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**Regulation of erythroid-specific 5-aminolevulinate synthase (ALAS2) by
hypoxia**

By

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A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Masters of Science

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ABSTRACT

5-Aminolevulinate synthase (ALAS) catalyses the rate-limiting step of heme synthesis. ALAS2 is expressed in erythroid cells and synthesizes heme specifically for hemoglobin. A database search revealed that ALAS2 contained a sequence of amino acids (LXXLAP), which may be hydroxylated under normoxic conditions (21% oxygen) and target the enzyme for ubiquitination and degradation by the proteasome. We examined protein degradation of ALAS2 when protein synthesis was inhibited by cycloheximide under different conditions. ALAS2 was completely degraded by approximately 36 hours of normoxia. Hypoxia, proteasome inhibition and mutation of a key proline within the LXXLAP sequence of ALAS2 stabilized the enzyme. The von Hippel-Lindau protein was also shown to interact with ALAS2, suggesting that ALAS2 is targeted for ubiquitination by E3 ubiquitin ligases. *In vitro* ubiquitination studies showed that ALAS2 could be ubiquitinated under normoxia. The results suggest that regulation of ALAS2 is post-translational and occurs via a mechanism involving proline hydroxylation.

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5. LIST OF ABBREVIATIONS

ALAS2: Aminolevulinic Acid Synthase 2

ALAD: Aminolevulinate Delta-Dehydratase

ARNT: Aryl Hydrocarbon Receptor

CBP: CREB Binding Protein

CBZ: Proteasome Inhibitor (Cbz-Leu-Leu-Leu-al)

CHX: Cycloheximide

CO: Carbon Monoxide

DAF: 2,7-Diaminofluorine

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl Sulfoxide

EKLF: Erythroid Kruppel-Like Factor

Epo: Erythropoietin

EpoR: Erythropoietin Receptor

ER: Endoplasmic Reticulum

FCS: Fetal Calf Serum

HIF: Hypoxia Inducible Factor

HRP: Horse Radish Peroxidase

IMDM: Isocove's Modified Dulbecco's Media

IRE: Iron Responsive Element

IRP: Iron Responsive Protein

K562: Human Chronic Myelogenous Leukemia Cells

NaBu: Sodium Butyrate

PAS: Period/ARNT/Single-minded

PBGD: Porphobilinogen Deaminase

PBS: Phosphate Buffered Saline

PHD: Prolyl Hydroxylase Domain

PMSF: Phenylmethylsulfonyl fluoride

RBC: Red Blood Cell

ROS: Reactive Oxygen Species

RT-PCR: Reverse Transcriptase-Polymerase Chain Reaction

Ub: Ubiquitin

UTR: Untranslated Region

VEGF: Vascular Endothelial Growth Factor

VHL: von Hippel Lindau protein

XLSA: X-Linked Sideroblastic Anemia

6. INTRODUCTION

6.1. Hypoxia

Oxygen is one of the most abundant gases in Earth's atmosphere, comprising 21% of the air that we breathe (Park *et al.*, 2004). The importance of oxygen is due to its role as the main electron acceptor in the electron transfer chain (Park *et al.*, 2004). Since the efficiency of this process is highly dependant on the availability of molecular oxygen, this makes oxygen homeostasis an important factor in the development and physiology of higher organisms. Atmospheric (21%) oxygen is called normoxia; increases or decreases from this level are known as hyperoxia and hypoxia respectively. For example, organisms encounter different oxygen levels as a result of a) environment and b) developmental stage. People who live at high altitudes are exposed to low oxygen levels on a daily basis. The human fetus is also exposed to relative hypoxia during development *in utero* until birth.

Organisms have developed different ways to survive under oxygen-deficient conditions. Exposure to hypoxia suppresses the expression of genes encoding enzymes whose function is dependant on oxygen availability, such as those of the citric acid cycle and the electron transport chain (Safran and Kaelin, 2003). Other genes are induced to survive the hypoxic condition. These genes are mainly involved in increasing blood flow to tissues (erythropoietin, vasoendothelial growth factor, inducible nitric oxide synthase), switching from aerobic to anaerobic energy production (lactate dehydrogenase A, phosphofructokinase L, phosphoglycerate kinase 1, pyruvate kinase M) (Mircea *et al.*,

2001) and the transport and storage of iron in cells (transferrin, transferrin receptor, ceruloplasmin) (Wenger, 2002). The regulation of most hypoxia-inducible genes is under the control of a single transcription factor known as Hypoxia-Inducible Factor (HIF), which binds to a conserved sequence in the promoter regions of hypoxia-regulated genes. To date, approximately 40 hypoxia-inducible genes, whose expression is directly regulated by HIF have been found (Park *et al.*, 2004).

6.2. Hypoxia-Inducible Factors

The past decade has seen enormous progress in the understanding of oxygen homeostasis at the molecular level. A growing number of physiologically-relevant genes that are upregulated by decreases in the oxygen levels through a novel oxygen sensing mechanism have been identified (Safran and Kaelin, 2003). This mechanism was the focus of a long-standing debate amongst scientists promoting different hypotheses of oxygen sensing (Mircea *et al.*, 2001; Livingston and Shivdasani, 2001; Panu *et al.*, 2001; Safran and Kaelin, 2003). The importance of the HIF-dependent oxygen-sensing pathway is demonstrated by the fact that it is ubiquitous amongst all higher eukaryotes. Two groups, one led by William Kaelin Jr. at Harvard and the other led by Peter Ratcliffe at Oxford, published simultaneously in *Science* in 2001 the oxygen-sensing pathway that leads to the degradation of the HIF-1. Their results showed that part of the regulation of HIF involved hydroxylation (an oxygen-dependent post-translational modification of proteins) of two key proline residues in the protein sequence leading to the degradation of

the alpha subunit of the HIF heterodimer (HIF-1 α) by the proteasome (Figure 1) (Livingston and Shivdasani, 2001).

HIF is the most important transcription factor mediating cellular response to physiological decreases in oxygen tension. Three isoforms of this factor have been identified (HIF-1, HIF-2 and HIF-3) (Safran and Kaelin, 2003). These proteins are heterodimers, composing one of three α subunits (HIF-1 α , HIF-2 α and HIF-3 α) that dimerizes with one of three β subunits (HIF-1 β , HIF-2 β , and HIF-3 β , also known as the Aryl hydrocarbon Receptor Nuclear Translocator or ARNT) (Figure 2). HIF is a member of the basic helix-loop-helix (bHLH) super family of DNA-binding proteins (Mircea *et al.*, 2001; Panu *et al.*, 2001). The HLH domain of proteins are involved in the dimerization of subunits (HIF- α and HIF- β in this case) while the basic domain binds to DNA. HIF-1 α contains a PAS domain that facilitates the heterodimerization of HIF- α with β . The name of the PAS domain is derived from the first proteins in which this domain was first discovered Period/ARNT/Single-minded. PAS domains play an important role in monitoring changes in light, redox-potential, oxygen and overall energy level of the cell (Taylor and Zhulin, 1999).

HIF target genes play a critical role in mediating cellular survival of low oxygen conditions. Examples of these genes include vasoendothelial growth factor (VEGF), glucose transporter-1 (GLUT1) and erythropoietin (Epo). The basic regions of the HIF heterodimer binds to a cis-regulatory DNA sequence (5'-TACGTG-3') known as the Hypoxic Response Element (HRE) that is present in the enhancer regions of the promoters of hypoxia-inducible genes. The transcriptional activation of HIF-induced

Figure 1: Stabilization of HIF-1 α under hypoxia leads to hypoxic gene expression. In the presence of oxygen (normoxia) HIF-1 α is hydroxylated at two prolines, allowing for recognition by and binding of vHL and the E3 ubiquitin ligase complex. Ubiquitination of HIF-1 α subsequently ensues, targeting this protein for proteolytic degradation by the proteasome. In the absence of oxygen (hypoxia), HIF-1 α is stabilized and can travel to the nucleus where it dimerizes with its partner protein, HIF-1 β (ARNT). Dimerization allows HIF-1 to bind to HREs present upstream of hypoxia-inducible genes. HIF-1 interacts with the basal transcription machinery through CBP/p300 to increase the rate of transcription of downstream genes (with permission from Willmore 2004).

Figure 2: Structure of the heterodimeric transcription factor HIF. HIF is composed of one of three alpha subunits (HIF-1 α , HIF-2 α and HIF-3 α) and one of three beta subunits (HIF-1 β , HIF-2 β and HIF-3 β). Both subunits contain a DNA-binding (basic) domain and a dimerization domain composed of helix-loop-helix and PAS (Period/ARNT/Single-minded) domains. HIF- α contains N-terminal (NAD) and C-terminal (CAD) transactivation domains while HIF- β contains only a CAD. The oxygen dependent degradation domain (ODD) containing the two LXXLAP sequences of HIF- α is shown (taken from Willmore 2004).

genes involves the recruitment of other coactivator proteins such as CREB-binding protein (CBP) or p300, which works as a bridging protein between HIF and the basal transcription machinery (Safran and Kaelin, 2003).

HIF function is controlled, by oxygen tension, at multiple levels. The central mechanism by which HIF is activated by hypoxia is by the stabilization of its α subunit. Under normoxia, two proline residues in HIF- α (Pro-402 and Pro-564) are constitutively hydroxylated by oxygen-dependent enzymes known as prolyl hydroxylases. These prolines are found in a short peptide recognized by prolyl hydroxylases with the sequence LXXLAP where L is Leucine, X is any amino acid, A is Alanine and P is proline. Proline hydroxylation of the two LXXLAP sequences of HIF- α allow for optimal protein-protein interactions with the von Hippel-Lindau tumour suppressor protein (vHL) (Bruick, 2004). vHL forms the substrate recognition subunit of the mammalian E3 ubiquitin ligase complex, a complex responsible for ubiquitinating hydroxylated HIF- α (Grunea *et al.*, 1996). The binding of vHL to hydroxylated HIF- α results in its rapid ubiquitination by the E3 ubiquitin ligase complex, which is followed by degradation of HIF- α by the 26S proteasome. The 4-hydroxyl group of hydroxylproline and residues in the vHL protein form a hydrogen bond that is critical for the recognition of this post-translational modification. The site-specific hydroxylation of the proline residues in HIF- α is catalyzed by a family of proline hydroxylases, prolyl hydroxylase domain (PHD) containing proteins (PHD1/HPH3, PHD2/HPH2, and PHD3/HPH1), which will be referred to as PHD herein. These enzymes bear homology to the collagen hydroxylases, which are responsible for the production of stable triple-helical collagen. PHDs employ

molecular oxygen, ascorbic acid, Fe^{2+} and 2-oxoglutarate as substrates in addition to the hydroxyl acceptor proline residue (Figure 3). In this reaction, 2-oxoglutarate undergoes stoichiometric decarboxylation that is coupled to hydroxylation of the proline substrate. Ascorbic acid serves as a distinct co-substrate, providing an alternative oxygen acceptor in uncoupled decarboxylation cycles. This enzymatic reaction provides a means by which oxygen tension can be coupled to HIF- α stabilization. Under normoxic conditions, proline hydroxylation occurs constitutively, thereby promoting the vHL-mediated degradation of HIF- α . Under hypoxic conditions, proline hydroxylation of HIF- α does not proceed, allowing HIF- α to escape degradation, travel to the nucleus, dimerize with HIF- β and activate hypoxic gene expression.

6.3. The Proteasome

Most proteins are stable molecules with a half-life of many hours or days. However, about 10–20% of cell proteins are short-lived, with turnover rates of a few minutes. The lysosome was once believed to be the main site of degradation of slow turnover proteins. Proteasome inhibitors were found to block breakdown of long-lived proteins in mammalian cells (Lee and Goldberg, 1998). In most cultured mammalian cells, under optimal nutritional conditions, 80–90% of protein breakdown occurs by the proteasome and is sensitive to proteasome inhibition or ATP depletion (Figure 4). The proteasome removes denatured, damaged or improperly translated proteins from cells and regulates the level of specific proteins like cyclins and certain transcription factors (Lee

Figure 3: The proposed order of binding of co-substrates and prime substrate by the HIF hydroxylases. Following binding of dioxygen, one oxygen atom is incorporated into the hydroxylated HIF residue and the other oxidizes 2-oxoglutarate forming succinate and CO₂ (taken from Masson and Ratcliffe, 2003)

Figure 4: Pathways of protein breakdown in eukaryotic cells. **A)** In mammalian cells, most proteins are degraded by the ubiquitin–proteasome pathway (some of the products are utilized for class I antigen presentation), while the breakdown of extracellular and membrane proteins is by the lysosome-endosomal pathway (which provides peptides for class II antigen presentation). **B)** In yeast, the slow degradation of long-lived proteins occurs in the vacuole, whereas short-lived and abnormal proteins are hydrolysed by the ubiquitin-proteasome pathway (taken from Lee and Goldberg, 1998).

and Goldberg, 1998). The protein substrate is first conjugated to multiple molecules of ubiquitin in a reaction involving ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). The ubiquitinated substrate is rapidly hydrolysed by the 26S proteasome, an ATP- and ubiquitin-dependent complex containing the core 20S proteasome enclosed by two 19S regulatory complexes (Lee and Goldberg, 1998).

The barrel-shaped 20S proteasome is an abundant enzyme, comprising about 1% of the total protein within a cell. It is made up of four stacked rings of six subunits per ring that enclose a central water cavity where proteolysis occurs (Safran and Kaelin, 2003). Subunits of the inner β -rings contain multiple proteolytic active sites that function together in protein degradation. These proteolytic sites include two chymotrypsin-like, two trypsin-like and two caspase-like (i.e. cleave after acidic residues) activities. The outer two α -rings, which are essential for assembly of the 20S particle, surround a small opening through which polypeptide substrates enter. The proteasome utilizes the hydroxyl group of the terminal threonine residue of the β -subunit as the catalytic nucleophile that attacks peptide bonds. Ubiquitinated polypeptide substrates are completely degraded in a highly processive fashion into small peptides of 3–20 residues which, in turn, are further hydrolysed to amino acids by peptidases (Shringarpurea *et al.*, 2001).

Ubiquitinated proteins are recognized by the 19S complexes, as well as enzymes that depolymerize ubiquitin chains. The 19S complex also contains six distinct ATPases that appear to unfold the substrate and facilitate its entry into the 20S particle. Degradation of some proteins by the 26S proteasome also occurs without ubiquitination (Shringarpurea *et al.*, 2001). This is also true for Gram-positive bacteria and

archaebacteria that lack ubiquitin, but contain a 20S proteasome that catalyses protein breakdown in association with an ATPase complex that is similar to the eukaryotic 19S complex (Shringarpurea *et al.*, 2001) (Figure 5).

6.4. Role of Hypoxia in Erythrocyte Maturation

Anaemia is a condition in which the blood cannot deliver enough oxygen to the cells and tissues, resulting in systemic hypoxia. Many of the hypoxic adaptations in eukaryotes are based on transcriptional regulation by the hypoxia-inducible factors HIF-1 and HIF-2 (Masson and Ratcliffe, 2003). One of the body's natural HIF-mediated responses to hypoxia is increased erythropoiesis, the process whereby new oxygen-carrying red blood cells are formed in the bone marrow. When blood delivered to the kidney does not contain enough oxygen, HIF activates the production of the Epo gene, which stimulates red blood cell production, as well as the genes involved in iron metabolism and hemoglobin synthesis; processes essential for proper red blood cell formation and maturation. By coordinating the entire erythropoietic process, HIF promotes the production of properly formed mature red blood cells, helping to restore delivery of sufficient oxygen to the body. Exposure to high altitude, for example, regularly leads to increased red blood cell mass, as shown by several studies of mountain climbing expeditions (Park *et al.*, 2004).

Figure 5: The ubiquitin–proteasome pathway of protein degradation. Long chains of ubiquitin are linked to denatured proteins (polyubiquitinated) by E1 (ubiquitin activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligase) enzymes, targeting them to the 26S proteasome (of which the 20S proteasome forms the core). Proteolytic cleavage occurs within the 20S core. Ubiquitin is stripped off of the protein and recycled. The resulting peptides can be further broken down to form amino acid precursors or for antigen presentation (taken from Lee and Goldberg, 1998).

6.5. Heme Synthesis and the Heme Synthesis Pathway

One of the main proteins that is important in red blood cell function is hemoglobin. Hemoglobin is composed of two parts, the globin protein and the heme cofactor. Heme plays a central role as the prosthetic group of many heme proteins including reactions involving molecular oxygen (and other diatomic gases) and electron transfer. It is a complex of iron and protoporphyrin IX (Jordan *et al.*, 2003; Rytter and Tyrrell, 2000). The heme biosynthetic pathway in animals is comprised of eight nuclear-encoded enzymes. These enzymes are divided between the cytoplasm and the mitochondria (Figure 6). Aminolevulinic acid synthase (ALAS), localized in the mitochondria, is responsible for the first and rate-limiting step of heme synthesis in cells (Dzikaita *et al.*, 2003). It catalyses the formation of 5-aminolevulinate (ALA) from glycine and succinyl CoA in a reaction that is dependent on the cofactor pyridoxal phosphate (Sadlon *et al.*, 1999). Subsequently, ALA is converted to coproporphyrinogen III in four cytosolic reactions, coproporphyrinogen III then re-enters the mitochondrion where the final three steps in heme synthesis occur. Heme produced through this pathway can then be utilised for mitochondrial cytochromes or transported to the cytosol for use as a prosthetic group in other cellular hemoproteins. The reason for the compartmentalisation of heme synthesis is not known (Sadlon *et al.*, 1999).

Figure 6: The heme biosynthetic pathway in mammals. The enzymes involved in heme synthesis are compartmentalized between the mitochondria and cytosol, with the first (ALAS) and the last three enzymes being located in the mitochondria. ALAS catalyzes the conversion of succinyl CoA and glycine to aminolevulinate. Two aminolevulinates combine to form porphobilinogen, four of which combine to form the porphorin ring. The final enzyme, ferrochelatase, catalyzes the insertion of iron into protoporphyrin IX to form heme (with permission from Willmore 2004)

6.6. Aminolevulinate Synthase (ALAS)

ALAS exists in two forms, encoded by two separate genes on different chromosomes. The ALAS1 gene is located on human chromosome 3 and encodes a ubiquitously expressed enzyme that serves a housekeeping role by providing heme for respiratory cytochromes and other hemoproteins. ALAS1 expression plays a negligible role in producing heme for hemoglobin synthesis (Sadlon *et al.*, 1999). The expression of ALAS1 is repressed by heme and can be induced in the liver by phenobarbital and other chemical toxins that induce cytochrome P450 proteins. The second ALAS isozyme, ALAS2, is located on the X chromosome. ALAS2 is the erythroid specific isozyme that is responsible for the production of heme for hemoglobin. In contrast to ALAS1, drugs do not induce this gene, although it may be repressed by heme. Interest in ALAS2 arises from the role it plays in various types of X-linked sideroblastic anaemia (Conboy *et al.*, 1992). The two isozymes have similar amino acid sequences and they both catalyze the same activity, but the genes encoding them possess distinctly different promoters.

The necessity of two ALAS isozymes can be explained by the negative feedback action of heme on ALAS1 gene transcription and mRNA stability (May *et al.*, 1995). The evolution of an erythroid-specific ALAS gene may have arisen as a result of this fine control of ALAS1 levels by heme. Although heme does not inhibit transcription of the ALAS2 gene or alter ALAS2 mRNA stability, there is evidence for several mechanisms to coordinate heme production with its utilization in erythroid cells. Overproduction of heme, as expected, will lead to feedback repression of its synthesis (Ponka *et al.*, 1997). Heme binds to the leader sequence of the premature protein and inhibits the import of

ALAS2 precursor into mitochondria (Lathrop *et al.*, 1993) (Figure 7). Heme can also inhibit the translation of human ALAS2 mRNA in immature erythroid cells through an unknown mechanism (Smith *et al.*, 1997). As well, high levels of heme appear to block iron uptake from transferrin either by inhibiting endocytosis of iron release from transferrin or its release (Ponka *et al.*, 1997). Together, these processes enable the cell to avoid an excessive build up of potentially toxic heme.

6.7. Regulation of ALAS

The human *ALAS2* gene is 22 kB in length and contains 11 exons (Conboy *et al.*, 1992). The initiation site of translation is located in exon 2, which also encodes for the mitochondrial localization sequence. The catalytic domain is encoded by exon 5-11 (Figure 8). In humans, a second transcript that lacks exon 4 arises by alternative splicing, which generates an ALAS2 isoform with a loss of 37 amino acids near the N-terminus of the mature form (Conboy *et al.*, 1992). It is not clear what biological role this sequence plays in humans especially because dogs and mice lack this exon. The promoter sequence of ALAS2 has been partially characterized. Some of the control elements found in the ALAS2 promoter are two GATA-1-binding sites located at -100 and -124 b.p. upstream of the transcriptional start site.

Figure 7: Regulation of ALAS2 expression in erythroid cells in response to Epo. Epo, through the erythropoietin receptor, stimulates transcription of the ALAS2 gene. Translation of ALAS2 mRNA is prevented by the binding of the iron regulatory protein (IRP) but relieved by iron (+), which enters as a complex with transferrin via the transferrin receptor. Following translation, the precursor protein (pre-ALAS2) is imported into mitochondria where it is proteolytically activated. Heme may inhibit translation of ALAS2 mRNA (-) and import of the precursor protein (-) but is necessary for globin mRNA translation (+) (adapted from Sadlon., *et al.*, 1999).

Figure 8: The human ALAS2 gene early promoter and intron/exon structure. Promoter control sequences important for expression, notably GATA boxes and a CACCC box that likely binds EKLF (Erythroid Kruppel-Like Factor), are shown. The GATA site at -27 can bind GATA-1 or TATA Binding Protein (TBP). Exons are shown as vertical lines: exon 1 encodes the iron response element (IRE), exon 2 the mitochondrial localization sequence, exons 3 and 4 are of unknown function and exons 5–11 encode the catalytic domain. Erythroid-specific enhancer sequences are present in introns 1 and 8. An alternatively spliced erythroid mRNA lacks exon 4. Mutations identified in XLSA are located in exons 5–11 and are shown as dots (taken from Sadlon., *et al.*, 1999).

The human ALAS2 gene promoter also contains a non-canonical TATA box (Figure 8) that can bind either GATA-1 or TATA box Binding Protein (TBP) *in vitro*. Two overlapping CACCC box-like motifs are found to be important for ALAS2 promoter activity (Figure 8) (Surinya *et al.*, 1997). GATA-1 sites and CACCC box elements have been identified in the promoters of a number of erythroid-specific genes including globins, GATA-1 (Tsai *et al.*, 1991), erythropoietin receptor (EpoR) (Yousoufian, 1994), PBGD (Porphobilinogen Deaminase) (Mignotte *et al.*, 1989), erythroid pyruvate kinase (Max-Audit *et al.*, 1993) and glycophorin B (Terajima *et al.*, 1995).

Erythropoiesis is the process of red blood cell development through several stages of cell division and differentiation. Erythropoiesis is initiated by the commitment of pluripotent hematopoietic stem cells to become erythroid progenitor cells (Figure 9) After going through proliferation and differentiation, erythroid progenitor cells produce mature red blood cells (Cross *et al.*, 1997). These processes are regulated by different growth factors via a specific combination of transcription factors. One of the key regulators of red blood cell production is the cytokine Epo which functions through EpoR on the surface of erythroid progenitor cells stimulating proliferation, differentiation and cell survival. To accommodate the increased demand for heme during the latter stages of erythropoiesis, there is an increase in the expression of ALAS2, together with the other enzymes of the heme pathway (Fujita *et al.*, 1991; May *et al.*, 1995) in erythroid progenitor cells.

Increased heme synthesis during erythropoiesis requires increased iron uptake by erythroid progenitor cells. Iron uptake and storage are carried out by transferrin and

Figure 9: Erythropoiesis is the process of development of red blood cells through several stages of cell division and differentiation. Erythropoiesis occurs in the bone marrow and involves series of development. All blood cells are derived from an initial stem cell known as the hemocytoblast. Commitment to erythrocyte development occurs at the proerythroblast stage. Various stages of development occur (erythroblast, normoblast, reticulocyte) with the loss of the nucleus and the synthesis of hemoglobin, arriving to the final mature red blood cell (erythrocyte) (taken from Online Anatomy and Physiology II@PGCC, Alex Imholtz, Prince George's Community College, Online Biology 206, http://academic.pgcc.edu/~aimholtz/AandP/206_ONLINE/Blood/Blood2.html).

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ferritin respectively, both of which are regulated by a feedback mechanism that is mediated at the post-transcriptional level by cytoplasmic factors known as iron-regulatory proteins-1 and -2 (IRP1 and 2) (Sadlon *et al.*, 1999). Certain genes have stem-loop RNA structures that are known as the iron-responsive elements (IREs) (Sadlon *et al.*, 1999). These structures are found at the 5' UTRs of the ferritin mRNA and 3' UTRs of the transferrin mRNA. When iron is scarce, IRPs will bind to IREs in the 5' UTRs and the 3' UTRs of the ferritin and the transferrin mRNAs respectively. Such a binding inhibits translation of ferritin mRNA and stabilizes the mRNA for transferrin receptors. When iron is plentiful the protein becomes the cytosolic enzyme aconitase. This pathway prevents the deleterious hazardous effects of iron accumulation while maintaining sufficient concentrations of the metal for heme synthesis (Sadlon *et al.*, 1999). In addition to transferrin and ferritin, IREs are found in the mRNAs of erythropoietin, *m*-aconitase, and succinate dehydrogenase. ALAS2 also has an IRE in its 5' UTR and this forms an important means of regulating the expression of ALAS2 at the level of translation. By complexing an IRP upstream of the translated region, IREs block progression of the translational machinery (Figure 7). One can readily appreciate why low iron levels should dictate suppression of iron storing (ferritin), transporting (transferrin), or utilizing (ALAS2) proteins (Sadlon *et al.*, 1999).

As iron depletion is known to inhibit ALAS2 translation, hypoxic induction of ALAS2 transcription has probably evolved to be HIF-1-independent, as the HIF-1 pathway is activated by iron depletion (Bruick 2003). This was shown by a recent study, which shows that the putative HIF-1 binding site in ALAS2 promoter is not active (Hofer *et al.*, 2003). Epo interacts with EpoR on immature erythroid cells and stimulates their

proliferation and maturation as well as their synthesis of hemoglobin. Epo is responsible for the stimulation of the enzymes involved in heme biosynthesis (Sadlon *et al.*, 1999). Through the interaction with its receptor, Epo activates the transcription factor GATA-1, a key regulator of erythrocyte differentiation. GATA-1 stimulates syntheses of alpha and beta-globins, as well as the enzymes of heme biosynthesis. GATA-1 is also responsible for the activation of its own gene as well as that of the Epo receptor (positive feedback) (Sadlon *et al.*, 1999).

The model system used for ALAS2 studies presented in this thesis is the human chronic myelogenous leukaemia cell line K562. These cells can be triggered, in culture, to differentiate along the erythrocytic or the granulocytic pathway in response to a variety of stimulatory agents. In the presence of NaBu, these cells differentiate to erythroblasts and acquire the capability to synthesize hemoglobin (Mark *et al.*, 2000).

6.8. LXXLAP Database Search

A database search on the LXXLAP peptide sequence revealed over 5000 different proteins, across multiple phyla, which contained the sequence. ALAS2, but not ALAS1, contained the sequence in many mammalian species. Hydroxylation of this sequence could be critical for the targeting of ALAS2 to the proteasome under normoxic conditions. Hypoxia would suppress the hydroxylation of ALAS2 by removing one of the key substrates of the reaction; oxygen. This would, in turn, increase ALAS2 activity in erythrogenic blood cells. It is believed that the upregulation of this protein is an essential adaptation to hypoxia where more red blood cells are required for oxygen transport. This

upregulation of ALAS2 by hypoxia is similar to the upregulation of this gene in the placenta during gestation. Heme synthesis is upregulated at this stage of development to supply a greater amount of oxygen to fetal circulation in response to the rapidly growing fetus.

6.9. Hypothesis

HIF-1-independent pathways of hypoxic protein function may exist. Other proteins may be degraded under normoxic conditions in a manner similar to that of HIF-1 α . Hydroxylation may not only lead to degradation, but can affect protein function (without degradation) under normoxic conditions. The removal of oxygen may stabilize these proteins and thereby increase their function. These proteins would play a key role in pathways that are HIF-independent (i.e. not regulated by HIF-dependent gene expression) but are just as crucial to hypoxia survival of the cell. The hypothesis is that other proteins are regulated in a similar manner to HIF-1 α and are more stable under hypoxic conditions. This thesis examines ALAS2 protein stability under hypoxic conditions. ALAS2 is the perfect candidate protein for HIF-1-independent hypoxic regulation, not only because of its involvement in oxygen transport to tissues, but also because it contains the same LXXLAP sequence present as two copies in HIF-1 α . The LXXLAP sequence in ALAS2 is phylogenetically conserved, which makes it highly likely that its degradation is regulated by prolyl hydroxylation and the E3 ubiquitin ligase/proteasome pathway.

7. MATERIALS AND METHODS

7.1. Database search for LXXLAP

SWISS-PROT and TrEMBL databases (Boeckmann *et al.*, 2003) were searched for proteins containing the LXXLAP sequence of amino acids and the results were sorted using a Perl-based algorithm (Wall 1987-2001). Proteins were grouped according to identity, number of species the protein appeared in and possible involvement in oxygen-sensing pathways.

7.2. Chemicals and Cell Culture

All chemicals and kits were purchased from Sigma (St. Louis, MO) unless specified below. Human chronic myelogenous leukemia cells (K562) were purchased from the American Type Culture Collection (Manassas, VA) and maintained in IMDM media supplemented with 10% fetal calf serum, 100 Units/mL penicillin, 100 µg/mL streptomycin and 0.25 mg/mL amphotercin B (Invitrogen, Carlsbad, CA). Cultures were maintained at 1×10^6 cells/mL at 37°C and 5% CO₂ in a humidified tissue culture incubator (Thermo Forma, Marietta, OH). Erythroid differentiation of K562 cells was induced with NaBu (1.5 mM) for 24 hours according to the protocol of Davis *et al* (1985). The concentration of NaBu used for cell treatment was optimized for maximal haemoglobin production and cell viability. Cells treated under hypoxic conditions (1% O₂, 5% CO₂, 37°C) were placed in a triple-gas incubator (Thermo Forma, Marietta, OH).

for the time of the treatment. Proteasome activity of K562 cells was inhibited with 10 μ M MG-132 (Cbz-Leu-Leu-Leu-al; referred to from here on as CBZ) (Lee and Goldberg 1998).

7.3. ALAS Gene Expression

RT-PCR was performed with the Access RT-PCR System (Promega, Madison, WI). Total RNA was extracted from K562 cells using RNeasy kit (Qiagen, Mississauga, ON). The oligonucleotides used for RT-PCR were "mature ALAS2 forward" with the FLAG epitope tag (underlined; referred to as FLAG from here on) and a BamHI site (bold):

5'-TGTGGATCCATGGACTACAAGGACGATGACAAACATATGCAAATCCACCTTAAGGCAAC-3'

and "mature ALAS2 reverse" with an EcoRI site (bold):

5'-TTAGGCTGAATTCAGCTAGAGGCACACAA-3'

FLAG is an eight amino acid epitope tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), the encoding sequence of which was linked in frame with the mature ALAS2 gene so that it could be easily detected and purified using anti-FLAG antibodies. One microgram of total RNA was used in each RT-PCR reaction. The integrity of the total RNA was confirmed by total RNA formaldehyde gel electrophoresis.

7.4. ALAS2 Cloning

PCR products were TA cloned into TOPO-pCR2.1 (cloning vector) (Invitrogen, Carlsbad, CA) and digested with BamHI/EcoRI to excise the insert and ligated into

pCR3.1 (expression vector) (Invitrogen, Carlsbad, CA) using T4 DNA ligase. After transformation and isolation of plasmids, constructs were sequenced for proper insert orientation and full length. Plasmids from selected positive clones were purified using the GenElute kit from Sigma. DNA was sequenced by Macrogen Inc. (Seoul, Korea), confirming the identity of the cloned gene as ALAS2 and the proper orientation in pCR3.1. Sequences were analysed using the Basic Local Alignment Search Tool 2.0 (BLAST) algorithms at the National Centre for Biotechnology Information (NCBI) using default settings. Human vHL was previously cloned by the author from a human hepatoma cell line (Hep3B), N-terminally FLAG-tagged and placed into the mammalian expression vector pCR3.1 for cotransfection studies.

7.5. Site-directed Mutagenesis

Proline 520 of the premature form of ALAS2 was mutated to alanine (P520A) using the QuikChange Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA) according to the manufacturer's protocol. Mutagenesis primers were as follows:

P520A(A)ALAS2: 5'-CTCCTGCGCTTGGCAGCCTCCCCCACCACA-3'

P520A(B)ALAS2: 5'-TGTGGTGGGGGAGGCTGCCAAGCGCAGGAG-3'

The resulting clones were screened for the presence of the mutation by sequence analysis (Macrogen Inc., Seoul, Korea).

7.6. Total ALAS Enzyme Activity Assay

The enzymatic activity of ALAS was measured in K562 cells using a continuous spectrophotometric kinetics assay described by Hunter and Ferreira (1995). The reaction couples the production of coenzyme A (CoASH) by ALAS (reaction 1) to the production of succinyl-CoA by α -ketoglutarate dehydrogenase (reaction 2) by the following reactions:

The production of CoASH by ALAS is coupled to the reduction of NAD^+ by α -ketoglutarate dehydrogenase and activity is monitored as the increase in absorbance at 340 nm. ALAS kinetics was followed using a Cary 100 spectrophotometer (Varian, Victoria, Australia) at 30°C over a period of 5 min. The reaction mixture contained 20 mM HEPES (pH 7.2), 1 mM α -ketoglutarate, 1 mM NAD^+ , 3 mM MgCl_2 , 0.25 mM thiamine pyrophosphate, 100 mM glycine, 20 μM succinyl-CoA and 0.25 Units α -ketoglutarate dehydrogenase. Cells were homogenized in 20 mM HEPES (pH 7.2). PMSF was added to inhibit protease activity. The reaction was initiated with the addition of cell lysate. Protein concentration was determined using the Bio-Rad protein dye reagent (Bio-Rad, Hercules, CA) and bovine serum albumin as a standard. One unit of

enzyme activity is defined as the activity to convert 1 nmol of substrate to product per hour at 30°C.

7.7. Hemoglobin Measurements

Hemoglobin was measured according to the assay of Bichet *et al* (1999). Briefly, K562 cells were lysed in 550 to 600 μ L 0.01% (v/v) Igepal (Nonidet P-40 substitute, USB, Cleveland, OH) in water. One aliquot of the cell lysate was used for protein determination (Bio-Rad, Hercules, CA). The other aliquot was used to determine the hemoglobin concentration in the dissociated cells. A mixture of 15 μ L of 1% 2,7-diaminofluorene (DAF) (w/v) in 90% glacial acetic acid with 1.5 mL of assay buffer (100 mM Tris phosphate buffer, pH 7.0, 6 M urea, 0.18% w/v H₂O₂) was added to 500 μ L of the supernatant of the lysed cells. After incubation for 12 min. at 25°C, the absorbance was measured at 610 nm. Bovine hemoglobin was used as a standard for determining hemoglobin concentration (1-40 μ g hemoglobin/mL).

7.8. ³⁵S Metabolic Labelling and Protein Synthesis Inhibition

Proteins from K562 cells were pulse labelled with ³⁵S-methionine/cysteine mixes and treated with a wide range of CHX concentrations (0.01-20 μ g/mL of media) to inhibit protein synthesis. Cells were resuspended in pulse-labelling media (DMEM without L-glutamine, sodium pyruvate, L-methionine or L-cysteine; Invitrogen, Carlsbad, CA) at a concentration of 5 X 10⁶ cells/mL for 15 min. at 37°C water bath to deplete intracellular

pool of methionine and cysteine. Cells were washed and resuspended in pulse-labelling media containing ^{35}S -methionine/cysteine (final concentration of 0.2 mCi/mL) and incubated for 30 min. in a 37°C . Cells were washed, resuspended in chase media (complete DMEM) containing CHX and incubated at 37°C for up to 24 hours. CHX was dissolved in DMSO and added to the media at concentrations of DMSO below 0.05%. The amount of ^{35}S incorporated into proteins was determined by TCA precipitation followed by scintillation counting. Scintillation counting was performed using a LS 6500 Multi-Purpose liquid scintillation counter on a ^{35}S channel and Ready Safe liquid scintillation cocktail (Beckman Coulter Inc. Fullerton, CA). The effects of CHX on cell viability were determined using the MTT assay (thiazolyl blue tetrazolium bromide) according to Mosmann (1983) and Denizot and Lang (1986).

7.9. MTT Assay

The reduction of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to a blue-black formazan product by living (but not by dead) cells can be used as a measure of cell viability (Mosmann 1983). MTT enters the mitochondria of living cells where mitochondrial dehydrogenases cleave this substrate to form the coloured product. Cells are then lysed in DMSO, which solubilizes the product. The colour can then be quantified spectrophotometrically.

K562 cells were treated in a 24-well plate at a density of 0.5×10^6 cells/mL. A MTT (Sigma St. Louis, MO) stock solution was prepared at a 5 mg/mL concentration in PBS, filtered and stored at 4°C . MTT was added to the wells at a volume equal to one

tenth of the original media volume. The plate was incubated at 37°C for 1 hour. Suspension cells were spun in the microplate benchtop centrifuge. The media was replaced with the same volume of DMSO and incubated at room temperature for about 5-10 min. The absorbance was then measured in a 96-well microplate using a SpectraMax 340PC microplate reader at a wavelength of 570 nm with background subtraction at 630 nm.

7.10. Transient Transfections of K562 Cells and ALAS2 Expression

Transient transfections of ALAS2 expression constructs into K562 cells were done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transfections were performed in 24-well plates over 48 hours. The optimal concentration of DNA was found to be 1.2 µg of DNA/0.5 mL of K562 cells at a concentration of 1.2×10^6 cells/mL. Cells were then lysed and total protein subjected to immunoprecipitation (see Coimmunoprecipitation Assays (Section 7.11) below) with anti-FLAG M2 beads. Purified proteins were then run on silver stained 10% Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) to confirm expression and purity.

7.11. Coimmunoprecipitation Assays

FLAG-ALAS2 and FLAG-vHL expression vectors were transiently transfected separately into K562 cells. Cells were lysed in Buffer 0.5 (20 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 10% glycerol, 0.1 % Tween 20, 0.5 M KCl, 0.07 % β-mercaptoethanol, 0.1

M PMSF and inhibitor cocktail tablet (Complete Mini, Roche Diagnostics, Laval, Quebec)) for 2 h at 4°C with continual shaking. The protease inhibitor tablets in Buffer 0.5 inhibit a broad spectrum of serine, cysteine and metalloproteases as well as calpains. Anti-FLAG M2 agarose beads (Sigma, St. Louis, MO) were washed in Buffer 0.5 three times. Cell lysates were added to the washed anti-FLAG M2 beads and incubated overnight at 4°C. Bound proteins were eluted by incubation with FLAG peptide (1mg/mL) for 30 min at 4°C in Buffer 0.5. Purified protein samples were then reduced by addition of SDS sample buffer to prepare them for Western blotting.

For crosslinking studies, dithiobis[succinimidylpropionate] (DSP; Pierce, Rockford, IL) was added to cell cultures at a final concentration of 2 mM for 30 min. at room temperature. DSP is a water-soluble compound that penetrates cell membranes and reversibly crosslinks primary amines. DSP can only crosslink amine amino acid side chains that are 12 Å apart and therefore can only form interprotein or intraprotein crosslinks of proteins that are in complexes. Cells were then quickly washed in 1 X PBS (10 mM phosphate buffered saline, 138 mM NaCl, 2.7 mM KCl, pH 7.4), lysed, and supernatants subjected to immunoprecipitation. Crosslinkage was cleaved by reduction with 5% β-mercaptoethanol at 100°C for 5 min. Concentrations of β-mercaptoethanol in Buffer 0.5 were not high enough to reduce crosslinker disulfide bonds at 4°C during immunoprecipitation.

7.12. Western Blot Analysis

After transfection, K562 cells were harvested by centrifugation and then washed twice with cold PBS and lysed using cell lysis buffer (PBS containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS, and 0.01% protease inhibitor cocktail). Five μ g of crude protein from the different treatments was boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA), subjected to 10% SDS-PAGE, and proteins transferred to a nitrocellulose membrane. Prestained broad range markers (New England BioLabs Beverly, MA), were used as protein standards on SDS-PAGE. Western blot analysis for ALAS2 was performed using monoclonal horseradish peroxidase-labeled mouse anti-FLAG M2 antibodies (1:2000 dilution; Sigma, St. Louis, MO). Western blot analysis for vHL, β -tubulin and HIF-1 α was performed using mouse anti-human vHL antibodies (1:200 dilution; Calbiochem, San Diego, CA), mouse anti-human β -tubulin (1:2000 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA) and mouse anti-human HIF-1 α (1:800 dilution, Novus Biologicals, Littleton, CO) and horseradish peroxidase-labeled anti-mouse IgG secondary antibody (1:5000 dilution; DakoCytomation Inc., Mississauga, ON).

7.13. *In Vitro* Ubiquitination Assay

In vitro ubiquitination of FLAG tagged ALAS2 was performed using 1 μ g of purified FLAG-ALAS2 in 10 μ l of ubiquitination mix containing the full component of purified conjugating enzyme (E1, E2s and E3s) that are found in Fraction II of HeLa cells

(Ubiquitin-Protein Conjugation Kit, Boston Biochem, Cambridge, MA), 4 μ M ubiquitin, 4 μ M ubiquitin aldehyde and an ATP-regenerating system (MgCl_2 , ATP and ATP regenerating enzymes to recycle hydrolyzed ATP (AMP, ADP) to ATP) and 5 μ M of CBZ. Transiently transfected cells were harvested at different time points post-transfection (0, 2, 4, 6, 12, 24 and 48 h) and assayed for ubiquitin conjugation. Whole cell lysates or immunoprecipitated proteins were incubated in the *in vitro* ubiquitination system at different time points at 37°C up to a maximum of 2.5 h. Samples were subjected to SDS-PAGE and immunoblotting using anti-FLAG antibodies.

8. RESULTS

8.1. Database Search for LXXLAP Subsequence

A Perl-based search (Wall, 1987-2001) of SWISS-PROT and TrEMBL (Boeckmann *et al.*, 2003) for the LXXLAP amino acid sequence uncovered over 5000 different proteins containing the target sequence. The list was narrowed to proteins of known function, b) proteins that occurred in multiple organisms (phylogenetically conserved), c) proteins with an easily measurable function (eg. enzyme activities) and d) proteins that could potentially be involved in oxygen-sensing pathways. One top candidate was ALAS2. An alignment of amino acid sequences of ALAS1 and ALAS2 enzymes revealed that ALAS2 contains the same oxygen-dependent sequence of amino acids (LXXLAP) as HIF-1 α (Figure 10). Moreover, this sequence is phylogenetically conserved. Unlike HIF-1 α , the LXXLAP sequence occurred only once in ALAS2.

8.2 Down-Regulation of ALAS Activity in Undifferentiated K562 Cells

Differentiation of the K562 cells with NaBu induces the expression of the erythroid specific form of ALAS (Fujita *et al.*, 1991). Before differentiation, total ALAS activity is comprised of both ALAS1 and ALAS2 activities. The total activity of ALAS was measured before and after chemical induction of differentiation by NaBu. Prior to

Figure 10: Multiple alignments of ALAS2 and ALAS1 amino acid sequences from multiple organisms showing conservation of the LXXLAP sequence in ALAS2 but not ALAS1.

	<u>LXXLAP</u>	
Human ALAS2	509 VPRGEELLRLAPSPHHSP	526
Rat ALAS2	509 VPRGEELLRLAPSPHHSP	526
Mouse ALAS2	509 VPRGEELLRLAPSPHHSP	526
Beluga Whale ALAS2	504 VPRGEELLRLAPSPHHSP	521
Zebrafish ALAS2	504 VPRGEELLRLAPSPFHNP	521
Human ALAS1	563 VPRGEELLRIAPTPHHTP	580
Rat ALAS1	565 VPRGEELLRIAPTPHHTP	582
Mouse ALAS1	564 VPRGEELLRIAPTPHHTP	581
Beluga Whale ALAS1	563 VRRGEELLRIAPTPHHTP	580
Zebrafish ALAS1	536 VARGEELLRIAPTPHHTP	553
Drosophila ALAS	459 VARGQEKRLAPTPFHTE	576
Human HIF-1 alpha (a)	391 KKEPDALTLLAPAAGDTI	408
Human HIF-1 alpha (b)	553 QDLDLEMLAPYIPMDD	570

differentiation, K562 cells treated with hypoxia for 24 hours had diminished total activity of ALAS. ALAS activity in K562 treated with 10 μ M of proteasome inhibitor was also reduced to approximately 60% of normoxic values (Figure 11).

8.3 Increase of Total ALAS Activity in Differentiated K562 Cells Under Hypoxia

Differentiation of the K562 cells with NaBu induces the expression of the erythroid specific form of ALAS (Fujita *et al* 1991; Kawasaki *et al* 1996; Kawasaki *et al* 1998; Zoller *et al* 2002). It is assumed that the increase in total ALAS activity after differentiation is due to an increased expression of the erythroid-specific form of ALAS (Fujita *et al* 1991). In general, total ALAS activity increased after differentiation with NaBu (Figure 12). ALAS activity increased about 2-fold over 72 hours of hypoxia compared to normoxia. Proteasome inhibition resulted in a larger increase in the specific activity of ALAS, increasing from 2- to 25-fold over 24 to 72 hours respectively compared to normoxia (Figure 13).

8.4 Hemoglobin Content of K562 Cells During Differentiation

To determine if K562 show the classical features of differentiated erythroid cells in response to NaBu, the hemoglobin content of cells was measured over a time course of NaBu treatment (Figure 14). K562 produced more than 2-fold more hemoglobin after 72 hours of treatment of NaBu. Moreover, differentiated K562 showed a 4-fold increase in

Figure 11: Enzyme activity of ALAS under different treatments. Total activity of ALAS in undifferentiated K562 cells treated for 24 hours with normoxia (N; 21% O₂, 5% CO₂, 37°C), hypoxia (H; 1% O₂, 5% CO₂, 37°C), 100 µM MnCl₂ (Mn), 100 µM NiCl₂ (Ni), 100 µM CoCl₂ (Co), 100 µM desferrioxamine (DFO) and 10 µM proteasome inhibitor (CBZ). B is blank without glycine and succinyl CoA.

Figure 12: Total ALAS activity in K562 cells treated with either normoxia or hypoxia for up to 72 h after differentiation with 1.5 mM NaBu. NG = activity with no glycine; NS = activity with no succinyl CoA.

Figure 13: ALAS activity from K562 cells, differentiated with 1.5 mM NaBu, after 24, 48 and 72 hours of treatment with normoxia, hypoxia and CBZ.

Figure 14: Hemoglobin content of K562 cells after differentiating them using 1.5 mM NaBu and treating them with normoxia, hypoxia and CBZ for 24 and 48 and 72 hours.

hemoglobin in response to hypoxia and proteasome inhibition. This concurs with the idea that the major form of ALAS induced under differentiation and hypoxia is ALAS2. The data also agree with the upregulation of specific activity of ALAS2 in response to these two treatments (Figure 13).

8.5 Cell Viability With CHX Treatment

In order to completely inhibit protein synthesis, without affecting cell viability, we examined the viability of K562 cells treated with different concentrations of CHX for 24 hours using the MTT assay. In this assay, a linear relationship between cell number and absorbance is established thereby enabling accurate, straightforward quantification of changes in cell proliferation. The reduction of tetrazolium salts is a safe, accurate alternative to radiometric testing.

Cell viability decreased as the concentration of CHX increased (Figure 15). Different cell densities did not affect the profile of CHX toxicity to cells; the curve only shifted upwards as the initial formazan absorbance increased. Viability decreased to approximately 50% of initial (untreated) values at a CHX concentration of 1 $\mu\text{g/mL}$ and remained the same for all higher concentrations. Concentrations lower than 0.5 $\mu\text{g/mL}$ were subsequently chosen to test for protein synthesis inhibition.

Figure 15: Cell viability (measured by MTT) with the addition of increasing amounts of CHX to K562 cells in their exponential growth phase ($0.5\text{-}1.0 \times 10^6$ cells/mL) over 24 hours of treatment.

8.6 Inhibition of Protein Synthesis By CHX Treatment

In order to eliminate the influence of transcriptional and translational controls on the determination of ALAS2 protein turnover, K562 cells were metabolically labelled with a ^{35}S methionine/cysteine mix and treated with increasing concentrations of CHX (Figure 16). Protein synthesis was inhibited at all concentrations of CHX by 24 hours of cell treatment. Concentrations of CHX less than $0.1\text{ }\mu\text{g/mL}$ were found to have no effect on cell viability (Figure 15). A concentration of $0.05\text{ }\mu\text{g/mL}$ CHX was therefore used for all subsequent studies.

8.7 Cell Viability With CBZ Treatment

Inhibition of the proteasome can be toxic to cells since it results in the build-up of damaged proteins in the cytosol. Currently, most studies use a concentration of $10\text{ }\mu\text{M}$ CBZ in cell media to inhibit the proteasome. The MTT assay was used to assess cell viability under different CBZ concentrations. Figure 17 shows the effect of different CBZ concentrations on cell viability compared to untreated cells. Treatment of cells with 15 and $20\text{ }\mu\text{M}$ CBZ lead to complete cell death after 72 hours. Treatment of cells with $10\text{ }\mu\text{M}$ CBZ resulted in a 24% decrease in cell viability by 72 hours. Since untreated samples also showed a slight decrease in cell viability at this time point, it was concluded that loss of cell viability was due to overgrowth of cells.

Figure 16: Inhibition of protein synthesis in K562 using CHX. K562 were metabolically labelled with ^{35}S Met/Cys and treated with varying concentrations of CHX (0-4 $\mu\text{g/mL}$). Protein synthesis was completely inhibited by approximately 24 hours of treatment with all concentrations. CHX results were combined with viability tests to determine the appropriate concentrations of CHX for further studies.

Figure 17: Cell viability of K562 cells (0.3×10^6 cells/mL) treated with different concentrations of CBZ (0-20 μ M CBZ).

8.8 Optimization of DNA Concentration for Transfection into K562 Cells

The amount of DNA transiently transfected into cells is important in controlling the level of protein expression. DNA overload causes intracellular acidic conditions and decreased cell viability. Total ALAS activity was used to optimize for the amount of DNA used for transfections. According to the manufacturer's protocol (Invitrogen, Carlsbad, CA), 0.8 μ g of DNA is required for a 0.5 mL reaction. After testing different DNA concentrations, the optimal DNA concentration for transfections was found to be 1.2 μ g of DNA per 0.5 mL transfection reaction (Figure 18). Total ALAS activity of transfected cells increased 19-fold compared to nontransfected cells.

8.9 ALAS2 Protein Expression and Immunoprecipitation

The gene for the mature form of ALAS2, which lacks the mitochondrial localization sequence, was cloned into mammalian expression vector pCR3.1 and N-terminally FLAG-tagged. ALAS2 identity and orientation in the vector was confirmed by N- and C-terminal sequencing through the entire gene as well as diagnostic restriction digests (data not shown). Proline 520 was mutated to alanine and confirmed with sequencing. In order to ensure that both wildtype and mutant proteins were expressed from constructs, vectors expressing ALAS2 were transiently transfected into K562 cells and FLAG-tagged ALAS2 was immunoprecipitated using anti-FLAG agarose beads and run on SDS-PAGE. Figure 19 shows the expression profile of tagged protein, immunoprecipitated from erythroid cells. Transient transfection resulted in a robust

Figure 18: Total ALAS activity of K562 transiently transfected with different DNA concentrations of FLAG tagged mature ALAS2 in pCR3.1. Cells are transfected for 48 hours in a 0.5 mL reactions.

Figure 19: **A)** Silver-stained 10% SDS-PAGE of immunoprecipitated FLAG-tagged mature ALAS2 overexpressed in K562 cells. **Lane 1:** untransfected K562; **Lane 2:** K562 transfected with empty pCR3.1 vector; **Lanes 3 and 4:** K562 transiently transfected with pCR3.1 expressing FLAG-tagged mature ALAS2 (two isolations). **B)** Coomassie blue-stained 10% SDS-PAGE of immunoprecipitated FLAG-tagged ALAS2 overexpressed in K562 cells. **Lane 1:** Prestained Broad Range Protein Markers (New England BioLabs); **Lane 2:** wildtype mature FLAG-tagged ALAS2; **Lane 3:** P520A mutant mature FLAG-tagged ALAS2. Arrows indicate the location of mature FLAG-ALAS2 on each gel.

expression of tagged mature ALAS2, with trace amounts of less abundant proteins coimmunoprecipitating with the tagged protein (detectable only with silver-staining, Figure 19A). The calculated molecular weight of the FLAG-tagged mature protein is 60 kDa, corresponding to the band revealing the highest density on the protein gel. This band was confirmed to be the overexpressed protein by anti-FLAG Western blots of the same gels (data not shown).

8.10 Protein Synthesis Inhibition Studies

Protein synthesis in K562 was inhibited and ALAS2 protein degradation was examined. Figure 20A shows the Western blots of FLAG-tagged ALAS2 under different conditions after inhibiting protein synthesis using CHX. No commercial antibody is available for ALAS2, and therefore the FLAG tag assists in both detection of the protein as well as its purification. ALAS2 expression was normalized to β tubulin expression on the same Western blot. ALAS2 is completely degraded after 48 hours of CHX treatment under normoxic conditions. ALAS2 protein was stabilized for 48 hours by both hypoxia (Hyp) and proteasome inhibition (CBZ). Mutation of proline 520 in the LXXLAP sequence also caused the stabilization of ALAS2 under normoxia after treatment with CHX, suggesting that post-translational modification of this amino acid assists in the normoxic breakdown of the protein. Westerns for HIF-1 α were also performed on the same cell extracts to show stabilization of an oxygen-regulated protein under conditions of hypoxia and proteasome inhibition in K562 cells (Figure 20B).

Figure 20: **A)** Anti-FLAG (top panels) and anti-tubulin (bottom panels) Western blots of wildtype and mutant FLAG-tagged ALAS2 over time normalized to β -tubulin gene expression. K562 cells were transiently transfected with mammalian expression vectors containing wildtype or mutant ALAS2 constructs and treated with normoxia (21% O₂, 5% CO₂, 37°C), hypoxia (Hyp; 1% O₂, 5% CO₂, 37°C) or proteasome inhibitor (CBZ; 10 μ M). Protein synthesis was inhibited in each treatment using CHX (0.05 μ g/mL). **B)** Anti-HIF-1 α (top panel) and anti-tubulin (bottom panel) showing stabilization of HIF-1 α in K562 cells under hypoxia and CBZ treatment.

8.11 ALAS2 and vHL Interaction

It was hypothesised that vHL, the substrate recognition subunit of the E3 ubiquitin ligase, would interact with hydroxylated but not the nonhydroxylated form of ALAS2. Hydroxylation of wild type ALAS2 by prolyl hydroxylases that recognize the LXXLAP sequence will occur under both normoxia and proteasome inhibition. Figure 21 shows the coimmunoprecipitation of vHL with ALAS2 using Western blots probed with either anti-vHL (Figure 21A) or anti-FLAG (Figure 21B) antibodies. Small amounts of vHL coimmunoprecipitated with wildtype FLAG-ALAS2 under hypoxic and normoxic conditions (Lanes 5 and 6 respectively). Larger amounts of vHL were pulled down with FLAG-ALAS2 under normoxic conditions when cells were treated with proteasome inhibitor or when cellular proteins were crosslinked (Lanes 1 and 7 respectively). It was not possible to pull vHL down when the mutant ALAS2 form was used. This is an indication that the proline in the LXXLAP sequence of ALAS2 is important for the association between the hydroxylated protein and vHL. Empty vector and no vector transfections did not immunoprecipitate FLAG-ALAS2 or vHL. FLAG-tagged vHL was used as a positive control for immunoprecipitations.

8.12 *In Vitro* Ubiquitination of ALAS2

Immunoprecipitated wildtype FLAG-tagged mature ALAS2 and vHL were used for *in vitro* ubiquitination studies. Figure 22 shows the difference between the

Figure 21: Western blot of immunoprecipitated proteins using anti-FLAG agarose beads. The Western blot was probed with anti-vHL antibodies **A)** as well as anti-FLAG antibodies **B)**. All transfections were carried out for 48 hours. **Lane 1:** wild type mature FLAG-ALAS2 transfected into K562 treated with proteasome inhibitor; **Lane 2:** untransfected K562 (negative control); **Lane 3:** K562 transfected with empty pCR3.1 (negative control); **Lane 4:** K562 transfected with mutant P520A FLAG-ALAS2; **Lane 5:** wild type mature FLAG-ALAS2 transfected into K562 treated with hypoxia; **Lane 6:** wild type mature FLAG-ALAS2 transfected into K562 treated with normoxia (non-crosslinked); **Lane 7:** wild type mature FLAG-ALAS2 transfected into K562 treated with normoxia (crosslinked); **Lane 8:** K562 transfected with wild type FLAG-vHL (positive control).

Figure 22: A) Anti-FLAG Western blot of *in vitro* ubiquitination of immunoprecipitated wildtype FLAG-tagged ALAS2 (WT), P520A mutant FLAG-tagged ALAS2 (MT) and FLAG-vHL (vHL). Ubiquitin (Ub) was omitted from non-ubiquitinated control reactions. FLAG-tagged proteins were isolated from transiently transfected cells treated with normoxia (N) or a combination of hypoxia and 100 μ M cobalt (H). B) Anti-FLAG Western blot of *in vitro* ubiquitination of mature FLAG-ALAS2 produced at different time points post-transfection in K562 cells. Cells were harvested at different time points up to 48 hours (Hr) after transfection and whole cell lysates subjected to *in vitro* ubiquitination. Mature FLAG-ALAS2 was not seen in K562 cells 6 hours post-transfection (data not shown). Lane 1: non-ubiquitinated control FLAG-ALAS2 produced for 48 hours. C) Timecourse of *in vitro* ubiquitination of FLAG-ALAS2 produced in K562 cells for 48 hours. Non-ubiquitinated control FLAG-ALAS2 (Lane 1) and FLAG-ALAS2 *in vitro* ubiquitinated for 1.0 (Lane 2), 1.5 (Lane 3), 2.0 (Lane 4) and 2.5 (Lane 5) hours respectively.

nonubiquitinated and ubiquitinated forms of ALAS2. Since ubiquitin is an 8 kDa protein, and target proteins are usually polyubiquitinated, it was expected that multiple bands would represent subsequent additions of ubiquitin units to ALAS2. This laddering effect is seen in Lanes 7 in Figure 22A, Lanes 2 to 4 in Figure 22B and Lanes 2 to 5 in Figure 22C.

Control ubiquitination experiments utilizing mutant ALAS2 show no ubiquitination of this protein when proline 520 is changed to alanine (Figure 22A, Lane 4). Furthermore, protein produced under conditions that would inhibit prolyl hydroxylases in cells (hypoxia and cobalt co-treatment) (Figure 22A, Lane 5) did not show *in vitro* ubiquitination. Wildtype FLAG-ALAS2 produced in normoxic K562 showed a robust ubiquitination by this *in vitro* system (Figure 22A, Lane 7). This further suggests that proline 520 is key to the ubiquitination process and that oxygen-dependent hydroxylation is a possible mechanism. vHL, a protein known not to be ubiquitinated, did not show presence of this modification in the *in vitro* system (Figure 22A, Lane 1). When Ub was omitted from these experiments, normoxic ubiquitination of FLAG-ALAS2 was not seen (Figure 22A, Lane 6).

A time course of the potential for ubiquitination of ALAS2 *in vivo* was followed (Figure 22B). K562 cells were transiently transfected with mammalian vectors expressing FLAG-ALAS2 and harvested at various time points up to 48 hours (0, 2, 4, 6, 12, 24 and 48 hours). Whole cell lysates were taken through the *in vitro* ubiquitination system and Western blotted for anti-FLAG. Prior to 6 hours, insufficient amounts of ALAS2 are produced in transiently transfected K562 cells to detect by anti-FLAG Western blots (data not shown). Ubiquitination of FLAG-ALAS2 occurs within the first two hours of *in*

vitro ubiquitination (Figure 22C). Normoxic degradation of mature FLAG-ALAS2 *in vivo* occurs at a much slower rate (48 hours, Figure 20A). The results suggest that putative hydroxylation and ubiquitination of ALAS2 occur at a rapid rate while the degradation of ubiquitinated ALAS2 occurs over a much longer period of time.

9. DISCUSSION

This study shows that oxygen-dependent hydroxylation of ALAS2 LXXLAP sequence targets the enzyme for proteolytic breakdown by the proteasome under normal atmospheric oxygen conditions. Hypoxia suppress the hydroxylation of ALAS2 by removing oxygen, which acts as a substrate for the prolyl hydroxylase enzymes, stabilizing ALAS2 and, as a result, increasing its activity in erythrogenic blood cells. It is believed that the upregulation of this protein is an essential adaptation to hypoxia where synthesis of mature red blood cells is required for increased oxygen transport to tissues in the face of limiting O₂ (Bruick, 2003).

9.1 Putative Hydroxylation Sites Search

A database search for the LXXLAP peptide sequence revealed over 5000 different proteins, across multiple phyla, which contained the sequence. The aim of this search was to find potential proteins that have the LXXLAP sequence and can be regulated by oxygen. This sequence is found twice in HIF- α proteins allowing them to be recognized by vHL and hydroxylated to promote rapid HIF- α degradation. ALAS2, but not ALAS1, contained the LXXLAP peptide in mammalian species where the gene has been sequenced. Hydroxylation of this sequence could be critical for the targeting of ALAS2 to the proteasome under normoxic conditions. The lack of hydroxylation of ALAS2 under hypoxic conditions would stabilize this protein and work in tandem with other regulatory mechanisms to increase the pool of this protein in erythrogenic cells. As

ALAS2 plays a critical role in hemoglobin synthesis, upregulation of this enzyme would be required during hypoxic erythropoiesis and essential for increasing the blood supply (and thus the amount of oxygen) to tissues.

9.2 Differentiation of the K562 Cells

K562 cells are multipotential, hematopoietic malignant cells that spontaneously differentiate into recognizable progenitors of the erythrocytic, granulocytic and monocytic series (Fujita et al., 1991). In response to NaBu treatment, K562 cells differentiate into erythrocytes (Davis et al., 2000). Differentiation of erythroid cells switches the major intracellular form of ALAS from ALAS1 to ALAS2 (Fujita et al., 1991). Differentiation of K562 in this study was confirmed by measuring hemoglobin synthesis spectroscopically (Figure 14). This is a sensitive colorimetric method that relies upon the specific oxidation of DAF by the pseudoperoxidase activity of hemoglobin (Hb). Generation of fluorene blue (FB), the chromophore generated upon oxidation of DAF, is linearly related to Hb concentration (Worthington *et al.*, 1987).

Differentiation of the K562 cells is essential to stimulate their production of ALAS2. The differentiation and maturation of red blood cells progenitors involves the coordinated interaction of two organ systems in the body. The first is the bone marrow which produces erythroblasts. The second is the kidney which produces the Epo. Hypoxia prompts the kidney to synthesize and release Epo in a process that is HIF-dependant. Epo then travels via the bloodstream to the bone marrow where it activates red blood cell

production. This increases the oxygen carrying capacity of the blood flowing to tissues in an attempt to return the organism to oxygen homeostasis.

Zoller *et al* (2002) demonstrated that Epo directly stimulates heme biosynthesis. In a time-dependent manner, Epo increased the expression and ALAS2 activity. The regulation of ALAS2 by Epo occurs by two different mechanisms. First, Epo increased ALAS2 transcription after incubation of erythroid cells with Epo for at least 24 hours. Epo also affects the levels of ALAS2 protein produced from its mRNA by reducing the binding affinity of IRP-2 to the ALAS IRE.

9.3 Protein Synthesis Inhibition

The increase in ALAS2 activity during hypoxia may be due, in part, to increased ALAS2 gene expression. Previous studies found the ALAS2 gene to be induced by hypoxia in a HIF-1-independent manner (Hofer *et al.* 2003). To remove the influences of transcription and translation, we utilized CHX to inhibit translation, allowing only degradation of ALAS2 to be observed. CHX treatment completely inhibited protein synthesis by 24 hours, with no effect on cell viability, at a concentration of 0.05 µg/mL (Figure 16). CHX is an antifungal antibiotic that inhibits protein translation in eukaryotes but not prokaryotes. It interacts directly with ribosomal translocase enzyme interfering with its translocation during translation (Obrig *et al.*, 1971). CHX treatment completely inhibited protein synthesis by 24 hours (Figure 16), with no effect on cell viability (Figure 15), at a concentration of 0.05 µg/mL. This concentration was used for all subsequent studies involving protein synthesis inhibition.

9.4 Cell Viability

The MTT assay was used to assess cell viability under different proteasome inhibitor concentrations (Figure 17) as well as determining cell viability under different CHX concentrations (Figure 15). Since the proteasome is responsible for breakdown of damaged or inactive proteins, inhibiting its activity will cause the buildup of damaged proteins and eventually cell death. The optimal concentration of CBZ used in other studies was 10 μ M (Mircea *et al.*, 2001; Panu *et al.*, 2001). We tested two points below and above this concentration to assess cell viability under these conditions. The adverse affects of inhibiting the proteasome were clear as it reduced cell viability compared to the untreated cells. Although the 10 μ M CBZ concentration did slow down the growth of the cells, it did not completely kill the cells. Since that was the optimal concentration to inhibit the proteasome activity it was used in further studies.

Measurements of cell viability and growth provide valuable tools in a wide range of research areas. Several approaches have been used in the past such as trypan blue. Trypan blue staining is a simple way to evaluate cell membrane integrity (and thus cell death) but the method is not sensitive and cannot be adapted for high-throughput screening (Denizot and Lang, 1986). Lactate dehydrogenase release is another method of measuring cell death (Denizot and Lang, 1986). The MTT assay is a colorimetric dye system that measures the reduction of the yellow tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) to a blue-black formazan product by living (but not by dead) cells (Mosmann 1983). MTT enters the mitochondria of living cells where mitochondrial dehydrogenases cleave this substrate to form the

coloured product. Cells are then lysed in DMSO which solubilizes the product. The colour can then be quantified spectrophotometrically (Mosmann 1983).

The MTT assay was used to assess cell viability under different CHX concentrations. CHX treatment has profound effects on cells as it inhibits all protein synthesis including essential proteins that are critical for the survival of the cell. Cell viability experiments were used in combination with pulse-chase labelling experiments to determine the optimal CHX concentration that will completely inhibit the protein synthesis without affecting cell viability. According to the initial results, we decided to use concentrations of CHX below 20 $\mu\text{g/mL}$. These results provided a range of CHX concentrations that were not immediately lethal to K562 cells and could be used for subsequent treatments.

9.5 Pulse-Chase Labelling of Cellular Proteins

In order to track protein synthesis in cells, proteins from K562 cells were pulse-labelled with [^{35}S] L-methionine and [^{35}S] L-cysteine mixes and treated with a wide range of CHX concentrations (0.01-20 $\mu\text{g/mL}$ of media). Protein labelling was combined with cell viability data to determine the optimal CHX concentration that completely inhibited protein synthesis while still maintaining living cells. For pulse experiments, cells were depleted of sulfur-containing amino acids by incubating them in methionine and cysteine-free media (pulse media). Cells were then grown in medium in which the only sulfur-containing amino acids were radiolabeled. Cells were "pulsed" for a brief period of time, washed with buffer to remove unincorporated isotope and then "chased"

in media containing non-labelled amino acids. Incorporation of label into proteins peaked at 4 hours and then slowly declined to no label incorporation by 24 hours (Figure 16). The decline represents protein degradation when synthesis is completely inhibited by CHX.

9.6 Mature ALAS2 Protein Profile

Over-expression of FLAG-tagged mature ALAS2 in cells resulted in a robust production of the fusion protein as determined by immunoprecipitation and SDS-PAGE (Figure 19). According to Western blot, recombinant ALAS2 expressed in K562 had a normoxic turnover time of approximately 36 hours (Figure 20). Hypoxia and proteasome inhibition resulted in stabilization of recombinant ALAS2 beyond 36 hours. Mutation of the key proline in the LXXLAP sequence of ALAS2 also stabilized the protein under normoxia. This evidence suggests that ALAS2 is hydroxylated under normoxic conditions and is subsequently ubiquitinated by vHL E3 ubiquitin ligase complex.

Fusion proteins have simplified the way in which we characterize, detect and purify proteins. To create a fusion protein that can be effectively detected and purified, the added tag should not interfere with the native folding of the protein of interest to which it is attached (Einhauer and Junbauer, 2001). It is important that the tag is water soluble so that it is exposed on the surface of the protein, presenting the epitope to antibodies. It will also be advantageous to have a marker that is suitable for mild, single-step affinity purification and that can be easily removed. We have chosen FLAG, an eight amino acid peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys), for N-terminal tagging of our

protein of interest (mature ALAS2). It is known that aromatic amino acids are the main factors in the interaction between an antibody and its antigen. The placement of Tyr at position 2 flanked by charged amino acids will make the antibody binding to Tyr much higher (Einhauer and Junbauer, 2001). The hexapeptide sequence (Lys-Asp-Asp-Asp-Asp-Lys) is hydrophilic. As many other tags, the FLAG peptide can be added to the N- or the C-terminus. The C terminal tag has the advantage of ensuring full-length proteins as it will not be synthesized unless the full protein is synthesized. There are a number of commercial anti-FLAG antibodies available in the market (M1, M2 and M5) (Einhauer and Junbauer, 2001). We used the HRP-conjugated anti-FLAG antibody as it negates the need for a secondary antibody. FLAG allowed for a single step affinity purification of recombinant ALAS2 without compromising its function, as measured by total ALAS enzyme activity. Total ALAS activity increased 19-fold in transfected cells compared to non-transfected cells (Figure 18). These results showed that FLAG did not interfere with the function of the recombinant protein produced in cells.

9.7 ALAS2/vHL Interaction

Hydroxylation of the two LXXLAP sequences in HIF-1 α allows for association with vHL, the substrate recognition subunit of E3 ubiquitin ligases. vHL does not associate with non-hydroxylated LXXLAP. The substrate protein is subsequently polyubiquitinated, which targets it for degradation by the proteasome. To test whether vHL associates with hydroxylated ALAS2, K562 were transiently transfected with either FLAG-ALAS2 or FLAG-vHL, immunoprecipitated and Western blotted for the

associated protein (Figure 21). FLAG-ALAS2 pulled down vHL under normoxic conditions (crosslinked) or proteasome inhibition. No vHL was pulled down by mutant ALAS2 immunoprecipitation. Only minute quantities of vHL were pulled down under hypoxic conditions or under normoxic conditions without crosslinking. This suggested that the hydroxylated ALAS2/vHL interaction is weak and that crosslinking was required to pull both proteins in the complex down. According the hypothesis that ALAS2 and vHL interact, it was expected that more vHL would be pulled down with ALAS2 under normoxic than hypoxic conditions. However, only small amounts of vHL coimmunoprecipitated with wildtype FLAG-ALAS2 in both cases. A much larger amount of vHL was pulled down when cellular proteins were crosslinked or when the proteasome was inhibited. From this result, it was concluded that interaction between putatively hydroxylated ALAS2 and vHL is weak and transitory. The other possibility is that cells were overgrown and overcrowded (Figure 21, Lane 6) leading to hypoxia. The former is confirmed by no interaction between mutant ALAS2 and vHL, as well as the crosslinking studies which show that interaction occurs. Chemical crosslinking studies are commonly used in proteomics to show protein-protein interactions and DSP can only crosslink primary amines (side chains of lysines and arginines) that are 12 Å apart (Joshi and Burrows,1990). Increased vHL pulldown with ALAS2 during proteasome inhibition may be due to (overall) more FLAG-ALAS2 present within the cell (Figure 21, Lane 1, bottom panel; compare to Lanes 4, 5, 6 and 7) and the six-fold increase in ALAS activity (indicating increased stability) with 48 hours of proteasome inhibition (Figure 13). These results suggest that the interaction between ALAS2 and vHL occurs and is dependent upon proline 520 modification.

9.8 ALAS2 Ubiquitination

In vitro ubiquitination systems test for the potential ubiquitination of substrate protein through ubiquitin-proteasome pathway. This kit depends on the use of Fraction II from HeLa cells. Fraction II is the protein fraction of a cell extract that binds to an anion exchange resin. It is essentially free of ubiquitin and ATP. FII contains E1, most E2s, some E3s, UCHs, and the proteasome (20S and 26S).

Ubiquitination is the first step for targeting select substrate proteins for proteolytic degradation. The ubiquitin-proteasome pathway is the cell's main mechanism for selectively degrading non-functional proteins. Ubiquitination has a significant role in the regulation of many critical cellular processes like transcription, oncogenesis and cell cycle progression (Lee and Goldberg, 1998). The mechanism by which proteins are selected to be ubiquitinated is not very well understood. Some proteins contain amino acid sequences that are recognized by the ubiquitination machinery. One such sequence is known as the PEST sequence, a short stretch of approximately eight amino acids that is rich in proline, glutamic acid, serine, and threonine. This is very important in the control of the overall amount of protein present in the cell under certain conditions. In some cases, the ubiquitination signal can be masked by an interacting protein if the ubiquitinated protein is part of a protein complex (Lee and Goldberg, 1998). The ubiquitination signal can also be masked by posttranslational modifications, such as phosphorylation. Masking allows for better control of the degradation of select proteins, as a proteins ubiquitination signal need only be unmasked to target it for destruction by

the proteasome. Most ubiquitination signals are usually buried in the hydrophobic core of the protein. This is why partially folded, abnormal, or mutant proteins are more prone to degradation (Lee and Goldberg, 1998).

To test whether ALAS2 has the potential to become ubiquitinated, purified FLAG-ALAS2 was incubated *in vitro* with a ubiquitination system comprised of E1 (activating), E2 (conjugating) and E3 (ligase) enzymes in Fraction II of HeLa cells, ubiquitin, ubiquitin aldehyde, an ATP-regenerating system and proteasome inhibitor. ALAS2 became increasingly ubiquitinated over time with ubiquitination occurring as early as 1 hour of treatment (Figure 22).

Different ubiquitinated proteins have different rates of degradation by the proteasome, dependent largely upon the amino acids present in their amino terminus (Baboshina et al. 2001). In transiently transfected K562 cells, ALAS2 appears to be ubiquitinated as rapidly as it is produced (Figure 8B). This agrees with the finding that ubiquitination of ALAS2 occurs as rapidly as it does with HIF-1 α but degradation occurs at a much slower rate. Under normoxic conditions, the turnover time of HIF-1 α is a matter of minutes, whereas ALAS2 requires hours for degradation. The difference may be due to an additional leucine found approximately 10 amino acids downstream of the hydroxylated proline in HIF-1 α that is a requirement for prolyl hydroxylase recognition (Kageyama et al. 2004). This study found that, although the 9 residue spacing between the proline and leucine in HIF-1 α was not obligatory, the degree of prolyl hydroxylation is enhanced by alteration of the spacing. Conserved leucines occur downstream of the proline 520 in ALAS2, but are separated from the prolines by 15 amino acids. The

extended distance may mean that hydroxylation of ALAS2 occurs at a rate that is slower than HIF-1 α .

Hydroxylation may play a broad role in HIF-1-independent responses to hypoxia. Hydroxylation, under normoxic conditions, may alter the activities of enzymes, transcription factors, receptors, etc. involved in pathways that maintain oxygen homeostasis. The LXXLAP sequence is probably involved in the hydroxylation of other proteins. A recent study (Kuznetsova et al. 2003) has shown that the hyperphosphorylated form of the major subunit of RNA polymerase II, RPB1, is hydroxylated by the HIF-1 α prolyl hydroxylase and is ubiquitinated by the vHL E3 ubiquitin ligase. RPB1 contains the HIF-1 α LXXLAP motif. Hyperphosphorylated RPB1 disappears rapidly from hypoxic cells and the role of phosphorylation in RPB1 hydroxylation remains to be elucidated. Further studies may reveal other proteins regulated in a similar fashion that are involved in maintaining oxygen homeostasis.

The present study is the first to examine the potential regulation of ALAS2, at the level of the protein, by hydroxylation. Although the results shown in this thesis provide good evidence of ALAS2 hydroxylation and vHL/ALAS2 interaction occurs, it is not possible to say with certainty that ALAS2 is hydroxylated. Further experiments that include mass spectroscopy on recombinant proteins are required to substantiate the role of hydroxylation. Currently, there are three human prolyl 4-hydroxylases which regulate HIF by hydroxylating the LXXLAP motif. It is not clear, at this stage, which one of these enzymes is responsible for the potential hydroxylation of ALAS2 in erythroid cells. RNA interference can be used to knock down the cellular expression of individual hydroxylases involved in destabilizing ALAS2. Another aspect that can be further

investigated is the nature of the vHL/ALAS2 interaction (Figure 23). Various methods of measuring protein-protein interactions can be utilized to show that vHL/ALAS2 interaction occurs. As well, other components of the E3 ubiquitin ligase can be examined.

Interest in the *ALAS2* gene comes from its pivotal role in the heme synthesis pathway and in its role in XLSA disease, which is caused by mutations in the *ALAS2* gene. More than 25 different mutations in the erythroid *ALAS2* gene have been identified in XLSA patients (May, 1998). All of these mutations have been single base substitutions changing the amino acid composition or reducing ALAS2 activity and/or stability (May, 1998). ALAS2 deficiency in patients with XLSA lead to a decreased supply of protoporphyrin IX. This will lead to the accumulation of iron in erythroblasts. Iron overload caused by this ALAS2 deficiency will disturb cellular reduction-oxidation state (due to the overgeneration of intracellular ROS) and may result in the shortening of the cellular lifetime. These findings reveal the critical role that ALAS2 has in the cellular differentiation and survival of erythroid cells (May, 1998). In addition, ALAS2 deficiency has been associated with the disruption of protein synthesis, leading to apoptosis of the cell (May, 1998). This makes ALAS2 an excellent therapeutical target which requires further investigation. Future studies may determine the importance of ALAS2 protein regulation in oxygen homeostasis of the organism as well as identify other novel targets of hydroxylases involved in HIF-independent pathways of adaptation to hypoxia.

Figure 23: Hydroxylated ALAS2 a substrate for mammalian E3 ubiquitin ligases?
Proposed mechanism for the breakdown of ALAS2 under normoxic conditions. Association of hydroxylated ALAS2 promotes recognition by vHL and the E3 ubiquitin ligase system composed of CUL-2, elongins B and C, Rbx-1 (a RING-finger protein) and E2 ubiquitin conjugating enzyme. ALAS2 is polyubiquitinated which targets the protein for degradation by the 26S proteasome. Ubiquitin is recycled in the process.

10. REFERENCES

- Baboshina, O.V., Crinelli, R., Siepmann, T.J., and Haas, A.L. 2001. N-end rule specificity within the ubiquitin/proteasome pathway is not an affinity effect. *J. Biol. Chem.* 276: 39428-39437.
- Bichet, S., Wenger, R.H., Camenisch, G., Rolfs, A., Ehleben, W., Porwol, T., Acker, H., Fandrey, J., Bauer, C., and Gassmann, M. 1999. Oxygen tension modulates β -globin switching in embryoid bodies. *FASEB J.* 13: 285–295.
- Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M.C., Estreicher, A., Gasteiger, E., Martin, M.J., Michoud, K., O'Donovan, C., Phan, I., Pilbout, S., and Schneider, M. 2003. The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic Acids Res.* 31(1):365-70
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
- Bruick, R.K. 2003. Oxygen sensing in the hypoxic response pathway: regulation of the hypoxia-inducible transcription factor. *Genes & Dev.* 17: 2614-2623.
- Bruick, R.K., and McKnight, S. L. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science.* 294: 1337-1340.
- Conboy, J.G., Cox, T.C., Bottomley, S.S., Bawden, M.J. and May, B.K. 1992. Human erythroid 5-aminolevulinate synthase. *J. Biol. Chem.* 267 (26): 18753-18758.
- Cross, M.A. and Enver, T. 1997. The lineage commitment of hematopoietic progenitor cells. *Curr. Opin. in Gene. Dev.* 7: 609–613.

- Davis, R.G., Kawai, Y., and Arinze, I.J. 2000. Involvement of G_iα2 in sodium butyrate-induced erythroblastic differentiation of K562 cells. *Biochem. J.* 346: 455-461.
- Denizot, F., and Lang, R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods.* 89(2): 271-277.
- Dreger, M. 2003. Proteome analysis at the level of subcellular structures. *Eur. J. Biochem.* 270: 589-599.
- Dzikaite, V., Hultcrantz, R. and Melefors, O. 2003. The regulatory effect of heme on erythroid aminolevulinate synthase in natural erythroid cells. *Biochimica et Biophysica Acta.* 1630: 19-24.
- Einhauer, A. and Jungbauer, A. 2001. The FLAGTM peptide, a versatile fusion tag for the purification of recombinant proteins. *J. Biochem. Biophys. Methods.* 49: 455-465.
- Ferreira, G.C. 1999. 5-Aminolevulinate synthase and mammalian heme biosynthesis. In: Ferreira, G.C., Moura, J.J.G., Franco, R. (Eds.), *Iron Metabolism. Inorganic Biochemistry and Regulatory Mechanisms.* Wiley-VHC, Weinheim, pp. 15-34.
- Fujita, H, Yamamoto, M., Yamagami, T., Hayashi, N., Sassa, S. 1991. Erythroleukemia differentiation. Distinctive responses of the erythroid-specific and the nonspecific δ-aminolevulinate synthase mRNA. *J. Biol. Chem.* 266: 17494-17502.
- GoodFellow, B.J., Dias, J.S., Ferreira, G.C., Henklein, P., Wray, V., and A.L. Macedo. 2001. The solution structure and heme binding of the presequence of murine 5-aminolevulinate synthase. *FEBS Letters* 503: 325-331.

- Grunea, T., Reinheckel, T., Davies, K.J.A. 1996. Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. *J. Biol. Chem.* 271: 15504–15509.
- Hofer, T., Wenger, R.H., Kramer, M.F., Ferreira, G.C. and Gassmann, M. 2003. Hypoxic up-regulation of erythroid 5-aminolevulinate synthase. *Blood*. 101: 348-350.
- Hunter, A.G. and Ferreira, G.C. 1994. A continuous spectrophotometric assay for 5-aminolevulinate synthase that utilizes substrate cycling. *Anal. Biochem.* 226: 221-224.
- Ihara, N., Akagi, R., Ejiri, K., Kudo, T., Furuyama, K., Fujita, H. 1998. Developmental changes of gene expression in heme metabolic enzymes in rat placenta. *FEBS Letters*. 439:163-167.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G Jr. 2001. HIF α targeted for vHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science*. 292: 464-468.
- Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R. C., Conaway, J. W., Klausner, R. D., and Pause, A. 1999. Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. *Proc. Natl. Acad. Sci. USA*. 96: 12436-12441.
- Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H, Pugh, C. W., and Ratcliffe, P. J. 2001. Targeting of HIF-1 α to the von Hippel

- Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science*. 292: 468-472.
- Jordan, P.M.S., Al-Daihan, S., Alexeev, D., Baxter, R.L., Bottomley, S.S., Kahari I.D., Roy, I., Sarwar, M., Sawyer, L., and Wang S.F. 2003. 5-Aminolevulinic acid synthase: mechanism, mutations and medicine. *Biochim. Biophys. Acta*. 1647: 361-366.
- Kageyama, Y., Koshiji, M., To, K.K.W., Tian, Y., Ratcliffe, P.J., and Huang, L.E. 2004. Leu-574 of human HIF-1 α is a molecular determinant of prolyl hydroxylation. *FASEB J*. 18: 1028-1030.
- Kawasaki, N., Morimoto, K., Tanimoto, T., and Hayakawa, T. 1996. Control of haemoglobin synthesis in erythroid differentiating K562 cells. I. Role of iron in erythroid cell heme synthesis. *Arch. Biochem. Biophys*. 328(2): 289-94.
- Kawasaki, N., Morimoto, K., and Hayakawa, T. 1998. Control of haemoglobin synthesis in erythroid differentiating K562 cells. II. Studies of iron mobilization in erythroid cells by high-performance liquid chromatography-electrochemical detection. *J. Chromatogr. B. Biomed. Sci. Appl*. 705(2): 193-201.
- Klingmuller, U. 1997. The role of tyrosine phosphorylation in proliferation and maturation of erythroid progenitor cells-signals emanating from the erythropoietin receptor, *Eur. J. Biochem*. 249: 637-647.
- Kramer, M.F., Gunaratne, P., and Ferreira, G.C. 2000. Transcriptional regulation of the murine erythroid-specific 5-aminolevulinate synthase gene. *Gene* 247: 153-166.
- Kuznetsova, A. V., Meller, J., Schnell P. O., Nash, J. A., Ignacak, M. L., Sanchez, Y., Conaway, J. W., Conaway, R. C., and Czyzyk-Krzeska, M. F. 2003. Von Hippel-

- Lindau protein binds hyperphosphorylated large subunit of RNA polymerase II through a proline hydroxylation motif and targets it for ubiquitination. *Proc. Natl. Acad. Sci.* 100: 2706-2711.
- Lathrop, J.T. and Timko, M.P. 1993. Regulation by heme of mitochondrial protein transport through a conserved amino acid motif. *Science* 259: 522–525.
- Lee, D.H., Goldberg A.L. 1998. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol.* 8: 397-403.
- Lisztwan, J., Imbert, G., Wirbelauer, C., Gstaiger, M., and Krek, W. 1999. The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. *Genes & Dev.* 13: 1822-33.
- Livingston, D.M., and Shivdasani, R. 2001. Toward mechanism-based cancer care. *JAMA.* 285(5): 588-93.
- Magness, S.T., Tugores, A., Diala, E.S. and Brenner, D.A. 1998. Analysis of the human ferrochelatase promoter in transgenic mice. *Blood.* 92: 320–328
- Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. 2001. Independent function of two destruction domains in hypoxia-inducible factor- α chains activated by prolyl hydroxylation. *EMBO J.* 20: 5197-5206.
- Masson, N. and Ratcliffe, P.J. 2003. HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O(2) levels. *J. Cell Sci.* 116: 3041-3049.
- May, B.K., Dogra, S.C., Sadlon, T.J., Bhasker, C.R., Cox, T.C and Bottomley, S.S. 1995. Molecular regulation of heme biosynthesis in higher vertebrates. *Prog Nucleic Acid Res Mol Biol.* 51:1–51.

- Max-Audit, I., Eleouet, J.F. and Romeo, P.H. 1993. Transcriptional regulation of the pyruvate kinase erythroid-specific promoter. *J. Biol. Chem.* 268: 5431–5437.
- Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. 1999. The tumour suppressor protein vHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature.* 399: 271-275.
- Maxwell, P.H. 2004. HIF-1's relationship to oxygen: simple yet sophisticated. *Cell Cycle* 3: 156-159.
- Meguro, K., Igarashi, K., Yamamoto, M., Fujita, H., and Sassa, S. 1995. The role of erythroid-specific δ -aminolevulinic synthase gene expression in erythroid heme synthesis. *Blood.* 86 (3): 940-948.
- Metzen, E. and Ratcliffe, P.J. 2004. HIF hydroxylation and cellular oxygen sensing. *J. Biol. Chem.* 385: 223-230.
- Mignotte, V., Eleouet, J.F., Raich, N. and Romeo, P.H. 1989. *Cis*- and *trans*-acting elements involved in the regulation of the erythroid promoter of the human porphobilinogen deaminase gene. *Proc. Natl. Acad. Sci.* 86: 6548–6552.
- Mircea, I., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G. 2001. HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science.* 292: 464-468.
- Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* 65 (1-2): 55-63.

- Nagai, T., Igarashi, K., Akasaka, J., Furuyama, K., Fujita, H., Hayashi, N., Yamamoto, M. and Sassa, S. 1998 Regulation of NF-E2 activity in erythroleukemia cell differentiation. *J. Biol. Chem.* 273: 5358–5365.
- Panu, J., Mole, D.R., Tian, Y.M., Wilson M.I., Gielbert, J., Gaskell, S.J., Von Kriegsheim, A., Hebestreit, H.F., Mukeherji, M., Schofield, C.J., Maxwell, P.H., Pugh C.W. and Ratcliffe, P.J. 2001. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science*. 292: 468-472.
- Park, J.W., Chun, Y.S., and Kim, M.S. 2004. Hypoxia-inducible factor 1-related diseases and prospective therapeutic tools. *J Pharmacol Sci.* 94:221-232.
- Ponka, P. 1997. Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells. *Blood*. 89: 1-25.
- Ryter, S.W., and Tyrrell, R.M. 2001. The heme synthesis and degradation pathways: role in oxidant sensitivity. *Free Rad. Biol. Med.* 28 (2): 289-309.
- Sadlon, T.J., Dell'Oso, T., Surinya, K.H. and May, B.K. 1999. Regulation of erythroid 5-aminolevulinate synthase expression during erythropoiesis. *Int. J. Biochem. Cell Biol.* 31: 1153-1167.
- Safran, M. and Kaelin Jr., W.G. 2003. HIF hydroxylation and the mammalian oxygen-sensing pathway. *J. Clin. Invest.* 111: 779-783.
- Semenza, G. 1999. Regulation of mammalian O₂ homeostasis by hypoxia-inducible factor 1. *Annu. Rev. Cell Dev. Biol.* 15: 551-578.
- Semenza, G. 2002. Signal transduction to hypoxia-inducible factor 1. *Biochem. Pharm.* 64: 993-998.

- Shibahara, S., Yoshizawa, M., Suzuki, H., Takeda, K., Meguro, K. and Endo, K. 1993. Functional analysis of cDNAs for two types of human heme oxygenase and evidence for their separate regulation. *J. Biochem.* 113: 214–218.
- Shringarpurea, T., Grunea, T., Davies, K.J.A. 2001. Protein oxidation and 20S proteasome-dependent proteolysis in mammalian cells. *Cell. Mol. Life Sci.* 58: 1442–1450.
- Smith, S.J. and Cox, T.M. 1997. Translational control of erythroid delta-aminolevulinate synthase in immature human erythroid cells by heme. *Cell. Mol. Biol.* 43:103–114.
- Surinya, K.H., Cox, T.C. and May, B.K. 1997. Transcriptional regulation of the human erythroid 5-aminolevulinate synthase gene. *J. Biol. Chem.* 272: 26,585–26,594.
- Taylor B.L., and Zhulin I.B. 1999. PAS domains: internal sensors of oxygen, redox potential and light. *Microbiol. Mol. Biol. Rev.* 63 (2): 479-506.
- Templeton D.M., and Liu Y. 2003. Genetic regulation of cell function in response to iron overload or chelation. *Biochimica et Biophysica Acta.* 1619: 113-124.
- Terajima, M., Nemoto, Y. and Obinata, M. 1995. Inducible expression of erythroid-specific mouse glycophorin gene is regulated by proximal elements and locus control region-like sequence. *J. Biochem.* 118: 593–600.
- Tsai, S.F., Strauss, E. and Orkin, S.H. 1991. Functional analysis and in vivo footprinting implicate the erythroid transcription factor GATA-1 as a positive regulator of its own promoter. *Genes Dev.* 5: 919–931.
- Wall, L. 1987-2001. Perl for Windows v.5.6.1.

- Wenger, R.H. 2000. Mammalian oxygen sensing, signalling and gene regulation. *J. Exp. Biol.* 203: 1253-1263.
- Wenger, R.H. 2002. Cellular adaptation to hypoxia: O₂-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O₂-regulated gene expression. *FASEB J.* 16: 1151-1162.
- Willmore, W.G. 2004. Control of Transcription in Eukaryotic Cells. In: *Functional Metabolism: Regulation and Adaptation*. Kenneth B. Storey (ed). Wiley-Liss, Inc., Hoboken, NJ.
- Worthington, R.E., Bossie-Codreanu, J., and Van Zant, G. 1987. Quantitation of erythroid differentiation in vitro using a sensitive colourimetric assay for hemoglobin. *Exp Hematol.* 15(1):85-92
- Youssoufian, H. 1994. Further characterization of *cis*-acting regulatory sequences in the genomic locus of the murine erythropoietin receptor-evidence for stage-specific regulation. *Blood.* 83: 1428–1435.
- Yu, F., White, S.B., Zhao, Q. and Lee, F.S. 2001. HIF-1 α binding to vHL is regulated by stimulus-sensitive proline hydroxylation, *Proc. Natl. Acad. Sci. USA* 98: 9630-9635.
- Zoller, H., Decristoforo, C. and Weiss, G. 2002. Erythroid 5-aminolevulinate synthase, ferrochelatase and DMT1 expression in erythroid progenitors: differential pathways for erythropoietin and iron-dependent regulation. *Br J Haematol* 118 (2):619-26

11. APPENDIX

11.1. Gene Cloning Into Expression Vectors

This is a general guide for cloning genes into plasmids using total RNA as starting material. The example given is the cloning of a specific gene into a mammalian expression vector. This guide may also be used to clone genes for the sole purpose of sequencing (for novel proteins of which the sequence is unknown), or to express proteins in insect or bacterial cells. The procedure provided below outlines the steps, from the isolation of total RNA, to sequencing the final ligated product. The methods outlined in each step are not exclusive; each may be substituted with others that the researcher is more familiar with. The methods listed have been proven effective by the current investigator for cloning from a wide variety of cell types.

11.1.1. Total RNA Preparation by TRIzol Method (Invitrogen)

Precautions When Working With RNA

RNA isolation must be carried out under conditions that eliminate the chance of RNase contamination. Oak ridge tubes, Eppendorf tubes, pipet tips, and containers should be treated with 0.1 % diethylpyrocarbonate (DEPC) in water overnight and then autoclaved the next day. The water itself can also be autoclaved and used to resuspend total RNA. Wear clean gloves to handle the RNA sample. Do not talk or breathe heavily

towards the sample. Keep the sample on ice when aliquoting and return it to the -80°C as soon as possible. RNase-free tips and tubes should be used whenever possible.

1. Homogenize cells using the following volumes of TRIzol
 - a) Tissues: 1 mL per 50-100 mg tissue using a glass-Teflon or power homogenizer
 - b) Plates: 8.75 mL per large 15 cm plate and pass cell lysate several times through a pipette (two plates must be used for each treatment in order to get 100 µg of total RNA per treatment (see below))
 - c) Suspension: pellet cells by centrifugation
: 1 mL per $5-10 \times 10^6$ cells (animal, plant, yeast) or per 1×10^7 cells (bacteria)
2. Incubate homogenized samples 5 min. at room temperature.
3. Add 0.2 mL chloroform per 1 mL TRIzol reagent (1.75 mL for large plates).
4. Shake gently for 15 sec.
5. Incubate at room temperature for 3 min. For best results (A_{260}/A_{280} close to 2) leave to settle at room temperature for 30 min.
6. Centrifuge at 12,000 X g for 15 min. at 4°C.
7. Remove aqueous phase to a fresh DEPC-treated tube. Add 0.5 mL isopropanol per 1 mL TRIzol used initially (4.38 mL for large plates).
8. Incubate samples at room temperature for 10 min.
9. Centrifuge at 12,000 X g for 10 min. at 4°C.

10. Remove supernatant. Wash with 75% DEPC ethanol (add 1 mL 75% ethanol per 1 mL TRIzol reagent used initially) without resuspending pellet (8.75 mL for large plates).
11. Centrifuge at 7,500 X g for 5 min. at 4°C.
12. Remove wash. Briefly air dry. Resuspend in 20 µL of DEPC-treated water. Read A_{260} and A_{280} nm (ratio should be > 1.6) (1 A_{260} unit of ssRNA = 40 µg/mL).

11.1.2. RNA Isolation Using RNeasy Method (QIAGEN)

1. The cells must be at a density of 1.3×10^6 cells/mL.
2. Add 3 mL of the cell suspension to an Eppendorf tube and centrifuge at 1000 R.P.M.
3. Remove the supernatant and freeze the cell pellet in liquid nitrogen.
4. Take 0.5 mL of the RLT concentrate buffer and add 5 µL of β-mercaptaethanol.
5. Loosen the cells pellet by flicking the tube. Add 350 µL of RLT buffer.
6. Vortex the sample or pipet it up and down to loosen the pellet.
7. Pipet the lysate onto a QIAshredder spin column placed in 2 mL collection tube.
8. Centrifuge for 2 min at maximum speed.
9. Add 350 µL of 70% ethanol to the homogenized lysate in the collection tube and mix well by pipeting up and down.
10. Apply up to 700 µL of the lysate (including the precipitate that has formed) to an RNeasy minicolumn placed in 2 mL collection tube.
11. Centrifuge the sample at high speed for 15 sec. Discard the flow-through liquid.

12. Add 700 μ L buffer RW1 to the RNeasy column.
13. Centrifuge the sample at high speed for 15 sec. Discard the flow through and collection tube.
14. Transfer the RNeasy column into a new collection tube. Add 500 μ L of RPE buffer into the column and centrifuge the sample at high speed for 15 sec. Discard the flow through liquid.
15. Add 500 μ L of RPE buffer into the column and centrifuge the sample at high speed for 2 min.
16. Transfer the RNeasy column to a new 1.5 mL collection tube. Add 30-50 μ L of RNase-free water. Centrifuge the sample at high speed for 1 min. Read A_{260} and A_{280} nm. Save the flow-through and store at -80°C .

11.1.3. Total RNA Formaldehyde Gel

1. Wash beakers, gel tray, cell and combs with 3 % H_2O_2 . Rinse with dd H_2O and rinse with 100% methanol. Let air dry.
2. Remove a volume of RNA solution that will give a total of 16 μ g of total RNA and place in a 0.5 mL Eppendorf tube. Bring the volume up to 5 μ L with RNase-free water.
3. Melt 3 gm of agarose in 186.4 mL of DEPC-treated water for large gel format in the microwave.
4. Leave the melted agarose in a 60°C water bath.

5. After cooling to 60°C, add 50 mL of 5 X formaldehyde gel running buffer and gently stir it in.
6. Add 13.6 mL of 37 % formaldehyde and gently stir it in. TRY TO AVOID CREATING BUBBLES WHILE STIRRING.
7. Pour mix into the gel tray and let it solidify.
8. While the gel is solidifying, premix the following sample buffer in an RNase-free Eppendorf tube:
 - a) Number of samples X 3 μ L of 5X formaldehyde gel running buffer per sample.
 - b) Number of samples X 5.25 μ L of 37 % formaldehyde buffer per sample.
 - c) Number of samples X 15 μ L of formamide per sample.
9. Do not place the sample buffer on ice. Formaldehyde in aqueous solutions will precipitate at 4°C.
10. Add 17 μ L of the above premix to each sample of 5 μ L of RNA and to the 0.24-0.95 Kb RNA ladder (use 7 μ l of the 1 μ g/ μ L stock (Invitrogen))
11. Incubate samples and standards for 15 min. at 55°C.
12. Place samples immediately on ice for 2 min. and then microfuge at maximum speed.
13. Alternatively, you can heat RNA at 65-70°C for 3-4 min., place samples on ice and then add the 17 μ L of the premixed sample buffer.
14. It is best to tape the gel tray to the gel running apparatus.

15. Pre-run the gel for 10 min. at 90 V. This eliminates residual RNases that may have been on the gel comb.
16. Add 4 μ L of sterile DEPC-treated formaldehyde loading buffer to each sample and standard.
17. Immediately load the samples and standards into the gel. Run the gel in 1 X formaldehyde gel running buffer at 90 V until the dye front has migrated 8 cm from the bottom of the gel (approximately 2/3 of the way down the gel).
18. After the run is complete cut off the bottom, unused portion of the gel as well as just above the gel wells.
19. Rinse the gel 3 times in normal ddH₂O before staining.
20. Stain in 400 mL of normal ddH₂O containing 30 μ L of a 10 mg/mL stock solution of ethidium bromide. Stain for 30 min. at room temperature with gentle shaking.
21. Destain in normal ddH₂O for 30 min. at room temperature with gentle shaking.
22. Wash in 400 mL of 10 X SSC for 20 min. Take a picture of the gel.

Solutions for Total RNA Formaldehyde Gels

10 X MOPS Buffer

500 mL. Bring to a final pH of 5.5-7.0. Store in the dark.

0.2 M MOPS (3-N-morpholino-propanesulfonic acid)

$$0.2 \text{ moles/L} \times 0.5 \text{ L} \times 209.3 \text{ g/mole} = 20.93 \text{ g}$$

10 mM EDTA

$$0.01 \text{ moles/L} \times 0.5 \text{ L} \times 372.24 \text{ g/mole} = 1.861 \text{ g}$$

50 mM Sodium acetate

$$0.050 \text{ moles/L} \times 0.5 \text{ L} \times 136.08 \text{ g/mole} = 3.402 \text{ g}$$

5X Formaldehyde Gel Running Buffer

1 L. Store at room temperature.

0.1 M MOPS

$$0.1 \text{ moles/L} \times 1 \text{ L} \times 209.3 \text{ g/mole} = 20.93 \text{ g}$$

5 mM EDTA

$$0.005 \text{ moles/L} \times 1 \text{ L} \times 372.24 \text{ g/mole} = 1.861 \text{ g}$$

40 mM Sodium acetate

$$0.040 \text{ moles/L} \times 1 \text{ L} \times 136.08 \text{ g/mole} = 5.443 \text{ g}$$

Dissolve 20.93 g of MOPS in 800 mL of DEPC-treated water 50 mM sodium acetate. Adjust the pH to pH 7.0 with 2 N HCl. Adjust the volume to 1 L with DEPC-treated water.

Pass through a sterile 0.2-micron Millipore filter.

20 X SSC Buffer

1 L. Store at room temperature.

3M NaCl

$$3 \text{ moles/L} \times 1 \text{ L} \times 58.44 \text{ g/mole} = 175.32 \text{ g}$$

0.3 M Na₃ citrate•2H₂O

$$0.3 \text{ moles/L} \times 1 \text{ L} \times 294.1 \text{ g/mole} = 88.23 \text{ g}$$

Adjust pH to 7.0 with 1 M HCl

Formaldehyde Gel Loading Buffer

Store at -20°C .

0.72 mL Formamide

0.16 mL 10 X MOPS buffer

0.26 mL Formaldehyde

0.2 mL DEPC-treated water

0.08 mL glycerol

0.08 mL bromophenol blue from 0.5% w/v saturated solution

11.1.4. Reverse Transcription Using Oligo dT Primer

First-strand (complementary DNA or cDNA) synthesis is carried out using oligo (dT)₁₈ as a primer. This converts all mRNA to cDNA as opposed to gene-specific primers which only convert one gene to cDNA. Many kits are available for first-strand synthesis (MBI Fermentas RevertAid H Minus kits work well). The RevertAid H Minus reverse transcriptase is a genetically engineered version of the Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV RT) which lacks ribonuclease H activity specific to RNA in RNA-DNA hybrids. Therefore, the original RNA template is not removed during first strand cDNA synthesis, resulting from higher yields of full length cDNA from longer templates.

1. Use 10 ng to 5 µg of total RNA in the following mixture:

Oligo (dT) ₁₈ primer (0.5 µg/µL)	1 µL
DEPC-treated water	up to 12 µL

2. Mix gently and spin down for 3-5 sec. in a microcentrifuge.
3. Incubate mixture at 70°C for 5 min., chill on ice and collect drops by brief centrifugation.
4. Place the tube on ice and add the following components in the indicated order:

5 X Reaction Buffer	4 µL
Ribonuclease inhibitor (20 U/µL)	1 µL
10 mM dNTP mix	2 µL
5. Mix gently and collect drops by brief centrifugation.
6. Incubate at 37°C for 5 min.
7. Add: RevertAid H Minus M-MuLV-RT (200 U/µL) 1 µL
8. Incubate mixture at 42°C for 60 min.
9. Stop the reaction by heating at 70°C for 10 min. Chill on ice.
10. Ethanol precipitate the synthesized single stranded cDNA (ss-cDNA). Mix with 5 µL of 3 M sodium acetate and 125 µL of 100 % ethanol. Place at -80°C for 10 min. Centrifuge at full speed (~14000 X g) for 10 min. at 4°C.
11. Rinse ss-cDNA pellet with 100 µL of 70 % ethanol and speed dry for 1 min.
12. Resuspend ss-cDNA pellet in 20 µL of sterile water. Store at -20°C.

11.1.5. PCR Using Gene-Specific Primers With Adaptors

1. On ice, add the following components to a sterile 0.5 mL Eppendorf tube:

Components	Volume	Final Concentration
10 X PCR buffer minus Mg	10 μ L	1 X
10 mM dNTP mixture	2 μ L	0.2 mM each
50 mM MgCl ₂	3 μ L	1.5 mM
Primer Mix (10 μ M each)	5 μ L	0.5 μ M
Template DNA	1-20 μ L	
Taq DNA polymerase (5 U/ μ L)	0.5 μ L	2.5 Units
Autoclaved distilled water	Up to 100 μ L	

2. If desired, a master mix can be prepared to minimize reagent loss and to enable accurate pipetting. Mix contents of tube. Cap tubes and centrifuge briefly to collect the contents to the bottom.
3. Incubate tubes in a thermal cycler at 94°C for 3 min. to completely denature template.
4. Perform 25-35 cycles of PCR amplifications as follows:

Denature	94°C for 45 sec.
Anneal	55°C for 30 sec.
Extend	72°C for 1 min. and 30 sec.
5. Incubate for additional 10 min. at 72°C and maintain the reaction at 4°C. The sample can be stored at -20°C until use.
6. Analyse the amplification products by agarose gel electrophoresis and visualize by ethidium bromide staining. Use appropriate DNA molecular weight standards.

11.1.6. TOPO TA Cloning Into pCR2.1 (Invitrogen)

The TOPO TA cloning kit (Invitrogen) provides a one step direct ligation of a *Taq* polymerase amplified PCR product into the mammalian expression vector pCR2.1. TA cloning is made possible by the non-template dependent terminal transferase activity of *Taq* that adds a single deoxyadenosine (A) to the 3' ends of PCR products. The pCR2.1 vector has a single, overhanging 3' deoxythymidine (T), which allows it to ligate to the PCR product. It is important to have a fresh solution of the PCR product (less than one day old) to start the TOPO TA cloning into the vector.

The table below describes how to set up your TOPO® Cloning reaction (6 µl) for eventual transformation into chemically competent TOP10 or TOP10F' One Shot® *E. coli*.

1. Set up the following 6 µL reaction:

Reagent	For Chemically Competent <i>E. coli</i>
Fresh PCR Product	0.5 to 4 µL
Salt Solution	1 µL
Sterile Water	add up to a total volume of 5 µL
TOPO® vector	1 µL
Final Volume	6 µL

2. Mix reaction gently and incubate for 5 min. at room temperature (22-23°C).
3. Place tubes on ice. Proceed to One Shot® Chemical Transformation.

Procedure for Transformation

1. Centrifuge the vials containing the ligation reactions briefly and place them on ice.
2. Thaw (on ice) one 50 μ L vial of frozen One Shot[®] TOP10F' competent cells for each ligation/transformation reaction.
3. Pipette 2 μ L of each ligation reaction directly into the competent cells and mix by stirring gently with the pipette tip. Do not pipette up and down.
4. Incubate the vials on ice for 30 min. Store the remaining ligation mixtures at -20°C.
5. Heat shock for exactly 30 sec. in a 42°C water bath. Do not mix or shake.
6. Remove the vials from the 42°C water bath and immediately place on ice for 2 min.
7. Add 250 μ L of SOC medium (at room temperature) to each tube.
8. Shake the vials at 37°C for 1 hour at 225 rpm in a shaking incubator.
9. Spread 10-50 μ L from each transformation vial on separate, labelled LB agar plates containing either 25 μ g/mL kanamycin or 50 μ g/mL ampicillin. Make sure all the liquid is absorbed, then invert the plates and place them in a 37°C incubator overnight. **Note:** Plate two different volumes to ensure that at least one plate has well-spaced colonies. When plating small volumes (< 20 μ L), add 20 μ L SOC to ensure even spreading.
10. You should obtain 100-400 colonies per plate depending on the volume plated.

11.1.7. Restriction Analysis

11. Pick at least 10 colonies and inoculate into 3 ml of LB containing 50 µg/mL ampicillin or 25 µg/mL kanamycin.
12. Isolate plasmid DNA and analyse by restriction mapping or sequencing for the presence and orientation of the insert. Use universal primers (T7, T3, M13), the priming sites of which are found on the vector, to do sequencing.
13. Once you have confirmed that you have the correct clone, be sure to prepare a glycerol stock (50% bacteria and 50% of 50% sterile-filtered glycerol) for long-term storage at -80°C.

11.2. Site Directed Mutagenesis (Stratagene QuikChange Kit)

11.2.1 PCR Mutagenesis

Ensure that the plasmid DNA template is isolated from a *dam*⁺ *E. coli* strain. The majority of the commonly used *E. coli* strains are *dam*⁺. Plasmid DNA isolated from *dam*⁻ strains (e.g. JM110 and SCS110) is not suitable. To maximize temperature-cycling performance, Stratagene strongly recommends using thin-walled tubes, which ensure ideal contact with the temperature cycler's heat blocks. The following protocols were optimized using thin-walled tubes.

1. Synthesize two complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence.

2. Prepare the **control reaction** as indicated below:

5 μL of 10 X reaction buffer

2 μL (10 ng) of pWhitescript 4.5-kb control plasmid (5 ng/ μL)

1.25 μL (125 ng) oligonucleotide control primer #1 [34-mer (100 ng/ μL)]

1.25 μL (125 ng) oligonucleotide control primer #2 [34-mer (100 ng/ μL)]

1 μL of dNTP mix

39.5 μL of double-distilled water (ddH₂O) to a final volume of 50 μL

3. Then add 1 μL of *PfuTurbo* DNA polymerase (2.5 U/ μL).

4. Prepare the **sample reaction(s)** as indicated below:

Note Stratagene recommends setting up a series of sample reactions using various concentrations of ds-DNA template ranging from 5 to 50 ng (e.g., 5, 10, 20, and 50 ng of ds-DNA template) while keeping the primer concentration constant.

5 μL of 10 X reaction buffer

X μL (5–50 ng) of ds-DNA template

X μL (125 ng) oligonucleotide primer #1

X μL (125 ng) oligonucleotide primer #2

1 μL of dNTP mix

ddH₂O to a final volume of 50 μL

14. Then add 1 μL of *PfuTurbo* DNA polymerase (2.5 U/ μL).

5. Parameters for thermal cycler

Segment	Cycles	Temperature	Time
1	1	95°C	30 sec
2	12	95°C	30 sec
		55°C	1 min
		68°C	7 min

For the control reaction, use a 5-min. extension time and run the reaction for 12 cycles.

- Following temperature cycling, place the reaction on ice for 2 min. to cool the reaction to approximately 37°C.

Note: If desired, amplification may be checked by electrophoresis of 10 μ L of the product on a 1% agarose gel. A band may or may not be visualized at this stage.

In either case proceed with Dpn I digestion and transformation.

11.2.2. Dpn I Digestion of the Amplification Products

- Add 1 μ L of the *Dpn* I restriction enzyme (10 U/ μ L) directly to each amplification reaction.
- Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times.
- Spin down the reaction mixtures in a microcentrifuge for 1 min. and immediately incubate each reaction at 37°C for 1 hour to digest the parental (i.e., the nonmutated) supercoiled dsDNA).

11.2.3. Transformation of XL1-Blue Supercompetent Cells

Note: If the mutagenized plasmid contains only the tetR resistance marker, an alternative tetracycline-sensitive strain of competent cells must be used.

1. Gently thaw the XL1-Blue supercompetent cells on ice. For each control and sample reaction to be transformed, aliquot 50 μ L of the supercompetent cells to a *prechilled* Falcon[®] 2059 polypropylene tube.
2. Transfer 1 μ L of the *Dpn* I-treated DNA from each control and sample reaction to separate aliquots of the supercompetent cells.
3. Swirl the transformation reactions gently to mix and incubate the reactions on ice for 30 min.
4. Heat pulse the transformation reactions for 45 sec. at 42°C and then place the reactions on ice for 2 min. *Note This heat pulse has been optimized for transformation in Falcon 2059 polypropylene tubes.*
5. Add 0.5 mL of NZY+ broth preheated to 42°C and incubate the transformation reactions at 37°C for 1 hour with shaking at 225–250 R.P.M.
6. Plate an appropriate volume of each transformation reaction (50-200 μ L) on agar plates containing the appropriate antibiotic for the plasmid vector.
7. For the mutagenesis and transformation controls, spread cells on LB–ampicillin agar plates containing 80 μ g/mL X-gal and 20 mM IPTG.
8. Incubate the transformation plates at 37°C for >16 hours.

11.3. Mini-Prep Plasmid Isolation (GenElute, Sigma)

Before you start thoroughly mix reagents. Examine reagents for precipitation. If any reagent forms a precipitate, warm at 55-65°C until the precipitate dissolves and allow to cool to room temperature before use.

Resuspension Solution: Spin the tube of the RNase A Solution briefly to collect the solution in the bottom of the tube. Add 13 μ L (for 10 prep package), 78 μ L (for 70 prep package) or 500 μ L (for 350 prep package) of the RNase A Solution to the Resuspension Solution prior to initial use. Store at 4 °C.

Wash Solution: Dilute the Wash Solution Concentrate with 10 mL (10 prep package), 100 mL (70 prep package) or 300 mL (350 prep package) of 95-100% ethanol prior to initial use. After each use, tightly cap the diluted wash solution to prevent the evaporation of ethanol.

1. **Prerun.** Prior to using the kit, run 500 mL of 1 N sodium hydroxide through the GenElute Miniprep Binding Column.
2. **Harvest cells.** Pellet 1-5 mL of an overnight recombinant *E. coli* culture by centrifugation. The optimum volume of culture to use depends upon the plasmid and culture density. For best yields, follow the instructions in the note below. Transfer the appropriate volume of the recombinant *E. coli* culture to a microcentrifuge tube and pellet cells at $\geq 12,000 \times g$ for 1 min. Discard the

supernatant. Note: For best results with recombinant *E. coli* grown in LB (Luria Broth), use 1-3 mL of culture for high copy plasmids or 1-5 mL of culture for low copy plasmids. With recombinant *E. coli* grown in rich media such as TB (Terrific Broth) or 2 X YT, use only 1 mL of culture. Higher volumes can cause a reduction in yield.

3. **Resuspend cells.** Prior to first time use, be sure to add the appropriate volume of the RNase A Solution to the Resuspension Solution. Completely resuspend the bacterial pellet with 200 μ L of the Resuspension Solution. Vortex or pipet up and down to thoroughly resuspend the cells until homogeneous. Incomplete resuspension will result in poor recovery.
4. **Lyse cells.** Lyse the resuspended cells by adding 200 μ L of the Lysis Solution. Immediately mix the contents by gentle inversion (6-8 times) until the mixture becomes clear and viscous. Do not vortex. Harsh mixing will shear genomic DNA, resulting in chromosomal DNA contamination in the final recovered plasmid DNA. Do not allow the lysis reaction to exceed 5 min. Prolonged alkaline lysis may permanently denature supercoiled plasmid DNA that may render it unsuitable for most downstream applications.
5. **Neutralize.** Precipitate the cell debris by adding 350 μ L of the Neutralization/Binding Solution. Gently invert the tube 4-6 times. Pellet the cell debris by centrifuging at 12,000 X g or maximum speed for 10 min. Cell debris, proteins, lipids, SDS, and chromosomal DNA should fall out of solution as a cloudy, viscous precipitate. If the supernatant contains a large amount of floating

particulates after centrifugation, recentrifuge the supernatant before proceeding to Step 7.

6. **Prepare Column.** Insert a GenElute Miniprep Binding Column (with a blue o-ring) into a provided microcentrifuge tube, if not already assembled. Add 500 μL of the Column Preparation Solution to each miniprep column and centrifuge at 12,000 X g for 30 sec to 1 min. Discard the flow-through liquid. **Note:** The Column Preparation Solution maximizes binding of DNA to the membrane resulting in more consistent yields. Load cleared lysate. Transfer the cleared lysate from Step 5 to the column prepared in Step 6 and centrifuge at 12,000 x g for 30 sec. to 1 min. Discard the flow-through liquid.
7. **Wash column.** Prior to first time use, be sure to add ethanol to the concentrated Wash Solution. Add 750 μL of the diluted Wash Solution to the column. Centrifuge at 12,000 X g for 30 sec. to 1 min. The column wash step removes residual salt and other contaminants introduced during the column load. Discard the flow-through liquid and centrifuge again at maximum speed for 1 to 2 min. without any additional Wash Solution to remove excess ethanol.
8. **Elute DNA.** Transfer the column to a fresh collection tube. Add 100 μL of Elution Solution or molecular biology reagent water to the column. For DNA sequencing and other enzymatic applications, use water or 5 mM Tris-HCl, pH 8.0, as an eluant. Centrifuge at 12,000 X g for 1 min. The DNA is now present in the eluate and is ready for immediate use or storage at $-20\text{ }^{\circ}\text{C}$. **Note:** If a more concentrated plasmid DNA preparation is required, the elution volume may be

reduced to a minimum of 50 μ L. However, this may result in a reduction in the total plasmid DNA yield.

11.4. Gene Cleaning DNA From Agarose Gels Using Glassmilk

After PCR or restriction digestion, run the DNA on a 1% agarose gel. Then proceed with gene cleaning with sodium iodide and glassmilk.

1. Cut the DNA insert or any piece of interest from the gel on UV box and place in a preweighted Eppendorf tube.
2. Determine the weight of the gel.
3. Add 3 volumes of NaI solution and heat at 55°C for about 5 min.
4. While waiting for the agarose to dissolve make sure that the glassmilk is suspended in sterile water.
5. After making sure that all the agarose is melted, cool it down on ice for 3 min.
6. Add 5 μ L of well suspended glassmilk solution and mix well.
7. If the size of the insert is above 1000 b.p. then leave on ice for 15 min. If it is less than 1000 b.p. then leave it for about 5 min. only.
8. Microcentrifuge at maximum speed for 30 sec.
9. Remove the supernatant completely.
10. Wash pellet with 400 μ L of NewWash solution, suspend by pipetting up and down or by tapping on the tube.
11. Microfuge at maximum speed for 25 sec.
12. Repeat steps 10 and 11 one more time.

13. Remove the NewWash solution completely.
14. Centrifuge and remove the traces of the NewWash solution with a 10 μ L pipette tip.
15. Resuspended the pellet in 5 μ L of sterile water using the pipette tip to gently resuspend the pellet.
16. Heat at 55°C for 3 min.
17. Microcentrifuge at max speed for 60 sec.
18. Remove the supernatant to a new sterile Eppendorf tube. Avoid taking up any of the glassmilk.
19. Repeat steps 15-18 once more to have 10 μ L of gel cleaned product.
20. Microcentrifuge at max speed for 60 sec.
21. **VERY CAREFULLY** remove the supernatant to a new sterile Eppendorf tube. Avoid taking up any of the glassmilk (some volume may need to be left behind to avoid the glassmilk). These added steps ensure that **NO** residual glassmilk is left in your sample which could interfere with subsequent processes.

11.5. Metabolic Labelling of Proteins

11.5.1. Metabolic Labelling with ^{35}S Methionine and Cysteine

1. Thaw [^{35}S] methionine-cysteine at room temperature and prepare a 0.2 mCi/mL working solution in prewarmed (37°C) pulse-labelling medium.

2. Harvest 2×10^7 cells in suspension by centrifuging for 5 min. at 500 RPM and room temperature.
3. Resuspend cells with 10 mL of prewarmed pulse-labelling medium. Centrifuge for 5 min. at 500 R.P.M. at room temperature and aspirate off the supernatant.
4. Resuspend cells by simply tapping the bottom of the tube and repeat wash.
5. Resuspend cells at 5×10^6 cells/mL in a prewarmed pulse-labelling medium and incubate 15 min. in a 37°C water bath to deplete intracellular pool of methionine and cysteine. Resuspend cells periodically.
6. Centrifuging at 500 R.P.M. at room temperature and aspirate off the supernatant.
7. Resuspend cells in a fresh 15 mL centrifuge tube using 2 mL [^{35}S] methionine-cysteine working solution.
8. Incubate cells 30 min. in a 37°C water bath resuspending frequently by gentle inversion of the tubes.
9. Centrifuging at 500 R.P.M. (4°C) and aspirate off the supernatant. Wash once with 10 mL chase medium.
10. Incubate cells for the desired time at 37°C in chase medium. Suspension cells are incubated at 2×10^6 cells/mL if harvested within 2 hours. If incubated for longer times, cells are suspended at 0.5×10^6 cells/mL.
11. Incubate for the desired time and then harvest 0.5 mL of the cell suspension into an Eppendorf tube and spin at maximum speed (13,000 R.P.M.) for 5 min. and then wash once with ice-cold PBS and freeze in liquid nitrogen.
12. Determine the amount of ^{35}S incorporation by TCA precipitation.

11.5.2. TCA Precipitation of Label Incorporation

1. Add 0.1 mL BSA/ NaN_3 to the frozen cell pellet and thaw them out. Place on ice.
2. Add 1 mL ice-cold 10% (w/v) TCA solution. Vortex vigorously. Take 500 μL of suspension and put in a scintillation vial with 5 mL scintillation fluid and incubate the rest on ice for 30 min. on ice. This is used to measure the total amount of radioactivity. TCA can be stored up to three months at 4°C.
3. Filter 500 μL of the suspension onto 2.5 cm glass microfiber filter disks in a filtration apparatus under vacuum.
4. Wash the disk twice with 5 mL ice cold 10% w/v TCA solution and twice with ethanol.
5. Air dry for 30 min
6. Transfer disk from 4 and 2 to 20 mL scintillation vials, add 5 mL of scintillation fluid and measure in a scintillation counter.
7. Calculate the ration of TCA-perceptible label to total radioactivity. Take the ratio of the sample radioactivity in step 4 to total radioactivity in step 2.

11.5.3. Dialysis of Fetal Calf Serum

11.5.3.1 Washing Dialysis Tubes

1. Cut the dialysis tubing into strips.

2. Place the dialysis tubing strips into a beaker of 10 mM EDTA PBS. Bring the solution to 80°C and incubate for 30 min.
3. Pour off the liquid and add fresh 10 mM EDTA PBS.
4. Incubate the solution and tubing over night at room temperature.
5. Pour off the solution and immerse the tubing in 95% Ethanol for 30 min.
6. Pour off the Ethanol and store the dialysis tubing strips in Dialysis Storage Buffer (5 mM EDTA and 47% (v/v)).

11.5.3.2. Dialysis of Protein Sample

Dialysis tubing can be purchased in two forms: one arrives as a "dry" roll in a box while the other is wet and stored in a liquid buffer. The dry roll must be pre-treated before use. This involves soaking the membrane in ddH₂O, heating the membrane to 60°C in Bicarbonate Solution (2% (w/v) NaH₂CO₃ and 1 mM EDTA), and rinsing in ddH₂O. The wet membrane has already been pre-treated for use and only needs to be rinsed in ddH₂O to remove preservatives.

1. Cut the right size of tubing and place the protein sample inside it.
2. Insert the dialysis tubing containing your protein into Dialysis Buffer in a large beaker (PBS). The volume of the buffer should be at least 100 times the original volume of the protein sample.
3. Stir the buffer slowly with a stir bar and magnetic stir plate for at least 1.5 hours.
4. Discard the PBS and replace with the same volume of fresh PBS stir overnight.

The exchange of solution components between the protein sample and the Dialysis Buffer is most efficient if carried out for longer than 1.5 hr. This should be empirically determined. However, an overnight incubation is often optimal for removal of small molecules from a protein sample.

Solutions to be Made for Protein Dialysis

Pulse labelling medium: Use cysteine and methionine free DMEM with 10% dialyzed PBS. Add 25 mM HEPES (with pH adjusted to 7.4 with NaOH). Store at 4°C for two weeks.

Chase medium: Normal medium (10% FBS, 1% PSA and 89% of specified medium) with 150 mg/L of methionine and cystine added to it.

11.6 Total ALAS Enzyme Activity Assay

Assay Components (in cuvette; total volume = 1.0 mL)

<u>Final Concentration</u>	<u>μL Stock Solution</u>
20 mM HEPES, pH 7.2	100
1 mM α-ketoglutarate	100
10 mM NAD ⁺	100
3 mM MgCl ₂	100
0.25 mM thiamine pyrophosphate	100
100 mM glycine	100

20 μ M succinyl-CoA 10

0.25 Units α -ketoglutarate dehydrogenase 40

Add water to a final volume of 1000 μ L.

Stock Solutions For Total ALAS Enzyme Activity Assay

Stock Solution 1

Make 50 mL in water.

Add 100 μ L per 1000 μ L reaction.

200 mM HEPES, pH 7.2

$$0.2 \text{ moles/L} \times 0.05 \text{ L} \times 238.3 \text{ g/mole} = 2.3830 \text{ g}$$

Stock Solution 2

Make 50 mL in water.

Add 100 μ L per 1000 μ L reaction.

10 mM α -ketoglutarate

$$0.01 \text{ moles/L} \times 0.05 \text{ L} \times 190.1 \text{ g/mole} = 0.0951 \text{ g}$$

Stock Solution 3

Make 1 mL in 20 mM Tris-HCl.

Add 100 μ L per 1000 μ L reaction.

100 mM NAD⁺

$$0.100 \text{ moles/L} \times 0.005 \text{ L} \times 663.4 \text{ g/mole} = 0.3317 \text{ g}$$

Stock Solution 4

Make 50 mL in water.

Add 100 μL per 1000 μL reaction.

30 mM MgCl_2

$$0.03 \text{ moles/L} \times 0.05 \text{ L} \times 95.21 \text{ g/mole} = 0.1428 \text{ g}$$

Stock Solution 5

Make 50 mL in water.

Add 100 μL per 1000 μL reaction.

2.5 mM thiamine pyrophosphate

$$0.0025 \text{ moles/L} \times 0.05 \text{ L} \times 460.8 \text{ g/mole} = 0.0576 \text{ g}$$

Stock Solution 6

Make 5 mL in water.

Add 100 μL per 1000 μL reaction.

1 M glycine

$$1 \text{ moles/L} \times 0.005 \text{ L} \times 75.07 \text{ g/mole} = 0.3750 \text{ g}$$

Stock Solution 7

Make 5 mL in water.

Add 10 μL per 1000 μL reaction.

Sold as 5 mg in total.

2 mM succinyl-CoA

$$0.002 \text{ moles/L} \times 0.00288 \text{ L} \times 867.6 \text{ g/mole} = 0.005 \text{ g}$$

Stock Solution 8

40 μL α -ketoglutarate dehydrogenase (Sigma K1502)

Added directly to reaction.

Homogenize cells in 20 mM HEPES, pH 7.2. Add α -ketoglutarate dehydrogenase **JUST** prior to doing assay. Initiate reaction with addition of 5-aminolevulinic acid synthase (cell lysate). Run blanks with cell lysate and everything else except glycine and succinyl-CoA. Subtract blank rates from full assay rates. The background is very high, especially when unpurified cell lysate is used. The background is due to the presence of dehydrogenases present in cell lysate that will use NAD^+ as its substrate (lactate dehydrogenase and others). To overcome this problem the cell lysate should be very high in protein. If you are growing K562 cells, then this should be around 10 mL of 10^6 to 10^7 cells/mL. Start the treatment at a cell density of about 0.5×10^6 cells/mL. Cells are treated with 0.5 mM of NaBu to differentiate them. Measure rate of reaction at 340 nm [$\epsilon_{340} = 6560$] at 30°C over a period of 10 min. One unit of enzyme activity is defined as the activity to convert 1 nmol of substrate to product per hour at 30°C. Activities are reported as nanomoles of NADH produced per hour.

11.7. Hemoglobin Measurement in K562 Cells

This assay is modified for K562 cells from the original protocol which was published in Analytical Biochemistry by Kaiha *et al.* (1985). K562 cells are grown in IMDM media with 10% fetal calf serum and 1% antibiotic/antimycotic. K562 cells are differentiated with NaBu. In our hands, two different concentrations of NaBu were tested (1.5 and 0.5 mM). The time points for this assay are 0, 24, 48 and 72 hours after NaBu treatment. Cells are plated at 0.5×10^6 cells/mL. Five mL of cells at that density are harvested and washed with PBS and stored at -80°C .

1. Lyse cells in 550 to 600 μL of 0.01% (v/v) NP-40 in water.
2. One aliquot of the cell lysate is used for protein quantitation (Bio-Rad Protein Assay Dye Reagent).
3. Add 15 μL of 1% DAF (w/v)/1.5 mL of assay buffer/sample depending on the number of samples.
4. Added the above mix to 500 μL of the supernatant of the lysed cells.
5. After incubation for 12 min. at 25°C, the absorbance was measured at 610 nm.

Use bovine hemoglobin (Sigma) as a standard for determining the hemoglobin concentration in samples. The points for the standard curve can range from 1-40 μg bovine hemoglobin/mL.

Stock Solutions For Hemoglobin Measurements

Lysis Buffer

0.01 % NP-40 (v/v) in water

Add 10 μL of NP-40 to 100 mL of water.

2,7-Diaminofluorene (DAF)

Make 1 mL in 90% glacial acetic acid

Stable for a week when stored in the dark at 4°C.

1% DAF

Add 10 mg for 1 mL of 90% glacial acetic acid.

Make 10 mL of 90% glacial acetic acid by adding 1 mL of water to 9 mL of glacial acetic acid.

Assay Buffer (pH 7.0) 50 mL

Make 50 mL in water.

100 mM Tris Phosphate

$$0.1 \text{ moles/L} \times 0.050 \text{ L} \times 340.3 \text{ g/mole} = 1.7015$$

6M Urea

$$6 \text{ moles/L} \times 0.05 \text{ L} \times 60.06 \text{ g/mole} = 18.02 \text{ g}$$

0.18% H₂O₂ (w/v)

Make from 30% H₂O₂ (w/v) stock solution.

Add 300 μ L of the 30% stock to the 50 mL of assay solution.