A METABOLOMICS ANALYSIS TO DEFINE THE SOURCE OF HEME PRECURSORS DURING ERYTHROPOIESIS

by

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(Under the Direction of Harry A. Dailey)

ABSTRACT

Heme is a cofactor essential to a vast array of metabolic and regulatory pathways. Although the eight enzymatic steps of heme synthesis have been thoroughly characterized, the source of carbon for succinyl-CoA, a substrate for the first step in heme synthesis, has received only modest attention. During erythropoiesis, the demand for heme to supply hemoglobinization necessitates a robust means to replenish succinyl-CoA. To address this question we carried out a metabolomics-based approach that employed ^{13}C labeling. These experiments demonstrated that glutamine efficiently supplies carbons for heme synthesis and that inhibition of glutamine metabolism with a pan transaminase inhibitor halts erythroid differentiation by impairing heme synthesis. Comparing activities of tricarboxcylic acid (TCA) cycle enzymes in differentiated versus undifferentiated mouse erythroleukemia cells revealed that α-ketoglutarate dehydrogenase (KDH) activity increases during erythroid differentiation. Furthermore, we identified a

protein-protein interaction between KDH and aminolevulinic acid synthase 2 (ALAS2), the erythroid specific form of the first enzyme in the heme synthesis pathway. This interaction increased the activity of both KDH and ALAS2 when low levels of CoA were used. Our data illustrates that there is a metabolic shift during erythroid differentiation and that this shift relies heavily upon glutamine metabolism.

INDEX WORDS: Heme biosynthesis, Aminolevulinic acid synthase, Glutamine,Succinyl-CoA, α-ketoglutarate dehydrogenase, α-ketoglutarate

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

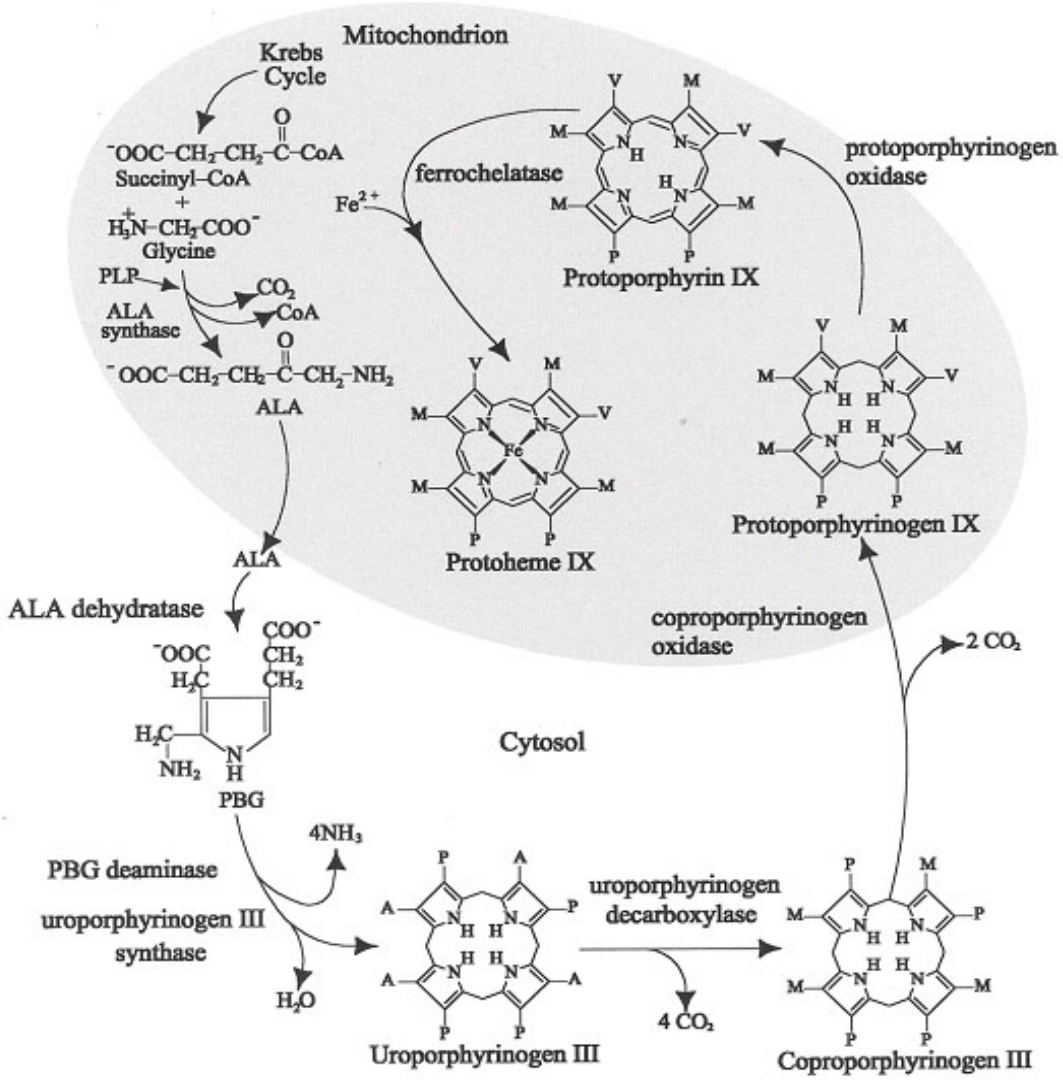
Introduction

Because of its diverse role in many biological processes, heme, an iron containing tetrapyrrole, is a cofactor vital to most organisms (Ajioka, Phillips et al. 2006, Severance and Hamza 2009). The importance of heme is demonstrated by a variety of biochemical pathways including oxidative phosphorylation, oxygen transport, drug metabolism, circadian rhythm, microRNA processing, and gas sensing that all require heme (Gilles-Gonzalez 2001, Dioum, Rutter et al. 2002, Faller, Matsunaga et al. 2007). In mammals, heme cannot be acquired from the diet, but must be synthesized *de novo* in an eight-step pathway. Any heme absorbed in the gut is degraded to extract the iron and the cleaved tetrapyrrole is discarded. The large amount of heme produced during erythropoiesis for hemoglobin synthesis creates a metabolic challenge for the cell. Although the substrates and products for the eight enzymes comprising this pathway have been clearly identified and studied, the impact of heme synthesis upon cellular metabolism has not been adequately examined. Below, a review will be given of the heme synthesis pathway, the regulation of heme synthesis, the metabolism of erythropoiesis, and the disorders associated with deranged heme synthesis.

Heme Synthesis Pathway

The heme synthesis pathway consists of eight enzymes found both in the cytoplasm and the mitochondria (Figure 1.1) (Dailey and Meissner 2013). During this process eight succinyl-CoAs, eight glycines and one iron are converted into a single heme. This process is highly regulated due to potential detrimental effects of excess pathway intermediates. Derangement of heme synthesis becomes medically relevant in certain anemias and porphyrias. Our current understanding of how heme is synthesized has changed how doctors treat some anemic and porphyric patients, but there are still large gaps in our knowledge that hinder physicians from fully understanding the intricacies of how heme synthesis and regulation impact health.

The heme synthesis pathway begins with the condensation of succinyl-CoA and glycine to form aminolevulinic acid (ALA) (Laver, Neuberger et al. 1958). This reaction occurs in the mitochondria and is catalyzed by the enzyme 5-aminolevulinic acid synthase (ALAS) (Hunter and Ferreira 2011). ALAS is unique in the fact that it is the only heme synthetic enzyme for which there are two genes (May, Dogra et al. 1995, Medlock and Dailey 2009, Dailey and Meissner 2013). ALAS1 is the “house keeping” form and is ubiquitously expressed while ALAS2 is specific for erythroid tissue and is only up regulated during erythropoiesis. The *ALAS1* gene is found on chromosome 3, and the *ALAS2* gene is located on the X chromosome. In both non-erythroid and erythroid heme synthesis, ALAS is considered to be the rate-limiting enzyme



(Dailey and Meissner 2013). ALAS is a

Figure 1.1 The Heme Synthesis Pathway. Image provided by HAD.

pyridoxal phosphate dependent homodimer. The only crystal structure available for ALAS is from the bacterium *Rhodobacter capsulatus (Astner, Schulze et al. 2005).* Fortunately the bacterial enzyme is similar to the human form of ALAS2

with the main difference being the addition of a C-terminal tail of approximately 25 residues in the human version. Like all enzymes in the heme synthesis pathway, the genes for both forms of ALAS are nuclear encoded (Medlock and Dailey 2009).

Next, ALA is transported out of the mitochondria by a yet to be determined exporter. Abcb10 (Bayeva, Khechaduri et al. 2013), a mitochondrial ABC transporter, and SLC25a38 (Guernsey, Jiang et al. 2009), a putative mitochondrial glycine transporter, have been proposed to fulfill this role; however, conclusive studies are still needed. Once in the cytoplasm, ALA is converted into coproporphyrin III by four enzymatic reactions before being transported back into the mitochondria for the three final reactions. In the first cytoplasmic reaction, porphobilinogen synthase (PBGS) (formerly ALA dehydratase) catalyzes the formation of the monopyrrole porphobilinogen from two ALAs (Dailey and Meissner 2013). PBGS is a homo-octomer consisting of a tetramer of homodimers (Erskine, Senior et al. 1997). Each of the PBG monomers bind one zinc atom with four of these zincs being used during catalysis and the other four being used for protein structure purposes. During chronic lead exposure, lead atoms replace these zinc atoms rendering PBGS inactive thus causing decreased porphyrin synthesis and ultimately anemia (Bergdahl, Grubb et al. 1997).

After formation of the monopyrrole by PBGS, four molecules of porphobilogin are converted into the linear tetrapyrrole hydroxymethylbilane by

hydroxymethylbilane synthase (HMBS) in the cytoplasm (Dailey and Meissner 2013). This reaction releases four of the glycine nitrogens as ammonium. During its first catalytic cycle apo-HMBS forms a hexameric linear polypyrrole that remains bound to HMBS. Once the initial tetrapyrrole hydroxylmethylbilane is cleaved a dipyrromethane molecule remains bound to HMBS and functions as an essential cofactor for future catalysis.

Hydroxymethylbilane can undergo spontaneous chemical conversion to uroporphyrinogen I, a cyclic tetrapyrrole. This product, however, is a metabolic dead end since it is not a suitable substrate for the terminal steps of the heme synthesis pathway. Instead, hydroxymethylbilane is enzymatically converted into uroporphyrinogen III by uroporphyrinogen synthase (UROS) (Dailey and Meissner 2013). This spiro reaction flips the final, or D ring, prior to cyclization

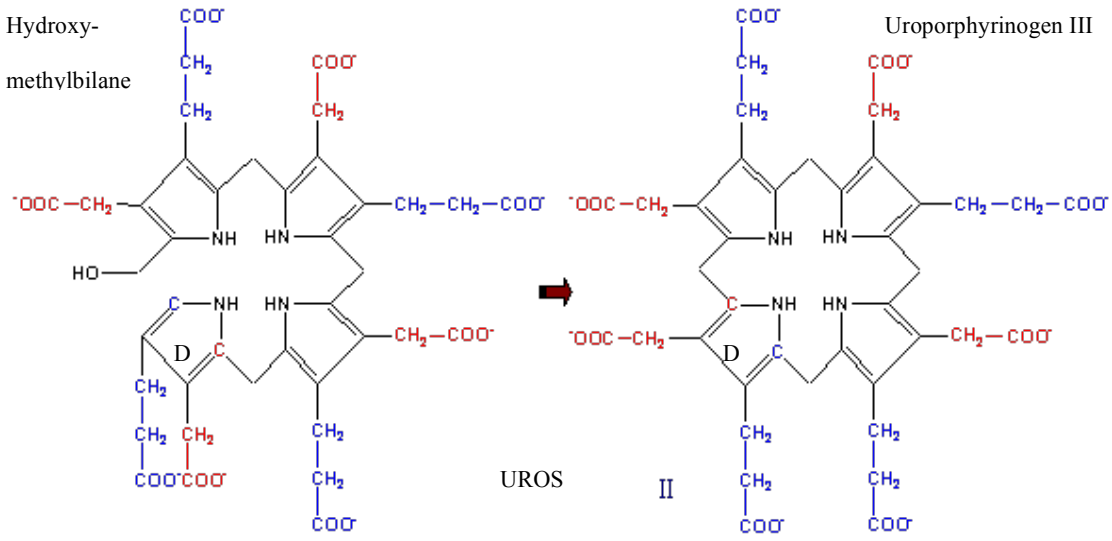


Figure 1.2: Enzymatic Reaction Catalyzed by UROS. The linear tetrapyrrole hydroxyl-methylbilane undergoes cyclization and inversion of the D-ring Adapted from http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/heme.htm

to form the cyclic tetrapyrrole uroporphyrinogen III (Figure 1.2). As is true with a number of other pathway enzymes, UROS does not use a cofactor. UROS functions as a monomer but does contain two separate structural domains that are coupled by a central flexible linker (Mathews, Schubert et al. 2001).

The next step in heme synthesis and the final cytoplasmic reaction is the decarboxylation of the four acetic acid side chains (Dailey and Meissner 2013). This reaction, catalyzed by uroporphyrinogen decarboxylase (UROD), produces coproporphyrinogen III and four molecules of CO_2. UROD forms a homodimer and similar to UROS does not require any cofactors. Uroporphyrinogen I produced non-enzymatically from hydroxymethylbilane can serve as a substrate for UROD resulting in the production of coproporphyrinogen I.

Once formed in the cytoplasm, coproporphyrinogen III must be transported back into the mitochondria for the remaining heme synthesis pathway. ATP-binding cassette, sub-family B, member 6 (Abcb6), an outer mitochondrial membrane transporter without a clearly defined substrate, has been proposed to fulfill this role (Krishnamurthy, Du et al. 2006, Krishnamurthy and Schuetz 2011). ABCB6 levels increase during erythroid differentiation (Nilsson, Schultz et al. 2009) and ABCB6 has been shown to bind porphyrins and heme. Competition assays indicated that coproporphyrin III, the planar, oxidized form of coproporphyrinogen III, is transported by ABCB6; however, the transport of nonplanar coproporhyrinogen III was not investigated. Additionally, ABCB6 has been shown to localize to the plasma membrane, the golgi, and lysozymes

questioning its role as a mitochondrial porphyrin transporter (Paterson, Shukla et al. 2007, Tsuchida, Emi et al. 2008). Current thought is that a protein other than Abcb6 is responsible for transport since mutations in Abcb6 do not result in a phenotype reflective of a coproporphyrinogen III transporter.

Once in the intermembrane space, coproporphryinogen III is converted to protoporphyrinogen IX by the oxidative decarboxylation of the A and B ring propionates resulting in vinyl groups (Dailey and Meissner 2013). This reaction, catalyzed by coproporphyrinogen oxidase (CPOX), requires two molecules of O_2 without the aid of any cofactors, and produces two molecules of CO_2 as byproducts. CPOX forms a homodimer, associates with the outer surface of the inner mitochondrial membrane, and has a relatively long mitochondrial localization sequence that may function in its regulation (Dailey and Meissner 2013).

Recently, transmembrane protein 14C (TMEM14C), a putative inner mitochondrial membrane transporter, has been identified as the erythroid specific protoporphyrinogen IX transporter (Yien, Robledo et al. 2014). Knock out of TMEM14C in a mouse model and multiple cell culture models of erythropoiesis causes accumulation of coproporphyrin III and decreased protoporphyrin IX levels. TMEM14 knockout mice die in utero with a pale phenotype, and cell culture models have impaired heme synthesis that can be rescued by deuteroporphryin, a membrane permeable analog of protoporphryin IX. Due to technical limitations, direct evidence of protoporphyrinogen transport by

TMEM14C was not obtained and the data could support alternate models in which TMEM14C facilitates heme synthesis by anchoring CPOX to the inner mitochondrial membrane or potentially assembling an interaction between CPOX and protoporphyrinogen oxidase (PPOX), the subsequent heme synthesis enzyme.

Inside the mitochondrial matrix, PPOX catalyzes the last step in porphyrin synthesis by oxidizing protoporphyrinogen IX into protoporphyrin IX (Dailey and Meissner 2013). In *in vitro* assays three molecules of O_2 are used in this FAD dependent reaction producing three molecules of hydrogen peroxide as byproducts. PPOX functions as a homodimer and associates with the inner mitochondrial membrane. PPOX is unique in that it requires both an amino terminal and internal mitochondrial localization sequence (Morgan, Errington et al. 2004, Dailey, Woodruff et al. 2005, Davids, Corrigall et al. 2006).

The heme synthesis pathway culminates with ferrochelatase catalyzing the insertion of ferrous iron in protoporphyrin IX (Dailey and Meissner 2013). This reaction lies at the intersection of the porphyrin synthesis pathway and the iron transport pathway. Ferrochelatase is a homodimeric protein with a [2Fe-2S] cluster (Wu, Dailey et al. 2001). The iron sulfur cluster does not play a direct role in catalysis: instead it plays a regulatory role modulating ferrochelatase activity in response to redox conditions (Shah, Takahashi-Makise et al. 2012). During catalysis extensive rearrangements occur at ferrochelatase's active site to facilitate product release. The structure of ferrochelatase has been captured in

three conformations: resting with its active site open, substrate bound with its active site closed on protoporphyrin, and product release with a π-helix unwound to open up the active site (Medlock, Swartz et al. 2007, Medlock, Dailey et al. 2007, Medlock and Dailey 2009).

Heme Synthesis Regulation

In general, ALA synthesis is considered to be rate limiting and the major site of regulation for the heme synthesis pathway. In support of this premise, ALAS is the only heme synthesis enzyme for which there are two separate genes for an erythroid and a house keeping isoform (May, Dogra et al. 1995, Medlock and Dailey 2009, Hunter and Ferreira 2011). However, several of the other heme synthesis enzymes have alternate mRNA splicing patterns for erythroid versus house keeping transcripts, but these differences usually affect the promoter region and result in the same mature protein (Dailey and Meissner 2013). HMBS is an exception with the housekeeping isoform having an additional 17 amino acids at the amino terminus (Grandchamp, De Verneuil et al. 1987). The purpose of this short extension is unknown.

In addition to the presence of housekeeping and erythroid forms, ALAS activity is regulated at the transcriptional, translational, and post-translational level. Considering varying and temporal needs for heme such as hormone synthesis in the adrenal glands, xenobiotic detoxification in the liver, or simply respiration common to most cells, ALAS1 expression is regulated in a tissue-specific manner (May, Dogra et al. 1995, Medlock and Dailey 2009). The half-life

for ALAS1 is only a few hours, allowing for rather rapid fluctuations that can reach as much 100-fold changes (Dailey and Meissner 2013). Additionally, heme levels regulate ALAS1 import into the mitochondria via “CP” motifs (Yamauchi, Hayashi et al. 1980, Lathrop and Timko 1993, Zhang and Guarente 1995, Dailey, Woodruff et al. 2005). When heme levels are sufficient, heme binds to cysteine residues of the CP motif and prevents translocation into the mitochondria to provide negative feedback on ALAS mitochondrial translocation. Finally, heme is also a competitive inhibitor of ALAS1.

Regulation of ALAS2 is rather distinct from that of ALAS1. Foremost, ALAS2 expression is regulated by hypoxia and various transcription factors of which GATA1 is the most prominent (Surinya, Cox et al. 1998). ALAS2 mRNA has a 5’ iron regulatory element (IRE) allowing for coordination of ALA synthesis with iron availability (Bhasker, Burgiel et al. 1993, Melefors, Goossen et al. 1993). In conditions of inadequate iron availability, an iron free iron-regulatory protein (IRP) binds to the IRE motif preventing translation of ALAS2. When iron supplies are sufficient to support erythroid heme synthesis, a [4Fe-4S] binds to IRP and prevents it from associating with IREs. Thus, translation of ALAS2 is synchronized with iron supplies to prevent porphyrin synthesis when sufficient cellular iron is absent.

FECH is another key site of regulation since it represents the convergence of the porphyrin synthesis pathway and the iron transport pathway. FECH expression is regulated by the transcription factors Sp1, NF-E2, and GATA1

(Tugores, Magness et al. 1994). The necessity of a [2Fe-2S] cluster for FECH enzyme activity provides a means to correlate activity with cellular iron availability as has been proposed (Crooks, Ghosh et al. 2010), but it also allows FECH activity to be regulated by the redox potential of the mitochondria (Shah, Takahashi-Makise et al. 2012). As seen in the *pinotage* zebrafish, FECH activity decreases when the redox potential of the mitochondria becomes more negative, affording a mechanism to decrease heme synthesis when redox conditions are unfavorable.

The current dogma in the field is that ALAS is rate limiting and that other pathway enzymes are at excessive levels; however, there are several points to consider regarding this theory. First, not only ALAS, but all heme synthesis enzymes are up regulated during erythropoiesis. Second, in photodynamic therapy in which exogenous ALA is supplied, protoporphyrin IX accumulates indicating that FECH can become rate limiting (Sopena, Ferramola de Sancovich et al. 2008, Takahashi, Ikeda et al. 2011). Finally, X-linked protoporphyria (XLP) caused by increased ALAS2 activity results in the accumulation of protoporphyrin IX and zinc protoporphyrin IX (Whatley, Ducamp et al. 2008). These later two points are consistent with a model in which ALAS2 is indeed rate limiting but only by a narrow margin under FECH (Dailey and Meissner 2013).

Recently, a heme metabolism complex has been identified (Medlock, Shiferaw et al. 2015). Direct protein-protein interactions have yet to be determined, but ALAS2, PPOX, and FECH were all found to be members of a

large complex. Since intermediates in the heme synthesis pathway are cytotoxic, a heme synthesis complex could facilitate the direct transfer of intermediates to the subsequent enzymes preventing the need for “free intermediates” in the cell.

Metabolism of Heme Synthesis

Many aspects of the heme synthesis pathway have been thoroughly studied in bacteria, plants, and metazoans. Each enzyme has been characterized kinetically and structures are available for all enzymes. Various accessory proteins essential for erythropoiesis have been identified for mitochondrial iron transport (mitoferrin) (Shaw, Cope et al. 2006), iron-sulfur cluster assembly (glutaredoxin 5) (Wingert, Galloway et al. 2005), mitochondrial glycine transport (SLC25a38) (Guernsey, Jiang et al. 2009), mitochondrial porphyrin transport (TMEM14C) (Yien, Robledo et al. 2014), and cofactor delivery to ALAS2 (CLPX) (Kardon, Yien et al. 2015). However, even though some aspects of heme synthesis have received a great deal of attention, other features of the pathway have been poorly characterized. One such area is how succinyl-CoA is generated for heme synthesis and how developing erythroblasts compensate for the large metabolic demand of heme synthesis.

As an intermediate in the TCA cycle, succinyl-CoA can be formed either by the irreversible oxidative decarboxylation of α-ketoglutarate catalyzed by α-ketoglutarate dehydrogenase (KDH) or by the reversible conversion of succinate catalyzed by succinyl-CoA synthetase (SCL) (Figure 1.3). During the heme synthesis pathway eight succinyl-CoAs and eight glycines form eight ALAs. In

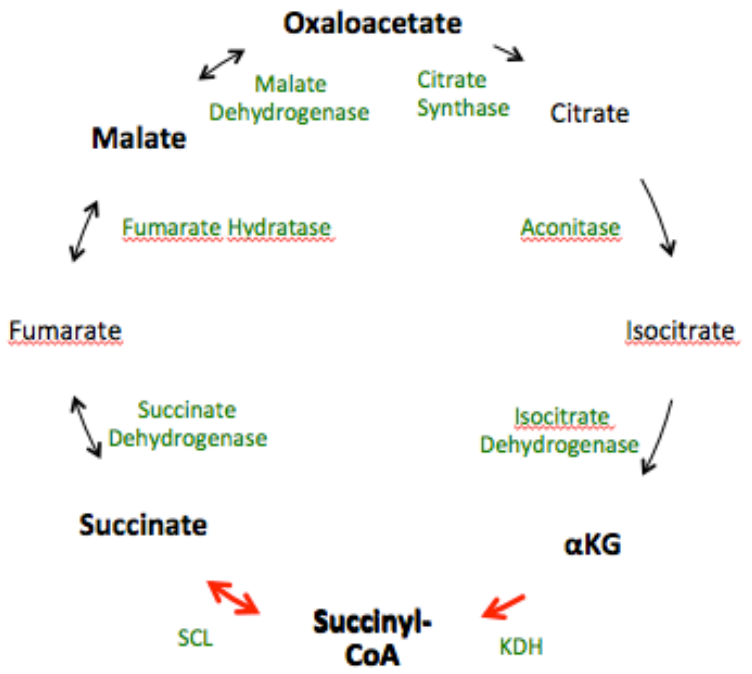


Figure 1.3: The TCA Cycle. Succinyl-CoA can be synthesized either by the irreversible oxidative decarboxylation of α-ketoglutarate catalyzed by KDH or by the reversible conversion of succinate catalyzed by SCL.

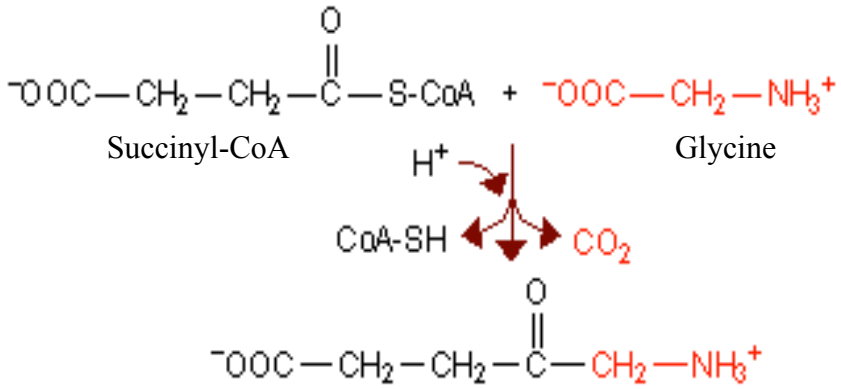


Figure 1.4. Reaction Catalyzed by ALAS. The condensation of glycine with succinyl-CoA forms ALA and CO_2. Image from

http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/heme.htm

this condensation reaction the carboxyl carbon from glycine is lost (Figure 1.4). Uroporphyrinogen III consists of 40 carbons, 32 originating from succinyl-CoA and eight originating from glycine. In the remaining decarboxylation reactions of the heme synthesis pathway, six of the eight terminal carbons originating from succinyl-CoA are lost. Hence, heme is comprised of 34 carbon atoms and four nitrogen atoms. 26 of these carbons arise from succinyl-CoA and eight from glycine (Figure 1.5).

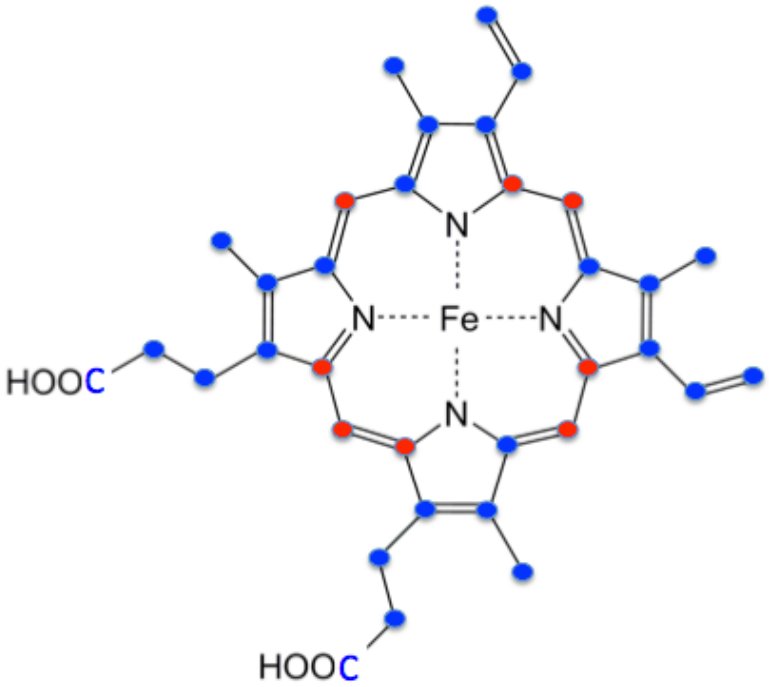


Figure 1.5. Heme. Carbons derived from succinyl-CoA are highlighted in blue, and carbons derived from glycine are highlighted in red.

Early studies by the Shemin group using duck red blood cells determined that all carbons except those arising from the methylene position of glycine could

be derived from exogenously supplied acetate (Radin, Rittenberg et al. 1950, Shemin and Wittenberg 1951). Using ^{14}C carboxy labeled acetate and ^{14}C methyl labeled acetate, Shemin's group postulated that heme synthesis initiated with the condensation of an "unsymmetrical succinyl compound" from the TCA cycle. In the early stages of this work succinyl-CoA had not yet been identified as an intermediate in the TCA cycle or as the substrate for ALA synthesis. Based upon the labeling pattern, Shemin was able to determine that most of "succinyl compound" arose directly from α-ketoglutarate and some arose from the reversible conversion of succinate into the "succinyl compound." ALA synthesis involving an intermediate between α-ketoglutarate and succinate was later confirmed by Shemin using α-ketoglutarate labeled with ^{14}C labeling at 1 and 2 positions or the 5 position (Wriston, Lack et al. 1955).

All of the work conduct by Shemin's group established that an asymmetric compound arising from either α-ketoglutarate or succinate in the TCA cycle provides 26 carbons to heme synthesis. In 1958 the Neuberger group was able to isolate and identify ALA as the first product in the heme synthesis pathway (Laver, Neuberger et al. 1958). The isolation of ALA in previous heme synthesis models was hindered by the fact that relatively little ALA was being made, and once made it was rapidly converted to the next intermediate. To overcome this challenge Neuberger isolated a "particulate fraction" from the reticulocytes of anemic chickens. When supplied with glycine, CoA, pyridoxal phosphate (PLP), $MgCl_2$, and phosphate they found that both α-ketoglutarate and succinate could

be converted into ALA at detectable levels. When either 1.7 mM α-ketoglutarate or 1.7 mM succinate was used in these assays, activity was 6 times higher when α-ketoglutarate was used. When succinyl-CoA was used in lieu of α-ketoglutarate or succinate, detectable levels of ALA were not produced. However, when the consistency of this particulate fraction was disrupted by freeze-drying or homogenization, the substrate requirement for ALA synthesis changed. Succinyl-CoA became necessary and α-ketoglutarate and succinate were ineffective for ALA synthesis (Gibson, Laver et al. 1958). Providing either KDH or succinyl-COA ligase (SCL) restored the ability of these particles to use α-ketoglutarate or succinate, respectively. The authors explained this phenomenon as a "permeability barrier" that prevented succinyl-CoA access to the active site in the intact particles, and that freeze-drying or homogenization removed this barrier. These results could be explained by KDH and SCL interacting with ALAS2 in the intact particles. This would require the formation of a complex that allows either α-ketoglutarate or succinate to enter the active sites of KDH or SCL, respectively, and following catalysis the transfer of succinyl-CoA to the active site of ALAS2. Furthermore, this complex would have to block succinyl-CoA from solution access to the active site of ALAS2. For this to be true KDH and SCL in this system would have to be more susceptible to degradation during freeze-drying or homogenization than ALAS2. No data was presented to verify the presence of KDH or SCL in the intact particles.

Although inconclusive, the work by Neuberger is consistent with protein-protein interactions between KDH, SCL, and ALAS2. Additional data has been gathered describing protein-protein interactions between KDH and SCL as well as between SCL and ALAS2. An interaction between pig heart KDH and SCL was shown *in vitro* using polyethylene glycol precipitation, ultracentrifugation, and gel chromatography (Porpaczy, Sumegi et al. 1983). K_m^{CoA} and V_{max} values were determined for KDH alone, SCL alone, KDH in a coupled reaction with SCL, and SCL in a coupled reaction with KDH. In the coupled reaction in which α-ketoglutarate was converted into succinyl-CoA by KDH and then converted into succinate by SCL, the K_m^{CoA} for KDH was modestly lowered compared to KDH alone. However, the $K_m^{succinyl\text{-}CoA}$ for SCL was 30-fold lower in the coupled reaction. Therefore, the KDH and SCL interaction facilitates the transfer of newly synthesized succinyl-CoA from KDH to SCL in a manner that drastically decreased the concentration of succinyl-CoA that is necessary for effective SCL activity. Succinyl-CoA is a highly reactive molecule and can participate in non-enzymatic succinylation of lysine residues (Weinert, Scholz et al. 2013). In addition to altering enzyme kinetics, an interaction between KDH and SCL could also be useful to minimize labile succinyl-CoA to prevent such undesired reactions.

When this work was published in 1983, it was not known that there are two isoforms of the β-subunit of SCL. Suclg2 is a SCL β-subunit that is specific for GDP/GTP, and Sucla2 is a SCL β-subunit that is specific for ADP/ATP. In

these experiments it is not clear which form of SCL was being tested or whether both were present. In the assays, GTP or GDP were the supplied nucleotides indicating Sucgl2 is more probable, but it is also known that Sucla2 interacts with nucleoside diphosphate kinase (Kadrmas, Ray et al. 1991), which could catalyze the conversion of GTP into ATP if any contaminating nucleotide diphosphate was present in the SCL purification. Even if it was Sucgl2 in these experiments, it is plausible that Sucla2 would also interact with KDH even though no experimental data is available at this time.

In addition to interacting with KDH, the Sucla2 component of SCL interacts with ALAS2. This was shown using a yeast 2-hybrid screen to identify protein-protein interactions between ALAS2 and proteins derived from a human bone marrow cDNA library (Furuyama and Sassa 2000). This interaction with Sucla2 was specific for ALAS2, with ALAS1 failing to interact in this assay. Additionally, ALAS2 did not interact with Suclg2, the GTP specific β-subunit. A connection with X-linked sideroblastic anemia (XLSA) was hypothesized based upon the fact that Sucla2 did not interact with an ALAS2 variant, D190V. *In vivo* the D190V mutation causes ALAS2 to be unstable leading to sideroblastic anemia (Furuyama, Fujita et al. 1997). It was postulated that this instability could be related to the loss of interaction with Sucla2 suggesting that Sucla2 might protect ALAS2 from degradation. On the other hand, the lack of evidence supporting an interaction between Sucla2 and D190V ALAS2 could also be explained by D190V ALAS2 being intrinsically unstable and upon degradation there is nothing

anything available for Sucla2 to interact with. Based upon the interaction of Sucla2 and ALAS2 is was proposed that SCL (containing Sucla2 as the β-subunit) converts succinate into succinyl-CoA and transfers the newly synthesized succinyl-CoA to ALAS2 for ALA production, but no additional data was provided to support this hypothesis. Work by the Bishop laboratory using affinity chromatography in which Sucla2 was immobilized to amylose resin using a maltose binding protein (MBP) confirmed that Sucla2 binds to ALAS2 *in vitro* (Bishop, Tchaikovskii et al. 2012). Subsequently applied untagged ALAS2 interacted with Sucla2, remained attached to the amylose resin, and co-eluted with Sucla2. This interaction was also demonstrated with complete SCL (containing Sucla2 as the β-subunit). ALAS2 was immobilized to the amylose resin with an MBP-tag and His-tagged SCL was applied to the column. SCL co-eluted with ALAS2 confirming that ALAS2 can interact with Sucla2 even when Sucla2 is apart of the SCL heterodimer. In addition to validating the Sucla2/ALAS2 interaction, the Bishop group also identified two XLSA causing ALAS2 mutations linked to this interaction. When characterized *in vitro* M567V and S568G ALAS2 had normal activity, kinetics, and stability; however, these two mutants failed to interact with Sucla2 or SCL. Based upon these results it was proposed these mutations cause XLSA by disrupting the ALAS2-SUCLA2 protein-protein interaction and prevent the acquisition of succinyl-CoA by ALAS2 from SCL. Once again no data was provided to illustrate the role of SCL in providing succinyl-CoA to ALAS2.

Other than the interaction between ALAS2 and Sucla2, the only evidence supporting SCL playing a crucial role for succinyl-CoA synthesis to support ALAS function came in 1965 when the Labbe group characterized SCL in mice livers (Labbe, Kurumada et al. 1965). 2-allyl-2-isopropryl-acetamide (AIA) was used to produce a porphyric-like state in mice. Using ^{14}C labeling they were able to determine the extent to which succinate or α-ketoglutarate was incorporated into heme. [1-^{14}C] succinate, [2,3-^{14}C] succinate, and [5-^{14}C] α-ketoglutarate were used. Succinate can be converted directly in succinyl-CoA by SCL or by cycling through the TCA cycle. In the latter route, carbons 1 and 4 of succinate are lost in the decarboxylation reactions of isocitrate dehydrogenase and KDH; therefore, any labeling from [1-^{14}C] succinate is due to SCL catalyzed conversion of succinate directly into succinyl-CoA. [2,3-^{14}C] succinate will label heme identically if it is directly converted into succinyl-CoA or if it traverses the TCA cycle once. The radioactivity of extracted heme was measured when the ^{14}C substrates were injected intraperitoneally in both control mice and mice treated with AIA to induce a porphyric state in which there is an increased flux of ALA into the pathway. It was found that the radioactivity arising from [1-^{14}C] succinate and [2,3-^{14}C] succinate increased by the same amount when mice were treated with AIA and that the radioactivity arising from [5-^{14}C] α-ketoglutarate remained the same. Based upon this data it was proposed that succinate was being directly converted into succinyl-CoA to meet the increased demand of ALA synthesis in the AIA treated mice. If succinate was traversing the TCA cycle

before being converted into succinyl-CoA the radioactivity for [2,3-^{14}C] succinate would have been higher than [1-^{14}C] succinate since carbon 1 is lost in the decarboxylation reaction catalyzed by isocitrate dehydrogenae or KDH. However there were two issues with their data. First, [1-^{14}C] succinate was reported to be able to label 10 carbons in heme. This is incorrect. Eight succinates will be converted into eight succinyl-CoAs followed by eight ALAs and

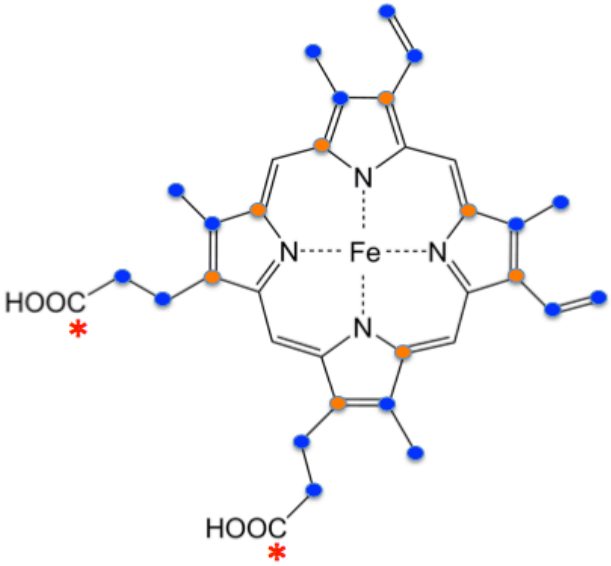


Figure 1.6: Heme Labeling Patterns from [1-^{14}C] Succinate, [2,3-^{14}C] Succinate, and [5-^{14}C] α-ketoglutarate. Being a symmetrical molecule, [1-^{14}C] succinate can become either [1-^{14}C] succinyl-CoA or [4-^{14}C] succinyl-CoA. This means [1-^{14}C] succinate labels 2-8 carbons in heme. Carbons labeled by [1-^{14}C] succinyl-CoA are colored orange. Carbons labeled by [2,3-^{14}C] succinate are colored blue. Carbons labeled by [5-^{14}C] α-ketoglutarate and [4-^{14}C] succinyl-CoA are marked with a red (*).

finally one heme molecule. If there is only one ^{14}C per succinate in [1-^{14}C] succinate then it should only label 8 carbons in heme (Figure 1.6). [1,4-^{14}C] succinate will label 10 heme carbons and it is unclear whether this substrate was actually used. [2,3-^{14}C] succinate labels 16 carbons in heme with ^{14}C and [5-^{14}C] α-ketoglutarate labels 2 carbons in heme with ^{14}C. For comparison radioactivity was normalized to the number of carbons labeled for each succinate substrate. The normalized values for [5-^{14}C] α-ketoglutarate were not reported or addressed. In both the control mice and the porphyric mice, [5-^{14}C] α-ketoglutarate normalized counts per minute were much higher than either succinate value. An attempt to remove red blood cells from the liver is not mentioned in the methods section. In this experiment, the increased production of succinyl-CoA by the liver is being measured via the incorporation of ^{14}C into heme. The effect of AIA on succinyl-CoA synthesis or heme synthesis in tissues other than the liver was was not determined. It is conceivable that different tissues respond to an increased demand for heme and succinyl-CoA through different mechanisms. Since red blood cells have a much higher concentration of heme per cell, if there was any contamination of red blood cells in the liver tissue ^{14}C labeling of red blood cell heme might be detectable. One possible explanation for this data is that red blood cells preferentially used α-ketoglutarate for heme synthesis and liver cells directly converted succinate into succinyl-CoA for heme synthesis. Heme synthesis in erythroid tissue being unaffected by AIA can account for ^{14}C incorporation remaining relatively unchanged when [5-^{14}C] α-

ketoglutarate was injected in the control versus porphyric mice. It would also explain how [5-^{14}C] α-ketoglutarate could have the highest normalized counts per minute since red blood cells synthesize heme at a higher rate than liver cells. Although this data has apparent issues and unexplained results, it does suggest that increased succinyl-CoA synthesis by SCL is used to compensate for increased porphyrin synthesis in liver tissue.

In 1988, the discovery of an ATP dependent SCL (containing Sucla2 as the β-subunit) (A-SCL) and a GTP dependent SCL (containing Suclg2 as the β-subunit) (G-SCL), the role of nucleotide specificity of SCL in heme synthesis was investigated (Jenkins and Weitzman 1988). SCL activities in liver cell lysates from control mice and mice treated with 3,5-dicarbethoxy-1,4-dihyrocollidine (DDC) to induce hepatic porphyria were determined with either ADP or GDP present. In control mice the activity arising from GDP was over 2 fold higher than the activity when ADP was present. Upon induction with DDC, activity when GDP was the utilized nucleotide doubled while activity with ADP remained unchanged. These data suggest that in the liver G-SCL is the predominant form of SCL both in normal conditions and in a chemically induced porphyric state. This also verifies previous findings that SCL activity increases in the liver during DDC treatment. These results, however, contradict expression profiles obtained both by RT-PCR and Northern blot analysis in which A-SCL appears to be predominant SCL isoform in bone marrow (Furuyama and Sassa 2000).

Congenital deficiency of Sucla2 results in an encephalomyopathic mitochondrial DNA depletion syndrome with methylmalonic aciduria (Ostergaard 2008). The first two patients characterized with mutations to Sucla2 had chronic mild microcytic anemia, but studies were not conducted to link the anemia seen in these patients to ineffective ALA synthesis (Elpeleg, Miller et al. 2005). Furthermore, 50 patients with Sucla2 deficiency have now been identified (Carrozzo, Verrigni et al. 2016). Anemia has not been reported in any subsequent case questioning whether Sucla2 is essential for ALA synthesis during erythropoiesis.

Although inadequately answered, the question of which TCA cycle reaction produces succinyl-CoA for heme synthesis has at least been addressed. On the other hand, the question of how the TCA cycle intermediates are replenished when succinyl-CoA is diverted into heme synthesis has not been asked. In general it is assumed that succinyl-CoA can be utilized for heme synthesis without any detriment to the TCA cycle or respiration since the amount of heme synthesis is rather minor. This may be the case in most cells that rely on heme mostly for cytochrome production that are not rapidly turned over, but in the context of erythropoiesis the amount of heme being synthesized will almost certainly exceed the abundance of TCA cycle intermediates. This is illustrated in a mouse model of acute intermittent porphyria (AIP). AIP is a congenital disorder caused by a defect in HMBS and results in the increased rate of ALA production leading to accumulation and excretion of ALA and porphobilinogen. Increased

flux of succinyl-CoA into ALA synthesis impairs the function of respiratory complexes and TCA cycle enzymes presumably by depleting TCA cycle intermediates and preventing the reduction of NAD^+ (Homedan, Laafi et al. 2014). This suggests that developing red blood cells must have efficient anaplerotic mechanisms to prevent energetic failure during erythropoiesis.

Recently glutamine and glucose metabolism have been shown to be important for lineage specification of $CD34^+$ hematopoietic stem cells. $CD34^+$ stem cells are human, pluripotent and can differentiate into all forms of white blood cells, red blood cells, and megakaryocytes. shRNA mediated knockdown of the glucose transporter Glut1 or the glutamine transporter Asct2 impaired erythropoietin dependent erythroid differentiation and promoted myeloid differentiation of $CD34^+$ stem cells (Oburoglu, Tardito et al. 2014). Treating $CD34^+$ stem cells with aminooxy acetic acid, a PLP dependent transaminase inhibitor, or 6-diazo-5-oxo-L-norleucine (DON), a metabolic inhibitor that nonspecifically targets glutamine dependent reactions, resulted in the same effect as knock down of Asct2. Impaired erythroid differentiation caused by AOA could be rescued by the addition of dimethyl- α-ketoglutarate (DMK), a cell-permeable analog of α-ketoglutarate. DON inhibition of erythroid differentiation, however, could not be rescued by the addition of DMK but could be partially rescued by supplementation with a nucleoside cocktail. Additionally, ^{13}C and ^{15}N incorporation into nucleotides increased upon erythropoietin stimulation but was blocked by DON treatment. DON is known to affect various glutamine dependent

pathways and has been tested in clinical trials as a chemotherapeutic. Two important pathways inhibited by DON are the conversion of glutamine into glutamate catalyzed by glutaminase and the *de* novo synthesis of nucleotides. Its role in nucleotide synthesis is proposed to be the essential role for glutamine during erythropoiesis based mostly upon the studies with DON. However, there was not any data presented to suggest that during erythropoiesis the rate of nucleotide synthesis is increased. The addition of nucleosides being able to rescue erythroid differentiation in DON treated cells only proves that DON inhibits nucleotide synthesis and that erythroid differentiation is a process that depends upon nucleotides. Impaired erythroid differentiation from shRNA knockdown of Glut1 or Asct2 supports an important role for glutamine during erythropoiesis, but the experiments presented failed to thoroughly evaluate roles for glutamine other than in nucleotide synthesis. A role for glucose or glutamine in heme synthesis was not investigated even though heme synthesis creates an enormous metabolic demand during erythroid differentiation.

A connection between glucose or glutamine metabolism and heme synthesis has not been previously made. Cellular concentration of heme in the form of hemoglobin in mature red blood cells is approximately 80 mM. Considering that eight ALAs and therefore eight succinyl-CoAs are necessary for each heme molecule synthesized, erythropoiesis creates a large demand for succinyl-CoA. At concentrations around 0.5 mM glutamine is the most abundant amino acid in plasma and plasma concentration of glucose are ~5 mM in healthy

adults. High plasma concentration and efficient transporters for glucose and glutamine in developing red blood cells make both metabolites plausible anaplerosis candidates.

Disorders of Heme Synthesis

In general, congenital disorders of the heme synthesis pathway fall into two main categories: mutations that impair ALA synthesis resulting in sideroblastic anemia and mutations to heme synthesis enzymes after ALAS resulting in porphyria.

Sideroblastic Anemia

The term sideroblastic anemia arises from the presence of iron-laden mitochondria surrounding the nucleus of developing red blood cells in the bone marrow. In these disorders the rate of ALA production is uncoupled from the increased rate of iron transport into the mitochondria in developing red blood cells. Sideroblastic anemias can be either inherited or acquired with the latter usually being caused by adverse drug effects, excessive alcohol consumption, lead toxicity, or copper deficiency (Bergmann, Campagna et al. 2010).

Mutations to ALAS2 are the most common causes for congenital sideroblastic anemia (CSA), but mutations to multiple proteins related to ALA synthesis have also been identified. In a cohort of 83 patients with CSA, 37% of cases were found to be XLSA arising from a mutation to ALAS2 (Bergmann, Campagna et al. 2010). To date over 40 disease causing mutations of ALAS2 have been identified. Most of these mutations disturb ALAS2's catalytic domain,

but one mutation has been identified in the ALAS2 promoter region (Bergmann, Campagna et al. 2010). However, the latter finding is in question since the same mutation has been found in unaffected individuals. XLSA is characterized by a microcytic, hypochromic anemia with iron overload. The exact mechanism of iron overload is unknown. Several mutations leading to XLSA affect PLP binding and can be treated by supplementation with pyridoxine, a PLP precursor.

The second most common cause of CSA is from mutations to SLC25a38, a putative mitochondrial glycine transporter (Bergmann, Campagna et al. 2010). Mutations to SLC25a38 cause a severe microcytic anemia with systemic iron overload that is refractory to pyridoxine treatment (Guernsey, Jiang et al. 2009). Although not conclusively shown, it is postulated that impaired glycine transport into the mitochondria impedes the glycine dependent synthesis of ALA.

X-linked sideroblastic anemia with ataxia (ALSA/A) results from mutation of ATP-binding cassette sub-family B, member 7 (Abcb7) (Bekri, Kispal et al. 2000). The exact function of Abcb7 is controversial, but it appears to be necessary for proper cytosolic iron-sulfur cluster assembly (Bekri, Kispal et al. 2000). Mutations to Abcb7 cause a mild hypochromic, microcytic anemia with spinocerebellar ataxia and cerebellar hypoplasia. Without sufficient cytosolic iron sulfur clusters, IRP1 activation prevents translation of ALA2.

Similar to the sideroblastic anemia caused by ineffective cytosolic iron sulfur cluster assembly, glutaredoxin 5 related sideroblastic anemia results from impaired mitochondrial iron sulfur cluster assembly. This mutation has only been

found in one patient but is corroborated by the zebrafish model, *shiraz (*Camaschella*,* Campanella *et al. 2007)*. It is proposed that mitochondrial accumulation of iron leads to a cytosolic iron deficit that activates IRP2 preventing ALAS2 translation.

Two mutations have been identified in pseudouridine synthase (PUS-1) that cause mitochondrial myopathy, lactic acidosis and sideroblastic anemia (MLASA) (Bykhovskaya, Casas et al. 2004, Zeharia, Fischel-Ghodsian et al. 2005, Fernandez-Vizarra, Berardinelli et al. 2007). These patients experienced muscle weakness, lactic academia, and normocytic anemia. PUS-1 is necessary for pseudouridylation of tRNAs and these mutations are believed to impair translation of mitochondrially encoded genes thus perturbing mitochondrial function (Bykhovskaya, Casas et al. 2004, Patton, Bykhovskaya et al. 2005, Zeharia, Fischel-Ghodsian et al. 2005).

Pearson marrow-pancreas syndrome (PMPS) is characterized by mitochondrial DNA abnormalities including deletions, rearrangements or duplications (Rotig, Colonna et al. 1989, Bergmann, Campagna et al. 2010). Clinical symptoms include macrocytic anemia, metabolic acidosis, and pancreatic insufficiency. The cause of sideroblastic anemia is unclear but similar to MLASA widespread mitochondrial dysfunction is plausible.

The clinical triad of diabetes, deafness, and megaloblastic anemia is found in thiamine responsive megaloblastic anemia (TRMA). TRMA is caused by mutation to SLC19A2 ,a high-affinity thiamine transporter (Neufeld, Fleming et al.

2001). Most tissues are unaffected because of redundant thiamine transporters, but these symptoms arise in tissues solely dependent upon SLC19A2 for thiamine transport. Thiamine is an essential cofactor for pyruvate dehydrogenase, KDH, branch chain amino acid dehydrogenase, and transketolase. The megaloblastic aspect of the anemia in this syndrome is caused by decreased transketolase activity and resulting nucleotide synthesis similar to the megaloblastic anemia seen with folate and B12 deficiency (Boros, Steinkamp et al. 2003). In addition to megaloblastic features, patients commonly present with sideroblastic rings. KDH activity was decreased in lymphoblasts isolated from TRMA patients, and it is believed that impaired production of succinyl-CoA by KDH is the cause of the sideroblastic aspects of the anemia in these patients (Abboud, Alexander et al. 1985).

Porphyrias

Malfunction of each enzyme in the heme synthesis pathway can result in a distinct porphyria (Table 1.1) (Balwani and Desnick 2012). Symptoms are caused by a buildup of pathway intermediates with a deficit of heme and anemia being uncommon. The eight porphyrias can be classified using two different schemes. First, the porphyrias can be organized based upon the presence of photosensitivity, neurovisceral attacks or both (Table 1.2). Alternatively, the porphyrias can be categorized based upon whether the pathway intermediate accumulates in the liver or in bone marrow erythroid cells (Table 1.3). The latter system will be used here.

Table 1.1: The Porphyrias. Adapted from (Balwani and Desnick 2012)

Porphyria	Affected Enzyme	Enzyme active (% of Normal)	Intermediate(s) Accumulated
X-linked Protoporphyria (XLP)	ALAS2	>100	Protoporphyrin Zinc- protoporphyrin
ALAD Deficiency Porphyria (ADP)	ALAD	~5	ALA, Coproporphyrin III
Acute Intermittent Porphyria (AIP)	HMBS	~50	ALA, Porphobilinogen Uroporphyrin III
Congenital Erythropoietic Porphyria (CEP)	UROS	1-5	Uroporphyrin I Coproporphyrin I
Porphyria Cutanea Tarda (PCT)	UROD	<20	Uroporphyrin III
Hereditary Coproporphyria (HCP)	CPOX	~50	ALA, Porphobilinogen Coproporphyrin III
Variegate Porphyria (VP)	PPOX	~50	ALA, Porphobilinogen Coproporphyrin III Protoporphyrin
Erythropoietic Protoporphyria (EPP)	FECH	~20-30	Protoporphyrin

Table 1.2: Clinical Features of the Prophyrias

Photosensitivity	Both	Neurovisceral Attacks
CEP, PCT, EPP, XLP	HCP, VP	ADP, AIP

Table 1.3: Site of Accumulated Intermediates in the Porphyrias

Erythrocytes	Liver
XLP, CEP, EPP	AIP, HCP, VP, ADP, PCT

The hepatic porphyrias include acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), variegate porphyria (VP), ALAD deficient porphyria (ADP) (also known as Doss porphyria), and porphyria cutanea tarda (PCT). PCT is the most common porphyria and ADP the most rare with less than ten cases being reported (Balwani and Desnick 2012). The most critical feature of AIP, HCP, VP, and ADP are acute neurological manifestations while abdominal pain and digestive issues (constipation, diarrhea, etc.) are the most common symptoms. With AIP, HCP, and VP, ALA and PBG are present in urine while only ALA is present in the urine with ADP. At this time it is believed that the neurological symptoms are caused by elevated levels of ALA or PBG.

The acute attacks usually result from hormonal, drug or dietary stimulation of hepatic ALAS activity. Hormone production and drug detoxification both require cytochrome P450s thus inducing ALAS activity. Hepatic ALAS1 is regulated by the peroxisome proliferator-activated receptor-coactivator 1α

(Handschin, Lin et al. 2005). This regulation causes ALAS1 activity to be up regulated during fasting and down regulated by carbohydrate loading. Acute attacks are treated by repressing ALAS1 activity; mild acute attacks can be treated with carbohydrate loading by the mechanism just described, and moderate to severe attacks can be treated with hemin therapy (Anderson KE 2005). PCT is the only hepatic porphyria in which photosensitivity is the predominate symptom (Balwani and Desnick 2012).

There are two forms of PCT: sporadic and familial. In the familial form UROD protein levels both in the liver and red blood cells are about 50% of normal. UROD activity levels are about 50% of normal in familial PCT red blood cells but only about 25% in liver cells. In sporadic PCT, UROD protein levels are normal but UROD activity is only about 25% of normal. This was recently explained by the discovery that uroporphomethene, the oxidized form of uroporphyrinogen, inhibits UROD exacerbating already low activity levels (Phillips, Bergonia et al. 2007). Although not completely understood at this time, iron excess and resulting oxidative stress in the liver appears to be associated with production of uroporphomethene exclusively in the liver (Egger, Goeger et al. 2002, Wickliffe, Abdel-Rahman et al. 2011, Ryan Caballes, Sendi et al. 2012). Excessive amounts of uroporphyrinogen are produced in the liver, released into the plasma, and finally excreted into the urine. It is the presence of uroporphyrin in the blood stream that causes photosensitivity.

The erythropoietic cutaneous porphyrias consist of congenital erythopoietic porphyria (CEP), erythropoietic protoporphyria (EPP), and X-linked protoporphyria (XLP). These disorders result in increased red blood cell levels of porphyrin intermediates both in circulation and in the bone marrow. Clinically these disorders present in childhood with cutaneous photosensitivity. CEP results from mutations to UROS. With deficient UROS activity, uroporphyrinogen I and coproporphyrinogen I accumulation occurs from non-enzymatic conversion of HMB (Desnick and Astrin 2002). Accumulation of these non-physiological porphyrins leads to hemolytic anemia, splenomegaly, and photosensitivity. Mutations to *Fech* that decrease activity to less than 35% of normal cause EPP (Chiabrando, Mercurio et al. 2014). Commonly, one *Fech* allele is mutated and the second allele is a hypomorphic *Fech* allele that is prevalent in unaffected populations. Decreased FECH activity causes an accumulation of PPIX leading to photosensitivity and potentially hepatobilliary disease or hepatic failure (Lecha, Puy et al. 2009). 20-60% of EPP patients also have a microcytic, hypochromic anemia which may include ringed sideroblasts (Holme, Worwood et al. 2007). XLP is unique in the porphyrias in that it is caused by a gain of function mutation that results in ALAS2 being over active (Whatley, Ducamp et al. 2008). Increased ALA production causes a buildup of PPIX and zinc protoporphyrin as iron availability becomes limiting. Clinically, XLP presents identical to EPP with photosensitivity being the main symptom. In XLP ALAS2 is truncated, but the mechanism of how this increases ALAS2 activity is still unresolved. Of interest,

Bishop *et al.* characterized an Alas2 truncation, Q548X, that causes X-linked protoporphyria (XLP) (Bishop, Tchaikovskii et al. 2013). This mutant does not bind to Sucla2, yet it has increased activity *in vitro* and causes XLP *in vivo.* Since Alas2 mutants that are overactive cause XLP *in vivo*, it appears that aberration of Sucla2 binding to this mutant does not decrease activity *in vivo*.

Many aspects of the heme synthesis pathway have been thoroughly studied. Each individual enzyme has been characterized and transcriptional regulation of the pathway during erythropoiesis has been defined. Succinyl-CoA metabolism during erythropoiesis has received less attention. In the following study we will investigate how succinyl-CoA is generated for erythroid heme synthesis and determine if glucose or glutamine metabolism play a role in erythroid heme synthesis.

CHAPTER TWO

A METABOLOMICS ANALYSIS TO DEFINE THE SOURCE OF HEME PRECURSORS DURING ERYTHROPOIESIS[1]

[1]To be submitted to *Blood*

ABSTRACT

Heme, iron inserted into protoporphyrin IX, is a cofactor essential to numerous metabolic and regulatory pathways, making it obligatory to most organisms. Heme is synthesized in an eight-step pathway of which many aspects have been thoroughly characterized. The demand for heme during erythropoiesis creates a metabolic challenge that has not been studied. A robust means to supply succinyl-CoA during erythropoiesis must be present, and to address this question we used ^{13}C labeling to determine that glutamine efficiently supplies carbons for heme synthesis in an erythroid cell culture model. Additionally, inhibition of glutamine metabolism with metabolic inhibitors halts erythroid differentiation by impairing heme synthesis. A protein-protein interaction between α-ketoglutarate dehydrogenase (KDH) and the erythroid form of the first heme synthesis enzyme, aminolevulinic acid synthase 2 (ALAS2), was identified. Kinetic parameters of both KDH and ALAS2 were altered by this interaction. Our data reveal new aspects of erythroid heme synthesis that illustrates a metabolic shift occurring during erythropoiesis that relies heavily upon glutamine metabolism.

INTRODUCTION

As an essential cofactor in numerous vital pathways, almost all organisms require heme (Ajioka, Phillips et al. 2006, Severance and Hamza 2009). In vertebrates heme must be synthesized *de novo* in an eight-step process. In the first reaction, which is accepted to be rate limiting and a site of complex

regulation, aminolevulinic acid synthase (ALAS) forms aminolevulinic acid (ALA) from succinyl-CoA and glycine(Sadlon, Dell'Oso et al. 1999). In the remainder of the pathway, eight ALAs form a porphyrin ring into which ferrous iron is inserted to form heme (Dailey and Meissner 2013).

It is known that succinyl-CoA is both a precursor to heme synthesis and an intermediate in the TCA cycle. The main routes by which succinyl-CoA is generated are through the oxidative decarboxylation of α-ketoglutarate to succinyl-CoA catalyzed by KDH, and the reversible reaction of succinate to succinyl-CoA catalyzed by succinyl-CoA ligase (SCL). Both of these reactions have been implicated to be important for heme synthesis. Anemia caused by impaired thiamine transport in thiamine responsive megaloblastic anemia is believed to be at least partially due to decreased production of succinyl-CoA by KDH, which requires thiamine as a cofactor (Abboud, Alexander et al. 1985). On the other hand, an interaction between Sucla2, the ATP specific beta subunit of SCL, and ALAS2 has been reported (Furuyama and Sassa 2000, Bishop, Tchaikovskii et al. 2012). This interaction is disrupted by several ALAS2 mutations that cause X-linked sideroblastic anemia supporting the hypothesis that succinate is converted into succinyl-CoA to support erythroid heme synthesis. To date, there have not been any conclusive studies illustrating whether both pathways participate in succinyl-CoA synthesis during erythropoiesis or if one pathway dominates.

Whether generated from α-ketoglutarate or succinate, the utilization of succinyl-CoA for heme synthesis removes intermediates from the TCA cycle. Using a mouse model of acute intermittent porphyria, it was recently shown that increased porphyrin synthesis can deplete the TCA cycle and cause detrimental downstream effects to respiration and energy production (Homedan, Laafi et al. 2014). In the context of erythropoiesis, succinyl-CoA has been simply assumed to be siphoned from the TCA cycle, but considering the quantity of heme that is rapidly produced a means to replenish the TCA cycle must exist.

Here, we explore potential routes to supply the necessary carbon flux for heme synthesis during erythropoiesis. We show that glutamine effectively provides carbons for ALA and subsequently heme synthesis. We further show that this is accomplished by an increased flux of glutamine through α-ketoglutarate indicating that KDH may be an important enzyme for erythropoiesis. Using purified enzymes we identify protein-protein interactions between KDH, SCL, and ALAS2 that impact the kinetics of both KDH and ALAS2.

RESULTS

^{13}C Labeling of Heme

During erythropoiesis the large demand for succinyl-CoA necessitates a means to replenish the TCA cycle. Recently, glucose and glutamine have been shown to be essential for erythroid differentiation of $CD34^+$ hematopoietic stem cells (Oburoglu, Tardito et al. 2014). Both glucose and glutamine can readily supply the TCA cycle by the anaplerotic conversions of pyruvate into

oxaloacetate and glutamate into α-ketoglutarate, respectively. In order to determine if either of these anaplerotic reactions supplies carbons for heme synthesis, differentiating mouse erythroleukemia (MEL) cells were labeled with ^{13}C uniformly labeled glucose, glutamine, and diethylsuccinate (DES). DES, a cell-permeable analog of succinate, was included to determine if succinyl-CoA used for heme synthesis was preferentially synthesized from succinate compared to α-ketoglutarate. At 48 hours of erythroid differentiation, MEL cells were labeled for 24 hours with ^{13}C substrates. This led to 23%, 8.3%, and 2.5% of heme carbons being labeled by ^{13}C glutamine, ^{13}C glucose, and ^{13}C diethylsuccinate, respectively as determined by LC/MS analysis of extracted heme (Table 2.1). Heme has a total of 34 carbons with 26 carbons being derived from succinyl-CoA and eight carbons being derived from glycine. Since the main route of *de novo* synthesis of glycine begins with 3-phospho-glycerate, a glycolytic intermediate, it is impossible to determine whether labeling of heme from ^{13}C glucose is due to glycine or succinyl-CoA being labeled in this experimental setup.

Table 2.1: ^{13}C Incorporation into Heme

^{13}C Substrate	% Heme Carbons Labeled
Glucose	8.4 ± 1.7
Glutamine	23.0 ± 0.3
Diethylsuccinate	2.5 ± 0.4

Metabolic Inhibitors

Aminooxy acetic acid (AOA), a broad-spectrum transaminase inhibitor, halts erythroid differentiation of $CD34^+$ hematopoietic stem cells (Oburoglu, Tardito et al. 2014). This phenomenon can be reversed by the addition of dimethyl- α-ketoglutarate (DMK), a cell permeable analog of α-ketoglutarate. Given that glutamine serves as source of carbons for heme synthesis (Table 2.1), we questioned whether AOA impairs erythroid differentiation by blocking heme synthesis. MEL cells were treated with AOA, and as previously reported erythroid differentiation was impaired in a concentration dependent manner when measured by hemoglobin content (Figure 2.1). Cells treated with AOA at the initiation of erythroid differentiation had only 24% the hemoglobin content as compared to untreated cells (Figure 2.2). Supporting the role of impaired heme

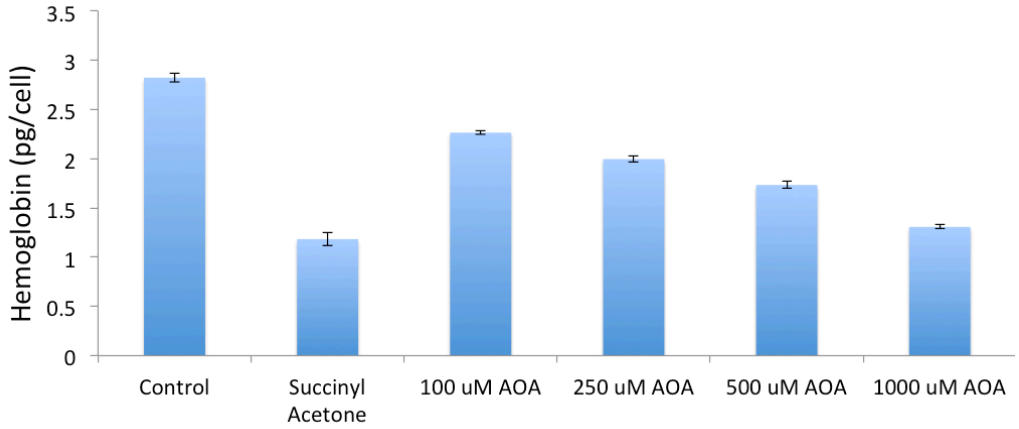


Figure 2.1: Concentration Dependent Inhibition of Hemoglobinization by AOA. Succinyl acetone is an inhibitor that block heme synthesis. MEL cells were treated with AOA and supplements at 48 hours post induction of differentiation. n=2 technical replicates. Error bars represent ± standard deviation.

synthesis as the culprit of altered erythroid differentiation by AOA, the addition of either DMK or ALA rescued hemoglobinization (Figure 2.2). Supplementing AOA treated cells with DES did not rescue hemoglobinization suggesting that succinyl-CoA for heme synthesis during erythropoiesis must arise from α-ketoglutarate.

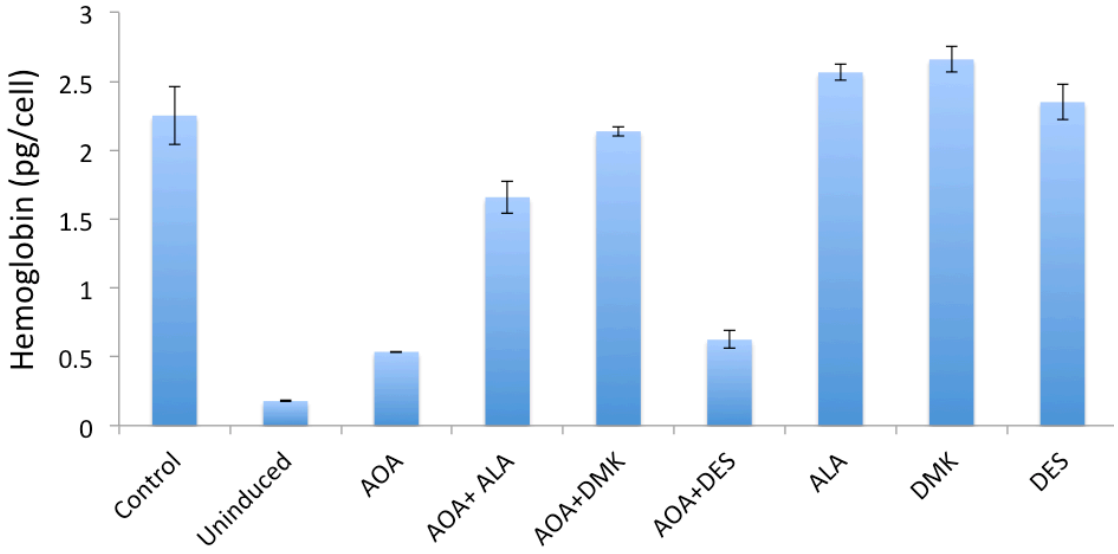


Figure 2.2: Rescue of Hemoglobinization by ALA and DMK. MEL cells were treated with AOA at the initiation of erythroid differentiation. ALA and DMK but not DES rescued hemoglobinization. n=3 technical replicates. Error bars represent ± standard deviation.

Erythroid differentiation of MEL cells is a biphasic process in which heme up-regulates some of the terminal heme synthetic enzymes (Lake-Bullock and Dailey 1993). To diminish the possibility that the rescue by ALA was due to any transcriptional feedback, we treated MEL cells with AOA and various supplements at 48 hours post-induction. Succinyl acetone (SA) is a potent

inhibitor of aminolevulinic acid dehydratase, the second enzyme in the heme synthesis pathway (Ebert, Hess et al. 1979). SA was included to measure the amount of hemoglobin that had already been produced by 48 hours of differentiation. Similar trends were seen as compared to when AOA and supplements were added at the initiation of differentiation (Figure 2.3). This data is consistent with a model in which substrates for heme synthesis are being disrupted by AOA in a transcription feedback independent manner.

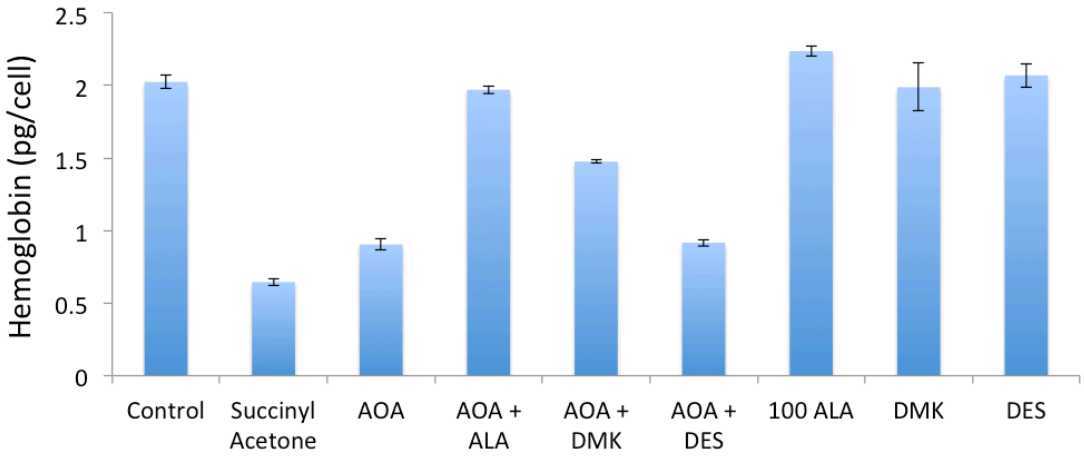


Figure 2.3: Rescue of Hemoglobinization by ALA and DMK at 48 Hours Post Erythroid Differentiation Induction. MEL cells were treated with AOA at 48 hours post induction of erythroid differentiation. ALA and DMK but not DES rescued hemoglobinization. n=3 technical replicates. Error bars represent ± standard deviation

To eliminate the possibility that AOA was altering iron metabolism to cause decreased heme synthesis, iron dextran was include in a parallel

experiment. As seen in Figure 2.4, excessive iron did not impact the effect of AOA, DMK, or ALA.

AOA inhibits transaminases; therefore, it blocks the conversion of glutamate into α-ketoglutarate. Next, we examined whether blocking the conversion of glutamine into glutamate would affect MEL cell erythropoiesis. Bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide (BPTES) is a specific inhibitor for glutaminase 1, the principal enzyme that catalyzes the deamination

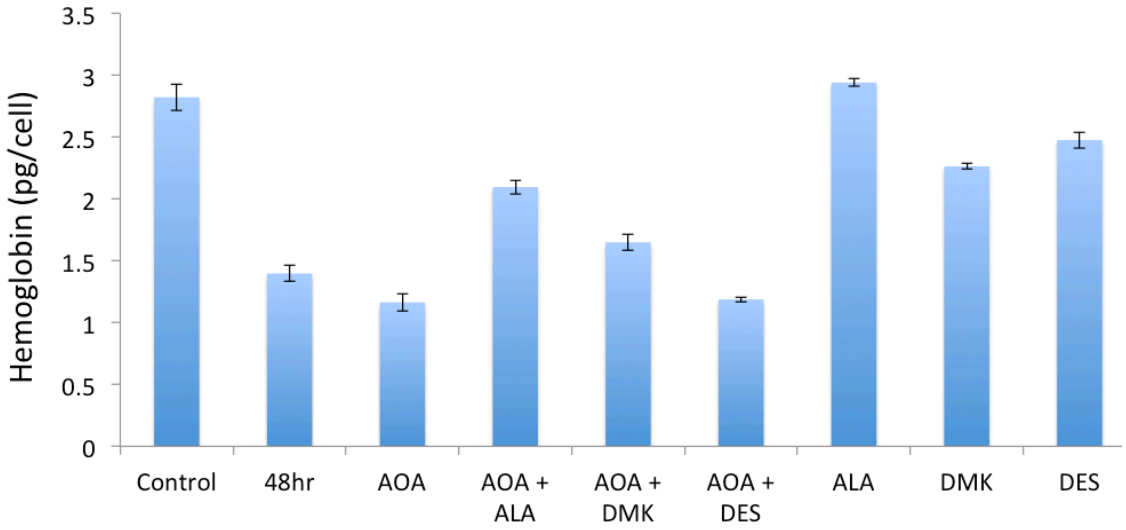


Figure 2.4: Rescue of Hemoglobinization by ALA and DMK with Excess Iron at 48 Hours Post Erythroid Differentiation Induction. MEL cells were treated with AOA at 48 hours post induction of erythroid differentiation. ALA and DMK but not DES rescued hemoglobinization. Hemoglobin levels were measured at the time of AOA and other additives (48 hours) as a control to show the amount of hemoglobin already synthesized. n=3 technical replicates. Error bars represent ± standard deviation.

of glutamine to form glutamate. Treatment of MEL cells with BPTES caused a modest decrease in hemoglobinization in a concentration dependent manner (Figure 2.5) indicating that glutamine metabolism is critical for hemoglobinization.

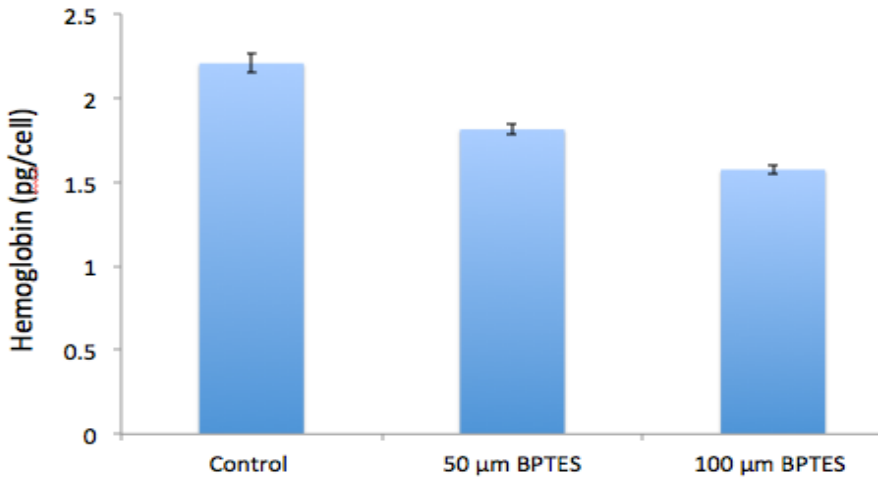


Figure 2.5: Concentration Dependent Inhibition of Hemoglobinization by BPTES. MEL cells were treated with BPTES at the initiation of differentiation. n=2 technical replicates. Error bars represent ± standard deviation.

We verified that our results with AOA and ALA would translate into a more physiologically relevant model. Similar to what has already been published we evaluated erythroid differentiation using erythroid cell surface marker glycophorin A, CD36, and CD71 on $CD34^{+}$ hematopoietic stem cells treated with AOA and supplements. As expected AOA impaired erythroid differentiation in a manner that could be rescued by DMK (Figure 2.6). Comparable to the results obtained with MEL cells, ALA was able to partially rescue erythroid differentiation but DES was not able to rescue. These results are consistent with AOA impairment of

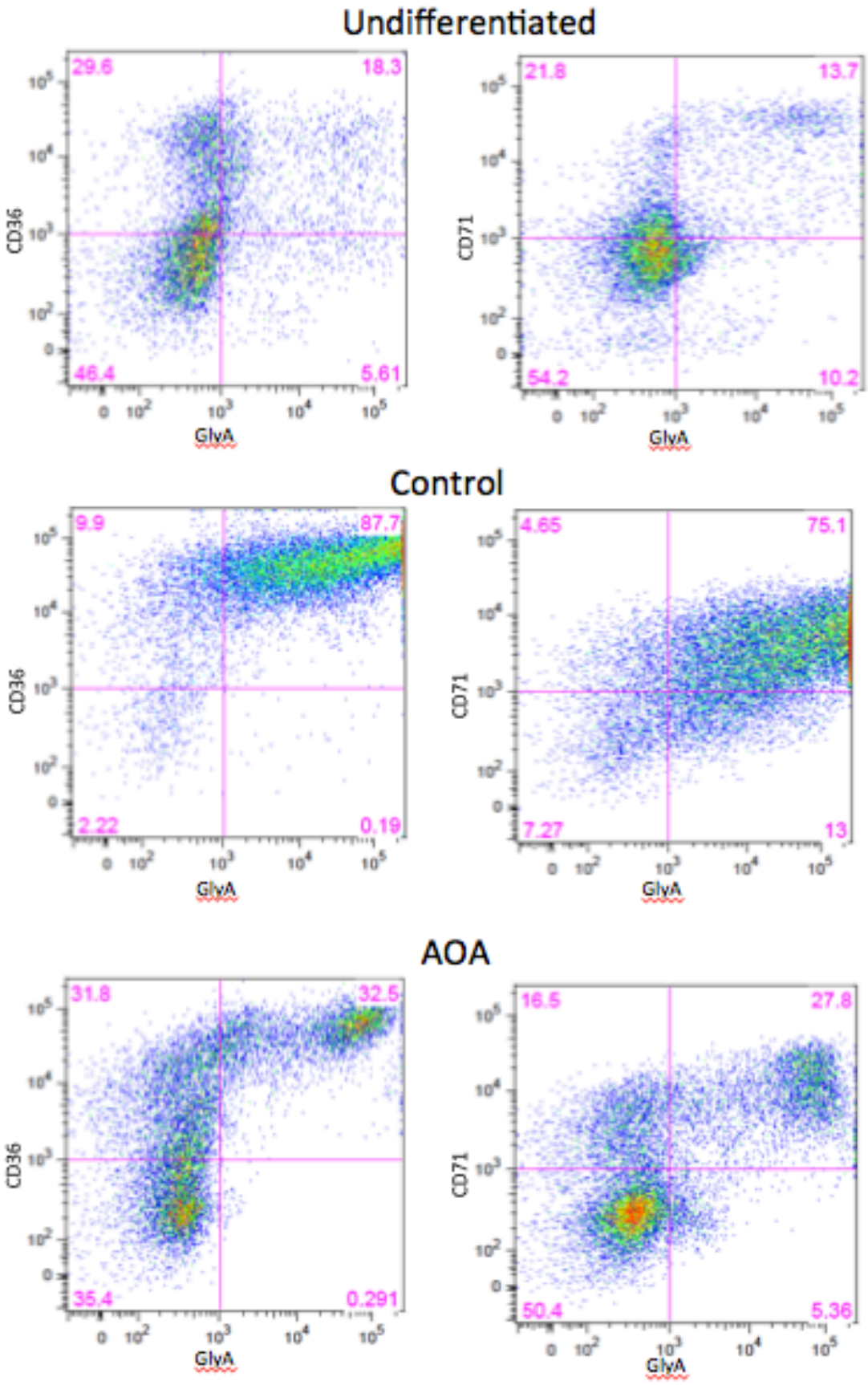
erythroid differentiation being at least partially due to compromised heme synthesis.

TCA Cycle Activity

Since succinyl-CoA is an intermediate in the TCA cycle, we considered the need of increased activity of TCA cycle enzymes during erythropoiesis to facilitate increased production of succinyl-CoA for heme synthesis. We determined the activity of six TCA cycle enzymes in both undifferentiated and differentiating MEL cell lysates. The activity of aconitase, isocitrate dehydrogenase, and KDH were all increased during differentiation with the activity of KDH being increased the most dramatically (Figure 2.7). Although the increase in activity for citrate synthase was not statistically significant ($p=0.11$), the activity of all the enzymes necessary to convert pyruvate derived from glycolysis into succinyl-CoA are increased and the activity of two TCA cycle enzymes after succinyl-CoA (SCL and succinate dehydrogenase) were unchanged. As would be expected if both glucose and glutamine were providing carbons for succinyl-CoA synthesis, KDH had the largest change in activity as would be necessary to handle α-ketoglutarate produced from isocitrate dehydrogenase in the TCA cycle as well as α-ketoglutarate produced from glutaminolysis.

KDH

Next we began questioning how MEL cells increase KDH activity during differentiation. KDH is a complex consisting of twelve oxoglutarate


Undifferentiated
29.6
18.3
46.4
5.61
CD36
GlyA
21.8
13.7
54.2
10.2
CD71
GlyA
Control
9.9
87.7
2.22
0.19
CD36
GlyA
4.65
75.1
7.27
13
CD71
GlyA
AOA
31.8
32.5
35.4
0.291
CD36
GlyA
16.5
27.8
50.4
5.36
CD71
GlyA

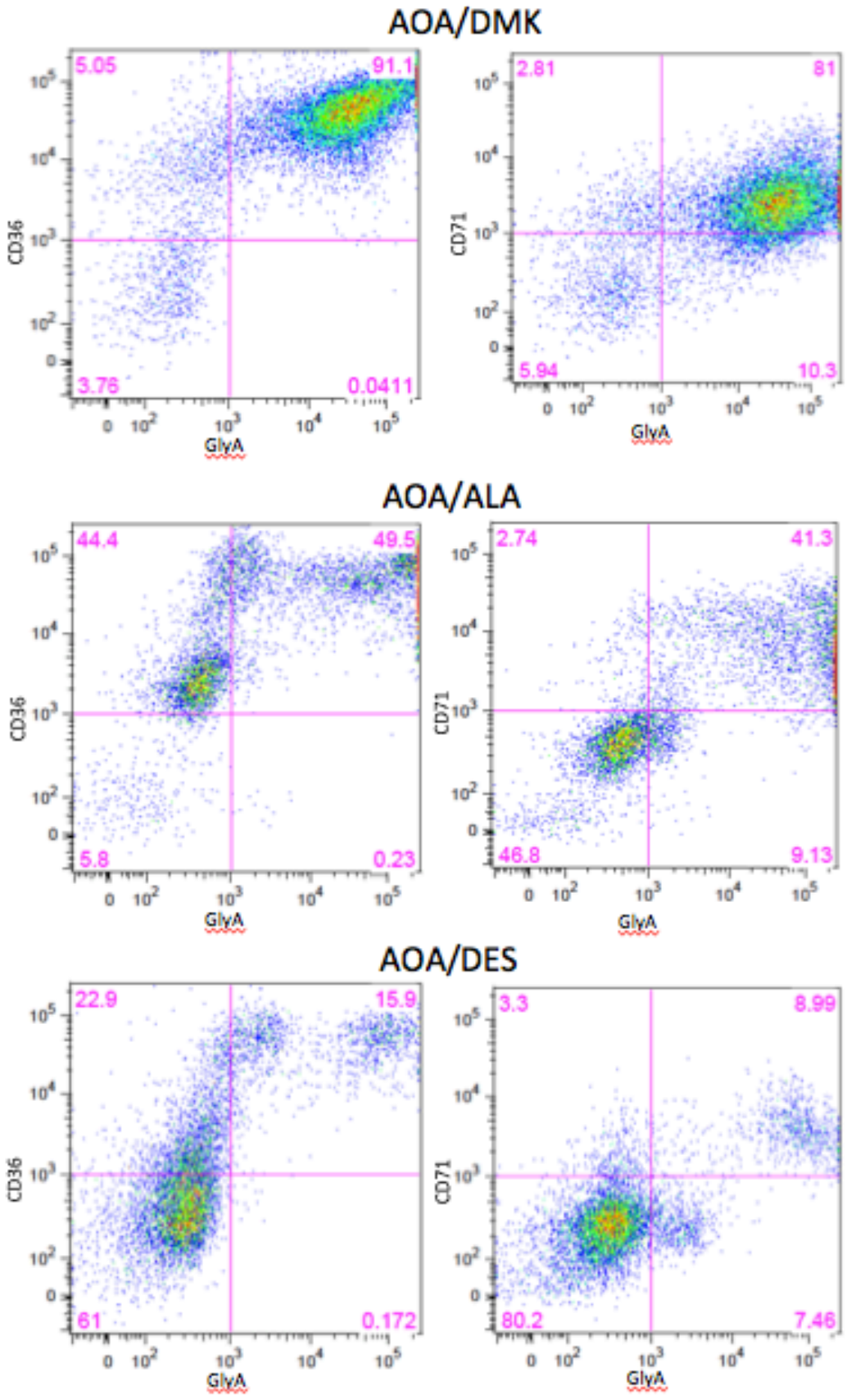

AOA/DMK
5.05
91.1
3.76
0.0411
CD36
GlyA
2.81
81
5.94
10.3
CD71
GlyA
AOA/ALA
44.4
49.5
5.8
0.23
CD36
GlyA
2.74
41.3
46.8
9.13
CD71
GlyA
AOA/DES
22.9
15.9
61
0.172
CD36
GlyA
3.3
8.99
80.2
7.46
CD71
GlyA

Figure 2.6: Rescue of Erythroid Differentiation by ALA and DMK in AOA Treated $CD34^+$ Hematopoietic Stem Cells. Erythroid differentiation of $CD34^+$ hematopoietic stem cells treated with AOA was measured using erythroid cell surface markers Glycophorin A, CD71 (transferrin receptor) and CD36 (fatty acid translocase). ALA and DMK but not DES rescued erythroid differentiation.

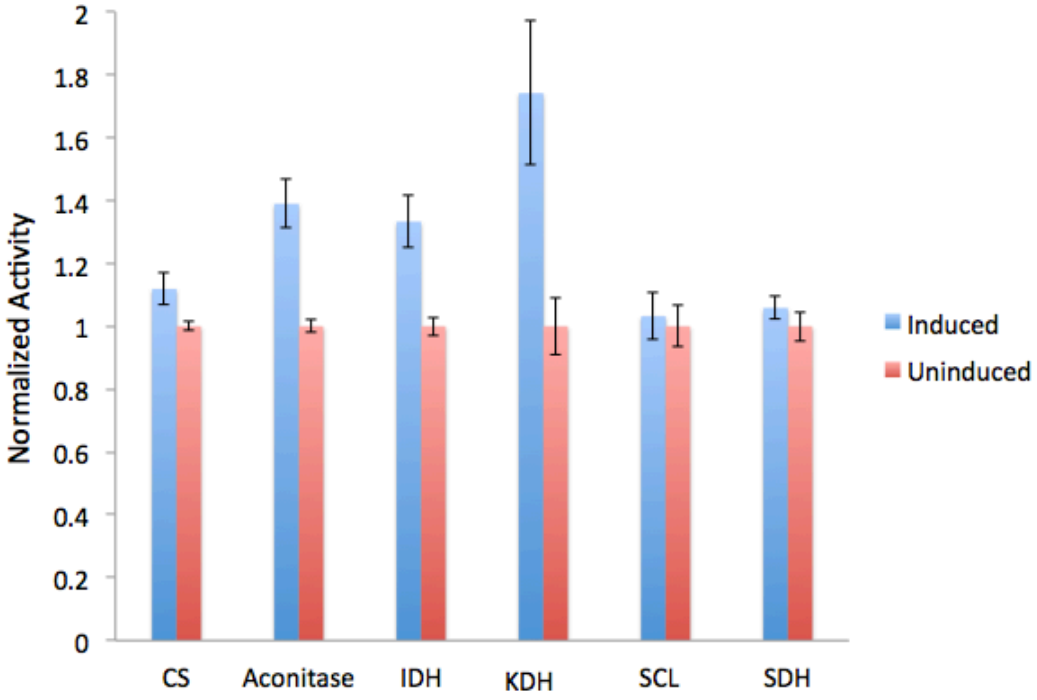


Figure 2.7: Activity of TCA Cycle Enzymes During Erythroid Differentiation. Activity of six TCA cycle enzymes was determined in mitochondrial lysates from undifferentiated and differentiating MEL cells. Activity of cells induced towards erythroid differentiation was normalized to the activity of uninduced cells. CS, citrate synthase; IDH, isocitrate dehydrogenase; SDH, succinate dehydrogenase. n=3 technical replicates. Error bars represent ± standard deviation. P-values for induced versus uninduced were CS .016; Aconitase .0011; IDH .0027; KDH .0064; SCL .6132; and SDH .1579.

dehydrogenase (E1) subunits, twenty-four dihydrolipoamide s-succinyltransferase (E2) subunits, and twelve dihydrolipoamide dehydrogenase (E3) subunits. Western blot analysis of MEL cells harvested at each day of erythroid differentiation showed that E1 expression increases during differentiation (Figure 2.8). Increased E1 protein levels explain how KDH activity increased by erythroid differentiation and further supports KDH as a critical enzyme for erythroid differentiation.

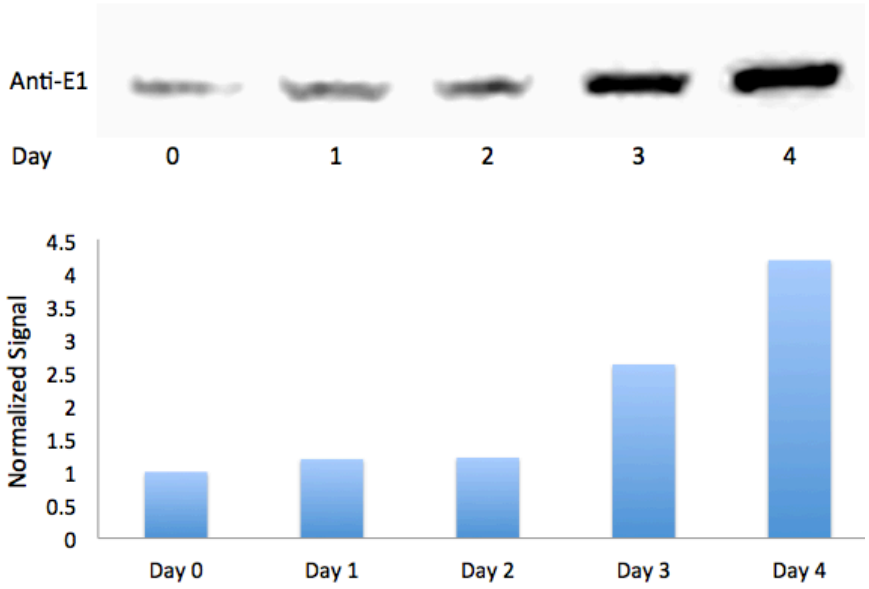


Figure 2.8: Protein Levels for the E1 Component of KDH during Erythroid Differentiation of MEL Cells. MEL cells were harvested every 24 hours and E1 protein levels detected by western blotting and a representative blot shown (top). Intensity was quantitated by densitometry and normalized to day 0 levels (bottom).

The enzymes of the TCA cycle like other metabolic pathways form a metabolon in order to facilitate substrate channeling(Robinson, Inman et al. 1987, Wu and Minteer 2015). KDH has been proposed to be the nucleating protein serving as the core to which other TCA cycle enzymes attach. In this metabolon KDH directly interacts with SCL to decrease the Km of SCL for succinyl-CoA by 30 fold (Porpaczy, Sumegi et al. 1983). Knowing that KDH interacts with SCL to deliver succinyl-CoA for succinate synthesis, we questioned how ALAS2 would have access to succinyl-CoA. Using recombinantly purified ALAS2 with a His-tag and untagged KDH purified from porcine hearts we tested for a protein-protein interaction. The E1 component of KDH co-elutes with ALAS2 when ALAS2 and KDH are mixed in solution and applied to a cobalt resin (Figure 2.9). This illustrates that at least *in vitro* the E1 component of KDH interacts with ALAS2.

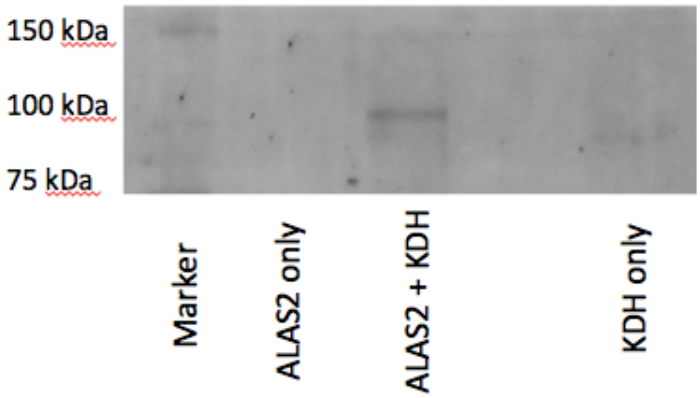


Figure 2.9: *In Vitro* Protein-Protein Interaction Between ALAS2 And KDH. Anti-E1 western blot showing Sucla2 interacts with ALAS2 immobilized on a cobalt resin column.

Having demonstrated a direct protein-protein interaction between KDH and ALAS2, we investigated the implication this interaction may have on KDH and ALAS2 activity. The rate of conversion of α-ketoglutarate into succinyl-CoA was monitored by measuring the reduction of NAD+ with excessive amounts of α-ketoglutarate and limiting amounts of CoA present. Adding recombinant SCL or ALAS2 alone increased the activity of KDH *in vitro*, but the addition of both SCL and ALAS2 resulted in the greatest increase in KDH activity (Figure 2.10). Similarly, adding KDH or SCL increased ALAS2 activity, but when both KDH and SCL were added there was no increase in activity (Figure 2.11). ALAS2 activity was determined by measuring the amount of ALA synthesized from succinyl-CoA. Although the best comparison of ALAS2 activity would be made by comparing the rate of ALA formation from succinyl-CoA in the absence of KDH to the rate of ALA formation from α-ketoglutarate in a coupled reaction with KDH, this was not possible. In these assays, CoA, as itself or in the form of succinyl-CoA, is the rate-limiting factor, and assaying ALA formation in a coupled reaction would allow for the replenishing of CoA upon ALA production. In these assays cofactors essential only to the enzyme being assayed were present rendering the other enzymes added catalytically inactive. From these assays it appears KDH is most active when interacting with both SCL and ALAS2 and that ALAS2 is most active when interacting with either SCL or KDH but not both enzymes. Analogous to the trend seen with KDH, we expected ALAS2 activity to be highest when interacting with both SCL and KDH. This could be due to the fact we were

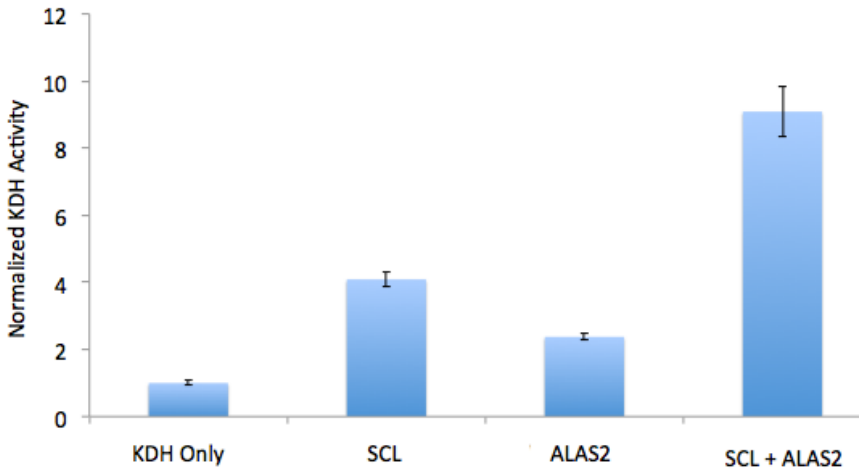


Figure 2.10: The addition of recombinant SCL or ALAS2 increased the activity of purified KDH. The addition of both SCL and ALAS2 resulted in the highest activity. Activities are normalized to KDH only. n=3. technical replicates. Error bars represent ± standard deviation.

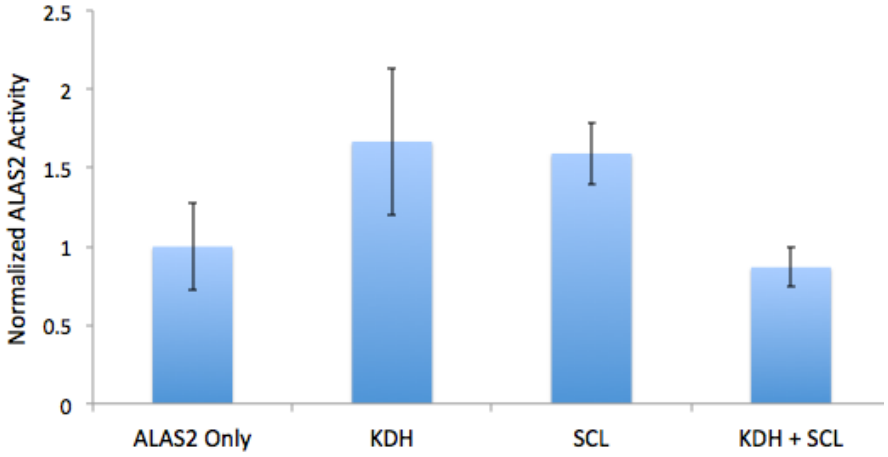


Figure 2.11: ALAS2 Activity With KDH And SCL Added. The addition of purified KDH or ALAS2 increased the activity of recombinant ALAS2. The addition of both KDH and SCL resulted in the highest activity. Activities are normalized to ALAS2 only. n=3 technical replicates. Error bars represent ± standard deviation.

required to use succinyl-CoA as the substrate when assaying ALAS2. Our data are consistent with a model in which ALAS2 binds both SCL and KDH in a manner that introduces steric hindrance at the active site of ALAS2. In this model α-ketoglutarate would be converted into succinyl-CoA by KDH and channeled directly to ALAS2 for ALA synthesis. It is expected that ALAS2 activity would be highest when interacting with both SCL and KDH if a coupled assay using α-ketoglutarate was used. As previously mentioned, doing so would regenerate CoA and prevent any worthwhile comparison.

Metabolic flux of ^{13}C

Having established that glutamine is preferentially used as a carbon source for heme synthesis and that α-ketoglutarate is the route by which this flux occurs, we traced incorporation of heavy isotopes from ^{13}C uniformly labeled glutamine, ^{13}C uniformly labeled glucose, and glutamine labeled by ^{15}N at the alpha amino group into two fumarate, malate, glutamine, and glutamate (Figure 2.12). Knowing that glutamine is a better source of carbons for heme synthesis, we expected ^{13}C from glutamine to be more readily incorporated into TCA cycle intermediates fumarate and malate. A comparison of heavy isotope incorporation shows that ^{13}C glucose labels fumarate and malate as well if not better than ^{13}C glutamine. This suggests that α-ketoglutarate originating from glutamine and being used for heme synthesis is distinct from α-ketoglutarate in the cycling in the TCA cycle. Instead of ALAS siphoning off succinyl-CoA from the TCA cycle a separate pathway may be present to produce α-ketoglutarate independent of the

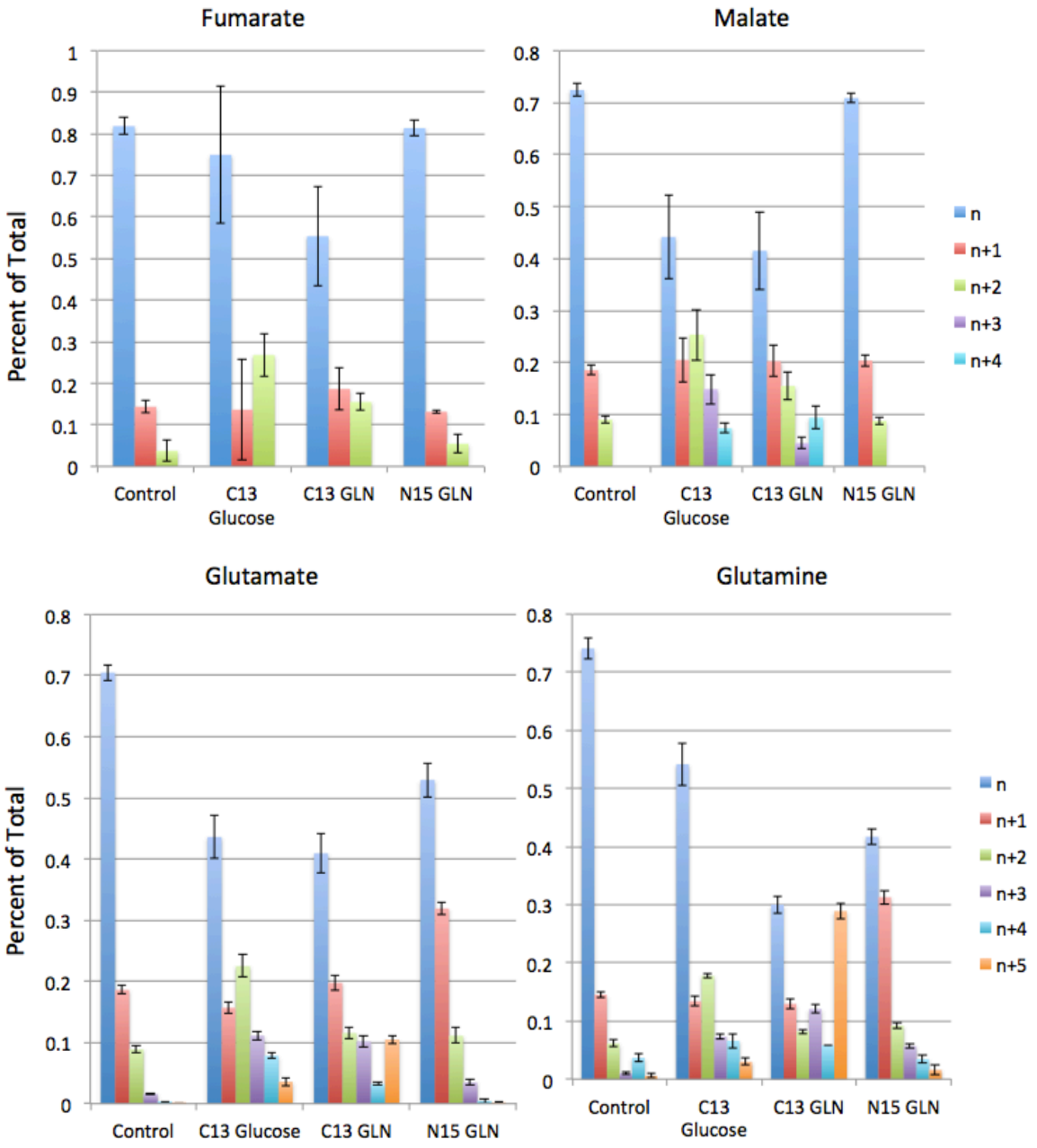


Figure 2.12: ^{13}C and ^{15}N Incorporation Into Various Metabolites. MEL cells were incubated with ^{13}C uniformly labeled glutamine, ^{13}C uniformly labeled glucose, and glutamine labeled by ^{15}N at the alpha amino group. Each bar represents an isotopologue with increasing heavy isotope incorporation. n=3 technical replicates. Error bars represent ± standard deviation.

TCA cycle. This alternate pathway may use KDH in a “moon-lighting” role and could potentially participate in a heme synthesis metabolon that has been recently proposed (Medlock, Shiferaw et al. 2015).

DISCUSSION

In 1958 Neuberger and colleagues identified succinyl-CoA as a substrate for ALAS (Laver, Neuberger et al. 1958), and to date it is accepted that heme synthesis relies on the TCA cycle. Although heme is necessary for various key processes common to most cells, the amount of heme synthesized is relatively small since heme, as a prosthetic group, is not rapidly turned over. It is conceivable that in most cells when heme is needed the TCA cycle supplies succinyl-CoA without compromising itself; however, given the quantities of heme necessary during erythropoiesis it is implausible the TCA cycle would be able to function without a robust means to resupply intermediates.

Realizing there must be a compensatory flow of carbons into the TCA cycle during erythropoiesis, we began to consider potential anaplerotic sources. Glucose and glutamine metabolism have been shown to be essential for erythroid lineage commitment of $CD34^{+}$ hematopoietic stem cells (Oburoglu, Tardito et al. 2014). Knockdown of the glucose transporter Glut1 or the glutamine transporter ASCT2 in CD34+ stem cells decreased erythroid differentiation. Since glutamine is the most abundant amino acid in plasma, and plasma glucose levels in a healthy person range from 4-6 mM, both metabolites are readily available to developing erythroblasts. Using ^{13}C labeled substrates

we determined that both glucose and glutamine supply carbons for heme synthesis during erythroid differentiation of MEL cells. Although glutamine only labeled 23% of the eligible carbons in heme, two points should be taken into consideration. First, ^{13}C substrates were not added until 48 hours post induction of differentiation. 30-40% of the total cellular heme had already been synthesized by this point preventing ^{13}C incorporation (See succinyl acetone controls in which heme synthesis was inhibited at 48 hours post induction in Figure 2.1 and 2.3). Second, GC/MS analysis reveals that only one third of the glutamine in the cell after 24 hours of incubation is uniformly ^{13}C labeled (See Figure 2.12). There is an equal amount of completely unlabeled glutamine at this time. Unlabeled glutamine could arise from various sources including the FBS in the media, glutamine synthesized by the cell, or glutamine imported into the cell before the addition of ^{13}C labeled substrates. With these two factors taken into consideration, the magnitude of carbon flux from glutamine into heme is probably drastically undervalued in our data. Other anaplerotic sources cannot be ruled out, but our data indicates that glutamine can efficiently provide carbons to meet the increased need of succinyl-CoA synthesis for heme production.

The essential role of glutamine metabolism in erythropoiesis has been attributed to nucleotide synthesis (Oburoglu, Tardito et al. 2014), but the impact of glutamine metabolism on heme synthesis was not investigated. Using the pan-transaminase inhibitor, AOA, we linked glutamine metabolism specifically to heme synthesis. Along with prior studies, we show that AOA inhibits erythroid

differentiation of $CD34^+$ hematopoietic stem cells and decreases hemoglobinization of differentiating MEL cells. This inhibition can be reversed with DMK consistent with α-ketoglutarate fulfilling an essential role in erythropoiesis. ALA was able to partially rescue impaired hemoglobinization or erythroid differentiation in MEL cells and $CD34^+$ hematopoietic stem cells treated with AOA, respectively. The ability of ALA, a committed intermediate in the heme synthesis pathway, to partially rescue suggest that heme synthesis is at least one essential aspect of glutamate and α-ketoglutarate metabolism.

It has also been shown by others that 6-diazo-5-oxo-L-norleucine (DON) inhibits erythroid differentiation (Oburoglu, Tardito et al. 2014). DON is known to inhibit a variety of reactions that use glutamine including reactions necessary for nucleotide synthesis, NAD synthesis, and glutaminolysis. Oburoglu et al. also reported that a "nucleoside mix" was able to partially rescue DON impairment of erythroid differentiation whereas DMK was ineffective. It should be noted that they did not report the impact of this rescue on overall cell viability or cell counts. In our own preliminary studies, treating differentiating MEL cells with the same concentration of DON proved to be toxic. Most cells were killed and the few remaining could not be accurately quantitated due to the presence of cell debris and dying cells. Without an accurate cell count, hemoglobin concentration per cell could not be calculated to access the effects of DON on hemoglobin synthesis. Additionally, the same nucleoside mix referenced was unable to rescue the effects of DON in MEL cells. Further studies are necessary to access

the impact of DON on heme synthesis in $CD34^+$ hematopoietic stem cells since prior studies only examined erythroid differentiation with erythroid cell surface markers. While nucleotide synthesis is an essential role for glutamine during erythropoiesis, our data indicate that it is essential for heme synthesis as well.

Succinyl-CoA production for heme synthesis has been suggested to come from both α-ketoglutarate (Abboud, Alexander et al. 1985) and succinate (Furuyama and Sassa 2000). At only 2.5% above unlabeled controls, incorporation of ^{13}C into heme from labeled DES was only marginally greater than natural abundance. However, incorporation of ^{13}C from labeled glucose and glutamine, both of which use α-ketoglutarate as an intermediate en route to succinyl-CoA, labeled heme more efficiently. In addition to the ^{13}C studies, experiments using AOA also support α-ketoglutarate as the sole route of succinyl-CoA synthesis during erythroid differentiation since DMK but not DES effectively reversed impaired hemoglobinization in MEL cells and erythroid differentiation of $CD34^+$ hematopoietic stem cells.

We have identified a previously unreported interaction between KDH and ALAS2. Since KDH interacts with SCL it is unclear if there is a labile pool of succinyl-CoA, and an interaction with KDH may provide ALAS2 access to succinyl-CoA. Alternatively, substrate channeling may decrease ALAS2's K_m for succinyl-CoA similar to the interaction between KDH and SCL (Porpaczy, Sumegi et al. 1983). Using *in vitro* assays we showed that SCL or ALAS2 increases KDH activity and that there is a synergistic increase in activity with both SCL and

ALAS2. Adding KDH or SCL increases ALAS2 activity, but when both KDH and SCL are added there is not an increase in activity. The latter trend is similar to data obtain by the Neuberger group in 1958 (Gibson, Laver et al. 1958, Laver, Neuberger et al. 1958). In their work ALA production was assayed from a "particulate fraction" isolated from anemic chicken reticulocytes. Both α-ketoglutarate and succinate were effective substrates for ALA synthesis, but succinyl-CoA was not a suitable substrate. When homogenized or freeze-dried the substrate requirement for ALA synthesis changed: succinyl-CoA became necessary and α-ketoglutarate and succinate became ineffective for ALA synthesis. This was explained as a "permeability barrier" that disappeared upon freeze-drying or homogenization. Their data is explainable by KDH, SCL, and ALAS2 forming a protein complex. When intact, α-ketoglutarate or succinate could be converted into succinyl-CoA and channeled to ALAS2 for ALA synthesis. Access to ALAS2's active site would be blocked by the interactions with KDH and SCL preventing ALA synthesis from succinyl-CoA in solution. Upon disruption of the complex, succinyl-CoA would become the superior substrate. Analogously, our data is consistent with both KDH and SCL increasing ALAS2 activity in a three-enzyme complex that blocks the entry of succinyl-CoA into the active site of ALAS2. Residual ALAS2 activity can be explained by incomplete formation of three-enzyme complexes allowing succinyl-CoA into ALAS2's active site similar to when only KDH or SCL is present. The role of SCL in this complex is not clear at this time. An interaction between KDH

and SCL is advantageous for α-ketoglutarate to succinate conversion in the TCA cycle and ALAS2 attaching to this complex to divert succinyl-CoA to heme synthesis is plausible.

METHODS

Chemicals

All chemicals were purchased from Sigma, St. Louis, MO unless otherwise stated.

^{13}C Labeling

DS19 MEL cells (Singer, Cooper et al. 1974, Ohta, Tanaka et al. 1976) were cultured in DMEM (Cellgro) with 5 mM glucose, 1 mM glutamine, 1mM DES, 10% FBS (Atlanta Biologicals) and 1% penicillin/streptomycin (Cellgro). These concentrations were chosen to resemble physiological concentrations where glucose levels range from 4-6 mM and glutamine levels are ~0.5 mM. Although not physiologically relevant, DES concentration was set equal to glutamine for comparison. Erythroid differentiation was induced by 1.5% dimethylsulfoxide being included in the growth media. At 48 hours post induction, unlabeled media was replaced with a media in which one of the three substrates (glucose, glutamine, or DES) was uniformly ^{13}C labeled. Cells were grown for an additional 24 hours, washed with phosphate buffered saline (PBS), harvested, and frozen by liquid nitrogen. Heme was then extracted by adding etheyl acetate and acetic acid to cell pellets. Final concentration of ethyl acetate, acetic acid, and sample was 4:1:1. Samples were homogenized and cell debris

removed by centrifugation. Incorporation of ^{13}C was determined by LC/MS analysis using an Agilent 6550 quadrupole time-of-flight mass spectrometer. Percentage of heme carbons labeled was determined by using a weighted average of the percent labeling of each isotopologue. It was assumed each substrate could only label the 26 carbons derived from succinyl-CoA. ^{13}C substrates were purchased from Cambridge Isotopes.

Conditions and experimental setup for analysis of ^{13}C labeling to various metabolites was similar to conditions for heme analysis with the following exceptions. Metabolites were extracted by suspending cells in 90% methanol to a final concentration of 80% methanol assuming that samples were 75% water. Samples were homogenized and cell debris removed by centrifugation. ^{13}C incorporation was analyzed using a Waters GCT Premier gas chromatograph-mass spectrometer (GC-MS).

Metabolic Inhibitors

MEL cells were cultured in DMEM with 25 mM glucose, 1 mM sodium pyruvate, and 4 mM glutamine (Cellgro) supplemented with 10% FBS, and 1% penicillin/streptomycin. 1 mM AOA, 3mM DMK, 3 mM DES, 100 μM ALA, 100 μM iron dextran and 500 μM succinyl acetone were added at the initiation of differentiation or at 48 hours post induction as indicated. BPTES was used at 50 μM or 100 μM as indicated. At 72 hours post induction, cells were washed with phosphate buffered saline, harvested and counted using a Scepter automated

cell counter (Millipore). Hemoglobin concentration was determined using a CLARiTY Spectrophotometer (Olis) as described (Marcero, in press).

$CD34^+$ hematopoietic stem cells isolated from peripheral blood were obtained from the University of Utah Stem Cell Core facility. Cells were expanded in StemSpan media (StemCell Technologies Inc.) with StemSpan CC100 expansion supplements (StemCell Technologies Inc.) for three days. Then cells were induced to erythroid differentiation using StemSpan erythroid expansion supplements (StemCell Technologies Inc.). 1 mM AOA, 3 mM DMK, 3 mM DES, and 1 mM ALA were added at erythroid differentiation induction as indicated. Media and substrates were replenished every three days. After nine days of erythroid differentiation and AOA treatment, cells were collected and erythroid differentiation accessed by flow cytometry detection of erythroid cell surface markers glycophorin A, CD71 (transferrin receptor), and CD36 (fatty acid translocase) as previously described (Oburoglu, Tardito et al. 2014).

TCA Cycle Activity

MEL cells were cultured in DMEM with 25 mM glucose, 1 mM sodium pyruvate, and 4 mM glutamine (Cellgro) supplemented with 10% FBS and 1% penicillin/streptomycin. Mitochondria were isolated from both undifferentiated cells and cells differentiated for 72 hours as previously published (Spector DL 1999). Protein concentration was determined using a BCA kit (Thermo Fisher Scientifc). Activities of TCA cycle enzymes were determined using assay

conditions described (Goncalves, Paupe et al. 2010). Activities from differentiated cells were normalized to the activity of undifferentiated cells.

Recombinant Protein-Protein Interactions

KDH purified from porcine hearts was purchased from Sigma. ALAS2 was recombinantly expressed in *Escherchia coli* using the pTrcHisA vector (Invitrogen). Cultures were grown to an OD of 1.0 in Circlegrow media (MP Bio medicals) and induced with 0.1 mM IPTG for ten hours. ALAS2 pellets from one liter of liquid culture were resuspended in solubilization buffer consisting of 50 mM Tris-MOPS, pH 8.0, 100 mM KCl, and 1% sodium cholate. Cell were lysed by sonication and clarified by ultracentrifugation at 100,000xg for 20 minutes. 0.7 mg of KDH solution was added to the resulting ALAS2 supernatant. An equal amount of KDH solution was also added an equal volume of solubilization buffer. Supernatants with ALAS2 only, ALAS2 with KDH added, and KDH in solubilization buffer were applied to HisPur Cobalt Resin (Thermo Fisher Scientifc) columns. The column was then washed with 15 mM imidazole solubilization buffer. Bound proteins were eluted with 250 mM imidazole solubilization buffer. Presence of the E1 component of KDH was detected using western blots. Proteins were separated using Mini-PROTEAN TGX Stain-Free gels (BioRad) and then transferred by Transblot semi-dry blotting (BioRad) to nitrocellulose membranes (BioRad). Anti-E1 rabbit polyclonal antibody (Gene-Tex) was used at a 1:1000 dilution. Anti-Rabbit IgG (H+L) HRP conjugate (Promega) secondary antibody was used at a 1:50,000 dilution. SuperSignal

West Pico Chemiluminescent substrate (Thermo Fisher Scientific) was used and visualized using a ChemiDoc imaging system (BioRad).

Recombinant Enzyme Assays

Suclg1 and Sucla2 were co-expressed in *E. coli* on two separate plasmids. Suclg1 was expressed using the pET28a vector (Invitrogen) with kanamycin resistance for selection, and Sucla2 was expressed using the pET14b vector (Invitrogen) with ampicillin resistance. One-liter cultures were grown to an OD of 1.0 and induced with 1 mM IPTG for five hours.

Both ALAS2 and SCL (Suclg1 and Sucla2 co-expressed) were purified using His-tag affinity chromatography using methods previously described (Burden, 1999). KDH activity was determined by monitoring the reduction of NAD^+ at 340 nM using a ClarioStar microplate reader (BMG Labtech). Assay conditions were as previously described except CoA concentration was 100 µM (Porpaczy, Sumegi et al. 1983). ALAS2, SCL, or both were in solubilization buffer with 250 mM imidazole. The volume of buffer added to each reaction was constant for each condition. Activities were normalized to the activity of KDH alone. Only the substrates for KDH were present and substrates necessary for ALAS2 or SCL were excluded. ALAS2 activity was determined using the modified Erhlich reaction (Shoolingin-Jordan 1997). Succinyl-CoA was synthesized using succinic anhyrdride and Coenzyme A (Simon 1953, Lawrence 1972). KDH was diluted into solubilization buffer with 250 mM imidazole.

Statistical Analysis

Statistical analysis was performed using an unpaired student *t*-test with a P value of <0.05 being significant. Error bars represent +/- standard deviations. For MEL cell experiments, technical replicates represent a separate cultured split from the same original culture.

CHAPTER THREE

CONCLUSION

The heme synthesis pathway has been thoroughly studied. The process of characterizing this pathway began in the 1940s and 1950s by identifying each enzyme and intermediates. Over time details of regulation and the effects of deranged heme synthesis emerged. Crystal structures and mechanisms of catalysis for each enzyme were solved. In the last decade, advances to our understanding of the heme synthesis pathway have been made by identifying accessory proteins that perform diverse processes essential for heme synthesis. Although not directly involved in the conversion of succinyl-CoA and glycine into heme, enzymes including ClpX, Glrx5, TMEME14c, ABCB10, ABCB7 all fulfill roles that if not performed have the potential to result in a disease state. In this work we have continued this effort by examining the source of succinyl-CoA for ALA synthesis during erythropoiesis.

Prior publications have presented conflicting hypotheses of whether α-ketoglutarate or succinate is converted into succinyl-CoA for heme synthesis during erythropoiesis (Abboud, Alexander et al. 1985, Furuyama and Sassa 2000, Neufeld, Fleming et al. 2001, Bishop, Tchaikovskii et al. 2012). Using MEL cells we show not only that α-ketoglutarate is the preferred route by which succinyl-CoA is made for erythroid heme synthesis but also that increased

succinate levels are not able to compensate for impaired α-ketoglutarate metabolism.

The second question we asked was what anaplerotic sources are used to replenish the vast amounts of succinyl-CoA consumed by heme synthesis during erythropoiesis. Knowing that glucose and glutamine metabolisms are indispensable for erythropoiesis (Oburoglu, Tardito et al. 2014), we tracked incorporation of carbons from ^{13}C labeled glucose and glutamine into heme. Labeling from glutamine was abundant indicating that it is responsible for a large portion of heme carbon during erythropoiesis. There was some incorporation of ^{13}C from glucose, but it is unclear the route by which glucose carbons were incorporated into succinyl-CoA for two reasons. First, 3-phospoglycerate is generated from glucose in the glycolytic pathway and eventually converted into glycine. The experimental setup employed cannot differentiate between ^{13}C incorporated via glycine or succinyl-CoA. Second, ^{13}C glucose labels the TCA cycle intermediates fumarate and malate as well if not better than ^{13}C glutamine, yet ^{13}C glutamine labels heme more efficiently. If α-ketoglutarate produced in the TCA cycle is used for erythroid succinyl-CoA production, any substrate that labels TCA cycle intermediates would be expected to label heme as well and the magnitude of heme labeling should parallel the magnitude of labeling of TCA cycle intermediates. However, if a separate “pool” of α-ketoglutarate is used for erythroid succinyl-CoA synthesis other pathways may be involved. The reactions to convert glutamine into α-ketoglutarate are reversible allowing for α-

ketoglutarate to be converted into glutamine. Since ^{13}C glucose labels glutamate and glutamine, it raises the possibility that labeling from glucose involves glutamine as an intermediate. α-ketoglutarate formed in the TCA consisting of carbons derived from glucose could be converted into glutamine that is then converted into succinyl-CoA in a spatially separated pathway specific for erythroid heme synthesis. This would mean glucose does supply extracellular carbons for heme synthesis, but it would also imply that there is more complexity to the process of succinyl-CoA synthesis for heme synthesis.

The current model for heme synthesis assumes that succinyl-CoA is acquired from the TCA cycle (Figure 3.1). We propose a model in which succinyl-

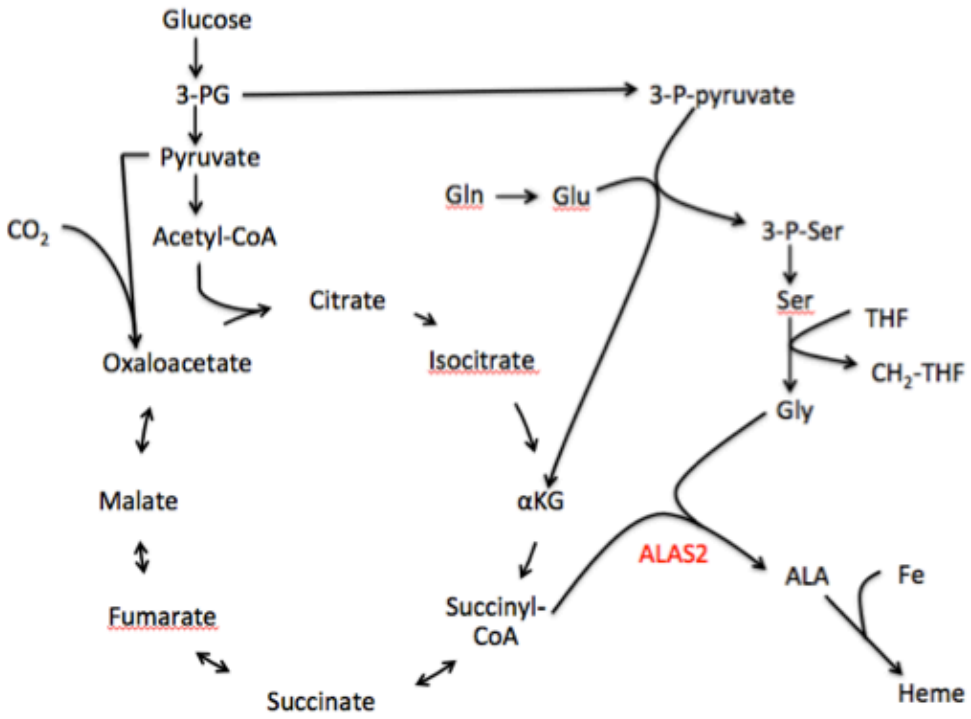


Figure 3.1: Currently Accepted Model For Erythroid Heme Synthesis. Succinyl-CoA is assumed to be removed from a labile pool in the TCA cycle.

CoA synthesized heme synthesis is coupled to glutamine metabolism and is distinct from succinyl-CoA produced in the TCA cycle (Figure 3.2). There is evidence that both the TCA cycle and heme synthesis form metabolons. Using protein-protein interactions and substrate channeling our model proposes that KDH not associated with the TCA cycle converts α-ketoglutarate into succinyl-CoA that is directly transferred to ALAS2 for ALA production. This model can explain the anomaly mentioned previously regarding labeling from ^{13}C glucose

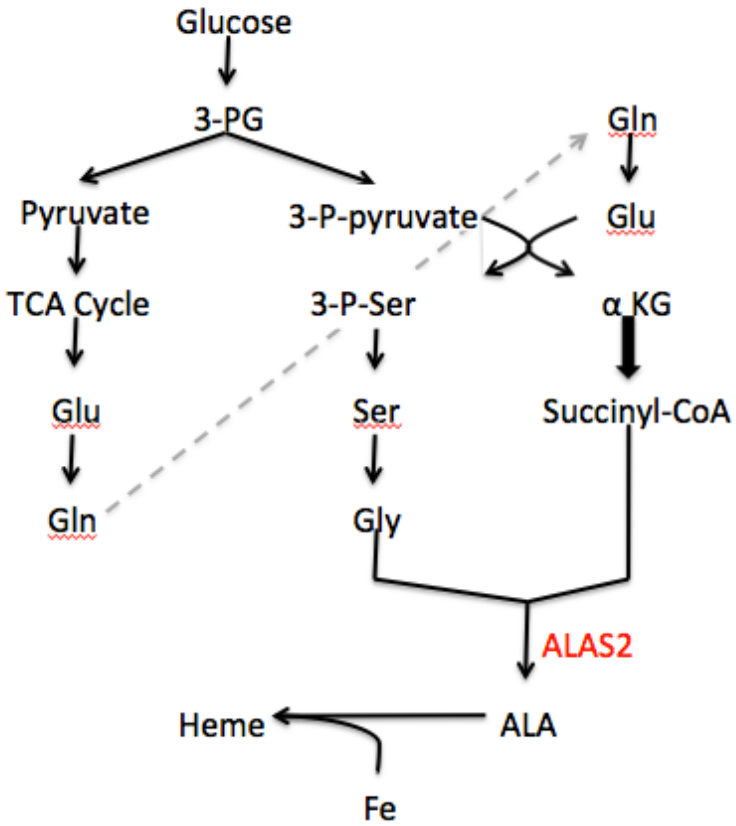


Figure 3.2: Proposed Model For Erythroid Heme Synthesis. Glutamine is principle source of carbons for heme synthesis in a pathway independent of the TCA cycle utilizing KDH in a moonlighting role.

and is supported by increased KDH activity and protein levels during MEL cell erythroid differentiation. In our model glutamine being converted into succinyl-CoA is viewed as a process completely separate from the TCA cycle where KDH plays a “moonlighting” role secondary to its function in the TCA cycle.

In a cohort of 83 patients, the mutations responsible for over 40% of CSAs are unknown (Bergmann, Campagna et al. 2010). In this cohort 15% of the CSA causing mutations are in SLC25a38, the putative mitochondrial glycine transporter. Similar to glycine, disrupting succinyl-CoA synthesis may result in CSA. Based upon our findings, KDH, Asct2, and various transaminases essential for glutamine/glutamate metabolism should be investigated for mutations in patients with idiopathic CSA.

A question not addressed by our work is whether succinyl-CoA for heme synthesis is generated by the same mechanism in non-erythroid tissue. Prior work suggests that succinate is converted directly into succinyl-CoA in the liver when mice are induced into a chemical hepatic porphyria (Labbe, Kurumada et al. 1965). This question is of interest specifically in the liver since it is the site of increased porphyrin production in the non-erythropoietic porphyrias. Determining how the increased demand for succinyl-CoA is met may offer therapeutic possibilities for these disorders.

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