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Exploration of mutations in erythroid 5-aminolevulinate synthase that lead to increased porphyrin synthesis

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Exploration of Mutations in Erythroid 5-Aminolevulinate Synthase that Lead to Increased Porphyrin Synthesis

by

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A dissertation submitted in partial fulfillment of the requirement of the degree of Doctor of Philosophy
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Keywords: x-linked sideroblastic anemia, x-linked erythropoietic protoporphyria, heme, isoniazid, photodynamic therapy

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DEDICATIONS

I dedicate this dissertation to my grandparents, Frederick and Katrina Richfield, two of the most hardworking, loving, and inspiring people I will have ever known. I also dedicate this work to my amazing parents, Donald Douglas Fratz and Naomi Richfield-Fratz, who inspire me to follow my dreams and support me through all of my endeavors. Lastly, I dedicate this dissertation to the love of my life, and the most fantastic human being I know, Michael Berilla, who inspires me to make every day the best it can be.
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# TABLE OF CONTENTS

List of Tables ......................................................................................................................... iv

List of Figures ........................................................................................................................ v

Abbreviations ........................................................................................................................ viii

Abstract .................................................................................................................................. xii

Chapter One: Introduction ................................................................................................. 1
  Biosynthesis of 5-aminolevulinate and heme ................................................................. 1
  5-Aminolevulinate synthase .............................................................................................. 5
  Regulation of ALAS2 transcription .................................................................................. 7
  Regulation of ALAS2 translation .................................................................................... 13
  Stability of ALAS2 ........................................................................................................... 17
  Mitochondrial import of ALAS2 ..................................................................................... 20
    Heme inhibition of ALAS mitochondrial import .......................................................... 22
    Heme regulatory motifs of ALAS ................................................................................ 25
  Diseases associated with mutations in ALAS2 .............................................................. 29
  Content of the dissertation ............................................................................................... 31
  References ......................................................................................................................... 32

Chapter Two: Expression of murine 5-aminolevulinate synthase variants
causes protoporphyrin IX accumulation and light-induced cell
dearth in mammalian cells .................................................................................................... 56
  Abstract ............................................................................................................................ 56
  Introduction ....................................................................................................................... 57
  Materials and Methods .................................................................................................... 60
    Materials ........................................................................................................................ 60
    Plasmids ........................................................................................................................ 61
    Cell Culture .................................................................................................................... 61
    Stable transfection of K562 human erythroleukemia cells ......................................... 62
    Transient transfection of HeLa cells .............................................................................. 63
    Preparation of cells for FACS and quantitation of PPIX ............................................ 64
    Light exposure assays .................................................................................................. 65
    Cell viability assays ...................................................................................................... 65
    Confocal fluorescence microscopy .............................................................................. 67
  Results ............................................................................................................................... 67
Transient expression of mALAS2 variants causes accumulation of PPIX in HeLa cells ........................................... 67
Supplementation of cell culture medium with glycine leads to increased PPIX accumulation in mALAS2-expressing HeLa cells ...................................................... 70
Glycine and deferoxamine increase PPIX accumulation in mALAS2-expressing K562 cells ........................................... 73
PPIX primarily accumulates and localizes at the plasma membrane in HeLa cells expressing R433K .................. 76
ALAS2-induced PPIX accumulation followed by light exposure combined with paclitaxel treatment causes cell death ................................................................. 78
Supplementation of cell culture medium with glycine enhances phototoxicity and cell death in mALAS2-expressing HeLa cells ...................................................... 79
Discussion .............................................................................. 81
References .............................................................................. 92

Chapter Three: Mutations in the C-terminus of human erythroid 5-aminolevulinate synthase associated with x-linked erythropoietic protoporphyria alter enzyme structure, kinetics, and ex vivo activity ................................................................. 104
Abstract .................................................................................. 104
Introduction ............................................................................. 105
Materials and Methods ................................................................. 108
  Reagents .................................................................................. 108
  Plasmids for mammalian expression and protein purification .... 108
  Cell culture ............................................................................. 111
  Transient transfection of HeLa cells ........................................ 111
  Transient transfection of K562 human erythroleukemia cells ..... 112
  Preparation of cells for FACS and quantitation of PPIX ........... 113
  Protein purification ................................................................. 114
  Spectrophotometric determination of ALAS activity ................. 116
  Thermostability Assays .......................................................... 116
  Acrylamide Fluorescence Quenching ....................................... 117
  Circular dichroism (CD) spectroscopy ....................................... 118
Results ....................................................................................... 118
  Kinetic characterization of the XLEPP variants at 37°C .......... 118
  HeLa and K562 cells expressing XLEPP variants accumulate more PPIX than cells expressing wild-type hALAS2 ................................................................. 119
  Glycine supplementation of the culture medium only increases PPIX accumulation in HeLa cells expressing XLEPP variants with an increased affinity for glycine ........ 120
  The XLEPP variants are more thermostable than wild-type hALAS2 ................................................................ 120
The XLEPP variants undergo significant changes in secondary structure upon succinyl-CoA binding .................... 122
The tertiary structures and PLP-binding properties of the XLEPP variants are distinct from those of wild-type hALAS2 .............................................................. 124
Discussion ........................................................................................................... 128
References ........................................................................................................ 134

Chapter Four: Isoniazid inhibits human erythroid 5-aminolevulinate synthase ................................................................. 141
Abstract ............................................................................................................ 141
Introduction ........................................................................................................ 142
Materials ............................................................................................................ 144
  Reagents .......................................................................................................... 144
Methods ............................................................................................................. 145
  Plasmids for mammalian expression and protein purification .......... 145
  Cell Culture .................................................................................................... 146
  Transient transfection of HeLa cells ............................................................. 146
  Preparation of cells for FACS and quantitation of PPIX .................... 147
  Confocal fluorescence microscopy ............................................................... 147
  Protein purification .......................................................................................... 147
  ALAS colorimetric activity assay ................................................................. 148
  Circular dichroism (CD) spectroscopy ........................................................... 149
Results ................................................................................................................ 149
  INH reduces PPIX accumulation in HeLa cells expressing wild-type hALAS2 and XLEPP variants .................... 150
  INH reduces PPIX accumulation in both HeLa cells expressing delAGTG and those adjacent to delAGTG-expressing HeLa cells ......................................................... 151
  INH inhibition of ALAS2 activity is not affected by pyridoxine ...... 152
  PLP and PMP reduce the effect of INH on ALAS2 inhibition ............... 154
  INH is not a competitive inhibitor for glycine .......................................... 156
  The hydrazine moiety of INH is necessary, but not sufficient for complete ALAS2 inhibition ......................................................... 158
  INH induces changes in the tertiary structure and PLP-binding to ALAS2 ..................................................................................... 159
Discussion ......................................................................................................... 162
References ....................................................................................................... 168

Chapter Five: Summary and Conclusions ................................................. 177
References ....................................................................................................... 184

About the Author .............................................................................................. End Page
LIST OF TABLES

Table 1.1. Genes encoding heme biosynthesis enzymes implicated in inherited porphyrias................................................................. 31

Table 2.1. Plasmid constructs used to express murine ALAS2 variants in mammalian cell lines................................................................. 68

Table 3.1. C-terminal amino acid sequences resulting from XLEPP mutations. ................................................................. 107

Table 3.2. Kinetic parameters for wild-type hALAS2 and XLEPP variant enzymes at 37°C................................................................. 119
LIST OF FIGURES

Figure 1.1. The heme biosynthetic pathway. ................................................................. 4

Figure 1.2. Biosynthesis of ALA via the C4 and C5 pathways. .............................. 5

Figure 1.3. The IRE located in the 5'-UTR of ALAS2 mRNA is a specific binding site for IRP and is sufficient to mediate iron-dependent translational regulation................................. 16

Figure 1.4. Multiple alignment of ALAS2 and ALAS1 C-terminal amino acid sequences from multiple organisms................................. 18

Figure 1.5. The N-terminal amino acid sequence of human ALAS1 and ALAS2 indicating the location of the HRMs. ......................... 26

Figure 1.6. Depiction of human ALAS1 and ALAS2 protein domains ............... 28

Figure 2.1. Murine ALAS2 protein schematic indicating mutated amino acid residues................................................................. 69

Figure 2.2. Porphyrin accumulation in transiently transfected HeLa cells with mALAS2 WT- and variant-encoding plasmids ................... 71

Figure 2.3. Glycine supplementation of culture medium increases PPIX accumulation in HeLa cells expressing murine wild-type, HPVT, and R433K ALAS2 ......................................................... 74

Figure 2.4. Stable expression of murine ALAS2 variants in K562 cells results in PPIX accumulation when culture medium is supplemented with either glycine or deferoxamine. .................. 75

Figure 2.5. Fluorescence microscopy of HeLa cells expressing GFP and the R433K mALAS2 variant.............................................................. 77

Figure 2.6. Light-induced cell death of HeLa cells expressing mALAS2 variants .................................................................................. 79

Figure 2.7. Light-induced cell death in murine ALAS2 variants-expressing
HeLa cells cultured in a medium supplemented with glycine. .......... 81

Figure 3.1. Expression of XLEPP variants in mammalian cells results in accumulation of PPIX ..................................................... 121

Figure 3.2. Glycine supplementation of the culture medium only increases PPIX accumulation in cells expressing XLEPP variants with an increased affinity for glycine................................. 122

Figure 3.3. Thromostability profiles vary for hALAS2 and XLEPP variants...... 123

Figure 3.4. Circular dichroism spectra of hALAS2 and the XLEPP variants in the far-UV nm region ............................................. 124

Figure 3.5. Stern-Volmer plots displaying fluorescence quenching of buried tryptophan residues in hALAS2 variants ......................... 125

Figure 3.6. Circular dichroism spectra of hALAS2 and the XLEPP variants in the near-UV and visible region .................................. 126

Figure 3.7. Circular dichroism spectra of hALAS2 and the XLEPP variants in the near-UV and visible region in the presence of 50 μM succinyl-CoA ........................................................................ 127

Figure 3.8. The citric acid cycle and heme synthesis .............................. 129

Figure 4.1. INH reduces PPIX accumulation in HeLa cells expressing wild-type hALAS2 and XLEPP variants ........................................ 150

Figure 4.2. INH reduces PPIX accumulation in both HeLa cells expressing delAGTG and those adjacent to delAGTG-expressing HeLa cells................................................................. 152

Figure 4.3. INH inhibition of PPIX accumulation in HeLa cells expressing either wild-type hALAS2 or XLEPP variants is not affected by pyridoxine. ......................................................... 153

Figure 4.4. Either PLP or PMP supplementation of the culture medium of HeLa cells expressing wild-type hALAS2 or XLEPP variants reduces the inhibitory effect of INH on PPIX accumulation ................................................................. 154

Figure 4.5. INH inhibits the activity of purified hALAS2 ......................... 156

Figure 4.6. INH does not compete with glycine to bind hALAS2............. 157
Figure 4.7. Pyrazinamide and 1,1-dimethylyhydrazine do not inhibit hALAS2 ................................................................. 160

Figure 4.8. INH induces changes in the tertiary structure of ALAS2 and binding of the PLP cofactor................................................................. 161

Figure 4.9. INH induces changes in the tertiary structure of delAGTG and binding of the PLP cofactor.......................... 162

Figure 4.10. Chemical mechanism of the ALAS2-catalyzed reaction .......... 166
LIST OF ABBREVIATIONS

A549  human lung adenocarcinoma cells
AIP  acute intermittent porphyria
ALA  5-aminolevulinate
ALAD-P  ALA dehydratase deficient porphyria
ALAS  5-aminolevulinate synthase
ALAS1  housekeeping 5-aminolevulinate synthase
ALAS2  erythroid-specific 5-aminolevulinate synthase
CD  circular dichroism
CEP  congenital erythropoietic protoporphyria
CoA  coenzyme A
COPROIII  coproporphyrinogen III
CP  cysteine-proline
DAPI  4',6-diamidino-2-phenylindole
EMSA  electrophoretic mobility shift assay
EPO  erythropoeitin
EPP  erythropoietic protoporphyria
ERK  extracellular signal-regulated kinase
FACS  fluorescence-activated cell sorting
FBS  fetal bovine serum
FSC  forward-scatter
GLRX5  glutaredoxin 5
H1299  human non-small cell lung carcinoma cells
hALAS2  human ALAS2
HAT  histone acetylase
HCP  hereditary coproporphyria
HDAC1  histone deacetylase 1
HEK293  human embryonic kidney cells
HeLa  human cervical carcinoma cells
HIF-1  hypoxia-inducible factor 1
HIF-1α  hypoxia-inducible factor 1 α
HIF-1β  hypoxia-inducible factor 1 β
HMB  hydroxymethylbilane or pre-uroporphyrinogen
HMBA  hexamethylene bisacetamide
HRM  heme-regulatory motif
INH  isoniazid, isonicotinic acid hydrazide
IRE  iron-responsive element
IRES  internal ribosomal entry site
IRP  iron regulatory protein
IRP1  iron-responsive element-binding protein 1
IRP2  iron-responsive element-binding protein 2
K562  human erythroleukemic cells
LONP1  Ion-peptidase 1
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPKs</td>
<td>mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MEL</td>
<td>murine erythroleukemia cells</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NaBu</td>
<td>sodium butyrate</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>murine embryonic fibroblasts</td>
</tr>
<tr>
<td>PBG</td>
<td>porphobilinogen</td>
</tr>
<tr>
<td>PBGD</td>
<td>porphobilinogen deaminase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>Pct</td>
<td>paclitaxel</td>
</tr>
<tr>
<td>PCT</td>
<td>porphyria cutanea tarda</td>
</tr>
<tr>
<td>PDT</td>
<td>photodynamic therapy</td>
</tr>
<tr>
<td>PLP</td>
<td>pyridoxal 5’-phosphate</td>
</tr>
<tr>
<td>PMP</td>
<td>pyridoxamine 5’-phosphate</td>
</tr>
<tr>
<td>PPGIX</td>
<td>protoporphyrinogen IX</td>
</tr>
<tr>
<td>PPIX</td>
<td>protoporphyrin IX</td>
</tr>
<tr>
<td>siRNA</td>
<td>silencing RNA</td>
</tr>
<tr>
<td>SCS</td>
<td>succinyl-CoA synthetase</td>
</tr>
<tr>
<td>SCS-A</td>
<td>ATP-specific succinyl-CoA synthetase</td>
</tr>
<tr>
<td>SCS-βA</td>
<td>ATP-specific succinyl-CoA synthetase β subunit</td>
</tr>
<tr>
<td>SCS-α</td>
<td>succinyl-CoA synthetase α subunit</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SSC</td>
<td>side-scatter</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
</tr>
<tr>
<td>TfR1</td>
<td>transferrin receptor 1</td>
</tr>
<tr>
<td>UROIII</td>
<td>uroporphyrinogen III</td>
</tr>
<tr>
<td>UROS</td>
<td>uroporphyrinogen III synthase</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VP</td>
<td>variegate porphyria</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>XLEPP</td>
<td>X-linked erythropoietic protoporphyria</td>
</tr>
<tr>
<td>XLSA</td>
<td>X-linked sideroblastic anemia</td>
</tr>
</tbody>
</table>
ABSTRACT

5-Aminolevulinate synthase (ALAS; EC 2.3.1.37) is a pyridoxal 5’-phosphate (PLP)-dependent enzyme that catalyzes the first committed step of heme biosynthesis in animals, the condensation of glycine and succinyl-CoA yielding 5-aminolevulinate (ALA), CoA, and CO₂. Murine erythroid-specific ALAS (mALAS2) variants that cause high levels of PPIX accumulation provide a new means of targeted, and potentially enhanced, photosensitization. Transfection of HeLa cells with expression plasmids for mALAS2 variants, specifically for those with mutated mitochondrial presequences and a mutation in the active site loop, caused significant cellular accumulation of PPIX, particularly in the membrane. Light treatment of HeLa cells expressing mALAS2 variants revealed that mALAS2 expression results in an increase in cell death in comparison to aminolevulinic acid (ALA) treatment producing a similar amount of PPIX. Generation of PPIX is a crucial component in the widely used photodynamic therapies (PDT) of cancer and other dysplasias. The delivery of stable and highly active mALAS2 variants has the potential to expand and improve upon current PDT regimes.

Mutations in the C-terminus of human ALAS2 (hALAS2) can increase hALAS2 activity and are associated with X-linked erythropoietic protoporphyria (XLEPP), a disease phenotypically characterized by elevated levels or PPIX and
zinc protoporphyrin in erythroblasts. This is apparently due to enhanced cellular hALAS2 activity, but the biochemical relationship between these C-terminal mutations and increased hALAS2 activity is not well understood. HALAS2 and three XLEPP variants were studied both in vitro to compare kinetic and structural parameters and ex vivo in HeLa and K562 cells. Two XLEPP variants, delAGTG, and Q548X, exhibited higher catalytic rates and affinity for succinyl-CoA than wild-type hALAS2, had increased transition temperatures, and caused porphyrin accumulation in HeLa and K562 cells. Another XLEPP mutation, delAT, had an increased transition temperature and caused porphyrin accumulation in mammalian cells, but exhibited a reduced catalytic rate at 37°C in comparison to wild-type hALAS2. The XLEPP variants, unlike wild-type hALAS2, were more structurally responsive upon binding of succinyl-CoA, and adopted distinct features in tertiary and PLP cofactor-binding site. These results imply that the C-terminus of hALAS2 is important for regulating its structural integrity, which affects kinetic activity and stability.

XLEPP has only recently been identified as a blood disorder, and thus there are no specific treatments. One potential treatment involves the use of the antibiotic isonicotinic acid hydrazide (isoniazid, INH), commonly used to treat tuberculosis. INH can cause sideroblastic anemia as a side-effect and has traditionally been thought to do so by limiting PLP availability to hALAS2 via direct inhibition of pyridoxal kinase, and reacting with pyridoxal to form pyridoxal isonicotinoyl hydrazone. We postulated that in addition to PLP-dependent inhibition of hALAS2, INH directly acts on hALAS2. Using FACS and confocal
microscopy, we show here that INH reduces protoporphyrin IX accumulation in HeLa cells expressing either wild-type human hALAS2 or XLEPP variants. In addition, PLP and pyridoxamine 5’-phosphate (PMP) restored cellular hALAS2 activity in the presence of INH. Kinetic analyses with purified hALAS2 demonstrated non-competitive or uncompetitive inhibition with an apparent $K_i$ of 1.5 µM. Circular dichroism studies revealed that INH triggers structural changes in hALAS2 that interfere with the association of hALAS2 with its PLP cofactor. These studies demonstrate that hALAS2 can be directly inhibited by INH, provide insight into the mechanism of inhibition, and support the prospective use of INH in treating patients with XLEPP and potentially other cutaneous porphyrias.
CHAPTER 1: INTRODUCTION

Biosynthesis of 5-aminolevulinate and heme

Heme plays a central role in nearly all living organisms due to its vital functions in biological processes such as, respiration, gas sensing (1,2) and oxygen metabolism and transport (3). Heme also functions as an essential cofactor for cytochromes P450, catalases, and peroxidases (4). Heme biosynthesis begins with the condensation of succinyl-CoA and glycine, catalyzed by 5-aminolevulinate synthase (ALAS), to form 5-aminolevulinic acid (ALA) (5). The reaction proceeds as follows:

\[
\text{Glycine + Succinyl-CoA } \rightarrow \text{ CoA + CO}_2 + \text{ALA.}
\]

In the final reactions of the pathway, protoporphyrinogen IX is oxidized to protoporphyrin IX (PPIX), into which ferrous iron (Fe\(^{2+}\)) is inserted to form heme, a reaction catalyzed by ferrochelatase (Figure 1.1) (6,7).

One molecule of heme is made from eight molecules of ALA, the common precursor to all naturally occurring tetrapyrroles, in either eight or nine enzymatic steps, depending on the organism (8). In nature, ALA is synthesized by two distinct routes. Mammals, fungi, and α-proteobacteria synthesize ALA from glycine and succinyl-CoA, via the C4 pathway, while in plants, archaea, and most
bacteria, ALA is synthesized from glutamyl-tRNA via the C5 pathway (Figure 1.2). In all organisms except one, the photosynthetic phytoflagellate *Euglena gracilis* (9-13), production of ALA for tetrapyrrole synthesis is carried out by only one of these two pathways.

The first evidence for the utilization of glycine as one of the initial precursors of heme was presented in 1945 by David Shemin when he infamously synthesized and then ingested 66g of 32% $^{15}$[N] glycine in addition to his usual diet (14). He observed that the isotopic nitrogen of glycine was incorporated in his blood’s heme, and he concluded that the nitrogen atoms of heme are derived from the NH$_3$ group of glycine (14). The initial proposal that glycine represents one of the early precursors of heme was confirmed and expanded to animal subjects, demonstrating that among various amino acids, glycine was directly utilized as the predominant source for the nitrogen atoms of heme (15). It was evident that the nitrogen atoms of heme were derived from glycine, but there remained the question of to what extent glycine was utilized as a source for the heme carbon atoms. Altman *et al.* (16) demonstrated that only the $\alpha$-carbon of glycine was incorporated into heme, whereas its carboxyl carbon was not (17). Thus, the carboxyl group of glycine had to be lost during some stage of heme synthesis.

Since the $\alpha$-carbon of glycine accounted for only 8 of the 32 carbon atoms of heme (18), the search for the potential substrate(s) other than glycine intensified. At this time, several groups suggested the involvement of an intermediate from the TCA cycle as a possible precursor of heme, with $\alpha$-
ketoglutarate as the most plausible candidate (18,19). However, Shemin and Wittenberg (20) proposed that the precursor used for heme formation was a four-carbon, asymmetric compound, and therefore they rejected any direct involvement of α-ketoglutarate or succinate in heme biosynthesis. Their conclusion for the involvement of a four-carbon asymmetric compound was based on the observed isotopic distribution patterns of the individual carbon atoms in heme synthesized in the presence of labeled acetate. Briefly, heme was synthesized by incubating avian erythrocytes with acetate whose carboxyl group was labeled with $^{14}$C and by incubating with acetate whose methyl group was labeled with $^{14}$C (20). Following its synthesis and isolation, heme was degraded into individual chemical components in order to identify the positions of the isotopic carbon atoms. The observed isotopic distribution patterns of the individual carbons strongly argued against direct involvement of α-ketoglutarate or succinate in heme biosynthesis; instead, the isotopic distribution patterns suggested a four-carbon, asymmetric compound serving as the precursor for all the non-glycine-derived carbon atoms of heme (20). It was proposed that the four-carbon, asymmetric compound was most likely a succinyl-coenzyme complex derived from the TCA cycle (20), and the validity of this proposal was corroborated several years later with the observation that the synthesis of ALA in bacterial and avian extracts exhibited dependence on succinyl-CoA (21,22).
Figure 1.1. The heme biosynthetic pathway. Numbers designate individual enzyme-catalyzed steps of heme biosynthesis and are as follows: 1) ALA synthase, 2) ALA dehydratase, 3) uroporphyrinogen synthase, 4) uroporphyrinogen-III cosynthase, 5) uroporphyrinogen decarboxylase, 6) coproporphyrinogen oxidase, 7) protoporphyrinogen oxidase, and 8) ferrochelatase. Abbreviations are as follows: ALA, 5-aminolevulinate synthase; PBG, porphobilinogen; HMB, pre-uroporphyrinogen; UROIII, uroporphyrinogen III; COPROIII, coproporphyrinogen III; PPGIX, protoporphyrinogen IX; PPIX, protoporphyrin IX.
Figure 1.2. Biosynthesis of ALA via the C4 and C5 pathways. ALA is the common committed precursor to tetrapyrrroles and is biosynthesized by two routes. Plants and most bacteria synthesize ALA from glutamyl-tRNA\textsuperscript{Glu} by reduction of glutamate to glutamate-1-semialdehyde. This is then converted to ALA by glutamate-1-semialdehyde aminotransferase. Non-plant eukaryotes and the α-subclass of purple bacteria synthesize ALA by the C4 pathway, which requires the respiratory intermediate succinyl-CoA. *Euglena gracilis*, an extraordinary unicellular organism with properties of both plants and animals, has the capacity to synthesize ALA by both pathways.

5-Aminolevulinate Synthase

There are two isoforms of ALAS expressed by two separate genes in animals (23). Human *ALAS1*, the housekeeping gene, is located on chromosome 3p21 (24) and the first complete nucleotide sequence of *ALAS1* was obtained using chick embryo liver (25), which represented the first complete nucleotide sequence of ALAS to be cloned from any source. After this initial report of the
full-length cDNA clone, Elliott and colleagues (26) proceeded to isolate the chicken ALAS gene and its putative control regions. The chicken ALAS gene spans 6.9 kb of DNA and alignment with the chick embryo cDNA sequence (25) shows that it is divided into 10 exons ranging from 156-280 bp, split by 9 introns of more variable length (26). The 10 exons contain 2103 nucleotides, which encode an ALAS precursor protein of 635 amino acids.

ALAS2, the erythroid-specific gene, is located on the X-chromosome (23,24). The mouse ALAS2 gene consists of 11 exons and 10 introns, spanning approximately 24 kb (27). The first exon consists of 37 base pairs of non-coding sequence and is separated from the body of the gene by a 6 kb intron. Consistent with the similarity observed between the mouse ALAS2 cDNA sequence and the chicken ALAS1 sequence (25,28), the location of the intron/exon boundaries 3' of exon 3 are virtually identical in chicken ALAS1 (26). A notable difference is the absence of a distant and non-coding first exon in the chicken. Also, the chicken ALAS1 introns are generally more modest in size, as the chicken gene spans only 6.9 kb.

Like mouse ALAS2, human ALAS2 also consists of 10 protein-coding exons and a 5'-untranslated exon (29) that contains sequence coding for an iron-responsive element important for the regulation of mRNA translation (30,31). The human gene structure bears remarkable similarity to the mouse ALAS2 (27). An examination of the immediate promoter of the human ALAS2 gene by Cox et al. (30) revealed several interesting putative cis-acting motifs. Unlike that of the mouse erythroid ALAS gene, the human promoter contains a consensus TATA
box at position -27 and a consensus CCAAT box at position -87. Cox et al. (30) also identified a possible GATA-1 binding site located at position -100. At position -40 there is a perfect consensus for NF-E2, another erythroid-specific factor found in the promoter of the erythroid porphobilinogen deaminase (PBGD) gene (32,33) and upstream of β-globin (34-36).

**Regulation of ALAS2 Transcription**

*ALAS1* and *ALAS2* each have evolved distinct transcriptional regulatory processes, likely correlated with the stringent regulation necessary for the large heme requirement during erythropoiesis that is not required in non-erythroid cells (37). Hence, during erythropoiesis, transcription of all genes for ALAS2 and the other heme pathway enzymes and globin (38) are clearly up-regulated (39). Transcription of human *ALAS2* is regulated by erythroid-specific transcription factors that act at the promoter and intronic enhancer sites (37,40), as well as downstream of the gene in the 3'-flanking region (41). In 1989, Schoenhaut and Curtis (27) published the structure of the mouse *ALAS2* gene and identified the DNAse I hypersensitivity sites. Since many enhancer-like sequences associated with other erythroid-specific sequences are marked by DNAse I hypersensitivity, in 1998 Surinya *et al.* (31,40) set out to determine whether the intronic sequences corresponding to DNAse I-hypersensitivity in the mouse *ALAS2* gene play a role in human *ALAS2* transcriptional regulation. The significance of introns 1, 3, and 8 on *ALAS2* expression were assessed by analysis of the effects of intron deletions, revealing that intron 3 is marginally inhibitory and introns 1 and 8
are stimulatory. Intron 8 conferred the strongest stimulation of promoter activity in K562 and MEL cells, and was found to be orientation-dependent. Specifically a 239 bp region located within intron 8, which is highly conserved in the human, mouse, and dog ALAS2 genes, was identified as the region conferring enhancer activity. In K562 cells, a 25-fold level of stimulation was observed when the enhancer was located upstream of the promoter and oriented in the same direction as the promoter, while only a 4-fold increase was observed when the enhancer was in the reverse direction. In this region, two CACCC box binding sites and four GATA-1 binding sites were identified, and functional contributions were assessed through site-directed mutagenesis experiments and gel shift assays to determine binding of individual transcription factors. Mutational analysis demonstrated that the two CACCC boxes and one of the GATA motifs were functional. The CACCC boxes each bind Sp1, but not erythroid Krüppel-like factor (EKLF) or basic Krüppel-like factor (BKLF), and the GATA motif binds GATA-1 in vitro (40).

In 2000, Kramer et al. (42) further examined the role of GATA-1 in the transcriptional regulation of murine ALAS2. They investigated the function of the 5'-flanking region of the murine ALAS2 gene in erythroid and non-erythroid cells, as well as in erythroid cells chemically induced to undergo differentiation. Transient transfections of mammalian cell lines with different sections of the murine ALAS2 promoter indicated that the first 700 bp of the murine promoter were required to give maximal expression. Specifically, it was found that the first 700 bp of the mouse ALAS2 promoter are essential for maximal expression in
murine erythroleukemia (MEL) cells and human myelogenous leukemia (K562) cells, although these regions stimulated similar expression in the human cervical carcinoma (HeLa) cells and mouse embryonic fibroblasts (NIH 3T3). Furthermore, in the mouse ALAS2 5'-flanking region, there are two critical GATA-1 binding sites within the first 300 bp; however additional upstream cis-acting elements are also necessary for maximal transient transcriptional activation. A region located within -518 to -315 bp is crucial for transcriptional activation during the chemically induced differentiation of MEL cells. The GATA-1 interaction with the two GATA-1-binding sites in the proximal ALAS2 promoter, located at -118/-113 and -98/-93, is also critical to confer erythroid cell specificity. This conclusion was supported both in vitro, by the elimination of GATA-1 binding upon mutation of either of the two GATA-1 elements using electrophoretic mobility shift assays (EMSA) and, in vivo, by the stimulation of the ALAS2 promoter activity of mutated GATA-1 binding sites-containing constructs in transiently transfected erythroid cells.

Hypoxia indirectly affects heme production by inducing the erythropoiesis via erythropoietin and transferrin (43). Hypoxia-inducible factor 1 (HIF-1), originally identified as a nuclear factor that can bind to the human erythropoietin gene, plays a vital role in sensing and responding to hypoxia, but has also been shown to work through direct induction of ALAS2. HIF-1 is implicated in the transcriptional upregulation of erythropoietin (EPO), transferrin (44), and transferrin receptor (45), and thus leads to an increase of erythropoiesis and hematopoietic iron supply. There are two basic helix-loop-helix subunits that
compose HIF-1, HIF-1α and HIF-1β. HIF-1α is constitutively expressed, but rapidly degraded under normoxic conditions, while HIF-1β is both constitutively expressed and stable (46). Thus, it is the amount of HIF-1α that determines the overall HIF-1 level in a cell. HIF-1 functions by binding HIF-1-responsive elements in the 5' regulatory regions of these genes and thereby mediating transcriptional activation. In 2000, Kramer et al. (42) identified a putative HIF-1-responsive element (CACGTG) within the 5'-flanking region of the mouse ALAS2 promoter at position -318 to -323. Since hypoxia induces several HIF-1 target genes involved in erythropoiesis, and ALAS2 is the rate-limiting enzymes in heme synthesis, in 2003 Hofer et al. (47) performed the first functional analysis of oxygen regulation of ALAS2. Nevertheless, despite an upregulation of ALAS2 mRNA by hypoxia as expected, the putative HIF-1-binding site, located at -323/-318 of the ALAS2 promoter, did not play a functional role. Thus, hypoxia induction of ALAS2 was concluded to be HIF-1-independent.

However, in 2011, Zhang et al. (41) looked downstream of ALAS2 and identified three putative HIF-1-responsive elements located 611, 621, and 741 bp beyond the ALAS2-encoding region and within the 3'-UTR of the human ALAS2 gene. They found that HIF-1 did bind to all three of the sites, overexpression of HIF-1α increased ALAS2 expression, and knockdown of HIF-1α by RNA interference decreased the level of ALAS2 expression. Thus, hypoxia results in a transcriptional upregulation of ALAS2 that is in fact HIF-1-dependent through HIF-1 binding sites downstream of the coding region of the ALAS2 gene (41). The hypoxic upregulation of ALAS2 expression contributed significantly to an
increased level of heme synthesis and likely represents an adaptive response to optimize heme biosynthesis under hypoxic conditions (41). Taken all together, evidence clearly supports that HIF-1 binds to elements in the 3’-flanking region of the human ALAS2 gene, and HIF-1 binding directly mediates the hypoxic induction of the human ALAS2 gene.

It is well established that oxygen level plays a key role in systemic control of erythropoiesis through feedback regulation of EPO production, which is increased by tissue hypoxia and decreased by return to normoxia. However, it appears that hypoxia influences erythropoiesis beyond the known mechanism, in that red blood cell production is enhanced by low oxygen concentration at many stages of development. The actions caused by hypoxia on erythroid progenitors include enhanced amplification and acceleration of their proliferation, differentiation, and maturation (48). These effects are associated with a hypoxia-induced gene expression profile, which includes increases in gene expression of ALAS2 in both cord blood and peripheral blood cell cultures, although increase is more prominent in cord blood cell cultures (48). GATA-1 expression is also increased by low oxygen in cord blood culture, and may be part of the mechanism linking ALAS2 expression to hypoxia. Globin expression is increased as well (48), which indicates that oxygen concentration is involved in the synchronization of heme and globin synthesis to efficiently generate hemoglobin.

Sodium butyrate (NaBu) has also been shown to directly regulate ALAS2 transcription. NaBu was first recognized to induce differentiation of erythroleukemic cells in 1975 by Leder et al. (49). In 2008, Han et al. (50)
examined the molecular basis underlying the NaBu activation of the ALAS2 gene. They confirmed the effect of NaBu not only on transcriptional activation of ALAS2 in K562 cells, but also in the non-hematopoietic cell lines 293T human embryonic kidney epithelial cells and A549 human lung adenocarcinoma cells. They went on to identify Sp1 binding sites in the ALAS2 promoter that mediated responsiveness to NaBu activation, but that mutations of the GATA-1 sites did not affect ALAS2 activation by NaBu (50). Furthermore, NaBu treatment led to an accumulation of Sp1 protein on the ALAS2 promoter, and it is speculated that histone deacetylase 1 (HDAC1) may be the target of NaBu inhibition that is responsible for ALAS2 expression (50). This is supported by previous work showing that the transcription cofactor p300 plays a role in ALAS2 transcription and activity of its histone acetyltransferase (HAT) is essential (51), and it was further shown that Sp1 and p300 synergistically upregulated ALAS2 expression, whereas HDAC1 reduced the synergy dose-dependently (50).

MEL cells can be induced to undergo erythroid differentiation by a variety of agents including DMSO, hexamethylene bisacetamide (HMBA) and hemin (52-57). The involvement of mitogen-activated protein kinases (MAPKs) in the regulation of erythroid differentiation has been studied in mammalian cell lines using these inducing agents (58-61) and demonstrates that MAPKs have an essential function in erythrocyte maturation. Both DMSO (52) and HMBA (53) increase ALAS2 expression in MEL cells. Treatment of HMBA-induced MEL cells with U0126, an extracellular-signal-related kinase (ERK) pathway inhibitor, further increases ALAS2 expression (62). In contrast, treatment with the p38
MAPK pathway inhibitor SB202190 slightly decreased ALAS2 expression over time (62). Thus, the ERK signaling pathway suppresses, and the p38 MAPK signaling pathways promotes the HMBA-induced erythroid differentiation of MEL cells. It is unclear how MAPKs are acting to control ALAS2 levels, but since MAPKs can function by regulating the activity of specific transcription factors through phosphorylation (63), they may be exerting effects through control of expression, DNA-binding, or transcriptional activity of transcription factors (62).

Regulation of ALAS2 Translation

Discovery of the intriguing properties of ferritin (64), a ubiquitous iron storage protein, stimulated many insightful studies that helped elucidate the major mechanisms for sensing and controlling cellular and organismal iron homeostasis. Initial studies demonstrated that ferritin abundance and synthesis were iron-responsive (65-67) and that mammalian cells contained a cytosolic iron sensor that controlled ferritin iron-responsive (65-67) and that mammalian cells contained a cytosolic iron sensor that controlled ferritin synthesis (68-70). In 1976, the Munro and coworkers (71) demonstrated that iron stimulated ferritin synthesis by activating the translation of ferritin mRNAs. The next critical step in studies of ferritin expression involved the identification of an evolutionarily conserved 28 nucleotide sequence, termed the Iron-Responsive Element (IRE), which was present in the 5′-UTR of ferritin mRNAs. The IRE proved to be necessary and sufficient for iron-dependent control of ferritin mRNA translation (72-74). These observations occurred simultaneously with the discovery of
cytosolic iron-regulated RNA binding proteins, IRP1 and IRP2, which recognize the IRE in a sequence and structure specific manner (75,76). A comparison of the data on ferritin regulation with data on transferrin receptor 1 (TfR1) from Kühn and associates (77,78), who showed that TfR1 mRNA stability was iron-regulated and that the regulatory element responsible was present in the 3'-UTR, suggested the idea of a general post-transcriptional regulatory network controlling mammalian iron metabolism (79). Further, Klausner and colleagues (80) then established that IRE-dependent post-transcriptional control was central to the regulation of cellular iron metabolism in vertebrates (81,82).

A standard IRE stem structure (Figure 1.3) consists of variable sequences that form base pairs of moderate stability, and folds into an α-helix that is distorted by the presence of a small bulge in the middle, caused by either an unpaired C residue or an asymmetric UGC/C bulge/loop (83). The loop at the top of this stem contains a conserved 5'-CAGUGX-3' sequence, in which "X" represents either an A, C, or U (83). This top loop most likely functions as a molecular ruler that orients and correctly distances the bulge C from residues in the loop, allowing flexible residues to participate in sequence-specific interactions between the IRE and IRPs (84).

In 1991, examination of the immediate promoter of the human ALAS2 gene revealed a putative IRE in the 5'-UTR of the human ALAS2 mRNA that is not present in the ALAS1 mRNA (30). This IRE motif was shown through gel retardation analysis to form a protein-RNA complex with cytosolic extracts from human K562 cells and this binding was strongly competed with IRE transcripts.
from ferritin or transferrin receptor mRNAs (30). Furthermore, a transcript of the ALAS2 IRE mutated in the conserved loop of the IRE did not readily produce this protein-RNA complex in gel retardation analyses. These results supported that the IRE in the ALAS2 mRNA is indeed functional and that translation of the mRNA during erythropoiesis is regulated by the availability of cellular iron (30). It was also later confirmed by a separate group that the IRE motif contained in ALAS2 mRNA is sufficient to confer translational control to a reporter mRNA both in transfected MEL cells and *in vitro* (85). Thus, the level of cellular iron during erythropoiesis plays a significant role in dictating the translation of ALAS2 mRNA, and this occurs through the interaction with the IRE motif in ALAS2, a motif not present in ALAS1 mRNA.

Considering that ALAS2 catalyzes the initial reaction for heme biosynthesis in erythroid cells, the translational repression of its mRNA by IRPs associates the IRE-IRP system with systemic iron utilization and homeostasis. This physiological response may be critical to inhibit the accumulation of toxic protoporphyrin IX when iron is scarce, as is illustrated by two disease phenotypes. *Shiraz* mutant zebrafish, which have a genetic defect in glutaredoxin 5 (GLRX5), a gene required for Fe-S cluster assembly, exhibit severe hypochromic anemia, and show impairment to the regulation of IRP1 that eventually leads to the translational repression of ALAS2 (86). A comparable biochemical phenotype is seen in human patients with sideroblastic anemia in which ALAS2 mRNA translation is suppressed by both IRPs because the
deregulation of IRP1 was associated with cytosolic iron depletion and concomitant induction of IRP2 (87).

Figure 1.3. The IRE located in the 5′-UTR of ALAS2 mRNA is a specific binding site for IRP and is sufficient to mediate iron-dependent translational regulation. The IREs found in (A.) murine ALAS2 mRNA and (B.) human ALAS2 mRNA. (C.) In the presence of iron, IRPs do not bind to the IRE, allowing for translation of ALAS2 mRNA. (D.) In iron-depleted cells, IRP binding to the IRE in the 5′-UTR interferes with translational initiation.

In 2010, Ye et al. (87) hypothesized that transcriptional remodeling in response to cell stress may account for systolic iron depletion, which activates IRPs, repressing ALAS2 synthesis, and thus inhibiting heme synthesis in GLRX5 deficiency. They found that in GLRX5-deficient cells, [Fe-S] cluster biosynthesis
was impaired and the IRE-binding activity was IRP1 was activated. Also, they observed increased IRP2 levels, indicative of relative cytosolic iron depletion, together with mitochondrial iron overload. Decreased ALAS2 levels attributable to IRP-translational repression were seen in erythroid cells in which GLRX5 expression had been downregulated using siRNA (87). The IRE-IRP system and its ability to regulate heme synthesis via ALAS2 help to explain why GLRX5 deficiency results in anemia in the zebrafish model and sideroblastic anemia in humans.

**Stability of ALAS2**

Regulation of ALAS2 at the protein level have been most notably studied in terms of oxygen level effects and also in terms of mutations seen in x-linked sideroblastic anemia (XLSA) patients. In 2005, Abu-Farha et al. (88) noted that ALAS2 contains a single, highly conserved LXXLAP sequence located at amino acid residues 515-520, that is not found in the corresponding sequence in ALAS1 (Figure 1.4). This LXXLAP motif, where L is leucine, A is alanine, P is proline, and X is any amino acid, is a motif found in HIF1-α, which was previously discussed regarding its ability to upregulate ALAS2 transcription (41), is destabilized in normoxic conditions, leading to rapid hydroxylation, ubiquitination, and proteasomal degradation (89,90). A defining feature of this HIF-1α degradation involves its two LXXLAP oxygen regulatory motifs, and its degradation process is known to be regulated through prolyl-4-hydroxylases that can hydroxylate the proline residues in the presence of oxygen, which leads to
ubiquitination of HIF1-α by E3 ubiquitin ligases (89-93). Through this LXXLAP motif, ALAS2 is regulated by hypoxia at the protein level and thus stabilized in low oxygen conditions much like HIF-1α (88). Not only is ALAS2 stabilized, but it is also more resistant to degradation under hypoxic conditions in comparison to normoxia. Mutation of proline 520 of the LXXLAP motif also caused stabilization of ALAS2 under normoxic conditions, suggesting that post-translational modification under normoxic conditions contributes to the degradation of ALAS2. Furthermore, mutation of proline 520 in vitro eliminated the ability of ALAS2 to be ubiquitinated in normoxic conditions (88). The mutation of proline 520 mimicked the lack of ubiquitination and stability of wild-type ALAS2 observed under hypoxic conditions. Thus, through its LXXLAP motif, ALAS2 can be stabilized under hypoxic conditions and resist ubiquitination and subsequent proteasomal degradation (88).

**Figure 1.4.** Multiple alignment of ALAS2 and ALAS1 C-terminal amino acid sequences from multiple organisms. The LXXLAP motif is well-conserved in ALAS2, but not ALAS1. The extended C-terminus is not present in *R. capsulatus* ALAS, for which there is the only solved crystal structure of ALAS, and thus the tertiary structure of the extended C-terminus is unknown.
Another noteworthy region of ALAS2 is within the C-terminus of ALAS2, of which the final 33 amino acids are conserved in higher eukaryotes, but are not present in prokaryotes (Figure 1.4). Since the only crystal structure of ALAS2 that has been solved is of *R. capsulatus* (94), the tertiary structure of the C-terminus remains a mystery. However, it appears that the ALAS2 C-terminus may have a function in higher eukaryotes, as mutations in ALAS2 corresponding to the C-terminus have been associated with both XLSA and x-linked erythropoietic porphyria (XLEPP) (95-97). In 2012, Kadirvel *et al.* (97) studied novel missense mutations in the 11th exon of the human ALAS2 gene associated with XLSA along with a human ALAS2 variant with a complete deletion of its C-terminus. Using a combination of experiments measuring *in vitro* enzymatic activity using bacterially expressed recombinant proteins and *in vivo* experiments evaluating catalytic activity by comparing the accumulation of porphyrins in eukaryotic cells stably expressing mutant ALAS2 enzyme tagged with FLAG, they concluded that the C-terminus of the human ALAS2 protein acts as an intrinsic regulator of catalytic activity and protein stability (97). Although there has been speculation of allosteric regulation, potentially through binding of iron or heme at a particular conserved “C-X-X-C” motif (98), and speculation of the potential for post-translational modifications in the C-terminus (98), it is still unclear how the C-terminal region is able to suppress the enzymatic activity, and how mutations in the C-terminus can cause changes in that activity and the stability of the enzyme.
Mitochondrial Import of ALAS2

In mammalian cells, anabolic and catabolic pathways are normally confined to a single cellular compartment unless the process involves import or export of products or substrates. The tetrapyrrole biosynthetic pathway is atypical, as its first step and its last three steps are catalyzed by mitochondrial enzymes, whereas the intermediate four steps are catalyzed by cytosolic enzymes (99). All eight of the pathway enzymes are encoded in the nucleus and are synthesized in the cytosol with the mitochondrial located proteins being post-translationally translocated to their appropriate mitochondrial space. For example, coproporphyrinogen oxidase is located in the space between the inner and outer mitochondrial membranes (100,101), protoporphyrinogen oxidase is thought to be either in the space between the inner and outer mitochondrial membranes or associated with the matrix side of the inner mitochondrial membrane (102), and ferrochelatase is associated with the matrix side of the inner mitochondrial membrane (103). ALAS is located in the mitochondrial matrix where its substrates, glycine and succinyl-CoA, are found (31,104). A pre-enzyme of ALAS is synthesized by cytosolic ribosomes, which is then imported to and processed within the mitochondria to yield the mature form of the enzyme (105-107). This pre-enzyme ALAS has a mitochondrial import sequence on its N-terminus that is vital for recognition by the protein import machinery located in the mitochondrial membrane (108).

Most mitochondrial proteins are encoded in the nucleus and synthesized in the cytosol as precursor polypeptides, larger than the corresponding mature
forms due to the additional amino-terminal residues comprising leader peptides. These precursor proteins are post-translationally recognized by mitochondria, translocated across one or both mitochondrial membranes by an energy-dependent mechanism, and processed to their mature size by proteolytic removal of the amino-terminal leader peptides (109,110). The leader peptides are required for mitochondrial import, supported by the fact that precursors incubated with isolated mitochondria are imported, whereas the corresponding mature proteins either fail to be taken up or interfere with uptake of the precursors (110,111). Whether leader sequences function independently or in concert with sequences in the mature portions of the precursors was unknown until 1985, when Horwich et al. (112) demonstrated that the leader peptide is sufficient to direct mitochondrial import. Researchers employed gene fusion techniques to join the coding sequence of the precursor of the mitochondrial matrix enzyme ornithine transcarbamylase with the coding sequencing for the cytosolic enzyme dihydrofolate reductase. They showed that the chimeric protein was recognized by mitochondria, translocated by mitochondria, and proteolytically processed (112). Since this discovery, the mitochondrial import field has expanded significantly and become more complex than previously imagined, including the characterization of several classes of precursor proteins containing distinct targeting signals that are directed to different import routes (113). However, mitochondrial import facilitated by an amino-terminal presequence on the cytosolic protein remains the most recognized means of mitochondrial protein targeting, and it is by this method that ALAS is imported to
the mitochondria.

Human ALAS2 is synthesized as a precursor of molecular mass 65 kDa with an amphipathic presequence of 49 amino acids (114). In general, mitochondrial presequences do not share any primary sequence identity (108), but they do show a statistical bias of positively charged amino acid residues, provided mostly through arginine residues (115). The positive charges in the presequences have the potential to interact with the negatively charged surface of mitochondrial membranes (116), the mitochondrial import receptor (117), and the mitochondrial processing peptidase (118,119). Mitochondrial leader sequences also share an ability to form an amphiphilic, α-helix (115,120,121). As much of heme synthesis depends on proper translocation of the enzymes active in the mitochondria, the mechanism of mitochondrial import of ALAS has been investigated in great detail, especially in terms of a role for feedback inhibition by heme.

**Heme Inhibition of ALAS Mitochondrial Import**

Researchers of ALAS in the 1960s and 1970s noted that the intracellular concentration of ALAS1 is normally low (122), but that an extensive selection of xenobiotics and steroids significantly increase its activity in mammalian (123) and avian liver cells (122,124-126), and cause the synthesis of high concentrations of porphyrins (127). Granick and Urata (122) produced chemically-induced porphyria in chick embryo liver cells that resembled human acute intermittent porphyria biochemically and noted that a number of chemical inducers are known
to precipitate attacks of the disease in susceptible individuals. Immunological studies later showed that, in experimentally induced porphyria, the synthesis of ALAS occurs de novo (128) and heme, the final product of the pathway, inhibits enzyme induction (125-127,129).

Although there was a general agreement that heme plays an important role in the physiological control of ALAS activity (126,127,129,130), there was no such consensus on the mechanism of its action, and many different schemes were suggested. Granick (127) originally proposed that heme acts as a co-repressor for ALAS transcription, and that chemical inducers acted as heme analogues that compete for the repressor protein, thus preventing heme from repressing ALAS mRNA transcription (127). However, later studies by Sassa and Granick (131) and Tyrrell and Marks (129) suggested that, contrary to this transcriptional theory, heme exerted its effect at a post-transcriptional stage. The finding that there was apparently no concentration-dependent competition between heme and porphyria-inducing chemicals (124,131) further argued against the original concept of Granick (127). The groups of Ohashi and Kikuchi (130) and Hayashi et al. (132) proposed that, in adult chickens, heme exerts its effect by controlling transport of cytoplasmically synthesized ALAS into the mitochondria. However, heme did not affect ALAS transport in embryonic chick liver (126,129,131).

In 1980, Srivastava et al. (133,134) reevaluated the effects of heme on ALAS induction by using a newly developed system of isolated chick-embryo liver cells. In this system, they showed that initiation of ALAS induction requires
the presence of a chemical inducer, in this case 2-allyl-2-isopropylacetamide, but that continued presence of this inducer was not necessary, provided heme synthesis is prevented. This was compatible with a scheme in which 2-allyl-2-isopropylacetamide causes a depletion of intracellular heme, and in which heme is the principal controlling agent in the induction of ALAS. Their data conflicted with those of Tyrrell and Marks (129) and showed that, at a concentration of 20 nM, hemin will completely inhibit ALAS induction and that this effect is primarily at the level of transcription.

Furthermore, Sassa and Granick (131) had estimated that the intracellular concentration of heme in chick embryo liver cells is in the order of 50-100 nM, which was sufficient to completely inhibit ALAS synthesis and to prevent induction of hepatic porphyria. At this time, the conclusion was that in chick-embryo liver cells, induction of ALAS synthesis was being primarily regulated by the intracellular concentration of heme and that the effect of heme on the synthesis of ALAS is at the level of transcription. Also, they felt that translational effects seen at higher concentrations were more likely related to heme toxicity. However, through the discovery of a short amino acid sequence to which heme binds, termed a heme regulatory motif (HRM), scientists were able to specifically examine effects of heme on transport of precursor ALAS into the mitochondria (105,135,136).
Heme Regulatory Motifs of ALAS

Both precursor ALAS1 and ALAS2 in mammals each contain three heme-binding HRMs consisting of CP-based short amino acid sequences (Figure 1.5) (137), a motif found in many proteins and shown to function as heme-oxygen sensors in bacteria (138), yeast (139,140), and mammals (136,141,142). The HRMs in ALAS were initially identified to contain the sequence R/L/N-C-P-L/V/I/F-L/M, where the cysteine and proline residues are invariable (136), although some minor substitutions are present in some species (143). Two of these motifs are contained in the region of the leader sequence that is proteolytically removed in the mitochondria, and the third is contained in the N-terminus of the mature enzyme (Figure 1.6). In human ALAS1, the motif locations are referred to by their cysteine residues and are at amino acid positions C8, C33, and C108. On the basis of in vitro translocation experiments with isolated mitochondria and ALAS2 protein, the two heme-binding motifs in the leader sequence have been proposed to bind heme and prevent translocation of precursor ALAS2 into the mitochondrion (136). Dailey et al. (144) studied the putative roles of all three HRMs in human ALAS1 in mitochondrial import, revealing both that each of the motifs play an independent functional role as a heme sensor, and that the HRMs function synergistically to achieve maximal heme inhibition. They demonstrated, through a collection of cysteine to serine mutations in human ALAS-GFP expressed in murine hepatoma cells, that intracellular translocation of ALAS1 is heme-sensitive and that this sensitivity is dependent on the presence of at least one of the heme-binding motifs. They had
not expected the third HRM located in the mature protein to be functional due to its distance, over 50 amino acid residues, from the targeting sequence. They hypothesized that given that little heme is present in the mitochondrial matrix compartment, it is plausible that any regulatory role for this particular HRM would be for translocation and not for enzyme inhibition or inhibition of cofactor binding.

Figure 1.5. The N-terminal amino acid sequence of human ALAS1 and ALAS2 indicating the location of the HRMs. In human ALAS2, shown in black, the three HRMs, shown in bolded red, are located at C11, C38, and C70. In human ALAS1, shown in blue, HRMs are located at C8, C33, and C108. The N-terminus is the least similar domain between ALAS1 and ALAS2.

by the precursor protein. Furthermore, it may be that the binding of heme at this site prevents the protein from folding properly, which would make it more susceptible to proteolytic processing in the cytoplasm (144).

Lathrop and Timko (136) were the first to show that the conserved cysteines of the HRMs in murine ALAS2 are involved in heme inhibition of transport into the mouse mitochondria. In mouse ALAS2, the motif locations are...
at amino acid positions C11, C38, and C70. In Lathrop and Timko’s initial experiments, the deletion of the third HRM (C70) that is normally present in N-terminus of the mature form of ALAS2 did not significantly affect mitochondrial transport, and thus they continued experimentation of only the C11 and C38 HRMs. Since conserved cysteine residues are often involved in the coordination of heme to its apoprotein (145,146), they mutagenized the C11 and C38 residues to serines and evaluated the effects of various hemin concentrations up to 25 μM on mitochondrial transport. Using hemin concentrations of 10 μM and 25 μM, they found that mutations of cysteines at both sites completely eliminated the heme inhibition of transport, while mutation of only either C11 or C38 resulted in a 75 to 82% inhibition of transport using 25 μM of hemin (136).

Goodfellow et al. (114) reinforced the functional role of the HRMs in ALAS2 by providing the structural data demonstrating that a heme-peptide interaction occurs between hemin and the presequence of murine ALAS2. However, some discrepancies on the functional role of the HRMs in ALAS2 still exist. In 2004, Munakata et al. (147) compared the role of the HRMs in the heme-regulated transport of the ALAS1 and ALAS2 in vivo. They constructed a series of mutants of rat ALAS1 with mutated cysteines and expressed the constructs in quail QT6 fibroblasts through transient transfection to examine mitochondrial import of the enzymes in the presence of hemin (147). While they found both that hemin inhibited the mitochondrial import of wild-type ALAS1 and that this inhibition was reversed by the mutation of all three HRMs in the enzyme, exogenous hemin did not affect the mitochondrial import of the ALAS2 under the
Figure 1.6. Depiction of human ALAS1 and ALAS2 protein domains. Region A represents the presequence, region B represents the catalytic core domain, and region C represents the mature mitochondrial protein. The dotted lines represent the locations of the three HRMs in the respective ALAS proteins. In the entire precursor protein, 56.2% of the amino acid residues are identical. However, the sequences are more similar in their catalytic core regions, in which 72.2% of amino acids are identical. The presequence is the most variable region of amino acid sequence.

The differences between ALAS1 and ALAS2 may be attributed to the difference in their biological roles, observing that it may be the activity of ALAS1 is regulated through the inhibition of mitochondrial import by the intracellular concentration of heme, whereas the activity of ALAS2 is mainly regulated translationally by the intracellular concentration of iron. Additionally, free heme in erythroid precursors may never reach high enough concentrations to be able to inhibit mitochondrial import of ALAS2, since most heme molecules are bound to globin. In conclusion, the ability of heme to control mitochondrial import is well established for ALAS1, but heme inhibition on mitochondrial import for ALAS2 remains controversial.
Diseases associated with mutations in ALAS2

Although there are no known genetic diseases associated with a mutation in the gene encoding ALAS1, there are two disease states associated with mutations in the gene encoding ALAS2—XLSA, the most common inherited sideroblastic anemia (148), and XLEPP (96). In XLSA, there is a decreased function of the ALAS2 enzyme. XLSA is characterized by anemia, which is a lack of healthy erythrocytes, and iron accumulation in perinuclear mitochondria of erythroblasts in the bone marrow, called ring sideroblasts, which are present as a result of impaired iron utilization (95,149). The first case of microcytic, hypochromic anemia with an X-linked pattern of inheritance was described in 1945 (150), but it was not until 1992 that Cotter et al. (151) established that the genetic basis for XLSA lies on ALAS2 genetic mutations. While, as expected, XLSA predominantly affects males, females are also affected when the mutant allele is expressed as a result of lyonization (152).

Management of XLSA symptoms involves not only treatment of anemia, but also prevention and treatment of iron overload (153). Approximately 75% of XLSA patients have mutations in ALAS2 that affect its binding to its PLP cofactor. These patients, termed pyridoxine-responsive, can be placed on a pyridoxine regimen to improve the function of the ALAS2 (152,153). Iron overload is treated based on the severity of the anemia. If the anemia is mild, then regular phlebotomies are performed as a preventive measure against iron overload; however, if the anemia is severe, then iron chelating agents, such as deferoxamine, are the preferred treatment (152).
In contrast to XLSA, XLEPP in characterized by an increase in function of the ALAS2 enzyme (96). These gain-of-function mutations result in an increase of PPIX in the bone marrow and circulating erythrocytes, and cause extreme skin photosensitivity. However, XLEPP was only recently identified because the symptoms of XLEPP are nearly identical to those of erythropoietic porphyria (EPP), which is caused by mutations in ferrochelatase that impair its function. Discovery of mutations in ALAS2 leading to porphyria demonstrated the association of each of the eight enzymes of heme biosynthesis with a unique porphyria (Table 1.1). Acute porphyrias, also known as hepatic porphyrias, primarily affect the nervous system. Cutaneous porphyrias, also called erythropoietic porphyrias, primarily affect the skin resulting in photosensitivity. The biochemical distinction between XLEPP and EPP that ultimately led to the discovery of XLEPP is the accumulation of zinc-protoporphyrin in red blood cells of XLEPP patients, an increase not seen in EPP patients (96). The high zinc-protoporphyrin concentrations can be explained considering that ferrochelatase can use Zn(II) as an alternate metal ion substrate (154), and the overactive ALASs present in XLEPP create an imbalance in the ratio of Fe(II) to protoporphyrin. Due to the substrate stoichiometric mismatch, Zn(II) is incorporated into protoporphyrin (155). Thus, measurement of zinc-protoporphyrin levels is the key for diagnosis of XLEPP.

All mutations that have been characterized from patients with XLEPP are located at the C-terminus of the ALAS2 mature protein, which is highly conserved in mammals, implying that the C-terminal region of ALAS plays a vital
role in its regulation, activity, and /or stability. The increased catalytic efficiencies and substrate affinities of purified, recombinant human ALAS2 variants harboring some of the known XLEPP mutations versus those of wild-type ALAS2 were consistent with the proposal that the XLEPP mutations in ALAS2 produced enzyme variants more active than ALAS2 in healthy individuals (156,157). In addition, the identification of a C-terminal, 33-amino acid sequence as the minimal size domain contributing to the gain-of-function of ALAS2 (156,158) supports the previously proposed molecular mechanism for the control of ALAS activity (98). However, the mechanism by which mutations in the C-terminus result in increased ALAS2 activity have yet to be fully elucidated.

Table 1.1. Genes encoding heme biosynthesis enzymes implicated in inherited porphyrias.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Porphyria</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAS2</td>
<td>X-linked erythropoietic protoporphyria (XLEPP)</td>
<td>Cutaneous</td>
</tr>
<tr>
<td>ALAD</td>
<td>ALA dehydratase deficient porphyria (ALAD-P)</td>
<td>Acute</td>
</tr>
<tr>
<td>HMBS</td>
<td>Acute intermittent porphyria (AIP)</td>
<td>Acute</td>
</tr>
<tr>
<td>UROS</td>
<td>Congenital erythropoietic porphyria (CEP)</td>
<td>Acute</td>
</tr>
<tr>
<td>UROD</td>
<td>Porphyria cutanea tarda (PCT)</td>
<td>Cutaneous</td>
</tr>
<tr>
<td>CPOX</td>
<td>Hereditary coproporphyria (HCP)</td>
<td>Cutaneous</td>
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<td>PPOX</td>
<td>Variegate porphyria (VP)</td>
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</tr>
<tr>
<td>FECH</td>
<td>Erythropoietic protoporphyria (EPP)</td>
<td>Cutaneous</td>
</tr>
</tbody>
</table>

Content of this dissertation

This dissertation focuses on mutations in ALAS2 that lead to an increased activity of the enzyme. First, we evaluate whether mALAS2 variants with
increased activity could be useful for production of the photosensitizer PPIX for PDT. Secondly, to understand the mechanisms behind the symptoms of XLEPP, XLEPP variants with mutations in the ALAS2 C-terminus are characterized in terms of their catalytic activity, substrate affinities, thermostability, cellular activity, and structure. Thirdly, INH, an antibiotic used to treat tuberculosis, is evaluated as an inhibitor for human ALAS2. The conclusions presented in each chapter add to the general knowledge of the ALAS-catalyzed reaction, provide insight into the mechanism of XLEPP, and provide evidence for INH as an inhibitor of ALAS2, which has potential for treating porphyria patients.

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CHAPTER 2: EXPRESSION OF MURINE 5-AMINOLEVULINATE SYNTHASE VARIANTS CAUSES PROTOPORPHYRIN IX ACCUMULATION AND LIGHT-INDUCED CELL DEATH IN MAMMALIAN CELLS

Abstract

5-Aminolevulinate synthase (ALAS; EC 2.3.1.37) catalyzes the first committed step of heme biosynthesis in animals. The erythroid-specific ALAS isozyme (ALAS2) is negatively regulated by heme at the level of mitochondrial import and, in its mature form, certain mutations of the murine ALAS2 active site loop result in increased production of protoporphyrin IX (PPIX), the precursor for heme. Importantly, generation of PPIX is a crucial component in the widely used photodynamic therapies (PDT) of cancer and other dysplasias. ALAS2 variants that cause high levels of PPIX accumulation provide a new means of targeted, and potentially enhanced, photosensitization. In order to assess the prospective utility of ALAS2 variants in PPIX production for PDT, K562 human erythroleukemia cells and HeLa human cervical carcinoma cells were transfected with plasmids encoding ALAS2 variants with greater enzymatic activity than the wild-type enzyme. The levels of accumulated PPIX in ALAS2-expressing cells were analyzed using flow cytometry with fluorescence detection. Further, cells expressing ALAS2 variants were subjected to white light treatments (21-22 kLux) for 10 minutes after which cell viability was determined. Transfection of HeLa cells with expression plasmids for murine ALAS2 variants, specifically for those
with mutated mitochondrial presequences and a mutation in the active site loop, caused significant cellular accumulation of PPIX, particularly in the membrane. Light treatments revealed that ALAS2 expression results in an increase in cell death in comparison to aminolevulinic acid (ALA) treatment producing a similar amount of PPIX. The delivery of stable and highly active ALAS2 variants has the potential to expand and improve upon current PDT regimes.

**Introduction**

The first committed step of heme biosynthesis in non-plant eukaryotes and some prokaryotes, the pyridoxal 5’-phosphate (PLP)-dependent condensation of glycine and succinyl-coenzyme A to generate 5-aminolevulinate (ALA), coenzyme A (CoA), and CO₂, is catalyzed by 5-aminolevulinate synthase (ALAS) (1,2). This reaction is directly coupled to the citric acid cycle via the substrate succinyl-CoA and is the key regulatory step of heme biosynthesis (3). In mammals, two chromosomally distinct genes each encode an ALAS isoenzyme, and the two isoenzymes are differentially expressed in a tissue specific manner (4). The human gene for the non-specific or housekeeping isoform, ALAS1, is located on chromosome 3 (5,6), and is expressed ubiquitously in all tissues (7). The gene encoding the erythroid specific isoform, ALAS2, is located on the x-chromosome (6,8) and is expressed only in developing erythroblasts (7).

The two ALAS isoenzymes are translated as precursor proteins with N-terminal mitochondrial matrix import signal sequences that are proteolytically
cleaved following importation to yield the mature enzymes (9-12). The activity of the enzyme is only manifested upon localization to the mitochondrial matrix, as this is where the substrate succinyl-CoA is produced (13-16). An important aspect of the import sequences in both ALAS1 (17) and ALAS2 (18) are the presence of heme-regulatory motifs (HRMs), which consist of short amino acid sequences characterized in part by adjacent cysteine-proline (CP) residues (19). HRMs confer heme-binding properties and have been shown to function as heme-oxygen sensors in bacteria (20), yeast (21) and mammals (18,22,23). In in vitro translocation experiments with isolated mitochondria and ALAS2 precursor protein, the two heme-binding motifs in the leader sequence, corresponding to C11 and C38 in murine ALAS2 (mALAS2), were reported to bind heme and prevent translocation of precursor ALAS2 into the mitochondrion (18). Structural and biochemical data have also demonstrated that a heme-peptide interaction occurs between hemin and the presequence of ALAS2 (24), further indicating the potential of heme to act as a feedback inhibitor of the pathway by preventing the mitochondrial import of precursor ALAS2 when heme levels are sufficient for cellular requirements.

Much of what we know about the chemical and kinetic mechanisms of ALAS2 comes from in vitro enzymatic assays that have helped establish and define the microscopic steps of the ALAS-catalyzed reaction, including the rates of glycine and succinyl-CoA binding, formation of the quinonoid intermediates, and product release (2,25-28). These studies, performed using mALAS2 purified from E. coli cells expressing the recombinant mature enzyme, have led to an
understanding of the importance of specific regions and single amino acid residues in the intrinsic activity of ALAS2 (29-35). Generally, a mutation made to an amino acid predicted to be of functional importance causes a decrease in activity of the enzyme. For example, K313 of mALAS2 was identified as the amino acid involved in the Schiff base linkage with the PLP cofactor (36), and mutations in K313 completely abolish measurable activity of mALAS2 under standard assay conditions (29,37). However, some mutations in mALAS2 cause significantly increased activity of the enzyme, as demonstrated both using purified enzyme (31,35), and in bacteria when expressing plasmids encoding the variant enzymes (35). These mutations are all located in an extended conformation region termed the active site loop (Y422-R439), which is predicted to act as a “lid” over the active site following substrate binding (35). Thus, ALAS is thought to undergo a conformational change from an open conformation, in which the substrates glycine and succinyl-CoA can bind in the active site, to a closed conformation, during which the products ALA, CoA, and CO$_2$ are formed (25,28). When the reaction is complete, the active site loop reopens, and the products are released. It is the opening of the active site loop to allow product release that limits the overall rate of the enzymatic reaction (2,25,27). Based on a combination of kinetic (25,28) and structural modeling studies (38), it was proposed that mutations in the active site loop can lead to hyperactive forms of ALAS, defined as those with at least a 10-fold increase in catalytic efficiency toward one or both substrates, by accelerating reversion to the open loop conformation upon product formation (35).
Since ALAS catalyzes the rate-determining step of tetrapyrrole biosynthesis in mammals (2,25), overexpression of ALAS in prokaryotic (35) and eukaryotic cells (39) results in accumulation of the photosensitizing heme precursor, protoporphyrin IX (PPIX). This property has potential for applications of ALAS or ALAS variants in photodynamic therapy of tumors and other non-malignant dermatological indications, such as acne vulgaris, psoriasis, and scleroderma (40,41). In this study, we transfected mammalian cells with mALAS2 variants and measured PPIX accumulation using fluorescence activated cell sorting (FACS). We identified the R433K variant with additional mutations of the HRMs in the presequence as the variant causing the most cellular PPIX accumulation. Subsequently, we used the variants causing the most PPIX accumulation to study the cell death caused by the PPIX toxicity and photosensitization.

**Materials and Methods**

*Materials*—5-Aminolevulinic acid hydrochloride (ALA) was purchased from Acros Organics (Morris Plains, NJ), and dissolved in distilled water at a concentration of 10 mg/mL. Glycine, purchased from Fisher Chemical (Fairlawn, NJ), was dissolved in phenol red-free culture medium purchased from Mediatech, Inc. (Manassas, VA) to give a stock concentration of 1 M. Paclitaxel (6 mg/mL stock in DMSO) was graciously provided by the laboratory of Dr. Scott Antonia (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL), and was diluted in culture medium directly before use. Deferoxamine mesylate, purchased
from Sigma (St. Louis, MO), was dissolved in distilled water to create a 10 mM stock solution. Propidium iodide, 4’,6-diamidino-2-phenylindole (DAPI) and kanamycin sulfate were purchased from Acros Organics (Morris Plains, NJ), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO). BmtI and BamHI restriction enzymes were obtained from New England BioLabs, Inc. G418 sulfate was procured from Mediatech, Inc. (Manassas, VA).

**Plasmids**—The precursor ALAS2 cDNAs were individually subcloned into the multiple cloning site of the pIRES2-ZsGreen1 vector (purchased from Clontech Laboratories, Inc. Mountain View, CA) using the BmtI and BamHI restriction sites. Digested ALAS2-encoding fragments were ligated into the digested pIRES2-ZsGreen1 vector using T4 DNA ligase and ligase buffer (Thermo Scientific Fermentas (Waltham, MA)). Electrocompetent BL21(DE3) cells were transformed by electroporation with the ligated plasmid DNA and selected by spreading them on LB agar medium containing 10 μg/mL kanamycin sulfate. Plasmid DNA was purified from a single colony using a QiaPrep Spin Miniprep kit (Qiagen Inc.; Germantown, MD), and the sequence of the cloned DNA was verified by Genewiz, Inc. (New Brunswick, NJ).

**Cell culture**—K562 human immortalized myelogenous leukemia cells (ATCC) were maintained in RPMI-1640 culture medium, purchased from Mediatech, Inc. (Manassas, VA), with 10% fetal bovine serum (FBS; purchased
from Thermo Scientific Waltham, MA), gentamicin (50 μg/mL), penicillin (60 μg/mL) and streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO₂. HeLa human cervical carcinoma cells (ATCC) were maintained in DMEM culture medium with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Mediatech, Inc.; Manassas, VA), with 10% fetal bovine serum (FBS), gentamicin (50 μg/mL), penicillin (60 μg/mL) and streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO₂.

**Stable transfection of K562 human erythroleukemia cells**—K562 cells were transfected with Lipofectamine™ LTX and PLUS™ Reagent, purchased from Invitrogen (San Jose, CA), according to the supplier’s optimized protocol for K562 cells. On the day of transfection, a hemocytometer and trypan blue staining were used to count the cells and determine culture density and viability. In a 6-well plate, K562 cells (5 x 10⁵ cells per well) were seeded in a 6-well plate at a volume of 2 mL of RPMI-1640 growth medium with 10% FBS 30 minutes prior to transfection. For each transfection, 2.5 μg of DNA was added into 500 μL of RPMI medium without serum. 2.5 μL of PLUS™ reagent (at a 1:1 ratio to DNA) was then added directly to the diluted DNA. After gentle mixing and a 10 minute incubation at room temperature, 10 μL of Lipofectamine™ LTX was added into the diluted DNA solution, mixed gently and incubated for 35 minutes at room temperature to form DNA-Lipofectamine™ LTX complexes. The DNA-Lipofectamine™ LTX complexes were added dropwise to each well containing cells and mixed gently by manually rocking the plate back and forth. 24 hours
after transfection, the cells were pelleted by centrifugation at 400xg and resuspended in RPMI-1640 culture medium with 10% FBS, gentamicin (50 μg/mL) and the selection antibiotic, G418 sulfate (500 μg/mL). Every 2-3 days, the cells were pelleted and resuspended in fresh medium with 10% FBS, gentamicin (50 μg/mL), and G418 sulfate (500 μg/mL). Assays were performed 2-6 weeks after the start of selection.

**Transient transfection of HeLa cells**—On the day prior to transfection, HeLa cells were trypsinized and counted. Approximately 2 x 10⁴ cells were seeded into each well of a 24-well plate in 0.5 mL of DMEM. Cell density was ~30-50% confluent on the day of transfection. For each transfection, 250ng of DNA was diluted into 100 μl of DMEM without serum. 1 μl of Lipofectamine™ LTX was added into the diluted DNA solution, mixed gently and incubated for 30-45 minutes at room temperature to form DNA-Lipofectamine™ LTX complexes. The DNA-Lipofectamine™ LTX complexes were added dropwise to each well containing cells and mixed gently by manually rocking the plate back and forth for a few seconds. After 4 hours of incubation with the DNA-Lipofectamine LTX complexes, the medium was aspirated out of each well and fresh DMEM with 10% FBS, gentamicin (50 μg/mL), penicillin (60 μg/mL) and streptomycin (100 μg/mL) was added to each well of cells. Cells were incubated at 37°C in a CO₂ incubator for 24 or 48 hours post-transfection before assaying.
Preparation of cells for FACS and quantitation of PPIX—While K562 cells were suspended in phenol red-free medium, HeLa cells were washed, scraped and resuspended in phosphate-buffered saline (PBS; 80 mM disodium hydrogen orthophosphate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, pH 7.5) before pipetting into BD Falcon tubes with cell strainer caps. Preparation of either K562 or HeLa cells for FACS was done under very low light conditions (1-2 Lux as measured by a Pyle PLMT68 light meter) in order to minimize phototoxicity caused by PPIX accumulation. FACS analyses were performed using a BD LSR II Analyzer (Becton, Dickinson, and Company) and FACSDiva Version 6.1.3 software. ZsGreen1 emission was measured between 515 nm and 545 nm (530/30BP filter) when cells were excited using the 488 nm laser. In order to eliminate any background red fluorescence, the 633 nm-red laser was blocked during the collection of the PPIX emission data. PPIX emission was determined in the 619 nm and 641 nm range (630/22BP filter) when cells were excited with the 405 nm laser. Forward-scatter (FSC) versus side-scatter (SSC) dot plots were used to gate the whole cells and thus remove the contribution of the cell debris from the population being examined. A minimum of 10,000 of the gated whole cells were then depicted in dot plots of SSC versus ZsGreen1 fluorescence, and the “green-fluorescent population” gate was defined based on untransfected HeLa cells as negative controls. Dot plots of SSC versus PPIX fluorescence were used to define the PPIX-accumulating cells for both the “green-fluorescent” and the “non-green fluorescent” populations. The PPIX gating was based on the negative control for PPIX, the pIRES2-ZsGreen1 vector-
expressing cells. PPIX fluorescence values were normalized for transfection efficiency using the corresponding ZsGreen1 fluorescence value. Normalized fluorescence values were obtained by first dividing the mean PPIX fluorescence by the mean ZsGreen1 fluorescence for each cell population and secondly dividing by the mean PPIX fluorescence/ZsGreen1 fluorescence ratio of the pIREs2-ZsGreen1-transfected cells.

Light exposure assays—HeLa cells in 24-well plates were transfected 48 hours prior to FACS analysis. Cells were washed twice with PBS and placed on ice (to prevent overheating) underneath a Sylvania incandescent flood lamp (150W, 120V) for 10 minutes. Light intensity at the samples was measured before each light exposure experiment using a Pyle PLMT68 light meter, and it was between 21-22 kLux for all experiments. The samples were resuspended and pipetted into tubes with cell strainer caps and analyzed first for ZsGreen1 and PPIX fluorescence (as described above), and then for cell viability. In order to determine the statistical significance of cell death directly attributable to mALAS2-induced PPIX accumulation and phototoxicity, samples were compared to the ZsGreen1 controls during one-way ANOVA evaluations.

Cell viability assays—Cell death was assessed by measuring incorporation of either propidium iodide or DAPI into nuclear DNA, as these fluorescent dyes cross the plasma membrane in dying cells much more efficiently than in live cells. HeLa cell cultures were independently incubated with the fluorescent DNA-binding dyes propidium iodide (10 μg/mL) and DAPI (10 μg/mL)
for 5 min at 22°C. The cells were then analyzed by determining the fluorescence intensities using FACS. Fluorescence emission of nucleic acid-bound propidium iodide was measured between 585 nm and 625 nm (605/40BP filter) upon excitation of the cells using the 488 nm laser, while DNA-bound DAPI emission was measured between 440 nm and 460 nm (450/20BP filter) following excitation of the cells at 375 nm. Dot plots of SSC versus propidium iodide or DAPI fluorescence were used to define the respective gates, which were based on the control samples in which no propidium iodide or DAPI were added. Cell viability was independently determined using the MTT dye reduction assay as described elsewhere (42,43). HeLa cells (5 x 10³ cells per well) were seeded in 96-well plates, and on the following day were transfected with plasmids encoding mALAS2 variants as described above. Twenty-four hours after plasmid transfection, or 4 hours after addition of 100 μM ALA, cells were exposed to light for 10 minutes. The culture medium was pipetted out, 50 μL of 2 mg/mL MTT was added to each well, and cells were incubated for an additional 4 hours at 37°C. Upon solubilization of the cells with DMSO (100 μL/well) during a 10 minute incubation, the solubilized, MTT-treated cells were thoroughly mixed by pipetting several times, and absorbance was measured at 540 nm using a μQuant plate reader (Bio-Tek Instruments, Inc.). For control and ALA-treated cells, cell death was calculated by dividing the number of DAPI-stained cells by the total number of cells. For ZsGreen1-, WT-, HPVT-, and R433K-expressing cells, cell death was calculated from the ZsGreen1-positive cell populations by
dividing the number of DAPI-stained cells by the total number of ZsGreen1-positive cells.

Confocal fluorescence microscopy—HeLa cells were grown in Thermoscientific™ Nunc™ Labtek™ sterile 4-well chambered coverglass until 50% confluent, and then transfected as described above. Six hours later, the culture medium was supplemented with glycine to yield a final concentration of 100 mM, and immediately before obtaining confocal fluorescence microscopy images of the cells, the medium was removed from the wells and the cells were washed with PBS three times. Live cell imaging was performed using a 3i-Olympus spinning disk confocal microscope operated by Slidebook 5 software and equipped with a Photometrics Evolve EMCCD camera. The filter block used consisted of a 350/50 nm excitation filter, a BS400 beamsplitter, and a 630/75 nm emission filter.

Results

Transient expression of mALAS2 variants causes accumulation of PPIX in HeLa cells—HeLa cells were transfected with mALAS2-encoding plasmids with or without mutations in the mALAS2 presequence and/or mature enzyme sequence (Table 2.1, Figure 2.1). Where indicated, the mALAS2 presequence was mutated at three cysteine residues, C11, C38, and C70, in order to yield nonfunctional HRMs and thus eliminate heme feedback inhibition of mitochondrial import (18,40). The plasmids were designed such that a single
bicistronic mRNA, encoding both mALAS2 and the fluorescent protein ZsGreen1, separated by an internal ribosomal entry site (IRES), would be produced. Transcriptional expression of mALAS2 and ZsGreen1 was under control of the constitutively active cytomegalovirus (CMV) promoter (44). Twenty-four hours post-transfection, wild-type mALAS2 with a wild-type presequence (WT\textsuperscript{\textit{w}}) expression caused a slight, but statistically significant as defined by Student's t-test (p<0.05), increase in PPIX accumulation (Figure 2.2A). The K313A mutation in ALAS2 leads to undetectable enzymatic activity values \textit{in vitro} (45), and under \textit{ex vivo} conditions this also appears to be the case, as no PPIX accumulated in K313A-expressing cells (K313A\textsuperscript{\textit{w}}), a result similar to that of the mammalian cells harboring the pIRES2-ZsGreen1 vector (ZsGreen1) alone (Figure 2.2A). In

<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Mutations in mALAS2 Presequence</th>
<th>Mutations in Mature mALAS2 Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIRES2-ZsGreen1</td>
<td>N/A\textsuperscript{1}</td>
<td>N/A\textsuperscript{1}</td>
</tr>
<tr>
<td>pEF21</td>
<td>---\textsuperscript{2}</td>
<td>---\textsuperscript{2}</td>
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<tr>
<td>pEF23</td>
<td>---\textsuperscript{2}</td>
<td>K313A</td>
</tr>
<tr>
<td>pEF25</td>
<td>C11S, C38S and C70S</td>
<td>K313A</td>
</tr>
<tr>
<td>pEF26</td>
<td>C11S, C38S and C70S</td>
<td>R433K</td>
</tr>
<tr>
<td>pEF31</td>
<td>C11S, C38S and C70S</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1}N/A, non-applicable; \textsuperscript{2}---, no mutations.
comparison to WT\textsuperscript{\textsuperscript{2}}, the heptavariant mutations in the active site loop alone (HPVT\textsuperscript{\textsuperscript{2}}) and mutations in the presequence alone (WT) caused a statistically significant (p<0.05) increase in PPIX production in expressing cells (Figure 2.2A).

The combination of the heptavariant mutations in the active site loop and mutations in the presequence (HPVT) resulted in variable PPIX production that was not statistically significant. Somewhat unexpectedly, it was expression of the mALAS2 variant with a mutated presequence containing the R433K mutation (R433K) that caused the largest accumulation of PPIX. Mutation of R433 residue to a lysine in the purified, mature enzyme results in an increase in activity to twice that of the wild-type enzyme (\textit{i.e.}, a 2-fold increase in the $k_{\text{cat}}$ value and a

\textbf{Figure 2.1. Murine ALAS2 protein schematic indicating mutated amino acid residues.} The green dotted lines represent the relative locations of the three cysteines in the HRMs of ALAS2. C11 and C38 are in the ALAS2 presequence. K313A, R433K, and the heptavariant mutations and their respective positions are indicated according to the amino acid numbering previously described for mature mALAS2 (31,35,36). The amino acid positions according to the numbering for the precursor enzyme are written above the diagram.
1.65 to 1.85-fold enhancement in the specificity constants for glycine and succinyl-CoA over those of wild type, mature mALAS2) (31). Similarly, the increase in PPIX accumulation in HeLa cells expressing the R433K precursor with a mutated presequence was approximately 2.5-fold compared to the cells expressing WT* and 2-fold in comparison to cells expressing WT (Figure 2.2A).

Because PPIX can be used as a photosensitizing agent for PDT of cancers, we examined the effect of combining mALAS2-induced PPIX accumulation with the chemotherapeutic drug paclitaxel, a well-characterized inhibitor of mitosis (46-48). Only mALAS2 variants with mutated presequences were used in the drug combination experiments, since those variants presented the most potential for PPIX accumulation, and thus, subsequent cell death resulting from light-induced phototoxicity. A dose response curve revealed that the concentration of paclitaxel that killed 25% of HeLa cells in 48 hours (IC25) was 10 nM (data not shown), and this concentration was chosen as the desired concentration for the PDT combination experiments. Paclitaxel had no statistically significant effect, as defined by Student’s t-tests, on the amount of accumulated PPIX in the mammalian cells transfected with any of the ALAS2 variants after 48 hours (Figure 2.2B). The largest increase in PPIX fluorescence was observed with the R433K variant, regardless of whether or not paclitaxel was included in the culture medium (Figure 2.2B).

Supplementation of cell culture medium with glycine leads to increased PPIX accumulation in mALAS2-expressing HeLa cells—Given that PPIX is a
photosensitizer (49) and the Michaelis-Menten constant ($K_m$) of mALAS2 for glycine, at 25±4 mM (3,25,34), is significantly higher than its intracellular concentration of approximately 2.5 mM (50), it is plausible that supplementation of the cell culture medium with glycine would lead to enhanced synthesis of ALA, and consequently, PPIX, which in turn might increase the efficacy of PPIX-induced phototoxicity. To examine whether increased glycine concentration caused enhanced PPIX accumulation, HeLa cells transfected with mALAS2 (wild
type and variants) were grown in medium with different glycine concentrations (Figure 2.3A). Samples with culture medium supplemented with 100 μM ALA for 4 hours served as a control for PPIX accumulation independent of glycine concentration (Figure 2.3B). The concentration and treatment times for ALA were chosen based on experiments indicating that the extent of PPIX accumulated in HeLa cells supplemented with 100 μM ALA was similar to that of HeLa cells expressing R433K, the mALAS2 variant that induces the highest levels of PPIX accumulation in these cells. HeLa cells expressing WT, HPVT or R433K in culture medium supplemented for 18 hours with 10 mM or 100 mM glycine exhibited significant increases in PPIX in comparison to the “no glycine” controls, with R433K again demonstrating the highest PPIX accumulation (p<0.01) (Figure 2.4A). Supplementing the culture medium with either 10 mM or 100 mM glycine for cells expressing WT resulted in approximately 4- and 6-fold increases in PPIX accumulation, respectively, as compared to no glycine supplementation. For cells expressing R433K, glycine supplementation more than tripled the PPIX accumulation, representing a fourteen-fold increase over the control HeLa cells supplemented with glycine. Cells expressing HPVT were also affected by addition of either 10 mM or 100 mM glycine, increasing the PPIX by 1.4-fold and 2.5-fold, respectively. Glycine elicited no effect on PPIX production in HeLa cells alone, those treated with 100 μM ALA, or HeLa cells expressing the pIRES2-ZsGreen1 vector (Figure 2.3B).
**Figure 2.3.** Glycine supplementation of culture medium increases PPIX accumulation in HeLa cells expressing murine wild-type, HPVT, and R433K ALAS2. (A) PPIX fluorescence 24 hours after transfection and 18 hours after supplementing culture medium with 10 mM glycine or 100 mM glycine. Normalized PPIX fluorescence values were obtained by dividing the mean PPIX fluorescence by the mean ZsGreen1 fluorescence for each cell population. Mean PPIX fluorescence values are representative of three separate experiments ± standard deviation (\( **p<0.01 \), one-way ANOVA) [a.u., arbitrary units]. (B) ZsGreen1 fluorescence versus PPIX fluorescence in HeLa cell populations supplemented with 100 mM glycine as measured by flow cytometry. In each dot plot, cells in the green-fluorescent range are represented in green and to the right of the vertical black line, while cells that are not green-fluorescent are in blue. Cells that are red-fluorescent due to PPIX accumulation are above the horizontal black line, while cells that are not red-fluorescent are below the horizontal black line. The percentage of cells in each quadrant is written in the corners of each dot plot. “Control” refers to non-transfected HeLa cells.

_Glycine and deferoxamine increase PPIX accumulation in mALAS2-expressing K562 cells_—We generated stable K562 human myelogenous erythroleukemia cell lines expressing mALAS2, expecting that these cells might accumulate high amounts of PPIX, due to their similarity to undifferentiated
erythrocytes (51). We expressed only WT and HPVT in order to test if HPVT could have higher activity in a cell line in which ALAS2 in normally expressed. Initially, the stable cell lines expressing WT did not show an increase in PPIX fluorescence as compared to regular K562 cells (Figure 2.4A), and we postulated that glycine availability was again a limiting factor, as we had observed in HeLa cells (Figure 2.3). Another possible limitation upon PPIX accumulation could be a more effective conversion of PPIX into heme in K562 cells, in which case the inclusion of an iron-specific chelator such as deferoxamine should increase the PPIX fluorescence by reducing or even preventing this conversion. To address why the expression of mALAS2 alone did not cause a larger increase in PPIX and what could limit the ALAS2-induced PPIX accumulation, the culture medium supplemented with glycine to yield final concentrations of 10 mM or 100 mM glycine (Figure 2.4A and 2.4C) or deferoxamine mesylate to yield a final concentration of 100 μM deferoxamine mesylate (Figure 2.4B and 2.4D). Expression of WT in K562 cells did not increase the cellular PPIX fluorescence, but PPIX fluorescence increased by more than 5– and 10–fold when the culture medium contained 10 mM glycine and 100 mM glycine, respectively (Figure 2.4A). No significant differences were seen in cells not expressing mALAS2 (Figure 2.4C) or in cells expressing HPVT when treated with 10 mM glycine (Figure 2.4A). However, in contrast to HeLa cells, when 100 mM glycine was used, cells expressing HPVT did exhibit an increase in PPIX fluorescence by approximately 3-fold (Figure 2.4A).
Deferoxamine is a well-characterized iron-specific bacterial siderophore with a long history of clinical use in iron chelation therapy. Deferoxamine has the

Figure 2.4. Stable expression of murine ALAS2 variants in K562 cells results in PPIX accumulation when culture medium is supplemented with either glycine or deferoxamine. (A) PPIX fluorescence after supplementation of the culture medium with glycine. (B) PPIX fluorescence after supplementation of the culture medium with deferoxamine (+D). (C) PPIX fluorescence of K562 cell populations supplemented with glycine. The blue area represents cells that do not express ZsGreen1, and the green area represents the population that expresses ZsGreen1. K562 cells that express ZsGreen1 are not affected by glycine supplementation of the culture medium (upper plots), while cells that express WT exhibit a significant increase in PPIX fluorescence when the culture medium is supplemented with glycine (lower plots). (D) PPIX fluorescence of K562 cell populations grown in culture media containing deferoxamine. Cell growth in the presence of deferoxamine significantly increases the PPIX fluorescence in K562 cells expressing WT, but does not affect PPIX fluorescence in cells expressing only ZsGreen1 or HPVT, as indicated by the number of cells within the red dotted-line boxes.
potential to increase PPIX by decreasing the cellular iron concentration, thereby inhibiting the conversion of PPIX to heme (52,53). Treatment of K562 cells with deferoxamine for 18 hours caused no change in PPIX fluorescence in cells not expressing mALAS2 or cells expressing HPVT. Deferoxamine did cause a significant increase in PPIX in cells expressing WT, in which case the mean PPIX fluorescence increased from 1.4-fold over cells expressing ZsGreen1, to 2.4-fold with deferoxamine (Figure 2.4B and 2.4D).

*PPIX primarily accumulates and localizes at the plasma membrane in HeLa cells expressing R433K*—To evaluate cellular intactness and accumulated PPIX distribution in the transfected HeLa cells, we used confocal microscopy to visualize the fluorescent PPIX in individual HeLa cells (Figure 2.5A) and HeLa cells transfected with either the pIRES2-ZsGreen1 vector (Figure 2.5B) or pEF31 (harboring R433K) (Figure 2.5C). Transfected cells were grown in medium supplemented with 100 mM glycine for 18 hours in preparation for imaging. The outline of intact cells, and thus their morphological integrity, was evident in the three cases. As expected, green fluorescence was visualized in the transfected (Figures 2.5B and 2.5C) but not control HeLa cells (Figure 2.5A). In fact, green fluorescence, arising from the soluble ZsGreen1 green fluorescent protein was observed evenly distributed throughout the cytoplasm of the HeLa cells transfected with either pIRES2-ZsGreen1 (Figure 2.5B) or the R433K-expression plasmid (Figure 2.5C). While the enzyme protoporphyrinogen oxidase catalyzes PPIX production exclusively within the mitochondrion, PPIX accumulated
primarily within the plasma membranes of HeLa cells expressing R433K, as
visualized by the characteristic red fluorescence; however, PPIX build-up was
also apparent within the cells (Figure 2.5C). Thus, it appears that much of the

Figure 2.5. Fluorescence microscopy of HeLa cells expressing GFP and the
R433K mALAS2 variant. From left to right for each row, the panels correspond to (1)
brightfield, (2) green fluorescence, (3) red fluorescence, (4) superimposed green and (5)
red fluorescence, and superimposed brightfield, green fluorescence, and red
fluorescence images. (A) HeLa cells grown to 100% confluency in culture medium with
100 mM glycine. (B) HeLa cells transfected with pIRES2-ZsGreen1 and grown in culture
medium to 100% confluency with 100 mM glycine. (C) HeLa cells transfected with
pEF31, which expresses the R433K variant from the CMV promoter in the pIRES2-
ZsGreen1 vector (Table 2.1), and grown to 100% confluency in culture medium with 100
mM glycine. (D) HeLa cells transfected with pEF31 and grown in culture medium to 80%
confluency with 100 mM glycine. Pictures were obtained 24 hours after transfection and
18 hours after glycine supplementation. ZsGreen1 (green) is present throughout the cell,
while PPIX (red) accumulates at the plasma membrane of cells expressing R433K and
surrounding cells.
PPIX produced in the mitochondria eventually accumulates in the plasma membrane, entirely consistent with the fact that PPIX is a relatively lipophilic molecule. Of note, PPIX accumulated in not only transfected cells, but also in surrounding cells, indicating that PPIX could leave the transfected cells and be taken up by nearby cells (Figure 2.5C). It is very likely that, additionally, PPIX accumulated in organelle membranes within the cell, such as in the mitochondrion, but since PPIX photobleached within a few seconds under the conditions utilized here, it was not possible to obtain high-resolution organelle images with our current microscopic parameters.

*ALAS2-induced PPIX accumulation followed by light exposure combined with paclitaxel treatment causes cell death*—Both propidium iodide and DAPI staining were utilized to assess cell viability after mALAS2-induced PPIX accumulation, light exposure, and paclitaxel treatment, based on the specific fluorescence emission of each dye when bound to the DNA of intact cells. Stains were added to cell samples 48 hours after transfection, and the fluorescence of DNA-bound propidium iodide and DAPI were measured by flow cytometric analysis using the respective fluorescence emission maxima of 613 nm and 460 nm. Expression of WT or HPVT did not significantly increase cell death following light exposure. However, expression of R433K caused a statistically significant (p<0.05) increase in cell death of up to 30%, as measured both by propidium iodine (Figure 2.6A) and DAPI (Figure 2.6B) staining, in comparison to expression of ZsGreen1 alone. Addition of paclitaxel increased cell death in all
samples, including controls, by 10-25%. HeLa cells expressing R433K and treated with paclitaxel exhibited the highest percentage of up to 50% cell death (Figure 2.6A-B). Combination of paclitaxel with mALAS2-induced PPIX accumulation and light treatments exhibited an additive effect in causing death in HeLa cells.

Figure 2.6. Light-induced cell death of HeLa cells expressing mALAS2 variants. (A) Cell death measured by changes in DNA-bound propidium iodide fluorescence. (B) Cell death measured by changes in DNA-bound DAPI fluorescence. HeLa cells were transfected with expression plasmids for either wild type (WT) or mALAS2 variants and treated with 10 nM paclitaxel 4-hour post-transfection. 48 hours after transfection, cells were stained and analyzed by flow cytometry. Cell death percentage values were compared to that of the pIRES2-ZsGreen1-transfected cells control. Each data set represents three separate experiments ± standard deviation (*p<0.05 and **p<0.01, Student's t-test).

Supplementation of cell culture medium with glycine enhances phototoxicity and cell death in mALAS2-expressing HeLa cells—The phototoxicity and subsequent cell death caused by mALAS2-induced PPIX accumulation, when the culture medium was supplemented with glycine, were
also investigated in HeLa cells. Cell viability was measured using propidium iodide (data not shown), DAPI staining, and MTT assays (Figure 2.7) 24 hours post-transfection, 18 hours after glycine addition, and 4 hours after ALA addition. Significant cell death was ascribed to those samples that exhibited more cell death compared to the pIRES2-ZsGreen1-transfected cells with the same glycine concentration as determined by one-way ANOVA (p<0.05). Transfection of HeLa cells with the pIRES2-ZsGreen1 plasmid caused a decrease in cell viability by an average of 30%, which can be attributed to the mild cytotoxicity of the transfection reagents (54).

Expressing WT, regardless of the glycine concentration in the culture medium, caused approximately 70% cell death following light exposure, as measured by DAPI staining (Figure 2.7A-B). In the MTT assays, expression of WT only caused statistically significant cell death (p<0.05) with supplementation of 100 mM glycine, in which case only an average of 15% of cells remained viable (Figure 2.7C). HeLa cells expressing HPVT or R433K did exhibit a glycine-dependent increase in cell death when the glycine concentration reached 100 mM as measured by both DAPI staining and MTT assays. When 100mM glycine was added, cells expressing HPVT decreased from an average of 81% viable to 13% viable, and cells expressing R433K decreased from 58% viable to 25% viable as measured by MTT assays (Figure 2.7C). ALA treatment did not cause significant cell death (p>0.05), regardless of glycine concentration, when measured by either DAPI staining or MTT assays.
Figure 2.7. Light-induced cell death in murine ALAS2 variants-expressing HeLa cells cultured in a medium supplemented with glycine. (A) Cell death, as assessed by DAPI fluorescence, are representative of three separate experiments ± standard deviation (*p<0.05 and **p<0.01, one-way ANOVA). (B) Cell death in HeLa cell populations grown in culture medium supplemented with glycine. The percentage of cells in each quadrant is written in the corners of each dot plot. (C) Cell viability as measured by metabolism of MTT. Cells were transfected 24 hours before light treatment, and 100 mM glycine was added 18 hours before light treatment. Cell viability percentage values are normalized to MTT metabolism in healthy, untreated HeLa cells and are representative of at least three separate experiments ± standard deviation (*p<0.05, one-way ANOVA). “Control” refers to non-transfected HeLa cells.

Discussion

Photodynamic therapy (PDT) is a widely utilized clinical procedure for many types of cancer, as well as dermatological conditions such as psoriasis and schleroderma (55-57). PDT is often initiated by application of the pro-drug 5-
aminolevulinic acid (ALA) to the patient in order to photosensitize the tissue to be treated (49). ALA-PDT is very effective, has minimal side effects and is being tested in numerous clinical trials on a wide-spectrum of cancers (49). There are, however, some limitations to ALA-PDT and other PDTs that provide opportunities for improvement. For example, there is no specific mechanism that targets PPIX to tumor cells after ALA has been applied, other than a slightly preferential uptake of photosensitizers by hyperproliferating cells (58). Consequently, normal tissue can be damaged from the procedure due to unintentional exposure to light resulting in pain, and conversely, not all hyperproliferating cells necessarily become highly sensitized (55,59). In an attempt to restrict photosensitvity by biological, rather than just chemical or physical means, an adenovirus expressing human ALAS2 with mutated HRMs was generated and H1299 lung carcinoma cells were successfully infected (40). The adenovirus-infected H1299 cells accumulated more PPIX and became more photosensitive than cells supplied with ALA in their media, indicating that delivery of ALAS to tumors could potentially be a useful tool for PDT not only for better targeting, but also for greater photosensitivity (40). However, the cell death was only 26% even in the presence of deferoxamine (40), leading us to postulate that one or more highly active variants of mALAS2 (31,35) would increase PPIX accumulation, phototoxicity, and targeted cell death sufficiently to make the approach more clinically attractive.

To explore the ability of mALAS2 variants to cause accumulation of PPIX in mammalian cells, we transfected cells, of both erythroid and non-erythroid
lineages, with murine ALAS2-expressing plasmids, and quantified the PPIX fluorescence using FACS analysis. In HeLa cells, we transfected plasmids encoding mALAS2 both with and without mutated presequences and with or without mutations in the mature enzyme sequence (Table 2.1, Figure 2.1). As with many mitochondrial proteins, ALAS2 is synthesized in the cytosol and contains a sequence at its N-terminus that targets ALAS2 for mitochondrial import after its synthesis (18). Within the N-terminus of the ALAS2 precursor, there are three HRMs as recognized by adjacent cysteine-proline residues that have the potential to bind heme. The HRMs located within the presequence of ALAS2 (C11 and C38) have been shown to bind heme (24) and subsequently inhibit the mitochondrial import of ALAS2 (18). Our experiments support the existing data that the cysteines in the HRMs bind heme, and that mutation of these HRMs relieve the inhibition of mitochondrial import, thus resulting in increased mature, functional ALAS2, as reflected by increased cellular concentrations of PPIX when the HRMs are mutated (Figure 2.2A). In our study, when the HRMs in the presequences of the mALAS2 constructs were mutated to relieve heme inhibition on mitochondrial import, there were significant increases in PPIX accumulation in HeLa cells expressing WT, HPVT, and R433K (Figure 2.2A-B).

We tested several mALAS2 variants, covering a range of in vitro activity from undetectable to higher than wild-type, for capacity to stimulate PPIX accumulation (31,35,36,60). Transfection of HeLa cells with the negative control plasmid harboring K313A resulted in no PPIX increase, as expected (Figure
2.2A). The mutation of K313 leads to undetectable enzymatic activity \textit{in vitro}, attributable to the role of K313 in formation of a Schiff-base linkage with the PLP cofactor (36), and its additional function as a general base catalyst during the ALAS-catalyzed reaction (3,28,36,61). In preliminary studies with HeLa cells, it was observed that expression of HPVT\textsuperscript{vii}, which has seven mutations in its active site loop, yielded significantly more accumulated PPIX than expression of the pIRES2-ZsGreen1 vector control plasmid. HPVT\textsuperscript{vii} was chosen for this study as the seven mutations of non-conserved residues in the active site loop resulted in the most active recombinant protein isolated from a variant library at 20°C (35). Lendrihas \textit{et al}. (35) hypothesized that these mutations in the active site loop, which increase both hydrophilicity and basicity, destabilize the loop by both increasing solubility and eliminating hydrophobic and electrostatic interactions that would typically act to stabilize the loop in its closed confirmation. However, the hyperactivity of HPVT\textsuperscript{vii} was temperature-dependent; while at a temperature of 20°C these seven mutations increased the $k_{\text{cat}}$ value of the recombinant, mature enzyme to more than 10 times of that of the WT enzyme, at 35°C the $k_{\text{cat}}$ values were nearly the same (35). While the levels of accumulated PPIX in HeLa cells expressing HPVT were significantly affected by supplementation with glycine ($p<0.01$), those in K562 cells expressing HPVT were only modestly increased when the medium was supplemented with 100 mM glycine. However, the HPVT-promoted PPIX concentration enrichments were much lower than those in cells expressing WT or R433K (Figures 2.3 and 2.4). The relatively low levels of PPIX associated with HPVT expression are presumably due to the
temperature-dependent activity profile of this particular mALAS2 variant. HPVT was isolated from a library of mALAS2 variants engineered to possess greater enzymatic activity than wild type mALAS2 by targeting the active site loop to acquire different degrees of mobility (35). Since the mutations in HPVT destabilized the active site loop and altered the protein conformation (35), it would not be surprising if this variant had a decreased cellular stability. Additionally, the HPVT mutations may affect protein-protein interactions, specifically the ability of mALAS2 to interact with a known binding partner, succinyl-CoA synthetase (62).

The simple addition of the non-toxic substrate glycine to the cell media increased PPIX production by WT, HPVT, and R433K significantly. When the culture medium was supplemented to a final concentration of 10 mM or 100 mM glycine, PPIX production in HeLa cells expressing WT was increased by more than 4- and 6-fold for each concentration, respectively (Figure 2.3). In K562 cells expressing WT, the effect was even larger, as PPIX increased by more than 5- and 10-fold for 10 mM and 100 mM glycine, respectively (Figure 2.4A and 2.4C). The slightly higher increases in PPIX concentrations in the K562 cells when treated with glycine could be due to the stable expression of ALAS2 versus the transient transfection used in the HeLa cells. However, the ability of the K562 cells to tolerate higher expression of WT and production of PPIX, in comparison to HeLa cells, might also be attributable to their erythroid lineage. K562 cells are of the erythroleukemic cell type, and bear some proteomic resemblance to undifferentiated erythrocytes (51,63) and express endogenous ALAS2. Thus
while expressing ALAS2 in HeLa cells introduces an enzyme that does not normally exist in epithelial cells, K562 cells may adapt more easily to the expression and up-regulation of the heme biosynthetic pathway. It seems likely that the capacity of mALAS2 to accumulate PPIX will be found to vary in other mammalian cell types as well.

Supplementing the culture medium of K562 cells expressing WT with 100 μM deferoxamine, an iron-chelator previously shown to increase the amount of PPIX in ALAS adenovirus-infected H1299 cells (40), resulted in increase in the mean PPIX fluorescence per cell (Figure 2.4B and 2.4D), but it was much less than with glycine supplementation. However, the modest increase was similar to what Gagnebin et al. (40) observed in the ALAS adenovirus-infected cells, suggesting the much larger increases reported here with R433K and glycine supplementation represent substantial advancements in our ability to overproduce PPIX in mammalian cells. R433K is only twice as active as WT \textit{in vitro}, and it is reasonable to believe that significantly more active variants could be readily produced via directed evolution, and that these variants would facilitate even greater levels of PPIX accumulation, perhaps to the point of expanding the clinical applicability of PPIX-based PDT.

The HeLa cells that accumulated the highest amount of PPIX were those expressing R433K. The R433K mutation was analyzed in 1998 by Tan et al. (31) in a study that identified the nearby R439 as being a conserved residue in many α-family PLP-dependent enzymes, as well as being involved in binding of the amino acid substrate during catalysis (31). In that study, the kinetic parameters
were defined for R439L, R439K, R433L, and R433K. Although not the primary focus of the article, the kinetic parameters for R433L were found to be comparable to those of the wild-type at 37°C, while for R433K the $k_{\text{cat}}$ was increased by two-fold, with no effect on the Michaelis constants, resulting in a doubling of catalytic efficiency for both substrates (31). In the results detailed here, expression of R433K in HeLa cells produced a 2- to 3-fold increase in PPIX in comparison to WT, and a 4- to 6-fold increase in PPIX fluorescence in comparison to cells transfected with the pIRES2-ZsGreen1 vector control or HeLa cells alone (Figure 2.2). When the culture medium was supplemented with glycine, PPIX accumulation increased by 13- to 15-fold in comparison to cells transfected with the pIRES2-ZsGreen1 vector control or HeLa cells alone, and represented the conditions for the highest cellular PPIX accumulation. Given that within these samples there were cells fluorescing with PPIX that were not ZsGreen1-expressing (Figure 2.3B), some of the PPIX produced in the R433K- and WT-expressing HeLa cells appears to have been transported out of the cells into the culture medium, where it was then taken up by non-transfected cells. These populations of red-fluorescing cells were comparable to those cells in culture medium supplemented with ALA (Figure 2.3B). This is a very important finding in terms of the potential of these constructs for photodynamic therapy, as it indicates that the PPIX produced within a delivery cell could be transported into surrounding diseased tissue and accumulate to clinically relevant levels.

The subcellular distribution of a photosensitizing agent might have important consequences in regards to PDT efficacy (64). Fluorescence
microscopy was successfully utilized to visualize PPIX accumulation in R433K expressing HeLa cells. Due to the extremely short time (a few seconds) it took for PPIX to photobleach, the resolution could not be optimized for visibility of individual organelles such as mitochondria and nuclei. However, it was possible to observe that PPIX had accumulated in the plasma membranes of the R433K expressing cells; and this fluorescence was not seen in the pIRES2-ZsGreen1 vector-expressing cells (Figure 2.5). It also appears that PPIX can exit the transfected cells, potentially via ABC transporters such as ABCB6 (65,66) or ABCG2 (67,68), and enter surrounding cells. Presumably, the lipophilic PPIX accumulated in the organelle membranes as well, but direct microscopic confirmation of PPIX accrual in organelle membranes awaits approaches resulting in better resolution than we were able to achieve here.

In order to study the photosensitivity of cells that accumulated PPIX (caused by expression of WT, HPVT, and R433K), we performed light treatments using an incandescent lamp, which emits light in the visible and infrared spectral regions (400-1000 nm). The cells were exposed for 10 minutes before FACS analysis of PPIX and cell death. Expression of the ZsGreen1 protein alone caused a toxicity that was independent of light treatment, and thus significant cell death was measured against the pIRES2-ZsGreen1 vector-transfected cells (p<0.05) as opposed to HeLa cells alone (Figure 2.7). Regardless of glycine concentration, the expression of WT led to a significant increase in cell death by 1.4- to 2-fold. R433K and HPVT, when the culture medium was supplemented with 100 mM glycine, also caused significant increases in cell death by 2.1- and
1.6-fold, respectively. With no or 10 mM glycine supplementation the error was too high for any of the increases to be significant, although there was a trend towards increased cell death. For WT and R433K, increased PPIX accumulation correlates with increased cell death after light treatment. In spite of the more modest increases in PPIX accumulation, light-induced death also occurred in HPVT-expressing cells. This finding led us to suggest that toxicity inherent to the overproduced HPVT protein is the other contributing factor to cell death. Although purified recombinant, mature HPVT is more active than WT at 37°C (with an enhancement of approximately 1.5 in the $k_{cat}$ value at 37°C vs. a 15.5-fold increased $k_{cat}$ at 20 °C) (35), when expressed in mammalian cells, HPVT is not as active as WT, as determined by PPIX fluorescence. Since expression of HPVT paired with glycine supplementation caused significant cell death (Figure 2.7), the potential role of another toxic heme pathway intermediate, such as the pro-oxidant ALA (69-72), in causing cell death, is plausible. However, at present, different levels of protein expression [Quantification of ALAS2 and variant protein levels was not feasible due to the unavailability of a functional ALAS2 antibody.] and distinct modulation of the enzymatic activity between ALAS2 and variants cannot be ruled out as possibilities. Treatment with 100 μM ALA, a positive control for PPIX accumulation, caused a similar cell death after light exposure to expression of the ZsGreen1 protein alone. In summary, the highest cell death was seen in light-treated HeLa cells expressing ZsGreen1 and either WT, HPVT, or R433K, in culture medium supplemented with 100 mM glycine. The total cell death was observed to be as high as 90% in the cells expressing both ZsGreen1
and R433K. This represents a substantial improvement upon the 26% cell death reported previously (40), and indicates that this approach, if carefully developed, may eventually find some clinical utility.

Because ALAS2, especially highly stable and active variants of ALAS2, would be useful in the development of multiplex cancer treatments involving PDT, we experimented with combination PDT and drug treatments of HeLa cells. Paclitaxel (Taxol) is currently approved in the United States for the treatment of AIDS-related Kaposi sarcoma (73), breast cancer (74), non-small lung cell lung cancer (75), and ovarian cancer (76). Paclitaxel induces apoptosis in cancer cells by binding to tubulin and inhibiting the disassembly of microtubules, thereby resulting in the inhibition of cell division (46,77). As expected, paclitaxel did not affect PPIX accumulation, as indicated by the similar mean PPIX fluorescence values between untreated and paclitaxel-treated cells (Figure 2.2B). However, paclitaxel had an additive effect to PDT and increased cell death in all samples by 10-25%. HeLa cells expressing R433K treated with paclitaxel exhibited the highest percentage of cell death (Figure 2.6A-B). Ever since PDT was first shown to be able to elicit an immune response (78), many recent advances in PDT are aiming toward creating PDT-generated cancer vaccines (79). Using highly active and stable ALAS2 variants as part of a vaccine strategy for photosensitization may be useful for this vaccine approach. Further experiments, both in cell culture and live animals, are necessary to test for potential immune response stimulated by ALAS2-PDT.
In this study, we have shown that transfecting mammalian cells, of both erythroid and non-erythroid lineages, with mALAS2 variants, is an effective way to stimulate cellular PPIX accumulation. Furthermore, supplementing the culture medium with glycine vastly increases the intracellular PPIX levels when cells express either WT or R433K. These data offer new ways to accumulate high levels of the photosensitizer PPIX in cancer cells for more targetable and efficient PDT. Human ALAS2 variants with higher than normal activity are now known to occur in nature, and are associated with a form of erythropoietic protoporphyria known as X-linked dominant protoporphyria, which is characterized by a 24-fold increase in erythrocyte PPIX concentrations (39). It would be appropriate if these variants were eventually utilized in PDT, and could thereby allow clinicians to exploit one disease to treat or cure others.

References


the first enzyme of heme biosynthesis, and its link to XLSA in humans. *EMBO J.* **24**, 3166-3177


CHAPTER 3: MUTATIONS IN THE C-TERMINUS OF HUMAN ERYTHROID 5-AMINOLEVULINATE SYNTHASE ASSOCIATED WITH X-LINKED ERYTHROPOIETIC PROTOPORPHYRIA ALTER ENZYME STRUCTURE, KINETICS, AND EX VIVO ACTIVITY

Abstract

Five different frameshift mutations in the final coding exon of the human erythroid ALAS (hALAS2) gene are associated with X-linked erythropoietic protoporphyria (XLEPP), a disease phenotypically characterized by elevated levels or protoporphyrin IX (PPIX) and zinc protoporphyrin in erythroblasts. This is apparently due to enhanced cellular ALAS2 activity, but the biochemical relationship between these C-terminal mutations and increased ALAS2 activity is not well understood. Mutations in the hALAS2 C-terminus associated with XLEPP were characterized using purified recombinant hALAS2 to compare kinetic and structural parameters and studied ex vivo in HeLa and K562 cells. Two XLEPP variants, delAGTG and Q548X, when compared to wild-type hALAS2, exhibited higher catalytic constants and affinity for succinyl-CoA, increased transition temperatures, and caused porphyrin accumulation in HeLa and K562 cells. Another XLEPP mutation, delAT, had an increased transition temperature and caused porphyrin accumulation in mammalian cells, but exhibited a reduced catalytic constant at 37°C in comparison to wild-type hALAS2. The XLEPP variants were more structurally responsive upon binding of
succinyl-CoA, and adopted distinct features in tertiary and PLP-cofactor binding site structure. These results imply that the C-terminus of hALAS2 is important for regulating its structural integrity, which affects kinetic activity and stability.

Introduction

5-Aminolevulinate synthase (ALAS) is a homodimeric, pyridoxal-5'-phosphate (PLP)-dependent enzyme that catalyzes the first and key regulatory step of heme biosynthesis in non-plant eukaryotes, as well as the α-subclass of purple bacteria (1-3). The reaction involves the condensation of glycine and succinyl-CoA to produce 5-aminolevulinate (ALA), CoA, and carbon dioxide (1). Animal genomes encode two highly conserved but differentially expressed ALAS genes, a housekeeping gene (ALAS1) and an erythroid-specific gene (ALAS2) (4). In humans, mutations in ALAS2 can result in two very different diseases, X-linked sideroblastic anemia (XLSA) and X-linked erythropoietic protoporphyria (XLEPP).

In XLSA, the mutation of ALAS2 leads to a decrease in activity, and thus a decrease in porphyrin synthesis, resulting in iron accumulation in the mitochondria of erythroblasts and anemia (5-8). ALAS2 mutations associated with XLSA have been identified in exons 4-11 of the ALAS2 gene, which encode the highly conserved catalytic core region of the enzyme (9). The discovery of XLSA began with reports that ALAS activity in the bone marrow of inherited sideroblastic anemia patients is decreased, indicating that impairment of heme biosynthesis may induce the onset of certain sideroblastic anemias (10-12).
Shortly after ALAS2 was mapped to region Xp11.21 of the X chromosome (13,14), the first sideroblastic anemia patient with a mutation in ALAS2 was reported (15), supporting the theory that ALAS2 deficiency results in both a decreased supply of heme for hemoglobin and iron accumulation in the mitochondria of erythroblasts. Since the identification of the first XLSA patient, at least 61 different mutations in ALAS2 in patients with XLSA have been identified (9,16-18). In more than 50% of XLSA patients, the mutation in ALAS2 decreases the affinity of ALAS2 for its cofactor PLP, and these patients are responsive to treatment with oral pyridoxine, which increases the amount of available PLP (9,17). In patients not responsive to pyridoxine, the ALAS2 mutations can create a premature stop codon or alter enzyme stability, leading to a decrease in protein level (9). ALAS2 protein levels can also be decreased as a result of mutations in the ALAS2 promoter, which decrease the transcription of ALAS2 resulting in XLSA (19). It is clear that a single amino acid change in a variety of locations in ALAS2 can decrease its function in vivo, and the mechanism by which the activity is reduced can be due to a decreased affinity for its PLP cofactor, affinity for its substrate/s, stability, and/or transcription.

Yet, in 2008, C-terminal mutations in patients that lead to an increase in ALAS2 function were discovered (20), raising the question of how certain mutations in the C-terminus of ALAS2 cause decreased function, while other mutations in the same region result in an increased function. In XLEPP, ALAS2 mutations in the C-terminal region lead to increased activity, and thus an accumulation of the heme precursor, protoporphyrin IX (PPIX), as well as zinc-
protoporphyrin (20). In contrast to XLSA, all mutations known associated with XLEPP are located in the final exon, exon 11, which encodes the far C-terminus (20-22) (Table 3.1). The final 33 amino acids of ALAS2 are highly conserved and yet have diverged from the ALAS1 isoform, suggesting that these residues may have an erythroid-specific function (20). Additionally, the C-terminal amino acids are conserved in higher eukaryotes but not present in prokaryotes, and deleting the final 33 amino acids of recombinant hALAS2 results in an increase in the catalytic activity (21). Given that mutations at the C-terminus can result in either a decrease (9,16) or increase (20,22,23) in the function of hALAS2, the mechanisms by which the C-terminus of hALAS2 can determine its activity and/or stability are of particular interest.

Table 3.1. C-terminal amino acid sequences resulting from XLEPP mutations.

<table>
<thead>
<tr>
<th>Genetic mutation</th>
<th>Protein name</th>
<th>hALAS2 sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.1699-1700</td>
<td>DelAT (M567E)</td>
<td>542 AVGLPLQDVSVAACNFCRRPVHFELMSEWERSYFGNMGQPQYVTYA</td>
</tr>
<tr>
<td>c.1706-1709</td>
<td>DelAGTG</td>
<td>542 AVGLPLQDVSVAACNFCRRPVHFELMSGNVPTSGTWGPSMSPPMPPEKAA</td>
</tr>
<tr>
<td>c.1642C&gt;T</td>
<td>Q548X</td>
<td>542 AVGLPLQ</td>
</tr>
</tbody>
</table>

Here we characterize the kinetics, thermostability, and structure of the XLEPP variants by evaluating ex vivo activity in HeLa and K562 cells expressing hALAS2 and XLEPP variants, and using purified recombinant hALAS2 for kinetic analyses, acrylamide fluorescence quenching, and CD spectroscopy. We
demonstrate that the C-terminus of hALAS2 is important not only for controlling its enzymatic activity, but also for maintaining structural integrity.

**Materials and Methods**

*Reagents*—Aprotinin, pepstatin, leupeptin, PMSF, ampicillin, β-mercaptoethanol, pyridoxal 5’-phosphate, succinyl-CoA, thiamine pyrophosphate, and NAD⁺ were obtained from Sigma-Aldrich Chemical Company. Glucose, glycerol, acetic acid, methanol, glycine, disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, EDTA, MOPS, tryptone, yeast extract, sodium chloride, tricine, ammonium sulfate, and potassium hydroxide were purchased from Fisher Scientific. Centricon concentrators were from Millipore. Sodium dodecyl sulfate polyacrylamide gel electrophoresis reagents and Phusion DNA Polymerase were acquired from Thermo Scientific. 5-Aminolevulinic acid hydrochloride was purchased from Acros Organics. BmtI, SalI, BplI, and BamHI restriction enzymes were obtained from New England BioLabs, Inc. Oligonucleotides were synthesized by Integrated DNA Technologies. T4 DNA ligase and ligase buffer were obtained from Thermo Scientific Fermentas and a bicinchoninic acid protein determination kit was purchased from Thermo Scientific Pierce.

*Plasmids for mammalian expression and protein purification*—The following mutations: c. 1699-1700 AT deletion (delAT, which results in a frame-shift mutation leading to a truncated ALAS2 protein with a glutamate (E567) as the C-
terminal amino acid (20) and c.1705-1708 AGTG deletion (delAGTG, which results in the E569G mutation and a 23 amino acid, non-related ALAS C-terminus (20) were introduced into the cDNA of hALAS2 using the QuikChange method (Stratagene), and confirmed by DNA sequencing. The plasmids encoding the wild-type hALAS2, delAT, delAGTG, and Q548X sequences were pSD1, pSD2, pSD3, and pQ548X, respectively. The cDNAs for these proteins were under the control of the *Escherichia coli* alkaline phosphatase promoter (24). For expression plasmids used in the transfection of mammalian cells, the wild-type precursor hALAS2 cDNA (GenBank: X56352.1) was individually subcloned into the multiple cloning site of the plRES2-ZsGreen1 vector (purchased from Clontech Laboratories, Inc. Mountain View, CA) using the Bmtl and BamHI restriction sites. The digested hALAS2-encoding fragment was ligated into the digested plRES2-ZsGreen1 vector using T4 DNA ligase in ligase buffer at 16°C. Electrocompetent BL21(DE3) cells were transformed by electroporation with the ligated plasmid DNA and selected by spreading the transformed cells on LB agar medium containing 10 µg/mL kanamycin sulfate. Plasmid DNA was purified from a single colony using a QiaPrep Spin Miniprep kit (Qiagen Inc.), and the sequence of the cloned DNA was verified by Genewiz, Inc. in New Brunswick, NJ. The resulting plasmid was named pEF27. For the mammalian expression plasmids encoding the precursor XLEPP variants, the cDNAs encoding the mutated hALAS2 C-termini were retrieved from pSD2 and pSD3 and subcloned into pEF27 using the Bpll and BamHI restriction sites. The
resulting plasmids were named pEF28 and pEF29, encoding delAT and delAGTG, respectively.

In order to purify hALAS2 and the XLEPP variant proteins using nickel affinity chromatography, we added six histidine codons to the cDNA sequence encoding the mature hALAS2 N-terminus. The mature wild-type hALAS2 was amplified from pSD1 using the oligonucleotides hALAS22 (5’- CGT GTC GAC GAT GCA CCA TCA CCA CCA TCA CGG GAA GAG CAA GAT TGT GCA GAA G-3’) and r-hALAS23 (5’-AAG TGG TAA AGA TGA AGC CTG CAG CAT- 3’) as forward and reverse primers, respectively. The SalI site and the codons for the six histidines are indicated in *italics* and *bold*, respectively, in the sequence for the hALAS22 primer (above). The r-hALAS23 reverse primer was designed to anneal to the DNA coding strand, 531 bp downstream of the BlpI site. The generated PCR product (1040bp) was digested with SalI and BlpI to yield a 505bp-DNA fragment that was subsequently subcloned into the previously digested pSD1, pSD2, pSD3, and pQ548X vectors using T4 DNA ligase in ligase buffer (Thermo Scientific Fermentas) at 16°C. Electrocompetent BL21(DE3) cells were transformed by electroporation with the ligated plasmids and selected by spreading the transformed cells on LB agar medium containing 50µg/mL ampicillin. Plasmid DNA was purified from a single colony using a QiaPrep Spin Miniprep kit (Qiagen Inc.). The N-terminal ALAS- and histidine-encoding sequences were verified by DNA sequencing using the primer r-hALAS17 (5’-CTG GTC ATA ACT GAA GAC-3’), which anneals complementarily to the DNA coding strand and 208 bp downstream of the initiation and histidine codons.
introduced in the sequences for the mature hALAS2 and XLEPP variants. The resulting plasmids, pEF40, pEF41, and pEF42, were used for the expression of histidine-tagged wild-type hALAS2, delAT, and delAGTG, respectively.

Cell culture—K562 cells were maintained in RPMI-1640 culture medium (Mediatech, Inc.), with 10% FBS (Thermo Scientific™ HyClone™), gentamicin (Mediatech, Inc., 50 μg/mL), penicillin (Mediatech, Inc., 60 μg/mL) and streptomycin (Mediatech, Inc., 100 μg/mL) at 37°C in a humidified incubator with 5% CO₂. HeLa cells were maintained in DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Mediatech, Inc.), with 10% FBS, gentamicin (50 μg/mL), penicillin (60 μg/mL) and streptomycin (100 μg/mL) at 37°C in a humidified incubator with 5% CO₂.

Transient transfection of HeLa cells—On the day prior to transfection, HeLa cells were trypsinized and counted. Approximately 2 x 10⁴ cells were seeded into each well of a 24-well plate in 0.5 mL of DMEM. Cell density was ~30-50% confluent on the day of transfection. For each transfection, 250 ng of DNA was diluted into 100 μL of DMEM without serum. One μL of Lipofectamine™ LTX was added into the diluted DNA solution, mixed gently and incubated for 30-45 minutes at room temperature to form DNA-Lipofectamine™ LTX complexes. The DNA-Lipofectamine™ LTX complexes were added dropwise to each well containing cells and mixed gently by manually rocking the plate back and forth for a few seconds. After 4 hours of incubation with the DNA-
Lipofectamine LTX complexes, the medium was aspirated out of each well and fresh DMEM with 10% FBS, gentamicin (50 μg/mL), penicillin (60 μg/mL) and streptomycin (100 μg/mL) was added to each well of cells. Cells were incubated at 37°C in a CO₂ incubator for 24 hours post-transfection before assaying. For cells treated with glycine, glycine dissolved in DMEM (1M) was added to the culture medium 4 hours post-transfection.

*Transient transfection of K562 human erythroleukemia cells*—K562 cells were transfected with Lipofectamine™ LTX and PLUS™ Reagent, purchased from Invitrogen (San Jose, CA), according to the supplier's optimized protocol for K562 cells. On the day of transfection, a hemocytometer and trypan blue staining were used to count the cells and determine culture density and viability. In a 6-well plate, K562 cells (5 x 10⁵ cells per well) were seeded in a 6-well plate at a volume of 2 mL of RPMI-1640 growth medium with 10% FBS 30 minutes prior to transfection. For each transfection, 2.5 μg of DNA was added into 500 μL of RPMI medium without serum. 2.5 μL of PLUS™ reagent (at a 1:1 ratio to DNA) was then added directly to the diluted DNA. After gentle mixing and a 10 minute incubation at room temperature, 10 μL of Lipofectamine™ LTX was added into the diluted DNA solution, mixed gently and incubated for 35 minutes at room temperature to form DNA-Lipofectamine™ LTX complexes. The DNA-Lipofectamine™ LTX complexes were added dropwise to each well containing cells and mixed gently by manually rocking the plate back and forth. Cells were incubated at 37°C in a CO₂ incubator for 24 hours post-transfection before
assaying. For cells treated with glycine, glycine dissolved in RPMI (1 M) was added to the culture medium 4 hours post-transfection.

*Preparation of cells for FACS and quantitation of PPIX*—HeLa and K562 cells were washed, scraped and resuspended in PBS (80 mM disodium hydrogen orthophosphate, 20 mM sodium dihydrogen orthophosphate, 100 mM sodium chloride, pH 7.5) before pipetting into BD Falcon tubes with cell strainer caps. Preparation of cells for FACS was done under very low light conditions (1-2 Lux as measured by a Pyle PLMT68 light meter) in order to minimize phototoxicity caused by PPIX accumulation. FACS analyses were performed using a BD LSR II Analyzer (Becton, Dickinson, and Company) and FACSDiva Version 6.1.3 software. ZsGreen1 emission was measured between 515 nm and 545 nm (530/30BP filter) when cells were excited using the 488 nm laser. In order to eliminate any background red fluorescence, the 633 nm-red laser was blocked during the collection of the PPIX emission data. PPIX emission was determined in the 619 nm and 641 nm range (630/22BP filter) when cells were excited with the 405 nm laser. Forward-scatter (FSC) versus side-scatter (SSC) dot plots were used to gate the whole cells and thus remove the contribution of the cell debris from the population being examined. A minimum of 10,000 of the gated whole cells were then depicted in dot plots of SSC versus ZsGreen1 fluorescence, and the “green-fluorescent population” gate was defined based on untransfected HeLa cells as negative controls. Dot plots of SSC versus PPIX fluorescence were used to define the PPIX-accumulating cells for both the
“green-fluorescent” and the “non-green fluorescent” populations. The PPIX gating was based on the negative control for PPIX, the pIRES2-ZsGreen1 vector-expressing cells. Normalized PPIX fluorescence values were obtained by dividing the mean PPIX fluorescence by the mean ZsGreen1 fluorescence for each cell population.

Protein purification—Similarly to methods previously described (24), recombinant human ALAS2 and the XLEPP variants were purified from BL21(DE3) E. coli cells containing the overexpressed protein. BL21(DE3) cells harboring the expression plasmids for histidine-tagged hALAS2 were grown in two 125 mL flasks with 50 mL of LB medium containing 100 µg/mL ampicillin at 37°C for 8 hours. In each of eight 2 L flasks, 10 mL of bacterial culture was added to MOPS medium containing 100 µg/mL ampicillin for a total volume of 1 L per flask. The bacterial cultures were grown for 16 hours at 37°C. The cells were then harvested by centrifugation and stored at -20°C until the day of purification.

All purification procedures were carried out at 4°C as described previously (25) with minor changes. For proteins purified for activity assays, the cell pellet was resuspended in 30-40 mL of resuspension buffer (20 mM tricine, pH 8.0 containing 5 mM β-mercaptoethanol, 20 µM PLP, 10% glycerol, and 0.2% Triton X-100). The following protease inhibitors were also added in all buffers just before usage: aprotinin, leupeptin, and pepstatin at 1 µg/mL, and PMSF at 10 µg/mL. Following resuspension, the cells were homogenized and lysed by passing the cells twice through a French press at 10,000 psi. Cell debris was
removed by ultracentrifugation in a swinging bucket rotor at 104,000 \times g for 1 hour. The supernatant containing the protein was then loaded onto an affinity chromatography column (1.5 x 10cm) packed with HisPur™ Ni-NTA resin (Thermo Fisher Scientific Inc.) previously equilibrated with an “equilibration buffer” (20 mM tricine, pH 8, containing 20 µM PLP, and 10% glycerol). The resin was subsequently washed with 100 mL of buffer A (20 mM tricine, pH 8, containing 5 mM β-mercaptoethanol, 20 µM PLP, and 10% glycerol), 50 mL of buffer A, pH 8, with 0.5 M NaCl, 50 mL of a 25 mM imidazole buffer (i.e., 25 mM imidazole, pH 7.2, containing 5 mM β-mercaptoethanol, 20 µM PLP, and 10% glycerol), and finally 50 mL of a 50 mM imidazole buffer (i.e., 50 mM imidazole, pH 7.2, containing 5 mM β-mercaptoethanol, 20 µM PLP, and 10% glycerol). The protein (either hALAS2 or XLEPP variants) was eluted with 50 mL of a 100 mM imidazole buffer (i.e., 100 mM imidazole, pH 7.5, containing 5 mM β-mercaptoethanol, 20 µM PLP, and 10% glycerol). The eluted protein was immediately concentrated in protein concentrators with a 20,000 molecular weight cutoff (Thermo Scientific™ Pierce™). During concentration, the buffer was gradually exchanged to 20 mM tricine buffer, pH 8, containing 20 µM PLP, and 10% glycerol.

For the purification of proteins used in structural studies, glycerol and free PLP was eliminated from the buffers. Briefly, the cell pellet was resuspended in 30-40 mL of 20mM sodium phosphate, pH 8.0, containing 300 mM sodium chloride, 20 µM PLP, and 0.2% Triton X-100. The supernatant containing the protein was then loaded onto an affinity chromatography column (1.5 x 10cm)
packed with HisPur™ Ni-NTA resin previously equilibrated with a phosphate equilibration buffer (20 mM sodium phosphate, pH 7.4, 300 mM sodium chloride, and 10 mM imidazole). The resin was first washed with phosphate equilibration buffer, followed by washes with increasing imidazole concentrations of 25 mM and 50 mM. The protein was eluted with 50 mL of a 150 mM imidazole buffer (i.e., 20 mM sodium phosphate, pH 7.4, containing 300 mM sodium chloride, 150 mM imidazole). The eluted protein was immediately concentrated and gradually exchanged with 20 mM phosphate buffer, pH 8.

Purity of the protein was assessed by SDS-PAGE. Protein concentration was determined by the bicinchoninic acid (BCA) assay and bovine serum albumin as the standard. Protein concentrations are reported on the basis of subunit molecular masses of 57.3 kDa, 55.0 kDa, and 57.3 kDa, for the wild-type hALAS2, delAT, and delAGTG variants, respectively, calculated using the amino acid sequences and ProtPram (26).

*Spectrophotometric determination of ALAS activity*—ALAS steady-state activity was determined at 37°C using a continuous coupled enzyme assay on a Shimadzu UV2100 UV/Vis spectrophotometer as described previously for wild-type ALAS (27). The ALAS steady-state kinetic parameters of wild-type hALAS2 and delAT activities were also determined at 30°C.

*Thermostability Assays*—ALAS2 samples were placed in a thermocycler and heated to the desired temperature for 3 minutes, after which the sample was
cooled to 37°C and used in the ALAS activity assay as described above. All temperatures for each enzyme variant were performed in triplicate.

*Acrylamide Fluorescence Quenching*—Fluorescence emission spectra were collected on a Shimadzu RE-5301 PC spectrofluorophotometer using an excitation wavelength of 295 nm and protein concentrations of 2 μM in 20 mM phosphate buffer, pH 8.0. Blank fluorescence spectra were collected from samples containing all components except enzyme immediately prior to the measurement of samples containing enzyme and were subtracted from the spectra of samples containing enzyme. Acrylamide, made just prior to running the experiments as a 2 M stock, was added to yield final volumes in 100 μM increments, and the spectra were recorded after each addition. To account for a significant dilution of the enzyme concentration as acrylamide was added to a final concentration of 1 M, emission intensities were multiplied by the appropriate dilution factor. To obtain dynamic quenching constants for each ALAS2 variant, emission intensities at 330 nm were graphed against the acrylamide concentration in Stern-Volmer plots to linear Equation 3.1

\[
\frac{F_0}{F} = (k_q)(\tau_0)[Q] + 1 = (K_{SV})[Q] + 1
\]

in which \(F_0\) is the fluorescence intensity without quencher, \(F\) is the fluorescence intensity with quencher, \(k_q\) is the quencher rate coefficient, \(\tau_0\) is the lifetime of the emissive state of tryptophan without quencher present, \(Q\) is the concentration of
the quencher acrylamide, and $K_{SV}$ is the dynamic quenching constant of acrylamide for the tryptophans of hALAS2.

**Circular dichroism (CD) spectroscopy**—CD spectra were recorded using a JASCO J-815 spectrometer. For far-ultraviolet (UV) CD, the proteins were at 0.1 mg/mL in 20 mM sodium phosphate, pH 8.0 in a cuvette with a 1.0 mm path length. Spectra covered a 260-190 nm range and were collected at 25°C and a scan speed of 20 nm/minute with a 0.1 nm step size and 1.0 nm bandwidth. Four spectra were accumulated and averaged for each sample. For near UV and visible CD, the proteins were prepared at 1.0 mg/mL in 20mM sodium phosphate, pH 8.0, in a cuvette with a 1.0 cm path length. Spectra were recorded between 500-260 nm at 25°C at a scan speed of 20 nm/minute. Three spectra were accumulated and averaged for each sample.

**Results**

*Kinetic characterization of the XLEPP variants at 37°C*—Steady-state kinetic parameters were determined with respect to substrates glycine and succinyl-CoA (Table 3.2) and compared to the published parameters, which were acquired at 30°C for wild-type hALAS2 and the XLEPP variants (22). The catalytic constant ($k_{cat}$) value determined for the wild-type enzyme was 0.27 s$^{-1}$, and the $K_m$ values for glycine and succinyl-CoA were 12.6 mM and 6.1 µM, respectively. DelAT, which exhibited a $k_{cat}$ value 3-fold higher than that of wild-
type hALAS2 at 30°C (22), had a 3-fold \( k_{\text{cat}} \) value than the wild-type hALAS2 at 37°C, indicating that delAT has remarkably different thermodynamic properties and may be less stable than wild-type hALAS2. The \( k_{\text{cat}} \) values for delAGTG and Q548X were increased in comparison to the wild-type enzyme by 1.7-fold and 1.4-fold, respectively. DelAGTG exhibited increased affinities for both glycine and succinyl-CoA, while Q548X only had an increased affinity for succinyl-CoA. Of the XLEPP variants tested alongside wild-type hALAS2, delAGTG, with its extended C-terminus, generated the greatest effects on \( k_{\text{cat}} \) values and substrate affinities.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( k_{\text{cat}} ) (sec(^{-1}))</th>
<th>( K_m^{\text{CoA}} ) (µM)</th>
<th>( k_{\text{cat}} ) ( K_m^{\text{CoA}} ) (sec(^{-1})µM(^{-1}))</th>
<th>( K_m^{\text{Gly}} ) (mM)</th>
<th>( k_{\text{cat}} ) ( K_m^{\text{Gly}} ) (sec(^{-1})mM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.27 ± 0.02</td>
<td>6.1 ± 3.7</td>
<td>0.05 ± 0.03</td>
<td>12.6 ± 0.2</td>
<td>0.021 ± 0.002</td>
</tr>
<tr>
<td>DelAT</td>
<td>0.09 ± 0.01</td>
<td>2.5 ± 1.3</td>
<td>0.04 ± 0.02</td>
<td>18.0 ± 7.6</td>
<td>0.005 ± 0.003</td>
</tr>
<tr>
<td>DelAGTG</td>
<td>0.45 ± 0.05</td>
<td>3.6 ± 2.0</td>
<td>0.13 ± 0.08</td>
<td>4.2 ± 1.3</td>
<td>0.108 ± 0.045</td>
</tr>
<tr>
<td>Q548X</td>
<td>0.39 ± 0.04</td>
<td>4.2 ± 2.2</td>
<td>0.09 ± 0.05</td>
<td>22.0 ± 3.8</td>
<td>0.018 ± 0.005</td>
</tr>
</tbody>
</table>

**HeLa and K562 cells expressing XLEPP variants accumulate more PPIX than cells expressing wild-type hALAS2**—The extent to which the XLEPP variants might lead to PPIX accumulation was evaluated ex vivo in HeLa and K562 cells. In HeLa cells, in comparison to expression of wild-type hALAS2, delAT, delAGTG, and Q548X increased PPIX by 2.9-fold (\( p<0.05 \)), 3.6-fold
(p<0.01), and 3.7-fold (p<0.01), respectively (Figure 3.1). In K562 cells, delAT, delAGTG, and Q548X increased PPIX by 1.6-fold (p<0.05), 1.6-fold (p<0.05), and 2.1-fold (p<0.01), respectively. Expression of delAT, delAGTG, or Q548X resulted in significantly increased PPIX accumulation in mammalian cells in comparison to expression of wild-type hALAS2, indicating that the XLEPP variants are stable in the cellular environment.

Glycine supplementation of the culture medium only increases PPIX accumulation in HeLa cells expressing XLEPP variants with an increased affinity for glycine—Since the XLEPP variants have variable affinities for glycine (Table 3.2), we set out to examine if glycine has a similar effect in mammalian cells expressing XLEPP variants. In cells expressing wild-type hALAS2, supplementation of the media with 100 mM glycine increased the PPIX by 2-fold (p<0.05) and in cells expressing delAGTG, PPIX increased by 1.5-fold (p<0.01) (Figure 3.2). However, the effect of glycine supplementation on cells expressing delAT and Q548X did not reach statistical significance (p>0.05). These data corroborate the steady-state kinetic characterizations at 37°C that indicate that delAGTG, in contrast to delAT and Q548X, has an increased affinity for the glycine substrate.

The XLEPP variants are more thermostable than wild-type hALAS2—To better understand the relationship between kinetics and temperature on the
XLEPP variants, we placed the enzymes at a range of temperatures prior to testing catalytic activity. Interestingly, the variants, especially delAT, were more sensitive to slight temperature increases up to 45°C (Figure 3.3). However, at temperatures above 50°C, wild-type hALAS2 lost all measurable activity whereas the delAT and delAGTG variants retained 7% and 11% of their initial activity, respectively, at 70°C. The $K_{1/2}$ values, defined as the observed temperatures at which the enzymes lost 50% of their initial activity, were 48.6°C, 52.5°C, 49.9°C, and 51.4°C, for wild-type hALAS2, delAT, delAGTG, and Q548X, respectively.

**Figure 3.1. Expression of XLEPP variants in mammalian cells results in accumulation of PPIX.** HeLa cells are indicated by vertical-striped bars, while K562 cells are represented by checkered bars. The hALAS2 variants shown are wild-type enzyme (blue), delAT (purple), delAGTG (pink), and Q548X (green). Mean PPIX fluorescence values are representative of three separate experiments ± standard deviation (*p<0.05 and **p<0.01, Student’s t-test) [a.u., arbitrary units].
Figure 3.2. Glycine supplementation of the culture medium only increases PPIX accumulation in cells expressing XLEPP variants with an increased affinity for glycine. Plus (+) signs indicate samples in which the culture medium was supplemented with 100 mM glycine, and minus (-) signs indicate samples in which no glycine was added. The hALAS2 variants shown are wild-type hALAS2 (blue), delAT (purple), delAGTG (pink), and Q548X (green). Mean PPIX fluorescence values are representative of three separate experiments ± standard deviation (*p<0.05 and **p<0.01, Student's t-test) [a.u., arbitrary units].

The XLEPP variants undergo significant changes in secondary structure upon succinyl-CoA binding—Structural changes among wild-type hALAS2 and
the XLEPP variants were evaluated using CD spectroscopy from 260 nm to 190 nm. ALAS undergoes a conformational change upon binding of its substrates (28-31), and thus we examined the change in secondary structure of wild-type hALAS2 and the XLEPP variants upon addition of succinyl-CoA. The CD spectra for wild-type hALAS2 and the XLEPP variants indicated presence high degree of α-helical content, consistent with the crystal structure of *R. capsulatus* ALAS (32,33) (Figure 3.4). Binding of succinyl-CoA resulted in a very minor change in secondary structure for wild-type hALAS2 (Figure 3.4A), but caused more significant alterations in the secondary structure of the XLEPP variants, as noted by the increases in mean residue molar ellipticity at 210 nm and 222 nm (Figure 3.4B-D).

Figure 3.3. Thermostability profiles vary for hALAS2 and XLEPP variants. Data are averages of three independent experiments and are fit to the sigmoidal equation $y = a/(1 + e^{-(x-x_0)/b})$. The observed $K_{1/2}$ values were 48.6°C, 52.5°C, 49.9°C, and 51.4°C, for wild-type hALAS2 (blue), delAT (green), delAGTG (pink), and Q548X (purple), respectively.
Figure 3.4. Circular dichroism spectra of hALAS2 and the XLEPP variants in the far-UV nm region. (A) Wild-type hALAS2, (B) delAT, (C) delAGTG, (D) Q548X. The spectra for the holoenzymes are shown in each panel in solid lines, and the spectra for the enzymes in the presence of 50 μM succinyl-CoA are in dotted lines. The concentrations of enzyme were 0.1 mg/mL in 20 mM phosphate buffer, pH 8.0.

The tertiary structures and PLP-binding properties of the XLEPP variants are distinct from those of wild-type hALAS2—Possible structural differences between hALAS2 and the XLEPP variants were also analyzed by quantifying the concentration of acrylamide required to quench the intrinsic fluorescence of buried tryptophan residues in the enzymes (Figure 3.5). Dynamic quenching constants were calculated using Stern-Volmer plots of the resultant data, as
defined by Equation 1, for wild-type hALAS2, delAT, delAGTG, and Q548X of 1.42 M$^{-1}$, 5.29 M$^{-1}$, 1.92 M$^{-1}$, and 2.54 M$^{-1}$, respectively. The increased $K_{SV}$ values observed with the XLEPP variants indicated that the tryptophans of the variants, especially those of delAT, were more accessible to the aqueous solvent than those of wild-type hALAS2.

![Stern-Volmer plots displaying fluorescence quenching of buried tryptophan residues in hALAS2 variants.](image)

**Figure 3.5.** Stern-Volmer plots displaying fluorescence quenching of buried tryptophan residues in hALAS2 variants. Wild-type hALAS2, delAT, delAGTG, and Q548X are shown in blue, green, pink, and purple, respectively. In each case the enzyme concentration was 2 µM, and buffer was 20 mM phosphate, pH 8.0.

The differences in tryptophan fluorescence as observed by acrylamide fluorescence quenching were further investigated by using near-UV CD and CD in the visible light region, to analyze tertiary structure and the structural integrity of the PLP binding site, respectively. Each of the XLEPP variants had a distinct CD spectrum in the near UV region, indicating slight changes in the tertiary
structure as visualized by changes in mean residue molar ellipticity bands at wavelengths corresponding to aromatic residues (260-295 nm) (Figure 3.6A). In the visible region, intense bands at 330 nm, indicative of PLP bound in the active site of ALAS2 were observed with the delAGTG and Q548X XLEPP variants, (Figure 3.6B). Wild-type hALAS2 and all three XLEPP variants displayed intense molar ellipticity bands around 430 nm, which also signified PLP bound in the active site.

Figure 3.6. Circular dichroism spectra in the near-UV and visible region. (A) Wild-type hALAS2, (B) delAT, (C) delAGTG, (D) Q548X are shown in blue, green, pink, and purple, respectively. The concentrations of enzyme were 1.0 mg/mL in 20 mM phosphate buffer, pH 8.0.
Succinyl-CoA binding caused secondary structural changes in the XLEPP variants to a greater extent than wild-type hALAS2 (Figure 3.4). CD was used to further assess the effects of succinyl-CoA binding on both the tertiary structures and integrity of the PLP-binding sites of the purified enzymes. While the tertiary structures of wild-type hALAS2 and delAT were only slightly changed upon binding of succinyl-CoA (Figure 3.7A-B), delAGTG and Q548X displayed right-shifted 330 nm bands and changes in positioning of the aromatic amino acids (Figure 3.7C-D), suggesting these variants undergo significant changes in the positioning of PLP in the active site.

Figure 3.7. Circular dichroism spectra in the near-UV and visible region in the presence of 50 μM succinyl-CoA. (A) Wild-type hALAS2, (B) delAT, (C) delAGTG, (D) Q548X are shown in blue, green, pink, and purple, respectively. The concentrations of enzyme were 1.0 mg/mL in 20 mM phosphate buffer, pH 8.0.
Discussion

Multiple molecular mechanisms, including changes in enzyme kinetics, stability, rate of degradation, structure, and/or interaction with the regulatory β-subunit of the ATP-specific isoform of succinyl-CoA synthetase (SCS-βA) can contribute to the decreases in ALAS2 activity observed in XLSA, (16,34), and each of these mechanisms might also be operative in the gain-of-function mutations resulting in XLEPP. Previously we (22) and others (21,23) have demonstrated that purified recombinant XLEPP variants are more active than the wild-type hALAS2, at least at 30°C. In this study, we report that the XLEPP variants differ in their substrate affinities, thermostabilities, secondary structures upon substrate binding, tertiary structures, and PLP-binding site integrity.

Deleting the C-terminal 33 amino acids of hALAS2, which are conserved in higher eukaryotes but not present in prokaryotes, results in increased catalytic activity (21). Furthermore, when this variant is expressed in HEK293 human embryonic kidney cells, there is an increase in protein stability as shown by lack of degradation in comparison to wild-type hALAS2 (21). In addition to the truncated variant, two XLSA variants with point mutations near the C-terminus, V562A and M567I, have been cloned and reported to have altered activities and stabilities (21). Interestingly, the purified V562A enzyme exhibits higher catalytic activity, but has a shorter half-life in transfected cells, while in contrast, the purified M567I enzyme loses 75% of catalytic activity in comparison to wild-type hALAS2, but has an extended half-life when expressed in human embryonic
Figure 3.8. The citric acid cycle and heme synthesis. ALAS activity couples heme production to aerobic respiration via common use of succinyl-CoA. The common usage of succinyl-CoA as substrate implies close proximity of the 2 enzymes in vivo.

kidney (HEK293) cells. These results emphasize that the hALAS2 C-terminal extension is clearly significant in determining the activity and stability of the mammalian enzyme.
The C-terminus is also important for protein-protein interaction of hALAS2 with SCS-βA (16) (Figure 3.8). Wild-type hALAS2 and two XLSA variants, R411C and M426V, associate with SCS-βA, while another XLSA variant, D190V, loses its ability to interact with SCS-βA (34). Two XLSA variants with mutations near the C-terminus, M567V and S568G, and a C-terminal truncated variant (F557X) were reported to have normal or enhanced activity and stability, but none of them bound to SCS-βA, whereas wild-type hALAS2 bound strongly (16). Intriguingly, delAT also leads to a mutation at the same methionine residue, M567E, which is followed by a premature stop codon. Unlike the M567V variant, delAT still strongly binds SCS-βA (16). These data, along with studies on other variants with mutations at the C-terminus (16), imply that the interaction of ALAS2 and SCS-βA is essential for full activity in vivo, and that one region vital for this interaction lies between R559 and S568. In a cellular environment, the interaction of the XLEPP variants and SCS-βA is likely critical for their increased activity and resulting PPIX accumulation (Figures 3.1 and 3.2).

The $k_{\text{cat}}$ values for the XLEPP variants measured using purified recombinant enzyme, determined at 30°C, ranged from 3- to 3.4-fold greater than that of wild-type hALAS2 and the specificity constants toward succinyl-CoA ($k_{\text{cat}} K_{\text{m}}^{\text{SCoA}}$) were increased up to fourteen-fold (22). The “gain-of-function domain,” was previously concluded to extend over a minimum of 33 amino acids of ALAS2 between residues G544 and G576 (22). Here we determined the $k_{\text{cat}}$ values for the XLEPP variants at 37°C, as experiments with hyperactive mouse ALAS2 variants have shown that activation energies among variants can vary...
widely, and elevated activity at lower temperatures does not always equate to elevated activity at physiological temperature (35). The $k_{\text{cat}}$ value for wild-type hALAS2 exhibited a 9-fold increase in activity when the temperature increased from 30°C to 37°C, whereas the $k_{\text{cat}}$ value for delAT remained similar between 30°C (22) and 37°C (Table 3.2). The $k_{\text{cat}}$ value for delAGTG increased by 4-fold and for Q548X increased by 4.3-fold. It is possible that the interaction of ALAS2 with the SCS-βA provides stabilization for ALAS2 in vivo, which could explain why we found delAT to be less active in vitro at 37°C in the absence of SCS-βA, but more active when HeLa cells and K562 cells were transfected with an expression plasmid for delAT than wild-type hALAS2.

The purified XLEPP variants are more stable at temperatures above 55°C (Figure 3.3), indicating that the increased thermostabilities of the variants is independent of interaction with SCS and proteolytic susceptibility. These results led us to examine the secondary and tertiary structures of wild-type hALAS2 in relation to those of the XLEPP variants using CD and fluorescence spectroscopies. The far-UV spectra for hALAS2 and the XLEPP variants are indicative of a predominantly α-helical structure, and are similar among the variant and wild-type proteins (Figure 3.4). However, upon addition of succinyl-CoA, the XLEPP variants undergo a much larger structural rearrangement than the wild-type enzyme. These enhanced structural changes may explain the increased affinities of the XLEPP variants for succinyl-CoA.

CD spectral signals in the near-UV region arise from the aromatic amino acids, and the intensities of these signals are sensitive to the tertiary structure of
the protein (32). Generally, tryptophan shows a spectral band close to 290 nm, tyrosine a band between 275 and 282 nm, with a shoulder at longer wavelengths often obscured by bands due to tryptophan, and phenylalanine shows weaker but sharper bands between 255 and 270 nm (32). The actual shape and magnitude of the near-UV CD spectrum of a protein will depend on the number of each type of aromatic amino acid present, their mobility, the nature of their environment (H-bonding, polar groups and polarizability) and their spatial disposition in the protein (32). Notably, wild-type hALAS2 and each XLEPP variant differ in the number of specific aromatic amino acids. Wild-type hALAS2 has 5 tryptophans, 15 tyrosines, and 26 phenylalanines, whereas delAT is missing 1 tryptophan, 3 tyrosines, and 1 phenylalanine. DelAGTG lacks the same aromatic amino acids as delAT, but has a tryptophan in its extended C-terminus, and Q548X lacks an additional 2 phenylalanines in comparison to delAT (Table 3.1). Each variant has a distinct near-UV spectrum that differs from that of the wild-type enzyme not only in intensity, but also in shape (Figure 3.6). The divergent tertiary structures of the variants (Figures 3.5 and 3.6) might help explain why the variants are more active than wild-type hALAS2. For instance, if the $k_{\text{cat}}$ value for hALAS2 is dependent on the conformation adopted by the protein, as has been shown to be the case for mouse ALAS2 (2), a looser, more conformationally dynamic structure in the XLEPP variants could result in higher activity. However, in the absence of high-resolution 3-dimensional structures of wild-type hALAS2 and the XLEPP variants, it is difficult to postulate exactly what these structural changes are and precisely why they are associated with increased activity.
Enzyme cofactors that absorb in the visible region of the electromagnetic spectrum, such as PLP, show CD signals in the visible region only when bound to their protein partner in sites which confer chirality. These CD signals are thus excellent indicators of the integrity of the cofactor-binding site (32). PLP-dependent absorption maxima at 330 and 420 nm are considered to be characteristic of different ionization states of the internal aldimine bond between the PLP and the ALAS, corresponding to the unprotonated and protonated forms, respectively (36-39). The binding of delAGTG and Q548X to PLP results in intense spectral bands around 330 nm that are not evident in the spectra of the wild-type hALAS2 or delAT (Figure 3.7A-B). The spectra of delAGTG and Q548X also display slightly higher intensity spectral bands between 420-430 nm (Figure 3.7C-D). PLP thus appears to bind these variants more tightly in both ionization states, which is again consistent with the enhanced activity of these variants in vivo. Upon addition of succinyl-CoA, the 330 nm-band observed in the spectra of the delAGTG and Q548X variants is red-shifted to a broader band from 340-400 nm, indicating a substantial change in the chirality of PLP in the active site when succinyl-CoA is bound.

In conclusion, these studies represent the first structural evaluations of the XLEPP variants of hALAS2. We provide evidence that XLEPP can be modeled in cell culture, and this model may aid in testing for direct action of potential therapeutics on hALAS2. We also found that the XLEPP variants are more thermostable, undergo more extensive conformational changes upon the addition of succinyl-CoA, and differ in terms of tertiary structure and PLP binding. Future
studies should aim to solve the crystal structure of hALAS2 and to discover effective therapeutics for the treatment of XLEPP.

References


CHAPTER 4: ISONIAZID INHIBITS HUMAN ERYTHROID 5-AMINOLEVULINATE SYNTHASE

Abstract

Mutations in the C-terminus of human erythroid 5-aminolevulinate synthase (hALAS2) can result in two very different diseases, X-linked sideroblastic anemia (XLSA) and X-linked erythropoietic protoporphyria (XLEPP). XLSA patients have hALAS2 mutations that decrease activity, while mutations associated with XLEPP result in a gain-of-function of hALAS2. XLEPP has only recently been characterized, and thus there are no specific treatments. One potential treatment involves the use of the antibiotic isonicotinic acid hydrazide (isoniazid, INH), commonly used to treat tuberculosis, as INH can cause sideroblastic anemia as a side-effect. INH has traditionally been thought to cause anemia by limiting PLP availability to hALAS2 via direct inhibition of pyridoxal kinase, and reacting with pyridoxal to form pyridoxal isonicotinoyl hydrazone. We postulated that in addition to PLP-dependent inhibition of hALAS2, INH directly acts on hALAS2. Using FACS and confocal microscopy, we show here that INH reduces protoporphyrin IX accumulation in HeLa cells expressing either wild-type human hALAS2 or XLEPP variants. In addition, PLP and PMP restored cellular hALAS2 activity in the presence of INH. Kinetic analyses with purified hALAS2 demonstrated non-competitive or uncompetitive inhibition with an apparent $K_i$ of
1.5 µM. Circular dichroism studies revealed that INH triggers structural changes in hALAS2 that interfere with the association of hALAS2 with its PLP cofactor. These studies demonstrate that hALAS2 can be directly inhibited by INH, provide insight into the mechanism of inhibition, and support the prospective use of INH in treating patients with XLEPP and potentially other cutaneous porphyrias.

**Introduction**

5-Aminolevulinate synthase (ALAS; EC 2.3.1.37) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the first and rate-limiting reaction in heme biosynthesis, the condensation of glycine and succinyl-CoA to form 5-aminolevulinate (ALA), CoA, and CO₂ (1-3). Two genes, ALAS1 and ALAS2, encode the housekeeping and erythroid-specific ALAS isoforms, respectively (4,5). ALAS2 is expressed specifically in developing erythrocytes, where high levels of heme are required for hemoglobin production. Mutations in human ALAS2 (hALAS2) are associated with two different diseases, X-linked sideroblastic anemia (XLSA; MIM# 300751) (6,7) and X-linked erythropoietic protoporphyria (XLEPP; MIM# 300751) (8,9). XLSA patients have mutations in hALAS2 that result in decreased activity (7,10); approximately 50% of these patients are pyridoxine-responsive (10-12). Mutations associated with XLEPP have only been observed in exon 11, which encodes the C-terminus, and result in a gain-of-function of hALAS2 (9). Accordingly, a "gain-of-function" domain has been identified in the C-terminus of hALAS2 with a minimal length of 33 amino
acids, ranging from G544 to G576 (13). XLEPP patients experience extreme, painful photosensitivity due to porphyrin accumulation in the skin and, in some cases, abnormal liver function that can lead to liver failure (9). XLEPP has only recently been characterized, as patients had been misdiagnosed with erythropoietic protoporphyria (EPP), which is most commonly caused by mutations in ferrochelatase, the final enzyme of heme biosynthesis (9).

Interestingly, administration of the antibiotic isonicotinic acid hydrazide (isoniazid, INH), which has been widely used for the treatment of tuberculosis, induces sideroblastic anemia in some patients (6,14-16). INH has been proposed to cause sideroblastic anemia through two mechanisms: inhibition of pyridoxal kinase activity (17,18) or INH reaction with pyridoxal to form an inactive pyridoxal hydrazone (19,20). Both mechanisms result in reduced PLP levels, and thereby lower hALAS2 activity. In 1979, Konopka and Hoffbrand tested the effect of isoniazid on hALAS2 in erythrocyte homogenates from normal bone marrow and found that INH inhibited the hALAS2 activity dose-dependently (21). Additionally, they examined bone marrow from a patient with INH-induced sideroblastic anemia and found that hALAS2 activity of bone marrow could be restored to normal levels in vitro with supplementation of PLP (21). Based on these findings the investigators concluded that INH functioned as a pyridoxine antagonist, and thus indirectly inhibited hALAS2 (21).

INH is currently in a clinical trial enrolling EPP and XLEPP patients, (NCT ID# NCT01550705), with the rationale that limiting vitamin B6 cofactor may reduce hALAS2 activity, thereby decreasing the amount of PPIX accumulation, but
the effect of INH on purified hALAS2 has not been reported. Since there are at least 56 different human genes known to encode PLP-dependent enzymes (22), and yet hALAS2 can apparently be selectively inhibited during INH treatment for tuberculosis, we hypothesized that INH might directly bind to and inhibit hALAS2. This hypothesis supports INH as a very logical treatment for cutaneous porphyrias like EPP and XLEPP, caused by disruption of heme biosynthesis and subsequent porphyrin accumulation.

Here we report that the results of cell culture studies support previous conclusions on the effect of INH on hALAS2 activity and the ability of PLP to reverse INH inhibition of hALAS2. Our studies using purified recombinant hALAS2 showed that INH can alter the tertiary structure of hALAS2, decrease the binding of PLP to hALAS2, and directly inhibit hALAS2 activity. We provide insight into its direct mechanism of inhibition and offer further evidence for using INH to treat patients with XLEPP and other cutaneous porphyrias.

**Materials**

*Reagents*—Aprotinin, pepstatin, leupeptin, PMSF, ampicillin, β-mercaptoethanol, pyridoxal 5'-phosphate, succinyl-CoA, thiamine pyrophosphate, and NAD⁺ were obtained from Sigma-Aldrich Chemical Company. Glucose, glycerol, acetic acid, methanol, glycine, disodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, DMSO, EDTA, MOPS, tryptone, yeast extract, sodium chloride, tricine, ammonium sulfate, and potassium hydroxide were purchased from Fisher Scientific. Centricon concentrators were from
Millipore. Sodium dodecyl sulfate polyacrylamide gel electrophoresis reagents and Phusion DNA Polymerase were acquired from Thermo Scientific. 5-Aminolevulinic acid hydrochloride, isonicotinic acid hydrazide, and pyrazinamide were purchased from Acros Organics. 1, 1-Dimethylhydrazine was purchased from SPEX Certiprep group. BmtI, Sall, BlpI, and BamHI restriction enzymes were obtained from New England BioLabs, Inc. Gentamicin sulfate and RPMI-1640 culture medium were procured from Mediatech, Inc. Oligonucleotides were synthesized by Integrated DNA Technologies. T4 DNA ligase and ligase buffer were obtained from Thermo Scientific Fermentas and a bicinchoninic acid protein determination kit was purchased from Thermo Scientific Pierce.

Methods

Plasmids for mammalian expression and protein purification—For expression plasmids used in the transfection of mammalian cells, the wild-type precursor ALAS2 cDNA (GenBank: X56352.1) was subcloned into the multiple cloning site of the pIRES2-ZsGreen1 vector (purchased from Clontech Laboratories, Inc. Mountain View, CA) using the BmtI and BamHI restriction sites as described previously (Chapter 3, pages 109-112). The resulting plasmid was named pEF27. For the mammalian expression plasmids encoding the precursor XLEPP variants, the cDNAs encoding the mutated hALAS2 C-termini were subcloned into pEF27 and the resulting plasmids were named pEF28 and pEF29, encoding delAT and delAGTG, respectively, as described previously (Chapter 3, pages 110-112). To purify hALAS2 and the XLEPP variant proteins
using nickel affinity chromatography, we added six histidine codons to the cDNA sequence encoding the mature N-terminus of hALAS2, and subcloned the digested PCR products into plasmids expressing wild-type hALAS2 and delAGTG, as described previously (Chapter 3, pages 111-112). The resulting plasmids, pEF40 and pEF42, were used for the expression of histidinetagged wild-type hALAS2 and delAGTG, respectively.

**Cell culture**—Human cervical carcinoma (HeLa) cells were maintained in DMEM with 4.5 g/L glucose, L-glutamine, and sodium pyruvate (Mediatech, Inc.), with 10% fetal bovine serum (FBS) (Thermo Scientific™ HyClone™), gentamicin (Mediatech, Inc.; 50 μg/mL), penicillin (Mediatech, Inc.; 60 μg/mL) and streptomycin (Mediatech, Inc.; 100 μg/mL) at 37°C under 5% CO₂ in a humidified incubator.

**Transient transfection of HeLa cells**—HeLa cells were transfected using DNA-Lipofectamine™ LTX transfection reagent as previously described (Chapter 3, page 112). For cells treated with INH, INH dissolved in DMSO (10 mg/mL) was added to the culture medium 24 hours post-transfection to allow for protoporphyrin IX (PPIX) accumulation caused by hALAS2 expression, as described with murine ALAS2 (E. J. Fratz, G. A. Hunter, and G. C. Ferreira, unpublished observations). As a control, DMSO (10 mg/mL) was added to non-transfected and transfected cells in culture medium at the same time point (*i.e.*, 24 h). Pyridoxine-HCl, PLP, and pyridoxamine-5′-phosphate (PMP) were dissolved in DMEM (10 mg/mL) and added to the culture medium 24 hours post-
transfection. The final pyridoxine, PLP and PMP concentrations were each 100 μg/mL in all experiments.

*Preparation of cells for FACS and quantitation of PPIX*—HeLa cells were prepared for FACS and PPIX was quantitated as previously described (Chapter 3, pages 114-115).

*Confocal fluorescence microscopy*—HeLa cells were grown in Thermoscientific™ Nunc™ Labtek™ sterile 4-well chambered coverglass until 50% confluent, and then transfected as described above. Twenty-four hours later, the culture medium was supplemented with INH to yield final concentrations of 100 μg/mL or 500 μg/mL. After an additional twenty-four hours, and immediately before obtaining confocal fluorescence microscopy images of the cells, the medium was removed from the wells and the cells were washed with PBS three times. Live cell imaging was performed using a 3i-Olympus spinning disk confocal microscope operated by Slidebook 5 software and equipped with a Photometrics Evolve EMCCD camera. The filter block used consisted of a 350/50 nm excitation filter, a BS400 beamsplitter, and a 630/75 nm emission filter.

*Protein purification*—Recombinant human ALAS2 and the XLEPP variants were purified from BL21(DE3) *E. coli* cells containing the overexpressed protein as described previously (Chapter 3, pages 115-117). Purity of the protein was
assessed by SDS-PAGE and protein concentration was determined by the bicinchoninic acid (BCA) assay and bovine serum albumin as the standard as described previously (Chapter 3, page 117).

**ALAS colorimetric activity assay**—ALAS activity was determined according to the method of Lien and Beattie (23) with minor modifications. Prior to the activity assay, purified recombinant wild-type hALAS2 in 25 mM HEPES, pH 7.2, containing 10% glycerol, was incubated with INH (0-1 mM), for 15 minutes at 37°C. After the addition of the succinyl-CoA and glycine substrates, the reactions proceeded, for 10 minutes at 37°C. The final concentrations of ALAS, succinyl-CoA and glycine were 1.5 µM, 100 µM and 100 mM for each assay. For the PLP-dependence assays, INH was first incubated with PLP for 15 minutes at 37°C and then the purified recombinant wild-type hALAS2, which is PLP-bound, was added for a 15 minute incubation at 37°C before beginning the ALAS activity assay as described above. The concentrations of added PLP ranged from 0 µM to 40 µM. For the glycine competition assays, ALAS2 activity was determined using glycine concentrations ranging from 10 mM to 150 mM, and INH concentrations from 0 µM to 100 µM. Succinyl-CoA and hALAS2 concentration were held constant at 100 µM and 1.5 µM, respectively. To determine the apparent $V_{\text{max}}$ and $K_m$ values for glycine, and the apparent $K_i$ for INH, the data were first fit using Sigmaplot graphing software to Equation 4.1 (24),
where $y$ is the activity as measured by rate of ALA formation, $x$ is either the concentration of glycine or INH, and $K_x$ is either the apparent $K_i$ for INH or $K_m$ for glycine. From these primary plots, the apparent maximal velocities were in turn used to construct secondary plots defining $V_{\text{max}}$ and $K_i$ values. In addition, the glycine competition data were independently analyzed using Dynafit model discrimination software (25,26) to compare competitive, mixed, and partial inhibitory mechanisms. For illustration purposes, glycine competition data were evaluated using a Lineweaver-Burke plot to display the nature of the inhibition. Also for illustration purposes, the raw data were fit using Sigmaplot graphing software to a sigmoidal Equation 4.2

\[
y = \frac{1}{2} \left( I_{\text{max}}^{-1} \times \sqrt{((x + 1 + K_x) - (x + 1 + K_x)^2 - 4x)} \right) \tag{4.1}
\]

\[
y = \frac{1}{2} \left( I_{\text{max}}^{-1} \times \sqrt{((x + 1 + K_x) - (x + 1 + K_x)^2 - 4x)} \right) \tag{4.1}
\]

where $y$ is the percentage of ALAS activity loss and $x$ is log of the INH concentration.

Circular dichroism (CD) spectroscopy—CD spectra were recorded using a JASCO J-815 spectrometer as described previously (Chapter 3, page 119).

Results
INH reduces PPIX accumulation in HeLa cells expressing wild-type hALAS2 and XLEPP variants—To test the ability of INH to reduce ALAS2 activity and PPIX production in cell culture, we transfected HeLa cells with expression plasmids encoding wild-type hALAS2 (WT) and two XLEPP variants, delAT and delAGTG, which have increased ALAS activity. At a concentration of 5 µg/mL, INH did not cause a significant decrease in PPIX fluorescence, and thus PPIX accumulation, in cells expressing WT, delAT, or delAGTG (Figure 4.1). An INH concentration of 50 µg/mL was sufficient to decrease PPIX levels in delAGTG-expressing cells (p<0.05), and INH concentrations of 100 µg/mL and 200 µg/mL...
further decreased the amount of PPIX accumulated by 36% and 53%, respectively (p<0.01). For WT-expressing cells, INH concentrations of 100 µg/mL and 200 µg/mL resulted in decreased PPIX accumulation (p<0.01), and delAT-expressing cells required an INH concentration of 200 µg/mL to reduce PPIX production and accumulation (p<0.05).

**INH reduces PPIX accumulation in both HeLa cells expressing delAGTG and those adjacent to delAGTG-expressing HeLa cells**—To examine the PPIX accumulation and the effect of the INH treatment in individual cells, we used confocal fluorescence microscopy to analyze ZsGreen1 fluorescence and PPIX fluorescence. We chose to monitor delAGTG expression because it resulted in the highest PPIX accumulation and largest percent decrease in PPIX when treated with INH (Figure 4.1). HeLa cells that were not transfected (Figure 4.2A) and HeLa cells that were transfected with the pIRES2-ZsGreen1 vector plasmids (Figure 4.2B) were used as controls for no PPIX fluorescence and ZsGreen1 fluorescence, respectively. In delAGTG-expressing cells, PPIX fluorescence was evident in nearly all cells, even though only approximately 25% of cells expressed ZsGreen1 and delAGTG (Figure 4.2C). When delAGTG-expressing cells were treated with either 100 or 500 µg/mL INH, PPIX was visibly decreased in all cells (Figure 4.2D and 4.2E).
INH inhibition of ALAS2 activity is not affected by pyridoxine—Since the two described mechanisms of INH inhibition of ALAS2 are PLP-dependent, and oral pyridoxine can be effective in treating sideroblastic anemia acquired from

![Figure 4.2](image)

**Figure 4.2.** INH reduces PPIX accumulation in both HeLa cells expressing delAGTG and those adjacent to delAGTG-expressing HeLa cells. Rows 1-4 of each panel correspond to the images of 1. superimposed bright-field and fluorescence, 2. green fluorescence 3. red fluorescence, and 4. overlaid red and green fluorescence, respectively. (A) Non-transfected HeLa cells. (B) HeLa cells transfected with pIRES2-ZsGreen1. (C) HeLa cells transfected with pEF29, which encodes both ZsGreen1 and delAGTG. (D) HeLa cells transfected with pEF29, which encodes both ZsGreen1 and delAGTG, treated with INH (100 μg/mL) for 24-hours. (E) HeLa cells transfected with pEF29, which encodes both ZsGreen1 and delAGTG, treated with INH (500 μg/mL) for 24-hours. “Control” refers to non-transfected HeLa cells.
prolonged INH treatment, we postulated that pyridoxine supplementation of the cell culture medium could reverse the INH inhibition of ALAS2. Pyridoxine (100 μg/mL) did not affect the PPIX levels in WT-, delAT-, or delAGTG-expressing cells (p>0.1) (Figure 4.3). In cells treated with INH (100 μg/mL), pyridoxine also did not exhibit any effects on PPIX fluorescence (p>0.1), indicating that the addition of pyridoxine is not sufficient to overcome the INH effect on ALAS2 activity.

FIGURE 4.3. INH inhibition of PPIX accumulation in HeLa cells expressing either wild-type hALAS2 or XLEPP variants is not affected by pyridoxine. Mean PPIX fluorescence values are representative of three separate experiments ± standard deviation [a.u., arbitrary units].
PLP and PMP reduce the effect of INH on ALAS2 inhibition—We next examined whether supplementation of the cell culture medium with PLP or PMP could reverse the INH inhibition of ALAS2 activity. In contrast to pyridoxine, both PLP and PMP (100 µg/mL) restored ALAS2 activity of INH-inhibited delAT and delAGTG-expressing HeLa cells (p<0.05) (Figure 4.4). A similar trend was seen in WT-expressing cells, but the changes did not reach statistical significance (p>0.05).

**FIGURE 4.4.** Either PLP or PMP supplementation of the culture medium of HeLa cells expressing wild-type hALAS2 or XLEPP variants reduces the inhibitory effect of INH on PPIX accumulation. Mean PPIX fluorescence values are representative of three separate experiments ± standard deviation (*p<0.05, Student’s t-test) [a.u., arbitrary units].
INH inhibits purified hALAS2—Since our findings with hALAS2- and XLEPP-expressing mammalian cells supported the proposition that inhibition of hALAS2 activity by INH involves PLP depletion, we set out to determine if INH could directly inhibit purified hALAS2, affect the availability of PLP, or if INH worked through a combination of both mechanisms. In the presence of excess of glycine (100 mM) and succinyl-CoA (100 µM) concentrations (i.e., over 10-fold the $K_m$ values of the two substrates (13,27)), INH inhibited hALAS2 activity (Figure 4.5A), and similarly, INH inhibited the ALAS activity of delAGTG (data not shown). To determine if the depletion of PLP and consequent inhibition of the purified hALAS2 resulted from the reaction between INH and the PLP cofactor or a decrease in available PLP for binding to hALAS2 and formation of the active holoenzyme, we included an additional step involving the incubation of INH with PLP prior to the assay reaction. The greater $K_i^{aPP}$ values for INH (from 2.7 to 10.6 µM) with increased PLP concentrations (Figure 4.5B) indicated that a portion of the INH reacts with PLP in such a way that INH can no longer inhibit hALAS2. At concentrations of INH of 10 µM and below, preincubation of INH with PLP reduced hALAS2 inhibition; however, 100 µM INH caused nearly 100% activity loss regardless of the concentration of PLP. In addition to these experiments in which INH was incubated with PLP prior to mixing with hALAS2, similar experiments were run in which hALAS2 (1.5 µM) was incubated with INH (0-100 µM) prior to mixing with PLP (0-40 µM) (data not shown). Regardless of the concentration of PLP added to the hALAS2-INH, the apparent $V_{max}$ was
unchanged, indicating that once INH is bound to hALAS2, PLP cannot reverse inhibition.

INH is not a competitive inhibitor for glycine—we next investigated whether INH competed with the glycine substrate for binding to the active site of hALAS2 (Figure 4.6A-B). INH decreased the $V_{\text{max}}$ for glycine and increased the

$K_m$ for glycine (Figure 4.6A). The decrease in the $V_{\text{max}}$ for glycine with increased INH concentration indicates that INH does not compete with glycine to bind to hALAS2. From the secondary plot of these data fit to Equation 1, we determined

FIGURE 4.5. INH inhibits the activity of purified hALAS2. A. Percent activity loss values are representative of three separate experiments ± standard deviation. Data were fit to Equation 2. B. INH inhibition of hALAS2 occurs in part independently of PLP availability. Enzymatic activity was determined over a range of INH concentrations (1 – 100 µM), at constant hALAS2 concentration (1.5 µM), and at constant PLP concentrations. Each data point represents the percentage activity loss either in the absence (blue) or in the presence of different added concentrations of PLP: 5 µM (green), 10µM (pink), 20 µM (cyan) and 40 µM (black). The corresponding colored curves are the best fits of the data to Equation 1, and the the respective estimated $K_i$ values for INH are 2.7 µM, 5.1 µM, 7.5 µM, 9.7 µM and 10.6 µM. Activity loss values are averages of three separate experiments.
the apparent inhibitory constant ($K_i^{\text{app}}$) to be 1.5 µM. Dynafit analysis of the kinetic data for INH inhibition excluded competitive and mixed inhibition, and supported partial inhibition of the type in Scheme 1,

$$E + S \xrightleftharpoons[K_{i}]{k_\text{cat}} ES \xrightarrow{k_\text{cat}'} E + P$$

where $E$ represents ALAS, $S$ is the substrate glycine, $P$ is the product, and $I$ is INH. The fitted parameters were 4.5 mM for the dissociation constant of glycine, 2.0 s$^{-1}$ for $k_\text{cat}$, 0.4 s$^{-1}$ for $k'_\text{cat}$, and 1.2 µM for the $K_i^{\text{app}}$ of INH.

We also performed similar experiments to assess competition for succinyl-CoA binding, but the enzyme concentration was close to that of the $K_m$ for succinyl-CoA (4.3 µM) (13), and the colorimetric assay was not sensitive enough to attain reliable signal to noise ratio at succinyl-CoA concentrations below 10 µM. Thus, there was a high error associated with the $K_i^{\text{app}}$ calculated from the secondary plot of these data. While the apparent $K_m$ for each concentration of INH could not be determined from the primary plot due to high error at succinyl-CoA concentrations less than 10 µM, a trend of decrease in the $V_{\text{max}}$ for succinyl-CoA as a result of increased INH concentrations was apparent (data not shown).
Figure 4.6. INH does not compete with glycine to bind hALAS2. A. INH concentrations are as follows: 0 µM (black), 2 µM (yellow), 5 µM (green), 10 µM (pink), and 50 µM (blue). Data were fit to Equation 4.1. B. Lineweaver-Burke reciprocal plot constructed using the data from primary plots. Activity values are averages of three separate experiments.

Taken together, these findings indicate that INH inhibits hALAS2 in a dose-dependent manner and does not directly compete for binding with either the glycine or succinyl-CoA substrates.

The hydrazine moiety of INH is necessary, but not sufficient for complete ALAS2 inhibition—Previously, using UV-visible absorption spectroscopy, we determined that the addition of INH (100 µM) to the PLP-glycine aldimine (100 µM PLP and 10 mM glycine) disrupted the Schiff base linkage as monitored by a decrease in absorbance at 420 nm (data not shown). Thus, we posited whether the reactive hydrazine moiety of INH would be sufficient to inhibit hALAS2. Pyrazinamide, an antibiotic also used to treat tuberculosis, has a similar size and structure to INH, but has an amide group on a pyrazine ring instead of a hydrazine group on a pyridine ring (Figure 4.7A, inset). To determine whether the
The hydrazine moiety of INH was the major contributor to the inhibition of hALAS2 by INH, we compared the hALAS2 activity in the presence of INH, pyrazinamide, or 1,1-dimethylhydrazine (Figure 4.7B, inset). Of significance, patients treated with pyrazinamide can also present anemia, which led us to hypothesize that hALAS2 might also be inhibited by this antibiotic (13,28). We found that while both pyrazinamide (Figure 4.7A) and 1,1-dimethylhydrazine (Figure 4.7B) could reduce hALAS2 activity, neither could inhibit hALAS2 to the same level as INH (Figure 4.7A-B) suggesting that both the hydrazine moiety and structure of INH are important for complete inhibition of hALAS2.

**INH induces changes in the tertiary structure and PLP-binding to ALAS2—**

Our activity assay data supported the hypothesis that INH can directly inhibit hALAS2, but it was still unclear where and how INH binds to hALAS2. Since INH was not a competitive inhibitor for glycine or succinyl-CoA, we hypothesized that INH binds at an alternate site which results in a structural rearrangement that lead to a diminishment in hALAS2 activity. Thus, we next examined the secondary and tertiary structural changes induced in hALAS2 upon binding of INH. CD spectroscopy revealed that while INH did not induce any major changes in secondary structure (data not shown). INH altered the tertiary structure of hALAS2 in a concentration-dependent manner, as visualized by changes in mean residue molar ellipticity bands at wavelengths corresponding to aromatic residues (260-295 nm) (Figure 4.8A). Specifically, INH decreases the tyrosine and tryptophan signals, which show bands between 275 and 282 nm and at 290
nm, respectively (29). INH also affected the visible region of the CD spectra; INH decreased the mean residue molar ellipticity band between 420-440 nm, which indicated that the orientation and positioning of PLP in the active site of hALAS2 was disrupted by INH (13,30) (Figure 4.9B). The decrease in mean residue molar ellipticity exhibited an INH concentration dependence described by a hyperbolic curve and a value of 53 μM for the INH concentration at which the mean residue molar ellipticity was one half of its maximal value. Similar to the activity of hALAS2, the structural changes induced by INH were time-dependent. Because we accumulated and averaged either 3 or 4 CD scans per variant, the structural changes within the first 30 minutes could not be distinguished. There were,
Figure 4.7. Pyrazinamide and 1,1-dimethylhydrazine do not inhibit hALAS2. Wild-type hALAS2 was incubated with INH (black), (A) pyrazinamide (pink), or (B) 1,1-dimethylhydrazine (blue) ranging from 0 to 100 µM before measuring ALAS activity. The structures for INH, pyrazinamide, and 1,1-dimethylhydrazine are provided as insets in each graph. Percent activity loss values are representative of three separate experiments ± standard deviation.

however, slight differences between the spectra of hALAS2 obtained immediately after INH addition and spectra of hALAS2 incubated with INH for 24 hours (data not shown), that indicated a time-dependence in the structural changes.
The CD spectra for the delAGTG variant (Figure 4.9) differed from those for wild-type hALAS2 (Figure 4.8A) in both the near UV and visible regions, with the most obvious difference being the presence of a mean residue molar ellipticity band at 330 nm for delAGTG (not present in the wild-type spectrum), often associated with PLP bound to its protein partner in a site which confers chirality (13,29). Similarly to wild-type hALAS2, INH altered the tertiary structure of the delAGTG variant in a concentration-dependent manner (Figure 4.9A). INH also affected the visible region of the CD spectra dose-dependently, as noted by decreased mean residue molar ellipticity at the wavelengths associated with PLP bound in the active site of hALAS2 (330nm and 420nm) (Figure 4.9B).

Figure 4.8. INH induces changes in the tertiary structure of ALAS2 and binding of the PLP cofactor. (A) CD spectra in the near UV and visible region for wild-type hALAS2 prior to reaction with INH (cyan) and after incubation with increasing concentrations of INH up to 100 µM (green). (B) The decrease in mean residue molar ellipticity at 420 nm plotted against the concentration of INH. Data were fit to Equation 4.1.
FIGURE 4.9. INH induces changes in the tertiary structure of delAGTG and binding of the PLP cofactor. CD spectra in the near UV and visible region for delAGTG prior to INH addition (—), and upon the addition of 60 μM INH (−−−) and 100 μM INH (····).

Discussion

Isoniazid has a long history of being used to treat tuberculosis infections, dating back to at least 1952 (31). Case studies reporting anemia as a common side effect were reported as early as 1962, and continued as doctors began expanding the use of isoniazid to treat tuberculosis patients (16,32-34). In some cases, patients recover from the acquired anemia when taken off of isoniazid, while others also require dietary pyridoxine supplementation to fully recover (16,21,34-36). In general, pyridoxine supplements are given to patients taking isoniazid in order to prevent onset of anemia (37). In addition to case studies in tuberculosis patients, the effects of INH on erythropoiesis have been
characterized in mice; when INH was administered during induced erythropoiesis in mice, heme synthesis and erythroid maturation were inhibited (38).

INH is well-studied in patients treated for tuberculosis (14,39,40), and, in *Mycobacterium tuberculosis*, INH is known to be activated by the katG enzyme and subsequently to target enzymes required for mycolic acid synthesis (41-47). However, the mechanism by which INH causes cell death remains elusive in *Mycobacterium tuberculosis* (42), and metabolism of INH in human cells is not well-investigated. Thus, in our studies, we explored the effects of INH in human cell lines, focusing on elucidating the mechanisms of INH-induced anemia. Our studies using mammalian cell cultures indicate that INH decreases PPIX accumulation in HeLa cells overexpressing hALAS2 (Figure 4.1), thus decreasing PPIX transported into surrounding cells (Figure 4.2), and demonstrate that INH is effective in reducing hALAS2 activity *ex vivo*. While pyridoxine did not lessen the effect of INH on PPIX levels (Figure 4.3), addition of either PLP or PMP completely reversed the effects of INH (Figure 4.4).

The results of these initial experiments might be interpreted by using the known PLP-dependent mechanisms of hALAS2 inhibition by INH, in which INH inhibits pyridoxal kinase (17,18), the enzyme that produces PLP, and also reacts with PLP to form pyridoxal isonicotinoyl hydrazones (19,20). However, it was still unclear if INH had a more direct effect on hALAS2 activity that would better explain the specific side effect of sideroblastic anemia. In order to reduce the number of variables, we began *in vitro* experiments in which we could more stringently control the experimental conditions. In doing so, we found that INH
inhibits purified hALAS2 regardless of the presence of excess PLP (Figure 4.5B). Excess PLP in the reaction buffer did affect the apparent $K_i$ values for INH, indicating that a small percentage of the INH might have reacted with PLP to form pyridoxal-hydrazones (20), thus reducing the amount of INH available to inhibit hALAS2. However, 100 µM INH caused nearly 100% activity loss regardless of the concentration of PLP (Figure 4.5B), suggesting a PLP-independent mechanism of hALAS2 inhibition by INH. We subsequently found that INH is not a competitive inhibitor for either the glycine or succinyl-CoA substrates, as it decreases the $V_{max}$ and increases the $K_m$ values for glycine, and decreases the $V_{max}$ for succinyl-CoA. These data suggest that INH binds to an alternate site distinct from the glycine-binding site and decreases the affinity of hALAS2 for glycine.

Other antibiotic drugs commonly prescribed to treat tuberculosis, such as pyrazinamide, chloramphenicol, and cycloserine, have also been associated with drug-induced sideroblastic anemia (6,48). However, it has been noted that some patients treated with a cocktail of antibiotics for tuberculosis can recover from sideroblastic anemia by withdrawal of only INH from the cocktail (14). Since the ability of pyrazinamide to induce sideroblastic anemia in patients is unresolved (14,28,48), we examined whether pyrazinamide similarly affected hALAS2 activity. Pyrazinamide did not inhibit hALAS2 activity at concentrations below 30 µM, whereas 30 µM INH resulted in greater than 80% inhibition of hALAS2 activity (Figure 4.7). Structural comparison of INH and pyrazinamide suggested that the hydrazine moiety of INH might be important for the inhibition of hALAS2.
In order to ascertain if hydrazine was sufficient to react with and inhibit hALAS2, we used 1,1-dimethylhydrazine in our activity assay. At concentrations below 10 µM, 1,1-dimethylhydrazine resulted in modest activity loss, but even at concentrations up to 500 µM, activity loss never reached 50%. Thus, both the hydrazine moiety and the linkage of the hydrazine moiety on the pyridine ring of INH appear crucial for complete inhibition of hALAS2.

The chemical mechanism of ALAS2 begins, in the absence of substrates, with active site lysine 313 of ALAS2 bound to PLP forming an internal aldimine (Figure 4.10). In the presence of substrates, the glycine binds first, followed by transaldimination with the active site lysine to yield an external aldimine (49). Next, the pro-R proton of glycine is abstracted by the active site lysine, followed by condensation with succinyl-CoA and CoA release to generate an α-amino-β-ketoadipate intermediate. This step is followed by decarboxylation resulting in an enol-quinonoid rapid equilibrium, then protonation of the enol to give an aldimine-bound molecule of ALA, and ultimately release of the product (ALA) (49). Despite the complexity of the proposed chemical mechanism, transient kinetic studies have led investigators to propose that the rate-determining step of the ALAS-catalyzed reaction is product release or a conformational change leading to product release (49,50). It is the movement of the active site loop (51) towards the open conformation that controls the rate at which ALA dissociates, and therefore the overall catalytic rate (30,49,50,52,53). Since INH does not compete with the substrates, one possibility is that binding of INH stabilizes the closed conformation of hALAS2. INH could bind both the hALAS2 holoenzyme and

glycine-bound hALAS2, which is consistent with the Dynafit prediction of partial inhibition (Scheme 1).

To better resolve the mechanism of inhibition, we examined the possibility of secondary and tertiary structural changes associated with binding of INH to hALAS2. We could not delineate significant secondary structural changes, but the tertiary structure exhibited a distinct, concentration-dependent change upon
incubation with INH. The changes in fluorescence of the aromatic residues of hALAS2 as seen in these CD spectra (Figure 4.8A) indicate that INH elicits a protein conformational change that renders the enzyme either less active or inactive. CD spectroscopy also revealed a concentration-dependent decrease in the mean residue molar ellipticity band between 420-440 nm, indicating that INH disrupts the hALAS2-PLP bond, apparently yielding an enzyme devoid of the Schiff base linkage (Figure 4.8A-B). Decreased PLP-binding of ALAS2 has been noted previously with mouse ALAS2 when serine 254 was mutated to an alanine residue (30). This active site mutation causes a change in the microenvironment of the PLP cofactor and disrupts the binding of succinyl-CoA (30). We also observed changes in tertiary structure and PLP-binding with the XLEPP variant delAGTG (Figure 4.9). One possibility is that INH causes a structural change that inhibits PLP binding, following either direct displacement or remote binding. This could explain why excess PLP cannot completely reverse activity loss caused by INH in vitro. In the cellular environment, ALAS2 is peripherally associated with the mitochondrial membrane and is known to bind at least one other enzyme partner, succinyl-CoA synthetase (12,54). INH might not bind as tightly or displace PLP as effectively when ALAS2 is membrane- and/or protein-bound, which might explain why PLP can reverse the INH inhibition ex vivo (21).

Here we provide evidence that INH directly inhibits purified hALAS2. Our data suggest that INH is an inhibitor that affects the functionality of the PLP cofactor. We propose a mechanism by which addition of INH to hALAS2 results in structural rearrangement of the protein and disruption in the hALAS2-PLP
linkage, thus inhibiting hALAS2 activity. To determine the position and location at which INH binds to hALAS2, further studies including x-ray crystallization of hALAS2 with INH are warranted. These data provide support for using INH to treat cutaneous porphyrias, especially for XLEPP, where hALAS2 is overly active. The direct effect of INH on hALAS2 activity should also be considered when treating tuberculosis patients who have developed sideroblastic anemia.

References


CHAPTER 5: SUMMARY AND CONCLUSION

This dissertation focuses on mutations in ALAS2 that lead to an increased activity of the enzyme. ALAS2 can be used to produce the heme precursor PPIX, which is used as a photosensitizer for PDT. Thus mutations in ALAS2 that increase its activity offer a means to increase PPIX photosensitization. We explored mutations located in the presequence known to alleviate the heme feedback inhibition of ALAS2 translocation into the mitochondrion (1) and mutations in the active site loop region known to increase ALAS2 activity (2,3), and assessed the ability of the ALAS2 variants to accumulate PPIX in mammalian cells and cause light-induced cell death. In humans, hALAS2 mutations in the C-terminus can result in increased activity and are associated with XLEPP. We characterized mutations in the hALAS2 C-terminus associated with XLEPP using purified recombinant hALAS2 and ex vivo in mammalian cells in terms of their catalytic activity, substrate affinities, thermostability, cellular activity, and structure. Since there are no specific treatments for XLEPP as of yet, we evaluated the use of a potential drug, INH, to treat XLEPP by inhibiting hALAS2. The conclusions presented add to the general knowledge of the ALAS2-catalyzed reaction, further elucidate the mechanism of XLEPP, and
provide evidence for isoniazid as an inhibitor of hALAS2, which has potential for treating porphyria patients.

To restrict photosensitivity by biological, rather than just chemical or physical means, we emulated an idea set forth by Gagnebin et al. (4) to deliver ALAS to tumors for PDT not only for better targeting, but also for greater photosensitivity (4). We used more highly active variants of mALAS2 (2,3) and postulated that these variants would increase PPIX accumulation, phototoxicity, and targeted cell death sufficiently to make the approach more clinically attractive. The HeLa cells that accumulated the highest amount of PPIX were those expressing R433K, an mALAS2 variant with mutated HRMs and the arginine residue at position 433 in the active site loop mutated to a lysine. Expression of R433K in HeLa cells produced a 2- to 3-fold increase in PPIX in comparison to WT, and a 4- to 6-fold increase in PPIX fluorescence in comparison to cells transfected with the pIRES2-ZsGreen1 vector control or HeLa cells alone. When the culture medium was supplemented with glycine, PPIX accumulation increased by 13- to 15-fold in comparison to cells transfected with the pIRES2-ZsGreen1 vector control or HeLa cells alone, and represented the conditions for the highest cellular PPIX accumulation. To study the photosensitivity of cells that accumulated PPIX, we performed light treatments using an incandescent lamp, which emits light in the visible and infrared spectral regions (400-1000 nm). The highest cell death of up to 90% was seen in light-treated HeLa cells expressing ZsGreen1 and R433K, in culture medium supplemented with 100 mM glycine. This represents a substantial improvement
over the 26% cell death reported previously (4), and indicates that this approach, if carefully developed, may eventually find some clinical utility.

Next we studied mutations in the C-terminus of hALAS2 associated with XLEPP that cause an increase in activity. Much like in XLSA, whereby multiple molecular mechanisms can contribute to a decrease in hALAS2 activity, we show that the XLEPP variants differ from wild-type and each other in their substrate affinities, thermostability, secondary structure upon substrate binding, tertiary structure, and integrity of the PLP-binding site. Kinetic analyses performed with purified recombinant hALAS2 variants at 37°C revealed that delAT, in comparison to wild-type hALAS2, exhibited a reduced catalytic efficiency for glycine, but the efficiency for succinyl-CoA was unaffected. DelAGTG exhibited increased catalytic efficiency for both substrates in comparison to wild-type, and Q548X had only a slight increase in catalytic efficiency for succinyl-CoA. While wild-type hALAS2 exhibited a sharp decrease in activity as the temperature increased from 45°C to 55°C, the decrease in activity for the variants was less abrupt and low levels of activity persisted up to 70°C. The purified XLEPP variants are more stable at temperatures above 55°C, indicating that the variants also have increased stability independent of interaction with SCS and resistance to proteolysis. These results led us to examine the structures of purified wild-type hALAS2 and the XLEPP variants.

The far-UV CD spectra for hALAS2 and the variants are dominated by α-helical structure, and are similar among the variants. However, upon addition of succinyl-CoA, the XLEPP variants exhibit a change in secondary structure that is
not evident with the wild-type enzyme. These CD spectra imply the possibility that a structural change that occurs upon binding of succinyl-CoA could be the reason behind the increased affinities of the XLEPP variants for succinyl-CoA. Each variant has a distinct near-UV CD spectrum that differs in intensity and shape from the wild-type enzyme, indicating that the positioning of the aromatic amino acids is altered in the XLEPP variants. The CD signals in the visible spectral region are excellent indicators of the integrity of the PLP cofactor-binding site (5) because, as with other PLP-dependent enzymes (6,7), absorption maxima at 330 and 420 nm are characteristic of different ionization states of the internal aldimine bond between the PLP and the ALAS, corresponding to the unprotonated and protonated forms, respectively (2,8). The binding of delAGTG and Q548X to PLP display slightly higher intensity bands between 420-430 nm, but exhibit large bands around 330 nm that are not evident in wild-type or delAT CD spectra. PLP appears to bind these variants more tightly, and specifically in both ionization states. Upon addition of succinyl-CoA, the band associated with the unprotonated internal aldimine for delAGTG and Q548X shifts to the right to become a broader band from 340-400 nm, indicating a change in the chirality of PLP in the active site when succinyl-CoA is bound. These studies are the first structural studies of the XLEPP variants of hALAS2 and they offer a new perspective to what is known about the molecular mechanism behind XLEPP. We provide evidence that XLEPP can be modeled in cell culture, and this model may aid in testing for direct action of potential therapeutics on hALAS2. We also found that the XLEPP variants are more thermostable, undergo distinct
conformational changes upon the addition of substrate, and differ in tertiary structure and PLP binding.

Using our developed cell culture model of XLEPP and purified recombinant proteins, we then explored the effects of INH, focusing on elucidating the mechanisms of INH-induced anemia and evaluating potential for XLEPP drug therapy. Our studies using mammalian cell cultures indicate that INH decreases PPIX accumulation in HeLa cells overexpressing hALAS2, and demonstrate that INH is effective in reducing hALAS2 activity *ex vivo*. While pyridoxine did not lessen the effect of INH on PPIX levels, addition of either PLP or PMP completely reversed the effects of INH. The results of these initial experiments might be interpreted by using the known PLP-dependent mechanisms of hALAS2 inhibition by INH, in which INH inhibits pyridoxal kinase (9,10), the enzyme that produces PLP, and also reacts with PLP to form pyridoxal isonicotinoyl hydrazones (11,12), but do not rule out the possibility of direct inhibition of hALAS2 by INH. We next used purified hALAS2 to test whether INH could directly inhibit hALAS2, and found that INH inhibits purified hALAS2 regardless of the presence of excess PLP. Excess PLP did, however, affect the apparent $K_i$ values for INH, indicating that a small percentage of the INH might have reacted with PLP to form pyridoxal-hydrazones (12), thus reducing the amount of INH available to inhibit hALAS2. However, 100 μM INH caused nearly 100% activity loss regardless of the concentration of PLP, suggesting a PLP-independent mechanism of hALAS2 inhibition by INH. We subsequently found that INH is not a competitive inhibitor for either the glycine or
succinyl-CoA substrates, as it decreases the $V_{max}$ and increases the $K_m$ values for glycine, and decreases the $V_{max}$ for succinyl-CoA. These data suggest that INH binds to an alternate site that is distinct from the glycine-binding site, and decreases the affinity of hALAS2 for glycine. Additionally, by evaluating the inhibitory effects of pyrazinamide and 1,1-dimethylhydrazine, we concluded that both the hydrazine moiety and the linkage of the hydrazine moiety on the pyridine ring of INH are crucial for complete inhibition of hALAS2.

Given that INH does not compete with the substrates, one possibility is that binding of INH stabilizes the closed conformation of hALAS2. INH could bind both the hALAS2 holoenzyme and glycine-bound hALAS2, which is consistent with partial inhibition of the enzyme. To better resolve the mechanism of inhibition, we examined the possibility of structural changes associated with binding of INH to hALAS2. The changes in fluorescence of the aromatic residues of hALAS2, as seen in CD spectra, indicate that INH elicits a protein conformational change that renders the enzyme either less active or inactive. CD spectroscopy also revealed a concentration-dependent decrease in the mean residue molar ellipticity band between 420-440 nm, indicating that INH disrupts the hALAS2-PLP bond, apparently yielding an enzyme devoid of the Schiff base linkage. Decreased PLP binding of ALAS2 has been noted previously with mouse ALAS2 when serine 254 was mutated to an alanine residue (13). This active site mutation causes a change in the microenvironment of the PLP cofactor and disrupts the binding of succinyl-CoA (13). Our data suggest that INH is an inhibitor of hALAS2 that affects the functionality of the PLP cofactor. We
propose a mechanism by which addition of INH to hALAS2 results in structural rearrangement of the protein and disruption in the hALAS2-PLP linkage, thus inhibiting hALAS2 activity. To determine the position and location at which INH binds to hALAS2, further studies including x-ray crystallization of hALAS2 with INH are warranted. These data provide support for using INH to treat cutaneous porphyrias, especially for XLEPP, where hALAS2 is overly active. The direct effect of INH on hALAS2 activity should also be considered when treating tuberculosis patients who have developed sideroblastic anemia.

In conclusion, this body of work explores mutations in ALAS2 that cause increased PPIX from two different perspectives. Firstly, we investigated mutations to be utilized as tools for developing ALAS-PDT, and found that mutation of the HRMs, in combination with the R433K mutation, results in significant cellular PPIX accumulation and up to 90% cell death upon light treatment. Secondly, we studied mutations in hALAS2 associated with the disease XLEPP, and elucidated structural changes that help to explain how the C-terminal mutations result in changes in catalytic activity of hALAS2, which in turn produce the symptoms of XLEPP. Furthermore, we presented INH as an inhibitor of hALAS2 and a promising therapy for XLEPP patients.
References


About the Author

Erica Fratz grew up in Gaithersburg, MD, where she had a passion for lacrosse, softball, and violin. She graduated from Lehigh University in Bethlehem, PA in 2009 with a Bachelor of Science degree in Biochemistry and a minor in Religion, but also loved playing on the club lacrosse and club rugby teams. Shortly after graduation, Erica moved to Tampa, Florida, a city to which she had never been, to begin graduate school at the University of South Florida, Morsani College of Medicine. In 2011, after successful completion of her comprehensive qualifying examination for PhD candidacy, she received a Master of Science degree in Medical Sciences from the University of South Florida. During her graduate career, in addition to scientific achievements, Erica trained for and completed one marathon, twelve half-marathons, three 10-milers, two 15Ks, five triathlons, three 200 mile overnight team relays, and numerous other endurance races.