Compatible Solute Binding to an Archaeal Inositol Monophosphatase

Author: Jessica Jade Chao

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COMPATIBLE SOLUTE BINDING TO AN ARCHAEAL INOSITOL MONOPHOSPHATASE

Jessica J. Chao

Thesis Advisor: Professor Mary F. Roberts

Abstract: Crystallization studies in presence of organic osmolytes were conducted to better understand the specific mechanism of compatible solute binding to *Archaeoglobus fulgidus*’s inositol monophosphatase. The synthesis of α-diglycerol phosphate, one of the natural osmolytes of *A. fulgidus*, was also completed for kinetic testing of its I-1-Pase thermoprotective properties and for crystallization trials.

List of Abbreviations .................................................................iii

List of Figures and Tables .........................................................iv

Chapter One: Background of archaeal inositol monophosphatase stress response and the resulting generation of osmolytes in archaea. .............................................1

Chapter Two: Materials and methods. .......................................20

Chapter Three: The binding of amino acids and other natural compatible organic solutes to the *A. fulgidus* inositol monophosphatase ........................................27

Chapter Four: Future directions ................................................46

References ..................................................................................48
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List of Abbreviations

Asp, aspartate

\(\alpha\)-DGP, \(\alpha\)-diglycerol phosphate

DIP, di-myo-inositol-1,1’-phosphate

Et\(_3\)N, triethylamine

EtOAc, ethyl acetate

Gln, glutamine

Glu, glutamate

I-1-Pase, inositol monophosphatase

K-Glu, potassium glutamate

PEG, polyethylene glycol

Pro, proline

THF, tetrahydrofuran
Figures

Figure 1: Osmolyte stabilization of proteins .................................................................10

Figure 2: Synthesis of myo-inositol ..............................................................................12

Figure 3: Overall dimer structure of *A. fulgidus* inositol monophosphatase ...............16

Figure 4: Comparison of dimer interfaces of *A. fulgidus* I-1-Pase and *M. jannaschii* I-1-Pase .................................................................18

Figure 5: Standard curve for BSA using Pierce Protein Assay ....................................22

Figure 6: General Synthesis of $\alpha$-diglycerol phosphate ........................................24

Figure 7: Crystal Structure of I-1-Pase with 25 mM Asp ...........................................45

Tables

Table 1: Structure and occurrence of organic osmolytes in diverse organisms .................2-7

Table 2: Kinetics of diverse inositol monophosphatases ..............................................14

Table 3: Crystallization trials of *A. fulgidus* I-1-Pase with different solutes ..................23

Table 4: Identification of major mass peaks in reaction mixtures of $\alpha$-DGP ..................31

Table 5: Major Peaks in $^{31}$P NMR spectra for reaction mixtures of $\alpha$-DGP ..............39
Chapter 1: Inositol Monophosphatases and the Effect of Compatible Solute

1.1. Introduction to Archaeal Stress Response

In recent years, many studies have focused on archaea and their specialized stress responses, since they can flourish in harsh, extreme environments that other organisms cannot tolerate. For example, archaea thrive in environments with pH, salinity, and temperature extremes\(^1\). Interest in the survival mechanisms of archaeal extremophiles stems from their unique adaptations, and understanding adaptation and growth in high salinity conditions has seen a particular emphasis\(^1\).

Researchers have proposed that archaea, and in fact all organisms, have two different basic strategies for osmoadaptation. The first strategy is the accumulation of inorganic ions like $K^+$ and $Cl^-$ in the cytoplasm of halobacteria. Unfortunately this method is not general or easily modified, since it requires highly specialized intracellular adaptations – notably evolution of proteins with high negative charge to counterbalance high intracellular $K^+$. Most cells employ the second strategy – accumulating small, water-soluble organic solutes, termed "compatible solutes," that have little effect on normal cell metabolism or enzyme function\(^2\). The majority of microorganisms, including archaea, bacteria, and eukaryotes accumulate organic solutes as part of their osmoregulatory response to external osmotic stress. Compatible solutes do not impede normal cell metabolism, even when they are present at very high concentrations within the cell.

These organic osmolytes are accumulated either by uptake from the cell media, or

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from *de novo* synthesis\(^3\). Cells accumulate organic osmolytes to restore normal turgor pressure and cell volume for maintaining their usual enzyme activity, cell division, and growth\(^4\). Accordingly, the compatible solutes have potential utility in biotechnology, pharmaceutical drugs, vaccine stabilizing agents, or even cosmetics\(^1\). While a few solutes are used by all types of cells, archaea synthesize several unique osmolytes not found in other organisms\(^3\). Osmolytes can be classified into three main categories: (i) neutral solutes that include polyols and sugars, (ii) anionic solutes, and (iii) zwitterions. Anionic solutes comprise the majority of archaeal osmolytes\(^3\). Santos and coworkers propose that in archaea, anionic solutes predominate to equilibrate the high concentration of inorganic cations within the cell\(^1\). Several examples of compatible solutes in archaea, eukaryotes, and bacteria are listed in Table 1 below.

**Table 1.** Structure and Occurrence of Organic Osmolytes in Diverse Organisms (adapted from reference 3)

<table>
<thead>
<tr>
<th>OSMOLYTE</th>
<th>OCCURRENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eukarya</td>
</tr>
<tr>
<td><strong>Neutral Solutes:</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Polyols:</strong></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>algae (Dunaliella sp.); fungi</td>
</tr>
<tr>
<td>Mannitol</td>
<td>yeast</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Compound</th>
<th>Organism/Location</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbitol</td>
<td>yeast</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>myo-inositol</td>
<td>Plants; mammalian brain</td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Carbohydrates:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sucrose</td>
<td>plants and animals</td>
<td>cyanobacteria: Anabaena sp. Phormidium autumnale, Chroococcidiopsis sp.</td>
<td>+ (by transport)</td>
</tr>
<tr>
<td>trehalose</td>
<td>plants and animals</td>
<td>cyanobacteria: Anabaena sp. Phormidium autumnale, Chroococcidiopsis R. marinus</td>
<td>Natronobacterium magadii, Sulfolobus solfataricus, S. ambivalens</td>
</tr>
<tr>
<td>mannosucrose</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Thermoplasma acidophilum Pyrobaculum aerophilum Thermoproteus tenax S. solfataricus Acidianus ambivalens</td>
</tr>
<tr>
<td><strong>α-mannosylglyceramide</strong></td>
<td>N.D.</td>
<td><strong>Rhodothermus marinus</strong>&lt;br/&gt;<strong>R. obamensis</strong></td>
<td>N.D.</td>
</tr>
<tr>
<td>---------------------------</td>
<td>------</td>
<td>-----------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td><img src="image" alt="α-mannosylglyceramide" /></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Other:**

<table>
<thead>
<tr>
<th><strong>urea</strong></th>
<th>cartilaginous fish;&lt;br/&gt;<strong>Drosophila melanogaster</strong></th>
<th>N.D.-</th>
<th>N.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="urea" /></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>N-acetylglutaminylglutamine</strong></th>
<th>N.D.</th>
<th><strong>Sinorhizobium meliloti</strong>&lt;br/&gt;<strong>Rhizobium guminosarum</strong></th>
<th>N.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="N-acetylglutaminylglutamine" /></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>N-α-carbamoyl L-glutamine 1-amide</strong></th>
<th>Halophilic bacteria:&lt;br/&gt;<strong>Ectothiorhodospira mobilis</strong>&lt;br/&gt;<strong>(marismortui)</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="N-α-carbamoyl L-glutamine 1-amide" /></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Zwitterionic Solutes**

<table>
<thead>
<tr>
<th><strong>glycine betaine</strong></th>
<th>+</th>
<th><strong>Transported by</strong> <strong>E. coli,</strong>&lt;br/&gt;<strong>Corynebacterium glutamicum</strong>&lt;br/&gt;<strong>Listeria monocytogenes</strong></th>
<th><strong>Methanohalophilus portocalensis</strong>&lt;br/&gt;<strong>Methanosarcina thermophila</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="glycine betaine" /></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>dimethylsulfoniopropionate</strong></th>
<th>N.D.</th>
<th>+</th>
<th>N.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="dimethylsulfoniopropionate" /></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ectoine</td>
<td>plants and algae</td>
<td><em>Ectothiorhodospira</em> sp. <em>Brevibacterium linens</em> <em>Bacillus</em> <em>Proteobacteria Vibrio costicola</em> <em>Micrococcus</em> (aerobic, halophiles from Mono Lake)</td>
<td>-</td>
</tr>
<tr>
<td>--------</td>
<td>------------------</td>
<td>-------------------------------------------------</td>
<td>----</td>
</tr>
<tr>
<td>proline</td>
<td>?</td>
<td>+</td>
<td>N.D.</td>
</tr>
<tr>
<td>β-glutamine</td>
<td>N.D.</td>
<td>N.D.</td>
<td><em>Methanohalophilus portucalensis</em></td>
</tr>
<tr>
<td>N-ε-acetyl-β-lysine</td>
<td>N.D.</td>
<td>N.D.</td>
<td><em>Methanosarcina thermophila</em> <em>Methanogenium cariaci</em> <em>Methanohalophilus sp.</em> <em>Methanococcus thermolithotrophicus</em></td>
</tr>
<tr>
<td>Trimethylamine oxide (TMAO)</td>
<td>Elasmobranchs; deep-living invertebrates; teleost fishes</td>
<td>?</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Anionic Solutes:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Phosphates:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-diglycerol phosphate (α-DGP)</td>
<td>N.D.</td>
<td>N.D.</td>
<td><em>Archaeoglobus fulgidus</em></td>
</tr>
<tr>
<td>Compound</td>
<td>Cell Type</td>
<td>Hyperthermophilic Archaea</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-----------</td>
<td>---------------------------</td>
<td></td>
</tr>
<tr>
<td>di-my o-1, 1'-inositol phosphate (DIP)</td>
<td>N.D.</td>
<td>Thermotoga maritima</td>
<td></td>
</tr>
<tr>
<td>cyclic-2,3-diphosphoglycerate (cDPG)</td>
<td>N.D.</td>
<td>Methanobacterium thermoautotrophicum, Methanopyrus kandleri, Methanothermus fervidus</td>
<td></td>
</tr>
<tr>
<td>Glycero-phospho-my o-inositol (GPI)</td>
<td>N.D.</td>
<td>Hyperthermophile archaea: Archaeoglobus, fulgidus, Aquifex</td>
<td></td>
</tr>
<tr>
<td>Carboxylates:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 3, 4, 6-hexanetetracarboxylic acid (HTC)</td>
<td>N.D.</td>
<td>Methanobacterium thermoautotrophicum Marburg and ΔH</td>
<td></td>
</tr>
<tr>
<td>α-glucosylglycerate</td>
<td>N.D.</td>
<td>Marine and freshwater cyanobacteria: Synechocystis sp, Microcystis filma; phototrophic eubacteria Rhodovulum sulfidophilum; Pseudomonas mendocina; P. pseudoalcaligenes; Stenotrophomonas</td>
<td></td>
</tr>
<tr>
<td>Compatible solute</td>
<td>Detected in</td>
<td>Organisms</td>
<td>Notes</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------</td>
<td>-----------</td>
<td>-------</td>
</tr>
<tr>
<td>β-mannosylglycerate</td>
<td>N.D.</td>
<td>Rhodothermus marinus, R. obamensis</td>
<td>A. fulgidus, Thermus thermophilus, Rhodothermus marinus (α-isomer)</td>
</tr>
<tr>
<td>α-mannosylglycerate (MG)</td>
<td>Red algae</td>
<td>Rhodothermus marinus</td>
<td>Archaea: Aeropyrum, Stetteria, Pyrococcus, Thermococcus, Archaeoglobus, Palaeococcus</td>
</tr>
<tr>
<td>alpha-glutamate</td>
<td>mammals, plants, yeast</td>
<td>E. coli</td>
<td>Halomonas elongata, Methanococcus sp. M. thermoautotrophicum, Natronococcus occultus</td>
</tr>
<tr>
<td>β-glutamate</td>
<td>N.D.</td>
<td>+</td>
<td>M. thermolithotrophicus, M. igneus, M. jannaschii (other methanogens)</td>
</tr>
<tr>
<td>Sulfate:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulfotrehalose</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Natronobacterium sp., Natronococcus occultus</td>
</tr>
<tr>
<td>Cationic Solute:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-galactopyranosyl-5-hydroxylysine</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Thermococcus litoralis</td>
</tr>
</tbody>
</table>

Key: N.D. = not detected, ? = not examined, (+) = observed, (-) = not observed

Compatible solutes can stabilize and maintain isolated protein structure\(^1\) by acting as thermoprotectants\(^4\). This is believed to be a role compatible solutes play in cells as
well. For example, a 20-fold increase in D-\textit{myo}-inositol 1,1’-phosphate (DIP) levels is observed in \textit{P. furiosus} at 101\textdegree C. This observation is consistent with the hypothesis that DIP stabilizes protein structure at extremely high temperatures\textsuperscript{4}. At such a high temperature, significant loss of protein secondary structure would normally occur. The increased DIP levels should provide thermoprotection, although the mechanism is not clear.

As a group, organic compatible solutes serve as stress protectants in environments with harsh osmotic conditions. Compatible solutes have been observed to protect proteins and cell components from osmotic stress-induced dehydration\textsuperscript{4}. They prevent desiccation, freezing, over-heating and stop the formation of damaging oxygen radicals in cell components. Glycerol and trehalose are two well-known solutes generated under stress that offer such protection. Glycerol is a yeast solute, and its increased accumulation in \textit{S. cerevisiae} was observed in response to a loss of intracellular water and turgor pressure\textsuperscript{5}. In contrast, trehalose was generated as part of the heat shock response \textit{in vitro}, and it prevents protein denaturation, and stops the aggregation of denatured firefly luciferase\textsuperscript{2}.

The general heat shock response primes the cell for survival under extremely hot conditions. Pre-treating cells at slightly raised temperatures induces this protective response. The cell survival rate is improved 100- to 10000-fold if pre-treated by gentle heating\textsuperscript{2}. The protective heat shock response first induces the production of heat shock proteins (HSP’s), also known as molecular chaperones. Heat shock proteins repair and insulate cells against damage induced by protein aggregation. To improve conditions for

overall cell survival, HSP’s also accelerate the degradation of substrates damaged beyond repair.

1.1.1. How Osmolytes Are Thought to Function

Osmolytes exhibit the osmophobic effect, a thermodynamically unfavorable interaction between the osmolyte and the peptide backbone in proteins\(^6\). The osmophobic effect is a type of solvophobic effect that is analogous to the hydrophobic effect in proteins, where contact between the nonpolar side chains and the solvent, water, increases the Gibbs free energy and makes this thermodynamically unfavorable\(^6\).

An increase in Gibbs free energy also resulting from thermodynamically unfavorable contact between the osmolyte and the peptide backbone drives the protein toward its native, folded state, as depicted in Figure 1. Addition of osmolyte to the buffer solution increases the Gibbs free energy slightly for the native folded protein. In comparison, a greater change in Gibbs free energy is observed when osmolyte is added to unfolded protein in buffer, much more than the change in \(\Delta G\) for the folded conformation. So, osmolyte presence greatly stabilizes protein by pushing it toward the native folded state, since adding an osmolyte to a folded protein is far more thermodynamically favorable.

The term osmophobic effect was first used\(^6\) by Bolen and coworkers. It has been observed that the osmophobic effect is stronger when proteins are denatured under environmental stress, since the denatured protein is more exposed to the solvent. Since

the difference in free energy of protein folding in presence of an osmolyte ($\Delta G_3$) is substantially larger than the difference in free energy for protein folding in aqueous buffer ($\Delta G_1$), the protein prefers its native folded conformation when an osmolyte is in solution. So, favorable thermodynamics is the reason why proteins revert to their folded state when an osmolyte is present.

**Fig. 1.** Osmolyte Stabilization of Proteins (adapted from reference (7))

\[ N_{\text{buffer}} = \text{native folded protein in buffer}; \ U_{\text{buffer}} = \text{unfolded protein in buffer}; \ \Delta G_1 \]
\[ \Delta G_2 = \text{Gibbs energy difference between native and unfolded protein in aqueous buffer}; \]
\[ \Delta G_3 = \text{Gibbs energy of unfolded protein in buffer}; \ N_{\text{osmolyte}} = \text{native folded protein in osmolyte solution}; \ U_{\text{osmolyte}} = \text{unfolded protein in osmolyte solution}; \]
\[ \Delta G_3 = \text{Gibbs energy difference for protein folding in osmolyte presence}; \ \Delta G_4 = \text{Gibbs energy for native folded protein in osmolyte solution}. \]

The protein is stabilized when the osmolyte cannot interact with the peptide backbone in the core of folded proteins. Osmolyte stabilization is significant and cumulative, since peptide bonds comprise the largest structural component of proteins.

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Accordingly, the peptide backbone contributes the most to the difference in free energy between the native and denatured protein conformations.

1.2. Catalysis, Kinetics, and Structure of the Inositol Monophosphatase Family

The inositol monophosphatases\(^8\) (I-1-Pases) are a family of phosphatases dependent on the cation Mg\(^{2+}\), and inhibited by Li\(^+\). They dephosphorylate the substrate inositol phosphate to produce myo-inositol and in eukaryotes play significant roles in phosphoinositide signaling pathways\(^9\). I-1-Pases are essential for generating free inositol in cells. However, inositol serves different purposes across the archaeal, bacterial, and eukaryotic kingdoms. In archaea, inositol is used to generate inositol 1-phosphate, which is used to synthesize the head group of different phospholipids. In these organisms, it can also be incorporated into thermoprotectants\(^9\). Inositol is rarely used by bacteria, but in actinobacteria, it can be a precursor in the synthesis of a protective cytosol thiol reductant\(^9\). Like archaea, eukaryotes also utilize inositol to produce components of their phosphoinositide membrane bilayers. Otherwise, in eukaryotes, inositol may be used as compatible solutes and intermediates in a number of different cellular processes\(^9\).

Inositol and its accompanying derivatives are found in all eukaryotes, most archaea and very few bacteria\(^9\). Inositol can either be synthesized from D-glucose 6-phosphate in the following pathway (depicted in Fig. 2), or organisms will import it from environmental sources using inositol transporters.

Organisms as diverse as eubacteria, eukaryotes and archaea have the same conserved biosynthetic pathway for the production of myo-inositol. First, D-glucose 6-}

\(^8\) "The National Center for Biotechnology Information", 2011.
phosphate (D-G6-P) is cyclized to L-inositol 1-phosphate (L-I-1-P), a transformation catalyzed by the enzyme myo-inositol-1-phosphate synthase (or Ino1 in yeast). Inositol monophosphatase (I-1-Pase) then dephosphorylates L-I-1-P to produce myo-inositol.

**Fig. 2. Synthesis of myo-inositol**

Across the bacterial, archaeal, and eukaryotic kingdoms, almost all inositol monophosphatases are symmetric homodimers. Inositol monophosphatases have been identified in thermophilic eubacteria, archaea like the *Archaeoglobus fulgidus* and *Methanocaldococcus jannaschi*, and humans, rats, and yeast, among other organisms. The inositol monophosphatase from a thermophilic eubacterium, *Thermotoga maritima*, is a tetramer, and is an exception to the general structural rule\(^\text{10}\). Otherwise, *T. maritima* has a similar domain and global secondary structure as the rest of the I-1-Pase family. I-1-Pases across the three kingdoms have a common 2-domain dimeric structure, and each domain subunit features an alternating \(\alpha\beta\alpha\beta\) layering pattern of \(\alpha\) helices and \(\beta\) sheets\(^\text{11}\). The loops that connect the \(\alpha\) helix and \(\beta\) sheet layers vary in length, depending on the I-1-Pase. Loop length is one of the major differentiating factors in the protein structure of the inositol monophosphatase family.


Another important variation among the I-1-Pases is the length of the amino acid peptide chain. Generally, each subunit is 29 kDa, but the number of residues within the peptide chain varies – from 252 residues for the I-1-Pase from \textit{M. jannaschi} and \textit{A. fulgidus}, and 256 residues for the \textit{T. maritima} I-1-Pase, to 400 residues for the inositol polyphosphate-1-phosphatase\textsuperscript{12}, an enzyme that prefers to act on multiply phosphorylated inositol compounds.

In terms of catalysis, I-1-Pases can be divided into two major classes – enzymes (i) sensitive or (ii) insensitive to Li\textsuperscript{+} inhibition. This sensitivity is related to the conformation and usage of the mobile loop that provides access to the catalytic site in each monomer of the homodimer. All I-1-Pases require Mg\textsuperscript{2+} or Mn\textsuperscript{2+} ions for activity. Two or three Mg\textsuperscript{2+} or Mn\textsuperscript{2+} ions and an activated water molecule that acts as the nucleophile are necessary for catalysis. In archaeal I-1-Pase catalysis, three Mg\textsuperscript{2+} or Mn\textsuperscript{2+} cations are necessary\textsuperscript{12}; the same number are also likely needed for bacterial and eukaryotic I-1-Pases as well.

The kinetics of these enzymes can be compared using the typical parameters for activity: maximum velocity (V\textsubscript{max}), the first order rate constant k\textsubscript{cat} = V\textsubscript{max}/[E], the Michaelis-Menten constant (K\textsubscript{m}), and the catalytic efficiency (k\textsubscript{cat}/K\textsubscript{m}). Data on the kinetics of several representative archaeal, bacterial, and eukaryotic inositol monophosphatases is presented in Table 2.

The activity assays to determine the kinetic parameters of the I-1-Pases in Table 2 were conducted at each organism’s optimal growth temperature: 37°C for bovine, recombinant human brain, and recombinant \textit{E. coli}; 85°C for \textit{A. fulgidus} and \textit{M.}\textsuperscript{12}  

and 95°C for the recombinant *T. maritima* I-1-Pase. In comparison with the other inositol monophosphatases, the enzyme from the eubacterium *T. maritima* has far higher catalytic efficiency and activity, since its $k_{cat}/K_m$ is $1.4 \times 10^6 \text{M}^{-1}\text{s}^{-1}$, at least ten times higher than $2.3 \times 10^5 \text{M}^{-1}\text{s}^{-1}$, the value for next most efficient I-1-Pase, the recombinant human enzyme. Aside from these two I-1-Pases, the archaeal, bacterial, and eukaryotic I-1-Pases are similar in catalytic efficiency, with a $k_{cat}/K_m$ of $1.4 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ for *A. fulgidus*, $4.9 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ for *M. jannaschi*, $4.0 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ for the bovine, and $9.0 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ for the *E. coli* I-1-Pase. The extremely high catalytic efficiency for *T. maritima*’s I-1-Pase could be related to its unique tetrameric structure, but the exact mechanism for its high activity is unknown.

**Table 2.** Kinetics of diverse inositol monophosphatases.

<table>
<thead>
<tr>
<th>I-1-Pase source</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (μmol min$^{-1}$mg$^{-1}$)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. fulgidus</em> (with Mg$^{2+}$)</td>
<td>0.11 ± 0.02</td>
<td>3.27 ± 0.67</td>
<td>$1.4 \times 10^4$</td>
<td>80</td>
</tr>
<tr>
<td>recombinant <em>T. maritima</em></td>
<td>0.148 ± 0.010</td>
<td>443 ± 10</td>
<td>$1.4 \times 10^6$</td>
<td>95</td>
</tr>
<tr>
<td>recombinant <em>M. jannaschi</em></td>
<td>0.091 ± 0.016</td>
<td>9.3 ± 0.45</td>
<td>$4.9 \times 10^4$</td>
<td>85</td>
</tr>
<tr>
<td>bovine</td>
<td>0.16 ± 0.02</td>
<td>13.3 ± 0.9</td>
<td>$4.0 \times 10^4$</td>
<td>37</td>
</tr>
<tr>
<td>recombinant human (brain)</td>
<td>0.075 ± 0.003</td>
<td>36.1 ± 1.0</td>
<td>$2.3 \times 10^5$</td>
<td>37</td>
</tr>
<tr>
<td>recombinant <em>E. coli</em></td>
<td>0.071 ± 0.008</td>
<td>13.3 ± 0.9</td>
<td>$9.0 \times 10^4$</td>
<td>37</td>
</tr>
</tbody>
</table>

As for the I-1-Pase maximum velocity, again, the *T. maritima* I-1-Pase is the fastest, at $443\pm10$ μmol min$^{-1}$mg$^{-1}$. The next highest $V_{max}$ is $36.1\pm1$, belonging to the recombinant human brain inositol monophosphatase. The maximum velocities of the rest of the I-1-Pases are quite similar. The inositol monophosphatase of *A. fulgidus* has a
$V_{\text{max}}$ of 3.27±0.67 μmol min$^{-1}$mg$^{-1}$, while the enzyme from *M. jannaschi* has a maximum velocity of 9.3±0.45 μmol min$^{-1}$mg$^{-1}$, the bovine enzyme has $V_{\text{max}} = 13.3±0.9$ μmol min$^{-1}$mg$^{-1}$, and recombinant human I-1-Pase’s maximum velocity is 13.3±0.9 μmol min$^{-1}$mg$^{-1}$.

In these representative inositol monophosphatases from bovine, recombinant human, *E. coli* (suhB), and *M. jannaschi*, there is no clear preference for the dephosphorylation of the $D$-inositol 1-phosphate over $L$-inositol 1-phosphate substrate. However, in *T. maritima*, the I-1-Pase has a twenty-fold preference for the $D$-inositol monophosphate over the $L$-inositol monophosphate$^{10}$. The mechanism and reasons for the selective dephosphorylation are still unclear, although it may serve to regulate the use of this enzyme in osmolyte generation. Also, *A. fulgidus* 2372, the I-1-Pase, has a 10-20 fold high $K_m$ for $L$-I-1-P over the $D$-I-1-P. However, there are only negligible differences$^{12}$ in $k_{\text{cat}}$ between the two substrates for the *A. fulgidus* I-1-Pase.

1.2.1. Detailed Background of the Inositol Monophosphatase from *A. fulgidus*

*Archaeoglobus fulgidus* is a halophilic hyperthermophile archaeon that lives in marine hydrothermal vents. Its preferred thermal environment is 60-90°C, and its optimum growth temperature is 83°C. *A. fulgidus* is a halophile, like most marine hyperthermophiles$^{13}$. Halophiles grow optimally in media containing 1.9% NaCl, and cannot grow in media above 5.5% NaCl. Several unusual organic osmolytes have been isolated from *A. fulgidus*, accumulated in response to heat or osmotic stress. *A. fulgidus* accumulates the phosphodiester $\alpha$-diglycerol phosphate ($\alpha$-DGP) under osmotic stress. In comparison, di-*$\text{myo}$*-inositol phosphate (DIP), a canonical phosphodiester osmolyte,

---

accumulates under thermal stress, at very high temperatures. Glycerophospho-myoinositol (GPI) is produced under a combination of heat and osmotic stress.

The inositol monophosphatase of *A. fulgidus* is a dimer, as pictured below in Figure 3.

**Fig. 3.** Overall Dimer Structure of *A. fulgidus* Inositol Monophosphatase

The dimer interface of each monomer domain has both $\beta$-strands and large loops\(^\text{12}\). A large mobile loop connects the two domains, and moves like a joint. Like the rest of the inositol monophosphatase family, the *A. fulgidus* I-1-Pase domains each have a layered $\alpha$-$\beta$-$\alpha$-$\beta$ alternating pattern of $\alpha$ helices and $\beta$ sheets. One domain has a 7 stranded sheet with two $\alpha$ helices on either side. The second domain has a 5 stranded sheet with one $\alpha$ helix in the top layer.
This I-1-Pase has similar binding affinity for the substrate from 55-75°C; there is a maximum of 2.5-fold variation in $K_m$ for binding to L-inositol-1-phosphate within this temperature range. However, a sharp drop in $K_m$ exists at 85°C. A conformational change in the inositol monophosphatase may be responsible for the preferred substrate binding and subsequent hydrolysis at high temperatures. Irreversible denaturation and a resulting loss of I-1-Pase catalytic activity occur at 87°C.

Figure 4 depicts the substrate, D-inositol-1-phosphate (D-I-1-P) bound in the structure of two different inositol monophosphatases and shows the close proximity of the dimer interface to the active sites. The I-1-Pase of *M. jannaschi* is in magenta, and *A. fulgidus*’s I-1-Pase is blue. The I-1-P is intact since Ca$^{2+}$ was added instead of Mg$^{2+}$. Two Ca$^{2+}$ ions in each monomer are pictured as gray spheres. Tyr155, in the *A. fulgidus* I-1-Pase dimer interfacial loop interacts with the oxygens in D-I-1-P’s inositol ring. Ser171 also participates in a hydrogen bonding network in the enzyme active site. The *A. fulgidus* I-1-Pase dimer interface is formed by the interaction of loop strands, while the interface interactions for *M. jannaschii* I-1-Pase occur between two helices (dashed lines between the domains in Fig. 4 indicate hydrogen bonds).

In contrast, the *M. jannaschii* enzyme has helices forming the interface – fewer hydrogen bonds exist, but interactions of the side chain residues aid in dimer stabilization. In both structures, the proximity of the active site to the dimer interface suggests that the dimer structure is needed for correct orientation of active site groups. Anything that destabilizes that interface, for example, heat, is likely to inactivate the enzyme.

1.3. Overview

For my thesis, the inositol monophosphatase from *A. fulgidus* was overexpressed in BL21(DE3)pLysS grown in LB broth media and then purified. This I-1-Pase was used in crystallization trials with amino acid solutes like aspartate (Asp), potassium glutamate (K-Glu), glutamine (Gln), and proline (Pro) to produce crystals suitable for X-ray diffraction and structural studies of the protein in the presence of osmolytes. These
crystal structures would then allow visualization of specific solute binding to I-1-Pase and elucidation of the mechanism of inhibition. One of *A. fulgidus*’s natural osmolytes, α-diglycerol phosphate (α-DGP), was also synthesized to test its thermoprotective properties with I-1-Pase and for eventual crystallization trials. The availability of the enzyme and the new solute will allow future studies of α-DGP effects of enzyme kinetics, and effect of this solute on protein thermostability. If crystals can be obtained, it will be interesting to see where this solute binds.
Chapter 2: Materials and Methods

2.1. Protein Expression and Purification

2.1.1. Overexpression and purification of *A. fulgidus* inositol monophosphatase

A recombinant plasmid prepared previously in the Roberts laboratory was transformed into BL21(DE3)pLysS competent cells for expression of the I-1-Pase. 2 µl of I-1-Pase recombinant plasmid was mixed with 150 µl BL21 competent cells and placed on ice for 30 minutes. The cells were transformed by heating at 42°C for 20 seconds, then storing the cells on ice for 2 minutes.

LB media broth (500 µl) was added to the tube, and the mixture incubated while stirring for 1 hour, at 37°C. The contents of the tube (300 µl) were spread on an AMP⁺ LB agar plate and incubated for approximately 17 hours at 37°C. This plate was stored at 4°C. Two single colonies of BL21(DE3)pLysS containing the I-1-Pase gene were each grown in a test tube containing 5 ml LB media, 5 µl ampicillin and 5 µl chloroamphenicol, and stirred at 37°C for 19 hours.

Next, the two 5 ml cultures of BL21(DE3)pLysS were each added to separate flasks containing 2L of LB broth, along with 2 ml ampicillin and 2 ml chloroamphenicol, and stirred at 200 rpm at 37°C for approximately 5 hours, until the OD₆₀₀ ~ 0.7-0.8. Overexpression of the recombinant protein in the culture was induced by adding IPTG (to a final concentration of 0.4 mM), and the flasks were incubated at 37°C, while stirring at 200 rpm for 3 hours. The cells were harvested by centrifuging the cultures at 5000 rpm, 4°C for 10 minutes. The cell pellets were stored at -20°C.

To break open the cells, the frozen cell pellets from one 2L LB media culture were thawed on ice, and suspended in 60 ml Buffer A (50 mM Tris-HCl, 1 mM EDTA,
all at pH 8.0). The frozen cell pellets were then lysed by sonicating the suspensions for 30 seconds each, 10 times, on ice. The cells were centrifuged for 30 minutes at 15000 rpm at 4°C to isolate the supernatant, which contains the I-1-Pase, from cell debris. The supernatant was heated to 85°C for 30 minutes to isolate the more heat-stable *A. fulgidus* inositol monophosphatase from *E. coli* host proteins. Centrifugation at 15000 rpm, 4°C, separated the soluble I-1-Pase (in the supernatant) from the denatured proteins in the precipitate. The supernatant was dialyzed overnight against a 4L bath of Buffer A.

The dialyzed supernatant (60 ml) was purified on a 2.5 x 12 cm Q-Sepharose Fast Flow (QFF) column. A linear gradient of 0-0.5M KCl in Buffer A (400 ml total) was used to elute the protein from the column. SDS-PAGE was used to monitor the purity of the protein fractions. The pure protein fractions were loaded onto a Vivaspin 20 filter column (MW 10000) and centrifuged to concentrate the I-1-Pase to about 4 mg/ml. Finally, the pure protein was stored at 4°C.

2.2. Assay of Protein Concentration

2.2.1. Pierce BCA Protein Assay

The Pierce BCA (bicinchoninic acid) Protein Assay protocol monitors the presence of a BCA/copper complex whose color is strongly affected by peptide residues. Cu$^{2+}$ is reduced to Cu$^{+}$ by protein in basic media, which is monitored by colorimetric assay of the Cu$^{+}$ cation via BCA. In the biuret reaction that occurs in basic media, copper chelates with the peptide (that has 3 or more amino acid residues), forming a light-blue chelate complex. In the next step, BCA reacts with the newly formed Cu$^{+}$ cation to produce a deep purple colored complex of two BCA molecules attached to one Cu$^{+}$ ion.
The purple BCA-Cu$^+$ complex is water soluble and displays a linear absorbance at 562 nm with increasing protein concentration (up to 1 mg/ml).

A standard curve of bovine serum albumin (BSA) at varying concentrations was prepared (Figure 5), and this chart of absorbances at 562 nm and protein concentrations was used to estimate the concentration of the I-1-Pase. At high protein concentrations (above 1 mg/ml), the curve deviates from linearity.

**Fig. 5. Standard Curve of Bovine Serum Albumin**

The absorbance of the I-1-Pase stock sample at 562 nm was measured at 10%, 25% and 50% dilutions to yield an estimated concentration of 2.87 mg/ml.

**2.3. Crystallization of A. fulgidus I-1-Pase**

Previously, crystallization trials$^{12}$ of the *A. fulgidus* I-1-Pase led to optimized conditions that gave crystals with good X-ray crystal diffraction. The crystals were
produced by vapor diffusion with 5 µl hanging drops. Optimized crystallization conditions for these previous trials\textsuperscript{12} were: 0.2M NH_{4}NO_{3} or 0.2M (NH_{4})_{3}PO_{4} and 30% PEG 3350. From this lead, variations were attempted in order to produce crystals for solving the structure of I-1-Pase with different solutes bound. Conditions of the optimized crystallization trials are detailed in Table 3. A wide range of glutamate concentrations was used, but for crystals with the other solutes only one or two concentrations were tested. All crystallization trays included the K_{2}SO_{4}, MgCl_{2} and PEG 3350 but with the addition of one of the solutes.

Table 3. Crystallization trials of \textit{A. fulgidus} I-1-Pase with Different Solutes

<table>
<thead>
<tr>
<th>Date of trial</th>
<th>K-Glu (mM)</th>
<th>Pro</th>
<th>Asp</th>
<th>Asn</th>
<th>Gln</th>
<th>K_{2}SO_{4} (M)</th>
<th>MgCl_{2}</th>
<th>% PEG 3350</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/19/2011</td>
<td>50-200</td>
<td>400</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>0.30</td>
<td>6</td>
<td>26-29</td>
</tr>
<tr>
<td>1/21/2011</td>
<td>50-200</td>
<td>400</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>0.30</td>
<td>6</td>
<td>26-29</td>
</tr>
<tr>
<td>1/21/2011</td>
<td>50-200</td>
<td>400</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>0.30</td>
<td>6</td>
<td>26-29</td>
</tr>
<tr>
<td>1/21/2011</td>
<td>50-200</td>
<td>400</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>0.30</td>
<td>5</td>
<td>21-24</td>
</tr>
<tr>
<td>1/24/2011</td>
<td>60-200</td>
<td>450</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>0.30</td>
<td>6</td>
<td>15-19</td>
</tr>
<tr>
<td>1/24/2011</td>
<td>60-200</td>
<td>450</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>0.30</td>
<td>6</td>
<td>25-30</td>
</tr>
<tr>
<td>1/31/2011</td>
<td>50-200</td>
<td>400</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>0.30</td>
<td>6</td>
<td>26-30</td>
</tr>
<tr>
<td>2/11/2011</td>
<td>60-200</td>
<td>450</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>0.30</td>
<td>6</td>
<td>15-19; 30-33</td>
</tr>
</tbody>
</table>

K-Glu = potassium glutamate, Pro = proline, Asn = asparagine, Gln = glutamine, PEG 3350 = polyethylene glycol (MW 3350)

2.4. \textit{General Synthesis of α-diglycerol phosphate}

\textit{α-Diglycerol phosphate is a rare organic solute that accumulates only in \textit{A. fulgidus} in response to organic stress. This osmolyte shows significant protein stabilizing}
thermoprotectant properties for a range of diverse dehydrogenases and rubredoxins\textsuperscript{15}. In this study, α-DGP was synthesized according to a previous protocol\textsuperscript{15} by Santos, since this procedure gave the fewest side products, and purification is a simple approach, completed on anion and cation exchange columns. Solketal, 1, is commercially available, meaning this synthesis could be easily reproduced on gram or kilogram scale for future thermoprotectant assays and other biological studies.

**Fig. 6. General Synthesis of α-diglycerol phosphate, 3**

![Synthesis of 2](image)

**Synthesis of 2**

To a solution of POCl\textsubscript{3} (0.66 ml) in anhydrous THF (12 ml) at 23°C, under nitrogen atmosphere was added a pre-mixed solution of solketal 1 (1.80 ml) in Et\textsubscript{3}N (12 ml), dropwise over ten minutes. A cloudy precipitate formed immediately upon addition. The reaction was then allowed to stir at 23°C for 22 hours, under nitrogen atmosphere.

Next, H\textsubscript{2}O (12 ml) was added to the reaction mixture, dropwise, and the reaction was allowed to stir at room temperature for four hours, under ambient atmosphere. The reaction mixture was extracted with EtOAc (2 x 20 ml), then the aqueous phase was extracted with CH\textsubscript{2}Cl\textsubscript{2} (2 x 20 ml). The organic layers were then dried over MgSO\textsubscript{4}, filtered, and concentrated by rotary evaporation to yield 2, a yellow-brown oil (6.68 g).

**Synthesis of 3**

To the crude product 2 in a round bottom flask was added THF (90 ml), followed by H₂O (54 ml). Concentrated HCl (30 drops) was then added to the solution, and the reaction was allowed to stir at 23°C for 21.5 hours, under ambient atmosphere. The reaction mixture was extracted with EtOAc, and the organic layers were washed with brine, dried over MgSO₄, filtered, and concentrated by rotary evaporation for analysis of components. The aqueous layers were concentrated to give desired compound 3, a powdery white solid (43.2 g, crude).

The synthesis of α-DGP was also examined using different ratios of solketal to POCl₃ to try and optimize diester formation. The ratio of solketal to POCl₃ was increased from 2:1 in the initial synthesis to 4:1 and then to 8:1. The rationale was that we might be forming significant amounts of the α-monoglycerol phosphate with a 2:1 ratio. Based on the mass spectra data, it was concluded that the original 2:1 ratio produced the highest percentage of desired α-DGP.

**2.4.1. General Procedure for Purification of α-DGP**

The purification of α-DGP from the 2:1 solketal to POCl₃ reaction was pursued, because its concentration of α-DGP was the highest compared to other species in the mass spectrometry analysis (see Chapter 3). The general purification procedure used is a modification of the Santos purification. First, ~30 mg AG1-8X anion exchange resin were added to ~20 mg of 3 dissolved in distilled H₂O. This resin should remove any inorganic phosphate in the aqueous fraction. The mixture was shaken in a vial to mix the
components, and then centrifuged at 13,000 rpm for 5 minutes to separate the solution from the resin. Next, the solution was rotovaped to give a crystalline white powder.

Following the first AG1-8X purification, 3 was further purified with AG 50W-8X cation exchange resin, to remove any triethylamine in the mixture. ~50 mg of AG50-8X cation exchange resin were added to 3 (already purified by AG1-8X resin) dissolved in distilled H₂O. The mixture was again shaken in a vial to mix the components, and then centrifuged at 13000 rpm for 5 minutes to separate the solution from the resin. Finally, the solution was rotovaped to yield a crystalline white powder, purified 3.
Chapter 3: Compatible Solute Binding to the *Archaeoglobus fulgidus* I-1-Pase

Research into the thermoprotective properties of the osmolyte α-diglycerol phosphate is part of the general question – do compatible solutes selectively bind to proteins? Organic osmolytes generally act through the osmophobic effect, by forcing proteins into their native folded conformations through thermodynamically unfavorable osmolyte-peptide backbone interactions. The Gibbs free energy of osmolyte-peptide backbone contact of unfolded protein in comparison to osmolyte-peptide interactions of folded protein is higher, pushing proteins toward their native folded form. The osmophobic effect is a solvophobic effect, analogous to the hydrophobic effect, the thermodynamically disfavored contact between water and nonpolar side chains. So, testing α-DGP as a thermoprotectant of *Archaeoglobus fulgidus* inositol monophosphate asks whether the enzyme specifically binds certain osmolytes, contrary to the usual protective mechanisms of osmolytes.

3.1. Does α-DGP protect the A. fulgidus I-1-Pase from heat denaturation?

The inositol monophosphatase enzyme of *Archaeoglobus fulgidus* was selected because as a halophilic hyperthermophile, *A. fulgidus* synthesizes unusual organic solutes (osmolytes) like α-diglycerol phosphate, di-myoinositol 1,1-phosphate (DIP) and glycerol-phospho-myoinositol in response to osmotic and heat stress. In previous studies by Santos and coworkers, these solutes have demonstrated considerable protein stabilization in other enzymes *in vitro*. The inositol monophosphatase of *A. fulgidus* 

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denatures irreversibly at 87°C, under thermal conditions only slightly higher than its optimal growth temperature of approximately 83-85°C. That makes it an interesting candidate for thermoprotection studies and how different solutes affect this enzyme.

Previously, the protein stabilization properties of other inorganic and organic compounds were tested with inositol monophosphatase. Inorganic ions like K⁺ are potentially important in preventing thermal denaturation above the I-1-Pase regular normal growth temperature. The organic osmolyte di-myoinositol phosphate (DIP) is strongly correlated with the cell response to high thermal stress. This compound was shown to indeed have a strong thermoprotective effect on the *A. fulgidus* I-1-Pase. The K⁺-salt of L-α-glutamate also reportedly improves inositol monophosphatase stability at high L-α-glutamate concentrations.

Santos and coworkers previously tested α-DGP for its protein stabilizing properties against an array of diverse dehydrogenases and rubredoxins. α-DGP had a strong thermally protective stabilizing effect against the heat inactivation of rabbit muscle lactate dehydrogenase, baker’s yeast alcohol dehydrogenase, and *Thermococcus litoralis* glutamate dehydrogenase. Santos and coworkers also discovered that α-diglycerol phosphate demonstrated significant thermoprotection of the *Desulfovibrio gigas* and *Clostridium pasteurianum* rubredoxins. On the other hand, α-DGP displayed no thermoprotective effect for the *Desulfovibrio desulfuricans* (ATCC 27774) rubredoxin.

α-DGP is only found in *A. fulgidus*. The primary cause of α-DGP accumulation is osmotic salt stress, and it is the major osmolyte in *A. fulgidus* at 76°C. At the I-1-Pase
denaturation temperature, 87°C, the concentrations of anionic solutes α-DGP and DIP are very similar.

For DIP, the inositol ring mimics the structure of the inositol monophosphatase substrate, inositol-1-phosphate. For structural and binding reasons, it possible that DIP improves inositol monophosphatase thermal stability by binding to the active site. In contrast, two free glycerols are esterified to the central phosphate group in α-DGP. The freedom of movement of the two glycerol groups may contribute to a different mode of binding to the I-1-Pase, compared to the constrained flexibility of the cyclic inositol rings attached to the central phosphate of DIP. With purified α-DGP in hand, we can compare its thermoprotective properties with other organic solutes, since different organic osmolytes have widely varying thermoprotective abilities\textsuperscript{19}. α-DGP may stabilize the I-1-Pase in an analogous fashion to DIP. Its synthesis is a relatively straightforward two-step procedure, whereas generating pure DIP in amounts large enough to have 0.1-0.5 M mixed with protein is exceptionally difficult.

The α-diglycerol phosphate (α-DGP) was synthesized based on a procedure\textsuperscript{15} by Santos and coworkers, with a 2:1 ratio of solketal to POCl\textsubscript{3} used in the first esterification step. 4:1 and 8:1 ratios of solketal to POCl\textsubscript{3} were also attempted, but analysis by mass spectrometry showed that the original 2:1 ratio gave the fewest side products, and highest percent of desired α-DGP. Taking the α-DGP forward in a purification procedure\textsuperscript{15} using an AG1-8X anion exchange resin, followed by purification on an AG50W-8X cation exchange resin gave the desired phosphodiester product.

The spectral characterization ($^1$H and $^{31}$P NMR) of the $\alpha$-DGP 3 is provided below. $^1$H NMR, $^{31}$P NMR, and mass spectrometry characterization of the crude $\alpha$-DGP products of the three synthetic trials (2:1, 4:1, and 8:1 ratios of solketal: POCl$_3$; abbreviated as 2:1, 4:1 and 8:1 for the remainder of this work) are also listed in the following section. Mass spectrometry confirms the synthesis of the crude $\alpha$-DGP and its Na$^+$-salt in all three conditions. $\alpha$-DGP produced by both 2:1 and 8:1 ratios of solketal to POCl$_3$ gave the highest percentage of desired $\alpha$-DGP product. The MS spectrum of the $\alpha$-DGP from the 2:1 mixture also revealed some methoxy-glycerol-phosphate as the major side product, the likely result of esterification of methanol during the transfer of protected product 2 between vials using methanol, before water was added to produce compound 3.

3.1.1. Spectral Characterization by $^1$H NMR, $^{31}$P NMR, and Mass Spectrometry

Mass Spectrometry

A Micromass LCT TOF (ES+) instrument was used for these analyses, run by Marek Domin (Boston College). 1 mg of each sample was dissolved in H$_2$O. The 4:1 and 8:1 samples of 3 also contained PBS (phosphate buffered saline) solution. The identification of the major peaks in the 2:1, 4:1 and 8:1 samples of 3 is listed in Table 4 below. Individual mass spectra are also attached on the following pages.
Table 4. Identification of Major Mass Peaks in 2:1, 4:1 and 8:1 samples of 3.

<table>
<thead>
<tr>
<th>Sample of 3</th>
<th>Major peaks</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:1</td>
<td>246/268 (α-DGP/Na⁺ salt), 183, 187 (methoxy-glycerol-phosphate)</td>
</tr>
<tr>
<td>4:1</td>
<td>239, 247/269 (α-DGP/Na⁺ salt), 205, 173/195 (mono-glycerol phosphate/ Na⁺ salt), 319 (tri-glycerol phosphate)</td>
</tr>
<tr>
<td>8:1</td>
<td>239, 247/269 (α-DGP/Na⁺ salt), 205, 173 (mono-glycerol phosphate),</td>
</tr>
</tbody>
</table>
Individual mass spectra are attached below.

HR-MS (ES+) calcd. for $\text{C}_{16}\text{H}_{15}\text{O}_8\text{P} \ [\text{M}+\text{H}]^+$: 246.2, found: 246.0 and $[\text{M}+\text{Na}^+]$: 268.0
HR-MS (ES+) calcd. for C_{16}H_{15}O_{8}P \ [M+H]^+: 246.2, found: 247.0 and [M+Na^+] : 269.0
HR-MS (ES+) calcd. for C₁₆H₁₅O₈P [M+H]+: 246.2, found: 247.0 and [M+Na⁺]: 269.0

NMR: ¹H and ³¹P

¹H and ³¹P NMR of 2:1, 4:1 and 8:1 compounds were obtained on a Varian Direct Drive NMR system (500 MHz). The samples were all prepared in D₂O and run at ambient temperature.

¹H NMR

2:1, after ion exchange treatment: ¹H NMR (D₂O, 500 MHz, nt=__, pH 4.3) δ3.60 (quint, 4H), δ3.72 (m, 6H), δ3.83 (m, 2.6H), δ3.98 (m, 2.4H), δ4.08 (m, 1.3H).

¹H NMR spectra attached on following pages.
2:1, crude
2:1, after ion exchange treatment
4:1, crude
8:1, crude
$^{31}$P NMR Spectra

$^{31}$P NMR spectra were taken to verify the presence of phosphodiester compound ($\alpha$-DGP) in the product mixtures.

**Table 5. Major Peaks in $^{31}$P NMR of 2:1, 4:1, and 8:1 Compounds**

<table>
<thead>
<tr>
<th>Solketal/POCl$_3$</th>
<th>Compound</th>
<th>Peak (ppm)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:1</td>
<td>$\alpha$-diglycerol phosphate</td>
<td>0.14</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>methoxy-glycerol phosphate</td>
<td>-0.47</td>
<td></td>
</tr>
<tr>
<td>4:1</td>
<td>$\alpha$-diglycerol phosphate</td>
<td>~0.10</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>$\alpha$-monoglycerol phosphate</td>
<td>~0.72</td>
<td></td>
</tr>
<tr>
<td>8:1</td>
<td>$\alpha$-diglycerol phosphate</td>
<td>~0.10</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>$\alpha$-monoglycerol phosphate</td>
<td>~0.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\alpha$-triglycerol phosphate</td>
<td>~0.50</td>
<td></td>
</tr>
</tbody>
</table>

Based on the NMR and mass spectrometry data for the crude and purified samples of 3 across the 2:1, 4:1 and 8:1 starting solketal/POCl$_3$ ratios, the original 2:1 ratio is the best synthetic procedure for obtaining $\alpha$-DGP. Using an AG1-8X resin, followed by AG50W-8X resin purification was a good way to remove the inorganic phosphate and most of the residual triethylamine from the reaction mixture, based on the $^1$H and $^{31}$P NMR spectra for the purified 2:1 sample. The actual $^{31}$P spectra are attached on the following pages.
2:1, after ion exchange treatment

*α-DGP = α-diglycerol phosphate, α-MGP = α-monoglycerol phosphate, α-TGP = α-triglycerol phosphate; MeOGP = methoxy glycerol phosphate
4:1, crude

Sample Name: 1J033-bet3-01p-8.1.11
 Appears directory: sample directory.
File Name: 1J033-bet3-01p-8.1.11
Pulse Sequence: Phosphorus (31p)
Solvent: D2O
Data collected on: Aug 1 2011
8:1, crude

Sample Name: J5EII-phi-lip-8.1.1.11
Archive directory:
Sample directory:
FileBase: J5EII-phi-lip-8.1.1.11
Pulse sequence: PHOSPHORUS (31P)
Solvent: D2O
Data collected on: Aug 1 2011

α-DGP

α-MGP

α-TGP
3.2 Observations of Osmolytes in Crystal Structures of the A. fulgidus I-1-Pase

If osmolytes selectively protect the A. fulgidus I-1-Pase from heat denaturation, specific solute-enzyme binding interactions are a possibility. Checking whether bound solutes are visible within the crystal structures of the I-1-Pase is one way to test this hypothesis. I tested crystallization conditions of the I-1-Pase with amino acid solutes at high and low concentrations. Table 4 (Chapter 2) outlines the diverse conditions of the crystallization trials. The results of the crystallization trials, including the shapes of crystals observed, are listed in Table 5.

Table 5. Crystallization Trials for A. fulgidus I-1-Pase with Diverse Solutes.

<table>
<thead>
<tr>
<th>record</th>
<th>K-Glu</th>
<th>Pro</th>
<th>Asp</th>
<th>Asn</th>
<th>Gln</th>
<th>K$_2$SO$_4$ (M)</th>
<th>MgCl$_2$</th>
<th>% PEG 3350</th>
<th>set-up</th>
<th>shape</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>0.30</td>
<td>6</td>
<td>26, 28</td>
<td>1/21/11</td>
<td>rectangle, hexagon</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.30</td>
<td>6</td>
<td>26</td>
<td>1/21/11</td>
<td>cube</td>
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<tr>
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<td></td>
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<td></td>
<td></td>
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<td>6</td>
<td>29</td>
<td>1/21/11</td>
<td>hexagon</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.30</td>
<td>6</td>
<td>26</td>
<td>1/21/11</td>
<td>rectangle, hexagon</td>
</tr>
<tr>
<td>1/24/11</td>
<td>100</td>
<td>400</td>
<td></td>
<td></td>
<td></td>
<td>0.30</td>
<td>6</td>
<td>28, 29</td>
<td>1/21/11</td>
<td>starbursts, rectangles</td>
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<tr>
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<td></td>
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<td>5</td>
<td>21, 23</td>
<td>1/21/11</td>
<td>rectangles</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.30</td>
<td>5</td>
<td>21</td>
<td>1/21/11</td>
<td>rectangles</td>
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<tr>
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<tr>
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<td>6</td>
<td>15, 30</td>
<td>1/19/11</td>
<td>cubes, hexagon</td>
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<td>6</td>
<td>25</td>
<td>1/19/11</td>
<td>hexagon</td>
</tr>
</tbody>
</table>
From these crystallization trials, two crystals of the *A. fulgidus* I-1-Pase of reasonable quality were produced. The crystallization trials of I-1-Pase with 50 mM K-Glu on 1/31/11 and the set-up of I-1-Pase with 25 mM Asp on 2/11/11 both produced crystals that diffracted\(^{20}\) to 2.80 Å. They were sent to the Argonne Synchrotron for data collection.

Following data processing\(^{21}\) by R. Goldstein, it was determined that the $R^2$ factor of all reflections in the crystal from the I-1-Pase crystallization with 50 mM K-Glu on 1/31/11 was 0.218, meaning the crystal quality was too poor for structure refinement. The other crystal with 25 mM Asp had an $R^2$ factor of 0.126, so Goldstein was able to produce a structure of the I-1-Pase with Asp (25 mM) bound. The structure is depicted in Figure 7.

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**Fig. 7.** Structure of I-1-Pase with 25 mM Asp

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\(^{20}\)X-Ray Diffractometer from Boston College: Rigaku MicroMax-07 HF high intensity microfocus rotating Cu anode X-ray generator.

\(^{21}\)Becca Goldstein, Roberts laboratory, unpublished work.
Four Asp residues are bound (indicated by arrows in Figure 7). Two Asp are bound in the active site of each monomer. It is likely that Asp protects the I-1-Pase from thermal denaturation, since this osmolyte sits in the active site of the enzyme. Compared with the amino acid glutamate, aspartate is a methylene unit smaller, so it is too small to bridge the dimer interface between the two monomers of I-1-Pase. Instead, a thermoprotective mechanism may be possible through activity at the enzyme’s active site instead.
Chapter 4: Future Directions

To elucidate the mechanism of α-diglycerol phosphate binding to the inositol monophosphatase from A. fulgidus, structures of this osmolyte added to the crystallization medium need to be obtained. A crystal structure of the I-1-Pase with the osmolyte α-DGP bound would greatly clarify the contacts and specific enzyme-solute interactions. R. Goldstein, in the Roberts laboratory\textsuperscript{21}, has obtained crystals of the I-1-Pase with 400 and 25 mM K\textsuperscript{+}-glutamate in the crystallization media. She found that in the dimer, 4 glutamate molecules are visible in the high glutamate structure and aligned along the dimer interface. Two other glutamates are both bound close to the active site – these are still seen in the low glutamate structure. Since there is no significant thermoprotection of activity at the low glutamate conditions, those glutamates near the active site are not likely to stabilize the protein toward thermal denaturation. However, the molecules lining the dimer interface could contribute to thermoprotection by enforcing a dimer structure.

Aspartate, although only a methylene unit shorter, is too small to bridge the interface. It is predicted that the crystals with aspartate will have molecules bound in the active site region, but not the dimer interface. Currently, R. Goldstein\textsuperscript{21} is refining the structure of a low aspartate crystal. Whether an osmolyte like α-DGP can bind to this inositol monophosphatase at sites analogous to the observed glutamate binding is a question we hope to address.

Another interesting question is whether the osmolyte binding observed in the crystal structures also exists in the high temperature enzyme assays. Other biophysical methods may work, although crystallography cannot address this problem. For example,
high resolution field cycling looking at \([1^{-13}\text{C}]\)glutamate (or labeled aspartate) with spin-labeled I-1-Pase may show a low field dispersions indicative of a discrete complex with a lifetime\(^{22, 23, 24}\) of >0.1 \(\mu\)s. This technique can be tested at temperatures up to 60\(^\circ\)C, and that may be sufficient to see if binding sites are lost at higher temperatures. If discrete binding still occurs, then this will be a novel mechanism for how osmolytes can stabilize proteins to heat denaturation.

\(^{31}\text{P}\) NMR, employing the same methodology, could be used to monitor the \(\alpha\)-DGP binding sites. For this naturally occurring osmolyte, kinetic tests are also needed, to show how \(\alpha\)-DGP affects the loss of activity at 90\(^\circ\)C. It is assumed that this osmolyte will stabilize the protein, but the enzyme inactivation in the absence and presence of this solute must be actively monitored to confirm this prediction. So far, I have supplied the compound for these studies, and future kinetic tests will be performed with the \(\alpha\)-DGP to decipher its thermoprotective activity.


References


