MOLECULAR STUDIES TO INVESTIGATE 4-HYDROXY-2-OXOGLUTARATE METABOLISM DEFECTS OF PRIMARY HYPEROXALURIA TYPE 3

BY

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## Contents

List of Abbreviations ........................................................................................................ v
Abstract ................................................................................................................................. vii

Chapter 1  Introduction ........................................................................................................ 1
  1.1  Primary Hyperoxaluria ............................................................................................... 1
  1.2  Hydroxyproline Metabolism .................................................................................... 1
  1.3  Types and Treatments of Primary Hyperoxaluria ....................................................... 3
  1.4  Statement of Purpose ................................................................................................. 5

Chapter 2  The crystallization of glyoxylate reductase in complex with an inhibitor, 4-hydroxy-2-oxoglutarate ........................................................................................................ 7
  2.1  Introduction ............................................................................................................... 7
  2.2  Methods ..................................................................................................................... 8
    2.2.1  Expression of 36/38R GR ................................................................................... 8
    2.2.2  Purification of 36/38R GR ................................................................................ 8
    2.2.3  Crystallization Screening and Optimization ....................................................... 9
  2.3  Results ....................................................................................................................... 10
  2.4  Discussion ................................................................................................................ 13

Chapter 3  The restoration of 4-hydroxy-2-oxoglutarate aldolase activity by chemical chaperones ....................................................................................................................... 17
  3.1  Introduction ............................................................................................................... 17
  3.2  Methods ..................................................................................................................... 20
    3.2.1  Expression of Mutant HOGA ......................................................................... 20
    3.2.3  HPLC Analysis of Lysate Samples .................................................................. 22
    3.2.4  Western Blotting of Lysate Samples ................................................................. 23
  3.3  Results ....................................................................................................................... 23
    3.3.1  Assay Development ...................................................................................... 23
    3.3.2.  Chemical Chaperone Analysis by HPLC ..................................................... 27
    3.3.3.  Chemical Chaperone Analysis by Western Blot .......................................... 29
  3.4  Discussion ................................................................................................................ 33

Conclusions ....................................................................................................................... 37
References ............................................................................................................................ 38
Curriculum Vitae ................................................................................................................. 43
List of Figures and Tables

Figure 1.1  Hydroxyproline degradation pathway ................................................................. 3

Figure 2.1  Asymmetric units of WT and 36/38R GR ............................................................. 11

Figure 2.2  Crystals resulting from conditions with 0.1 M MES pH 6.0, 1.0 M LiCl, and
             PEG 6000 ............................................................................................................. 12

Figure 2.3  Crystals resulting from conditions with 0.1 M HEPES pH 7.5 and PEG
             8000 ....................................................................................................................... 14

Figure 3.1  Representative chromatogram of sample containing 162 μM HOG, 200 μM
             glyoxylate, 250 μM pyruvate, and 278 μM 2-oxobutyrate ................................. 25

Figure 3.2  Assay development: pyruvate standard curve and time course ....................... 26

Table I      Pyruvate concentrations (μM) of lysates for each mutant by treatment .......... 28

Table II     Pyruvate concentrations (μM) generated by HOGA ........................................ 28

Table III    Mutant activity as a percentage of WT activity ............................................... 28

Figure 3.3  Western blot analysis of lysates from each chemical chaperone .................... 31

Figure 3.4  Ponceau S staining of representative blots ......................................................... 32

Figure 3.5  Location of residues whose PH3-causing mutants were studied .................... 34
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P5C</td>
<td>$\Delta^1$-pyrroline-3-OH-5-carboxylate</td>
</tr>
<tr>
<td>1P5CDH</td>
<td>$\Delta^1$-pyrroline-3-OH-5-carboxylate dehydrogenase</td>
</tr>
<tr>
<td>4-Hyp</td>
<td>4-hydroxyproline</td>
</tr>
<tr>
<td>AGT</td>
<td>alanine glyoxylate aminotransferase</td>
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<tr>
<td>AhpC</td>
<td>alkyl hydroperoxide reductase C</td>
</tr>
<tr>
<td>Asp AT</td>
<td>aspartate aminotransferase</td>
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<tr>
<td>CC</td>
<td>chemical chaperone</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
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<td>CBS</td>
<td>cystathionine β-synthase</td>
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</tr>
<tr>
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<td>glyoxylate reductase</td>
</tr>
<tr>
<td>GO</td>
<td>glyoxylate oxidase</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HOG</td>
<td>4-hydroxy-2-oxoglutarate</td>
</tr>
<tr>
<td>HOGA</td>
<td>4-hydroxy-2-oxoglutarate aldolase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HPOX</td>
<td>hydroxyproline oxidase</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-Morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide diphosphate</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PH</td>
<td>primary hyperoxaluria</td>
</tr>
<tr>
<td>PH1</td>
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</tr>
<tr>
<td>PH2</td>
<td>primary hyperoxaluria type 2</td>
</tr>
<tr>
<td>PH3</td>
<td>primary hyperoxaluria type 3</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal phosphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween</td>
</tr>
<tr>
<td>TMAO</td>
<td>trimethylamine-N-oxide</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
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Abstract

Primary hyperoxaluria (PH) is marked by recurrent calcium oxalate kidney stones that form due to the overproduction of oxalate in the liver. PH is caused by mutations affecting enzymes involved in hydroxyproline and glyoxylate metabolism. Of the three PH types, the third type, PH3, is the most recently classified and least thoroughly investigated. PH3 results when mutations prevent 4-hydroxy-2-oxoglutarate aldolase (HOGA) from cleaving 4-hydroxy-2-oxoglutarate (HOG) into pyruvate and glyoxylate. It is proposed that the consequences of PH3 could be lessened by preventing conditions that favor oxalate production downstream of deficient HOGA and by recovering activity of mutant HOGA directly by chemical chaperone treatments. Glyoxylate reductase (GR) is an enzyme with a role in glyoxylate metabolism downstream of HOGA and is inhibited by HOG. Attempts to crystallize GR in complex with HOG were insufficient to produce crystals that diffracted consistently beyond 8 Å. Additionally, a new HPLC assay was developed for monitoring HOGA activity in crude lysates. Results from this assay indicated that exposure to the chemical chaperones TMAO, glycerol, and DMSO during expression in E. coli did not increase the activity of several HOGA mutants, though treatment with 10% glycerol may have increased activity of the R70P mutant to ~80% of WT. Future studies should investigate alternative crystallization methods and additional chaperone treatments.
Chapter 1  Introduction

1.1  Primary Hyperoxaluria

Primary hyperoxaluria (PH) is an autosomal recessive disease marked by the overproduction of oxalate in the liver. First described in 1925, PH is due to metabolic defects in glyoxylate metabolism that result in an oxalate burden that cannot be excreted from the kidneys\(^1\). Accumulated oxalate crystallizes with calcium, eventually forming stones, and leads to inflammation and fibrosis that can result in end stage renal failure\(^2\). At this stage, plasma oxalate levels can rise beyond saturation\(^2\). Oxalate is deposited in other body tissues in a condition called systemic oxalosis, leading to complications that can result in death\(^2\). PH can manifest at any age; however, the median age of onset is 5.5 years, with the median age of end stage renal failure diagnosis being 24 years\(^1\). PH is caused by genetic mutations that result in dysfunctional enzymes involved in glyoxylate and hydroxyproline metabolism.

1.2  Hydroxyproline Metabolism

The aberrant metabolism of glyoxylate and 4-hydroxyproline (4-Hyp) leads to PH. Collagen turnover provides a major source of 4-Hyp, around 300-450 mg of 4-Hyp per day, and diet can also contribute to 4-Hyp levels\(^3\). 4-Hyp metabolism occurs primarily in the liver and kidney, though tissues like the small intestine and pancreas also express the necessary genes\(^4\). As indicated in Figure 1.1, 4-Hyp metabolism begins with the conversion of 4-Hyp to $\Delta^1$-pyrroline-3-OH-5-carboxylate (1P5C) by the enzyme hydroxyproline oxidase (HPOX) in the mitochondria. $\Delta^1$-pyrroline-3-OH-5-carboxylate
dehydrogenase (1P5CDH) then converts 1P5C into 4-hydroxyglutamate, which aspartate aminotransferase (Asp AT) uses to form 4-hydroxy-2-oxoglutarate (HOG). HOG is broken down by 4-hydroxy-2-oxoglutarate aldolase (HOGA) into pyruvate and glyoxylate. This glyoxylate has several potential fates. It can be converted to glycolate via glyoxylate reductase (GR) in either the mitochondria or cytoplasm. Glycolate is free to enter the peroxisome, where glycolate oxidase (GO) can convert it back to glyoxylate for use by alanine-glyoxylate aminotransferase (AGT) in the formation of glycine. Glyoxylate can also be converted to oxalate via lactate dehydrogenase (LDH). Under normal circumstances, glyoxylate metabolism poses no risk of significant oxalate production. However, in the case of PH, mutations in several enzymes of this pathway render them dysfunctional, forcing the glyoxylate to be converted to oxalate by LDH. The three classes of PH are distinguished based on which enzyme in this pathway is mutated.
1.3 Types and Treatments of Primary Hyperoxaluria

PH Type 1 involves mutations in AGT, the enzyme that catalyzes the conversion of glyoxylate to glycine in the peroxisome. In these patients, who often suffer the most severe phenotypes of any PH group, AGT is catalytically inactive or mistargeted. AGT deficiency allows the diffusion of glyoxylate from the peroxisome to the cytoplasm, where its accumulation promotes the production of oxalate by LDH.

PH Type 2 is marked by mutations in GR. Found in the mitochondria and cytosol, GR converts glyoxylate to glycolate using NADPH as a cofactor. GR deficiency also shunts the glyoxylate pool towards the pathway of oxalate production by LDH. Complications of PH2 are often less severe than those of PH1, thus symptoms of PH2...
may not present until adulthood; still, these patients can undergo renal failure and systemic oxalosis.

PH type 3 is the most recently categorized and, typically, the least severe class of PH. Patients with this PH type have mutations in HOGA, preventing HOG from being broken down to pyruvate and glyoxylate; the mechanism by which these defects lead to PH will be discussed in more detail in Chapter 3. One way to distinguish patients with PH3 from other PH patients is to measure the levels of HOG, 4-hydroxyglutamate, and 2,4-dihydroxyglutarate in their urine, as these compounds accumulate to a greater extent in these patients. PH3 does not usually lead to kidney failure, and no reports of systemic oxalosis due to PH3 exist to date. Nonetheless, multiple mutations in HOGA have been documented that lead to excess oxalate production and kidney stones.

Treatment of PH is prescribed based on class and severity. For some PH1 patients, treatment with pyridoxine (vitamin B6) can decrease oxalate excretion. Pyridoxine is a precursor of the AGT cofactor pyridoxal-phosphate (PLP) and has been shown to increase expression, proper localization, and activity of the G170R and F152I AGT mutants. As no other PH-causing enzymes utilize PLP, pyridoxine treatment is only effective for PH1 patients with these particular AGT variants. Doctors often recommend that PH patients consume more than three liters of water per day, and they may prescribe citrate and orthophosphate to increase the solubility of the kidney stones. Another potential treatment under investigation involves the administration of Oxalobacter formigenes, a species of bacteria that normally colonizes the human colon and degrades oxalate as a carbon source. O. formigenes colonization is correlated with lower recurrence of calcium oxalate stones and decreased urinary and plasma oxalate.
More exploration of this treatment is necessary, however, as one study has shown no significant reduction in urinary oxalate upon treatment with Oxabact, a capsule of lyophilized *O. formigenes* that is considered safe for human consumption\textsuperscript{12}. Ultimately, a combination transplant of both the kidney and liver is the only cure for PH1 patients with the most severe phenotypes\textsuperscript{1}; however, even this option can lead to poor outcomes if not performed early in the course of the disease\textsuperscript{5}. A significant need exists for further research into the molecular basis of PH so that new ideas for better treatment options can be generated.

1.4 Statement of Purpose

The classification of PH type 3 has only occurred recently, demonstrating our incomplete knowledge of PH and raising questions regarding potential treatments for this specific form of PH. The hypothesis instigating this work is that the effects of PH type 3 could be ameliorated by preventing the conditions that favor oxalate production downstream of deficient HOGA and by recovering activity of mutant HOGA directly. As described in Chapter 2, this work investigates the first facet of this hypothesis through efforts to crystallize glyoxylate reductase in complex with an inhibitor, 4-hydroxy-2-oxoglutarate, which is the substrate of HOGA that accumulates in PH3 patients. The second facet of the hypothesis is explored in Chapter 3 and involves testing the ability of chemical chaperones to restore activity to mutant HOGA. For the latter, an HPLC assay was developed and standardized to quantitate the amount of pyruvate formed through HOGA activity in crude lysates.

Overall, this thesis provides useful information on crystallization conditions for GR with HOG that can act as a foundation for the successful crystallization and structure
solution of this complex in the future. This research also lays the framework for a more comprehensive trial of chemical chaperones. The investigated chemical chaperones did not increase activity of three HOGA mutants at the experimental doses used, though treatment with 10% glycerol may have restored activity to the R70P mutant. Attempts to restore HOGA activity through chemical chaperones should be continued; several mutants found in PH3 patients were not tested, and a representative from the amino acid group of chemical chaperones was not used. With this foundation, the ultimate goals of these projects can be realized, providing insights into the mechanism of PH3 and new ideas for its treatment.
Chapter 2   The crystallization of glyoxylate reductase in complex with an inhibitor, 4-hydroxy-2-oxoglutarate

2.1   Introduction

Upon classification of PH3, the mechanism by which mutations in HOGA could lead to hyperoxaluria remained enigmatic; it was unclear how mutations that should reduce the production of glyoxylate, and thus oxalate, could result in increased oxalate concentrations. It was thought that perhaps another unidentified enzyme was capable of converting HOG to glyoxylate and that this pathway could lead to increased conversion of glyoxylate to oxalate. However, one potential candidate, the pyruvate aldolase N-acetyl-neuraminic lyase, was not capable of converting the necessary amounts of HOG to account for the PH phenotype\textsuperscript{13}. Another hypothesis, that HOG accumulates to inhibit another enzyme in the glyoxylate metabolism pathway, has since been validated; HOG is a specific inhibitor of GR with a $K_i = 1.8 \pm 0.9$ mM\textsuperscript{13}. Inhibition of GR by HOG would promote the formation of oxalate from glyoxylate originating from other sources, such as glyoxal metabolism\textsuperscript{14} or perhaps even from the breakdown of HOG by a currently unidentified enzyme or series of enzymes. Additionally, patients with PH3 have elevated levels of HOG in their urine, liver tissue, and serum compared to patients with other types of PH, suggesting that the inhibition of GR by HOG could be physiologically relevant\textsuperscript{13}.

Even with the realization of GR inhibition by HOG, a potential treatment for this condition cannot be developed without further knowledge of the mechanism of inhibition. The complexities of the observed mixed partial inhibition pattern make it unclear if HOG binds at the glyoxylate binding site, the cofactor binding site, or both locations\textsuperscript{13}. The
binding of HOG at both locations is possible considering NADPH binds to the co-
enzyme binding domain in a region adjacent to the substrate binding domain\(^\text{15}\). It is also
possible that HOG is binding at another location entirely. To elucidate the manner of
HOG binding to GR, the crystal structure of the complex will be solved, potentially
yielding information that could be used to create a drug or treatment to prevent the
inhibitory interaction.

2.2 Methods

2.2.1 Expression of 36/38R GR

C41(DE3) E. coli cells were transformed with a pET28b vector containing the
full-length human GR gene with two mutations: S36R and E38R. Growth was monitored
at 37 °C until the OD\(_{600}\) of the 11 L culture was between 0.8 and 1.0, at which time the
temperature was lowered to 16 °C. GR expression was then induced with 0.1 mM IPTG
and continued overnight. The cells were harvested via centrifugation, and the resulting
pellets were frozen at -80 °C.

2.2.2 Purification of 36/38R GR

The pellet was resuspended in buffer containing 50 mM HEPES pH 7.9, 500 mM
KCl, 5 mM imidazole, 10% glycerol, and 0.1% Triton X-100. PMSF and benzamidine
(0.67-1 mM), as well as DNase, were also added, and cells were lysed with an Avestin
Emulsiflex-C3 homogenizer. The sample was loaded onto a Ni-NTA (Novagen) column
or a HisPur cobalt column (Thermo Scientific) that was then flushed with the
resuspension buffer (without PMSF, benzamidine, or DNase). Following a wash with 25
mM HEPES pH 7.9, 500 mM KCl, and 5 mM imidazole, the protein was eluted by a 5-
250 mM imidazole gradient over 400 mL. SDS-PAGE analysis was used after this step, as well as after subsequent purification steps, to determine which fractions contained 36/38R GR. These fractions were pooled and dialyzed overnight against 25 mM HEPES pH 7.0, 10% glycerol, 2 mM EDTA, and 15 mM DTT. Biotinylated thrombin (Novagen) was also added (~0.2 U/mg) to cleave the His-tag during dialysis, and SDS-PAGE or mass spectrometry was used to confirm successful cleavage. After treatment with strepavidin beads (Novagen, ~32 μL/U thrombin) to remove the biotinylated thrombin, the sample, currently in dialysis buffer, was eluted from an S-Sepharose column using a 200-300 mL linear gradient to a buffer comprised of 25 mM HEPES pH 7.0, 2 mM EDTA, 5 mM DTT, 10% glycerol, and 500 mM NaCl. Fractions containing the protein were concentrated to ~5 mL and injected onto a Superdex 200 gel filtration column equilibrated with a buffer of 25 mM HEPES pH 7.0, 2 mM DTT, 5% glycerol, and 100 mM NaCl. The resulting fractions with GR were concentrated to 20.6-28.5 mg/mL, and UV spectroscopy was used to determine protein concentration (1 cm cuvette, ε = 27960 M⁻¹cm⁻¹ calculated using the ProtParam algorithm). Protein aliquots were flash frozen with liquid nitrogen prior to storage at -80 °C.

2.2.3 Crystallization Screening and Optimization

An Art Robbins Instruments Crystal Gryphon robot was used to set up 96 well plate crystallization screens (200 nL total drop size) of various solutions purchased from Qiagen, including the Joint Center for Structural Genomics Suites I, II, III, and IV as well as the pH Clear I, pH Clear II, Plus, and PACT suites. The addition of horse hair fragments (1:5 volume ratio of horse hair solution to total solution) was also utilized in some plates to aid in crystal nucleation. Using the vapor diffusion method, hits from
these screens were further optimized in 24 well plates by varying components of the hit solutions. Microseeding was performed by crushing the crystals with a cat whisker, by using a Seed Bead kit (Hampton Research) and making serial dilutions of crushed crystals, or by touching a cat whisker to a single crystal. In each case, drops were streak seeded using a cat whisker. Macroseeding was performed by transferring crystals to a holding solution of similar composition to the parent drop before selecting a single crystal for transfer to the new drop. An in-house Rigaku MicroMax-007 generator with either an R-AXIS IV image plate detector or a Saturn92 CCD detector was used to collect X-ray diffraction data, which was analyzed using Rigaku Crystal Clear 2.0 software.

2.3 Results

Because previous crystallization attempts of WT GR resulted in thin plates that diffracted poorly (unit cell dimensions of $a = 76.6$, $b = 66.9$, $c = 149.8$ Å, $\beta = 98.22^\circ$; space group $P2_1$), a 36/38R GR mutant was utilized for this work$^{15,17}$. Mutating serine 36 and glutamate 38 to arginine disrupts crystal contacts between asymmetric units (Figure 2.1), altering dimer stacking and producing crystals (unit cell dimensions of $a = 60.8$, $b = 120.9$, $c = 107.7$ Å, $\beta = 104.3^\circ$; space group $P2_1$) with better diffraction and NADPH occupancy$^{17}$. For the initial screens, the 36/38R GR protein was used at 10 mg/mL. HOG was added at 100 times the protein concentration, a value significantly higher than the $K_i$, so that most of the protein would be inhibited, and NADPH was added at 10 times the protein concentration because that value has been shown to provide full NADPH occupancy in a previous structure of 36/38R GR$^{17}$. 
Two of the most promising hits generated during screening with the crystallization robot were optimized extensively. A protein solution of 10 mg/mL 36/38R GR, 103 mM HOG (370 times the protein concentration), and 2.8 mM NADPH (10 times the protein concentration) combined with a solution containing 20% PEG 6000, 0.1 M MES pH 6.0, and 1.0 M LiCl resulted in the development of rod-like crystals (Figure 2.2A). Factors such as the concentration of PEG and LiCl, ratio of well solution to protein solution per drop, buffer type and pH, and concentration of HOG and NADPH were altered in attempts to improve crystal quality. Strategies such as microseeding, using well inserts for sitting drops, and changing the overall drop size were also utilized to increase crystal size. Despite these efforts, crystals remained very small and diffracted to only 9 or 10 Å (Figure 2.2B). In one room temperature exposure with a detector distance of 150 mm, it appears that two of the unit cell lengths are 49.3 Å and 97.2 Å (Figure 2.2B). Screening at low temperature (100 K) was attempted, resulting in slightly improved diffraction with the cryoprotectant paratone and little or no diffraction with

Figure 2.1\textsuperscript{17}. Asymmetric units of WT and 36/38R GR. (A) One WT GR dimer (purple) is turned 90° away from the other dimer (green). (B) Both 36/38R GR dimers (one in purple and one in green) stack in the same orientation. Image used with permission of figure author, Dr. Michael Murray.
synthetic mother liquor solution paired with 25% ethylene glycol or 25% glycerol. A 50:50 mixture of paratone and mineral oil was also tried but yielded similar results.

![Image](image1)

**Figure 2.2.** Crystals resulting from conditions with 0.1 M MES pH 6.0, 1.0 M LiCl, and PEG 6000. (A) The crystals are single and form long rods. (B) Diffraction pattern obtained from a similar crystal at room temperature after a 30 min exposure. At a detector distance of 150 mm, two of the unit cell lengths appear to be 49.3 Å and 97.2 Å. Red lines were drawn and measurements were obtained using the “measure” tool of the Rigaku Crystal Clear 2.0 software.
Another condition of 0.1 M HEPES pH 7.5 and 11% PEG 8000 was also optimized. Interestingly, the original hit found in the robot screens did not include HOG in the protein solution (10 mg/mL 36/38R GR, 2.8 mM NADPH), and the crystals formed thin plates. When 24.6 mM HOG was introduced in the 24 well plate screen, single, birefringent crystals were produced (Figure 2.3A). Previously listed strategies as well as the addition of reductants like DTT and the use of macroseeding were part of the optimization process. The resulting crystals displayed mixed morphology and remained very small. One crystal diffracted to ~10 Å at room temperature with a detector distance of 150 mm (Figure 2.3B). In this exposure, it appears that two of the unit cell lengths are 54.8 Å and 157.6 Å (Figure 2.3B). Data collection at 100 K was not possible because screening with several cryoprotectants, including glycerol with and without ethylene glycol, paratone, and a mixture of paratone and mineral oil, yielded little or no diffraction.

2.4 Discussion

Although attempts to crystallize GR in complex with HOG may have been successful, the crystals did not diffract to the extent necessary to solve the crystal structure. While the crystals from the first hit are similar in morphology to 36/38R GR crystals without HOG, the crystals from the second hit have a significantly different morphology, suggesting that HOG may have been incorporated into the crystals. This incorporation, however, cannot be confirmed without knowing the unit cell dimensions of the crystals. Though some of the unit cell dimensions can be predicted from the diffraction pattern image, they cannot be known with certainty without the analysis of a data set with multiple images, which were not obtained. Ultimately, the presence of
HOG in the crystals can only be established by examination of the electron density map of the structure.

Several key aspects with this project require further study. One important component that has yet to be completed is the determination of the $K_i$ with which HOG

Figure 2.3. Crystals resulting from conditions with 0.1 M HEPES pH 7.5 and PEG 8000. (A) The crystals are single, and different morphologies are present within the same drop. (B) Diffraction pattern obtained from a similar crystal at room temperature after a 30 min exposure. At a detector distance of 150 mm, two of the unit cell lengths appear to be 54.8 Å and 157.6 Å. Red lines were drawn and measurements were obtained using the “measure” tool of the Rigaku Crystal Clear 2.0 software.
inhibits the 36/38R mutant. Because the 36/38R GR mutant has similar activity to that of WT GR\textsuperscript{17}, it does not seem likely that HOG would behave differently in relation to the mutant. Time constraints prevented the 36/38R inhibition data from being included in this work, but it should be confirmed that HOG inhibits the mutant with the same parameters as it inhibits WT before continued efforts to crystallize this complex are made.

Additionally, the concentrations of HOG and NADPH may need to be adjusted in the protein solution. It is possible that the presence or excess of one ligand could be affecting the ability of the other to bind in the appropriate location. Initial screens conducted in the absence of each component yielded different hit conditions, so manipulating the concentration of each component could generate new conditions or could further improve current crystals, as was seen in this work.

There are also multiple techniques that could bring success in the future. The crystallization robot could be used to perform high throughput homogeneous microseeding, a process that has been shown to increase crystallization hits and quality\textsuperscript{18}. While horse hair was used to provide a nucleation point in some of the screens, materials such as seaweed and hydroxyapatite could also be used to induce nucleation, and the combination of these materials has an even greater effect on crystal formation\textsuperscript{16}. Finally, in light of the inability of many crystals to withstand long X-ray exposures at room temperature, additional cryoprotectants should be identified and tested so that the number of images obtained from each crystal is sufficient for determining a structure.
Although the overall goal of this project was not achieved, this work generated multiple hit conditions that can be explored in the future and highlighted key aspects of the project that need additional consideration.
Chapter 3  The restoration of 4-hydroxy-2-oxoglutarate aldolase activity by chemical chaperones

3.1 Introduction

Osmolytes are small molecules produced by a wide variety of organisms, from humans to bacteria, to combat the deleterious effects of environmental stresses. Some osmolytes act as chemical chaperones by promoting the folding of proteins into their native states. There are three classes of osmolytes that function as chaperones. The first class includes polyols, which consist of sugars like sucrose as well as glycerol. Polyols are known to protect against the effects of extreme temperatures and dehydration. They stabilize proteins especially well at low pH due to limited hydrogen bonding between polyols and protonated amino acids, favoring the exclusion of polyols from the surface of the protein. The second class of osmolytes is comprised of stabilizing amino acids like proline and glycine that protect against increased salinity regardless of pH. The final class of osmolytes is the methylamines, including sarcosine and trimethylamine-N-oxide (TMAO). These osmolytes protect against high concentrations of urea, a denaturant that accumulates, for example, in elasmobranchs.

The exact mechanism by which chemical chaperones induce protein folding has yet to be fully elucidated. It is accepted that when osmolytes interact with unfolded proteins, there is an increase in the free energy of the denatured state, and the native state is favored. This phenomenon, known as the osmophobic effect, induces correct folding to the native state, a state of lower free energy, and leads to the preferential exclusion of the osmolytes from the protein surface in a manner that does not alter the functionality of the refolded protein.
effect. One idea is that water interacts preferentially with the peptide backbone compared to the osmolyte TMAO such that TMAO is excluded from the protein surface; water is smaller than TMAO and can act as both a proton donor and acceptor while TMAO can only act as a proton acceptor\textsuperscript{22}. Additionally, it has been suggested that osmolytes, unlike denaturants, interact unfavorably with each other as well as the protein backbone to make their presence near the protein even more undesirable\textsuperscript{21}. TMAO has also been shown to destabilize the unfolded state by hydrogen bonding with exposed amide nitrogens, making the unfolded state entropically less favorable\textsuperscript{23}. To this end, intrachain hydrogen bonding, rather than bonds between backbone and osmolyte, is favored in solutions containing osmolytes\textsuperscript{24}. Some chemical chaperones may also exert their effects through the upregulation of endogenous molecular chaperones, though other studies indicate that no effect on molecular chaperone expression occurs in the presence of chemical chaperones and that glycerol can even decrease the expression of DnaK\textsuperscript{25,26,27}.

Chemical chaperones have been used successfully to restore function to several mutant proteins implicated in disease. Glycerol has been shown to promote correct folding of the ΔF508 mutant cystic fibrosis transmembrane conductance regulator\textsuperscript{28}, and 4-phenylbutyrate has also improved chloride transport in some patients with the ΔF508 mutation\textsuperscript{29,30}. Chemical chaperones have increased trafficking of mutant podocin erroneously retained in the endoplasmic reticulum to the plasma membrane\textsuperscript{31}. Mutant proteins that result in cancer, emphysema and liver disease, and nephrogenic diabetes insipidus have also been aided by chemical chaperone treatment\textsuperscript{32}.

Chemical chaperones have also led to the recovery of catalytic activity and native tetramerization of mutant tetrameric proteins. Maple syrup urine disease is caused by
mutations in the mitochondrial branched-chain α-ketoacid dehydrogenase complex, and treatment with TMAO leads to increased activity and native heterotetramerization of the E1 component involved in that complex. Missense mutations of cystathionine β-synthase (CBS) lead to improper folding and loss of activity, resulting in high plasma levels of homocysteine and methionine that can cause skeletal problems and mental disability. Expression of CBS mutants in the presence of chemical chaperones such as DMSO, ethanol, and TMAO have increased mutant activity and tetramerization. In another case, TMAO and glycerol increased activity and tetramerization while decreasing aggregation of mutant phenylalanine hydrolase, the defective enzyme responsible for phenylketonuria.

In light of these studies, chemical chaperones have the potential to restore catalytic activity to the 4-hydroxy-2-oxoglutarate aldolase (HOGA) variants found in PH3 patients. HOGA, which is similar in sequence and organization to bacterial dihydricipicolinate synthase, is a functional tetramer composed of a dimer of dimers. Potential consequences for patient mutations in HOGA have been predicted. Some substitutions (P190L) could affect the position of active site residues that are essential for catalysis. Others (R70P) may alter the positioning of a conserved G-x-x-G-E sequence that is involved in substrate binding. Finally, mutations (A243D, C257G) could affect the folding and tetramerization of HOGA. We hypothesize that the chemical chaperones will be most effective in restoring function to mutants with errors in folding or tetramerization, though chaperone-induced folding may be able to increase activity in mutants with altered active site positioning by shifting affected residues into their native locations.
3.2 Methods

3.2.1 Expression of Mutant HOGA

The genes for WT human HOGA and the HOGA variants P190L, A243D, C257G, and R70P were cloned into pMal vectors such that their mitochondrial targeting sequences had been removed and the resulting protein (residues 26-327) would be fused with a maltose binding protein (MBP) tag on the N-terminus. This cloning method was used previously to increase the solubility and yield of HOGA variants\(^3\). An engineered 3C protease site was also included to enable the removal of the MBP tag during purification.

BL-21 Gold (DE3) \textit{E. coli} cells were transformed with each vector encoding an HOGA gene as well as an empty vector control, plated on carbenicillin containing plates, and incubated overnight at 37 °C. One colony of each mutant was transferred to a culture of 10 mL LB with carbenicillin that incubated at 37 °C and 200 rpm overnight. A portion (0.5 mL) of each overnight culture was transferred to 30 mL of LB broth containing carbenicillin and the chemical chaperone tested. The cultures were grown at 37 °C and 200 rpm until the OD\(_{600}\) reached ~0.6, when the cultures were cooled on ice and 0.3 mM IPTG was added to induce HOGA expression. The cultures incubated at 16 °C for 18 hours. The cells were then pelleted via centrifugation (3000 rpm, 20 min, 4 °C), and the supernatant was discarded. Pellets were stored at -80 °C. The following treatments were tested: 1 mM and 10 mM TMAO (Sigma), 1% and 10% v/v glycerol (EMD), and 1% and 6% v/v DMSO (EMD). There were duplicate cultures for each mutant in every treatment, and duplicate WT HOGA cultures grown in the absence of any chaperone
were used as a control for each experiment. TMAO and glycerol solutions were filter sterilized before use.

Pellets were thawed and resuspended in a buffer of 100 mM Tris pH 8.5 and 0.5 mM EDTA. The cells were lysed via sonication using a Branson Sonifier 250 and microtip. Samples experienced three rounds of sonication pulses on ice for 1 min (50% duty cycle and output control level 6), with each round separated by 1 min in a different ice bucket with no sonication. The samples were centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was removed, 0.2 μM filtered, aliquoted, and stored at -80 °C. Bradford assays were used to determine the total protein concentration for each lysate using bovine serum albumin as a standard.

3.2.2 Determination of Lysate HOGA Activity

The HOGA activity of each lysate was determined by incubating 14 μg of total protein from each lysate with 400 μM HOG (made in-house\(^3\) and pH neutralized) in a 100 μL reaction with 100 mM Tris pH 8.5 and 0.5 mM EDTA buffer. The 25 °C reaction was quenched after 15 min with trichloroacetic acid added to a final concentration of 10% (12 μL of 100% v/v TCA stock solution). Sodium-2-oxobutyrate (Aldrich), which acted as the internal standard, was added to a final concentration of 278 μM (10 μL of a 3.4 mM stock solution), and samples were mixed and centrifuged at 14,000 rpm and 4 °C for 10 minutes. The sample (100 μL) was transferred to a new tube, partially neutralized with potassium hydroxide (3 μL of a 10 M stock solution), and centrifuged as before. The sample was removed (75-80 μL), and 30 μL was incubated at room temperature with 31.9 mM phenylhydrazine (5 μL of 223 mM stock in 35 μL labeling reaction) for at least 30 min in darkened vials. Phenylhydrazine (Aldrich) was made fresh daily in ethanol.
After phenylhydrazine labeling, samples were stored in a Waters 717 Plus autosampler at 4 °C and in the dark until injection (10 μL).

To ensure the accuracy of this method, the concentrations of the HOG stock as well as the pyruvate stock used to generate a standard curve were determined using a lactate dehydrogenase coupled assay. Serial dilutions were made for each compound based on the proposed concentration. For HOG standardization, samples containing HOG (proposed concentrations ranging from 14-118 μM) were incubated at 37 °C with 200 μM NADH and 200 mU LDH (Sigma) in a buffer of 100 mM Tris pH 8.5. The absorbance of the 200 μL sample was monitored at 340 nm. Six μM purified recombinant HOGA (2 μL) was spiked into the sample. The change in absorbance, reflecting the oxidation of NADH, was monitored as HOG was converted to pyruvate by HOGA, and pyruvate acted as a substrate for LDH in a reaction requiring NADH. Once the absorbance plateaued, indicating complete conversion, the final absorbance was subtracted from the initial absorbance multiplied by the dilution factor (200/202). The concentration of NADH used was determined using the extinction coefficient (ε = 6220 M⁻¹ cm⁻¹), and the dilution of each sample was accounted for. Similar procedures were used for the determination of the pyruvate stock concentration using pyruvate samples (proposed concentrations ranging from 25-100 μM) but without the use of HOGA.

3.2.3 HPLC Analysis of Lysate Samples

The analytes were separated over a Beckman Ultrasphere column (C18, 4.6 × 250 mm) coupled to a Waters 2487 Dual Wavelength Absorbance Detector monitoring at 325 nm. Running buffer consisted of 120 mM ammonium acetate, 13% acetonitrile, and 5% methanol flowing at 1 mL/min, and column temperature was set to 26 °C.
3.2.4 Western Blotting of Lysate Samples

Lysate proteins were separated via SDS-PAGE after loading 2 μg total protein on a 10% gel or 10 μg total protein on a 12% gel. After transferring the proteins to nitrocellulose membranes, some membranes were cut in order to probe for HOGA (MW: 77 kDa, including MBP tag) and AhpC (MW: 22 kDa). Milk (5% w/v in TBST) was used to block the membranes, and antibodies for human HOGA (rabbit, 1:2000-5000, generated by Lampire using recombinant hHOGA) and AhpC (rabbit35, 1:1000) were added to the appropriate membranes, which were shaken gently at 4 °C overnight. Following a series of three washing steps with TBST, an HRP-linked anti-rabbit IgG antibody (Cell Signaling Technology) was incubated with the membranes for two hours at a dilution ratio of 1:3000. Membranes were washed again several times. The proteins were visualized on film using chemiluminescent substrates from the Thermo Scientific SuperSignal West Dura and Pico kits. Reagents within each kit were mixed in equal ratios, and those solutions were mixed to make a 50% Dura and 50% Pico solution. After this step, Ponceau S staining solution (Sigma) was used to evaluate total protein loading.

3.3 Results

3.3.1 Assay Development

After achieving consistent separation of the analytes (Figure 3.1), the stability of the glyoxylate and pyruvate derivatives was assessed over 24 hours. After a 16 hour period, the ratio of the glyoxylate peak area to the internal standard (2-oxobutyrate) peak area was 88% of the area of the initial 0 hr sample. The ratio of the pyruvate peak area to the 2-oxobutyrate peak area was 98% of the original area after 16 hours, indicating the superior stability of the labeled pyruvate (data not shown). Based on these findings, a
reproducible standard curve was generated to determine the relationship between pyruvate concentration and the ratio of the pyruvate peak area to the 2-oxobutyrate peak area (Figure 3.2A). This standard curve was used to quantify the pyruvate concentration in each of the tested samples, and a sample containing 250 μM pyruvate was included with some of the sample sets to ensure that the standard curve was still applicable.

Additionally, a time course experiment was performed. It was determined that a 15 min incubation of 400 μM HOG with 14 μg total protein from lysate with WT HOGA resulted in the cleavage of approximately half of the original 400 μM HOG (Figure 3.2B). This 15 min incubation time was selected for use in each experiment because in a previous study, some chaperones increased mutant activity above that of WT25. If this phenomenon were to occur, a 15 min incubation of a more active mutant would likely leave some uncleaved HOG remaining at the end of the reaction time while still allowing less active mutants to produce a measurable amount of pyruvate in the time allotted.
Figure 3.1. Representative chromatogram of sample containing 162 μM HOG, 200 μM glyoxylate, 250 μM pyruvate, and 278 μM 2-oxobutyrate. The consistent retention times for the analytes are 2.6, 5.1, 5.8, and 7.7 min, respectively. Baseline peak separation was achieved with buffer conditions consisting of 120 mM ammonium acetate, 13% acetonitrile, and 5% methanol.
Figure 3.2. Assay development: pyruvate standard curve and time course. (A) Pyruvate standard curve for pyruvate concentrations 0-500 μM. (B) Time course depicting the amount of pyruvate generated by 14 μg total protein from lysate containing WT HOGA. At 15 min, 159 μM pyruvate had been produced, a conversion of nearly half of the 400 μM HOG present in the reaction.
3.3.2. Chemical Chaperone Analysis by HPLC

No dose of the three chemical chaperones appeared to increase HOGA activity for any mutant. Table I shows the amount of pyruvate in each culture as calculated from peak area analyses compared to the pyruvate standard curve. As indicated by color in the tables, the treatments were conducted in batches, with each batch having the same WT controls that were not treated with chemical chaperone (WT - no CC). In these experiments, the amount of pyruvate in the cultures transformed with the empty pMal vector represents the background pyruvate occurring as a result of factors other than HOGA activity. Table II depicts the pyruvate values after the background amount has been removed from the total, indicating the pyruvate concentrations generated by HOGA. The values are reported as a percentage of WT activity in table III, where WT activity from cultures receiving no treatment was set to 100%.

For each treatment, the presence of a chemical chaperone does not greatly affect the activity of WT HOGA compared to the WT activity in the absence of chaperone. The R70P mutant has ~30% WT activity, and this activity appears to be either slightly decreased or maintained in the presence of each chaperone. The P190L, A243D, and C257G variants do not have activity under any of the tested conditions, except perhaps a slight increase to 2-4% WT activity for the P190L mutant in the presence of 10 mM TMAO.
Table I. Pyruvate concentrations (μM) of lysates for each mutant by treatment.*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>pMal vector - no insert</th>
<th>WT - no CC</th>
<th>WT</th>
<th>R70P</th>
<th>P190L</th>
<th>A243D</th>
<th>C257G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no treatment</td>
<td>1 mM TMAO</td>
<td>10 mM TMAO</td>
<td>1% glycerol</td>
<td>10% glycerol</td>
<td>1% DMSO</td>
<td>6% DMSO</td>
</tr>
<tr>
<td>pMal vector</td>
<td>25 ± 2</td>
<td>26 ± 2</td>
<td>23 ± 1</td>
<td>31 ± 2</td>
<td>35 ± 3</td>
<td>38 ± 1</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>no insert</td>
<td>151 ± 54</td>
<td>129 ± 4</td>
<td>117 ± 4</td>
<td>157 ± 1</td>
<td>142 ± 3</td>
<td>135 ± 2</td>
<td>150 ± 18</td>
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<td>WT</td>
<td>121 ± 14</td>
<td>136 ± 9</td>
<td>127 ± 9</td>
<td>157 ± 1</td>
<td>142 ± 3</td>
<td>135 ± 2</td>
<td>150 ± 18</td>
</tr>
<tr>
<td>R70P</td>
<td>62 ± 15</td>
<td>36 ± 2</td>
<td>39 ± 0</td>
<td>40 ± 3</td>
<td>68 ± 4</td>
<td>43 ± 3</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>P190L</td>
<td>27 ± 1</td>
<td>25 ± 3</td>
<td>26 ± 1</td>
<td>29 ± 0</td>
<td>36 ± 2</td>
<td>33 ± 1</td>
<td>35 ± 0</td>
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<tr>
<td>A243D</td>
<td>20 ± 1</td>
<td>19 ± 2</td>
<td>24 ± 1</td>
<td>25 ± 0</td>
<td>30 ± 1</td>
<td>30 ± 1</td>
<td>33 ± 1</td>
</tr>
<tr>
<td>C257G</td>
<td>20 ± 1</td>
<td>20 ± 0</td>
<td>23 ± 0</td>
<td>26 ± 1</td>
<td>37 ± 0</td>
<td>32 ± 2</td>
<td>33 ± 1</td>
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</tbody>
</table>

*The WT no CC control applies to treatments with the same column color, as cultures for the two treatments were grown at the same time as the corresponding controls. Values represent the average pyruvate concentrations of two replicate cultures ± standard error.

Table II. Pyruvate concentrations (μM) generated by HOGA.**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>WT - no CC</th>
<th>WT</th>
<th>R70P</th>
<th>P190L</th>
<th>A243D</th>
<th>C257G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no treatment</td>
<td>1 mM TMAO</td>
<td>10 mM TMAO</td>
<td>1% glycerol</td>
<td>10% glycerol</td>
<td>1% DMSO</td>
</tr>
<tr>
<td>WT</td>
<td>126 ± 54</td>
<td>106 ± 4</td>
<td>96 ± 9</td>
<td>122 ± 3</td>
<td>104 ± 3</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>R70P</td>
<td>37 ± 15</td>
<td>10 ± 3</td>
<td>9 ± 4</td>
<td>33 ± 5</td>
<td>5 ± 3</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>P190L</td>
<td>2 ± -1</td>
<td>3 ± 1</td>
<td>-2 ± 2</td>
<td>1 ± 4</td>
<td>-5 ± 1</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>A243D</td>
<td>-5 ± 2</td>
<td>-7 ± 3</td>
<td>1 ± 1</td>
<td>-6 ± 2</td>
<td>-5 ± 3</td>
<td>-8 ± 1</td>
</tr>
<tr>
<td>C257G</td>
<td>-5 ± 2</td>
<td>-6 ± 2</td>
<td>0 ± 1</td>
<td>-5 ± 2</td>
<td>2 ± 3</td>
<td>-6 ± 2</td>
</tr>
</tbody>
</table>

**Pyruvate present in the cultures transformed with the empty vector has been subtracted from the values in Table 1. Values represent the new pyruvate concentrations ± standard error.

Table III. Mutant activity as a percentage of WT activity.***

<table>
<thead>
<tr>
<th>Mutant</th>
<th>WT - no CC</th>
<th>WT</th>
<th>R70P</th>
<th>P190L</th>
<th>A243D</th>
<th>C257G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no treatment</td>
<td>1 mM TMAO</td>
<td>10 mM TMAO</td>
<td>1% glycerol</td>
<td>10% glycerol</td>
<td>1% DMSO</td>
</tr>
<tr>
<td>WT</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R70P</td>
<td>29 ± 17</td>
<td>15 ± 1</td>
<td>8 ± 4</td>
<td>40 ± 21</td>
<td>6 ± 5</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>P190L</td>
<td>2 ± 2</td>
<td>0 ± 3</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>1 ± 2</td>
</tr>
<tr>
<td>A243D</td>
<td>0 ± 0</td>
<td>1 ± 1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>C257G</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2 ± 2</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

***The activity of WT with no chaperone treatment (no CC) has been set to 100%. In each treatment with the same column color, the activities of the variants are related to the same WT no CC controls. Values represent percentage of WT activity in the absence of chaperone ± standard error.
3.3.3. Chemical Chaperone Analysis by Western Blot

In light of the HPLC analysis results, it seemed possible that the mutants had low activity due to little or no expression. This possibility was tested using SDS-PAGE followed by western blotting. Figure 3.3 shows western blots for HOGA in each of the treatment groups. The HOGA expression level is similar for all the mutants in the “no chemical chaperone” group as well as the 1 mM TMAO and 1% glycerol groups. The level of R70P in one sample from the 10 mM TMAO group is decreased. HOGA levels vary in the 10% glycerol group, with more significant expression of WT with and without chaperone and decreased expression of the mutants. A similar trend is seen in the 1% and 6% DMSO treatments, where the WT seems to be expressed more readily than most of the mutants. In the 6% DMSO treatment, the A243D mutant did not express well, as its presence could not be visualized with the other mutants. The presence of lower molecular weight bands that have reacted with the HOGA antibody most likely indicates that HOGA degradation products are present in the lysates.

Originally, alkyl hydroperoxide reductase C (AhpC), a bacterial peroxiredoxin, was to be used as a loading control. Difficulties with detecting the protein arose, and time constraints did not allow for the optimization of its use. In lieu of a loading control, Ponceau S staining was used to visualize total protein on the nitrocellulose membrane. Figure 3.4A depicts a blot stained immediately post-transfer. Total protein loading is comparable across samples. Lane 1 does not stain in a similar pattern because it contains 0.01 μg purified recombinant HOGA. Additionally, lanes 4 and 5 contain the empty pMal vector samples, and the protein seen at around 44 kDa is MBP without protein
fused. Because such large concentrations of protein at a single molecular weight can affect the separation of other proteins running in close proximity on the gel, these samples were loaded at the end of the gel in subsequent experiments to minimize impact on the other samples. Figure 3.4B shows a representative blot stained with Ponceau S solution after the blot had been developed, and the total protein loading was similar across all samples. This evidence of consistent protein loading in combination with the blotting data shows that each HOGA mutant was expressed in every treatment, with the only exception being the A243D mutant in 6% DMSO.
Figure 3.3. Western blot analysis of lysates from each chemical chaperone treatment. Treatment labels are to the right of the blot. Blotting occurred after SDS-PAGE. Arrows indicate HOGA+MBP (77 kDa). (A) Lane order is as follows from left to right, with 1 or 2 indicating samples of replicate cultures: WT 1, WT 2, P190L 1, P190L 2, R70P 1, R70P 2, A243D 1, A243D 2, C257G 1, C257G 2, pMal only 1, pMal only 2. (B) Lane order is as follows from left to right: WT 1 no chemical chaperone (CC), WT 2 no CC, P190L 1, P190L 2, R70P 1, R70P 2, A243D 1, A243D 2, C257G 1, C257G 2, pMal only 1.
Figure 3.4  Ponceau S staining of representative blots. (A) Ponceau S stained nitrocellulose membrane of samples treated with no chemical chaperone. Lanes are as follows (left to right): Ladder, 0.01 μg purified HOGA, WT 1, WT 2, pMal only 1, pMal only 2, P190L 1, P190L 2, R70P 1, R70P 2, A243D 1, A243D 2, C257G 1, C257G 2. Staining was performed immediately after transfer. Image taken by LeAnn Rogers. (B) Ponceau S stained nitrocellulose membrane of samples treated with 1 mM TMAO. Lanes are as follows (left to right): WT 1 no CC, WT 2 no CC, WT 1, WT 2, P190L 1, P190L 2, R70P 1, R70P 2, A243D 1, A243D 2, C257G 1, C257G 2, pMal only 1, pMal only 2, ladder. Staining was performed after western blot analysis, and the blot had been cut so that the bottom portion could be used for AhpC detection.
3.4 Discussion

Chemical chaperones are small molecules known for their ability to induce proper protein folding, enabling protein activity. The chemical chaperones TMAO (1 mM, 10 mM), glycerol (1%, 10%) and DMSO (1%, 6%) did not seem to improve the activity of the tested HOGA variants. These results align with what was anticipated for the R70P and the P190L mutants. The mutation R70P could affect the catalytic site by altering the position of the G-x-x-G-E motif involved in pyruvate binding (Figure 3.5A)\textsuperscript{3}. The introduction of leucine instead of proline at residue 190 could also interrupt the correct positioning of the active site by affecting residues K196 and S198, which are important for catalysis (Figure 3.5B)\textsuperscript{3}. Both mutations involve the inclusion or exclusion of a proline residue, an amino acid with unique properties that are not easily mimicked. It may be that even if the chaperones did induce more native folding, they could not facilitate the positioning necessary for catalytic activity due to the proline substitutions. However, it should be noted that for the 10% glycerol treatment, the expression level of the mutant R70P was about half the level of WT HOGA in the absence of any chaperone. The HPLC results indicate that R70P had about 40% WT activity with this treatment, a value that could reasonably be doubled to 80% to account for the decreased mutant expression. This adjustment would represent a significant recovery of activity for the R70P mutant in the presence of 10% glycerol.

The results were in conflict with what was anticipated for the A243D and C257G mutants. Alanine 243 is facing toward the protein core and surrounded by hydrophobic residues (Figure 3.5C). Replacing this residue with aspartate could interfere with the
hydrophobicity of this region, perhaps leading to altered protein folding$^8$. Cysteine 257 is located near the dimer-dimer interface (Figure 3.5D), so its mutation to glycine could disrupt HOGA tetramerization$^3$. Because both mutations are far from the active site, refolding to the native state should be all that is required to regain activity, so treatment with chemical chaperones was expected to restore function.

It is not entirely clear if the chaperones were able to induce tetramerization of the mutants. While the mutants all had decreased activity compared to WT, western blotting indicated that some variants were expressed at lower levels than WT in certain
treatments. Though it would seem that the chaperones did not have an effect, if they were successful at increasing tetramerization of these variants, then perhaps the observed decreased activity was related to the decreased expression. This decreased expression could be responsible for an artificially low percentage of R70P activity in the 10% glycerol treatment group. Still, for the other mutants, the activity that would have been gained from equal expression would likely be negligible based on the absence of measured activity at the current expression level. Native gel electrophoresis should be used in future experiments to determine the level of tetramerization of each mutant with and without the chaperone treatments. Future blotting methods should include the optimization and use of an appropriate loading control, such as ribosomal protein L15, and protease inhibitors, such as phenylmethylsulfonyl fluoride or benzamidine, could be added during cell lysis to limit the formation of HOGA degradation products.

It should also be noted that in a previous study, there was no activity detected for the R70P mutant in experiments using the purified recombinant protein or in experiments using cell lysate from CHO cells transfected with the R70P HOGA DNA construct. Interestingly, this work shows that the R70P mutant exhibits activity above the background level in the absence of any chaperone. The cause of this discrepancy is unclear at this time but will be reassessed in the future. Additionally, experiments to replicate the restoration of R70P activity via treatment with 10% glycerol should be conducted.

Future directions for this work involve several options for expansion and refinement. No representative from the amino acid group of chemical chaperones was tested in this work, so a future treatment could involve proline or glycine. Several other
known osmolytes include ethanol\textsuperscript{25}, trehalose\textsuperscript{37}, and 4-phenylbutyrate, a drug that has received FDA approval for treatment of urea-cycle disorders\textsuperscript{38}. The osmolyte betaine (75 mM) has also been shown to increase solubility of mutant AGT\textsuperscript{39}. Additionally, the scope of this work was limited to four mutants, but there are several other HOGA variants that should also be studied\textsuperscript{3,8,9}. Finally, alternative concentrations of certain chaperones could be tested. The presence of some of the chaperones, particularly 10% glycerol and 6% DMSO, seemed to significantly slow the growth of the cultures, perhaps stressing the cells and affecting chaperone performance. In the future, a slightly lower concentration of these chaperones could be evaluated.

The overall goal of this project, to gain insight into the effects of chemical chaperones on the activity of HOGA variants, was achieved. A method was developed for testing the chaperones on a small scale while accommodating multiple cultures. Additionally, this work was instrumental in developing an HPLC-based assay for the determination of HOGA activity, an assay that will allow this project to proceed and will aid in other lab projects. This study suggests that activity may have been restored to the R70P mutant in the presence of 10% glycerol, indicating that the use of chemical chaperones to restore activity to mutant HOGA warrants further exploration and could reveal a new treatment for PH3.
Conclusions

Primary hyperoxaluria is a disease characterized by the overproduction of oxalate, which complexes with calcium to form kidney stones. In extreme cases, patients with PH can experience end stage renal failure and require combined kidney and liver transplants. Of the three classes of PH, PH3 is the most recently recognized and is caused by mutations in the enzyme HOGA that converts HOG to pyruvate and glyoxyxlate. By pursuing two main goals, this work sought to provide insight into the molecular mechanisms contributing to PH3.

The first goal of this work was to demonstrate how HOG, which accumulates when HOGA is deficient, binds to inhibit GR, another enzyme that is implicated in PH. Many optimization techniques were employed to crystallize GR in complex with HOG, and insight into how to move forward with this ambition was obtained.

The second goal of this work was to determine how chemical chaperones, or molecules that can induce proper protein folding, could aid in recovering the activity of HOGA mutants. An HPLC-based assay was developed and used to determine the activity of each mutant. TMAO, glycerol, and DMSO failed to restore activity to three tested mutants, but expression discrepancies revealed by western blotting suggest that treatment with 10% glycerol may have restored activity of the R70P mutant to ~80% of WT. Future studies will include alternative chaperones and a greater number of mutants.

Though nearly 90 years have passed since the first description of PH1, many aspects of the disease remain a mystery, especially the aspects of PH3. Continuing crystallization efforts and chaperone studies will advance the knowledge of this disease so that better treatments can be realized.
References


Curriculum Vitae

Jessie A. Breazeale

Education
Wake Forest University Graduate School of Arts and Sciences, Winston-Salem, NC
M.S. Biomedical Science 8/12-5/14

Meredith College, Raleigh, NC
B.S. Biology, summa cum laude 8/08-5/12
GPA: 4.0/4.0

Relevant Work Experience
Graduate Student, Wake Forest University, Winston-Salem, NC 8/12-5/14
Department of Biochemistry, Lab of Dr. Todd Lowther
- Expressed recombinant proteins on a bench top scale (30 mL and 10 L E. coli cultures).
- Purified glyoxylate reductase by affinity, ion-exchange, and size-exclusion chromatography.
- Crystallized glyoxylate reductase with its inhibitor in order to determine the structure of the complex using x-ray crystallography.
- Developed and standardized HPLC assay to evaluate the ability of chemical chaperones to recover activity of 4-hydroxy-2-oxoglutarate aldolase mutants implicated in disease.

Lab Technician, Novozymes, Franklinton, NC 5/12-7/12
- Anticipated and supported laboratory needs of the Biomass Application Development group as a part-time lab technician.
- Performed small and medium scale hydrolysis of pretreated bagasse and corn stover.

Peer Tutor, Learning Center, Meredith College, Raleigh, NC 3/09-5/12
- Assessed the learning styles and needs of undergraduate students seeking help with biology and writing.
- Implemented strategies to aid students in learning new concepts.

Intern, Biomass Application Development, Novozymes, Franklinton NC 5/11-8/11
- Performed small and medium scale hydrolysis of pretreated corn stover using enzymes involved in the conversion of cellulose to ethanol.
- Evaluated enzyme cocktails produced by different strains of a fungal organism to determine which cocktail was most effective in hydrolysing cellulose.
- Designed, implemented, and analyzed an experiment in which an aspect of first generation ethanol production was incorporated into cellulosic ethanol fermentation to determine if the addition boosted ethanol yield.
Intern, Duke University, Durham, NC 1/11-4/11
- Selected from pool of Meredith applicants to participate in a cooperative internship at Duke University under the mentorship of Dr. Marcelo Ardon in Dr. Emily Bernhardt’s lab.
- Studied the effects of artificial seawater and sulfate on the pH and microbial communities of wetland soils facing drought and flooded conditions.
- Tested soil sample pH and prepared samples for carbon and nitrogen analysis.
- Performed spectrophotometric phenol oxidase assays.

Intern, Biofuel from Grain, Novozymes, Franklinton, NC 5/10-8/10
- Tested the addition of booster chemicals in lab scale corn liquefaction and fermentation to determine their effects on ethanol yield.
- Optimized an assay designed to measure the starch composition of mashes after liquefaction.

Participant, Meredith College Undergraduate Research, Raleigh, NC 1/09-12/09
- Conducted research experiments to isolate and purify a potential HIV protease inhibitor from *Tyloosema fassoglensis*.
- Awarded summer research stipend to continue the research during the summer of 2009.
- Performed gravity-based size exclusion and ion exchange chromatography, Bradford assays, spectrophotometric trypsin assays, and SDS-PAGE.

Abstracts, Presentations, and Papers

“An Investigation of Enzymatic Applications in First and Second Generation Biofuel Production” 4/12
Meredith College Honors Thesis (Paper), Celebrating Student Achievement Day (Presentation)

“Future Biomass Cellulase Strain and Dose Profile” 7/11
Intern Presentations, Novozymes, Inc. (Presentation)

“Microbial Enzyme Activity in Response to Drought and Saltwater Intrusion in Wetland Soils” 4/11
Celebrating Student Achievement Day, Meredith College (Presentation)

“Truncation of Osmotic Stress Activated Soybean Protein Kinase” 12/10
Honors Presentation, Meredith College Biology Department (Presentation)

“Optimization of Starch Composition Assay and Study of Chemical Boosters in Fermentation” 7/10
Intern Presentations, Novozymes, Inc. (Presentation)

“HIV Protease Inhibition Abilities of *Tyloosema fassoglensis*” 11/09
State of NC Undergraduate Research and Creativity Symposium, UNC-Wilmington (Poster)
“HIV Protease Inhibition Abilities of *Tylosema fassoglensis*”  
Taste of Research, Meredith College (Poster)  
10/09

“HIV Protease Inhibition Abilities of *Tylosema fassoglensis*”  
Celebrating Student Achievement Day, Meredith College (Poster)  
4/09

**Awards**

**Academic Excellence Award, Meredith College**  
Awarded to students who complete their undergraduate studies with a 4.0 GPA on a 4.0 scale.  
4/12

**Deborah K. Smith Award for Achievement in Biology, Meredith College**  
Awarded to three graduating seniors in the biology department based on their scholarship, service, research, and leadership.  
4/12

**Deborah K. Smith Biology Scholarship, Meredith College**  
Awarded to a Meredith student who is pursuing a career as a scientist or medical professional.  
4/11

**USA South Academic All-Conference Team**  
2009-2011

**Novozymes Summer Intern Scholarship, Novozymes**  
Awarded to one summer intern who best displays Novozymes’ core values of spark, openness, and passion.  
7/10

**John Yarborough Award Recipient, Meredith College**  
Awarded to a rising junior and senior biology major who have high grades, show potential in biology, and have been of service to the department.  
4/10

**Betty Hyatt Biology Scholarship Recipient, Meredith College**  
Awarded to a biology major who demonstrates a determination to achieve her full potential in the field of biology.  
4/10

**Beta Beta Beta National Biological Honor Society, Meredith College**  
3/10

**President’s Report 2010, Meredith College**  
Selected to represent intellectual curiosity in Meredith College’s 2010 President’s Report.  
3/10

**Extracurricular Activities**

**Varsity Intercollegiate Soccer Team, Meredith College**  
8/08-5/12

**Meredith College Honors Program**  
8/08-5/12

**Co-Treasurer, Kappa Nu Sigma Honor Society, Meredith College**  
4/11-5/12