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5-Aminolevulinate Synthase and the First Step of Heme Biosynthesis

Gloria C. Ferreira[1,2,3] and Jian Gong[1,2]

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5-Aminolevulinate synthase catalyzes the condensation of glycine and succinyl-CoA to yield 5-aminolevulinate. In animals, fungi, and some bacteria, 5-aminolevulinate synthase is the first enzyme of the heme biosynthetic pathway. Mutations on the human erythroid 5-aminolevulinate synthase, which is localized on the X-chromosome, have been associated with X-linked sideroblastic anemia. Recent biochemical and molecular biological developments provide important insights into the structure and function of this enzyme. In animals, two aminolevulinate synthase genes, one housekeeping and one erythroid-specific, have been identified. In addition, the isolation of 5-aminolevulinate synthase genomic and cDNA clones have permitted the development of expression systems, which have tremendously increased the yields of purified enzyme, facilitating structural and functional studies. A lysine residue has been identified as the residue involved in the Schiff base linkage of the pyridoxal 5′-phosphate cofactor, and the catalytic domain has been assigned to the C-terminus of the enzyme. A conserved glycine-rich motif, common to all aminolevulinate synthases, has been proposed to be at the pyridoxal 5′-phosphate-binding site. A heme-regulatory motif, present in the presequences of 5-aminolevulinate synthase precursors, has been shown to mediate the inhibition of the mitochondrial import of the precursor proteins in the presence of heme. Finally, the regulatory mechanisms, exerted by an iron-responsive element binding protein, during the translation of erythroid 5-aminolevulinate synthase mRNA, are discussed in relation to heme biosynthesis.

KEY WORDS: Heme; 5-aminolevulinate; pyridoxal 5′-phosphate; mitochondria; heme metabolism.

INTRODUCTION

Tetrapyrroles are ubiquitous in biological systems. Among the major tetrapyrroles is heme, a ferrous iron-containing complex. The biosynthesis of tetrapyrroles is initiated from simple molecules and comprises only a few enzymatic steps. Moreover, the pathway is remarkably similar in all living systems, having 5-aminolevulinic acid (ALA) as the first committed intermediate. In animals, fungi, and some bacteria, the first enzyme in the pathway is ALA synthase (ALAS). The ALAS-catalzyed reaction involves the condensation of glycine and succinyl-CoA and is the rate-limiting step in the production of heme in the liver. ALAS is also probably the regulatory enzyme in the heme biosynthetic pathway in differentiating erythrocytes, although the regulatory mechanism(s) remain(s) unknown. In animals, two separate genes have been identified for ALAS; one encodes the erythroid-specific form (ALAS2 or ALAS-E) and the other, the house-keeping gene (ALAS1 or ALAS-H), encodes the hepatic form of ALAS. Recently, defects in the ALAS2 gene have been associated with X-linked sideroblastic anemia. In this minireview we will focus on molecular aspects

[1] Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, Tampa, Florida 33612.
[2] Institute for Biomolecular Science, University of South Florida, Tampa, Florida 33612.
[3] The H. Lee Moffitt Cancer Center and Research Institute, University of South Florida, Tampa, Florida 33612.

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of ALAS (at the RNA and protein levels) and their relationship to the function of the enzyme.

ISOLATION AND PURIFICATION

Since the discovery, in 1958, of ALAS in the bacterium *Rhodobacter spheroides* and in chicken erythrocytes in the laboratories of Shemin and Neuberger (Kikuchi *et al.*, 1958; Gibson *et al.*, 1958), respectively, the enzyme has been purified from diverse sources. These include *R. spheroides* (Warnick and Burnham, 1971; Jordan and Laghai-Newton, 1986), *Paracoccus denitrificans* (Tait, 1973), *Saccharomyces cerevisiae* (Voland and Felix, 1984), *Euglena gracilis* (Dzelzalns *et al.*, 1982), chicken liver (Borthwick *et al.*, 1983), and rat liver (Scholnick *et al.*, 1972; Ohashi and Kikuchi, 1979; Yomogida *et al.*, 1993). All of the purified ALASs appear to be homodimers, with subunit molecular masses of 40,000–70,000 (Warnick and Burnham, 1971; Scholnick *et al.*, 1972; Ohashi and Kikuchi, 1979; Nakakuki *et al.*, 1980; Borthwick *et al.*, 1983; Yomogida *et al.*, 1993). The early attempts at the isolation and purification of the enzyme were hindered by the low amount of ALAS in mitochondria, its susceptibility to proteolytic degradation during isolation, and the tendency of the enzyme to form aggregates. Since hepatic mitochondrial ALAS is strongly induced in animals by a wide range of drugs (e.g., 2-allyl-2-isopropylacetamide and 3,5-di-carbethoxy-1,4-dihydrocollidine), investigators recurred to isolation and purification of ALAS from drug-induced animals (Whiting and Granick, 1976; Srivastava *et al.*, 1982; Watanabe *et al.*, 1984; Borthwick *et al.*, 1986). More recently, with the development of expression systems, the isolation of ALAS became less of a problem, given the ready availability of recombinant protein (see section "Expression and Site-directed Mutagenesis").

KINETICS, SUBSTRATE SPECIFICITY, AND MECHANISM

ALAS has high specificity for one of the substrates, glycine. No other amino acid can replace glycine. Nevertheless, the reported K_m values for glycine are in the millimolar range. The requirement for the second substrate, succinyl-CoA, is less specific, but in contrast, the K_m values are in the micromolar range (Bishop *et al.*, 1981; Jordan and Laghai-Newton, 1986; Munakata *et al.*, 1993). All ALASs have pyridoxal 5′-phosphate (PLP) as an essential cofactor (Laghai and Jordan, 1976; Nandi, 1978a; Jordan, 1991). Fanica-Gaignier and Clement-Metral (1973), using steady-state kinetics, proposed an ordered bi-bi mechanism for ALAS, in which glycine binds first and 5-aminolevulinic acid dissociates last. As proposed for the *R. spheroides* enzyme (Kikuchi *et al.*, 1958; Zaman *et al.*, 1973), the first step in the synthesis of ALA involves the binding of glycine to the PLP-enzyme complex. In principle, two alternatives are possible for the generation of a stabilized carbanion at the C-2 of the glycine, either by loss of one of the α-hydrogen atoms or decarboxylation. Akhtar and Jordan (1968) demonstrated, unequivocally, that the proton with a pro-R configuration is removed from C-2 of the glycine-PLP-enzyme complex (Zaman *et al.*, 1973; Laghai and Jordan, 1976). This deprotonation step produces the carbanion to which succinyl-CoA is condensed. The decarboxylation of the intermediate, followed by the addition of H^+ at C-5 of ALA, maintains the prochirality of the ALA product (Emery and Akhtar, 1987). However, the ALAS residues involved in the catalysis remain to be identified.

CLONING AND SEQUENCING

Cloning and sequencing of cDNAs and genes encoding ALAS from different species permitted investigators to deduce and analyze the primary structure of the encoded proteins (Borthwick *et al.*, 1984; Leong *et al.*, 1985; Schoenhaut and Curtis, 1986; Bawden *et al.*, 1987; McClung *et al.*, 1987). In 1985, Yamamoto *et al.* isolated several cDNA clones from a chicken erythroid library, using an antibody raised against the hepatic ALAS. (Previously it had been demonstrated that the antibody cross-reacted with both the liver and erythroid ALAS forms.) Subsequently, Riddle *et al.* (1989) isolated and sequenced erythroid and liver ALAS cDNAs, demonstrating unequivocally that two separate genes encode the erythroid and the hepatic ALAS isozymes. Using functional complementation of a *hem1 S. cerevisiae* mutant, Urban-Grimal *et al.* (1984) cloned the yeast *hem1* ALAS gene. The protein predicted from the nucleotide sequence indicated that the ALAS precursor has 548 amino acids with an inferred molecular mass of 59.3 kDa. Similarly, ALAS genes and cDNAs

have been isolated and sequenced from *Bradyrhizobium japonicum* (McClung *et al.*, 1987), *Rhizobium meliloti* (Leong *et al.*, 1985), *Rhodobacter spheroides* (Neidle and Kaplan, 1993), *Rhodobacter capsulatus* (Biel *et al.*, 1988), mouse erythroid cells (Schoenhaut and Curtis, 1986, 1989), rat liver and erythroid cells (Yamamoto *et al.*, 1988; Srivastava *et al.*, 1988; Munakata *et al.*, 1993), and human liver and erythroid cells (Bawden *et al.*, 1987; Cox *et al.*, 1991). [An examination of the human erythroid ALAS gene, ALAS2, and its relationship to X-linked sideroblastic anemia is reviewed elsewhere in this volume by Bottomley *et al.* (1995).]

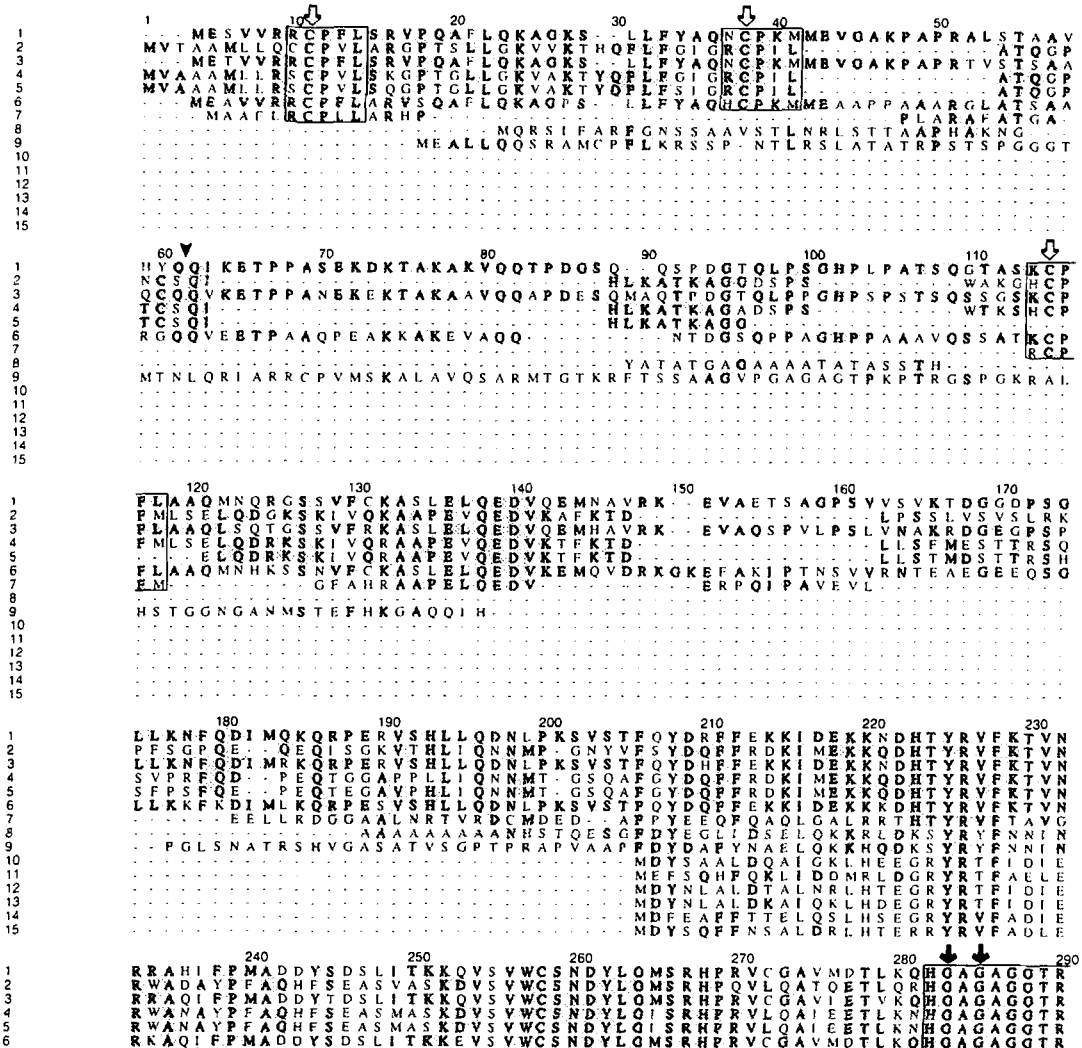
Comparison of the amino acid sequences of the different ALASs, ranging from bacteria to man, reveals extensive similarities, particularly centered in the C-terminal 75% of the mature proteins (Cox *et al.*, 1991; Neidle and Kaplan, 1993) (Fig. 1). In this conserved region the sequence similarity between mouse ALAS2 and human ALAS2 is 94%, whereas the human ALAS2 and human ALAS1 are 73% similar (Schoenhaut and Curtis, 1986; Bawden *et al.*, 1987; Cox *et al.*, 1991). The N-terminus of this conserved region corresponds to the N-termini of the bacterial enzymes (Leong *et al.*, 1985; McClung *et al.*, 1987; Cox *et al.*, 1991). Collectively, these observations suggest that the catalytic domain of all ALAS proteins is located at the C-terminal region (May *et al.*, 1990; Ferreira *et al.*, 1993). More recently, Munakata *et al.* (1993) confirmed this hypothesis. These investigators purified ALAS2 from rat erythroid cells and treated the purified enzyme with papain. The purified 48 kDa papain-resistant domain was shown to have catalytic activity, and sequence analysis indicated that it overlapped with the conserved C-domain (Munakata *et al.*, 1993). Significantly, the identification of the ALAS2 lysine-313, which is located in the C-terminal domain, as the residue involved in the Schiff base linkage of the PLP cofactor corroborates the assumption that the ALAS active site is, indeed, at the C-terminus of the enzyme (Ferreira *et al.*, 1993; see section "Identification of the PLP-Binding Peptide").

SYNTHESIS AND IMPORT INTO MITOCHONDRIA

ALAS is synthesized on cytosolic ribosomes as a pre-enzyme, which is then imported into and processed within the mitochondria to give the mature enzyme (Yamauchi *et al.*, 1980; Srivastava *et al.*, 1983; Volland and Urban-Grimal, 1988). Hayashi *et al.* (1983) reported the *in vitro* transport of ALAS precursor using isolated chicken liver mitochondria while Yamauchi *et al.* (1980) demonstrated that the rat liver ALAS precursor was translocated *in vivo* from the cytosol to the mitochondria. Human ALAS2 is synthesized as a precursor of molecular mass 65 kDa with an amphipathic presequence of 49 amino acids. During its import and processing, the presequence is removed to yield a mature enzyme with a molecular mass of 59.5 kDa.

Similarly, the yeast enzyme is initially synthesized as precursor protein with molecular mass of 59 kDa, whose presequence is cleaved by matrix proteases to yield a mature protein with molecular mass of 55 kDa (Urban-Grimal *et al.*, 1986; Volland and Urban-Grimal, 1988). Significantly, Volland and Urban-Grimal (1988) showed that the yeast ALAS presequence was not required to target the precursor protein to the mitochondria. However, the ALAS protein only reached its final destination, the mitochondrial matrix, when the presequence was present. Thus, it seems that the organelle-targeting information resides, at least in the yeast enzyme, in the mature protein sequence *per se*, whereas the subcompartmental (i.e., matrix) information is in the presequence. Further, using a series of yeast ALAS constructs carrying N-terminal deletions of the presequence to complement a *hem1* mutant yeast strain, Haldi and Guarente (1989) demonstrated that the targeting information is encoded in nonoverlapping regions of the presequence.

The import of the ALAS precursor into the mitochondria can be inhibited, however, by heme. High heme concentration can inhibit the mitochondrial import of ALAS (erythroid and hepatic enzymes) both *in vivo* and *in vitro* (Yamauchi *et al.*, 1980; Srivastava *et al.*, 1983; Hayashi *et al.*, 1983). Recently, Lathrop and Timko (1993) identified two heme-regulatory elements in the murine erythroid ALAS presequence (Fig. 1), which are involved in hemin inhibition of the mitochondrial import of the precursor. In contrast to the normal mitochondrial import of the human mitochondrial ornithine transcarbamoylase, the mitochondrial import of ALAS2 precursor was strongly inhibited in the presence of exogenous hemin. However, the replacement of the ornithine transcarbamoylase presequence with the ALAS2 presequence conferred the same pattern of hemin inhibition to the chimeric ornithine transcarbamoylase. Moreover, each of the heme-regulatory motifs



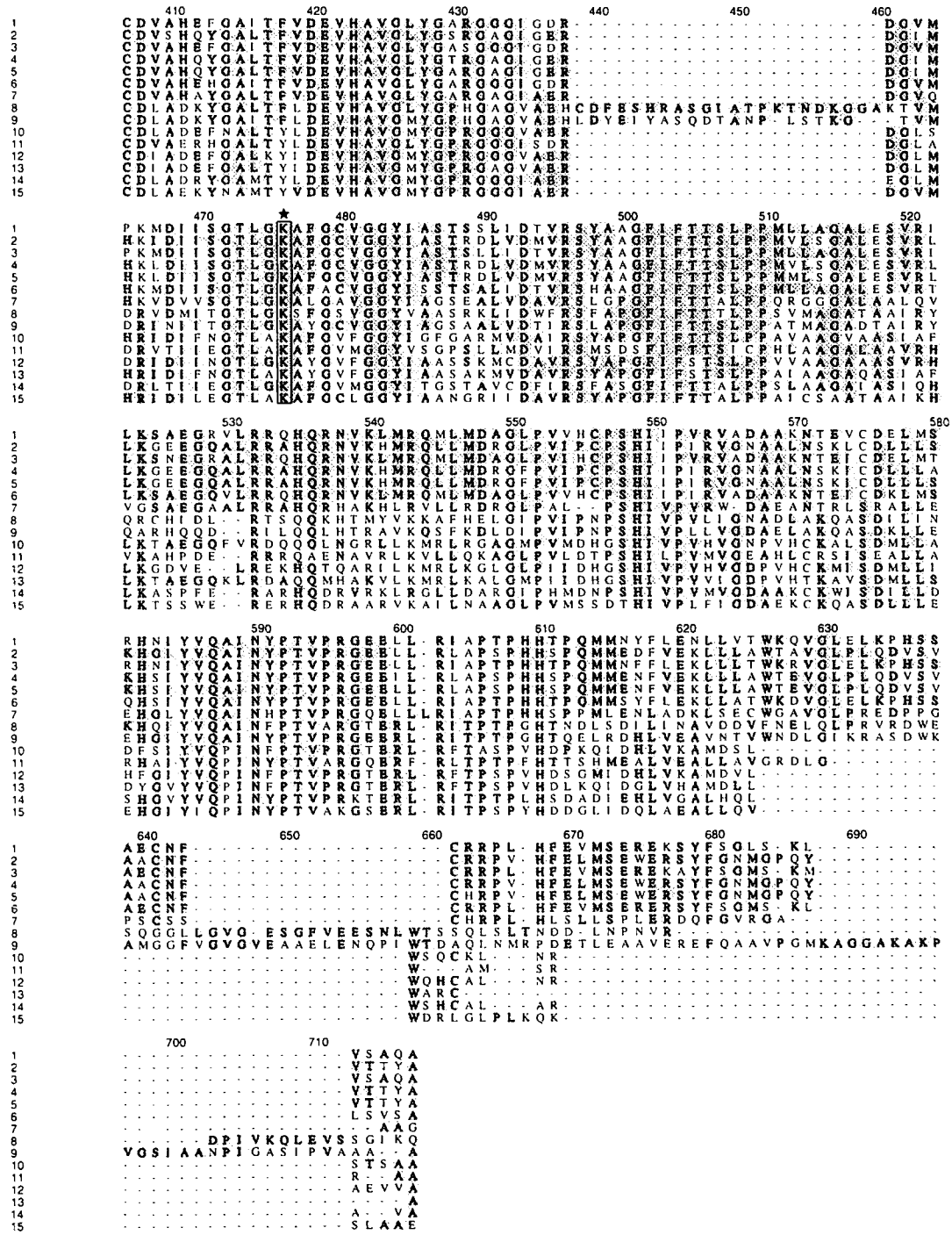


Fig. 1. Sequence alignment of eukaryotic and prokaryotic ALAS proteins. The ALASs sequences are: 1. human ALAS1 (Bishop, 1990); 2. human ALAS2 (Cox *et al.*, 1991); 3. rat ALAS1 (Yamamoto *et al.*, 1988); 4. rat ALAS2 (Munakata *et al.*, 1993); 5. mouse ALAS2 (Schoenhaut and Curtis, 1986, 1988); 6. chicken ALAS1 (Borthwick *et al.*, 1985); 7. chicken ALAS2 (Riddle *et al.*, 1989); 8. *Saccharomyces cerevisiae hem1* (Urban-Grimal *et al.*, 1986); 9. *Aspergillus nidulans* ALAS (Bradshaw *et al.*, 1993); 10. *Paracocus denitrificans* ALAS (Page and Ferguson, 1994); 11. *Rhodobacter spheroides hemT* (Neidle and Kaplan, 1993); 12. *Rhodobacter spheroides hemA* (Elliott *et al.*, 1989; Neidle and Kaplan, 1993); 13. *Rhodobacter capsulatus* ALAS (Wright *et al.*, 1991); 14. *Agrobacterium radiobacter* ALAS (Drolet and Sasarman, 1991); 15. *Bradyrhizobium japonicum* ALAS (McClung *et al.*, 1987). Residues conserved in all sequences are shaded. The lysine residue involved in the Schiff base linkage with the PLP cofactor is boxed and indicated with a star (Ferreira *et al.*, 1993). The glycine-loop motif, which is proposed to be involved in PLP binding, is boxed and the glycine residues are identified with closed arrows (Gong and Ferreira, 1995). The heme-regulatory elements involved in hemin inhibition of the mitochondrial import of the precursor are enclosed in boxes and the mutated cysteines, referred in the text, are designated with open arrows (Lathrop and Timko, 1993). The proteolytic cleavage site of the presequences is shown with a closed arrow head (Cox *et al.*, 1991) [Experimental evidence on the proteolytic cleavage site has only been determined for chicken and rat ALAS1 (Borthwick *et al.*, 1985; Srivastava *et al.*, 1988)].

contains a single cysteine residue. The deletion of the heme-regulatory motifs, as well as the mutation of the cysteine to serine residues, reversed the heme inhibition (Lathrop and Timko, 1993).

FUNCTION AND STRUCTURE STUDIES

Expression and Site-Directed Mutagenesis

Recently, a full-length cDNA encoding the murine ALAS2 was expressed in *E. coli*, yielding the recombinant ALAS as the major protein associated with the bacterial soluble fraction (Ferreira and Dailey, 1993). Basically, the investigators placed the ALAS2-encoding DNA fragment under the transcriptional control of the strong alkaline phosphatase promoter and transformed *E. coli* cells with the expression construct. Bacterial cells containing the ALAS expression plasmid were induced by growth in low phosphate concentration medium. The purified, overproduced recombinant ALAS exhibited physical and catalytic properties (i.e., a dimer of identical subunits and K_m values for both substrates) similar to those of ALAS isolated from nonrecombinant sources (Ferreira and Dailey, 1993). Wild-type and mutant forms of human ALAS2 have also been overproduced in *E. coli* by having the DNA fragments encoding these proteins placed under the transcriptional control of T7 RNA polymerase promoter (Cotter *et al.*, 1992; Cox *et al.*, 1994).

Identification of the PLP-Binding Peptide

Although ALAS has been known for quite a long time to be a PLP-dependent enzyme (Warnick and Burnham, 1971), the nature of the binding of the cofactor to the enzyme remained unknown until recently. Scholnick *et al.* (1972), using specific inhibitor and isotopic studies, proposed an initial formation of a thiohemiacetal between ALAS and PLP; Fanica-Gaignier and Clement-Metral (1973) suggested that a –SH group was present at the active site of the enzyme; and Jordan (1991) suggested that under neutral conditions PLP is bound to the enzyme, possibly as a carbinolamine through a –SH group rather than as a Schiff base. In contrast, Nandi (1978a,b) proposed that PLP is bound as an imine to an ϵ-amino group of a lysine residue. In order to determine whether PLP is bound to an ϵ-amino group of an active-site lysine residue by a Schiff base linkage and to define the ALAS active center, Ferreira *et al.* (1993) identified the amino acid residue that is involved directly in the binding of the cofactor. In essence, purified recombinant ALAS2 was reduced with tritiated sodium borohydride, alkylated with iodoacetamide, and proteolysed with trypsin. The labeled tryptic peptide was separated by HPLC and sequenced.

The labeled peptide (LDIISGTLGKAFGCV-GGYI) had 15 amino acids around lysine-313, to which PLP is bound. Significantly, the pyridoxyllysine peptide is conserved in all known ALAS sequences, from bacteria to man (Ferreira *et al.*, 1993) (Fig. 1). Mutagenesis of the murine ALAS2 lysine-313 (K313) to alanine, glycine, or histidine abolished enzyme activity in the mutated ALAS2 variants. Noncovalent interactions are also involved in the binding of the PLP cofactor, and the lysine residue involved in the Schiff base linkage with the cofactor appears to have also a catalytic role (Ferreira *et al.* unpublished results).

Informational Content of a Glycine-Rich Motif

Sequence alignment of all known ALASs indicated the presence of a conserved glycine-rich motif (Fig. 1; Gong and Ferreira, 1995). Interestingly, the consensus sequence GXGXXG, which forms a loop between a β-sheet and an α-helix, has been implicated as part of the cofactor binding site in some PLP-dependent enzymes (Weber *et al.*, 1978; Hyde *et al.*, 1988; Marceau *et al.*, 1988), and as the phospate-binding motif in numerous nucleotide-binding proteins (Branden and Tooze, 1991; Swindells, 1993). The glycine-loop has therefore been proposed to be part of the PLP-binding motif in ALAS (Gong and Ferreira, 1995).

Gong and Ferreira (1995) performed an informational content analysis on each residue within the 11 amino acid glycine-rich region of murine ALAS2 (141-HGAGAGGTRNI) (Fig. 1). Through partial random mutagenesis of this region, followed by an efficient biological selection using a *hemA*$^-$ *E. coli* strain, the investigators were able to recover functional unnatural enzymes. As indicated in Fig. 2, most residues in the glycine-loop can be replaced by several other amino acids, yet producing active enzymes. However, Gly-142 and Gly-144 could only tolerate alanine replacement, while Arg-149 was conserved in all functional enzymes (Fig. 2). The high informational content of these three residues suggests they have important functional roles. Subsequently, characterization of selected glycine-loop functional

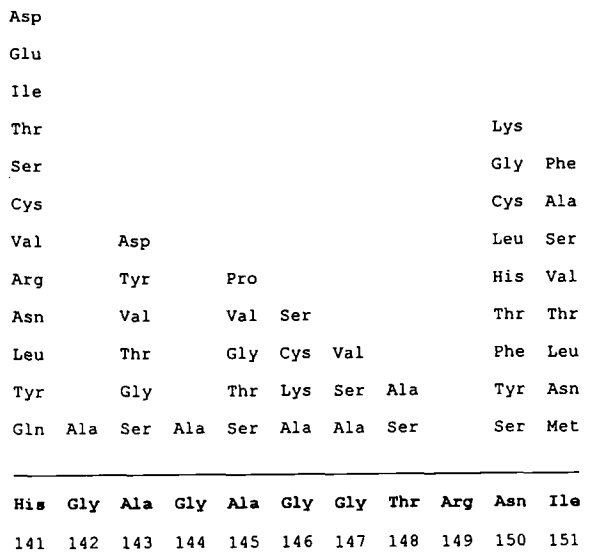


Fig. 2. Informational content analysis of the glycine-loop: functionally acceptable amino acid substitutions at each position. Wild-type murine ALAS2 glycine-loop sequence is shown below the line. Adapted from Gong and Ferreira (1995).

mutants indicated that they exhibited lower V_{max} and higher K_m values for the substrates, as compared to those of the wild-type ALAS. This indicates that both the catalysis and the binding of the substrates were affected in the mutants, suggesting that perturbations in the glycine loop may induce conformational changes and/or alterations of cofactor orientation, which affect residues involved in both catalysis and substrate binding (Gong and Ferreira, 1995). Even without the three-dimensional structure, one can now conceive that the aldehyde group of the PLP cofactor is bound to ALAS through a Schiff base linkage with the lysine-313 and, as with other PLP-dependent enzymes and nucleotide-binding proteins (Weber *et al.*, 1978; Hyde *et al.*, 1988), that the phosphate group of the PLP cofactor interacts (probably through hydrogen bonding and van der Waals contacts) with the glycine-rich loop.

Regulatory Mechanisms in Hepatic and Erythroid Cells

Two separate genes for ALAS, one encoding the erythroid-specific form (ALAS2) and the other, the house-keeping gene (ALAS1), encoding the hepatic form of ALAS have been identified in animal cells (Riddle *et al.*, 1989; Fujita *et al.*, 1991). [Curiously, two ALAS separate genes, *hemA* and *hemT*, have also been identified in *R. spheroides* (Neidle and Kaplan, 1993).] The ALAS2 gene is solely expressed in erythroid cells, whereas ALAS1 is ubiquitously expressed (Riddle *et al.*, 1989; Cox *et al.*, 1990; Bishop *et al.*, 1990). Moreover, the two genes for ALAS are under separate control mechanisms (Fujita *et al.*, 1991), and in man, they are localized in different chromosomes (Cox *et al.*, 1990; Bishop *et al.*, 1990). ALAS1 controls the production of heme for basic cellular functions and is feedback-regulated by heme (Dierks, 1990). Several mechanisms have been suggested to explain the inhibitory action of heme in the hepatic pathway: (1) direct inhibition of ALAS activity (Scholnick *et al.*, 1972; Whiting and Granick, 1976) [however, as pointed out by May *et al.* (1986), "it was not established in any of these studies that native ALAS was used"]; (2) repression of the synthesis of mRNA for ALAS (Yamamoto *et al.*, 1988); (3) repression of ALAS at the translational level (Yamamoto *et al.*, 1983; Dierks, 1990); (4) inhibition of the translocation of the cytosolic form of the enzyme into the mitochondrial matrix (Yamauchi *et al.*, 1980). In contrast to heme, porphyrinogenic drugs (e.g., 2-allyl-2-isopropylacetamide or phenobarbital), activate transcription of the ALAS1 gene (Yomogida *et al.*, 1993).

ALAS2 is mainly involved in the synthesis of heme for the production of hemoglobin in differentiating erythrocytes. Thus, erythroid differentiation requires a coordinate regulation of expression of globin genes and the genes for the enzymes of the heme biosynthetic pathway. In contrast to ALAS1, ALAS2 is neither induced by the porphyrinogenic effectors nor inhibited by the addition of hemin (Wada *et al.*, 1967; Srivastava *et al.*, 1989, 1990). Fraser and Curtis (1987) showed that upon induction of murine erythroleukemia (MEL) cells with DMSO, there was "a gradual, steady decrease in total transcriptional activity and mRNA content per cell, which by day 3 of DMSO treatment amounted to less than 50% of the level in the uninduced cell." During the same period, the investigators observed "increases in transcriptional activity for ALAS," coordinated with a "large increase in β-globin gene transcription" (Fraser and Curtis, 1987). Recently, it was demonstrated that while the transcription of the gene for ALAS1 was inhibited by heme, transcription of the gene for ALAS2 was induced by heme in MEL cells undergoing differentiation (Fujita *et al.*, 1991). It was, therefore, proposed that the genes encoding the two ALAS isoenzymes are under separate control mechanisms in erythrocytes and that the accumulation of ALAS2 mRNA is responsible for the

increased heme biosynthesis in differentiating erythrocytes of MEL cells (Fujita *et al.*, 1991).

Regulation of ALAS2 is, therefore, very complex and occurs at many different levels. These include regulation of (1) transcription of the ALAS2 gene (Fujita *et al.*, 1991; Braidotti *et al.*, 1993); (2) translation of the ALAS2 mRNA through interactions between an iron-responsive element (IRE) and IRE-binding protein (IRE-BP or IRP1) (Cox *et al.*, 1991; Dandekar *et al.*, 1991; Samaniego *et al.*, 1994); (3) post-transcription, through alternative splicing within ALAS2 (Schoenhaut and Curtis, 1989; Conboy *et al.*, 1992). An iron-responsive element (IRE), present in the 5′ untranslated region of the ALAS2 mRNA, seems to have an important role in the control of heme biosynthesis during erythroid differentiation through interaction with the IRE-binding protein (IRE-BP) (Cox *et al.*, 1991; Melefors *et al.*, 1993; Gray and Hentze, 1994). [No IRE has been identified in the ALAS1 mRNA (Braidotti *et al.*, 1993; Melefors *et al.*, 1993).] IRE-BP has structural homology with the iron-sulfur protein, cytosolic aconitase (Rouault *et al.*, 1992), and when bound to its specific IRE, functions as a repressor of translation of ALAS2 mRNA (Melefors *et al.*, 1993; Gray and Hentze, 1994). When the iron concentrations in the cell are low, the IRE-BP has its [4Fe-4S] cluster disassembled and exhibits high affinity for IRE binding, whereas when iron is abundant, IRE-BP has low affinity for IRE and high aconitase activity (Klausner *et al.*, 1993). Recently, Gray and Hentze (1994) proposed a molecular mechanism for the IRE-BP-mediated translational inhibition of ALAS2 mRNA. The authors demonstrated that the interaction of IRE-BP with IRE prevents the association of the 43S translational pre-initiation complex with the ALAS2 mRNA.

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