMR (500 MHz) spectrum illustrating the conversion of [5-^{13}C]G-6-P to product I-1-P; each resonance is labeled by the proton that gives rise to it. The large resonance at 3.68 ppm represents residual proteo-Tris. Note the ^{13}C-^{1}H splitting (J_{H-C} = 152 Hz) of the C-2 proton in I-1-P centered at 4.2 ppm.

The affinity of metal ions for IPS as measured by the dependence of specific activity (with 5 mM G-6-P and 0.5 μM NAD^+) on the concentration of metal ion was $Mn^{2+} \approx Zn^{2+} > Mg^{2+}$ with a $K_D \approx 1$ μM for the first two cations. Presumably, trace amounts of tightly bound Mn^{2+} or Zn^{2+} are responsible for the basal activity (no extraneous added metal ion). This residual activity could be eliminated by the addition of EDTA; 50% inhibition of basal IPS activity occurred at 2 μM EDTA (Figure 6B). Also, unlike all other known IPS enzymes, NH_4^+ had no effect on *A. fulgidus* IPS;

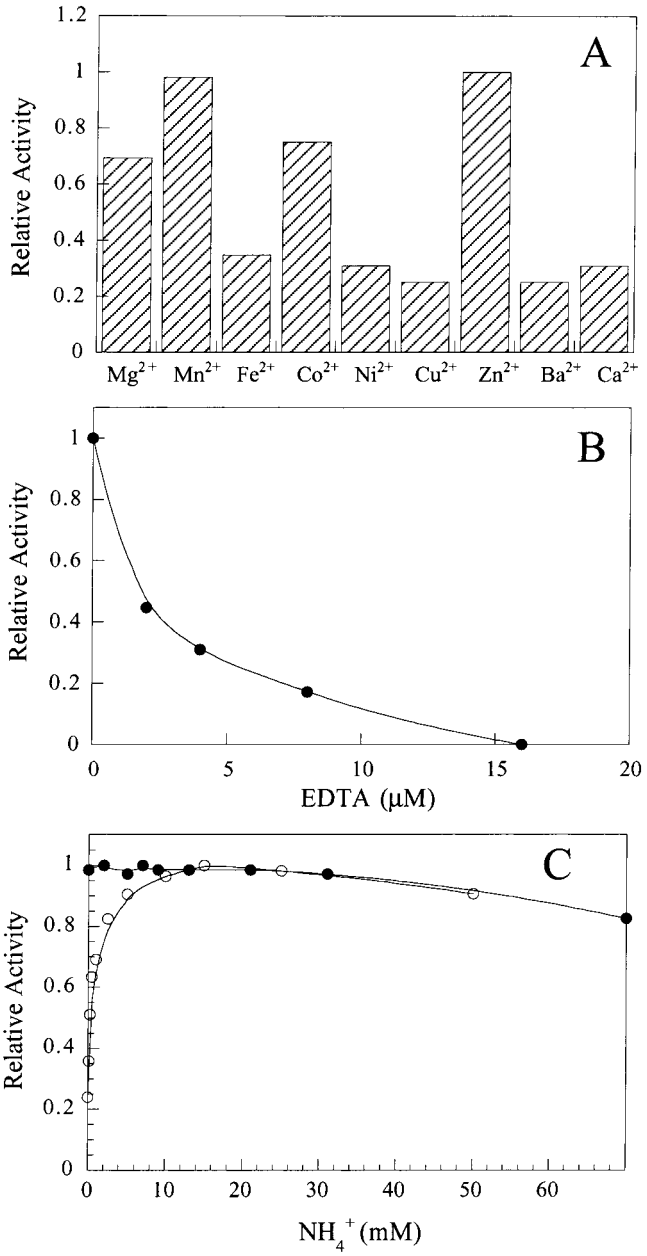


FIGURE 6: Relative activity of *A. fulgidus* IPS toward 5 mM G-6-P in 50 mM Tris acetate, pH 7.5, at 90 °C as a function of (A) different metal ions (20 μM) or (B) added EDTA. (C) Effect of NH_4^+ on the activities of the recombinant IPS from *A. fulgidus* (●) and yeast (○). In panel A, all specific activities were referenced to that for *A. fulgidus* IPS with saturating Zn^{2+} (13 μmol min^{-1} mg^{-1} under these conditions); the basal activity of IPS without added metal ion was 3.2 μmol min^{-1} mg^{-1}. In panel B, the specific activity of the IPS without added metal ion or EDTA was 3.2 μmol min^{-1} mg^{-1}; activities with EDTA added were referenced to that value to obtain the relative activity. In panel C, relative activities were determined by comparing the activity of each IPS enzyme with various concentrations of NH_4^+ to the specific activity of both proteins (the *A. fulgidus* IPS has with saturating Zn^{2+} added while the yeast IPS does not) without NH_4^+.

for comparison, the yeast IPS was stimulated 5-fold at 15 mM NH_4^+ (Figure 6C). The pH profile for *A. fulgidus* IPS (Figure 7A) shows an optimum activity at pH 8 at 85 °C; the drop in activity on the acidic side of this optimum suggests that a group with a p$K_a \approx 6.4$ must be deprotonated.

The activity of IPS (0.2 mg) was also examined toward 5 mM of D-glucose-6-sulfate (G-6-S) and 2-deoxy-G-6-P

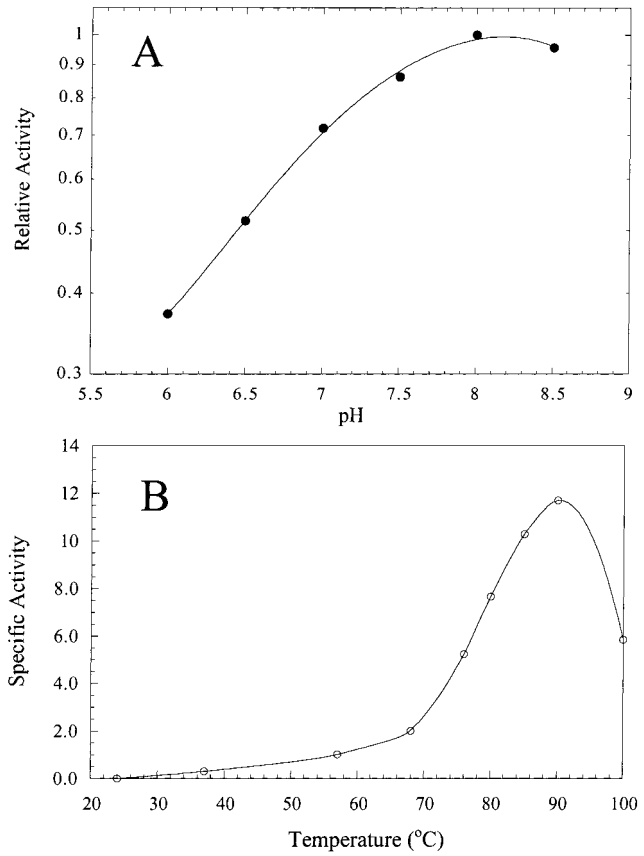


FIGURE 7: (A) pH and (B) temperature dependence of *A. fulgidus* IPS activity.

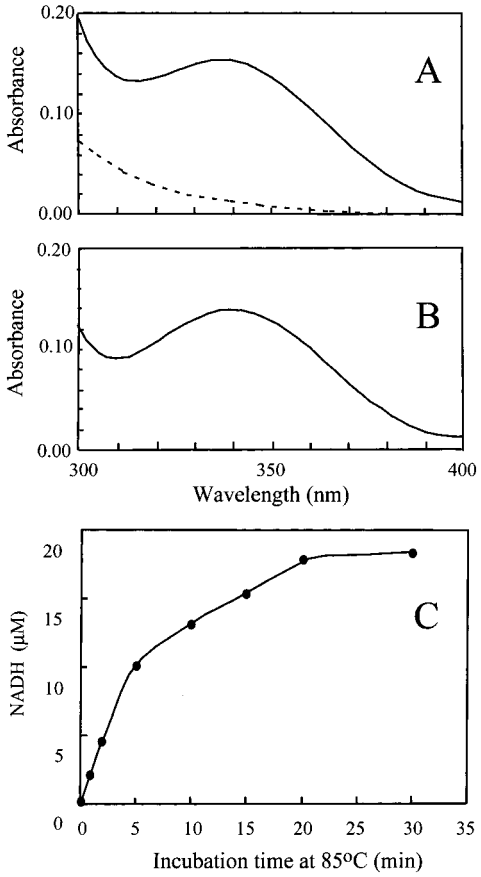


FIGURE 8: (A) Absorption spectrum (recorded at room temperature) of 20 μM IPS in the presence of 300 μM NAD^+, and 400 μM EDTA before (- - -) and after (—) the addition of 5 mM G-6-P and heating for 30 min at 85 °C. (B) The difference spectrum after heating with G-6-P minus the spectrum before G-6-P addition; note the band at 340 nm consistent with NADH production. (C) Time dependence of the reduction of NAD^+ (300 μM) to NADH (concentration determined by the absorbance at 340 nm) in the presence of 5 mM G-6-P and 400 μM EDTA. The sample was heated for the indicated time at 85 °C, then cooled to room temperature to obtain the UV spectrum. The IPS concentration was 21 ± 1 μM; limiting NADH produced was 18 ± 1 μM.

(assay conditions included 10 μM Zn^{2+}, 200 μM NAD^+, and incubation for 1 h at 85 °C). There was no inositol formation from G-6-S as judged by ^{1}H NMR spectroscopy (under the assay conditions used this would correspond to a specific activity <0.001 μmol min^{-1} mg^{-1}). A small ^{31}P resonance at ~4.2 ppm was observed after incubation of 2-deoxy-G-6-P (δ_P = 4.7 ppm) with IPS; this would correspond to a specific activity of 0.01 μmol min^{-1} mg^{-1}. However, in the ^{1}H spectrum, the chemical shift region for the very nonequivalent $-C(2)H_2-$ resonances of 2-deoxy-G-6-P shows no new resonances (C-2 becomes C-5 of the inositol analogue and the attached protons should exhibit altered chemical shifts), strongly suggesting that the small amount of material at 4.2 ppm in the ^{31}P spectrum can be attributed to contaminating G-6-P.

Thermal Stability of IPS. Since *A. fulgidus* is an extreme thermophile with an optimum growth temperature at 83 °C, the stability of IPS was examined at 90 °C. There was only a small decrease (less than 10%) in activity after 30 min preincubation at 90 °C, indicating that *A. fulgidus* IPS is extremely heat stable. Neither substrates (G-6-P or NAD^+), nor the compatible solute pair potassium glutamate had much effect on IPS activity when the enzyme was incubated at 90 °C for up to 1 h. Given the thermostability of the protein, the temperature dependence of IPS activity was also examined (Figure 7B). There was no detectable activity at 24 °C and very low activity below 65 °C under the assay conditions used. At 100 °C IPS activity decreased 50%. An Arrhenius plot of the IPS activity between 70 and 90 °C provided an estimate of 69 kJ/mol for the activation energy.

Catalytic Mechanism: A Class II Aldolase. IPS has been proposed to catalyze three reactions in the overall conversion of (D)-G-6-P to (L)-I-1-P with the second step an aldol condensation. *A. fulgidus* IPS requires divalent metal ions, and the likely role of the cation is to stabilize the carbanion formed prior to the aldol cyclization by IPS. If metal ions indeed play this role, then in the presence of EDTA the IPS should be able to convert (D)-G-6-P to 5-keto-G-6-P with concomitant reduction of NAD^+ to NADH, but not proceed further to *myo*-inosose-2 1-phosphate. Furthermore, if the NADH and 5-keto-G-6-P are tightly bound to the IPS, the amount of NADH generated should be stoichiometric with IPS. Both UV absorption and fluorescence spectra of the enzyme with EDTA and 300 μM NAD^+ present were examined before and after the addition of G-6-P. As shown in Figure 8A, in the absence of G-6-P, the enzyme (20 μM) has no distinct absorption feature at 340 nm, where NADH would be expected to observe. After the addition of 5 mM G-6-P and heating at 85 °C for 30 min, the IPS exhibited a new absorption maximum at 340 nm (the difference spectrum is shown in Figure 8B). This absorbance corresponds to the production of ~20 μM NADH. If the enzyme is heated to

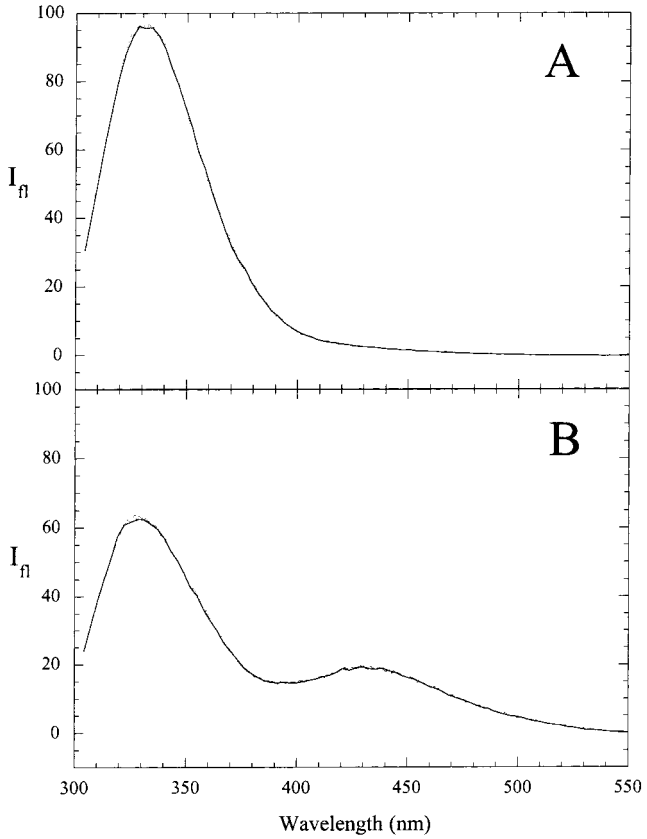


FIGURE 9: (A) Fluorescence spectrum of 45 μM IPS in the presence of 300 μM NAD^+ (A) before and (B) after the addition of 5 mM G-6-P and heating at 85 °C for 30 min. The protein was excited at 280 nm and fluorescence scanned from 300 to 600 nm with excitation and emission slit widths of 1.5 mm. The emission peak at 422 nm, characteristic of NADH, was not observed in samples heated without added G-6-P or in unheated samples of IPS, NAD^+, and G-6-P.

85 °C for 30 min in the absence of G-6-P, no NADH absorption band is produced. Reduction of NAD^+ to NADH by IPS in the presence of EDTA does not occur rapidly (Figure 8C). The absorbance of IPS (21 ± 1 μM as measured by Bradford assay) at 340 nm after heating for various times at 85 °C in the presence of 400 μM EDTA, 5 mM G-6-P, and 300 μM NAD^+ showed that about 20 min were needed to obtain maximum NADH formation. The NADH produced was equivalent in concentration (18 ± 1 μM) to the IPS in solution. A ^{31}P spectrum of the solution showed only G-6-P and NAD^+ resonances indicating that the NADH generated (and presumably the 5-keto-G-6-P) was tightly bound to the IPS and not released into solution. When an excess of Zn^{2+} was added to the solution and the temperature incubated at 85 °C for 30 min, the absorbance at 340 nm was lost. This treatment converted all of the G-6-P in solution to I-1-P as judged by ^{31}P NMR (spectrum not shown).

NADH also has a characteristic fluorescence maximum ~425 nm, whereas NAD^+ is nonfluorescent. Figure 9A shows the fluorescence spectrum of 45 μM IPS, excited at 280 nm, with 300 μM NAD^+ added. After adding G-6-P and heating the sample at 85 °C for 30 min, a new fluorescence peak was observed with a maximum wavelength between 420 and 430 nm (Figure 9B) that is consistent with NADH fluorescence. IPS intrinsic fluorescence (maximum at 330 nm) was also decreased indicating resonance energy transfer from tryptophan on the protein to the bound NADH. The NADH fluorescence band could also be specifically produced by exciting the sample at 340 nm. The maximum emission occurred at 422 nm under these conditions, consistent with generation of NADH tightly bound to the protein. After the addition of Zn^{2+} in excess to the EDTA and heating to 85 °C for 30 min, the NADH fluorescence spectrum was lost.

Both the IPS absorption and fluorescence results indicate that without the addition of divalent metal ions, an amount of NADH can be produced that is stoichiometric to the enzyme. The slow time course for NADH generation suggests that Zn^{2+} may also have other roles in the protein. Addition of Zn^{2+} abolishes the NADH peaks and converts all the G-6-P to I-1-P. This is consistent with *A. fulgidus* IPS functioning as a class II aldolase in the second step.

DISCUSSION

IPS has been identified in virtually all eukaryotic organisms as well as several bacteria. Studies of IPS in *Streptomyces griseus* (*23*), a Gram positive bacterium, suggest that the inositol biosynthesis pathway arose early in the evolution of life and the origin of IPS precedes the evolutionary divergence of modern eukaryotes and bacteria (*33*). Recent analyses of *INO1* homologues in protein sequence databases (*26*) suggest a distinct branch between eukaryotic and prokaryotic organisms, with greater homology between the considerably smaller archaeal and bacterial sequences (intermingling of archaeal IPS candidates with bacterial proteins also suggests horizontal transfer of genes). Our previous studies of the biosynthetic pathway of DIP (an osmolyte derived from inositol) in *M. igneus* (*34*) first confirmed IPS and I-1-Pase activities in a protein extract from that archaeon. IPS activity has also been detected in protein extracts of other hyperthermophiles including *P. furiosus*, *P. woesei*, and *T. maritima*, all organisms that synthesize and accumulate DIP (*44*, *45*). Genebank and Protein Databases sequence homology searches for archaeal IPS have resulted in several putative candidates in *Methanobacterium thermoautotrophicum*, *P. horikoshii*, *P. abyssi*, and *Aeropyrun pernix*, as well as AF1794 in *A. fulgidus* (interestingly, no homologue was identified in the *M. jannaschii* genome). I-1-Pase (the second enzyme in the de novo inositol biosynthesis pathway) homologues have been found in all the archaeal genomes that have been sequenced to date (*38*, *46*−*48*). If AF1794 really codes for an archaeal IPS, one might expect some mechanistic variations from the well-characterized eukaryotic IPS, particularly since the N-terminal domain of the protein, which is thought to have the NAD^+ binding site) is very different in eukaryotes versus bacteria and archaea (*26*). The *A. fulgidus* IPS is about 140 amino acids shorter than the yeast IPS with most of the missing residues occurring in the N-terminal region of the yeast protein. In the present work, we have confirmed that AF1794 indeed codes for IPS activity. Overexpression of the enzyme in *E. coli* provided large amounts of a very heat stable enzyme with three unique characteristics: (i) an absolute requirement for metal ions for catalytic activity (suggesting this archaeal IPS diverged from other members of the IPS gene family by evolving a different mechanism to stabilize the carbanion intermediate formed in the aldol condensation step); (ii) a much smaller subunit molecular weight (44 compared to 60 kDa) than the

eukaryotic IPS; and (iii) a k_{cat} at the growth temperature of the organism much higher than other known IPS.

An interesting characteristic of eukaryotic IPS is that there is a high degree of transcriptional regulation by inositol phospholipid precursors. At least one archaeon does have a modified inositol phospholipid as a major component of its membrane (*49*), although nothing is known about regulation of the synthesis of that phospholipid. *A. fulgidus* is a hyperthermophilic sulfate-reducing archaeon that grows optimally at 83 °C but can grow over a wide temperature range. We suggest that the major role of IPS in this organism is to synthesize I-1-P for conversion into the osmolyte DIP, a compatible solute that is accumulated in many different thermophiles to cope with environmental stress associated with growth at supraoptimal temperatures or growth in high salinity media. The intracellular concentration of DIP depends critically on the growth temperature and salt. Since IPS is the first step committing cellular resources to this pathway, it is likely to be a target for regulation.

The k_{cat} of *A. fulgidus* IPS at 90 °C is exceptionally high compared to that for other well-characterized IPS. Since DIP can be accumulated to very high intracellular concentrations, IPS may need a high specific activity. Intracellular DIP concentrations in *A. fulgidus* increased 20−30-fold when the growth temperature was raised from 76 to 87 °C. Yet the enzyme has substantial activity at 76 °C compared to 87 °C. If the regulation of DIP synthesis and accumulation in hyperthermophiles is controlled by the overall activity of IPS in the cell, then it is most probably regulated at the transcriptional level. One can also ask if the IPS from *A. fulgidus* is inherently more active than other IPS enzymes. Yeast IPS has an optimum activity at 55 °C [3.1 μmol min^{-1} mg^{-1} (*41*)]. Given an activation energy of 69 kJ/mol, the recombinant *A. fulgidus* IPS would have a specific activity of 1.1 μmol min^{-1} mg^{-1} at the same temperature. This indicates that at a temperature where both proteins are stable (at least on the time scale of the few minutes needed for the assay), the two enzymes have similar activity. However, the archaeal enzyme has evolved to carry out the conversion of G-6-P to I-1-P at significantly higher temperatures (e.g., 90 °C). Further insights into what makes the archaeal enzyme so thermostable will require detailed structural information for it as well as for the yeast IPS. On a more practical note, the high specific activity of *A. fulgidus* IPS makes the enzymatic production of optically pure (L)-I-1-P practical.

Perhaps the most striking difference for *A. fulgidus* IPS from other known IPS is that it requires metal ions for the aldol condensation step. The catalytic mechanism of this archaeal IPS is different from that invoked for IPS from animals, yeast, and plants, which belong to neither of the two known classes of aldolases since they are uniquely stimulated by NH_4^+. The class I aldolase relies on a specific active-site lysine residue to form a Schiff base intermediate with a keto group while the class II aldolase requires a divalent metal ion to form a complex with the reaction intermediate that stabilizes the carbanion that is formed prior to cyclization. There was one report that rat testes IPS was a class I aldolase (*50*), but later publications did not support the initial findings (*6*, *19*). Early work with the IPS from *Neurospora crassa* (*51*) and duckweed (*21*) indicated that those activities were inhibited by EDTA, suggesting possible divalent metal ion involvement. Inhibition of those IPS activities by 50% only occurred with 1−30 mM EDTA. On the basis of the early assay methods used (periodate oxidation of I-1-P to generate Pi), a slight amount of contaminating phosphatase activity (which is metal ion dependent) could result in a false EDTA inhibition pattern. More recent work with the *N. crassa* IPS indicated that divalent metal ions inhibited the activity of the enzyme while NH_4^+ produced a 2.5-fold activation (*22*). A possible explanation for monovalent cation (NH_4^+) stimulation of eukaryotic IPS enzymes is the interaction of the ion with a keto−enol tautomer intermediate to delocalize and stabilize the carbanion formed in the aldol condensation (*20*).

A detailed proposal for the catalytic mechanism of IPS by Floss and Beale (*52*) involved binding of the acyclic β-anomer of G-6-P and minor changes in G-6-P atomic positions as a single active-site base and its conjugate acid catalyzed multiple proton transfers. Lack of inhibition of IPS by conformationally restrained G-6-P analogues that hinder ring opening and the preference for (*E*)-vinylhomophosphonates versus (*Z*)-vinylhomophosphonates have modified that proposed mechanism by providing evidence that the dibasic phosphate ester of a transoid acyclic substrate is the base that catalyzes the intramolecular aldol condensation (*53*). Consistent with the involvement of the phosphate in intramolecular proton transfer, we have found that G-6-S, with its much lower pK_a, is not a substrate for the archaeal IPS. The inability of IPS to process 2-deoxy-G-6-P may result from hindered ring opening or an altered conformation of the acyclic form.

For the archaeal IPS, the activation by μM divalent metal ions and the inhibition by μM EDTA strongly suggest that *A. fulgidus* IPS is a class II aldolase. The first step of the IPS reaction, the reduction of NAD^+ to NADH concomitant with the oxidation of G-6-P to 5-keto-G-6-P, can be uncoupled from the aldol condensation when EDTA is present. That the NADH produced was stoichiometric with the IPS (while both G-6-P and NAD^+ were in excess) indicated that the substrate and cofactor were tightly bound to the enzyme and not able to dissociate into solution. Generation of the bound NADH under these conditions required heating the sample to 85 °C (room-temperature incubation produced no significant absorbance at 340 nm) and took 20−30 min to reach completion. This might suggest that metal ion has another role in the IPS, possibly helping the enzyme to adopt the correct conformation for optimal catalysis as well as stabilizing the carbanion. Clearly, the ability to stop the IPS reaction after the first step should allow more detailed studies of the archaeal IPS mechanism (and particularly the role of the divalent metal ion) in the future.

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BI001517Q