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Heme biosynthesis in mammalian systems: Evidence of a Schiff base linkage between the pyridoxal 5′-phosphate cofactor and a lysine residue in 5-aminolevulinate synthase

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Abstract

5-Aminolevulinate synthase is the first enzyme of the heme biosynthetic pathway in nonplant higher eukaryotes. Murine erythroid 5-aminolevulinate synthase has been purified to homogeneity from an *Escherichia coli* overproducing strain, and the catalytic and spectroscopic properties of this recombinant enzyme were compared with those from nonrecombinant sources (Ferreira, G.C. & Dailey, H.A., 1993, *J. Biol. Chem. 268*, 584–590). 5-Aminolevulinate synthase is a pyridoxal 5′-phosphate-dependent enzyme and is functional as a homodimer.

The recombinant 5-aminolevulinate synthase holoenzyme was reduced with tritiated sodium borohydride and digested with trypsin. A single peptide contained the majority of the label. The tritiated peptide was isolated, and its amino acid sequence was determined; it corresponded to 15 amino acids around lysine 313, to which pyridoxal 5′-phosphate is bound. Significantly, the pyridoxyllysine peptide is conserved in all known cDNA-derived 5-aminolevulinate synthase sequences and is present in the C-terminal (catalytic) domain.

Mutagenesis of the 5-aminolevulinate synthase residue, which is involved in the Schiff base linkage with pyridoxal 5′-phosphate, from lysine to alanine or histidine abolished enzyme activity in the expressed protein.

Keywords: 5-aminolevulinate synthase; heme biosynthesis; pyridoxyllysine; pyridoxal 5′-phosphate

5-Aminolevulinate (ALA) is the first committed intermediate in the tetrapyrrole biosynthetic pathway in all living systems, although in plants, algae, and some photosynthetic bacteria it is synthesized using a different mechanism than the one in animals, fungi, and other bacteria. In nonplant higher eukaryotes, ALA synthase (ALAS) (EC 2.3.1.37) is the first enzyme of the heme biosynthetic pathway and catalyzes the condensation of glycine and succinyl-CoA to yield ALA, CoA-SH, and carbon dioxide (Kikuchi et al., 1958; Jordan, 1991). Heme is used as the prosthetic group of various hemoproteins essential to cell function and, as would be expected, ALAS is expressed ubiquitously (May et al., 1990; Jordan, 1991). The highest levels of expression are found in erythrocytes and hepatocytes, where heme demands are greater due to the biosynthesis of hemoglobin or cytochrome P-450, respectively (May et al., 1990; Conboy et al., 1992).

Two separate genes for ALAS, one encoding the erythroid-specific and the other the hepatic (housekeeping) form of ALAS, have been identified (Riddle et al., 1989). The gene for human erythroid ALAS is located on the X-chromosome (Bishop et al., 1990; Cox et al., 1990), whereas the gene for human housekeeping ALAS has been localized on chromosome 3 (Sutherland et al., 1988;

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Abbreviations: ALA, 5-aminolevulinate; ALAS, 5-aminolevulinate synthase; AKB, 2-amino-3-ketobutyrate; HPLC, high-pressure liquid chromatography; KAPA, 7-keto-8-aminopelargonic acid; $NaBH_4$, sodium borohydride; PLP, pyridoxal 5′-phosphate; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

Bishop et al., 1990). Synthesis of ALAS occurs in the cytosol as a precursor form, which is then imported to its final mitochondrial location and processed to give the mature enzyme (Yamauchi et al., 1980; Urban-Grimal et al., 1986).

The murine erythroid ALAS has a molecular mass of approx. 56 kDa and consists of two apparently identical subunits (Ferreira & Dailey, 1993). Its complete amino acid sequence has been inferred from the sequence of partial cDNA clones encoding the mouse erythroid ALAS (Schoenhaut & Curtis, 1986). This ALAS is a PLP-dependent enzyme and is highly specific for one of its substrates, glycine (Jordan, 1991). However, the nature of the binding of the cofactor to the enzyme remains unknown.

In order to determine whether the PLP cofactor is bound to an ϵ-amino group of an active site lysine residue by a Schiff base linkage, and to delineate the ALAS active center, we identified the amino acid residue that is involved directly in the binding of PLP. In this paper we report the amino acid sequence of the PLP-binding peptide, its pyridoxyllysine nature, and its location in the primary sequence of the mouse erythroid ALAS. This residue is present in the carboxy domain of every known ALAS. In addition, site-directed mutated variants, in which the lysine residue involved in the Schiff base linkage of the PLP cofactor was replaced by either alanine or histidine, did not exhibit enzymatic activity. The consensus sequence reported below can now be used for elucidation of the architecture of the ALAS active site and determination of amino acid residues involved in binding of substrates and catalysis.

This is the first report determining the pyridoxyllysine peptide sequence in any ALAS and showing that an ALAS lysine residue is involved in a Schiff base linkage with PLP.

Results

Tryptic peptide map of the NaB^{3}H$_4$-reduced ALAS

Gel filtration analysis of the tryptic digest of the PLP-ALAS adduct gave a single peak of labeled material, centering on fraction 17 (Fig. 1). This fraction, and the two flanking fractions, 16 and 18, were applied directly to a reversed-phase column. To identify the peptide that contained label, UV-absorbing peaks were collected by hand, and approximately 25% of each peak was subjected to scintillation counting for ^{3}H. As expected, the majority of counts were found in fraction 17 (Fig. 2). The major labeled peak was distributed between fraction 16 (1,110 cpm) and fraction 17 (2,800 cpm), eluting at 19.4 min. Other, minor, radioactivity peaks were present in fractions 17 (560 cpm) and 18 (470 cpm). Other peptides had insignificant levels of radioactivity (<400 cpm) (Fig. 2).

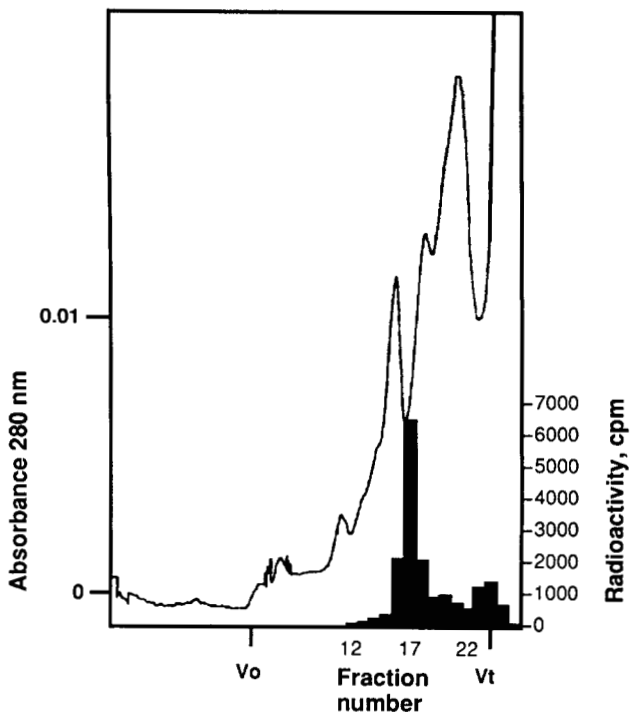

Fig. 1. Gel filtration separation of tryptic peptides from the PLP-ALAS adduct. The tryptic digest was applied to a Superdex 75 column, equilibrated in 4.0 M guanidine-HCl and monitored at 280 nm as described in the Materials and methods. Aliquots (25 μL) from each fraction (0.5 mL) were analyzed by scintillation counting. The majority of label was in fractions 16, 17, and 18, as shown by the solid vertical bars.

Purification and sequence determination of the pyridoxyllysine peptide

The major labeled peak from fraction 17 (Fig. 2) was sequenced and found to contain two peptides. The two sequences could be differentiated both by the relative yields of individual amino acids and by comparison of the sequence data with the published cDNA-derived sequence of murine ALAS (Schoenhaut & Curtis, 1986). One sequence (LDIISGTLGxAFGxVGGYIAxT . . . , approx. 15 pmol initial yield) corresponded to residues 304–326 in murine ALAS, and the other (SYAAGFIFTTSLPPMML SGAXE, approx. 4 pmol initial yield) corresponded to residues 334–358. The residues at positions 10 and 14 in the first peptide sequence were found to be lysine and cysteine, respectively, in the published sequence. We were unable to identify their PTH-amino acid derivatives directly.

Identification of ϵ-N-pyridoxyllysine

Part of the product of each cycle of sequencing (approx. 15%) was diverted to the fraction collector of the sequencer. Scintillation counting of released PTH-amino acids gave a large increase in counts, corresponding to the lysine residue (Fig. 3), followed by a higher than back-

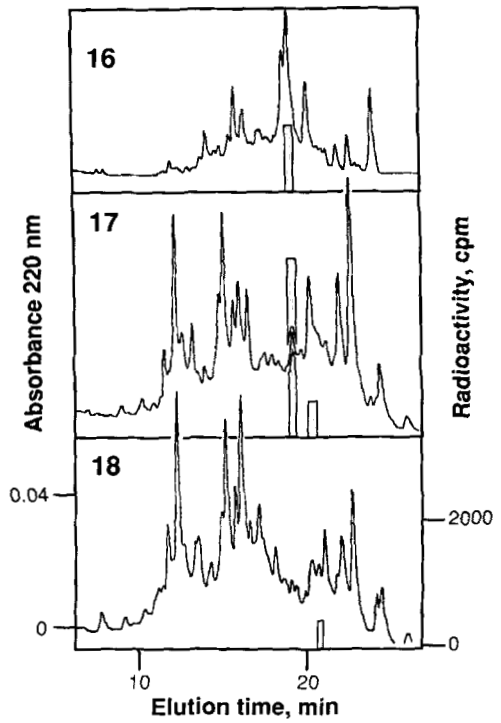

Fig. 2. Reversed-phase analysis of label-containing fractions from Figure 1. Individual fractions were analyzed by reversed-phase HPLC as described in the Materials and methods. Peak fractions were collected by hand and aliquots (approx. 25% of each peak) were analyzed by scintillation counting. Peaks with >400 cpm are identified graphically by vertical bars corresponding to their radioactivity. The majority of peaks were at or near background. The absorbance and radioactivity scales for fraction 18 are the same for fractions 16 and 17.

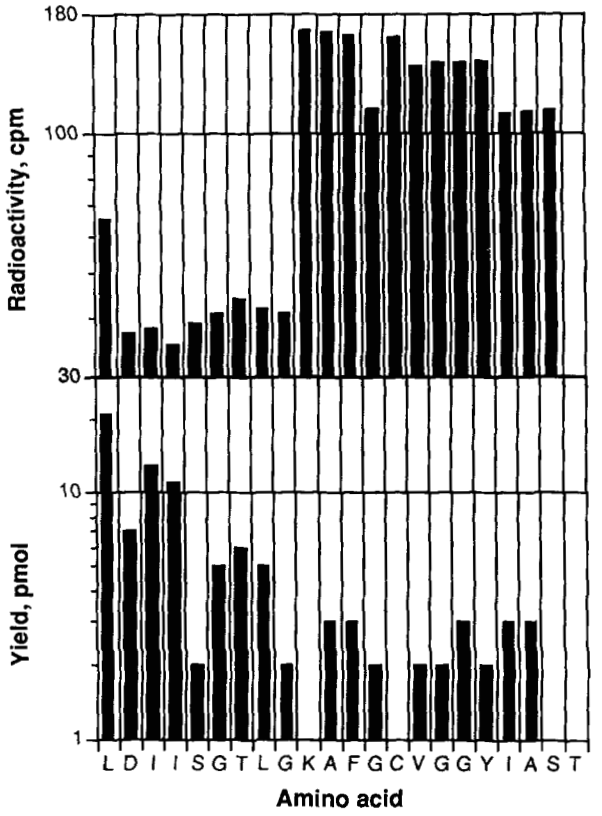

Fig. 3. Yields and cpm from sequence analysis of the major labeled tryptic peptide from the PLP–ALAS adduct. Yields for each cycle (in pmol after background correction) for the peptide LDII . . . (bottom panel) are shown together with the cpm for approximately 25% of the product of each sequencing cycle (top panel).

ground label in subsequent fractions. We are unable to explain completely the high background of counts after the release of the first labeled amino acid. It was not paralleled with a high lag in the HPLC analysis of released amino acids. Our explanation for this lag is the physical constraint imposed by collecting fractions on an Applied Biosystems 477A sequencer. Because only a small percentage of the PTH-amino acid is sent to the fraction collector (to ensure adequate sensitivity for HPLC detection of the amino acid derivative), the transfer line from the injection loop to the fraction collector is not washed thoroughly between cycles. We have observed this lag problem on other occasions when we have used the built-in fraction collector.

Overexpression of the erythroid ALAS wild-type and K-313 mutated variants

To assess the role of lysine 313 in the Schiff base linkage with PLP, this lysine residue was mutated to alanine and histidine (Table 1). The DNA sequence of each mutant was verified by dideoxyoligonucleotide sequencing, using a primer complementary to a region of ALAS approximately 150 nucleotides downstream from the region of mutation. The site-directed mutated ALAS-encoding fragments were then subcloned in the ALAS expression plasmid, pGF23, and the level of expression in *Escherichia coli* was examined. Cells harboring the ALAS wild-type and K313-mutated expression vectors, as well as the

Table 1. *Oligonucleotide-directed mutagenesis of the ALAS-encoding plasmid*[a]

	L	G	*K	A	F	G
Wild-type sequence (ALAS 311–316)	5′ ... CTT	GGC	AAG	GCC	TTT	GGT ... 3′
Replacement						
K313 → A	3′ ... GAA	CCG	**CG**C	CGG	AAA	AC. ... 5′
K313 → H	3′ ... GAA	CCG	**GTA**	CGG	AAA	CC. ... 5′

[a] Variants of the ALAS-encoding plasmid, pGF23, were constructed as described under Materials and methods using the oligonucleotides shown above. The PLP-binding lysine in the wild-type amino acid sequence is marked with an asterisk. The nucleotide substitutions introduced by mutagenesis are underlined and in bold.

expression vector without the ALAS-encoding DNA fragment, were induced by growth in low phosphate media (see Materials and methods). The wild-type ALAS was produced in *E. coli* as the major soluble protein (Fig. 4). Similarly, the ALAS variants (K313A and K313H) were produced at the same levels of expression (Fig. 4), as verified by densitometric scanning of the SDS-polyacrylamide gel of the bacterial cultures harboring the ALAS mutant and wild-type plasmids (data not shown).

Enzymatic activity of wild-type and variant ALAS proteins in bacterial extracts

The enzymatic activity of the mutant ALAS proteins was compared to that of the wild-type ALAS. Overnight-induced bacterial cultures, harboring the ALAS expression plasmid, pGF23, exhibited ALAS enzymatic activity, in contrast to the lack of activity shown in the K313A and K313H ALAS mutants (data not shown). These results indicate that mutations of lysine 313 (into alanine and histidine) abolish ALAS activity, although the enzyme is overproduced at the same high levels.

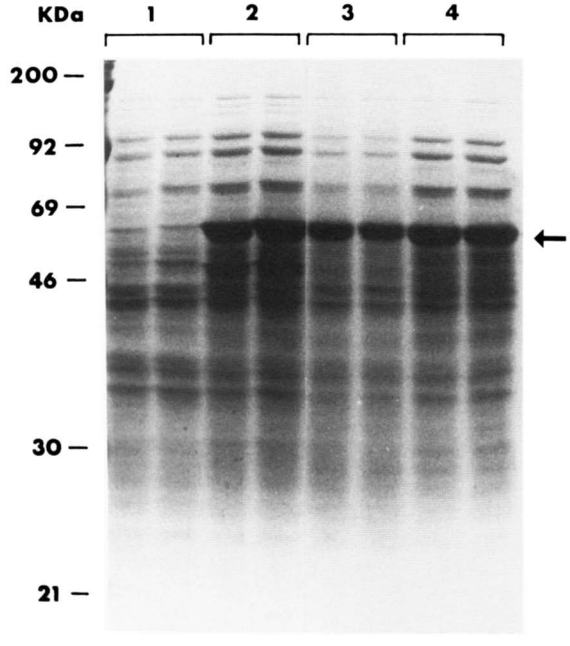

Fig. 4. Expression of wild-type mouse erythroid ALAS and lysine 313-directed ALAS mutants in *E. coli*. Proteins from *E. coli* cells harboring the ALAS expression plasmid, the ALAS K313 mutants, and the parent vector were separated by SDS-PAGE and detected by staining with fast stain (see Materials and methods). Lane 1, parent vector (i.e., without the ALAS-encoding fragment); lane 2, ALAS expression plasmid; lane 3, K313A expression plasmid (i.e., ALAS with lysine 313 mutated into alanine); lane 4, K313H expression plasmid (i.e., ALAS with lysine 313 mutated into histidine). The molecular masses are indicated on the left. The position of the overexpressed ALAS (wild-type and variants) is indicated by an arrow.

Discussion

Although ALAS has been known for over three decades to require PLP for activity (Neuberger, 1961), the nature of the ALAS–PLP linkage remains to be established. This is the first report on the isolation and identification of the ϵ-*N*-pyridoxyllysine of erythroid ALAS, establishing that the PLP cofactor is bound as a Schiff base linkage to an ϵ-amino group of a lysine residue of ALAS, and the first report documenting the identification of a PLP-binding peptide in any ALAS.

As with other PLP-dependent enzyme reactions, the initial step in the synthesis of ALA involves the binding of the first substrate, glycine, to the PLP–ALAS complex, forming a Schiff base between the glycine and the PLP–ALAS complex (Jordan, 1991). In contrast to other PLP-dependent enzymes, in which the enzyme–PLP linkage involves a protein-derived lysine ϵ-amino group, there have been different opinions concerning the amino acid involved directly in PLP binding with ALAS. Scholnick et al. (1972) and Fanica-Gaignier and Clement-Metral (1973) suggested that PLP is bound to the enzyme as a carbinolamine through an −SH group, forming a thiohemiacetal, whereas Nandi (1978) reported that it is bound as an imine to an ϵ-amino group of a lysine residue, and Mukherjee and Dekker (1990) found a high degree of homology between a stretch of 10 amino acids in chicken ALAS with a pyridoxyllysine peptide from AKB ligase.

The recently developed procedure for isolation and purification of recombinant erythroid ALAS from an *E. coli*-overproducing strain yields milligrams of purified enzyme, and has allowed the spectroscopic and kinetic characterization of the enzyme to be determined (Ferreira & Dailey, 1993). The 420-nm absorbance maximum observed with the ALAS holoenzyme probably corresponded to an internal aldimine between the PLP and the enzyme. Further, the 420 nm absorbance maximum is shifted to 333 nm upon reduction with sodium borohydride (Ferreira & Dailey, 1993). It was suggested that it might represent a Schiff base linkage between the C4′ of the cofactor and a nucleophile group on ALAS.

In this study the recombinant erythroid ALAS holoenzyme was reduced with $NaB[^3H]_4$, and then the tritiated PLP–enzyme adduct was digested with trypsin. The tryptic peptides were separated by gel filtration chromatography followed by HPLC reversed-phase chromatography. The majority of label appeared in a single peak (centered on fraction 17, Fig. 1). Another peak appeared in the V_t of the gel filtration column and probably represented traces of unincorporated label. HPLC analysis of fractions 16, 17, and 18 by reversed-phase HPLC gave the UV absorbance profiles presented in Figure 2. The majority of the label was found in a peak eluting at 19.3 min in fraction 17. This peak was sequenced and found to contain two peptides, one of 22 amino acids and the other of

24 amino acids. Residue 10, which we were unable to identify, proved to be labeled, as determined by scintillation counting of a fraction of the sequencer product (Fig. 3). Based on the published murine ALAS sequence, one of these peptides contained an internal lysine at residue 10 which, if it were derivatized, would not act as a cleavage site for trypsin. The lysine residue (Lys 313) of ALAS seems, therefore, to be involved in the Schiff base linkage between the cofactor and the enzyme. Further, when this lysine residue was mutated to alanine or histidine, the ALAS variants lost their catalytic competence, even though the mutated enzymes were produced at the same level of expression as the wild-type enzyme (Fig. 4).

Significantly, the lysine 313 residue of the mouse erythroid ALAS is conserved in all known ALAS sequences, ranging from bacteria to man (Fig. 5). It is worth noting that the mouse erythroid cysteine 317, the only cysteine residue in the PLP-binding peptide, is not a conserved residue; in chicken erythroid ALAS the corresponding residue is an alanine, whereas in *Saccharomyces cerevisiae* and *Rhodopseudomonas spheroides*, it is a serine and a valine, respectively (Fig. 5). As reported previously, the high degree of similarity between the pyridoxyllysine peptide (isolated from *E. coli* 2-amino-3-ketobutyrate ligase) and the chicken hepatic ALAS (Mukherjee & Dekker, 1990) is now extended to all the other ALAS proteins and is established to be the pyridoxyllysine peptide in ALAS. Likewise, a high degree of similarity is found between the PLP-binding peptides of ALAS and KAPA synthase (Fig. 5). In addition, comparison of the deduced amino acid sequences of different ALAS proteins revealed extensive similarity, particularly centered in the C-terminal 70% of the eukaryotic mature proteins. This region has been designated the catalytic domain of ALAS (Cox et al., 1991). The lysine 313 residue occurs in this domain.

The determination of the pyridoxyllysine peptide in mouse erythroid ALAS, and the high degree of similarity among all known ALAS proteins, raises questions about the catalytic role of active-site residues. Future experiments on the erythroid ALAS will be directed at determining crucial residues involved in catalysis.

Materials and methods

Restriction enzymes were obtained from New England Biolabs and Boehringer Mannheim and were used according to the suppliers' instructions. Sequenase was from United States Biochemicals; T4 DNA ligase was purchased from New England Biolabs. Deoxy- and dideoxynucleotide triphosphates were from United States Biochemicals. Trypsin, sequencing grade, was obtained from Boehringer Mannheim. The [^{35}S]dαATP was from ICN. Sodium boro[^{3}H]hydride and the oligonucleotide-directed in vitro mutagenesis kit were purchased from Amersham. The GeneClean II kit was a product of Bio 101, Inc. Acrylamide and gel reagents were purchased from Bio-Rad. Fast stain was from Zoion Research. The bicinchoninic acid protein assay reagents were obtained from Pierce Chemical Co. The DEAE-Sephacel was obtained from Sigma Chemical Co. and Ultrogel Aca 44 from IBF Biotechnics, Inc. All other chemicals were of the highest purity available. The oligonucleotide primers were synthesized in the Molecular Genetics Instrumentation Facility at The University of Georgia.

Purification of erythroid ALAS

The ALAS was purified from a bacterial overproducing strain as described in Ferreira and Dailey (1993). Basically, JM 109 cells harboring pGF23, an ALAS expression plasmid, were grown in low phosphate medium containing 100 μg/mL ampicillin at 37 °C for 20 h. Starvation for phosphate turns on the alkaline phosphatase promoter, which controls ALAS expression. The cells were then harvested by centrifugation. The purification procedure involves lysis of cells in a French press cell,

Mouse Erythroid ALAS	**304**	**L**	**D**	**I**	**I**	**S**	**G**	**T**	**L**	**G**	**K**	**A**	**F**	**G**	**C**	**V**	
Human Hepatic ALAS	**384**	**M**															**(Bawden *et al.*, 1987)**
Human Erythroid ALAS	**335**	**I**															**(Cox *et al.*, 1991)**
Rat Hepatic ALAS	**382**	**M**															**(Srivastava *et al.*,1988)**
Chicken Hepatic ALAS	**375**	**M**											**A**				**(Riddle *et al.*, 1989)**
Chicken Erythroid ALAS	**282**	**V**		**V**	**V**							**L**		**A**			**(Riddle *et al.*, 1989)**
***Saccharomyces cerevisiae* ALAS**	**305**	**V**		**M**		**T**						**S**		**S**			**(Urban-Grimal *et al.*,1986)**
***Bradyrhizobium japonicum* ALAS**	**238**	**I**			**L**	**E**									**L**		**(McClung *et al.*, 1987)**
***Rhodobacter sphaeroides* ALAS(Hem A)**	**75**	**I**				**N**							**Y**		**V**	**F**	**(Neidle & Kaplan, 1993)**
***E. coli* AKB ligase**	**235**	**V**				**T**							**L**		**G**	**A**	**(Mukherjee & Dekker,1990)**
***E. coli* KAPA synthase**	**227**	**P**	**E**	**L**	**L**	**V**	**V**		**F**			**G**			**V**	**S**	**(Otsuka *et al.*, 1988)**
***Bacillus sphaericus* KAPA synthase**	**243**	**I**		**F**	**T**	**V**				**S**			**I**		**A**	**E**	**(Gloeckler *et al.*, 1990)**

Fig. 5. Presence of a pyridoxyllysine peptide sequence in all known ALASs. Sequence alignment of the mouse erythroid ALAS PLP-binding peptide and peptides inferred from other ALAS cDNAs together with the *E. coli* AKB ligase and *E. coli* KAPA synthase PLP-binding peptides and *Bacillus sphaericus* KAPA synthase PLP-binding peptide. Amino acids that differ from murine ALAS are shown. The mouse erythroid ALAS amino acid in bold type corresponds to the lysine identified in this report as the residue involved in the Schiff base linkage with PLP. Note that this peptide is present in the conserved C-domain (catalytic domain) of all known ALASs.

ALAS precipitation with ammonium sulfate, gel-filtration (Ultrogel AcA 44), and ion-exchange (DEAE-Sephacel) chromatographies (Ferreira & Dailey, 1993).

Formation of the tritiated PLP–enzyme adduct

The $NaB[^3H]_4$ (90 μCi, 4.1 nmol) was added to 3.5 mL of ALAS solution (1 mg/mL), pH 7.2, and incubated with stirring at room temperature for 30 min. The reductive reaction proceeded for 60 min upon the addition of $NaBH_4$ (2.5 μmol). The yellow color characteristic of PLP disappeared after the addition of $NaB[^3H]_4$, accompanied by a shift in the absorption maximum to 330 nm, which is typical of a reduced azomethine linkage (Karpeisky & Dixon, 1986). The tritiated enzyme was dialyzed against eight changes of 1 L of 0.1 M NH_4HCO_3, pH 8.3 at 4 °C, at which point the dialysis buffer was reduced to background counts and then evaporated to dryness with a Speed-Vac concentrator.

Thiol alkylation of the reduced enzyme adduct and tryptic digestion

The pellet was resuspended in 1 mL of 1.2 M Tris-HCl, pH 8.1, containing 6 M guanidine-HCl. Dithiothreitol (3 mg) was added to the enzyme solution, followed by alkylation with iodoacetamide (8 mg) for 3 h as described in Esaki and Walsh (1986). The alkylated enzyme adduct was dialyzed overnight against several changes of 0.1 M NH_4HCO_3, pH 8.2. Trypsin, sequencing grade (45:1 mass ratio, ALAS subunit/trypsin), was then added, and proteolysis was carried out overnight at 37 °C. The reaction was stopped by the addition of glacial acetic acid (10% v/v).

Peptide mapping and purification

The tryptic digest of the PLP–ALAS was adjusted to 4 M with guanidine hydrochloride and applied in a volume of 0.5 mL to a Superdex-75 column (Pharmacia, 10 × 300 mm). Flow rate was 0.5 mL/min, and the column was equilibrated in 4 M guanidine-HCl, 50 mM Tris-HCl, pH 6.5. Eluant was monitored at 280 nm and aliquots (25 μL) of each 0.5-mL fraction were analyzed by scintillation counting. Individual label-containing fractions (Fig. 1, fractions 16–18) were applied to a reversed-phase column (Vydac C18, 2.1 × 15mm) equilibrated in 0.1% trifluoroacetic acid in water, using an Applied Biosystems 130A microbore as the HPLC equipment. The column was eluted with a gradient of acetonitrile (0.85% trifluoroacetic acid), 0–70% over 45 min at a flow rate of 0.25 mL/min. Peaks were collected by hand for scintillation counting (approx. 25% of each peak) and sequence analysis.

Peptide sequencing

Peptides were sequenced using FAST cycles on an Applied Biosystems 477A with an on-line 120A PTH-amino acid analyzer. Approximately 25% of the PTH-amino acid was collected in the built-in fraction collector for scintillation counting.

Oligonucleotide-directed mutagenesis

Lysine 313 (K313), the amino acid involved in the Schiff base linkage of the PLP cofactor, was mutated to alanine and histidine. Oligonucleotide-directed mutagenesis, using M13mp18 as the cloning vector, was carried out essentially as described in the Amersham oligonucleotide-directed in vitro mutagenesis kit directions. The mutagenesis primers used were 5′CCAAAGGCC(C/G)CGCCAAG3′ and 5′CCAAAGGCATGGCCAAG3′. The K313A and K313H mutations (i.e., lysine 313 mutated into alanine and histidine, respectively) were verified by sequencing according to the dideoxy chain-termination method (Sanger et al., 1977; Sambrook et al., 1989). The mutated ALAS DNA-encoding fragments were then subcloned in the ALAS expression plasmid, pGF23 (Ferreira & Dailey, 1993), replacing the wild-type region.

SDS-PAGE and protein determination

SDS-PAGE was performed as described by Laemmli (1970); 15% acrylamide and 1.5-mm-thick gels were used unless otherwise indicated. Aliquots of the bacterial cultures (100 μL) were heated for 3 min at 95 °C in SDS-mercaptoethanol loading buffer, and the proteins were visualized upon fast-staining. Protein concentrations were determined by the bicinchoninic acid assay, using bovine serum albumin as the standard.

Enzyme assay

Overnight-induced DH5 bacterial cultures (50 mL) containing the different expression plasmids were harvested, washed in 20 mM KP_i buffer, pH 7.2, and resuspended in 20 mM KP_i buffer, pH 7.2 (5 mL). The resuspended cells were sonicated for four 30-s intervals with a Branson sonicator set at 60 W. The ALAS activity of the cell extract was measured according to the method of Lien and Beattie (1982).

Acknowledgments

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