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The enzyme 4-hydroxy-2-oxoglutarate aldolase and its role in mitochondrial metabolism

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOLOGICAL SCIENCES

UNIVERSITY OF AUCKLAND

2015
Abstract

4-Hydroxy-2-oxoglutarate aldolase (HOGA) is a Class I aldolase implicated in the rare autosomal recessive disease Primary Hyperoxaluria Type 3 (PH3), characterized by excessive oxalate production and calcium oxalate stone formation. HOGA is expressed primarily in animal liver and kidney mitochondria, and has dual activities; (1) HOG aldolase activity involved in hydroxyproline catabolism; and (2) oxaloacetate decarboxylase (OAD) activity putatively influencing the tricarboxylic acid (TCA) cycle.

The mechanism whereby PH3 HOGA mutations lead to hyperoxaluria is uncharacterized. There are 26 HOGA mutations identified in PH3 patients to date, and this thesis investigates the two most common mutations, the deletion of glutamate residue 315 (Δ315) and the intronic in-frame insertion of 17 amino acids between exons 5 and 6 (c.700+5G>T). Following their recombinant expression in Escherichia coli, the mutant proteins Δ315 and c.700+5G>T HOGA aggregated, were thermally unstable and kinetically inactive. To investigate intracellular processing of PH3 HOGA mutants, Δ315 HOGA was transfected in Flp-In® HEK-293 T-REx (F-293) cells; this allowed tetracycline-inducible expression of HOGA. mRNA was upregulated similarly in wild-type and Δ315 HOGA F-293 cells following induction of transcription, however HOGA protein was not detected in Δ315 HOGA cell lysate, in contrast to wild-type HOGA F-293 cells. Intracellular degradation of the Δ315 HOGA protein was inhibited using the proteasome inhibitor PS-341, which permitted the detection of Δ315 HOGA protein by western blot.

It was hypothesized that PH3 mutations altered the quaternary structure of HOGA; however, this theory was invalid following the finding of misfolded recombinant PH3 HOGA mutant protein. The quaternary structure of wild-type HOGA formed a tetramer across a wide-concentration range.
In a kinetic investigation, HOG aldolase activity was 4.8-fold more efficient compared to OAD activity of wild-type recombinant human HOGA (hHOGA), due to a lower $K_M$ ($56\ \mu M$ and $130\ \mu M$ for HOG and oxaloacetate, respectively), and 2-fold greater turnover rate ($k_{cat}$ $0.5\ \text{s}^{-1}$ vs $1\ \text{s}^{-1}$ for HOG and oxaloacetate, respectively). HOG and oxaloacetate cleavage utilized the same kinetic mechanism, and the TCA cycle intermediate $\alpha$-ketoglutarate ($\alpha$KG) competitively inhibited OAD activity ($K_i = 2.8\ \text{mM}$), and HOG aldolase activity ($K_i = 22\ \text{mM}$).

As HOGA has OAD activity, it was hypothesised that it may contribute to TCA cycle regulation in malate-fuelled mitochondria, through turnover of malate to oxaloacetate by malate dehydrogenase and the subsequent oxaloacetate decarboxylase to form pyruvate. As the net reactions of HOGA and malate dehydrogenase are similar to malic enzyme (ME), the contribution of HOGA and ME to TCA cycle turnover was investigated in mitochondria isolated from: (1) F-293 cells vs F-293 cells overexpressing hHOGA, and (2) metabolically diverse rat organs with differing expression of HOGA and ME. Malate supported respiration was high in F-293 mitochondria, and not altered by HOGA overexpression. During blockage of the ME2 pathway with the novel inhibitor NPD-389, 80% of malate respiration was inhibited in F-293 cells, and this revealed 16% ($p<0.01$) higher malate respiration in F-293 cell mitochondria overexpressing HOGA. Malate respiration varied across metabolically diverse rat organs, however rat tissue was insensitive to the ME2 inhibitor, thus the contribution of ME2 and HOGA could not be determined in rat tissues.

In conclusion, the two most common PH3 HOGA mutations are confirmed to be loss of function mutations, and $\Delta 315$ HOGA protein is proteasomally degraded in vitro. The two activities of HOGA, HOG aldolase and OAD activity, proceed via the same catalytic mechanism, and HOG aldolase activity is 4-fold more efficient. Both activities are regulated
by the TCA intermediate αKG, and preliminary evidence shows that HOGA may be involved in TCA cycle regulation.
Acknowledgements

I would firstly like to acknowledge my supervisors Kerry, Tony, Ant, and Ted. Kerry was calm and supportive when I was stressed, and great at seeing the bigger picture. Tony, our local mitochondrial guru, was innovative as always, and still amazes me with his DIY inventions (hicto-scopes and the like).

SBS has been a great place to research, and everyone has always been there to give a helping hand. Adrian Turner patiently taught me microscopy techniques, and Fiona Clow from the Med School helped me with what she described as the most difficult case of biotin-SPR she has come across yet! Mike Griffin in Melbourne gave me a whole week of his time to run an experiment with my protein, which was a great overseas lab experience. I especially want to thank Jiwon in our lab, our overworked but amazing post-doc, who was always ready to outpour her molecular knowledge at request. A number of other post-docs at SBS have helped me along the way, including Harriet, Chris, Jodie, and Genevieve. I’d also like to thank Dill, a fellow PhD student I’ve been lucky enough to work alongside with on the HOGA project.

Doing a PhD has been a great experience, but also a bit of a roller coaster, and the greatest thing about it was being able to share the experience with fellow PhD students along the way. The frequent coffee breaks with Chris, Jiwon, Amelia, and Sarah helped keep me sane, and I am missing them already!

To my best non-uni friends Emma, Bex, and Sarah, you have been a great escape, and I hope to see more of you now that the Thesis is done!

The greatest supports in my life have been my parents, Sue and Bruce, and my fiancé Haydn, and I can’t give thanks enough for your love and support over this challenging journey.
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<tbody>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AGTX</td>
<td>Alanine: glyoxylate amino transferase</td>
</tr>
<tr>
<td>αKG</td>
<td>α-ketoglutarate</td>
</tr>
<tr>
<td>AUC</td>
<td>Analytical ultracentrifugation</td>
</tr>
<tr>
<td>AUC-SE</td>
<td>Analytical ultracentrifugation - sedimentation equilibrium</td>
</tr>
<tr>
<td>AUC-SV</td>
<td>Analytical centrifugation – sedimentation velocity</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BirA</td>
<td>Biotin ligase</td>
</tr>
<tr>
<td>Bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>COX-IV</td>
<td>Cytochrome-c oxidase / Complex IV</td>
</tr>
<tr>
<td>CS</td>
<td>Citrate synthase</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
</tr>
<tr>
<td>DFS</td>
<td>Differential scanning fluorimetry</td>
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<td>Dihydrodipicolinate synthase</td>
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<tr>
<td>DHDPSL</td>
<td>Dihydrodipicolinate synthase-like</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GO</td>
<td>Glyoxylate oxidase</td>
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GOI  Gene of interest
GRHPR  Glyoxylate reductase / hydroxypyruvate reductase
GSH  Glutathione
HBS  Hepes buffered saline
HEK  Human embryonic kidney
HEPES  (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HKB  4-Hydroxy-2-ketobutyrate
HOG  4-Hydroxy-2-oxoglutarate
HOGA  4-Hydroxy-2-oxoglutarate aldolase
HOGA1  4-Hydroxy-2-oxoglutarate gene
HPRT  Hypoxanthine-guanine phosphoribosyltransferase
HTK  Histidine-tryprophan-ketoglutarate
IPTG  Isopropyl β-D-1-thiogalactopyranoside
KDGA  2-keto-2-deoxygluconate
KDPGA  2-keto-3-deoxyphosphogluconate
Ki  Inhibitor constant
Km  Michaelis constant
Kcat  Turnover number
KOH  Potassium hydroxide
LB  Lysogeny broth
LDH  Lactate dehydrogenase
LDS  Lithium dodecyl sulfate
MBP  Maltose binding protein
MCT  Monocarboxylate transferase
MDH  Malate dehydrogenase
MB  Megabase
ME  Malic enzyme
MES  2-(N-morpholino)ethanesulfonic acid
MIM  Mendelian inheritance in Man
MiR05  Mitochondrial respiration medium
MW  Molecular weight
NAD+  Nicotinamide adenine dinucleotide
NADH  Nicotinamide adenine dinucleotide, reduced
<table>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced</td>
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<td>N-acetylneuraminate lyase</td>
</tr>
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<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<tr>
<td>OAD</td>
<td>Oxaloacetate decarboxylase</td>
</tr>
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<td>ORF</td>
<td>Open reading frame</td>
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<td>phosphate buffered salint-tween</td>
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</tr>
<tr>
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<tr>
<td>$V_{\text{max}}$</td>
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<tr>
<td>YWHAZ</td>
<td>Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein</td>
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1. INTRODUCTION

1.1 Genetics and phylogeny of HOGA

1.1.1 Discovery of *hoga1*, the gene encoding the enzyme HOGA

4-Hydroxy-2-oxoglutarate aldolase (HOGA, EC 4.1.3.16) is a bi-functional enzyme that reversibly cleaves 4-hydroxy-2-oxoglutarate (HOG), an intermediate of hydroxyproline catabolism, into glyoxylate and pyruvate, and can also decarboxylate oxaloacetate (Figure 1). Until 2010, HOGA was an orphan enzyme, i.e. an enzyme with extensive experimental data but no gene annotation.

In 2010, mutations were identified in the *hoga1* gene in patients with the autosomal recessive disease Primary Hyperoxaluria Type 3 (PH3, MIM 613616) (Belostotsky et al., 2010). This gene was originally annotated dihydrodipicolinate synthase-like (*dhdpsl*) due to its similarity with bacterial dhdps enzymes, which are pyruvate lyase enzymes involved in sialic acid metabolism and lysine biosynthesis. This *hoga1/dhdpsl* gene has since been expressed recombinantly, the crystal structure solved and biochemical 4-hydroxy-2-oxoglutarate aldolase activity confirmed (Riedel et al., 2011). The *dhdpsl* gene is now referred to as *hoga1*, located on chromosome 10, position q24.2 in humans.
1.1.2 Species dispersion

HOGA (uniprot Q86XE5 for H. sapiens) is found throughout the animal kingdom, from mammals (90% amino acid identity with B. Taurus Q0P515, 87% with R. norvegicus NP_001099825), amphibians (73% identity with X. laevis Q5XGL6), avian species (72% identity with G. gallus XP_001233352), reptiles (70% with A. carolinensis XP_008104732), and Actinopterygii (70% with D. rerio Q6NY77). Proteomic evidence also shows that HOGA is found in some species of filamentous fungi (33% identity with A. nidulcans, uniprot Q5B577) (Kim, Nandakumar, & Marten, 2007) (Figure 2).
Figure 2  Phylogenetic relationship of selected species for 4-hydroxy-2-oxoglutarate (HOGA), and other members of the dihydrodipicolinate synthase (DHDPS) / N-acetylneuraminic lyase (NAL) family. Including DHDPS, NAL, 2-keto-2-deoxygluconate (KDGA) as well as the distantly related but biochemically similar 2-keto-3-deoxyphosphogluconate (KDPGA) (created using GeniousPro 5.3.6 software).
1.1.3 Homology to known enzymes

HOGA is also known as dihydrodipicolinate synthase-like (DHDPSL) and is most closely related to the dihydrodipicolinate synthase (DHDPS) / N-acetylneuraminate lyase (NAL) family of enzymes. The DHDPS/NAL family of enzymes are Class I aldolases with a (β/α)₈ protein fold and conserved Schiff-base forming lysine residue (Choi et al., 2006).

Most, but not all members of the DHDPS/NAL family use pyruvate as either a substrate or product (Bunker, 2010).

Human HOGA (hHOGA) has relatively low homology with bacterial forms of DHDPS (Riedel et al., 2011). Phylogenetic comparison of HOGA with selected members of the DHDPS/NAL family (Figure 2), as well as the Class I aldolase KDPGA (which is not a member of the DHDPS/NAL family but catalyzes the similar reactions as HOGA) shows that HOGA is most closely related to DHDPS from Thermus thermophilus (pairwise identity 33 %), and the structure of this enzyme was used to solve the crystal structure of HOGA using molecular replacement (Bunker, 2010).

DHDPS is a bacterial enzyme involved in lysine synthesis and sialic acid metabolism, neither of which occur in humans. DHDPS therefore represents a potential antibiotic drug target (Mitsakos et al., 2008). HOGA does not retain DHDPS or NAL activity (Bunker, 2010). Many members of the DHDPS/NAL family are not found in humans; only N-acetylneuraminate lyase is present in humans (Wu et al., 2005). Reactions catalyzed by HOGA, DHDPS, KDGA and KDPGA are shown in Figure 3.
1.1.3.1 2-keto-3-deoxygluconate aldolase (KDGA)

Bacterial 2-keto-3-deoxygluconate aldolase (KDGA) displays less amino acid homology to HOGA than DHDPS, but more similarity in its substrate/reaction site chemistry, catalyzing the reversible cleavage of 2-keto-3-deoxygluconate or 2-keto-3-deoxygalactonate into pyruvate and D-glyceraldehyde via Schiff base formation (Theodossis et al., 2004). KDGA from Desulfotomaculum alkaliphilum displays 29% pairwise identity to HOGA and contains the same essential catalytic residues K196 and Y168 (Riedel et al., 2011). Additionally, KDGA can substitute glyoxylate for D-glyceraldehyde in the condensation reaction with pyruvate at an appreciable rate (40% for S. acidocaldarius and 77% for S. solfataricus versus the condensation rate with D-glyceraldehyde), which is the same condensation reaction as occurs with HOGA (Wolterink-van Loo et al., 2007).

1.1.3.2 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPGA)

The trifunctional bacterial enzyme 2-keto-3-deoxy-6-phosphogluconate aldolase (KDPGA) catalyzes the reversible cleavage of 2-keto-3-deoxy-6-phosphogluconate, in addition to the same reactions as HOGA: the reversible cleavage of HOG and decarboxylation of oxaloacetate. HOGA can form a Schiff-base with the KDPGA substrate 2-keto-3-deoxy-6-phosphogluconate (Kobes & Dekker, 1971b), and both enzymes show similar competitive inhibition with the same substrate analogs (Grady, Wang, & Dekker, 1981). While HOGA and KDPGA have similar active site stereochemistry, the enzymes differ in that: (1) HOGA is in the DHDPS/NAL family while KHG/KDPGA is not; (2) there is little apparent sequence or structural homology between families; (3) HOGA is a homotetramer while KDPGA is a homotrimer; and (4) KDPGA is stereoselective for (4S)-4-hydroxy-2-oxoglutarate, while HOGA exhibits no stereoselectivity (Nishihara & Dekker, 1972).
Hydroxyproline is not detectable in bacteria (Gupta & Dekker, 1984), therefore it may seem odd that bacterial KDPGA possesses HOG aldolase activity, which is involved in the hydroxyproline catabolism, as well as oxaloacetate decarboxylase activity. However, there is evidence that KDPGA is important in regulating cellular glyoxylate levels (Gupta & Dekker, 1984). KDPGA condenses glyoxylate and pyruvate to HOG, which is then converted to oxaloacetate through the concerted actions of α-ketoglutarate dehydrogenase, citrate synthase, and malate dehydrogenase. Oxaloacetate is then decarboxylated to pyruvate by KDPGA.

In support of this, KDPGA-catalyzed oxaloacetate decarboxylation and glyoxylate-pyruvate condensation reactions occur at similar rates (Carnie, Rowsell, Dabbaghian, Hobbs, & Rowsell, 1982; Grady et al., 1981), supporting an equal contribution of these activities for detoxifying one molecule of glyoxylate. This is in contrast to HOGA, which cleaves oxaloacetate at 10-50 % the rate of HOG (discussed in Section 1.5.2).
Figure 3  Reversible reactions of Class I aldolases with similar structures and functions to HOGA. (A) HOGA reaction (non-stereoselective); (B) two reactions catalyzed by structurally dissimilar KHG/KDPGA, not in the DHDPS/NAL family. The upper reaction is the same as HOGA but stereoselective for (4S)-4-hydroxy-2-oxoglutarate; (C) KDGA in the DHDPS/NAL family performs a similar reaction to KDPGA; and (D) DHDPS, which is structurally the most similar to HOGA and yet performs the most dissimilar reaction, as it favours the reverse condensation reaction. * HOGA and KDPGA also catalyze the decarboxylation of oxaloacetate, discussed in section 1.5.2.
1.2 Mitochondrial localization

HOGA localizes to mitochondria. Residues 1-25 of HOGA encode a mitochondrial targeting sequence (Bunker, Loomes, & Baker, 2011), and confocal microscopy of HOGA-transfected cells show an overlay of HOGA (GFP-tagged or labelled with anti-HOGA antibody) and mitochondrial signals (Mitotracker dye or cytochrome-c, Figure 4) (Beck et al., 2013; Riedel et al., 2012).

**Figure 4** Confocal microscopy of cos cells transiently transfected with human *hoga1* transcript. Labelled for (a) HOGA with fluorescent anti-HOGA antibody; (b) mitochondria with mitotracker, which accumulates dependent on mitochondrial membrane potential; (c) an overlay of a and b indicating that HOGA is localized to mitochondria. Reprinted by permission from Macmillan Publishers Ltd: *European Journal of Human Genetics* (2013) 21, 162–172; doi:10.1038/ejhg.2012.139, copyright © 2013.
1.3 Protein fold

The crystal structure of HOGA is a homotetramer made up of a dimer of dimers (Figure 5a) (Bunker, 2010; Riedel et al., 2011). Each monomer has an (α/β)_8 barrel-fold with three C-terminal α-helices (Figure 5b). The hydrophobic core of the monomer forms a solvent channel that opens up into the central core of the tetramer, which contains the active site Schiff-base forming lysine (K196). The monomers form a tetramer by two interfaces of interactions (Figure 5a). The major dimer interface between a/b (and c/d) consists of 44 residues from mainly loop regions of β-barrels, comprising hydrophobic and polar interactions. The minor dimer interface between a/c (and b/d) comprises 30 residues, predominantly hydrophobic interactions.

1.4 Quaternary structure

HOGA isolated from bovine kidney and liver forms a tetramer of 120-140 kDa, as shown with analytical ultracentrifugation-sedimentation equilibrium (AUC-SE), size exclusion chromatography (SEC) and sucrose density gradient centrifugation (Dekker & Kitson, 1992; Kobes & Dekker, 1969; Rosso & Adams, 1967). One study investigated the quaternary structure of human recombinant HOGA in solution and found an equilibrium between dimer and tetramer forms (K_D = 60 μM) using AUC-SE and SEC (Riedel et al., 2011). Based on this finding, the majority of HOGA used in enzymatic experiments in this study would be dimeric, and although the activity of dimeric vs. tetrameric hHOGA is unknown, the structurally similar DHDPS enzymes have marked differences in catalytic parameters between the dimeric and tetrameric forms (Griffin et al., 2008).
Figure 5  HOGA protein fold. (A) Tetrameric structure of HOGA. A dimer of identical dimers (a/b and c/d) make up the homotetramer, the major dimer interface occurs between a/b (and c/d). In (B), the monomer of subunit a can be observed looking down the centre of the β-barrel (purple), and three C-terminal α-helices in red.
1.5 Biochemical Function of HOGA

4-Hydroxy-2-oxoglutarate aldolase (HOGA, E.C. 4.1.3.16) catalyzes two reactions within mitochondria, the reversible aldolitic cleavage of 4-hydroxy-2-oxoglutarate (HOG) and the decarboxylation of oxaloacetate (Figure 1).

HOG aldolase activity is involved in hydroxyproline catabolism and the disease PH3 (section 1.6.2.3). The oxaloacetate decarboxylase activity of HOGA may putatively influence tricarboxylic acid (TCA) cycle turnover, gluconeogenesis and cancer cell metabolism (Section 1.7), due to its mitochondrial localization and involved metabolites, however there is currently little evidence for this.

1.5.1 HOG Aldolase activity of HOGA

HOGA is not stereo selective for HOG, with little difference found in $K_{M}$ or $V_{max}$ when comparing D-, L-, or DL-HOG (Kobes & Dekker, 1971a; Kobes & Dekker, 1971b; Lane & Dekker, 1969). The measured $K_{M}$ for HOG ranges from 11 $\mu$M (human recombinant enzyme), 26 $\mu$M (purified from bovine kidney) and 100 $\mu$M (purified from bovine liver) (Dekker & Kitson, 1992; Dekker, Lane, & Shapley, 1971; Riedel et al., 2011). The reaction is reversible but favours the direction of HOG cleavage, with measured equilibrium constants of 9-12 (Kobes & Dekker, 1971a; Maitra & Dekker, 1964; Rosso & Adams, 1967).

1.5.2 Oxaloacetate decarboxylase activity of HOGA

HOGA has a lower affinity for oxaloacetate compared to HOG, with a measured $K_{M}$ of 230 $\mu$M in rat liver (Dean & Bartley, 1973), and in bovine kidney 164 $\mu$M, which is 6 fold higher than the $K_{M}$ for HOG from the same preparation (26 $\mu$M) (Dekker & Kitson, 1992). There are conflicting reports in the literature about a mitochondrial oxaloacetate
decarboxylase, some crude preparations citing activity dependence on divalent metal cations Mg$^{2+}$ or Mn$^{2+}$ (Corwin, 1959; Morinaga & Shirakawa, 1971) and a lower pH optimum (pH 6.5-7.5, (Corwin, 1959; Wojtczak & Walajtys, 1974) than has been measured for pure HOGA. As a Class I aldolase, HOGA has no divalent metal cation requirement, and a pH optimum of 8.6-8.8 (Dekker & Kitson, 1992; Kobes & Dekker, 1971b; Maitra & Dekker, 1964). These crude preparations may have been contaminated by cytosolic or mitochondrial enzyme/s capable of decarboxylating oxaloacetate, for example malic enzyme (ME) or phosphoenolpyruvate carboxylase (PEPCK), which have a lower optimum pH and divalent metal cation requirement (Dean & Bartley, 1973). Regardless, pure HOGA isolated from mammalian liver or kidney decarboxylates oxaloacetate at an appreciable rate, 10-50% of the maximal rate of HOG cleavage (Dekker & Kitson, 1992; Dekker et al., 1971).

It is not known if the oxaloacetate decarboxylase reaction is reversible (pyruvate carboxylation). However, pyruvate carboxylation is energetically unfavourable and the only known enzyme to catalyze this, pyruvate carboxylase, requires ATP for catalysis.

1.5.3 Catalytic mechanism

HOGA is a pyruvate-lyase and the active site lysl residue of HOGA (K196A) has been crystallized as a Schiff base with pyruvate, but not yet with either substrate of HOGA, 4-hydroxy-2-oxoglutarate (HOG) or oxaloacetate (Bunker, 2010; Riedel et al., 2011). A simulation of HOG in the active site of HOGA is shown in Figure 6.

The catalytic mechanism for the aldolytic cleavage of HOG into glyoxylate and pyruvate by HOGA was proposed by Riedel et al. (2011) by combining biochemical studies of mutated active site residues and the elucidated crystal structure of human recombinant HOGA (hHOGA). Essential residues include K196 and Y168, which are conserved in all class I aldolases. Interestingly, Y140 was not essential for HOGA catalysis, in contrast to the closely related DHDPS enzymes (Dobson, Välegård, & Gerrard, 2004).
Briefly, HOG docks into the active site so that the 2-oxo-carbonyl group of HOG is adjacent to K196, involving hydrogen bond formation between HOG and S77, N78, S198 and G222 (Figure 6). A nucleophilic attack on the 2-oxo group of HOG by the ε-amino group of K196 forms a zwitterionic intermediate. Y168 and S77 form a proton relay causing proton addition followed by dehydration and Schiff-base formation with K196. The first product of the reaction, glyoxylate, is released concomitantly with a proton. Pyruvate is released after the remaining enamine tautomerizes, gains a water molecule, and loses a proton.

There is less literature on the catalytic mechanism of oxaloacetate decarboxylation. There is evidence that oxaloacetate is cleaved in the same way as HOG. Oxaloacetate is a competitive inhibitor of the HOG aldolase reaction (Ki=220 μM) (Anderson, Scholtz, & Schuster, 1985), implying it binds or has affinity for the HOG reaction site. Additionally, oxaloacetate decarboxylase activity is completely inhibited when the enzyme is previously incubated with either pyruvate or glyoxylate followed by treatment with sodium borohydride. Presumably, the resulting glyoxylate or pyruvate lysine adducts are irreversibly reduced to Schiff bases thereby preventing any further reaction with oxaloacetate (Kobes & Dekker, 1971a). This implies that the oxaloacetate decarboxylation is unable to occur because the reactive site lysine is unavailable.
1.5.4 HOGA inhibitors

Many analogs of pyruvate, glyoxylate and 4-hydroxy-2-oxoglutarate form Schiff-bases with the reactive ε-amino lysyl residue of HOGA purified from animal tissue (Dekker et al., 1971; Kobes & Dekker, 1971b). These analogs act as reversible competitive inhibitors of the HOG cleavage or condensation reaction by blocking the active site lysine, for example: bromopyruvate (Ki=18 μM); 2-ketobutyrate (Ki=31 mM); glyoxal (Ki=28 mM); and α-ketoglutarate (Ki=18 mM) (Anderson et al., 1985; Dekker et al., 1971; Grady et al., 1981).

The TCA cycle intermediates malate and succinate modestly inhibit hHOGA oxaloacetate decarboxylase activity at high concentrations (10-20 % at 5 mM) (Sabherwal, 2011). Acetyl CoA inhibited the oxaloacetate decarboxylase activity of HOGA isolated from
rat liver (Ki=50 µM) (Dean & Bartley, 1973), in contrast to human recombinant HOGA, which was not found to be inhibited by acetyl CoA (Sabherwal, 2011).

There is insufficient evidence for the inhibition of HOGA by divalent metal cations. In a continuous lactate-dehydrogenase (LDH)-coupled assay, human recombinant HOGA was inhibited by 50-100% with Ni^{2+}, Zn^{2+}, Mn^{2+} and Mg^{2+} (Sabherwal, 2011). HOGA isolated from bovine kidney was similarly inhibited with Mn^{2+} and Hg^{2+} (Dekker & Kitson, 1992). However, LDH is inhibited completely by the divalent metal cation Zn^{2+} at 10 mM (Dobryszycja & Owczarek), therefore inhibition of the coupling enzyme rather than inhibition of HOGA is also a likely mechanism. In support of this, there was no effect of the divalent metal cation Mg^{2+} on the oxaloacetate decarboxylation of rat liver in an assay not coupled to LDH (Wojtczak & Wałajtys, 1974).

1.5.5 Alternative HOGA substrates

There is evidence for HOGA catalyzed reversible cleavage of 4-hydroxy-2-ketobutyrate (HKB), a structural analog of HOG, to form pyruvate and formaldehyde, a structural analog of glyoxylate (Dekker et al., 1971; Hrubey, 1987). Similar to HOG aldolase activity, the forward cleavage reaction is favoured, however cleavage of HKB proceeds at only 1% of the rate of HOG cleavage, with a 30 fold higher K_M (3 mM vs 100 µM for HOG) and 3 fold lower V_max. The relevance of this is unknown, and there is no evidence that HKB is a physiological metabolite. However, formaldehyde is endogenously produced in humans and the blood concentration in healthy humans, monkeys and rats is around 100 µM and can be 2-4 fold higher in rat liver and nasal mucosa (Heck & Casanova, 2004). Additionally, 3C-substituted analogs of pyruvate can condense with glyoxylate in the condensation reaction, including oxaloacetate, 3-bromopyruvate, 2-ketoglutarate and 2-ketobutyrate (Scholtz & Schuster, 1984).
1.6 Metabolic pathways of HOGA

1.6.1 Hydroxyproline metabolism

The 4-hydroxy-2-oxoglutarate aldolase activity of HOGA plays a role in hydroxyproline catabolism. Hydroxyproline is a major component of collagen, and the turnover of endogenous collagen combined with a moderate dietary intake of animal protein results in an estimated >500 mg hydroxyproline per day (Jiang et al., 2012). Herbivores also have a significant intake of hydroxyproline, which is ubiquitous in the cell wall of plants and algae (Watanabe et al., 2012). Some animals require hydroxyproline for the production of other metabolites such as birds where hydroxyproline is necessary for glycine production. In ruminants, hydroxyproline is a significant source of pyruvate and glucose (Wu et al., 2011).

Hydroxyproline taken up by the mitochondria is metabolized by hydroxyproline oxidase, L-1-pyrroline-2-hydroxy-25-carboxylate dehydrogenase, aspartate amino transferase, and 4-hydroxy-2-oxoglutarate aldolase, releasing glyxoylate and pyruvate (Figure 7). The main form of glyoxylate removal is through (i) glyoxylate reductase (GRHPR), which converts glyoxylate to glycolate, and (ii) alanine:glyoxylate aminotransferase (AGTX), which converts glyoxylate to glycine in the peroxisomes (Figure 7). Glyoxylate can be oxidized in the mitochondria or cytosol to oxalate by lactate dehydrogenase (LDH), however oxalate, which is toxic in animals, cannot be further metabolized or stored. Disorders of glyoxylate metabolism, the “Primary Hyperoxalurias” are characterised by a build-up of oxalate, which precipitates with calcium resulting in kidney stone deposits, and extra-renal deposits depending on severity (Hoppe, 2012a).
1.6.2 The Primary Hyperoxalurias

The Primary Hyperoxalurias (PH) are rare autosomal recessive disorders of oxalate metabolism of which there are three recognized types (PH 1-3). Hyperoxaluria results from increased endogenous oxalate synthesis by the liver. The calcium salt of oxalate is insoluble, and excessive urinary oxalate causes recurrent urolithiasis (calculi or “stones” in the urinary system) and progressive nephrolithiasis (calcification of the kidney). Progressive renal damage leads to a decrease in glomerular filtration rate and chronic kidney disease. In severe cases, end-stage renal disease (ESRD) can eventually result. Additionally, the damaged kidney is less able to excrete oxalate, leading to the deposition of systemic calcium oxalate (oxalosis) in tissues such as the retina, myocardium, central nervous system, vessels, skin and bone (Hoppe, 2012a).

1.6.2.1 PH Type I

The most common form of PH is Type 1 (MIM 604296), accounting for 70-80% of all cases of PH with a prevalence of 1 to 3 per million population (Hoppe, Beck, & Milliner, 2009; Sonia, Justine, & Pierre, 2011). PH1 is the most severe form of Primary Hyperoxaluria, leading to ESRD in the first three decades of life, requiring combined liver/kidney transplantation in most cases (Hoppe, 2012a; Hoppe et al., 2009). PH1 is caused by deficient or mis-targeted activity of liver-specific peroxisomal AGTX, which normally transaminates (detoxifies) glyoxylate to glycine. AGTX deficiency causes glyoxylate to diffuse into the cytosol where it is both oxidized by cytosolic LDH to oxalate, and reduced to glycolate by GRHPR (Figure 7). The build-up of oxalate and glycolate is characteristic of PH1 (Danpure & Rumsby, 2004; Marangella, Petraruio, Vitale, Cosseddu, & Linari, 1992).
Figure 7  Glyoxylate metabolism indicating enzymes affected by the three forms of Primary Hyperoxaluria (PH 1-3). The main routes of glyoxylate removal through peroxisomal AGTX and mitochondrial GRHPR are perturbed in PH1 and PH2, respectively, leading to oxalate build up and calcium oxalate stone formation. The mechanism whereby HOGA mutations cause PH3 is currently unknown. HOGA: 4-hydroxy-2-oxoglutarate aldolase, GRHPR: Glyoxylate reductase/ hydroxy pyruvate reductase, LDH: Lactate dehydrogenase, AGTX: Alanine: glyoxylate amino transferase, GO: Glycolate oxidase, DAO: diamine oxidase, CaOx stones: Calcium-oxalate stones.
1.6.2.2 PH Type 2

PH2 accounts for roughly 10% of Primary Hyperoxaluria cases, although this number may be underestimated as some patients go undiagnosed due to the less severe clinical course (Hoppe, 2012a). PH2 involves a deficiency of the ubiquitously expressed glyoxylate reductase/hydroxy pyruvate reductase (GRHPR).

Decreased glyoxylate reductase activity of GRHPR in PH2 causes a build-up of glyoxylate, resulting in increased levels of oxalate levels similar to PH1. In contrast to PH1, decreased hydroxypyruvate reductase activity of GRHPR in PH2 leads to L-glycerate aciduria. Kidney transplantation in isolation is recommended over combined kidney-liver transplant for PH2 patients in renal failure as the enzyme deficiency is not liver specific (Hoppe, 2012b; Monico & Milliner, 2001).

1.6.2.3 PH Type 3

PH3 has an estimated prevalence of 5-10% of all Primary Hyperoxalurias (Moochhala & Unwin, 2014). This may be underestimated by an order of magnitude as the National Heart, Lung, and Blood institute (NHLBI) Exome Sequencing Project screened 5 known HOGA mutations and 10 additional predicted pathogenic HOGA mutations, and found a prevalence for hoga1 mutations of 1:135,866 (Hopp et al., 2015). This was similar to the prevalence of PH1 mutations, indicating that PH3 is underdiagnosed or not completely penetrant.

PH patients are generally diagnosed within the first decade of life, and for unknown reasons the clinical course appears to become less severe in adulthood (Hoppe, 2012a). There is only one documented case of ESRD in PH3, in an 8-year old patient heterozygous for two HOGA mutations (p.N103I and c.700+5G>T) (Hopp et al., 2015).
The cause of hyperoxaluria in these patients was unknown until a study published in 2010 provided evidence of an alteration at the level of 4-hydroxy-2-oxoglutarate aldolase (HOGA), referred to in the paper as DHDPS-Like due to its sequence similarity with bacterial DHDPS (Belostotsky et al., 2010). SNP microarray analyses and heterozygosity mapping of Ashkenazi Jew and European families with non-type 1/2 PH revealed a 0.6 MB fragment on chromosome 10. Within this region four Ashkenazi Jewish families contained a similar pattern of overlapping SNPs. The *dhdpsl* or *c10orf65* gene was selected as the most likely candidate from the 19 genes in this 0.6 MB region, based on its likely protein expression (liver/kidney) and function (glyoxylate metabolism). The product of the *dhdpsl* gene, now known as *hoga1*, was hypothesized to encode an enzyme possessing HOGA activity. Subsequently the kinetic parameters have been measured, and the crystal structure solved (Riedel et al., 2011).

### 1.6.2.3.1 Mutations identified in PH3 patients

To date, 26 *hoga1* mutations have been identified in patients with PH3 (Table 1) (Allard et al., 2015; Beck et al., 2013; Belostotsky et al., 2010; Hopp et al., 2015; Monico et al., 2011; Williams et al., 2012). The two most common *hoga1* mutations are the deletion of a glutamate residue (p.Glu315del or Δ315) and a splice site mutation resulting in the in-frame insertion of 51bp between exons five and six (c.700+5G>T).

The estimated allelic frequency of the c.700+5G>T and Δ315 mutations are 42-67 % and 24 %, respectively (Beck et al., 2013; Hopp et al., 2015; Riedel et al., 2012; Williams et al., 2012), with an apparent founder effect for the Δ315 mutation in Ashkenazi Jews (Belostotsky et al., 2010). A suggested diagnostic test for PH3 involves sequencing exons 5-7, which would include both the Δ315 and c.700+5G>T mutations (Williams et al., 2012). The other variants are much less common, accounting for 1-5 % of all PH3 mutations, some detected in only one family or individual (Table 1).
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<td>Active site</td>
<td>(Monico et al., 2011)</td>
</tr>
<tr>
<td>c.209G&gt;C</td>
<td>p.Arg70Pro</td>
<td>Active site</td>
<td>(Belostotsky et al., 2010)</td>
</tr>
<tr>
<td>c.569C&gt;T</td>
<td>p.Pro190Leu</td>
<td>Active site</td>
<td>(Monico et al., 2011)</td>
</tr>
<tr>
<td>c.227G&gt;A</td>
<td>p.Glu76Asp</td>
<td>Active site</td>
<td>(Hopp et al., 2015)</td>
</tr>
<tr>
<td>c.208C&gt;T</td>
<td>p.Arg70X</td>
<td>Truncated</td>
<td>(Williams et al., 2012)</td>
</tr>
<tr>
<td>c.117C&gt;A</td>
<td>p.Tyr39X</td>
<td>Truncated</td>
<td>(Williams et al., 2012)</td>
</tr>
<tr>
<td>c.346C&gt;T</td>
<td>p.Gln116X</td>
<td>Truncated</td>
<td>(Beck et al., 2013)</td>
</tr>
<tr>
<td>c.3G&gt;A</td>
<td>p.Met1&gt;Ile</td>
<td>Prevention of translation</td>
<td>(Allard et al., 2015)</td>
</tr>
<tr>
<td>c.289C&gt;T</td>
<td>p.Arg97Cys</td>
<td>Unknown</td>
<td>(Belostotsky et al., 2010)</td>
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<tr>
<td>c.107C&gt;T</td>
<td>p.Ala36Val</td>
<td>unknown</td>
<td>(Williams et al., 2012)</td>
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<td>c.839C&gt;T</td>
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</tr>
<tr>
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<tr>
<td>c.308A&gt;T</td>
<td>p.Asni103Ile</td>
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<td>(Hopp et al., 2015)</td>
</tr>
<tr>
<td>c.973G&gt;A</td>
<td>p.Glu325Ser</td>
<td>unknown</td>
<td>(Hopp et al., 2015)</td>
</tr>
</tbody>
</table>
1.6.2.3.2  Postulated effects of mutations on glyoxylate/oxalate metabolism

PH3 patients have elevated urinary and plasma oxalate concentrations (Monico et al., 2011; Williams et al., 2012) and 4-hydroxy-2-oxoglutarate (Belostotsky et al., 2012; Riedel et al., 2012), normal glyoxylate and L-glycerate (Monico et al., 2011), normal or sometimes raised glycolate (Monico et al., 2011; Williams et al., 2012) and intermittent hypercalcuria (Beck et al., 2013). The mechanism leading to hyperoxaluria in PH3 is still unclear, however initial studies have gained insight into the process.

Initially, the “loss of regulation/increased activity” theory proposed that mutations to HO\textsubscript{A} could increase the aldolytic cleavage of 4-hydroxy-2-oxoglutarate (HOG), resulting in a build-up glyoxylate and subsequently oxalate (Belostotsky et al., 2010). However, this mechanism is unlikely as 4 nonsense mutations have been found in PH3 patients, leading to a truncated protein that would be incapable of catalysis, and the finding of a missense mutation in the start codon that likely prevents initiation of translation (Table 1).

Furthermore, the clinical finding of normal glyoxylate and raised 4-hydroxy-2-oxoglutarate concentrations in PH3 patients supported a mechanism involving substrate build-up through loss of HO\textsubscript{A} activity in PH3 mutants (Monico et al., 2011).

The second theory proposed that HOG could leak from the mitochondria into the cytosol where an unspecific aldolase putatively cleaves HOG into glyoxylate, and subsequent oxidation to oxalate (Salido, Pey, Rodriguez, & Lorenzo, 2012). However, it has been debated whether this could in fact result in hyperoxaluria as the resulting glyoxylate should be detoxified normally by GRHP\textsubscript{R} and AGTX (Figure 7). In addition, the most likely cytosolic aldolase to cleave HOG to glyoxylate, NAL (Section 1.1.3), was found to cleave HOG at such a slow rate that it would be unlikely to cause hyperoxaluria (Riedel et al., 2012). Consequently, a third theory has been proposed that 4-hydroxy-2-oxoglutarate inhibits the glyoxylate-detoxifying enzyme glyoxylate reductase. In this theory, decreased activity of HO\textsubscript{A} leads to a build-up of mitochondrial HOG, which could in turn inhibit
GRHPR. The decreased activity of GRHPR could result in a build-up of glyoxylate and its oxidation product oxalate, and in support of this theory, Reidel et al. (2012) found a $K_i$ of 1.8 mM for HOG inhibition of human recombinant GRHPR with partial mixed type inhibition.

### 1.6.2.3.3 Effects of clinical PH3 mutations on HOGA structure

The speculated effects on protein structure are listed for some mutations in Table 1. These are logical conclusions based on the known structure and amino acid properties but have not yet been confirmed by structural studies. The two most common mutations are located in positions that could affect dimerization and the tetrameric organization of the protein.

The deletion of E315 causes a frame shift in the final 11C terminal residues on $\alpha$-helix-17 (Figure 8). This frame shift would cause a rotation of helix 17, which is amphipathic, and affect the packing of helix 17 into helix 15, leading to partial unfolding of the protein. The other common mutation results in the insertion of 17 amino acids in the region of the minor dimer interface, which is likely to disrupt the dimerization of dimers. It is also quite possible that these added residues greatly affect the folding of the monomer, resulting in a non-functional and/or aggregated protein.

The other 24 less common mutations are mostly missense mutations, with 4 nonsense mutations and 1 splice site mutation. The truncation R255X results in the 3 C-terminal $\alpha$-helices not being transcribed, and likely affects dimerization, as these are key components in both dimer interfaces. The other truncation mutants (p.Arg70X, p.Tyr39X, Gln116X) are unlikely to be functional, as the essential active site lysine would not be transcribed. The other mutations include possible alterations to the active site, mutations that may affect protein folding or the quaternary structure of the protein, and one mutation likely to prevent translation (c.3G>A).
Figure 8  Quaternary structure of HOGA, showing the location of the common PH3 clinical mutations, p.A315 (red) and c.700+5G>T (orange).

1.7 Pyruvate metabolism

HOGA has a role in hydroxyproline catabolism as a 4-hydroxy-2-oxoglutarate aldolase, (Section 1.6.1), and putatively influences TCA cycle turnover as an oxaloacetate decarboxylase. Pyruvate is a product of both HOGA-catalyzed reactions, and this thesis will focus on the metabolic role of HOGA in the contexts of pyruvate synthesis, and how HOGA may integrate with mitochondrial metabolism.

Pyruvate has roles in multiple metabolic pathways (Figure 9). In the cytosol, glucose breakdown (glycolysis) yields pyruvate where it may fuel anaerobic lactate production by lactate dehydrogenase, undergo transamination to alanine, or enter
mitochondria via the monocarboxylate transporter. In the mitochondrion, pyruvate may fuel (1) the tricarboxylic acid (TCA) cycle for oxidative ATP production; (2) or support anaplerosis of the TCA cycle intermediate oxaloacetate, which may fuel glucose production in gluconeogenic organs. Pyruvate may also be formed from malate within the mitochondria or cytosol by malic enzyme (ME, section 1.7.3). The cycling of pyruvate between the mitochondria and cytosol is thought to be a regulator of metabolism (section 1.7.4). Pyruvate metabolism can become perturbed in diseases such as cancer, and multiple anti-cancer drugs are being developed or in use targeting pathways involving pyruvate (section 1.7.7.3).

1.7.1 The TCA cycle

The TCA cycle provides reducing equivalents for the electron transport system (ETS) which produces the majority of cellular ATP requirements through oxidative phosphorylation. Citrate synthase (CS), which is the rate-controlling TCA cycle enzyme, condenses oxaloacetate and acetyl CoA to form citrate (Figure 9). TCA cycle flux is dependent on the cellular energy state, and CS is inhibited by ATP and NADH (Nunes-Nesi, Araújo, Obata, & Fernie, 2013) and its product citrate (Smith & Williamson, 1971). Oxaloacetate is a potent inhibitor of TCA cycle enzymes, predominantly Succinate Dehydrogenase (SDH) (Pardee & Potter, 1948; Wojtczak, 1969; Wojtczak, Wojtczak, & Ernster, 1969). Other regulators of the TCA cycle include ATP which inhibits isocitrate dehydrogenase, and calcium which stimulates αKG dehydrogenase and NAD-dependent isocitrate dehydrogenase (Tarasov, Griffiths, & Rutter, 2012).
Figure 9  Pathways involving pyruvate metabolism and potential roles of HOGA. These pathways include the TCA cycle, gluconeogenesis (dotted arrows), and pyruvate cycling (grey arrows). ME=malic enzyme, PC=pyruvate carboxylase, PEPCK=phosphoenolpyruvate carboxylase, PEP=phosphoenolpyruvate, HOGA=4-hydroxy-2-oxoglutarate (HOGA), PK=pyruvate kinase, MDH=malate dehydrogenase.
1.7.1.1 Malate in the malate-aspartate shuttle

The malate-aspartate shuttle allows oxaloacetate to indirectly enter the mitochondrial matrix, and also has a net redox effect, resulting in the production of NADH in the matrix and production of NAD+ in the cytosol (Figure 10). Malate is imported into the matrix via anti-port with αKG. Once in the cytosol, malate is oxidized to oxaloacetate by malate dehydrogenase, and NAD+ is reduced to NADH. This is the way that oxaloacetate enters the TCA cycle; oxaloacetate cannot be imported directly into the matrix. Oxaloacetate is converted to aspartate by aspartate aminotransferase, using the nitrogen donor glutamate, which is converted to αKG. Then aspartate exits the matrix via anti-port with glutamate, and in the cytosol aspartate aminotransferase performs the reverse reaction to regenerate oxaloacetate and glutamate.
Figure 10  The malate-aspartate shuttle, required to indirectly import oxaloacetate from the cytosol to the mitochondrial matrix.
1.7.2 The Electron Transport System

The electron transport system (ETS) in the inner mitochondrial membrane drives proton translocation from the matrix across the inner mitochondrial membrane into the intermembrane space. The proton accumulation mediates a charge and concentration gradient, or proton motive force, which mediates the production of ATP by oxidative phosphorylation (Figure 11). The reducing equivalents NADH and succinate from the TCA cycle provide electrons to Complex 1 (C1; NADH:ubiquinone oxidoreductase), and Complex 2 (C2; succinate dehydrogenase), respectively, which are passed through the electron carriers ubiquinone, Complex 3, and cytochrome-c, and finally reduce molecular oxygen (O$_2$) to form water (H$_2$O) at Complex 4 (Cytochrome-c oxidase). The simultaneous pumping of protons from the matrix to the inter-membrane space builds an electrochemical gradient to drive ATP synthesis by the F$_0$F$_1$ ATP synthase.
Figure 11  The electron transport system (ETS) of mitochondria. The pumping of protons into the inter-membrane space is coupled to electron transport to Complex IV, where oxygen is reduced to water. F$_0$F$_1$ ATPase harnesses the energy from the proton gradient to phosphorylate ADP. Note that protons are pumped or transferred at C1, C3 and C4, but not C2. C1 = Complex 1 or NADH-ubiquinone reductase, C2=succinate dehydrogenase, ub=ubiquinone, cyt-c=cytochrome-c, C3=coenzyme Q: cytochrome-c oxidoreductase, C4=Cytochrome-C oxidase.

1.7.2.1 Methods in mitochondrial oximetry

Oxidative metabolism is commonly studied by measurement of oxygen uptake/flux by tissues, isolated mitochondria, permeabilized cells and tissues. Different substrates, inhibitors and uncoupling agents can be informative of various pathway activities and the use of multiple substrates to reconstitute the TCA cycle provides an estimate of maximal flux or proportions of flux through different pathways. For example, the fractional contributions of Complex 1 (C1) and/or Complex 2 (C2) flux to the ETS and oxidative phosphorylation.

To measure C1 respiration in substrate-depleted mitochondria, a combination of malate and pyruvate or other NADH-linked TCA cycle intermediates is often added at saturating concentrations (Gnaiger, 2008, 2009). Malate is seldom added to substrate-depleted mammalian mitochondria alone, as oxaloacetate levels are expected to build-up due
to the lack of acetyl CoA, which citrate synthase would normally condense together with oxaloacetate to form citrate. Moreover, oxaloacetate inhibits C2 (Wojtczak et al., 1969). Few studies investigate malate as a single respiratory substrate in the ADP-fuelled state, and results vary markedly across species, tissues, and diseases such as cancer. Healthy mammalian tissues respire to a limited extent on malate alone, with skeletal muscle reportedly respiring at only 1.3 % of the flux generated with pyruvate and malate combined (Jackman & Willis), or 12 % of malate and glutamate (Elustondo et al., 2013). Heart mitochondria respire at 22 % of malate and pyruvate flux (Tarjan, 1971), and brain mitochondria 22 % of glutamate and malate (D'alecy, Myers, Brewer, Rising, & Shlafer, 1986).

Some species oxidize malate preferentially, and malate alone is able to provide maximal C1 flux in some plants (Coleman & Palmer, 1972), insects (Chamberlin & Phillips, 1983) and cold-adapted fish and crustaceans (Iftikar, MacDonald, Baker, Renshaw, & Hickey, 2014; Skorkowski, 1988). Immortalized cell lines and cancer cell lines respire well on malate alone, including Chinese hamster ovary (CHO) cells (Wahrheit, Nonnenmacher, Sperber, & Heinzle, 2015), L-1210 Ascites tumour cell mitochondria (Hansford & Lehninger, 1973), and mouse ascites tumour mitochondria (Sauer & Dauchy, 1978). Why some tissues, species, and various disease states vary in their ability to respire on malate alone has been mainly attributed to malic enzyme (ME).

1.7.3 Malic Enzyme

Malic enzyme (ME) oxidatively decarboxylates malate to pyruvate with the concomitant reduction of NAD+/NADP* to NADH/NADPH. There are three isoforms of malic enzyme, cytosolic ME1 and mitochondrial ME2 and ME3. ME1 and ME3 (E.C. 1.1.40) are similar, with specificity for NADP* and low K_M for malate. ME2 (E.C. 1.1.38) can utilize either NADP* or NAD*, with a higher affinity for NAD*, displays a higher K_M for
malate, and allosteric activation by fumarate and inhibition by ATP (MacDonald, Longacre, & Kendrick, 2009; Yang, Lanks, & Tong, 2002).

The malic enzyme reaction is reversible (pyruvate $\rightarrow$ malate), however it is widely thought to be in the direction of pyruvate formation in vivo (Veech, Eggleston, & Krebs, 1969). ME could fuel C1 of the ETS in two ways. Firstly, mitochondrial ME2 activity produces NADH, which could directly fuel C1, and secondly, ME could potentially allow the TCA cycle to turnover malate by producing pyruvate as a source of acetyl CoA. Acetyl CoA then should condense with the oxaloacetate produced from malate by malate dehydrogenase. This effectively reconstitutes the TCA cycle through the combined activities of malate dehydrogenase, ME, and pyruvate dehydrogenase (Figure 9).

1.7.3.1 ME and malate respiration

There is evidence that ME allows mitochondria to respire on malate alone, including inhibition studies, knockout studies and an association with ME activity and malate oxidation. Hydroxymalonate is an inhibitor of ME, and hydroxymalonate inhibited malate oxidation in rat heart mitochondria (Hiltunen & Davis, 1981) and cod heart mitochondria (Skorkowski, Aleksandrowicz, Ŝcisłowski, & Świerczyński, 1984), yet had no effect on combined malate and pyruvate oxidation. These results should be interpreted with caution, as hydroxymalonate is not a specific inhibitor, and has been shown to inhibit malate dehydrogenase (Emyanitoff & Kelly, 1982) and pyruvate oxidation (Pardee & Potter, 1949) at similar concentrations used in the ME inhibition studies.

ME2 knockdown in A549 non-small cell lung cancer cell lines resulted in malate accumulation, and decreased pyruvate, ATP, and triacylglycerol concentrations (Ren et al., 2014), and altered redox state in K562 erythroleukemia cells by increasing the NAD$^+$/NADH and NADP$^+$/NADPH ratios (Ren, Seth, Everett, Clish, & Sukhatme, 2010) (Ren et
al., 2014; Ren et al., 2010). ME2 overexpression is a common transition in some tumour types (discussed in section 1.7.7.2), for example hepatomas increase ME activity 10-20 fold, and ME activity is positively correlated with mitochondrial pyruvate production (Sauer, Dauchy, Nagel, & Morris, 1980).

1.7.4 Pyruvate cycling

Pyruvate cycling describes a loop of intracellular conversions of pyruvate between the mitochondria and cytosol and is sometimes described as “futile pyruvate cycling”. Pyruvate cycling has been measured by radiolabelling or NMR and reported as pyruvate kinase (PKM) plus ME flux relative to TCA cycle flux (Citrate synthase or CS). A process whereby pyruvate is formed from PEP, or malate through PKM and ME, could effectively dampen gluconeogenesis, as these intermediates could otherwise have contributed to glucose production (Figure 9). Labelling studies have shown that pyruvate cycling can be higher than TCA cycle flux; for example PKM+ME/CS is 1.2-3.2 in fasted rats (Jin, Beddow, Malloy, & Samuel, 2013; Jin et al., 2004), 3.5 in fasted elephant seals (Champagne, Houser, Fowler, Costa, & Crocker, 2012), and 1.4-5 in fasted humans (Befroy et al., 2014; Jones, Solomon, Cole, Sherry, & Malloy, 2001)

There is evidence that pyruvate cycling is increased in the obese/diabetic state, and may be a regulator of gluconeogenesis. Obese female cats, for example, display higher pyruvate cycling and TCA cycle flux, as well as increased fat oxidation than obese male cats, and they also show less risk of developing type 2 diabetes mellitus than obese male cats (Kley et al., 2009). Zucker Diabetic Fatty (ZDF) rats have increased pyruvate cycling and decreased gluconeogenesis relative to lean controls, and though endogenous glucose production is increased in these animals, this is attributed to a nine-fold increase in glycogenolysis (Jin, Park, Sherry, & Malloy, 2007). ZDF rats also have increased activity of
pyruvate cycling enzymes (malic enzyme and pyruvate kinase) (Jin, Burgess, Merritt, Sherry, & Malloy, 2005).

1.7.5 Oxaloacetate decarboxylase and pyruvate cycling

The existence of a mitochondrial oxaloacetate decarboxylase in a pyruvate cycling role has been alluded to in the literature. Oxaloacetate decarboxylase activity has been measured in mitochondrial fractions, however it is difficult to determine whether oxaloacetate decarboxylase contributes to pyruvate cycling in vivo, as other enzymatic pathways can link oxaloacetate to pyruvate. The main recognised pathway of oxaloacetate conversion to pyruvate involves the combined actions of PEPCK (oxaloacetate→PEP) and PKM (PEP→pyruvate, Figure 9). Additionally, there is evidence that ME1 and ME2 can decarboxylate oxaloacetate, however most measurements have been done at non-physiological low pH (4-5) (Norden & Matanganyidze, 1977; Ochoa, Mehler, & Kornberg, 1948; Weeda, 1981; Yamaguchi, 1979). Lastly, oxaloacetate decarboxylase activity of HOGA may potentially contribute to this cycling, however NMR studies using $^{13}$C and $^2$H$_2$O tracers have only included estimations of ME and (PEPCK)+PK. Early $^{13}$C studies in rat kidney cortex and hepatocytes suggest the possibility of an oxaloacetate decarboxylase, upon finding an inhibitor of PEPCK, 3-mercaptopicolinate, was unable to completely inhibit pyruvate cycling (Janssens, Hems, & Ross, 1980; Rognstad, 1979). However, this oxaloacetate decarboxylase activity was attributed to ME or PEPCK.

1.7.6 Gluconeogenesis

Gluconeogenesis, the production of glucose from non-carbohydrate precursors, is stimulated by the hormone glucagon and inhibited by insulin. In gluconeogenic tissues there is an up-regulation of gluconeogenic enzymes such as pyruvate carboxylase (PC) and PEPCK, fructose-1,6-bisphosphatase and glucose-6-phosphatase (Wallace & Barritt, 2005).
The first committed step of gluconeogenesis is catalyzed by pyruvate carboxylase (PC), which catalyzes the irreversible ATP-consuming carboxylation of pyruvate to oxaloacetate in the mitochondrial matrix, essentially the reverse reaction of HOGA-catalyzed oxaloacetate decarboxylation (Figure 9).

1.7.6.1 The gluconeogenic inhibitor FR225654 and HOGA

Evidence for the involvement of HOGA in gluconeogenesis comes from the patent literature. A fungal compound FR225654 (Figure 12) isolated from the fungus Phoma No. 00144 was identified as a novel inhibitor of gluconeogenesis and was subsequently found to bind HOGA specifically and irreversibly (Nakajima, Ohkubo, Yoshimura, Nishio, & Nishio, 2008).

FR225654 inhibits glucose production both in vitro and in vivo. In primary cultured rat hepatocytes, the IC$_{50}$ for FR225654 inhibition of gluconeogenesis was 0.11 µM, and in two mouse models of diabetes (streptozotocin-induced and db/db), plasma glucose was significantly lowered with FR225654 in a dose-dependent manner, with no effect on insulin levels (Ohtsu, Sasamura, Shibata, Hino, & Nakajima, 2005a; Ohtsu et al., 2005b). No effect on glycogenolysis or key enzymes in gluconeogenesis was apparent, and it was concluded that the anti-gluconeogenic effects of FR225654 occurred downstream of fructose bisphosphate aldolase.

HOGA was irreversibly bound to FR225654 following avidin-biotin chromatography in rat liver cells undergoing gluconeogenesis (Nakajima et al., 2008). The protein was denoted “35 kD protein,” and following mass spectrometry to determine its amino acid sequence, the protein was expressed recombinantly. The 35 kDa protein possessed Class I aldolase activity, and the expression of the protein is limited to liver and kidney, leading to the conclusion that the protein is involved in gluconeogenesis.
In summary, HOGA may be able to reverse the first step of gluconeogenesis catalyzed by ATP-dependent pyruvate carboxylase (pyruvate $\rightarrow$ oxaloacetate) potentially creating a futile energy wasting cycle. The gluconeogenic substrate $\alpha$-ketoglutarate inhibits HOGA, implying regulation of HOGA by this pathway. The gluconeogenic inhibitor FR225654 binds HOGA, though the significance of this is unknown.

1.7.7 Cancer cell metabolism

The metabolic alterations that occur in cancer cells, which allow high proliferative rates, include increased glucose and glutamine consumption. These metabolic changes promote nucleotide and lipid biosynthesis and anaerobic lactate production. These biosynthetic pathways deplete TCA cycle intermediates which can be replenished largely by glutamine and glucose (DeBerardinis, Sayed, Ditsworth, & Thompson, 2008) (Figure 13).

1.7.7.1 Glutaminolysis

Glutamine is the most abundant amino acid in the blood, and many cancer cells become dependent or “addicted” to glutamine, upregulating glutaminolytic flux and the glutaminolic enzymes glutaminase and malic enzyme (ME), Figure 13 (Daye & Wellen,
2012). Glutaminolysis integrates part of the TCA cycle with the malate/aspartate shuttle resulting in the production of pyruvate, lactate, alanine, ammonia, citrate, NADPH and nitrogen, fuelling lipid and nucleotide biosynthesis (DeBerardinis et al., 2008). Glutamine enters the TCA cycle through conversion to glutamate by glutaminase. Glutamate is then converted to α-ketoglutarate (αKG), which can either proceed through the TCA cycle to form oxaloacetate for citrate production and lipid biosynthesis, or malate can exit the cycle via mitochondrial malic enzyme (ME2), which oxidatively decarboxylates malate to pyruvate, and reduces NAD⁺/NADP⁺ to NADH/NADPH in the mitochondria. Alternatively, malate may exit the mitochondria for conversion to pyruvate by cytosolic malic enzyme (ME1).
Figure 13  The TCA cycle and pathways that increase during malignant transformation (dotted lines). Chemotherapeutic inhibitors are shown in blue (DCA=dichloroacetate, Q3S=quinolone-3-sulfonamide, NPD387, AZD-3965, TEPP-46). ME1/2=malic enzyme 1/2, HOGA=4-hydroxy-2-oxotutarate aldolase, gln=glutamine, glu=glutamate, PDCK=pyruvate dehydrogenase complex kinase, gln=glutamine, glu=glutamate.
1.7.7.2 Malic Enzyme (ME) and cancer

ME1 and ME2 are the major isoforms overexpressed in cancer (Jiang, Du, Mancuso, Wellen, & Yang, 2013), and down-regulation of ME1 or ME2 decreases glutamine uptake in a colon cancer cells (Ruiz-Perez et al., 2014). There is evidence that ME1 and ME2 are regulated by the tumour suppressor p53. p53 knockout in normal fibroblast and osteosarcoma cells increased ME1 and ME2 mRNA levels, protein expression, and enzymatic activity together with increased NADPH and triacylglycerol concentrations (Jiang et al., 2013).

ME2 may be more involved in tumour progression than ME1. ME2 knockdown resulted in a greater decrease in cellular NADPH and triacylglycerol levels compared to ME1 knockdown (Jiang et al., 2013), and during the progression to melanoma, ME2 mRNA and protein levels increased, while the expression of the other ME isoforms was unchanged (Chang et al., 2014).

ME2 knockdown and silencing studies support the role of ME2 in glutaminolysis and tumorigenesis. RNAi silencing of ME2 in erythroleukemic (K562) cells suppressed tumour formation in nude mice (Ren et al., 2014), and in vitro induced differentiation and apoptosis of erythroleukemic and non-small cell lung cancer (A549) cells (Ren et al., 2010). Glutaminolytic flux was suppressed with ME2 knockdown in vitro, resulting in malate accumulation, and decreases in pyruvate, ATP, and triacylglycerol concentrations (Ren et al., 2014; Ren et al., 2010). Recently, the novel molecule NPD-389 was identified as a potent, specific inhibitor of ME2. NPD-389 may offer a promising chemotherapeutic agent for glutamine-addicted tumour cells (Wen et al., 2014).

1.7.7.3 Pyruvate pathways targeted in cancer

Pyruvate is a key metabolite in cellular energy metabolism, involved in aerobic and anaerobic glycolysis, gluconeogenesis and glutaminolysis. Multiple chemotherapeutic agents
are targeted at pyruvate production and utilization, which often become dysfunctional in tumorigenesis (Figure 13).

Dichloroacetate (DCA) acts to increase the entry of pyruvate into the TCA cycle by inhibiting pyruvate dehydrogenase kinase, which allows pyruvate dehydrogenase complex (PDC) to remain active (Kankotia & Stacpoole, 2014; Michelakis, Webster, & Mackey, 2008).

Anaerobic flux of glycolysis can also be inhibited. LDH catalyzes the reversible reduction of pyruvate to lactate and simultaneous oxidation of NADH. The LDH-A isoform is upregulated in many glycolytic cancers (Chen, Lu, Garcia-Prieto, & Huang, 2007; Granchi, Fancelli, & Minutolo, 2014). Quinoline-3-sulfonamide (Q3S) is a potent inhibitor of LDH-A. Q3S inhibited lactate production in several cancer cell lines, and in highly glycolytic hepatocellular carcinoma Snu398 cells, decreased glucose consumption, increased oxygen utilization, and promoted apoptosis (Granchi et al., 2014). The monocarboxylate transporter-1 (MCT-1) is targeted by AZD-3965, which inhibits influx and efflux of cellular lactate and pyruvate, and is currently in phase 1 clinical trials (Chen et al., 2007; Granchi et al., 2014).

Pyruvate kinase (PKM) catalyzes the final step of glycolysis, the conversion of PEP to pyruvate, and many cancers upregulate the less active isoform PKM2 (Chen et al., 2007; Granchi et al., 2014). This may appear paradoxical, as generally glycolysis is upregulated in cancer cell metabolism, however slowing down the last step of glycolysis may allow glycolytic intermediates to build-up, which promotes biomass production through the pentose phosphate pathway. The small molecule TEPP-46 is an activator of PKM2, and inhibited xenograft tumor formation in nude mice, and decreased production of ribose phosphate and serine, products of the pentose phosphate pathway (Anastasiou et al., 2012).
1.7.7.4 HOGA

The only proteomic study identifying HOGA in cancer detected a 3-fold down regulation of HOGA in renal cell carcinoma (Siu et al., 2009).

1.8 Summary and scope of this thesis

The structure of human recombinant HOGA has recently been solved by two independent laboratories with similar findings. The quaternary structure of the human enzyme, however, has only been studied by one group, whose evidence suggests that in vivo, HOGA is a dimer and not a tetramer, which may have implications for catalysis. In Chapter 3, the quaternary structure of wild-type recombinant HOGA protein is characterized.

Mutations to the hoga1 gene have recently been implicated in Primary Hyperoxaluria Type 3 (PH3), however it is not yet understood how HOGA mutations cause disease. Initial evidence suggests a loss of function of HOGA. HOGA function will be further investigated in Chapter 4 with recombinant PH3 mutant HOGA proteins, and a stably transformed cell line induced to overexpress the PH3-mutant HOGA protein.

In addition to its involvement in hydroxyproline metabolism as a 4-hydroxy-2-oxoglutarate aldolase, HOGA has oxaloacetate decarboxylase activity. The kinetics of HOG aldolase and oxaloacetate decarboxylase activity are characterized in Chapter 5. Additionally, we show evidence that the TCA cycle intermediate α-ketoglutarate is a competitive inhibitor of the oxaloacetate decarboxylase activity of human recombinant HOGA, in agreement with kinetic studies of HOGA isolated from animal tissue.

As an oxaloacetate decarboxylase, HOGA could potentially be involved in pyruvate metabolism in vivo. HOGA oxaloacetate decarboxylase activity could allow mitochondria sustained respiration on malate, due to the concerted action of malate dehydrogenase
(malate $\rightarrow$ oxaloacetate), HOGA (oxaloacetate $\rightarrow$ pyruvate), and pyruvate dehydrogenase (pyruvate $\rightarrow$ acetyl CoA). Malate respiration is known to vary across species, tissues, and disease due to another enzyme, ME, which produces pyruvate from malate. In Chapter 6, the contribution of HOGA and ME to malate respiration is investigated in HEK cells overexpressing HOGA, and in animal tissues with high HOGA expression (liver, kidney) and low HOGA expression (brain, heart).
2. General Methods

2.1 Recombinant protein studies

2.1.1 Antibiotics, media and buffers

2.1.1.1 Antibiotics

Antibiotic stocks were made up in 1 ml aliquots and frozen at -20 °C then diluted 1:1000 for cloning, plasmid maintenance and protein expression (Table 2).

Table 2 Antibiotic formulations for molecular work

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<tr>
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<td>Cloning and Plasmid maintenance in <em>E. coli</em></td>
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</table>

2.1.1.2 Bacteria Culture Media

Culture media for plasmid maintenance and protein expression in *E. coli* were made in distilled water and autoclaved as described by Studier et al (Studier, 2005) (Table 3). For plated cultures, Lysogeny Broth (LB) agar was made by supplementing LB with 1.5 % agar.
### Table 3  
**Culture Media Formulations**

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB (Terrific Broth)</td>
<td>1.2 % Bacto Peptone, 2.4 % Yeast extract, 0.4 % (w/v) glycerol, 17 mM KH₂PO₄*, 72 mM K₂HPO₄*, 1 mM MgSO₄*, trace metals mix* (below)</td>
<td>Protein expression in BL21-DE3 <em>E. coli</em></td>
</tr>
<tr>
<td>LB-Lennox</td>
<td>1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl</td>
<td>Media used for plasmid midi-prep and plating</td>
</tr>
<tr>
<td>SOC</td>
<td>2 % Bacto Peptone, 0.5 % Yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂*, 10 mM MgSO₄*, 20 mM glucose*</td>
<td>Media used for transformation of <em>E. coli</em></td>
</tr>
<tr>
<td>ZYM-5052</td>
<td>1 % NZ Bacto peptone, 0.5 % yeast extract, 25 mM Na₂HPO₄, 25 mM KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄, 2 mM MgSO₄, 0.5 % glycerol, 0.05 % glucose, 0.2 % lactose, Trace metals mix (below)</td>
<td>Auto-induction of protein expression in BL21-DE3 <em>E. coli</em></td>
</tr>
<tr>
<td>Trace metals mix</td>
<td>50 µM FeCl, 20 µM MnCl₃, 10 µM Mn, 10 µM Zn, 2 µM Co, 2 µM Cu, 2 µM Ni, 2 µM Mo, 2 µM Se, 2 µM B</td>
<td>An additive in TB and ZYM-5052</td>
</tr>
</tbody>
</table>

*components added after media was autoclaved and cooled

### 2.1.1.3 Cell Lines

Four *Eschericia coli* cell lines were used in this study. Top10 chemically competent cells (Invitrogen) were used for plasmid maintenance and BL21 DE3 for protein expression (Invitrogen). The pRP plasmid encodes chloramphenicol resistance and contains several
tRNA genes. XL-1 Blue supercompetent cells (Stratagene) were used for site-directed mutagenesis, and Oneshot OmniMAX 2 T1 Phage-Resistant cells (Invitrogen) were used for Gateway cloning.

2.1.2 DNA manipulation and cloning

2.1.2.1 Plasmid DNA isolation

Plasmid DNA was isolated using a commercial “Miniprep” kit from Invitrogen, following the manufacturer’s instructions. DNA was eluted in either distilled water for sequencing, or TE (10 mM Tris-HCl pH 8, 0.1 mM EDTA) for subsequent experiments.

2.1.2.2 DNA gel electrophoresis

DNA gel electrophoresis was run in 1.2 % agarose in TAE buffer (40 mM Tris-Acetate, 1 mM EDTA). Samples were mixed 1:6 with DNA loading dye (30 % glycerol, 0.25 % bromophenol blue) and run alongside the molecular weight standard 1 kb plus ladder (Invitrogen). SYBR® Safe (Invitrogen) was diluted 10,000x and added to agarose before it set. DNA bands were visualized under UV light with an in-house gel documentation system (Biorad).

2.1.2.3 DNA concentration determination

DNA concentration was determined using a Nanodrop®ND-1000 spectrophotometer (Nanodrop technologies), which measures UV-visible absorption, and calculates DNA concentration using the Beer-Lambert Law.
2.1.2.4 DNA sequencing

DNA sequencing was done in house in the Centre for Genomics and Proteomics (GCP) lab in the School of Biological Sciences by Kristine Boxen.

2.1.2.5 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was carried out using Primestar HS Polymerase (Takara) using the 2-step protocol recommended by the manufacturers (Table 4), and buffer optimized for GC-rich templates. DNA primers (IDT technologies) were made up in distilled water. Reactions were generally supplemented with 4 % DMSO.

Table 4        Polymerase Chain Reaction cycling conditions

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Duration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>98 °C</td>
<td>10s</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Annealing and extension</td>
<td>68 °C</td>
<td>1min/kb</td>
<td></td>
</tr>
</tbody>
</table>

2.1.2.6 Transformation of chemocompetent cells

Generally, 1 µl of DNA was added to 50 µl of chemocompetent cells in a 15-ml round bottom tube on ice for 30 minutes. Cells were heat-shocked at 42°C for 45 seconds then 250 µl SOC (Table 3) was added and the cell suspension incubated on a rotating wheel
at 37 °C for 1h. Cells were then plated on LB plates containing the appropriate antibiotic for selection (Table 2).

2.1.2.7 Plasmid miniprep

Single colonies plated on Lysogeny Broth (LB) agar under antibiotic selection were picked using a sterile plastic loop, and inoculated into a 15 ml round bottom polypropylene tube containing 2 mL LB and the appropriate antibiotic for 24-48 hours at 37 °C on a rotating wheel. Plasmid DNA was then isolated from the cell suspension using a commercial plasmid miniprep kit.

2.1.2.8 Gateway Recombinant Cloning

Gateway® (Invitrogen) recombinant cloning was used to create most of the expression plasmids used in this study, using bacteriophage λ derived sequence-specific recombination as described by Moreland et al (Moreland et al., 2005). An entry vector is initially created using the BP recombination reaction (attB x attP → attL x attR), which can be used to shuttle the gene of interest into other destination vectors using the LR reaction (attR x attL → attB x attP, Figure 14). Generally, BP or LR reactions contained 50 fmol of DNA and 2 ul of BP or LR clonase made up to a final volume of 10 μl with TE. Reactions were incubated for two hours at 25 °C and terminated with 1 μl Proteinase K for 10 minutes at 37 °C.
2.1.2.9 *E. coli* expression plasmids

The *E. coli* expression plasmid for this project was cloned by Dr. Richard Bunker during his PhD using Gateway® Recombinant cloning (Bunker, 2010). These methods will be briefly described here. The “entry clone” vector was created containing the open reading frame (ORF) of *hoga1* using double-nested sequential PCR. The first round of PCR amplified the ORF using ORF-specific primer pairs containing recognition sites for the second round of PCR. This PCR product was purified and used as the template for the second round of PCR using “Generic” primers containing *att* sites for recombination and a human rhinovirus-14 3C protease recognition site. The PCR cycling conditions and primers are listed in Table 5 and Table 6, respectively. This PCR product was purified and inserted into the donor vector pDONR221 using BP clonase (BP reaction), which was then shuttled...
into the expression plasmid p-DESThisMBP using LR clonase (LR reaction), and screened for the desired insert. This insert was subsequently cloned into the pDEST-566 plasmid (Addgene 11517) by Dill Huang following difficulty with site-directed mutagenesis, possibly due to degradation of the template.
<table>
<thead>
<tr>
<th>Step</th>
<th>Duration (s)</th>
<th>Temperature (°C)</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First round</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation</td>
<td>120</td>
<td>94</td>
<td>1x</td>
</tr>
<tr>
<td>Denaturation</td>
<td>20</td>
<td>94</td>
<td>1x</td>
</tr>
<tr>
<td>Annealing</td>
<td>5</td>
<td>55</td>
<td>30x</td>
</tr>
<tr>
<td>Extension</td>
<td>100</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>180</td>
<td>72</td>
<td>1x</td>
</tr>
<tr>
<td><strong>Second round</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activation</td>
<td>120</td>
<td>94</td>
<td>1x</td>
</tr>
<tr>
<td>Denaturation</td>
<td>10</td>
<td>94</td>
<td>1x</td>
</tr>
<tr>
<td>Annealing</td>
<td>5</td>
<td>60</td>
<td>10x</td>
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<tr>
<td>Extension</td>
<td>100</td>
<td>72</td>
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<tr>
<td>Denaturation</td>
<td>10</td>
<td>94</td>
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<tr>
<td>Annealing</td>
<td>5</td>
<td>55</td>
<td>20x</td>
</tr>
<tr>
<td>Extension</td>
<td>100</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>180</td>
<td>72</td>
<td>1x</td>
</tr>
</tbody>
</table>
### Table 6  
Primer pairs for double-nested PCR and sequencing

<table>
<thead>
<tr>
<th>Primer pair (purpose)</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| **HOGA ORF specific**  | **Forward:**<br>5'-TTCCAAGGTCCGATGCTGGGTCCCCAAGTC-3’  
| (for first round of nested PCR) | **Reverse:**<br>5'-GAAAGCTGGGTGCTAGAGCAGCCAGCGTGTGCT-3’ |
| **Generic adaptors** | **Forward:**<br>5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCTCGAGGTACTCTTCCAAGGTCCG-3’  
| (for second round of nested PCR) | **Reverse:**<br>5'-GGGGACCACTTTGTACAAGAAAGCTGGGTG-3’ |
| **M13 primers** | **Forward:**<br>5'-GTAAAACGACGGCCAG-3’  
| (for sequencing) | **Reverse:**<br>5'-CAGGAAACAGCTATGACC-3’ |

2.1.2.10 Site-directed Mutagenesis

Point mutations were introduced into the *hoga*1 nucleotide reading frame in the entry vector pDONR-221 using overlapping primers (Table 7), with the exception of 700+5G>T and BirA mutants (see section 2.1.2.11). Primestar HS DNA polymerase was used for PCR with the PCR cycling conditions as set out in 2.1.2.4 with the exception that the number of cycles was limited to 14-16.

The original (unmutated) template DNA was then digested with 10 U DPN-1 (Invitrogen) for 2 hours at 37 °C. The restriction enzyme DPN-1 recognizes and cleaves methylated DNA, therefore digesting the template plasmid but leaving the PCR product intact. The DPN-digested PCR mix was transformed into XL-1 Blue Supercompetent cells (Stratagene) as detailed in 2.1.2.7, and plated on LB plates containing 50 μg/ml kanamycin.

To screen for clones containing the correct mutation, single colonies were grown in 2 ml LB containing 50 μg/ml kanamycin for two days at 37 °C on a rotating wheel, and plasmid DNA isolated using a commercial plasmid miniprep kit. In-house sequencing confirmed the mutated clone.
To transfer the mutated *hogaI* sequence from the entry clone (pDONR-221) into the expression clone (pDEST-566), the LR reaction was performed (2.1.2.5), using 2 µl LR clonase and 150 ng each of mutated pDONR-221 and pDEST-566. Following Proteinase-K termination, 1 µl of the LR reaction was transformed into One Shot OmniMAX 2T1 phage resistant cells (Invitrogen) as in 2.1.2.7 and cells plated on LB plates containing Ampicillin. Resultant colonies were grown in 2 ml LB for two days at 37 °C on a rotating wheel and plasmids isolated using a commercial plasmid miniprep kit. In-house sequencing confirmed the mutation in the pDEST-566 plasmid.
Table 7: Primers used for site-directed mutagenesis of the *hoga1* template

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Protein</th>
<th>Primers</th>
</tr>
</thead>
</table>
| Clinical (PH3) | Δ315    | **Forward** 5' - CCGCTGAGGAGGACTGCGCATGG - 3'  
**Reverse** 5' - CCGCAGTGCCCTGCTCAGCGG - 3' |
| Active-site | K196A   | **Forward** 5' - GAATATTGTGGCAGCGTGTTGG - 3'  
**Reverse** 5' - CACCACGCTGTGCCCATGCCCAACATATC - 3' |
| Active-site | Y168F   | **Forward** CCTGTGCTGTGCTCCACTGCTCCACCACAG  
**Reverse** GTCCGGGACTGAACAGCACCACAGG |
| Active-site | N78T    | **Forward** GGCTCCAGCGAGTTCCCTCCCT  
**Reverse** CTCGCACTGGACCTGGACCACGA |
| Active-site | S77A    | **Forward** CGTGGCCATGGGCAATGCGAGTT  
**Reverse** AACTCGCATTGGCCTGACCAGC |
| Active-site | S198A   | **Forward** GTGGGCATGAAGGACGCCGCTGTGATGAC  
**Reverse** GTCACTCACCCACCGCGTCTCATGCCCAC |
2.1.2.11 Round the horn Mutagenesis

Mutagenesis involving large insertions required a different approach. Two recombinant HOGA mutants required the insertion of 51 bp. The PH3 splice site mutant c.700+5G>T introduced 51 bp between c700 and 701 of the cDNA template, and the creation of biotin-tagged HOGA for ligand binding studies required the C-terminal addition of 51 bp, the “biotin acceptor peptide”. Long overlapping PAGE-purified 5’phosphorylated primers (IDT) were designed (Table 8). The forward primers containing the second half of the 51 bp insertion followed by 21-25 base pairs of the wild-type template, and the reverse primers containing the first half of the 51 bp insertion and 22 base pairs of the wild-type template (see Figure 15 for clarification of the method).

**Table 8** Primers used for large insertion mutagenesis

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH3 splice-site mutant c.700+5G&gt;T</td>
<td><strong>Forward</strong> 5’-GTCATGGGTGACCAAGAGATACCCAGGAGCTGTGGGGGGGTCTGCG-3’</td>
</tr>
<tr>
<td></td>
<td><strong>Reverse</strong> 5’-AATTTGAGACGATGGGCGGACTACCCCAAGGCATAGCTGGCCATCAG-3’</td>
</tr>
<tr>
<td>C-terminal biotin acceptor peptide</td>
<td><strong>Forward</strong> 5’-CTCAGAAATCGAATGCGACGAATTAACCCAGCTTTCTTGTACAAAGTGGT-3’</td>
</tr>
<tr>
<td>(HOGA-BAP)</td>
<td><strong>Reverse</strong> 5’-CTCAGAAGATGCTGCTAGGCAGCCACC*GAGCCAGCGTGGCTGGTGAAG-3’</td>
</tr>
</tbody>
</table>

The original HOGA template is underlined. *indicates the position where the stop codon on the original template C-terminus was omitted on the reverse primer. The bold italicised codon, TAA, is the new stop codon, immediately following the biotin acceptor peptide.

PCR was carried out using the 2-step Primestar protocol as outlined in 2.2.2.5 for 25 cycles, using the expression plasmid pDEST-556 containing wild-type HOGA sequence as template. The original template DNA was digested using 10 U DPN-I (Roche) for two hours at 37 °C. A commercial PCR clean-up kit (Invitrogen) was used to remove unwanted PCR
components (primers, DNA polymerase, salts, unincorporated dNTPs) prior to ligation. T4 DNA ligase (Invitrogen) was used to ligate the blunt ends using 150 ng DNA and 1 U T4 DNA ligase in the recommended volume of ligase reaction buffer for 20 hours at 14 °C. This ligation mix was used to transform chemocompetent Top10 cells (2.2.2.6), which were then plated on LB plates containing 100 µg/mL ampicillin. Resultant single colonies were grown in 2 ml LB and plasmid DNA isolated as described in 2.2.2.7. Plasmids were sequenced using the “generic adaptor” primers (Table 6) using in-house sequencing.

![Mutagenesis strategy](image)

**Figure 15** Mutagenesis strategy used for inserting 51 bp between residue 700 and 701 of the HOGA cDNA template.

### 2.1.3 Recombinant protein expression & purification

#### 2.1.3.1 Buffers

Buffer compositions used during protein purification are listed in Table 9. Buffers contained the reductant Tris(2-carboxyethyl)phosphine (TCEP), which was made up as a 0.5 M stock.
<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash buffer</td>
<td>50 mM Tris/HCl, 150 mM NaCl, pH 8.0</td>
</tr>
<tr>
<td>Lysis buffer</td>
<td>50 mM Tris/HCl, 150 mM NaCl, 40 mM EDTA, 0.5 mg.ml⁻¹ hen egg white lysozyme, 0.5 mM TCEP, pH 8.0</td>
</tr>
<tr>
<td>MBP Affinity Buffer</td>
<td>50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.5 mM TCEP, pH 8.0</td>
</tr>
<tr>
<td>MBP elution buffer</td>
<td>50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 10 mM maltose, 0.5 mM TCEP, pH 8.0</td>
</tr>
<tr>
<td>SEC buffer</td>
<td>50 mM Tris-HCl, 150 mM NaCl, 0.5 mM TCEP</td>
</tr>
</tbody>
</table>

### 2.1.3.2 Protein expression

Wild-type or mutant HOGA pDEST-556 plasmids were transformed in BL21 *E. coli* as described in 2.2.2.6 and colonies grown up in 2 L baffled flasks containing 500 ml of Terrific Broth (TB, Table 3), a rich broth optimized by Studier et al (2005) to increase protein yield. Ampicillin (100 μg.mL⁻¹) and chloramphenicol (34 μg.mL⁻¹) were added to select for pDEST-566 and the heat chaperone PrP, respectively. Cultures were grown overnight in flasks at 37 °C with shaking at 180 rpm followed by induction with 0.5 mM IPTG at 1 °C for 5-6 hours.
2.1.3.3 Cell harvesting

Cells were harvested immediately by centrifugation at 4000 rpm for 20 minutes at 4 °C. The pellet was re-suspended in 20 ml ice-cold TBS (Table 9), and spun again at 4000 rpm for 20 minutes at 4 °C. The pellet was immediately lysed, or stored at -20 °C.

2.1.3.4 Cell lysis & clarification

The cell pellet was resuspended in 5 ml g\(^{-1}\) lysis buffer (Table 9), and stirred at 4 °C for 30 minutes. Cells were lysed by sonication (Misonix Sonicator 3000) for 10 minutes using 0.5 s pulses at 3 W. To obtain the soluble fraction, the homogenate was spun at 16,500 rpm for 30 minutes at 4 °C. The soluble lysate was frozen at -20 °C at this point, as it was found that freezing reduced levels of the polydisperse aggregated protein in later purification steps. After defrosting, the lysate was again spun at 16,500 rpm for 30 minutes at 4 °C and filtered through 1 µM and 0.45 µM pore size membranes, respectively.

2.1.3.5 Maltose binding affinity & size exclusion chromatography

A simple two step purification was used to obtain MBP-HOGA fusion protein, using dextrin affinity and size exclusion chromatography (SEC) established by Richard Bunker (Bunker, 2010). The MBP fusion tag was utilized to separate MBP-HOGA from other soluble lysate proteins using two 5 ml MBP-Trap™ dextran affinity columns (GE Healthcare) in series. Ten volumes of MBP-affinity buffer was washed through the column before soluble lysate was loaded with a peristaltic pump. Affinity buffer was washed through the column until no protein was detected in the flow-through as detected by Bradford reagent. MBP-HOGA was eluted with elution buffer containing 10 mM maltose (Table 9). The eluate was centrifuged to remove large aggregates (16,100 xg, 4 °C, 30 minutes).
Size exclusion chromatography was then utilized to obtain a homogenous population of MBP-HOGA using a HiPrep™ 16/60 Sephacryl® S-300 R column (GE Healthcare). The homogenous peak was concentrated using Vivaspin® concentrators with a molecular weight cut-off of 10 kDa.

2.1.3.6 Cleavage of the fusion tag

HOGA was cleaved from the fusion tag MBP-his-3C along with the mitochondrial targeting sequence of HOGA using chymotrypsin, which cleaves at residue 26 of HOGA. The expression construct was initially created for 3C protease cleavage of the MBP tag from HOGA. However, full length HOGA was difficult to work with and was found to adsorb onto plastic-ware and consequently unable to be concentrated to greater than 1 mg.mL⁻¹ (Bunker, 2010). The optimization by Dill Huang (PhD student) to cleave HOGA using chymotrypsin led to a soluble protein that was able to be concentrated to >20 mg.mL⁻¹, presumably by removing the “sticky” mitochondrial targeting sequence.

Following SEC, MBP-HOGA was concentrated to 20 mg.mL⁻¹ and chymotrypsin added 1:5000 (i.e. 1 µg chymotrypsin: 5000 µg HOGA) from a 40 µg.mL⁻¹ chymotrypsin stock. This was incubated at 18 °C for 24 hours and gave complete cleavage of the fusion tag. The tag was separated from HOGA using SEC (Figure 17). This HOGA peak was collected and concentrated using Vivaspin® concentrators (MWCO 10 kDa).

In some cases, the requirement for very pure cleaved HOGA necessitated reverse MBP-purification to minimize any contaminating MBP-HOGA (e.g. anti-HOGA antibody generation in 2.2.4.3 and analytical ultra-centrifugation in 3.2.5). Dextran affinity purification proceeded as described in 2.2.3.5. However, the flow through was collected instead of the elution with maltose, as HOGA free from MBP tag displayed no affinity for the resin. Protein was concentrated using Vivaspin® 10 kDa concentrators and the concentration adjusted as required.
2.1.3.7 Protein Concentration

Protein concentration was estimated by one of two ways: (i) using the Beer Lambert law (Equation 1) and the theoretical extinction coefficient for the enzyme and the measured $A_{280}$; (ii) using Bicinchoninic acid. These two methods of measurement were comparable and were used consistently within one set of experiments.

**Equation 1:** Beer-Lambert Law

$$A = \varepsilon \cdot c \cdot l$$

Where $A$ is the absorbance of the protein solution at 280 nm, $\varepsilon$ is the theoretical extinction coefficient of the protein (109.125 M$^{-1}$cm for MBP-HOGA and 29.910 M$^{-1}$cm for cleaved HOGA, calculated using ProtParam software (Wilkins et al., 1999), $c$ is the protein concentration in molarity, and “$l$” is the pathlength (1 cm).

Where bicinchoninic acid (BCA) was used to measure protein concentration using a protein assay kit (Pierce), bovine serum albumin used as a standard. Copper (II) sulphate solution was mixed with BCA reagent and the combination mixed with 1:50 and 200 µl added per well of a 96 well microplate. 25 µl of BSA standard (125-1000 µg.mL$^{-1}$) or protein sample was then added to each well, and incubated at 37 °C for 30 minutes. The absorbance at 562 nm was read using a microplate reader (Spectramax 340PC, Molecular devices, USA), and the protein concentration calculated relative to the BSA standard curve.

2.1.4 Protein analysis

2.1.4.1 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed using NuPAGE® Novex® 4-12 % Bis-Tris 10 well gels (Invitrogen).

Generally, 15 µl protein sample was mixed with 4 µl of 4X NuPAGE LDS sample buffer (Invitrogen) with 1 µl of 0.5 M reducing agent Tris(2-carboxyethyl)phosphine (TCEP) and denatured by heating at 70 °C for 10 minutes. 7 ul of Seeblue2 Plus Pre-stained
Protein Standard (Invitrogen) was loaded along with 20 μl of sample and run for 35 minutes at 200 V in MES-SDS Running buffer (Invitrogen). Gels were washed three times for five minutes in distilled water and stained in SimplyBlue ® Safe Stain (Invitrogen). Background staining was minimized by rinsing the gel in distilled water.

2.1.4.2 Western Blot

SDS-PAGE was run as described in 2.2.4.1 with staining omitted. Recombinant protein was loaded at 0.4 μg and cell or tissue lysates loaded at 12.5-50 μg as determined by the BCA protein assay (2.2.3.7). Wet transfer was completed in transfer buffer (25 mM glycine, 192 mM glycine, 20 % methanol) for one hour at 30 V onto PVDF Invitrolon membrane using an X-Cell SureLock® Blot chamber (Invitrogen). Completion of transfer was confirmed by staining the membrane with Ponceau S, and subsequent repeated washing with PBS-T (phosphate buffered saline with 0.1 % tween-20). The membrane was blocked with 5 % BSA in PBST for one hour with shaking at room temperature. Primary antibody was added (see Table 10 for antibodies and dilutions) and incubated overnight at 4 °C with slow shaking (65 rpm). Following three ten-minute washes in PBST, a secondary anti-rabbit horseradish peroxidase (HRP) antibody was added 1:5000 in 5 % milk in PBST and the membrane incubated at room temperature for one hour with shaking. The detection agent was Westernbright™ ECL spray (Adansta), which was sprayed directly onto the membrane following three washes in PBST for five minute each. Blots were imaged on an ImageQuant® LAS4000 (GE Healthcare Sciences) on Chemiluminescent mode and relative intensity analyzed using ImageJ (NIH).
### Table 10  Primary antibody details

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Catalogue number</th>
<th>supplier</th>
<th>Dilution</th>
<th>diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HOGA</td>
<td>NA*</td>
<td>NA*</td>
<td>1:2000</td>
<td>5 % BSA in PBST</td>
</tr>
<tr>
<td>Anti-ME2</td>
<td>HPA008880</td>
<td>Sigma</td>
<td>1:250</td>
<td>3 % BSA in PBST</td>
</tr>
<tr>
<td>Anti-ME3</td>
<td>EPR10378</td>
<td>Abcam</td>
<td>1:2000</td>
<td>5 % milk in PBST</td>
</tr>
<tr>
<td>Anti-ubiquitin</td>
<td>AB7780</td>
<td>Abcam</td>
<td>1:2000</td>
<td>5 % BSA in TBST</td>
</tr>
<tr>
<td>Anti-COX-IV</td>
<td>AB16056</td>
<td>Abcam</td>
<td>1:2000</td>
<td>5 % BSA in PBST</td>
</tr>
<tr>
<td>(mitochondrial loading control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Anti-HOGA antibody was generated by AgResearch in Hamilton against the cleaved HOGA protein. IgG in serum was precipitated in 30 % ammonium sulphate at 4 °C for 2 hours stirring, then spun down at 14,500 rpm for one hour. The pellet was re-suspended in PBS and affinity-purified using a column loaded with glutaraldehyde-cross linked cleaved HOGA protein, then purified by SEC as described in 2.2.3.5 into PBS + 0.02 % azide.

2.1.4.3 Differential Scanning Fluorimetry

Differential Scanning fluorimetry (DSF) is a fluorescent method to estimate protein thermal stability. The fluorescent dye SYPRO orange has affinity for the (interior) hydrophobic parts of protein, and as the temperature increases, the protein unfolds leading to an increase in fluorescence (Niesen, Berglund, & Vedadi, 2007). It is commonly used to screen ligands or buffers that increase a protein’s thermal stability.

SYPRO orange (Invitrogen) was diluted to 10x and 2.5 μl added to a V-bottom PCR plate. 30 μM HOGA protein and 10 mM of ligand (if used) were typically added in a final volume of 25 μl in SEC buffer. The plate was briefly mixed and centrifuged, and a standard 10-95 °C or 25-95 °C melt program run on the My-iQ™ single-colour RT-PCR detection
system using the IQ™5 software (Biorad) with 575 nm emission and 490 nm excitation settings.

The inflection point of the transition curve (i.e. melting temperature $T_M$) was calculated using the analysis tools that are freely available at ftp://ftp.sgc.ox.ac.uk/pub/biophysics/ (Niesen et al., 2007), which incorporate the Boltzmann equation (Equation 2).

$$\text{Equation 2} \quad y = \frac{\text{LL} + (\text{UL} - \text{LL})}{1 + \exp \left( \frac{T_m - x}{a} \right)}$$

Where LL and UL are the minimum and maximum intensities, respectively, and “$a$” is the slope of the curve within LL and UL.

2.1.4.4 Kinetics

The production of pyruvate via the enzymatic cleavage of either the HOG or oxaloacetate substrates by HOGA was monitored by the addition of lactate dehydrogenase (LDH) and NADH. Lactate dehydrogenase reduces pyruvate to lactate, and the oxidation of NADH was followed by monitoring the absorbance at 340 nm in a Spectramax 340PC plate reader. The reaction mix composition is listed in Table 11. The reaction was initiated by the addition of the HOGA enzyme in a final volume of 200 µL using a 96 well flat-bottom plate. Enzyme activity was measured across a range of substrate concentrations (10-1000 µM), each measured in triplicate. The substrate oxaloacetate is known to spontaneously decarboxylate, therefore this background was measured at each substrate concentration without the HOGA enzyme, and subtracted from each enzyme activity measurement. After subtraction of background, enzyme activity was calculated using the Beer-Lambert law.
(Equation 1), using the measured change in absorbance over time, the extinction coefficient for NADH (6220 M.cm⁻¹), and the measured path length (0.51 cm).

GraphPad PRISM software was used to calculate the $K_M$ and $k_{cat}$ using the Michaelis-Menten model (Equation 3 and Equation 4).

**Equation 3 ($K_M$)**

$$Y = V_{max} \cdot X / (K_M + X)$$

**Equation 4 ($k_{cat}$)**

$$Y = E_t \cdot k_{cat} \cdot X / (K_M + X)$$

Where $V_{max}$ is the maximal velocity of the reaction, $X$ is the concentration of substrate, $K_M$ is the concentration at 50 % $V_{max}$ and $E_t$ is the concentration of catalytic sites (4 per mole of tetrameric HOGA).

**Table 11** Components of the LDH-plate based assay for HOGA activity

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl, pH 8.5</td>
<td>100 mM</td>
</tr>
<tr>
<td>NADH</td>
<td>200 µM</td>
</tr>
<tr>
<td>LDH</td>
<td>0.1 U.mL⁻¹</td>
</tr>
<tr>
<td>Oxaloacetic acid or 4-hydroxy-2-oxoglutarate</td>
<td>12.5-2000 µM</td>
</tr>
<tr>
<td>MBP-HOGA enzyme</td>
<td>10 µg</td>
</tr>
</tbody>
</table>
2.2  Mammalian Cell Culture Studies

To investigate intracellular processing of PH3 mutant HOGA, cultured mammalian cells were used to overexpress wild-type and Δ315 HOGA. Initially, transient transfection of GFP-tagged wild-type HOGA in a mammalian vector were tested in both HepG2 and cos-7 cells, using various transfection methods (lipofectamine, calcium phosphate, polyethylenimine), however low transfection yield and low cell viability led us to trial a stable transfection model.

2.2.1 The Flp-In System for stable expression

The Flp-In™ System (Life Technologies) can be utilized to integrate and constitutively express a gene of interest (GOI) at a specific genomic location in a mammalian cell line. To incorporate the GOI, cell lines must first be modified by the integration of Flp Recombination Targets (FRT) and a gene for zeocin resistance, or these modified cell lines can be obtained from Life Technologies. In this study, Flp-In™ HEK-293 cells (Life technologies), were transfected with two plasmids; (1) an expression plasmid (pcDNA5™/FRT) containing the hoga1 gene and a hygromycin resistance gene flanked by FRT sites; and (2) the pOG44 vector, which codes for FLP recombinase. Upon transfection of pcDNA5™/FRT and pOG44, FLP recombinase recombines between FRT recombination sites, inserting the GOI into the cell genome. Successful integrants become resistant to hygromycin and sensitive to zeocin, and thus all cells maintained in hygromycin media express the GOI.

This method was unsuccessful for constitutive HOGA expression in Flp-In™ HEK-293 cells. Following transfection of pOG44 and pcDNA5™/FRT, a foci of cells appeared to be resistant to hygromycin; however, these died over the following weeks.
Following the failure of transient transfection and constitutive HOGA over-expression in mammalian cell lines, it was hypothesized that HOGA over-expression may be toxic to cellular metabolism. Therefore, a modified version of the Flp-In System was used to inducibly express HOGA.

### 2.2.2 The inducible Flp-In System

#### 2.2.2.1 Overview of the Flp-in TREx system

To allow the study of “toxic” protein overexpression, a modified version of the Flp-In system incorporates tetracycline-regulated expression of the GOI. Flp-in™ T-REx™ cell lines (Life Technologies) contain a plasmid coding for the tet repressor protein and blasticidin resistance, in addition to genomic FRT recombination sites and zeocin resistance (Figure 16). The expression vector pcDNA5/FRT/TO contains a tetracycline responsive promotor upstream of the hygromycin resistance gene and the GOI, flanked by FRT sites. Therefore, following transfection and integration of the GOI, transcription is repressed by the tet repressor protein, until tetracycline is added to induce transcription, allowing the study of short term effects of GOI over-expression.

#### 2.2.2.2 Cell line

Flp-in™ HEK-293 T-REx™ (F-293) cells were a kind gift from David Christie’s laboratory. F-293 cells were maintained in zeocin and blasticidin containing media (Table 12) to maintain the genomic FRT sites and tet repressor plasmid, respectively.

#### 2.2.2.3 Plasmid propagation and purification

The pOG44 and pcDNA5/FRT/TO plasmids were transformed in Top10 E. coli, and plated on ampicillin (100 μg.mL⁻¹) LB plates as in section 2.1.2.7. Transformed colonies
were inoculated in flasks containing 200 mL LB media (Table 3), grown shaking for 18 hours at 37 °C, and plasmid DNA isolated using a plasmid midiprep kit (Macherey-Nagel).

2.2.2.4 Cloning hoga1 into pcDNA5/FRT/TO

The hoga1 expression vector pDEST-566 and the destination mammalian vector pcDNA5/TR were cut with the same restriction enzymes Hind-III and ECO-RV for 1 hour at 37°C. The hoga1 band was separated by gel electrophoresis (1.2% agarose in TBE), cut from the gel, and purified using a gel extraction kit (Invitrogen). The purified hoga1 band was then ligated into the cut pcDNA5/TR destination plasmid at a 3:1 ratio using T4 DNA ligase (Life Technologies) at 14°C overnight. The hoga1-pcDNA5/TR plasmid was transformed into E. coli Top 10 cells as in section 2.1.2.6, plated on LB plates containing ampicillin (100 μg.mL⁻¹), and propagated and purified as in section 2.1.2.7.

2.2.2.5 Transfection of Flp-In HEK-293 TREx cells

F-293 cells were transfected with pOG44 and HOGA-pcDNA5/FRT/TO vector in a 9:1 ratio (3.6 μg pOG44 and 0.4 μg pcDNA6/TR) with lipofectamine-LTX (Life Technologies) in OPTI-MEM media (Life Technologies). Two days later, hygromycin media was added to cells to select for stable integrants (Table 12).

Using this method, a foci of stable integrants resistant to hygromycin grew for both wild-type HOGA-transfected cells and Δ315 HOGA-transfected cells. These cells were maintained in hygromycin media, and for experiments on HOGA expression, tetracycline (1 μg.mL⁻¹) was added to media (Table 12), and maximal protein expression observed 24 hours after induction.
Table 12  Cell culture media used for F-293 cells

<table>
<thead>
<tr>
<th>Purpose</th>
<th>Base media</th>
<th>Additives</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Growth and maintenance of F-293 cells</strong></td>
<td>D-MEM (high glucose)</td>
<td>2 mM L-glutamine</td>
<td>100 μg.mL⁻¹ zeocin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 % fetal bovine serum</td>
<td>15 μg.mL⁻¹ blasticidin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 % Penicillin-Streptomycin</td>
<td></td>
</tr>
<tr>
<td><strong>Growth and maintenance of F293 HOGA cells</strong></td>
<td>D-MEM (high glucose)</td>
<td>2 mM L-glutamine</td>
<td>100 μg.mL⁻¹ hygromycin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 % fetal bovine serum</td>
<td>15 μg.mL⁻¹ blasticidin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 % Penicillin-Streptomycin</td>
<td></td>
</tr>
<tr>
<td><strong>Induction of HOGA expression</strong></td>
<td>D-MEM (-glucose)</td>
<td>5 mM glucose</td>
<td>1 μg.mL⁻¹ tetracycline</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mM L-glutamine</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 % fetal bovine serum</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 % Penicillin-streptomycin</td>
<td></td>
</tr>
</tbody>
</table>

F-293 = Flp-In™ HEK-293 T-Rex™ cells

2.3  Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

2.3.1 Sample Collection and RNA extraction from animal tissue

For animal studies, ~30 mg of liver, kidney, heart and brain were collected in 1 mL RNAlater® (Ambion) in RNase-free 1.5 mL tubes and stored at 4 °C for 1-4 weeks, then transferred to -80 °C for long term storage.

RNA was extracted using the Purelink® Mini RNA Kit (Ambion). Tissue was removed from the -80 °C and immediately lysed in 2 mL tubes containing 600 μL lysis buffer and a stainless steel ball bearing in the Tissue Lyser II (Qiagen) for two cycles of two minutes at 30 rps. RNA extraction proceeded as per the kit instructions, with the recommended on-column DNAsel (Ambion) digestion of genomic DNA. RNA was eluted in 40-80 μL RNAse free water.
2.3.2 Sample collection and RNA extraction from cultured cells

Cells were collected from T-75 flasks using a cell scraper and spun at 650 x g for five minutes at 4 °C, media aspirated and cell pellet stored at -80 °C.

RNA was extracted using the illustra™ RNaSpin Mini RNA Isolation Kit (GE Healthcare).

2.3.3 RNA concentration and integrity analysis

The RNA concentration was checked with Nanodrop, and the sample only assumed pure if the 260/280 nm ratio was larger than 2.

RNA integrity was assessed using the 2100 Bioanalyzer (Agilent) and the RNA6000 Nano kit, according to the manufacturer’s instructions. The RNA sample and RNA ladder were denatured at 70 °C for two minutes, and 1 μL loaded on to the microfluidic chip. The Agilent 2100 Expert software runs the RNA profile through an algorithm to determine the RNA Integrity Number (RIN), between 0 (completely degraded RNA) and 10 (completely intact RNA). Samples with an RIN above 8 were used for cDNA synthesis.

2.3.4 cDNA synthesis

cDNA was synthesized using Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer’s instructions, using 1 μg template RNA. A negative reverse-transcription control (-RT) was included, which contained 1 μg RNA without reverse transcriptase, to assess for genomic DNA contamination in subsequent PCR reactions.
2.3.5 Semi-quantitative real-time Polymerase chain Reaction (qRT-PCR)

qRT-PCR was run using EXPRESS SYBR® GreenER™ qPCR Supermix with Premixed ROX (Life Technologies), according to the manufacturer’s manual. Samples were added in triplicate to 384 well plates, each well containing 1 μL of 10-fold diluted cDNA and 9 μL Mastermix. Negative controls for each primer pair included a no-template control (NTC, dH2O in place of cDNA) and a –RT control. A standard cycling program was used (50 °C for 2 minutes, 95 °C for 2 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute) in the Applied Biosystems 7900 HT Fast Real-Time PCR System (Applied Biosystems). Melting curve analysis was included during primer optimization, to identify primer dimers.

2.3.5.1 Primer optimization

qRT-PCR primers used in rat studies and cell culture studies are shown in Table 13 and Table 14, respectively, all of which had an acceptable efficiency, single peak in disassociation curve, and single PCR product following electrophoresis (Figure 17 and Figure 18 for rat and cell culture studies, respectively).

2.3.5.2 Analysis

Ct values were determined by SDS 2.4 (Applied Biosystems) software. Housekeeping genes were chosen based on similar expression levels across samples, determined using the Ct values in Bestkeeper (Pfaffl, Tichopad, Prgomet, & Neuvians, 2004) and transformed Ct values (2^-Ct) using Normfinder (Andersen, Jensen, & Ørntoft, 2004). Data was normalized using the 2^ΔΔCt method, by first normalizing to the geometric
mean of the best selected housekeeping genes, and then normalizing to liver samples (in the case of animal tissue) or F-293 cells (in the case of cell culture studies).

Table 13  Rat primers for qRT-PCR primers and efficiencies.

<table>
<thead>
<tr>
<th>Target/reference gene</th>
<th>Gene</th>
<th>Accession</th>
<th>Primer sequence</th>
<th>Standard curve slope</th>
<th>R²</th>
<th>Efficiency (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target gene</td>
<td>HOGA1</td>
<td>NM_138413</td>
<td>Forward: 5’-AAAGAGAGTGTTTAATGGTG-3’&lt;br&gt;Reverse: 5’-CTCTCTGTATCTTCAG-3’</td>
<td>-3.44</td>
<td>0.974</td>
<td>95</td>
</tr>
<tr>
<td>Target gene</td>
<td>ME1</td>
<td>NM_012600</td>
<td>Forward: 5’-ATGGAGAGGAGTTATCAAAG-3’&lt;br&gt;Reverse: 5’-GGCTCTAGTTCTCTTCT-3’</td>
<td>-3.38</td>
<td>0.997</td>
<td>98</td>
</tr>
<tr>
<td>Target gene</td>
<td>ME2</td>
<td>XM_225729</td>
<td>Forward: 5’-GGCTTAAGCTGTATCTGTGA-3’&lt;br&gt;Reverse: 5’-TGAATTAGCAAGTGATGGGTAA-3’</td>
<td>-3.06</td>
<td>0.996</td>
<td>96</td>
</tr>
<tr>
<td>Target gene</td>
<td>ME3</td>
<td>NM_001108491</td>
<td>Forward: 5’-AGATAAGTGTTGATATAATGCCT-3’&lt;br&gt;Reverse: 5’-CATCATTGAAACATGCAGTATTTTG-3’</td>
<td>-3.587</td>
<td>0.991</td>
<td>100</td>
</tr>
<tr>
<td>Reference gene</td>
<td>YWHAZ</td>
<td>NM_013011</td>
<td>Forward: 5’-GATGAAGCCATTGCTGCT-3’&lt;br&gt;Reverse: 5’-GTCTCTTTGGATAGTGATGTC-3’</td>
<td>-3.509</td>
<td>0.992</td>
<td>93</td>
</tr>
<tr>
<td>Reference gene</td>
<td>PPIA</td>
<td>NM_017101</td>
<td>Forward: 5’-CCTAACAAAATGTTGTCCTCAGT-3’&lt;br&gt;Reverse: 5’-ATTCCCTGGACCCAAACGCT-3’</td>
<td>-3.465</td>
<td>0.998</td>
<td>94</td>
</tr>
<tr>
<td>Reference gene</td>
<td>HPRT</td>
<td>NM_012583</td>
<td>Forward: 5’-CAGTCCCAGCGTCTGATC-3’&lt;br&gt;Reverse: 5’-AGCAAGTCTTTCTCCCGTTC-3’</td>
<td>-3.29</td>
<td>0.996</td>
<td>101</td>
</tr>
<tr>
<td>Reference gene</td>
<td>TBP</td>
<td>NM_001004198</td>
<td>Forward: 5’-CACCCTGGAATCTTGGTGAATAC-3’&lt;br&gt;Reverse: 5’-CGAGTTGCGTGTCCTC-3’</td>
<td>-3.045</td>
<td>0.984</td>
<td>112</td>
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<tr>
<td>Reference gene</td>
<td>TFRC</td>
<td>NM_022712</td>
<td>Forward: 5’-GGATTTCTGTACATCATCAAGC-3’&lt;br&gt;Reverse: 5’-TCCAGGCTACAGAGGATATC-3’</td>
<td>-3.392</td>
<td>0.995</td>
<td>97</td>
</tr>
</tbody>
</table>

* Efficiency = [10^{(1/slope) - 1}] *100
Figure 17 DNA electrophoresis of qRT-PCR products from cDNA reverse-transcribed from liver RNA, showing the presence of a single band for each primer. ME1-3 = Malic enzyme 1-3, HOGA1 = 4-hydroxy-2-oxoglutarate aldolase, YWHAZ = Tyrosine 3-Monoxygenase/Tryptophan 5-Monoxygenase Activation Protein, PPIA = peptidylprolyl isomerase A, TFRC = transferrin receptor, HPRT = Hypoxanthine-guanine phosphoribosyltransferase, TBP = TATA box binding protein
| Target/ reference gene | Gene   | Accession | Primer sequence | Standard curve slope | R²   | Efficiency (%) *
<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Target gene</td>
<td>HOGA1</td>
<td>NM_138413</td>
<td>Forward: 5'-GGTCCCCAAGTCTGGTCTTCT -3' Reverse: 5'-GATACCCGCAATGTCCACCTT-3'</td>
<td>-3.413</td>
<td>0.996</td>
<td>93</td>
</tr>
<tr>
<td>Target gene</td>
<td>ME1</td>
<td>NM_002395</td>
<td>Forward: 5'-CTGCTGACACGGAACCCTC-3' Reverse: 5'-GATCTCCTGACTGTGAAGGAAG-3'</td>
<td>-3.462</td>
<td>0.993</td>
<td>94</td>
</tr>
<tr>
<td>Target gene</td>
<td>ME2</td>
<td>NM_002396</td>
<td>Forward: 5'-ATGTGTTCCCGTTAAGAGTAGT-3' Reverse: 5'-ATGTGTTCCCGTTAAGAGTAGT-3'</td>
<td>-3.487</td>
<td>0.992</td>
<td>94</td>
</tr>
<tr>
<td>Reference gene</td>
<td>PPIA</td>
<td>NM_017101</td>
<td>Forward: 5'-GTCAACCCCAACCGTCTT-3' Reverse: 5'-CTGCTGTCCTTTGGGACCTTG-3'</td>
<td>-3.494</td>
<td>0.997</td>
<td>93</td>
</tr>
<tr>
<td>Reference gene</td>
<td>HPRT</td>
<td>NM_012583</td>
<td>Forward: 5'-TGACACTGCGAAAAACATGCA-3' Reverse: 5'-CTGCTGTCCTTTGGGACCTTG-3'</td>
<td>-3.529</td>
<td>0.998</td>
<td>92</td>
</tr>
<tr>
<td>Reference gene</td>
<td>B2M</td>
<td>NM_004048</td>
<td>Forward: 5'-ATGATGATGCTGCGGTGTA-3' Reverse: 5'-GGCATCTCCAACCTCCATG-3'</td>
<td>-3.374</td>
<td>0.996</td>
<td>98</td>
</tr>
<tr>
<td>Reference gene</td>
<td>TBP</td>
<td>NM_003194</td>
<td>Forward: 5'-CAGGAACCACGGCAGTGATT-3' Reverse: 5'-TTTCTTGCTGCCAGTGCTGA-3'</td>
<td>-3.226</td>
<td>0.992</td>
<td>104</td>
</tr>
</tbody>
</table>

* Efficiency = \[10^{(-1/\text{slope})} - 1\] * 100
**Figure 18** DNA electrophoresis of qRT-PCR products from cDNA reverse-transcribed from F-293 cell RNA, showing the presence of a single band for each primer. HOGA1 = 4-hydroxy-2-oxoglutarate aldolase, ME1-2 = Malic enzyme 1-2, PPIA = peptidylprolyl isomerase A, HPRT = Hypoxanthine-guanine phosphoribosyltransferase, B2M = beta-2-microglobulin, TBP = TATA box binding protein, YWHAZ = Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, RPII = RNA polymerase II.
3. **Characterization of the quaternary structure of wild-type HOGA**

3.1 **Introduction**

Wild-type human HOGA crystallizes as a tetramer and in solution there is an apparent equilibrium between dimer and tetramer forms as suggested by analytical ultracentrifugation (Riedel et al., 2011). The relatively high $K_D$ (60 µM, ~2 mg.mL$^{-1}$) of tetramer disassociation into dimers in this study has implications for enzyme activity *in vivo* and *in vitro*. In fact, all kinetic measurements in the Riedel (2011) study used <100 nM of HOGA, indicating that at this lower concentration the majority of the enzyme present was in a dimeric form. The activity of dimeric HOGA vs tetrameric HOGA is not known. However, the structurally similar tetrameric DHDPS enzymes show markedly decreased catalytic activities and increased $K_M$ values in dimeric vs tetrameric enzyme forms (Griffin et al., 2008). For example, the catalytic efficiency ($K_M/k_{cat}$) in two dimeric mutants of *E. coli* DHDPS was less than 1 % of the wild-type tetramer (Griffin et al., 2008).

The two most common clinical HOGA mutations in patients with Primary Hyperoxaluria Type 3 (PH3) may lead to altered protein quaternary structure, due to the mutations location in the major (p.Δ315) and minor (c. 700+5g>T) dimer interfaces of the tetramer, respectively. Here it is hypothesized that these mutations might alter the association of monomers and/or dimers in the quaternary structure. The effect of the PH3 mutants is reported in Chapter 4. However, given the uncertainty as to whether dimeric and tetrameric forms of HOGA coexisted, there was first a clear requirement to first characterize the quaternary structure of wild-type HOGA.
3.2 Methods

3.2.1 Recombinant protein production

Recombinant wild-type HOGA was cloned, expressed and purified as detailed in 2.1. Cleaved HOGA was used for all experiments and protein concentrations were measured using the Beer-Lambert law (2.1.3.7) and an extinction coefficient of 29,910 M\(^{-1}\) cm for the cleaved HOGA.

3.2.2 Dynamic Light Scattering

Dynamic light scattering (DLS) was used to estimate protein homogeneity and the molecular weight of protein in solution. A Dynapro™ Titan (Wyatt) instrument was used, and analyses carried out using DYNAMICS® software (Wyatt).

Typically, 50 µL of protein was spun in a 1.5 mL tube containing a 0.2 µM centrifugal filter (Millipore) for 30 minutes at 16,100 xg to remove particulate matter. The upper 12 µL was put in a quartz DLS cuvette, and at least 500 auto-correlation functions (ACFs) measured. The DYNAMICS® software uses the cumulants method to calculate the diffusional coefficient (D\(_T\)), which then calculates the hydrodynamic radius (R\(_H\)) using the Stokes-Einstein equation (Equation 5).

Equation 5: Stokes Einstein Equation: \[ R_H = \frac{k_B T}{6\pi \eta D_T} \]

Where \( k_B \) is the Boltzmann constant (1.3806503 x 10\(^{-23}\) m\(^2\) kg s\(^{-2}\)), T is the temperature in Kelvin (291.15 K), \( \eta \) is the dynamic viscosity of water at 18 °C (1.002 x 10\(^{-3}\) kg m\(^{-1}\) s\(^{-1}\)), and \( D_T \) is the translational diffusion coefficient. The DYNAMICS® software also provides an estimate of molecular weight, using a standard curve of the hydrodynamic radius of common globular proteins versus their known molecular weight.
3.2.3 Hydrodynamic radius calculation using BEST

The program BEST was used to elucidate whether the DLS-measured hydrodynamic radius was indicative of a tetramer in solution. BEST uses the pdb coordinates from the crystal structure to calculate the hydrodynamic radius of HOGA, which crystallizes as a tetramer. If the hydrodynamic radius calculated from the tetrameric HOGA crystal structure using the program BEST matches the hydrodynamic radius measured using DLS, this indicates that the protein is in fact a tetramer in solution (Aragon, 2004).

Using the pdb coordinates from the .pdb file, BEST triangulates the molecular surface, and computes a translational diffusion coefficient for four different numbers of N (triangulations). The translational diffusion coefficient is then plotted against 1/N and the linear slope is extrapolated to N=\infty or 1/N = 0. The diffusion coefficient can then be used to calculate the hypothetical hydrodynamic radius ($R_H$) of the HOGA tetramer using the Stokes-Einstein equation (Equation 5).

3.2.4 SEC-MALS-RI

Size-exclusion chromatography (SEC) was the last purification step in isolating pure HOGA (2.1.3.5). This separation technique provides a rough estimate of molecular weight based on elution volume as smaller molecules traverse the pores of the column matrix more quickly than larger molecules. However, this method is dependent on shape as well as size that can lead to erroneous errors if the shape of the sample differs from the shape of the calibration standards (Oliva, Llabrés, & Fariña, 2001).

A more accurate way of determining molar mass is by combining SEC with two detectors inline and using multi-angle light scattering (MALS) and refractive index (RI). This method is based on the principle that (1) the light scattered is proportional to the
product of the weight average molar mass ($M_w$) and the concentration of the solute (c). The concentration can be measured using the refractive index detector (Equation 6).

**Equation 6:** Light Scattering Equation: \[
\frac{K^*c}{\Delta R\theta} = \frac{1}{M_w} \left( 1 + \frac{16\pi^2 s^2 \sin^2(\theta)}{3\lambda^2} + \ldots \right)
\]

Where $\Delta R\theta$ is the excess Rayleigh Ratio, or the change in scattered light relative to the buffer determined by MALS, $c$ is the concentration determined by RI, $\lambda$ is the wavelength of the light in a vacuum, $\theta$ is the scattering angle and $K$ is an instrument constant given by Equation 7.

**Equation 7:** \[
K = 4 \pi^2 n^2 \frac{dn}{dc} \left( \frac{\lambda_0}{N_A} \right)^2
\]

Here, $\lambda_0$ is the wavelength of light in a vacuum, $N_A$ is Avogadro’s number, $n$ is the solvent refractive index, and $dn/dc$ is the solvent refractive index increment. By plotting $Kc/\Delta R\theta$ versus $\sin^2(\theta)/2$, the molecular weight can be calculated from the slope.

### 3.2.5 Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) allows the accurate measurement of molecular weight in solution and the detection of interacting species, for example the presence of both tetrameric and dimer protein forms. By combining an ultra-centrifuge with UV-vis absorbance measurements, basic thermodynamic principles can be applied to calculate molecular weight (sedimentation velocity, 3.2.5.1) or association/disassociation of two molecules (sedimentation equilibrium, 3.2.5.2).
3.2.5.1 Sedimentation Velocity

Sedimentation velocity (SV) was used to calculate the sedimentation coefficient (s) and molecular weight of cleaved wild-type HOGA. Data was collected using an Optima XL-I Ultracentrifuge (Beckman Coulter) at the University of Melbourne, under the supervision of Dr. Mike Griffin. Double sector centrepieces for protein and solvent (reference buffer) with quartz windows were loaded with 380 μl of protein and 400 μl of reference buffer in an 8-hole An 50 Ti rotor and spun at 40,000 rpm at 20 °C. Scans were collected every five minutes for a total of 50-80 scans. The wavelength chosen for each experiment was based on an initial radial scan at 3,000 rpm between A200 and A400nm. Four different concentrations of wild-type HOGA protein were measured (3.2, 1.59, 0.8 and 0.1 mg.mL⁻¹).

The density (1.0056 g.mL⁻¹) and viscosity (0.01033 poise) of the solvent (50 mM Tris, 150 mM NaCl) at 20 °C, and the partial specific volume of cleaved wild-type HOGA (0.7367 mL.g⁻¹) based on the amino acid composition were calculated using SEDNTERP. The data was fitted to a continuous-size distribution model using the software SEDFIT (Schuck, 2000).

3.2.5.2 Sedimentation equilibrium

Sedimentation Equilibrium (SE) experiments was performed using the Optima XL-1 (Beckman Coulter) using an 8-hole An 50 Ti rotor. 120 μl of reference buffer and 100 μl of protein sample were loaded into double sector cells with quartz windows, and spun first at 7,500 rpm and then 10,500 rpm. Scans were collected at each speed after equilibrium was reached. Three concentrations of protein were used: 0.5, 0.15 and 0.05 mg.mL⁻¹.

Data was analyzed using SEDPHAT species analysis, using the partial specific volume and buffer density calculated using SEDNTERP as described in 3.2.5.1.
3.3 Results

3.3.1 Size Exclusion Chromatography of wild-type HOGA

Size Exclusion Chromatography (SEC) indicated that wild-type MBP-HOGA is approximately 227 kDa according to the standard curve of globular proteins (bovine serum albumin, maltose-binding protein and cytochrome-c). This molecular mass is lower than the expected size of tetrameric HOGA-MBP-fusion protein (monomer = 78.5 kDa, dimer = 157 kDa, tetramer = 314 kDa) and may indicate a dynamic equilibrium between dimeric and tetrameric forms. However, as SEC can inaccurately estimate the size of non-globular proteins, additional measures of molecular weight were undertaken (3.2.4 and 3.2.5).

Cleavage of the hisMBP tag with chymotrypsin resulted in >90% cleavage of the fusion tag from HOGA, and the elution volume during SEC indicated a molecular weight of 80 kDa, which is between a dimer and trimer (monomer = 32.5 kDa, tetramer = 130 kDa) (Figure 19). Cleavage of hisMBP and residues 1-25 of HOGA was confirmed in-house by Dr. Dave Greenwood using Electrospray Ionization-Fourier Transform Mass Spectrometry (ESI-FTMS), confirming an intact mass of 32.465 kDa. This is almost identical to the expected molecular mass calculated using PROTPARAM based on the amino acid sequence of residues 26-327. For analytical ultracentrifugation requiring high purity HOGA, the cleaved HOGA was subjected to reverse-MBP column purification to remove contaminating hisMBP fragments.
Overlaid size-exclusion chromatograms of wild-type MBP-HOGA fusion protein (dotted black line), HOGA following chymotrypsin cleavage of the his-MBP tag and the first 25 residues of HOGA (grey line) and HOGA after removal of residual MBP fragments by reverse-MBP purification (black line). Inset shows SDS-PAGE gel of MBP-HOGA and chymotrypsin-cleaved HOGA following SEC. $V_0 =$ void volume, MBP-HOGA

3.3.2 Dynamic Light Scattering of wild-type HOGA

Dynamic light scattering calculated a hydrodynamic radius (RH) of 4.0-4.1 nm for wild-type HOGA at three different concentrations (Table 15). Polydispersity was generally $<10\%$, except in the 0.5 mg.mL$^{-1}$ sample, indicating a monodisperse population. The molecular weight estimate of 87-92 kDa is close to the size of a HOGA trimer (97 kDa), a similar finding based on SEC elution volume (2.3.1), however both methods use standard curves derived from globular proteins to estimate molecular weight. These methods are
therefore error-prone if the shape of the protein of interest deviates from the spherical shape of the standard curve proteins (e.g. more or less hydrated, or elongated shape).

Table 15 Dynamic Light Scattering results for cleaved wild-type HOGA at three protein concentrations.

<table>
<thead>
<tr>
<th></th>
<th>0.5 mg.mL(^{-1})</th>
<th>1 mg.mL(^{-1})</th>
<th>2 mg.mL(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH (nm)</td>
<td>4.0 ± 0.023</td>
<td>4.1 ± 0.016</td>
<td>4.1 ± 0.002</td>
</tr>
<tr>
<td>% Pd</td>
<td>21.3 ± 1.246</td>
<td>9.9 ± 0.858</td>
<td>2.7 ± 0.134</td>
</tr>
<tr>
<td>MW (kDa)</td>
<td>86.9 ± 1.408</td>
<td>90.8 ± 0.896</td>
<td>92.4 ± 0.100</td>
</tr>
</tbody>
</table>

Results are reported as mean ± SEM. RH = hydrodynamic radius in nm, %Pd = percentage polydispersity, MW (kDa) = molecular weight in kilodaltons.

3.3.3 BEST-calculated hydrodynamic radius of HOGA

The pdb coordinates corresponding to the HOGA crystal structure were used to calculate the translational diffusion coefficient (5.2679x10\(^{-7}\) cm\(^2\).s\(^{-1}\)), which was then used to calculate the hydrodynamic radius using the Stokes-Einstein equation. The BEST-calculated hydrodynamic radius of hHOGA was 4.04 nm, very close to the DLS-measured hydrodynamic radius of 4.0-4.1 nm. BEST calculated the hydrodynamic radius of HOGA using the .pdb coordinates of crystallized HOGA, which is a tetramer. This indicates that HOGA is in fact a tetramer in solution. DLS calculates the molecular weight of proteins using “standard” globular proteins, therefore ellipsoid shaped proteins or differently hydrated proteins may under or overestimate molecular weight using DLS.
3.3.4 SEC-MALS-RI of wild-type HOGA

For three concentrations of HOGA (0.613, 1.25, 2.5 mg.mL\(^{-1}\)), SEC-MALS-RI calculated a molecular weight average of 132.5-136.8 kDa, within 4% of the expected tetrameric molecular weight of 130 kDa. A representative trace of a SEC-MALS-RI experiment is shown in Figure 20, showing a single symmetrical peak and the calculated molecular weight on the right y-axis. Overlaid traces of the three HOGA concentrations and the calculated molecular weight of the elution peak are shown in Figure 21.
**Figure 20**  Representative trace of SEC-MALS-RI for HOGA at 1.25 mg.ml\(^{-1}\), showing the refractive index (RI) and Molecular Weight average distribution.
Figure 21 Molecular weight average distribution of HOGA using SEC-MALS-RI over the concentration range 0.613-2.5 mg.ml\(^{-1}\) showing no variation in molecular weight average with concentration.
3.3.5 Analytical Ultra-centrifugation of wild-type HOGA

3.3.4.1 Sedimentation Velocity Experiments

Sedimentation velocity (SV) experiments clearly showed a single mono-disperse species of wild-type HOGA, with no evidence of subunit disassociation even at low protein concentrations (0.1 mg.ml\(^{-1}\)). A representative SV experiment along with residuals is shown in Figure 22. Wild-type HOGA has a standard sedimentation coefficient (s\(_{20,w}\)) of 6.9 S, which corresponds to a molecular weight of 124 kDa across the concentration range 0.1-3.2 mg.mL\(^{-1}\) (Figure 23).
**Figure 22** Representative sedimentation velocity experiment of wild-type HOGA (3.2 mg.ml$^{-1}$) showing the protein boundary (A280nm) across the radius of the cell as the solution is spun. The residuals are shown in the bottom panel.
Figure 23  Concentration distribution c[s] of wild-type HOGA sedimentation at four protein concentrations (0.1-3.2 mg.mL⁻¹) (A). The molecular weight shows no variation with protein concentration (B).

3.3.4.2 Sedimentation Equilibrium experiments

Sedimentation equilibrium experiments confirmed that wild-type HOGA is a tetramer and does not disassociate even at very low concentrations (0.5-0.05 mg.mL⁻¹).

Figure 24 shows the equilibrium profile at the three HOGA concentrations at two different velocities (7,500 and 10,500 rpm). The species analysis in SEDPHAT using a global fit of the three concentrations calculated a molecular weight of 118.1 kDa, which is lower than the expected molecular weight of a tetramer (130 kDa). Further analysis using a single fit for each concentration revealed the data was skewed by the high protein concentration data (0.5 mg.mL⁻¹, 117.9 kDa), whereas the two lower concentrations gave a value closer to what
would be expected for a tetramer (0.15 mg.mL$^{-1}$: 131.9 kDa, 0.05mg.mL$^{-1}$: 136.3 kDa). The experiment was not repeated as the other SEC-MALS-RI experiments confirmed the expected molecular weight of HOGA.

**Figure 24** Sedimentation equilibrium analysis of wild-type HOGA at three concentrations (0.5, 0.15, 0.05 mg.mL$^{-1}$), at two velocities (7,500 and 10,500 rpm).
3.4 Discussion

The biophysical data clearly show that wild-type human HOGA is a tetramer in solution, even at low concentrations (0.05 mg.mL\(^{-1}\)). The protein molecular weights calculated indirectly using SEC and DLS data alone indicate the possibility of a trimer or dimer-tetramer equilibrium. However, this study only highlights that these techniques are only estimates of molecular weight, as they assume similar hydrodynamic shapes of globular proteins as used in their respective standard curves. Accurate biophysical techniques for determining molecular weight and quaternary structure in solution include analytical ultracentrifugation (sedimentation velocity and sedimentation equilibrium) and size exclusion chromatography in line with light scattering and refractive index (SEC-MALS-RI). Both these techniques support a tetrameric HOGA. In addition, the crystal structure of HOGA is tetrameric, and using the pdb coordinates of the solved structure, the program BEST calculated a hydrodynamic radius (\(R_H\)) of 4.04 nm, which is within the range measured in DLS experiments (\(R_H=4.0-4.1\) nm). This confirms that the experimentally measured hydrodynamic radius of HOGA in solution (DLS) matches the dehydrated HOGA crystal hydrodynamic radius, and provides further evidence that HOGA is a tetramer in solution.
4. Structure & Function of HOGA Disease Mutations

4.1 Introduction

Mutations to the 4-hydroxy-2-oxoglutarate aldolase gene (*hoga1*) cause the autosomal recessive disease Primary Hyperoxaluria Type 3 (PH3) in humans (Beck et al., 2013; Belostotsky et al., 2010). 26 *hoga1* mutations have been identified in PH3 patients to date (Table 1), however it is still unclear whether these mutant proteins are actually expressed in the cell, correctly targeted to the mitochondria, or biochemically active.

The only study of recombinant PH3 HOGA mutants found poor expression in *E. coli*, evidence of aggregation during purification, and biochemically inactive protein for the nine PH3 mutants studied (R70P, R97C, P190L, R255X, C257G, T280I, G287V, R303C, Δ315) (Riedel, 2012).

Two studies have expressed recombinant human HOGA clinical mutants in mammalian cell lines. Mutant HOGA protein was targeted to the mitochondria but poorly expressed and biochemically inactive for the nine mutants listed above (Riedel, 2012). In cos-7 cells, HOGA mutants were targeted to mitochondria, and showed similar levels of expression relative to wild-type HOGA in five of the six mutants investigated (V74G, A148V, P190L, A243D, V245I), with the exception of the truncated mutant Q116X, which expressed no HOGA protein (Beck et al., 2013).

Many HOGA mutants are associated with PH3, most of which have been identified in only a few patients. We investigated the most prevalent clinical mutants (p.Δ315 and c.700+5G>T) and hypothesized that these mutations could interrupt the quaternary structure of the protein, due to the location of the mutations near the major and minor dimer interfaces, respectively (Figure 8).
The aims of this chapter are to investigate;

(1) The structure and function of recombinant PH3 HOGA mutants using a bacterial expression system, and

(2) The expression, targeting to mitochondria, and intracellular processing of PH3 HOGA mutants in transfected mammalian cells.

4.2 Methods

4.2.1 Recombinant protein mutagenesis, expression and purification

Wild-type protein was cloned, expressed, and purified as detailed in 2.1.3.

The \( \Delta 315 \) HOGA mutant was created using site-directed mutagenesis of the p221 DONR plasmid as detailed in 2.1.2.10, using the primers listed in Table 7. Once a successful candidate clone was identified by in-house DNA sequencing, the LR reaction shuttled the open reading frame into the pDEST-566 expression plasmid (2.1.2.8).

The c.700+5G>T HOGA mutant was created using “round the horn” mutagenesis, described in 2.1.2.11. Bacterial expression in BL21 \( E.coli \) and purification of MBP-HOGA proceeded as described in 2.1.3.

4.2.2 Differential Scanning Fluorimetry

Differential Scanning Fluorimetry of clinical mutants and wild-type HOGA was undertaken as described in 2.1.4.3, except a higher concentration of pyruvate (50 mM) was used with the less responsive \( \Delta 315 \) mutant protein.
4.2.3 Cell Culture Studies

The wild-type HOGA and Δ315 HOGA mutant sequences were cloned into F-293 cells as described 2.2.4, and maintained in standard cell culture media for F-293 HOGA cells (Table 12). This allowed for HOGA transcription only in the presence of tetracycline, which was added to induction culture media (Table 12) for 24h prior to harvesting cells.

4.2.3.1 qRT-PCR

RNA was isolated from cells as described in Section 2.3.2. RNA integrity was assessed as described in Section 2.3.3, and RNA from cells always gave RIN >9.5, indicating high quality, intact RNA. cDNA synthesis and qRT-PCR were as described in Section 2.3.4 and 2.3.5, respectively.

4.2.3.2 Cell harvesting and lysis for western blot

Cells were collected at 650 xg for 5 minutes in PBS, after aspirating media and scraping cells into 15 mL tubes, and cell pellets frozen at -80 °C. Cells were lysed at 4 °C in 100-300 μL RIPA buffer (50 mM Tris, 1 mM EDTA, 1 % Triton X-100, 0.1 % SDS, 0.25 % Na-deoxycholate, Complete EDTA-free mini protease inhibitor tablet [Roche], pH 7.4 @ 0 °C), by shaking at 65 rpm for 30 minutes, followed by 3-5 freeze-thaw cycles and sonication with a fine-tip. Protein was quantified by BCA as described in Section 2.1.3.7.

4.2.3.3 Western blot

Western blot were run as described in Section 2.1.4.2, and 25-50 μg cell lysate loaded per lane.
4.2.3.4 Immunofluorescence

Cells were plated on Poly-L-lysine (0.1 mg.mL⁻¹) coated coverslips in 6 well plates overnight, and induction media added the next day. Following 24 hours of tetracycline-induction, cells were washed in PBS and fixed in 4 % paraformaldehyde in PBS for 10 minutes at room temperature. Cells were permeabilized and blocked (10 % goat serum, 0.1 % Triton X-100 in PBS) for one hour, and incubated with primary antibodies (Anti-HOGA 1:1000 and Anti-TOM20 1:400) at 4 °C overnight, shaking at 65 rpm at. Primary antibodies were washed away with 3 washes in PBS for five minutes each, and secondary antibodies diluted 1:1000 in blocking buffer [Alexa Fluor® 488 Goat Anti-Rabbit IgG (H+L) and Alexa Fluor® 555 Goat Anti-Mouse IgG₂a (γ2a), [Molecular Probes]]. Coverslips were mounted on slides with Prolong diamond anti-fade reagent and sealed with nail polish. Immunofluorescence was imaged on Andor Revolution XD using 488 and 561 lasers, and images processed using ImageJ.

4.2.4 Proteasome Inhibition studies

The 26S proteasome inhibitor PS-341 (Bortezomib, Sapphire Biosciences) was added to cell culture induction media (100 nM) in T-75 flasks containing either wild-type HOGA F-293 cells (wtHOGA) or Δ315 HOGA F-293 cells. In negative control experiments, Δ315 cells were given induction media containing tetracycline but not PS-341. Cells were harvested at time 0, and after 8, 24 and 48 hours.

Two different proteasome-inhibition time-course experiments were run, and then followed by western blot analyses. Firstly, wild-type HOGA was compared to Δ315 HOGA following PS-341 proteasome inhibition during tetracycline induction, with samples taken at t =0, 8, 24 and 48 hours. Secondly, Δ315 cells were induced for transcription in the absence and presence of PS-341 at 0, 8, 24 and 48 hours.
Western blots were run as described in Section 2.1.4.2, except the primary antibody probed was anti-ubiquitin (Table 10), and following secondary antibody incubation and imaging, the membrane was stripped (62.5 mM Tris, pH 6.8, 2 % SDS, 100 mM β-mercaptoethanol) for 15 minutes at 60 °C, and re-probed with anti-HOGA and anti-COX-IV antibodies.

4.3 Results

4.3.1 Recombinant protein studies

4.3.1.1 SEC of clinical mutants

The two clinical mutants Δ315 and 700+5G>T expressed soluble HOGA in *E. coli* when expressed as a fusion with MBP. However, during SEC both these recombinant proteins eluted exclusively in the void volume (V₀), indicating polydisperse aggregates (Figure 25).

Attempts were made to stabilize the mutant proteins including: (1) the addition of pyruvate (10 mM) to all purification buffers, a ligand known to bind and stabilize HOGA; (2) a comprehensive 96-well buffer/additive screen in combination with differential scanning fluorimetry (2.1.4.3) to identify ligands/buffers/pH that increased HOGA thermal stability, and (3) refolding was trialled. However no attempts to prevent HOGA aggregation were successful.
Figure 25  Size-Exclusion chromatogram of wild-type HOGA (black line) and the clinical PH3 mutants Δ315 (red line) and 700+5G>T (orange). Both mutants elute in the void volume (Vo) indicating large polydisperse aggregates, while the majority of wild-type HOGA elutes at roughly the size of a trimer (227kDa). Inset shows a SDS-PAGE gel of both mutants following MBP affinity and SEC, showing that both MBP-HOGA mutant proteins elute in the void volume (Vo) while the monodisperse HOGA peak is absent.

4.3.1.2 Differential Scanning Fluorimetry

Differential Scanning Fluorimetry (DSF) is commonly used to identify conditions (ligands, buffers, pH) that alter a proteins thermal stability. DSF was used in this study to measure the thermal stability of wild-type HOGA and compare this to the PH3 mutants Δ315 and 700+5G>T.
4.3.1.2.1 DSF in Wild-type HOGA

Wild-type MBP-HOGA gave two melting peaks (54 °C and 75 °C) during differential scanning fluorimetry (DSF), indicating that MBP and HOGA have different thermal stabilities. To test which peak corresponded to which protein, 10 mM of known substrate for both proteins was added to separate wells: pyruvate for HOGA and maltose for MBP. The addition of these compounds identified the 54 °C peak as MBP, as indicated by a rightward shift of 10 °C in this peak following the addition of maltose. HOGA was identified as the more thermostable protein, as indicated by rightward shifting of the 75 °C peak by 8 °C with the addition of pyruvate (Figure 26).

DSF indicated that the MBP fusion protein could be used to identify ligands that caused a shift in the HOGA peak, however the melting temperature (T<sub>M</sub>, midpoint value) was not able to be calculated, as this required the minimum and maximum fluorescence intensities (Equation 2), which is largely interfered with by the first MBP unfolding peak. Cleaved HOGA was therefore used in assays aiming to screen low molecular weight inhibitors (Chapter 5). It was found that cleaved HOGA exhibits the same 75 °C melting peak as MBP-HOGA fusion protein and also a similar protein stabilization profile with pyruvate (Figure 26, lower panel). Fusion MBP-HOGA was used for protein analysis of the clinical mutants, as they formed large polydisperse aggregates that eluted from SEC exclusively in the void volume. Therefore, a pure preparation of monodisperse cleaved Δ315 or c.700+5G>T HOGA was impossible, due to its propensity to aggregate.
Figure 26  Differential Scanning Fluorimetry of wild-type MBP-HOGA fusion protein (A), identifying the melting peak of each protein using known ligands (maltose for MBP and pyruvate for HOGA). HOGA cleaved from the fusion tag shows the same 75 °C melting peak as MBP-HOGA and a similar rightward shift is seen with the ligand pyruvate (B).
4.3.1.2.2  Differential Scanning fluorimetry in PH3 HOGA mutants

Both the clinical mutants (Δ315and 700+5G>T) unfold at much lower temperatures than wild-type HOGA (peak at 24 °C, Figure 27). It is possible they are partially unfolded prior to the assay as the starting fluorescence was relatively high. Although small peaks at 24 °C and 55 °C indicate HOGA and MBP unfolding, respectively, these fluorescence intensities never reach above the starting fluorescence. The 24 °C mutant-HOGA unfolding peak was identified as HOGA by the addition of pyruvate, which caused a rightward shift in the peak (Figure 28).

Figure 27  DSF analysis of wild-type MBP-HOGA and PH3 clinical mutants Δ315 and 700+5G>T.
Figure 28  Differential Scanning Fluorimetry of HOGA Δ315 with and without pyruvate (50mM). Addition of pyruvate caused a 3 °C rightward shift in the unfolding peak.

4.3.1.3 Kinetics of PH3 HOGA mutants

The activity of recombinant MBP-fusion Δ315 and c. 700+5G>T HOGA was measured using the LDH-coupled assay described in 2.2.4.6. No activity above baseline was detected with 15 µM protein, or even when excess protein (100 µM) or substrate (10 mM oxaloacetate or HOG) was added to the reaction mix.
4.3.2 PH3 HOGA mutant cell culture studies

Recombinant Δ315 and 700+5G>T mutant HOGA aggregated, appeared partially unfolded and thermally unstable. This led to the hypothesis that if expressed in vivo, PH3 mutant protein may be degraded by the cells via ubiquitin-mediated proteolysis. Therefore, wild-type and Δ315 HOGA were cloned into an inducible-expression system in Flp-In™ T-REx™ 293 (F-293). Following 24 hours of transcription induction, gene expression, protein expression, targeting of HOGA to mitochondria, and ubiquitin-mediated turnover was measured in PH3 HOGA mutant expressing cells compared to wild-type HOGA expressing cells.

4.3.2.1 HOGA overexpression

4.3.2.1.1 Gene expression

F-293 cells express very low endogenous levels of hoga1 mRNA. F-293 cells transfected with wild-type hoga1 (wtHOGA) express an average of 99±16 fold (p <0.01) increased levels of hoga1 mRNA after induction of transcription with tetracycline (Figure 29), relative to F-293 cells. F-293 cells induced to express Δ315 HOGA1 gene (Δ315 HOGA) similarly overexpress hoga1 mRNA relative to reference genes (128±28 fold, relative to F-293 cells, p <0.0001).

Both wtHOGA and Δ315 HOGA F293 cells appear to express increased levels of hoga1 RNA without tetracycline induction of transcription (~25-fold increase relative to F-293 cells), however this increase was not significant, and may be due to basal levels of tetracycline in the fetal bovine serum contained in the culture media, or leaky transcription by the tet repressor system.
Figure 29  Fold expression increase in *hoga1* RNA in F-293 cells (-ve control) and F-293 cells with wild-type *hoga1* (wtHOGA) and Δ315 *hoga1* mutant gene, induced (+tet) and not induced (-tet) for gene transcription. Results reported relative to the geometric mean of PPIA and TBP housekeeping genes. ***p <0.001 for a significant difference compared to F-293 cells [One way ANOVA with post-hoc Tukey’s]. qRT-PCR was conducted in triplicate, from 4 independent cell passages for each cell line.
4.3.2.1.2 Protein expression

HOGA protein is absent in F-293 cells as shown by western blot (Figure 30), and analyses by confocal microscopy indicated a low signal with HOGA antibody (Figure 31, a-c). F-293 cells induced for wtHOGA expression for 24h upregulate HOGA protein expression, as shown in both western blot (Figure 30) and confocal microscopy (Figure 31, d-f). F-293 cells induced to express Δ315-HOGA showed no evidence of HOGA protein, both by western blot and confocal microscopy, where the low HOGA signal is similar to F-293 cells (Figure 32).
Figure 30  Representative western blot of HOGA and COX-IV loading control in F-293 cells, F-293 cells with wt-HOGA gene induced for transcription for 24h (+tet) and not induced for transcription (-tet), and mutant Δ315 HOGA gene induced for transcription for 24 h (50 μg of cell lysate loaded per lane).
**Figure 31** Confocal microscopy of F-293 cells (a-c) and F-293 cells induced to overexpress HOGA (wtHOGA, d-g). a,d HOGA (**green**), b,e TOM20 (**red**) mitochondrial marker, c,f merged. g brightfield image.
Figure 32  Confocal microscopy of F-293 cells induced to express Δ315 HOGA mutant. Merged images of HOGA (green) and TOM20 mitochondrial marker (red).
4.3.3 Ubiquitination of PH3 HOGA mutants

The proteasome inhibitor PS-341 did not result in an increase in ubiquitin signal in Δ315 HOGA F-293 cells induced for gene transcription, relative to wild-type HOGA expressing cells with PS-341, or Δ315 cells without PS-341 (Figure 33 A). However, after re-probing membranes with anti-HOGA, HOGA protein was detected in Δ315 induced cells after 24, whereas no HOGA protein was detected in Δ315 cells in the absence of PS-341(Figure 33 B). The amount of HOGA protein detected in Δ315 cells with PS-341 was much lower than in wild-type HOGA cells with PS-341 (16 ± 4.5 % relative to wt-HOGA, expressed relative to COX-IV loading control, p < 0.01, Figure 33 C-D).
Figure 33  Western blot of total ubiquitin signal and COX-IV loading control in F293 cells, F-293 cells expressing wild-type HOGA (wt), and F293 cells expressing Δ315 HOGA in the presence (+) and absence (-) of PS-341 over time (A). Western Blot of HOGA and COX-IV loading control in Δ315 HOGA expressing cells in the absence and presence of PS-341 over time, and the positive control wt HOGA expressing cells induced for 24h in the presence of PS-341 (B). Time-course of PS-341 proteasome inhibition in wt and Δ315 HOGA expressing cells (C). HOGA signal relative to COX-IV loading control in wt vs Δ315 HOGA cells, induced for 24h in the presence of PS-341 (D). **p <0.01 using 2-tailed students t-test, from 3 independent experiments conducted on different cell passages.
4.4 Discussion

The most prevalent HOGA mutants in PH3 patients, p. Δ315 and c.700+5G>T, are dysfunctional and inactive forms of the recombinant HOGA protein. This is in complete agreement with the only other study investigating recombinant PH3 mutant expression in *E. coli*, which found poor expression, aggregation, and inactivity of recombinant Δ315 HOGA (Riedel et al., 2012).

The inducible expression of Δ315 HOGA in F-293 cells resulted in similar transcription of HOGA compared to wild-type HOGA, however Δ315 HOGA protein was almost undetectable by western blot or immunofluorescence. This is in agreement with Riedel et al. (2012) who reported poor expression of transiently transfected Δ315 HOGA in CHO cells, and no detectable HOG aldolase activity in cell lysates.

These data led to the hypothesis that dysfunctional mutant protein was degraded via the proteasome, and by adding the proteasome inhibitor during induction of HOGA transcription, we confirmed this hypothesis. HOGA protein was detected in Δ315 HOGA expressing cells only in the presence of the proteasome inhibitor PS-341. This confirms that one of the most prevalent PH3 mutations, p.Δ315 is a “loss of function” mutation.

How the loss of HOGA leads to build-up of oxalate is not yet known. The two current theories as discussed in Section 1.6.23.2 are that; (1) HOG, the substrate of HOGA, leaks into the cytosol and is cleaved to glyoxylate by a non-specific aldolase, which is subsequently oxidized to oxalate by lactate dehydrogenase; or (2) HOG build-up inhibits glyoxylate reductase, the enzyme normally responsible for detoxifying glyoxylate. There is initial evidence for both theories as discussed in 1.6.23.2 and our lab is currently investigating the first theory of cytosolic HOG aldolase activity.
5. Kinetics and Regulation of HOGA

5.1 Introduction

HOGA isolated from bovine liver and kidney show dual HOG aldolase and oxaloacetate decarboxylase (OAD) activities, where OAD is 10-50% of HOG aldolase activity (Dekker & Kitson, 1992; Kobes & Dekker, 1967). Additionally, bovine liver HOG aldolase activity is inhibited by the TCA cycle intermediate α-ketoglutarate (αKG) (Dekker et al., 1971; Grady et al., 1981; Kobes & Dekker, 1971b). Initial kinetic studies of human recombinant HOGA (hHOGA) report a $K_M$ for its substrate HOG of 11 μM (Riedel et al., 2011). Our laboratory also confirmed OAD activity of hHOGA (Sabherwal, 2011). However, no studies of hHOGA have compared HOG aldolase and oxaloacetate decarboxylase activities, or reported inhibition of hHOGA activity with αKG.

Oxaloacetate is a key intermediate at a cross roads between the TCA cycle and gluconeogenesis. In the TCA cycle, citrate synthase condenses oxaloacetate and acetyl CoA to form citrate (Figure 13), and oxaloacetate is a key regulator of TCA cycle turnover (Pardee & Potter, 1948; Wojtczak, 1969). Oxaloacetate is also a substrate for gluconeogenic PEPCK, and the production of oxaloacetate from pyruvate by pyruvate carboxylase increases during gluconeogenesis (Jitrapakdee, Vidal-Puig, & Wallace, 2006). Therefore, oxaloacetate decarboxylase activity of HOGA may potentially have a role in the regulation of the TCA cycle and/or gluconeogenesis. The potential regulation of HOGA OAD aldolase activity by αKG further implicates a role of HOGA in the TCA cycle and/or gluconeogenesis, as αKG is a TCA cycle intermediate whose concentrations drops during gluconeogenesis (Siess, Brocks, Lattke, & Wieland, 1977; Williamson & Corkey, 1979). A further question is posed regarding pyruvate. As a product of HOGA OAD and HOG aldolase activity, and also a substrate in the reverse condensation reaction of pyruvate and glyoxylate, does pyruvate regulate HOGA OAD activity? The level of OAD inhibition at
physiological concentrations of pyruvate may further implicate HOGA regulation during gluconeogenesis, when the pyruvate supply diminishes (Parrilla, Jimenez, & Ayuso-Parrilla, 1976; Siess et al., 1977; Siess & Wieland, 1980).

In this chapter, a comparative study of hHOGA kinetics with the substrates HOG and oxaloacetate was undertaken. To compare the catalytic mechanism of HOG and oxaloacetate cleavage, active site residues were mutated and the effect on $K_M$ and $k_{cat}$ with both substrates was measured. The regulation of HOGA by the TCA cycle intermediate αKG was also investigated using enzyme kinetics and binding experiments. Additionally, product inhibition with pyruvate was investigated by measuring the effect of pyruvate on OAD activity of hHOGA.

5.2 Methods

5.2.1 Recombinant protein production

Recombinant human wild-type HOGA was cloned as described in 2.1.2.8 and expressed and purified as described in section 2.1.3.

Point mutations were made to active site residues using site-directed mutagenesis as described in section 2.1.2.10 using the primers listed in Table 7.

The wild-type HOGA expression plasmid pDEST566 was used as the template for PCR, and mutagenesis was confirmed by in-house DNA sequencing (8.1, Appendix). Bacterial expression in BL21 E.coli then proceeded as described in section 2.1.3.
5.2.2  Kinetic measurements

The LDH-coupled microplate assay was used for measuring the rate of HOG and oxaloacetate cleavage into pyruvate, as described in 2.1.4.4. The MBP-HOGA fusion protein was used for kinetic measurements, as the kinetic parameters (K_M, V_max, k_cat) were no different when compared with cleaved HOGA (Figure 34).

![Oxaloacetate decarboxylase activity of MBP-HOGA fusion protein (white circles) vs cleaved HOGA (filled circles). Each circle represents the average of triplicates measured in one day, and experiments were conducted on 2 separate days.](image)

Figure 34  Oxaloacetate decarboxylase activity of MBP-HOGA fusion protein (white circles) vs cleaved HOGA (filled circles). Each circle represents the average of triplicates measured in one day, and experiments were conducted on 2 separate days.

5.2.2.1  Kinetics with active-site mutants

The K_M and K_cat of wild-type HOGA together with the active site mutants K196A, Y168F, S77A, S198A and N78T were measured with oxaloacetate and HOG (12.5 μM-1000 μM), using the LDH-coupled assay as described in 2.1.4.4.
5.2.2.2 Reversibility of oxaloacetate decarboxylase activity

The ability of HOGA to catalyze the carboxylation of pyruvate to oxaloacetate, the reversal of oxaloacetate decarboxylation (**Equation 8**) was measured using a spectrophotometric 96-well plate malate-dehydrogenase coupled assay.

**Equation 8**

\[
\text{pyruvate} + \text{HCO}_3^- \rightarrow \text{oxaloacetate} \rightarrow \text{malate} \\
\text{NADH} \rightarrow \text{NAD}^+ 
\]

The assay contained excess malate dehydrogenase (Sigma, 4 U.μL\(^{-1}\)), 0.2 mM NADH, 500 mM NaHCO\(_3\), 50 mM Tris, pH 8, and the reaction was initiated by addition of purified cleaved HOGA (5-60 μg).

5.2.2.3 Inhibition experiments with αKG

To measure enzyme inhibition in hHOGA, the standard LDH-coupled assay (2.1.4.4) was used with varying concentrations of αKG (0-20 mM). αKG inhibition was also measured in mitochondrial lysates from HEK cells induced to overexpress hHOGA; measurement of HOG aldolase and OAD activities were undertaken as described in Section 6.2.9.1 and 6.2.9.2, in the presence and absence of 20 mM αKG.

5.2.2.4 Alternative kinetic assay for pyruvate inhibition

The continuous LDH-coupled kinetic assay could not be used to measure the effect of pyruvate, as pyruvate addition would interfere with LDH activity, as it is itself a substrate.
of LDH. Therefore, an alternative time-course assay was employed, where aliquots were
removed from the reaction mixture during the assay for measurement of remaining
oxaloacetate. Product inhibition of HOGA OAD activity was measured using a time-course
assay. The reaction contained 75 μg MBP-HOGA and pyruvate (0, 250, 500 or 1000 μM) in
Tris-HCl, pH 8, and the reaction was initiated with the addition of oxaloacetate (128 μM or
256 μM). Background spontaneous oxaloacetate decarboxylation was measured in parallel
with 128 or 256 μM oxaloacetate. 50 μL aliquots were removed from the reaction every 30
seconds, deproteinized (25 μl 8 % v/v perchloric acid) and neutralized (25 μL 8 % w/v
KOH), and spun for 2 minutes at 10,000 x g, 4°C. The amount of oxaloacetate in each aliquot
was then quantified in a 96-well plate using malate dehydrogenase (Sigma). 80 μL of each
aliquot was added to each well containing 120 μL reaction buffer (250 μM NADH, 1 mM
MgCl₂ in 100 mM K-phosphate, pH 7.5), and the absorbance at 340 nm (A340) and
pathlength measured. Excess malate dehydrogenase (450 mU) was then added and the A340
and pathlength re-measured, following brief mixing. The amount of oxaloacetate was
equivalent to the NADH consumed by malate dehydrogenase (Equation 9), after correction
for the dilution of the aliquot during deproteinization/neutralization and malate
dehydrogenase measurement.

**Equation 9**

\[
\text{mM Pyruvate} = \left( \frac{A340_{\text{final}}}{6.22 \text{ mM.cm}^{-1} \ast l_{\text{final}}} \right) - \left( \frac{A340_{\text{initial}}}{6.22 \text{ mM.cm}^{-1} \ast l_{\text{initial}}} \right)
\]

**5.2.3 Differential Scanning Fluorimetry**

Differential scanning fluorimetry experiments were undertaken as described in
2.1.4.3, with 30 μM HOGA and varying concentrations of αKG.
5.2.4 Ligand binding experiments

5.2.4.1 Mutagenesis to incorporate a biotinylation site into HOGA, protein expression and purification

Site-specific biotinylation of recombinant protein was produced by cloning a biotin acceptor peptide (BAP) into the C terminus of the expression plasmid (HOGA-BAP) as detailed in 2.1.2.11. Protein was expressed in BL-21DE3 cells and purified as detailed in 2.1.3. The hisMBP tag and first 25 residues of HOGA was cleaved with chymotrypsin (2.1.3.6) and purified by SEC into biotinylation buffer (10 mM Tris, pH8, 10 mM magnesium acetate). Cleaved HOGA-BAP was concentrated to 2 mg.mL\(^{-1}\) with a 10 kDa molecular weight cutoff vivaspin concentrator.

5.2.4.2 Biotinylation

Biotin ligase (BirA) catalyzes the ATP-dependent biotinylation at the specific recognition BAP introduced at the c-terminus of HOGA as described above. BirA was kindly purified as a GST-fusion protein by Fiona Clow at the Faculty of Medical and Health Sciences. Biotinylation was performed at 18 °C overnight with 100 µM HOGA-BAP, 5 mM ATP, 5 µM BirA and 1 mM D-biotin on a rotating wheel. SEC was used to separate HOGA and other reaction components in 100 mM Hepes, 150 mM NaCl, pH 7.1 (HBS). However the HOGA and BirA peaks overlapped and were further separated using reverse glutathione (GSH)-affinity. 5 mL of GSH resin was equilibrated with HBS and the protein mix (HOGA+BirA) was passed over the column. Eluate containing biotinylated HOGA was collected and BirA eluted with HBS containing 10 mM glutathione.

5.2.4.3 Surface Plasmon Resonance (SPR) binding experiments

SPR experiments were kindly run by Fiona Clow at the Faculty of Medical and Health Sciences at the University of Auckland. A BIACORE® 2000 (Biacore AB)
instrument was used to measure αKG binding to biotinylated-HOGA, using an SA chip (GE healthcare) coated with streptavidin, which was bound to the chip surface with carboxymethylated dextran. The chip was conditioned with 1 M NaCl in 50 mM NaOH. Biotinylated-HOGA was then immobilized on the chip surface (50 μg.mL⁻¹) until ~2000 relative response units (RU) was reached. αKG (pH 7) was then passed over the chip surface at varying concentrations (0-100 mM). Initial experiments demonstrated non-specific binding of αKG to the control flow-cell that didn’t contain immobilized HOGA, indicating that αKG had background affinity for the chip surface. Therefore, a “negative control” protein, biotinylated glucose-6-phosphate dehydrogenase (bt-G6PDH), was passed over the control flow cell, as this protein was of a similar size to HOGA, with no known affinity for keto acids like αKG. Bt-G6PDH was confirmed with DFS to show no thermal shift with αKG, and Bt-G6PDH proved effective as a negative control in SPR by preventing non-specific binding of αKG in the control flow cell. The change in RU with αKG, after adjustment of control-flow cell RU, was analysed in using GraphPad PRISM software using the Hill Slope.

5.3 Results

5.3.1 Wild-type HOGA kinetic parameters

Wild-type recombinant human HOGA was 4.8-fold less efficient at cleaving oxaloacetate compared to HOG (Table 16). This difference is a consequence of both a two-fold higher $K_M$ (130±8 μM vs 54±5 μM for oxaloacetate and HOG, respectively) and halved $k_{cat}$ (0.52 s⁻¹ vs 1.01 s⁻¹ for oxaloacetate and HOG, respectively).
5.3.2 Active-site mutant kinetics

Mutations to active site residues indicate that HOG and oxaloacetate cleavage proceeds using the same kinetic mechanism. The K196A and Y168F mutants were inactive with both substrates, supporting their essential role in schiff-base formation and proton shuttling during catalysis. The S77A mutant similarly affected HOG aldolase and OAD activities, increasing the $K_M$ and decreasing the $K_{cat}$, resulting in a 9.5-fold and 5.9-fold decrease in efficiency ($k_{cat}/K_M$) relative to wild-type for HOG and oxaloacetate, respectively (Table 16). N78 and S198 mutations had differing effects with HOG aldolase and OAD. The N78T mutant had no effect on OAD kinetics relative to wild-type. By comparison, the corresponding $K_M$ for HOG was increased 3-fold, while the $k_{cat}$ was increased 2-fold, resulting in no overall significant difference in efficiency relative to wild-type. These kinetic observations suggest that while N78 is important for HOG substrate binding, this long side-chain also causes steric hindrance, as shortening the N78 side-chain increased HOG turnover. Similarly, the S198A mutant had no effect on the $K_M$ for oxaloacetate, although the $k_{cat}$ was reduced by half. By comparison, the $K_M$ for HOG was increased 3-fold, while the $k_{cat}$ was again halved.
Table 16  Kinetic parameters for wild-type and active-site HOGA mutants with oxaloacetate or HOG as substrate (mean ± SEM).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mutant</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM.s$^{-1}$)</th>
<th>$k_{cat}/K_m$ relative to wild-type</th>
<th>Independ. Measurements (N)</th>
</tr>
</thead>
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<tr>
<td>oxaloacetate</td>
<td>wild-type</td>
<td>130±8</td>
<td>0.52±0.01</td>
<td>4.0±0.24</td>
<td>1.00</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>N78T</td>
<td>117±3</td>
<td>0.57±0.01</td>
<td>4.9±0.14</td>
<td>1.21</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>S77A</td>
<td>417±42</td>
<td>0.27±0.02</td>
<td>0.7±0.10</td>
<td>0.16</td>
<td>6</td>
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<tr>
<td></td>
<td>S198A</td>
<td>172±18</td>
<td>0.22±0.03</td>
<td>1.4±0.03</td>
<td>0.31</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>K196A</td>
<td>ND#</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
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<tr>
<td></td>
<td>Y168F</td>
<td>ND#</td>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>HOG</td>
<td>wild-type</td>
<td>56±4</td>
<td>1.01±0.05</td>
<td>19.0±1.61</td>
<td>1.00</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>N78T</td>
<td>152±4</td>
<td>1.97±0.07</td>
<td>12.2±0.18</td>
<td>0.64</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>S77A</td>
<td>156±4</td>
<td>0.31±0.01</td>
<td>2.0±0.08</td>
<td>0.11</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>S198A</td>
<td>139±6</td>
<td>0.46±0.02</td>
<td>3.3±0.15</td>
<td>0.17</td>
<td>5</td>
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<tr>
<td></td>
<td>K196A</td>
<td>ND#</td>
<td></td>
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<td></td>
<td>Y168F</td>
<td>ND#</td>
<td></td>
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</tr>
</tbody>
</table>

#ND = no detectable activity with excess enzyme and substrate (2 mM).

Experiments were conducted in triplicate on 2-3 independent days for each protein. Limited protein yield for S77A and S198A meant these measurements were conducted on 2 independent days, and for S198A with HOG substrate, one of these days was a duplicate measurement. The number of independent measurements are shown in the table for clarity, and data is displayed as mean ± SEM.
5.3.3 HOGA-catalyzed pyruvate carboxylation

The reversibility of the oxaloacetate decarboxylation reaction was measured with wild-type recombinant HOGA, and it was found that even with excess HOGA (60 μg) and excess pyruvate (100 mM), no pyruvate carboxylation occurred.

5.3.4 Inhibition of HOGA with α-ketoglutarate

For purified recombinant human HOGA, the TCA cycle intermediate αKG competitively inhibited HOGA OAD activity (Ki = 2.8±0.5 mM), and also inhibited HOG aldolase activity to a lesser extent (Ki=22±6.7 mM, Figure 35). The Ki was calculated using a global non-linear fit in GraphPad PRISM. OAD and HOG aldolase activity were also inhibited in mitochondrial lysates from HEK cells induced to overexpress wild-type HOGA (Figure 36). With 1 mM oxaloacetate, 20 mM αKG inhibited OAD activity ~50 % (p<0.01), however with the same concentration of HOG substrate, no inhibition of HOG aldolase activity was apparent. Inhibition of HOG aldolase activity with 20 mM αKG was only apparent when the concentration of HOG was closer to the K_M for HOG (50 % inhibition with 100 μM HOG, p<0.01).
Figure 35  Lineweaver Burk plots showing the competitive inhibition of HOGA oxaloacetate decarboxylase activity (A) and 4-hydroxy-2-oxoglutarate aldolase activity (B) with α-ketoglutarate. Experiments were conducted in triplicate on 3 separate days.
Figure 36 Oxaloacetate decarboxylase (OAD) activity (A) and HOG aldolase activity (B) in lysed mitochondria from F-293 cells overexpressing HOGA in the presence and absence of 20 mM αKG. **p<0.01 denotes a statistically significant difference without αKG using unpaired students t-test. Experiments were conducted in triplicate on 3 separate days.

5.3.5 Pyruvate inhibition of OAD activity

To gain insight into the effect of physiological concentrations of pyruvate that occur in mitochondria, pyruvate inhibition of HOGA OAD activity was measured with 128 μM oxaloacetate, the K_M value for HOGA. OAD activity was inhibited 30±8% with 250 μM pyruvate, 62±3% with 500 μM pyruvate, and 77±5% with 1000 μM pyruvate.
Figure 37  Inhibition of OAD activity with pyruvate with 128 μM oxaloacetate (K_M value for HOGA). Experiments were conducted in triplicate on 3 separate days.

5.3.6 αKG binding

Surface plasmon resonance determined an apparent K_D of 50 mM for αKG and hHOGA binding Figure 38 A). Thermal stability with DSF was another measure of αKG binding, and a dose-dependent increase in thermal stability was also observed with αKG Figure 38 B).
5.4 Discussion

5.4.1 hHOGA OAD vs HOG aldolase kinetics

Recombinant wild-type hHOGA was 4.8-fold less efficient at cleaving oxaloacetate as compared to HOG, similar to the finding with bovine HOGA that OAD decarboxylase activity was 10-50% of HOG aldolase activity (Dekker & Kitson, 1992; Kobes & Dekker, 1971b). We found an approximately 5-fold higher $K_M$ for HOG than has been measured previously for hHOGA (56 μM vs 11 μM by Riedel et al (2011)). While these data differ both are within range of $K_M$s measured for HOGA from bovine liver (100 μM) and bovine kidney (26 μM) (Dekker & Kitson, 1992; Dekker et al., 1971).

HOGA appears to use the same catalytic mechanism for OAD and HOG cleavage, and the effect of active-site mutations on HOG aldolase kinetics was similar to the only
other study of hHOGA (Riedel et al., 2011). K196 and Y168 are essential for OAD and HOG aldolase activities, and inactivity of K196A and Y168F with HOG was also found by Riedel et al. (2011). These residues are conserved across all Class I aldolases and necessary for Schiff-base formation and proton relay (Choi et al., 2006). S77 was not essential, but mutating this residue to alanine decreased the catalytic efficiency with HOG and oxaloacetate to 11-16% of wild-type values, similar to the finding of Riedel et al (2011), and this residue is proposed to be involved in the proton relay. Mutation of the S198 and N78 residues had differing effects with HOG and oxaloacetate. S198A and N78T mutants had no effect on the $K_M$ for oxaloacetate, but increased the HOG $K_M$ 3-fold, and this may be due to the longer length of the HOG molecule, which may permit maintenance of hydrogen bonding with N78 and S198 to increase its affinity, but not the shorter molecule oxaloacetate. The turnover rate of N78T with HOG was also doubled, so even though the $K_M$ was increased, the apparent turnover efficiency ($k_{cat}/K_M$) was not significantly different to the wild-type HOGA. This may be because hydrogen bonds between HOG and N78 are important for substrate binding, but the long side chain of N78 provides steric hindrance, which may then act to decrease the turnover rate with HOG.

5.4.2 Regulation of HOGA

$\alpha$KG was a competitive inhibitor of hHOGA, with approximately 10-fold more potency against OAD activity ($K_i=2.8$ mM) compared to HOG aldolase activity (22 mM). $\alpha$KG has similarly been found to competitively inhibit bovine HOGA HOG aldolase activity ($K_i=18$ mM) (Dekker et al., 1971) and the reverse condensation reaction ($K_i=52$ mM) (Grady et al., 1981). $\alpha$KG inhibition was also more potent with OAD activity compared to HOG aldolase activity in mitochondrial lysates from cells overexpressing wild-type HOGA, and only inhibited HOG aldolase activity to a similar extent when 10-fold lower concentrations of HOG were used (100 $\mu$M). This may reflect the higher affinity and faster turnover rate with HOG compared to oxaloacetate. The intra-mitochondrial hepatocyte
concentration of αKG is reportedly 2.7-3.2 mM, but drops markedly with glucagon (0.7-0.8 mM) (Siess et al., 1977; Williamson & Corkey, 1979). This may imply that αKG regulation of HOGA OAD activity varies depending on fed/fasted state, with potentially greater inhibition during in the fed state.

5.4.3 Physiological relevance of HOGA OAD activity

Intra-mitochondrial oxaloacetate concentrations are reported to vary from 5 to 30 μM (Iles, Cohen, Rist, & Baron, 1977; Siess et al., 1977; Williamson & Corkey, 1979), however concentrations of oxaloacetate were found to increase from 14 μM to 125 μM when mitochondria were incubated with malate alone (Garber & Salganicoff, 1973). It has been long held that malate alone is insufficient to fuel the TCA cycle, as oxaloacetate builds up in the absence of a source of acetyl CoA (eg. pyruvate) to condense with, leading to inhibition of upstream TCA cycle enzymes. (Gnaiger, 2009). We therefore investigated pyruvate inhibition of OAD activity with a relatively high level of oxaloacetate (128 μM), that may mimic the situation when mitochondria are respiring on malate alone. Pyruvate was found to inhibit OAD activity of hHOGA by 30 % with 250 μM pyruvate, and 77 % with 1000 μM pyruvate. This suggests that OAD activity of HOGA may be high when mitochondria are respiring on malate alone, but the re-introduction of pyruvate increasingly inhibits OAD activity. Pyruvate is both a product (OAD and HOG aldolase reactions) and a substrate (condensation of pyruvate and glyoxylate) for HOGA, and it is unsurprising that pyruvate inhibits HOGA OAD activity. Inhibition data suggests a level of regulation of hHOGA OAD activity by the TCA cycle, and this is further investigated in Chapter 6 in intact respiring mitochondria.
6. Contribution of HOGA and Malic Enzyme to TCA cycle turnover

6.1 Introduction

The cyclic nature of the TCA cycle involves the oxidation of malate to oxaloacetate by malate dehydrogenase, followed by the condensation of oxaloacetate and acetyl CoA by citrate synthase (Figure 39). As described in Section 1.7.12, mitochondria respiration with malate alone is expected to be minimal, as oxaloacetate build up in the absence of acetyl CoA inhibits upstream TCA cycles (Garber & Salganicoff, 1973; Gnaiger, 2009). Nevertheless, respiration with malate as the sole substrate does in fact occur, and is highly variable across species, tissue type, and disease status. This “residual” respiration is generally attributed to the variable expression of malic enzyme (ME).

The oxaloacetate decarboxylase activity of HOGA could also putatively contribute to malate respiration. However this possibility remains un-investigated in the literature, despite the original isolation of an enzyme in bovine and rat liver capable of both HOG aldolase and oxaloacetate decarboxylase activities (Kobes & Dekker, 1967; Lane & Dekker, 1972).

The few studies that have investigated malate respiration in healthy mammalian tissues report highly variable contributions of malate to combined pyruvate and malate (PM) or combined glutamate and malate (GM). Thus malate respiration was 22 % of PM mediated flux in rabbit heart mitochondria (Tarjan, 1971), 22 % of GM mediated flux in mouse brain mitochondria (D’alecy et al., 1986), 78 % of PM mediated flux in rat brain mitochondria (Amaral et al., 2012) and 80 % of PM mediated flux in guinea pig liver mitochondria (Garber & Salganicoff, 1973; Mikulski, Angielski, & Rogulski, 1972). In some non-mammalian species, malate alone is able to provide almost maximal C1 flux (section
1.7.3.1), including some plants, insects and cold-adapted fish and crustaceans (Chamberlin, 2004; Coleman & Palmer, 1972; Iftikar et al., 2014; Skorkowski, 1988). Additionally, some immortalized/tumour cells respire markedly on malate alone including Chinese hamster ovary (CHO) cells (Wahrheit et al., 2015), L-1210 Ascites tumour cell mitochondria (Hansford & Lehninger, 1973), and mouse ascites tumour mitochondria (Sauer & Dauchy, 1978).

This ability to respire on malate is generally attributed to mitochondrial ME (ME2 and or ME3), which oxidatively decarboxylates malate to pyruvate with concomitant reduction of NAD\(^+\)/NADP\(^+\) to NADH/NADPH. Malic enzyme thereby provides pyruvate, which is a source of acetyl CoA for oxaloacetate to condense with. The high \(K_M\) of ME2 for malate is suggested to allow TCA cycle function in the absence of ample pyruvate supply (Mandella & Sauer, 1975). In principle, the same pathway of pyruvate and NADH formation from malate could also occur through the concerted action of malate dehydrogenase and HOGA, Figure 39.

The aim of this chapter was to investigate the relative contribution of malic enzyme and HOGA to malate respiration in mammalian tissues. Two models were used: (1) cultured mammalian F-293 cells with no endogenous HOGA expression compared to F-293 cells induced to overexpress HOGA, as validated in section 4.4.3; and (2) healthy rat tissues with varying expression of HOGA and malic enzymes. In each model, the gene and protein expression of HOGA and mitochondrial malic enzymes ME2 and ME3, as well as enzymatic activities were measured. Then, phosphorylating malate respiration was measured, followed by pyruvate addition to enlighten the relative contribution of malate to combined malate and pyruvate flux. In an effort to segregate the contribution of HOGA and malic enzymes to malate respiration, a novel specific inhibitor of ME2 was utilized in a comprehensive substrate-inhibitor-uncoupler-titration oxygraphic protocol.
6.2 Methods

6.2.1 Experimental animals

Sprague-Dawley rats were maintained on standard rat chow and tap water ad libitum, and kept under a constant 12-hour light/dark cycle. Prior to tissue collection, rats were fasted overnight. All experiments were approved by the University of Auckland Ethics Committee.
6.2.2 Tissue collection

Tissue samples of liver, kidney, heart and brain were obtained from three month Sprague Dawley (SD) rats. Rats were anesthetized with isoflurane (3 L.min⁻¹) and euthanized by cardiac puncture. For isolation of functional mitochondria, roughly 1 g of heart apex, liver, kidney cortex and brain cortical tissue was rinsed in ice-cold PBS, and tissue minced and preserved in University of Wisconsin buffer (UW) for kidney and liver, and histidine-tryptophan-ketoglutarate (HTK) for heart and brain (Table 17) prior to mitochondrial isolation (6.2.7). For qRT-PCR, 20-50 mg tissue was preserved in RNA later (Ambion) and left at 4 °C for up to 1 month, and ~500 mg tissue was snap frozen and stored at -80 °C for enzyme assays.

6.2.3 HOGA overexpression in cultured cells

Flp-In™ HEK-293 T-REx™ (F-293) cells were used to inducibly express HOGA (F-293 HOGA) as described in 2.2.2. Media for the growth and maintenance of F-293 and F-293 HOGA cells are shown in Table 12. 24 hours prior to the collection of cells for each experiment, HOGA expression was induced in low glucose media containing tetracycline, and control F-293 cells were given the same media (Table 12). In experiments containing un-induced F-293 HOGA cells, low glucose media without tetracycline was added 24 h prior to cell collection.

6.2.4 qRT-PCR

RNA was isolated from animal tissue and cultured cells as described in 2.31 and 2.32, respectively. RNA concentration and integrity were measured as in 2.33, and cDNA synthesized as described in 2.34. RT-PCR was run as described in 2.35, using rat primers
listed in Table 13 or human primers listed in Table 14. Data was analysed using the $2^{\Delta\Delta C_{t}}$ method as described in 2.37.

6.2.5 Western Blot

Western blots were run as described in 2.2.42, loading 25 µg cell lysate or animal tissue lysate protein per well.

6.2.6 Mitochondrial isolation from rat tissues

Mitochondria were isolated by differential centrifugation. ~300 mg minced tissue in ice-cold preservation medium was transferred to 2 mL mitochondrial isolation medium (MIB, Table 17) and homogenized (OMNI-TH) for 20-30 seconds on medium speed. The first slow spin of the homogenate (1000 x g, 10 min, 4 °C), was followed by a second slow spin (1000 x g, 10 min, 4 °C) of the supernatant and the pellet, which was resuspended in MIB. The supernatants were then spun fast (14,000 x g, 10 min, 4 °C), and the mitochondrial pellet resuspended in ~30 µl mitochondrial respiration medium (MiR05, Table 17).

6.2.7 Mitochondrial isolation from cultured cells

Mitochondria from two T-175 flasks of F-293 cells were isolated by differential centrifugation. Media was aspirated and cells scraped in 15 mL PBS to two 50 mL falcon tubes. Cells were spun at 650 x g for 5 min at 4 °C, and the pellet resuspended in 1.5 mL MIB. Cells were manually homogenized by 30-40 strokes in a 2 mL Potter-Elvenjhem, and homogenate spun at 1000 x g (10 min, 4 °C). The supernatant was transferred to a 1.5 mL tube and the pellet resuspended in 750 µL MIB, and both spun again at 650 x g, 10 min, 4 °C. Supernatants were spun fast (14,000 x g, 10 min, 4 °C), and pellet resuspended in ~30 µL MiR05.
Table 17  Composition of buffers used in mitochondrial isolation

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Use</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>UW</td>
<td>Preservation of kidney and liver tissue</td>
<td>100 mM K-lactobionate, 30 mM raffinose, 25 mM H₂PO₄, 3 mM glutathione, 5 mM adenosine, 30 mM Na⁺, 125 mM K⁺, 5 mM Mg²⁺, 50 g HES, pH 7.4</td>
</tr>
<tr>
<td>HTK</td>
<td>Preservation of heart and brain tissue</td>
<td>30 mM mannitol, 1 mM ketoglutarate, 2 mM tryptophan, 180 mM histidine, 1 mM allopurinol, 15 mM Na⁺, 10 mM K⁺, 13 mM Mg²⁺, 0.25 mM Ca²⁺, pH 7.2</td>
</tr>
<tr>
<td>MIB</td>
<td>Isolation of mitochondria (cultured cells and animal tissue)</td>
<td>10 mM Tris-HCl, 250 mM sucrose, 0.5 mM EDTA, pH 7.4 @ 0 °C</td>
</tr>
<tr>
<td>Mir05</td>
<td>Oxygraphy and suspending mitochondrial pellet</td>
<td>60 mM K-lactobionate, 20 mM HEPES, 110 mM sucrose, 10 mM KH₂PO₄, 20 mM taurine, 0.5 mM EGTA, 3 mM MgCl₂, 1 mg.mL⁻¹ BSA, pH 7.1 @ 37 °C</td>
</tr>
</tbody>
</table>

UW: University of Wisconsin buffer, HTK: histidine-tryptophan-ketoglutarate, MIB: mitochondrial isolation buffer, Mir05 = mitochondrial respiration medium.

6.2.8 Protein quantification

Protein was quantified using BCA reagent, as described in 2.1.3.7. Mitochondrial suspensions were generally diluted 1:10, and BSA standards (100-1000 µg.mL⁻¹) were made up in MiR05.
6.2.9 Enzyme assays

Enzyme assays were performed using frozen mitochondria that were surplus to respirometry assays. Frozen mitochondria from one tissue or cell type were pooled, as individual remaining mitochondria from each sample were not sufficient for experiments. Pooled mitochondria were diluted to approximately 3 mg.mL$^{-1}$ in 100 mM Tris, pH 8, 0.5 % Triton X-100 on ice, and sonicated briefly. Protein was quantified using BCA (2.1.3.7), and aliquots frozen at -80 °C.

6.2.9.1 HOG aldolase

HOG aldolase was measured as in section 2.1.4.4 with a few modifications. Roughly 60 μg mitochondrial lysate and 1mM HOG were added to a 96 well plate in reaction buffer (100 mM Tris-HCl, pH 8, 0.1 U.mL$^{-1}$ LDH) and reaction initiated with 150 μM NADH in a final volume of 200 μL. The decrease in absorbance at 340 nm was followed in a Spectramax 340PC plate reader, and the linear rate subtracted from the background drop in NADH absorbance in mitochondrial lysate in the absence of HOG. The rate was calculated using the Beer-Lambert law (Equation 1), and normalized to protein content.

6.2.9.2 Oxaloacetate decarboxylase

Oxaloacetate decarboxylase activity in mitochondrial lysate could not be measured in a continuous LDH-coupled assay as it was for recombinant HOGA, as oxaloacetate and NADH will fuel malate dehydrogenase, which is abundant in mitochondria. Therefore, a staggered time-course assay was undertaken, in which mitochondrial lysate (~250 μg) was diluted to 150 μL in 100 mM Tris-HCl, pH 8, and allowed to equilibrate to 37 °C in a temperature-controlled mixing heat block (Thermomixer). An initial 50 μL aliquot was removed and immediately deproteinized (25 μL ice-cold 8 % perchloric acid) and later neutralized (25 μL 8 % KOH). The reaction was then initiated with 1 mM oxaloacetate, and
50 μL aliquots were removed every 5 or 10 minutes. Samples were staggered by 1 minute, and 3 background measurements were done in parallel containing only 1 mM oxaloacetate in 100 mM Tris-HCl, pH 8, for subtraction of background degradation of oxaloacetate to pyruvate at each time point. Each aliquot was then spun (1,000 xg, 2 min, 4 °C), and 80 μL supernatant transferred to a 96 well plate, to measure the formed pyruvate with LDH.

Reaction buffer (250 μM NADH, 100 mM Tris-HCl, pH 8) was added to the supernatant to a final volume of 200 μL, and the absorbance at 340 nm (A340) and pathlength measured in a Spectramax 340PC plate reader. Then LDH was added (11 U), and the A340 and pathlength re-measured after brief mixing. The amount of pyruvate was then calculated using Equation 10. After accounting for the dilution of the aliquot during deproteinization/neutralization and LDH measurement, the amount of pyruvate in the background experiments was subtracted, then normalized to the amount of protein in each aliquot.

\[
\text{mm Pyruvate} = \frac{(A340_{\text{final}} / 6.22 \text{ mM cm}^{-1} \times l_{\text{final}}) - (A340_{\text{initial}} / 6.22 \text{ mM cm}^{-1} \times l_{\text{initial}})}{}
\]

6.2.9.3 Malic enzyme

The activity of ME2 was measured as described previously (MacDonald et al., 2009), with a few modifications. Lysed mitochondria (30 μL, ~90 μg), 1 mM NADP⁺ and reaction buffer (6 mM MgCl₂, 0.5 mM TCEP in 100 mM Tris-HCl, pH 7.5) were added to a 96-well plate, and the reaction initiated with 10 mM malate and 5 mM fumarate. The production of NADPH was monitored by measuring the increase in absorbance at 340 nm. Beer’s law was used to calculate the production of NADPH (Equation 1), which has the same extinction coefficient as NADH. NADPH production was then normalized to protein content.
6.2.10 High-resolution respirometry

High-resolution respirometry was performed using OROBOROS Oxygraph-2K (Innsbruck, Austria) at 37 °C in 2 mL MiR05, and the air saturation of the medium was 215 nmol O₂.mL⁻¹ at 95 kPa barometric pressure. Generally, 0.1 mg of F-293 cell mitochondria or 0.25-0.5 mg of rat tissue mitochondria were added per 2 mL chamber. Two multi-substrate-inhibitor assays were completed to investigate malate respiration in F-293 cultured cells and animal tissues.

6.2.10.1 Malate respiration assay

In animal tissue mitochondria, malate respiration was generally quite low, therefore 0.5 mM ADP was added to ensure endogenous substrates were depleted prior to addition of malate. In F-293 cells, malate substrate addition alone achieved maximal C1 respiration, therefore mitochondria were left for ~15 minutes in the oxygraph to allow depletion of intramitochondrial substrates. Malate (2 mM) was then added followed by ADP (1.25 mM), then pyruvate (10 mM) for maximal C1 respiration. Background respiration was measured by the addition of rotenone (1 μM) and antimycin-a (2 μM) to poison C1 and C4, respectively.

6.2.10.2 Inhibition of malate respiration by the ME2 inhibitor NPD389

The specific ME2 inhibitor NPD-389 (Figure 40) was kindly gifted from the laboratory of Jia Li (Jiangsu University, Zhenjiang, China).

A more in-depth respirometry protocol was employed in F-293 cell mitochondria to determine the NPD-389 concentration required to cause maximal inhibition of malate respiration, with no effect on other components of the ETS. Mitochondria from F-293 cells (0.1 mg) were added to each of 6 oxygraph chambers, followed by NPD-389 at one of three different concentrations, equivalent to the IC₅₀ (5 μM), 2-fold the IC₅₀ (10 μM) and 5-fold the IC₅₀ (25 μM), based on the IC₅₀ found for recombinant ME2 by Wen et al. (2014). The
adjacent chamber acted as a vehicle control (DMSO). Following 15 minutes incubation, malate (2 mM) then ADP (1.25 mM) was added, followed by pyruvate (10 mM) and glutamate (10 mM) for maximal C1 respiration. Rotenone (1 μM) was added to inhibit C1, then C2 respiration stimulated with succinate (10 mM). ATP synthase was inhibited with oligomycin (5 μM) and maximal ETS flux measured by uncoupling mitochondria with Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.5 μM titrations).

Background respiration measured with antimycin-a (2 μM), which was later subtracted from other respiration measurements.

![Structure of the Malic Enzyme-2 inhibitor NPD-389](image)

**Figure 40**  Structure of the Malic Enzyme-2 inhibitor NPD-389
6.3 Results

6.3.1 Expression of HOGA and ME in F-293 cells

6.3.1.1 qRT-PCR Housekeeping genes

Five housekeeping genes were included in qRT-PCR experiments; PPIA, TBP, B2M, HPRT. The most stable housekeeping genes across tissues was determined using the Ct values in Bestkeeper software (Pfaffl et al., 2004) and transformed Ct values ($2^{-\Delta C_t}$) using Normfinder (Andersen et al., 2004). The two genes PPIA and TBP were identified as the best housekeeping genes to use in combination by Normfinder, with a combined stability value of 0.011. Bestkeeper identified PPIA and TBP as having both acceptably low standard deviation (<0.1) and high correlation coefficients, and the geometric mean of these two genes was used to normalize relative gene expression in HEK cells.

Table 18 Housekeeping gene parameters for HEK cells

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<tr>
<th>Housekeeping genes</th>
<th>PPIA</th>
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</table>
6.3.1.2 HOGA and ME2 expression

As described in 4.3.3.10, F-293 cells expressed very low endogenous levels of \textit{hoga1} mRNA, and HOGA protein was not detected in these cells (Figure 41 A, D). F-293 cells transfected with \textit{hoga1} show slightly increased levels of \textit{HOGA} mRNA and protein prior to induction with tetracycline, and this is increased significantly with 24 hours of tetracycline induction. To investigate whether other pathways of malate respiration were affected by HOGA expression, ME1 and ME2 gene expression were measured, and found not to change following HOGA expression Figure 41 B, C). ME2 protein expression was also unchanged with HOGA expression (Figure 41 D).

The measurement of ME3 transcript levels and protein expression by western blot were attempted. However ME3 was not detected, which is consistent with low expression of ME3 in other cell lines (Jiang et al., 2013; Pongratz, Kibbey, & Cline, 2009).
Figure 41  Relative gene (A-C) and protein expression (D) of hoga1 and malic enzymes in F-293 cells, and F-293 cells transfected with hoga1 gene, induced (+tet) and not induced (-tet) for HOGA expression. qRT-PCR experiments were conducted using cDNA from 5 different cell passages, each run in triplicate.
6.3.1.3 HOG aldolase and oxaloacetate decarboxylase activity in F-293 cell lysed mitochondria

In lysed mitochondria from F-293 cells overexpressing HOGA, HOG aldolase activity was increased ~20-fold (p<0.001) relative to F-293 cells (Figure 42 A), confirming that overexpressed HOGA protein was functional. Oxaloacetate decarboxylase activity was also increased in lysed mitochondria from F-293 HOGA cells, ~6-fold (p<0.05) relative to F-293 cells (Figure 42 B).

Figure 42  HOG aldolase (A) and oxaloacetate decarboxylase (B) activities of F-293 cells and F-293 cells induced to express HOGA. ***p<0.001 denotes a significant difference between F-293 cells and F-293 HOGA, *p<0.05, **p<0.01 denotes a difference in pyruvate concentration between F-293 and F-293 HOGA, #p<0.01 for a difference in slope between F-293 and F-293 HOGA.

Experiments were conducted using pooled mitochondrial lysate in triplicate, on 3-4 separate days.
6.3.1.4 Malate respiration in F-293 cells

Phosphorylating malate respiration was high in F-293 cells (Figure 43 A), and malate alone was able to support C1 respiration almost exclusively, with only a 10 % or less increase with pyruvate addition (Figure 43 B). In F-293 cells overexpressing HOGA, malate respiration was unchanged as was the relative contribution of malate to C1 respiration. A representative trace of F-293 cells is shown in Figure 43 C.
Phosphorylating malate respiration in F-293 cells and F-293 cells overexpressing HOGA (A), and ratio of malate:malate+pyruvate respiration (B). A representative oxygraph trace is shown in (B). 5 independent experiments were run, each in duplicate, using mitochondrial isolated from different cell passages.
6.3.1.5 Validation of malate respiration inhibition with NPD-389

Given that tumor cells are known to express high levels of ME2, we hypothesized that high malate respiration in these immortalized HEK cells measured in 6.2.3.5 was due to high ME2 expression. We therefore employed a strategy to inhibit ME2, which may have outcompeted HOGA activity, to determine whether HOGA activity may contribute to malate respiration in the absence of ME2 activity. A potent, specific inhibitor of ME2, developed against the human recombinant form of ME2, was kindly gifted to us by Professor Jingya Li (Wen et al., 2014).

A comprehensive substrate-uncoupler-inhibitor-titration (SUIT) protocol was employed to validate the inhibition of malate respiration with NPD-389, and confirm it did not interfere with other components of the ETS. NPD-389 inhibited malate respiration in F-293 cells in a dose-dependent manner, and upon addition of pyruvate respiration was restored to vehicle control levels, as shown in Figure 44. The IC\textsubscript{50} concentration of NPD-389 for ME2 activity (5 μM) found by Wen et al. (2014) inhibited malate respiration ~50 % relative to vehicle control. Increasing the concentration to 5-fold the IC\textsubscript{50} concentration (25 μM), inhibited 80 % of malate respiration, which was again completely restored with the addition of pyruvate. This finding implied that NPD-389 specifically inhibited malate mediated respiration through ME2, but that other components of the ETS were unaffected at the saturating concentrations used in the SUIT protocol. With 50 μM NPD-389, malate respiration was completely inhibited, however respiration was not restored upon the addition of subsequent C1 substrates pyruvate or glutamate or the C2 substrate succinate, implying global toxicity of the ETS at this concentration.

With increasing NPD-389 concentrations, F-293 cells became increasingly dependent on pyruvate to support C1 respiration (Figure 45), with a 4.5-fold increase in respiration with pyruvate seen with 25 μM NPD-389.
Figure 44 Representative plot of the inhibition of phosphorylating malate respiration (mal-ADP) by the ME2 inhibitor NPD-389 in F-293 cell mitochondria. Two concentrations are shown; the IC$_{50}$ for ME2 (5 μM) and 5-fold the IC$_{50}$ (25 μM). The green and red dotted lines indicate the inhibition by NPD-389 relative to vehicle control. Additional titrations show NPD-389 inhibition was specific to malate respiration, as the addition of pyruvate (pyr) restored Complex I respiration to vehicle control levels. Glutamate (glut) was added for maximal Complex I respiration. Complex 2 (succinate, succ) and uncoupled respiration (FCCP) were also unaffected by NPD389. Poisons have similar effects in control and NPD-inhibited mitochondria, including the Complex I inhibitor rotenone (rot), the ATP-synthase inhibitor oligomycin and (olig) and the Complex IV inhibitor antimycin-a (ant).
Figure 45  Dose-dependent increase in respiration with pyruvate (pyruvate+malate / malate) with NPD-389 compared to the equivalent volume of vehicle control (DMSO). ***p<0.001 denotes a significant difference between vehicle and NPD-389. Experiments were run in duplicate using mitochondria isolated from cells from different cell passages (n = 6).
6.3.1.6 Inhibition of malate respiration in F-293 HOGA cells

NPD-389 inhibited malate respiration to a greater extent in F-293 cells relative to F-293 cells overexpressing HOGA. At the highest concentration of NPD-389 used (25 µM), inhibition of malate respiration was on average 16 % lower in cells overexpressing HOGA (79±3.5 % vs 62±3.2 % in F-293 vs F-293 HOGA cells, p<0.01). Greater inhibition of malate respiration in F-293 cells with NPD-389 was also reflected by a greater increase in respiration with pyruvate in F-293 cells (5-fold vs 3.5-fold in F-293 cells vs F-293 HOGA cells, p<0.05, Figure 46).
Figure 46  Inhibition of malate respiration with NPD-389 in F-293 cells and F-293 cells overexpressing HOGA (A) and relative increase in respiration with pyruvate (B). *p<0.05 denotes a significant difference between F-293 and F-293 HOGA with 25 μM NPD, # p<0.05 denotes a significant difference in slope between F-293 and F-293 HOGA. Experiments were run in duplicate using mitochondria isolated from cells from different cell passages (n = 6).
6.3.2 Tissue expression of HOGA and ME

Five housekeeping genes were included in qRT-PCR experiments; YWHAZ, PPIA, TFRC, HPRT, TBP. The most stable housekeeping genes across tissues were determined using the Ct values in Bestkeeper software (Pfaffl et al., 2004) and transformed Ct values ($2^{-\Delta \Delta Ct}$) using Normfinder (Andersen et al., 2004). Bestkeeper identified two data sets as being outliers (one from a brain sample, one from a heart sample), and these datasets were excluded from further analysis. The two genes PPIA and TBP were identified as the best housekeeping genes to use in combination by Normfinder, with a combined stability value of 0.1, and Bestkeeper identified PPIA and TBP as having both acceptably low standard deviation (<0.1) and high correlation coefficients (Table 19).
Table 19  Housekeeping gene parameters

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<th>PPIA</th>
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6.3.2.1 HOGA1 and ME2 and ME3 gene and protein expression

*Hogal* gene and protein expression were similar in liver and kidney and almost undetectable in brain and heart, with >100-fold lower levels of relative *hogal* transcript relative to liver and kidney (Figure 47 A), and undetectable HOGA protein by western blot (Figure 47 B).

ME2 gene expression was similar in kidney, heart and brain, with liver expressing lower levels compared to kidney (p<0.01), however protein expression of ME2 protein expression was higher in brain relative to all other tissues (Figure 47 C and D). ME3 gene expression was 13-fold higher in heart relative to other tissues (p<0.001) while ME3 protein expression was higher in heart and brain relative to liver and kidney Figure 47 E and F). In tissues that express HOGA (liver and kidney) levels of *hogal* transcript appear to be higher than all malic enzyme isoforms, when normalized to housekeeping genes ($2^{-ΔCt}$, Figure 47 E).
Figure 47 Relative gene expression (A,C,E) and protein expression (B, D, F) of HOGA1 and ME2/3 in rat tissues. Gene expression is relative to the geometric mean of the housekeeping genes PPIA and TBP, and relative to the liver for each gene ($2^{-\Delta\Delta Ct}$). Results are expressed as mean ± SEM. Protein expression is relative to COX-IV intensity using western blot, and representative blots shown for each. Significant differences are depicted with a letter for each tissue (liver, L; kidney, K; heart, H, brain, B), by ANOVA with post-hoc Bonferroni pairwise correction. qRT-PCR experiments were run in triplicate (n = 6), and western blot samples were run on a single lane (n = 4).
6.3.2.2 HOG aldolase and oxaloacetate decarboxylase activity in mitochondrial lysate

HOG aldolase activity was higher in liver compared to kidney ($p<0.05$), which were both significantly higher than heart and brain activities ($p<0.001$ for liver vs heart and brain and $p<0.05$ for kidney vs heart and brain) (Figure 48A). Pyruvate production from oxaloacetate was similar in liver, kidney and heart, however oxaloacetate decarboxylase rates were not calculated as brain had a lag in pyruvate production, with initial consumption of pyruvate (Figure 48B).
Figure 48  HOG aldolase activity (A), malic enzyme activity (B), and oxaloacetate decarboxylase activity (C) of rat mitochondrial lysates. The amount of pyruvate produced per mg mitochondrial protein after 30 minutes is depicted for (C). ***p<0.001 for a significant difference between brain and all other tissues, *p<0.05 for a significant difference between kidney only. ME activity in brain was only able to be assessed once, therefore brain was excluded from statistics. Experiments were run using pooled mitochondrial lysate, run on 3-4 separate days in duplicate.
6.3.2.3 Malate respiration in rat mitochondria

Phosphorylating malate respiration was similar across rat tissues (**Figure 49**). With the addition of pyruvate for maximal Complex 1 respiration, the ratio of malate:malate+pyruvate respiration was calculated to reveal the proportion of C1 respiration supported by malate alone (**Figure 49B**). Malate supported the greatest relative amount of maximal C1 respiration in liver (45 %, p<0.01 vs kidney and p<0.001 vs heart), whereas malate supported 33 % of C1 respiration in brain (p<0.01 vs heart), and malate supported the least relative amount of C1 respiration in kidney and heart (26 % kidney, 15 % heart). A representative oxygraph trace is shown in **Figure 49 C**.
Figure 49  Phosphorylating malate respiration in rat liver, kidney, heart and brain mitochondria (A) and ratio of malate respiration:malate+pyruvate respiration (B). *p<0.01, ***p<0.001 using one-way ANOVA with post-hoc Bonferroni pairwise correction. A representative oxygraph trace of kidney mitochondria is show in (C). mal, malate; pyr, pyruvate; rot, rotenone. Experiments were run in duplicate (n = 6).
6.3.2.4 Malate respiration inhibition with NPD-389

Liver, kidney, heart and brain all relied on malate for C1 respiration to varying extents, and expressed varying amounts of HOGA and ME2/3. In an effort to segregate the contributions of malic enzyme and HOGA to malate respiration, the ME2 inhibitor NPD-389 was trialled in rat tissue mitochondria, as described in 6.3.1.5 for cells. In contrast to F-293 cultured cells, NPD-389 had no effect on malate respiration in isolated rat mitochondria, at a concentration (25 μM) that inhibited malate respiration by ~80 % in F-293 cells.

6.3.3 Complex 2 inhibition

In a preliminary investigation, malate inhibition of Complex 2 respiration and the effect of HOGA overexpression in F-293 cells were measured by titrating malate into succinate-respiring mitochondria (Figure 50). With 1 mM malate, succinate respiration was inhibited 10 % in F-293 mitochondria, and 9 % in F-293 HOGA mitochondria, and with supra-physiological 5.4 mM malate, respiration was inhibited 20 % in both cell types. Due to the lack of difference in cell types, no further experiments investigating C2 respiration were undertaken.
Figure 50  Representative traces of inhibition of succinate-respiration with malate in F-293 (A) and F-293 cell mitochondria overexpressing HOGA (B). In (C) the % inhibition is shown. Succ=succinate, rot=rotenone.
6.4 Discussion

6.4.1 Inhibition of ME2 in F-293 cells

Induced expression of hHOGA in F-293 cells increased HOG aldolase and OAD activities but made no difference to malate respiration compared to F-293 cells which expressed no endogenous HOGA. We hypothesized that high malate respiration in these cells may be due to ME2, an enzyme known to have high expression and activity in immortalized/transformed cells, which may have outcompeted any potential contribution of HOGA to malate respiration. We therefore utilized the novel, specific inhibitor of ME2, NPD-389, which dose-dependently inhibited malate respiration in F-293 cell mitochondria. With 5-fold the IC50 concentration (25 μM) of NPD-389 found for recombinant human ME2 (Wen et al., 2014), we found 80 % inhibition of malate respiration with no off-target effects on ETS function. This was the first study to suppress malate respiration specifically through NPD-389 inhibition of ME2, and informs as to the large contribution of ME2 for malate respiration in immortalized cells such as HEKs (Chang et al., 2014; Jiang et al., 2013; Ren et al., 2014; Wen et al., 2014).

6.4.2 Inhibition of ME2 in F-293 cells overexpressing HOGA

By almost completely inhibiting ME2-induced malate respiration with NPD-389, we showed that F-293 cell mitochondria overexpressing HOGA had higher malate supported respiration. Additionally, HOGA-expressing cell mitochondria showed a smaller relative increase in respiration with pyruvate addition, implying that these mitochondria were more able to respire on malate during blockage of the ME2 pathway. This is supportive of HOGAs potential contribution of OAD activity to pyruvate formation when high malate levels lead to a build-up of oxaloacetate, which allows TCA cycle turnover in the absence of externally added pyruvate. Though our measured $K_M$ for hHOGA is above the generally
measured intra-mitochondrial concentration of oxaloacetate (5-30 μM), oxaloacetate concentration is reported to increase to ~100 μM when malate is the sole substrate (Garber & Salganicoff, 1973).

### 6.4.3 Malate respiration in various animal tissues

Healthy rat mitochondria respired on malate relative to malate and pyruvate respiration to varying degrees in different tissues (in liver malate respiration was 45 % of PM, brain 33 % of PM, kidney 26 % of PM, and heart 15 % of PM). We were unable to segregate the potential contribution of ME2 and HOGA to malate respiration in rat tissues as we did in F-293 cells, as rat mitochondria appeared to be insensitive to NPD-389. NPD-389 was developed using human recombinant ME2 (Wen et al., 2014) and it is possible that species differences between rat and human ME2 isoforms explain the lack of response to NPD-389 in rats. For example, NAD+ binding is required for NPD-389 binding to human ME2, and residues in the second NAD+ binding site differ between the human and rat ME2 isoforms (residue 194 and 197) (Xu, Bhargava, Wu, Loeber, & Tong, 1999).

It is likely that HOGA and ME contribute to malate respiration to varying extents in different tissues. HOGA expression and activity were high in liver and kidney, and virtually absent in heart and brain, whereas mitochondrial ME activity was highest in brain and heart, with ME2 expression high in brain, and ME3 expression high in heart. ME2 is implicated in the literature as having a greater role in mitochondrial malate respiration than other ME isoforms, and it is suggested that the high $K_M$ of ME2 for malate allows TCA cycle turnover in the absence of pyruvate (Mandella & Sauer, 1975). It is possible that high ME2 expression in the brain and HOGA expression in liver and kidney allowed malate turnover, whereas heart had low malate turnover relative to malate and pyruvate combined flux.
6.4.4 Summary

In conclusion, overexpression of HOga allowed increased TCA cycle turnover on malate in an immortalized HEK cell line, only when the ME2 pathway was almost completely blocked. In healthy rat tissues, HOga and ME2/3 expression were variable, and the contribution of malate to C1 respiration was also variable, however the contribution of HOga and ME to malate respiration was unable to be segregated using the ME2 inhibitor NPD-389, potentially due to species differences in ME2 isoforms.
7. General Discussion

7.1 Summary of findings

The main findings of this thesis are that; (1) recombinant wild-type HOGA is a tetramer in solution; (2) the most common PH3 HOGA mutants are expressed in vitro, but degraded proteasomally; (3) the HOG aldolase activity of HOGA is 4-fold the efficiency of OAD activity, and these activities proceed via the same catalytic mechanism; (4) HOGA is inhibited by the TCA cycle intermediate αKG at a physiologically relevant concentration; (5) HOGA may influence TCA cycle turnover, as overexpression of HOGA in HEK cells increases malate turnover during inhibition of the ME2 pathway.

7.1.1 Quaternary structure of hHOGA

Human recombinant HOGA was a tetramer in solution across a wide range of concentrations. This is in contrast to the only other study of hHOGA in which disassociation of tetramer into dimers at a relatively high concentration was observed (K_D=60 μM) (Riedel et al., 2011). It is not known why our results with hHOGA differ with Riedel et al. (2011). Similar to our study of hHOGA, bovine HOGA is consistently found to be a tetramer in solution (Dekker & Kitson, 1992; Kobes & Dekker, 1969; Rosso & Adams, 1967).

7.1.2 Effect of PH3 mutations on HOGA structure/function

The most prevalent PH3 mutations, the deletion of glutamate residue 315 (Δ315) and in-frame insertion of 17 residues (c.700+5G>T) caused HOGA protein aggregation, thermal instability and inactivity when expressed recombinantly in a bacterial expression system, similar to the findings of Riedel et al. (2012) with Δ315 HOGA. In a mammalian cell expression system, Δ315 HOGA mutant RNA was upregulated but protein was not
detectable, and we have further shown that defective Δ315 HOGA protein was degraded intracellularly via ubiquitin-mediated proteasomal degradation in vitro. This study confirms that the most common PH3 mutants Δ315 and c.700+5G>T are loss of function mutations, which has previously been hypothesized due to increased levels of HOGA's substrate in PH3 patients, and the finding of truncation mutants of HOGA in PH3 incapable of catalysis (Beck et al., 2013; Monico et al., 2011; Williams et al., 2012).

7.1.3 Regulation of HOGA by the TCA cycle

The TCA cycle intermediate αKG competitively inhibited OAD activity of hHOGA at a physiologically relevant concentration (Ki=2.8 mM), and also inhibited HOG aldolase activity to a lesser extent (Ki=22 mM). Similar concentrations of αKG inhibit HOG aldolase activity of bovine liver HOGA (Dekker et al., 1971; Grady et al., 1981), however this is the first study to report αKG inhibition of human HOGA OAD activity. The Ki for αKG inhibition of HOGA OAD activity is within the physiological range of intra-mitochondrial α concentrations, which are reportedly 2.7-3.2 mM in hepatocytes in the normal fed state, and drop to 0.4-0.7 mM with glucagon (Siess et al., 1977; Williamson & Corkey, 1979), implying that regulation of HOGA through αKG may vary depending on fasted/fed state. Future studies could investigate HOGA expression and/or activity in the fasted versus fed state.

7.1.4 HOGA in the TCA cycle

While our measured KM of hHOGA for oxaloacetate (130 μM) is above the reported intra-mitochondrial concentrations of oxaloacetate (5-30 μM) (Iles et al., 1977; Siess et al., 1977; Williamson & Corkey, 1979), regional concentrations of oxaloacetate may vary, and oxaloacetate has been shown to increase up to 125 μM in malate-fuelled guinea-pig
mitochondria (Garber & Salganicoff, 1973). In malate-fuelled mitochondria, HOGA OAD activity may provide a pathway to decrease the build-up of oxaloacetate, by forming pyruvate and decreasing inhibition of complex 2. This would allow the TCA cycle to turnover in the absence of exogenous pyruvate. In support of this, blockade of the ME2 pathway with the novel inhibitor of ME2 NPD-389 showed that HOGA-overexpression allowed higher malate respiration in F-293 cells.

7.2 Physiological relevance of HOGA OAD activity

HOGA OAD activity may regulate TCA cycle turnover when malate concentrations are high, and the direction of effect likely depends on acetyl CoA concentrations. When acetyl CoA is limiting, HOGA-OAD activity may sustain TCA cycle turnover by providing pyruvate as a source of acetyl CoA. However, if acetyl CoA concentrations are sufficient, OAD activity may suppress TCA cycle turnover by depleting oxaloacetate. Potential physiological situations are discussed in the following sections.

7.2.1 Fasted/fed state

In glucagon-stimulated rat hepatocytes, the intra-mitochondrial concentration of malate increases and αKG decreases, which may lead to an intermittent increase in oxaloacetate potentially stimulating HOGA activity (Parrilla et al., 1976; Siess et al., 1977; Siess & Wieland, 1980; Williamson & Corkey, 1979). However, in the glucagon-stimulated state, fatty acid β-oxidation and gluconeogenesis are upregulated, leading to increased acetyl CoA and utilization of oxaloacetate (Siess et al., 1977; Williamson & Corkey, 1979). Therefore, HOGA OAD activity may potentially slow TCA cycle turnover and gluconeogenesis during fasting by depleting oxaloacetate necessary for both processes.
7.2.2 Glutamine utilization

Upregulated glycolysis and glutaminolysis are hallmarks of tumorigenesis (DeBerardinis et al., 2008; Ruiz-Perez et al., 2014), and some *in vitro* studies show that glucose deprivation is mitigated by glutaminolysis (Yang et al., 2014). Potentially glutamine-fuelled tumour cells deprived of glucose would have increased malate and oxaloacetate concentrations and a lack of acetyl CoA, however *in vitro* this is not apparent.

In isotope labelling studies of glioblastoma cells, glucose was the main supply of acetyl CoA and glutamine the main supply of oxaloacetate (DeBerardinis et al., 2007; Yang et al., 2009). However, during glucose deprivation, glutaminolysis appears to maintain acetyl CoA and citrate concentrations, and this effect has been ascribed to ME2, as siRNA silencing of ME2 in glioma (SFxL) cells decreased glutamine-derived citrate ~75 % (p<0.001) (Yang et al., 2014). Therefore, it appears that even during glucose deprivation, acetyl CoA and citrate levels are maintained by glutaminolysis in glioblastoma cells. This may be in part due to the activity of ME2, and also through reductive carboxylation of cytosolic αKG to citrate by IDH1, which may contribute to 7-25 % of citrate formed in lung cancer, breast cancer and HEK cell lines (Metallo et al., 2012).

The ME2 reaction in effect combines the net reactions of malate dehydrogenase and HOGA, therefore it is conceivable that HOGA could be involved in glutaminolysis and/or tumorigenesis. However, there is a lack of evidence that the MDH-HOGA pathway is involved in cancer metabolism. One proteomic study found 3-fold down-regulation of HOGA protein in colorectal cancer (Siu et al., 2009), and another finding down-regulation of mitochondrial MDH (White et al., 2014). It may be that the malic enzyme pathway is more beneficial for tumours, allowing for the production of NADPH necessary for lipid and glutathione synthesis. Although ME2 has traditionally thought to have preferred NAD+ over NADP+, knockdown experiments suggest otherwise (Jiang et al., 2013; Ren et al., 2014). ME2 knockdown decreased cellular NADPH levels in normal fibroblast IMR90 cells (Jiang
et al., 2013), and decreased the cellular NADPH/NADP+ ratio in A549 non-small cell lung
cancer cells (Ren et al., 2014). Additionally, overexpression of ME2 increased cellular
NADPH in IMR90 cells (Jiang et al., 2013).

7.2.3 ROS production

HOGA may have a role in the regulation of reactive oxygen species (ROS).
Succinate generally increases during ischemia across metabolically diverse tissues, and is
likely due to reversal of succinate dehydrogenase (fumarate \( \rightarrow \) succinate) (Chouchani et al.,
2014). Rapid succinate oxidation upon reperfusion leads to a large burst of mitochondrial
ROS, and it appears most likely that reverse electron transport to Complex 1 is the cause
(Chouchani et al., 2014; Dröse, 2013). Though the site of ROS production from succinate is
debated, it appears to depend on the concentration of succinate, with high succinate
concentrations causing reverse electron transport to Complex 1, and low succinate
concentrations causing ROS production at Complex 2 (Dröse, 2013). Regardless, succinate
build-up during ischemia causes excessive ROS production following reperfusion, which
may be augmented by inhibiting succinate buildup.

Malate concentrations reportedly increase in the succinate-fuelled state and during
ischemia (Papa, Lofrumento, Paradies, & Quagliariello, 1969; Pisarenko, Studneva,
Khlopkov, Solomatina, & Ruuge, 1988; Williamson & Corkey, 1979). By depleting malate
and oxaloacetate, ME2 and HOVA could potentially limit succinate build-up during
ischemia, and ameliorate the ROS burst upon reperfusion.

7.2.3.1 Oxaloacetate and brain ischemia

Oxaloacetate appears to be neuroprotective during ischemia, as its supplementation
prior to cerebral ischemia decreases infarct size (Campos, Sobrino, Ramos-Cabrera, &
Castillo, 2012; Nagy et al., 2009) by decreasing blood glutamate levels. Glutamate is an
established mediator of neuronal damage during ischemia, released in excessive amounts from neurons and astrocytes causing calcium overload. It is hypothesized that oxaloacetate decreases blood glutamate by increasing glutamate-oxaloacetate transaminase (GOT) activity, an enzyme that converts glutamate and oxaloacetate to alpha-ketoglutarate and asparatate. In support of this, supplementing GOT or oxaloacetate decreased blood glutamate levels and decreased infarct size in an animal model of ischemia (Pérez-Mato et al., 2014). The absence of HOGA in the brain is probably beneficial during cerebral ischemia, as HOGA could deplete neuroprotective oxaloacetate levels.

7.2.3.2 Malic enzyme and ROS

ME2 appears to ameliorate ROS production in normoxia, as ME2 knockdown in multiple cancer cell lines increased ROS production (Chang et al., 2014; Jiang et al., 2013; Ren et al., 2014; Woo et al., 2015 May ). ME2 may contribute to ROS depletion through NADPH production, which is necessary for antioxidants such as glutathione. Additionally, fumarate is an activator of ME2-catalyzed oxidative decarboxylation of malate (MacDonald et al., 2009), and enhanced malate turnover could therefore contribute to depletion of the malate precursor, fumarate. In our study, we found that malate inhibited succinate respiration by 10% with 1 mM malate, and 20% with 5.4 mM malate. HOGA expression did not alter these levels of inhibition. Previous studies show 50% inhibition of succinate respiration with 4 mM malate or 60 μM oxaloacetate in kidney preparations (Bienholz et al., 2014; Papa et al., 1969), comparatively lower than our results with HEK cells.

Potentially, high ME2 expression in F-293 cells allowed malate depletion and prevented oxaloacetate build-up, hampering succinate inhibition. High ME2 expression may also explain why we saw no effect of HOGA overexpression on malate-inhibition of succinate respiration. High ME2 turnover of malate could prevent sufficient oxaloacetate concentrations for HOGA OAD activity.
7.3 hHOGA OAD vs HOG aldolase activity

The present study shows that the OAD and HOG aldolase activities of HO GA utilize the same catalytic mechanism and thus compete for the same active site. However HO G has a higher affinity and faster turnover than oxaloacetate, suggesting that the HOG aldolase activity should predominate particularly when hydroxyproline flux is high. During hydroxyproline catabolism, oxaloacetate may intermittently decrease, as the enzyme upstream of HO GA aspartate aminotransferase transfers the amino group from 4-hydroxy-L-glutamate to oxaloacetate to form 4-hydroxy-2-oxoglutarate, the substrate for HO GA, and aspartate (Figure 51).

Hydroxyproline flux increases following ingestion of hydroxyproline-containing food. Ingestion of hydroxyproline-containing gelatin in humans increased plasma hydroxyproline and urinary excretion of hydroxyproline metabolites glycolate and oxalate concentrations (Jiang et al., 2012). Similarly, urinary excretion of hydroxyproline metabolites increased following hydroxyproline ingestion in rats relative to fasted rats, including glycolate, glycine and oxalate (Takayama et al., 2003). Rats fed a high-protein, high-fat diet increased activities of hydroxyproline-catabolising enzymes, including HO GA and AGTX, relative to a low-protein, low-fat diet (Carnie et al., 1982).

Increased flux through the hydroxyproline catabolic pathway suggests higher relative HOG aldolase activity of HO GA, which may decrease OAD activity of HO GA in the hydroxyproline-fed state. In turn, OAD activity may increase in the fasted state when hydroxyproline flux decreases and αKG concentration drops.
**Figure 51** Metabolic pathways of HOGA involvement. HOGA HOG aldolase activity in hydroxyproline catabolism (blue), and oxaloacetate decarboxylase activity in the TCA cycle (black), and the malate-aspartate shuttle for import of malate and oxaloacetate (grey) are shown.
7.4 Effect of OAD activity in PH3 patients

PH3 patients have loss of function \textit{HOGA1} mutations. Assuming that HOGA OAD activity dampens gluconeogenesis by depleting the gluconeogenic substrate oxaloacetate, PH3 patients may potentially be at increased risk of unregulated gluconeogenesis, a hallmark of type II diabetes (Magnusson, Rothman, Katz, Shulman, & Shulman, 1992). The Primary Hyperoxalurias are rare diseases with an estimated incidence of 1-3 per million of the population, of which 5-10\% are PH3 (Hoppe et al., 2009), therefore sparse information is available on these patients, including metabolic status or comorbidities.

In the non-PH population, glyoxylate, the product of HOGA HOG aldolase activity is raised prior to diagnosis of T2DM and is proposed as a biomarker for T2DM (Nikiforova et al., 2014; Padberg et al., 2014). One hypothesis is that increased glyoxylate is due to decreased activity of AGTX, which occurs in hypertension (Padberg et al., 2014). AGTX can turnover dimethylarginine, an inhibitor of the endogenous vasodilator nitric oxide that occurs naturally in blood plasma as a by-product of methylated-protein breakdown (Rodionov, Murry, Vaulman, Stevens, & Lentz, 2010). The hypothesis that increased glyoxylate is an early marker of AGTX-induced endothelial dysfunction associated with hypertension and diabetes development warrants further investigation in the non-PH population. AGTX is deficient or mis-targeted in PH1 (Danpure & Jennings, 1986), and while there are no reported associations of PH1 and T2DM or hypertension, this population may provide valuable information about the potential association between these comorbidities and AGTX dysfunction. Additionally, glyoxylate reductase has been found decreased in T2DM obese subjects compared to obese subjects (Valle et al., 2012).

Type 2 diabetics have increased urinary excretion of oxalate (Eisner, Porten, Bechis, & Stoller, 2010; Taylor & Curhan, 2008). However, the lipase inhibitor orlistat commonly prescribed to overweight diabetics, increases urinary oxalate, (reviewed in (Ahmed, 2010), and it is hypothesized that orlistat increases oxalate enteric absorption due to unabsorbed fat.
reacting with calcium in the colon. Most studies find similar hydroxyproline urinary excretion in T2DM and controls, but increases in nephropathy, associated with microalbuminuria (reviewed in (Starup-Linde & Vestergaard, 2015). T2DM increases the risk of renal stones of any type (Taylor, Stampfer, & Curhan, 2005), however, another study found only uric acid renal stones were associated with metabolic syndrome, and there was no association with calcium oxalate stone formation and metabolic syndrome (Cho, Jung, Myung, & Kim, 2013; Kadlec et al., 2012).

In summary, there is currently insufficient evidence that PH3 patients are at risk of elevated gluconeogenesis and T2DM. The collection of metabolic comorbidity data in this patient population is of interest due to the potential regulation of gluconeogenesis by HOGA which is defective in PH3. Conversely, the observations of elevated glyoxylate and oxalate in T2DM may suggest an association between elevated gluconeogenesis and hydroxyproline catabolism; however, this interaction may be complicated by medications such as orlistat, which appears to alter oxalate absorption.

7.5 Conclusions and scope for future studies

7.5.1 Primary Hyperoxaluria Type 3

This study confirms that the most common PH3 mutations are loss of function mutations, and demonstrates the mechanism of ubiquitin-mediated proteasomal degradation of the Δ315 HOGA mutant. However, the mechanism whereby loss of HOGA function leads to hyperoxaluria in PH3 is still uncharacterized. Our laboratory is pursuing the theory that HOG, the substrate of HOGA, leaks from the mitochondria to the cytosol where it could potentially be cleaved by an unspecific cytosolic aldolase. The group of Riedel et al (2012) is reportedly using the HOGA knockout mouse to further investigate the theory that HOG
inhibits cytosolic glyoxylate reductase, an enzyme normally responsible for detoxifying
glyoxylate.

### 7.5.2 HOGA oxaloacetate decarboxylase activity and the TCA cycle

This thesis has confirmed a novel pathway in mitochondria. The presence of a
mitochondrial oxaloacetate decarboxylase was measured decades ago, and debated by
others, and this study confirms that human recombinant HOGA decarboxylates oxaloacetate
at an appreciable rate. In intact mitochondria, overexpression of HOGA influences TCA
cycle turnover in the malate-fuelled, malic-enzyme 2-inhibited state in HEK cells. Further,
human HOGA is regulated by the TCA cycle intermediate α-ketoglutarate. Future study
could further elucidate the contributions of HOGA and ME2 to TCA cycle turnover. Isotope
labelling studies across metabolically diverse cell lines in which either HOGA or malic
enzyme isoforms were knocked down may elucidate the physiological contribution of each
pathway. Alternatively, the specific ME2 inhibitor NPD-389 used in this study could be used
in isotope-labelling studies. However, there is currently no specific inhibitor of HOGA. The
potential regulation of gluconeogenesis by HOGA also warrants further investigation. A
simple investigation could measure HOGA activity in starved vs fed rat liver. A more
involved approach could involve transfection of primary rat hepatocytes with HOGA, and
measure glucose output relative to the transfection control cells.
8. APPENDICES – DNA sequences

8.1 Wild-type HOGA in pDEST556 expression plasmid.
1.2 Δ315 in pDEST556 plasmid

1.3 c. 700+5G>T in pDEST556 plasmid
1.4 F-293 wild-type HOGA and Δ315 HOGA

![Diagram showing sequences and amino acid comparisons for wild-type and modified HOGA proteins.](image-url)
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