

Aminolevulinate synthase: Lysine 313 is not essential for binding the pyridoxal phosphate cofactor but is essential for catalysis

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Abstract

5-Aminolevulinate synthase is the first enzyme of the heme biosynthetic pathway in animals and some bacteria. Lysine-313 of the mouse erythroid aminolevulinate synthase was recently identified to be linked covalently to the pyridoxal 5'-phosphate cofactor (Ferreira GC, Neame PJ, Dailey HA, 1993, *Protein Sci* 2:1959–1965). Here we report on the effect of replacement of aminolevulinate synthase lysine-313 by alanine, histidine, and glycine, using site-directed mutagenesis. Mutant enzymes were purified to homogeneity, and the purification yields were similar to those of the wild-type enzyme. Although their absorption spectra indicate that the mutant enzymes bind pyridoxal 5'-phosphate, they bind noncovalently. However, addition of glycine to the mutant enzymes led to the formation of external aldimines. The formation of an external aldimine between the pyridoxal 5'-phosphate cofactor and the glycine substrate is the first step in the mechanism of the aminolevulinate synthase-catalyzed reaction. In contrast, lysine-313 is an essential catalytic residue, because the K313-directed mutant enzymes have no measurable activity. In summary, site-directed mutagenesis of the aminolevulinate synthase active-site lysine-313, to alanine (K313A), histidine (K313H), or glycine (K313G) yields enzymes that bind the pyridoxal 5'-phosphate cofactor and the glycine substrate to produce external aldimines, but which are inactive. This suggests that lysine-313 has a functional role in catalysis.

Keywords: 5-aminolevulinate synthase; heme biosynthesis; pyridoxal 5'-phosphate

5-Aminolevulinate synthase (EC 2.3.1.37), found in animals and some bacteria, is a pyridoxal 5'-phosphate-dependent enzyme that catalyzes the condensation of glycine and succinyl-CoA to yield ALA, CoA, and carbon dioxide (Kikuchi et al., 1958; Jordan, 1991). This reaction represents the first committed step in the heme biosynthetic pathway of these organisms (Jordan, 1991). ALAS has been isolated and purified from different sources, ranging from bacteria to man (Warnick & Burnham, 1971; Nakakuki et al., 1980; Borthwick et al., 1986; Munakata et al., 1993), and in all instances the enzyme is functional as a homodimer (Jordan, 1991; Munakata et al., 1993). In animals,

there are two ALAS isoforms, which are encoded by distinct genes and are differentially expressed: the erythroid form of ALAS (ALAS-E) only in erythroid cells and the housekeeping form (ALAS-H) nonspecifically in all tissues (Riddle et al., 1989; Bishop et al., 1990; Cox et al., 1990). Two ALAS isozymes, encoded by hemA and hemT, have also been identified in *Rhodospirillum rubrum* (Neidle & Kaplan, 1993).

Although ALAS has been known to be a PLP-dependent enzyme for several decades, only recently, with the overproduction of murine ALAS-E in *Escherichia coli* (Ferreira & Dailey, 1993), has it become possible to obtain enough purified enzyme for the determination of the PLP-binding lysine residue. The ϵ -amino group of lysine-313 was identified to form the Schiff base linkage with PLP in murine ALAS-E (Ferreira et al., 1993). Sequence alignment analysis of ALAS sequences from different species indicates that the pyridoxyllysine peptide and the ALAS active site are conserved among all organisms and are present in the C-terminal domain of the enzyme (Cox et al., 1990; Ferreira et al., 1993). The recent sequencing of the purified papain-resistant core domain of the rat ALAS-E has confirmed that

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Abbreviations: ALA, 5-aminolevulinate; ALAS, 5-aminolevulinate synthase; PLP, pyridoxal 5'-phosphate; MES, 2-[N-morpholino]ethanesulfonic acid; CHES, 2-[N-cyclohexylamino]ethanesulfonic acid; BSA, bovine serum albumin.

Table 1. Site-directed mutagenesis of the *ALAS*-encoding plasmid^a

ALAS-WT		L	G	K*	A	F	G			
ALAS-WT	5'	...	CTT	GGC	AAG	GCC	TTT	GGT	...	3'
K313A	3'	...	GAA	CCG	CGC	CGG	AAA	AC.	...	5'
K313H	3'	...	GAA	CCG	GTA	CGG	AAA	AC.	...	5'
K313G	3'	...	GAA	CCG	CCC	CGG	AAA	AC.	...	5'

^a K313-directed mutants were constructed as described in the Materials and methods using the oligonucleotides shown above. The PLP-binding lysine in the wild type (K313) is marked with an asterisk. Nucleotide substitutions introduced by mutagenesis are in bold.

the catalytic domain is located in the ALAS C-terminal region (Munakata et al., 1993).

The availability of a large amount of purified recombinant ALAS has allowed us to study the relationship between enzyme function and the structure of its active site. In this paper we report the effects of site-directed mutagenesis of the active-site lysine residue (Lys-313) to which the PLP cofactor is covalently bound. The results provide evidence that, although Lys-313-directed mutants (e.g., K313A, K313H, and K313G) bind non-covalently the PLP cofactor and the glycine substrate to produce external aldimines, Lys-313 covalently bound to PLP is required for catalytic activity.

Results

Purification and enzymatic activity of *ALAS-E* wild-type and K313 mutated variants

To define the functional role of the lysine residue that forms an internal aldimine with PLP in the active site of ALAS, we compared some critical catalytic and spectroscopic properties of

the wild-type enzyme (ALAS-WT) and ALAS mutant variants, in which Lys-313 was replaced by alanine (K313A), histidine (K313H), and glycine (K313G) (Table 1). The DNA sequences of the different mutants were 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 subcloned in the ALAS expression plasmid, pGF23 (Ferreira et al., 1993). The expression in *E. coli* and purification of the K313 mutant enzymes were performed as described for the wild-type enzyme (Ferreira & Dailey, 1993). The purified enzymes, both wild-type and K313 mutants, were yellowish in color, which is typical of PLP-dependent enzymes. The yields and the degree of purification for the K313-directed ALAS mutants were similar to those for the wild-type ALAS (Fig. 1A). The amount of enzyme from 2 L of overproducing bacterial cells (approximately 8 g of cell paste) was usually about 45 mg. However, in contrast to the wild-type ALAS, the K313 mutants exhibited no detectable activity under the standard assay conditions (Fig. 1B).

pH dependence of *ALAS-WT* activity and PLP content of *ALAS-WT* and K313 mutant enzymes

Purified ALAS-WT was examined for the effect of pH on enzyme activity. ALAS-WT has a pH optimum of 8.5 (Fig. 2). The stoichiometric ratios of PLP in the ALAS-WT and K313 mutants were established by determining the amount of PLP present in the Amicon cell-concentrated enzymes (see the Materials and methods) using the method of Adams (1979). Values ranging from 1.2 to 1.7 mol of PLP/mol of enzyme subunit were found for both the wild-type and mutant enzymes. (Values of 1.2 mol of PLP/mol of enzyme subunit were obtained for ALAS-WT and K313G, 1.5 mol of PLP/mol of enzyme subunit for K313A, and 1.7 mol of PLP/mol of enzyme subunit for K313H.)

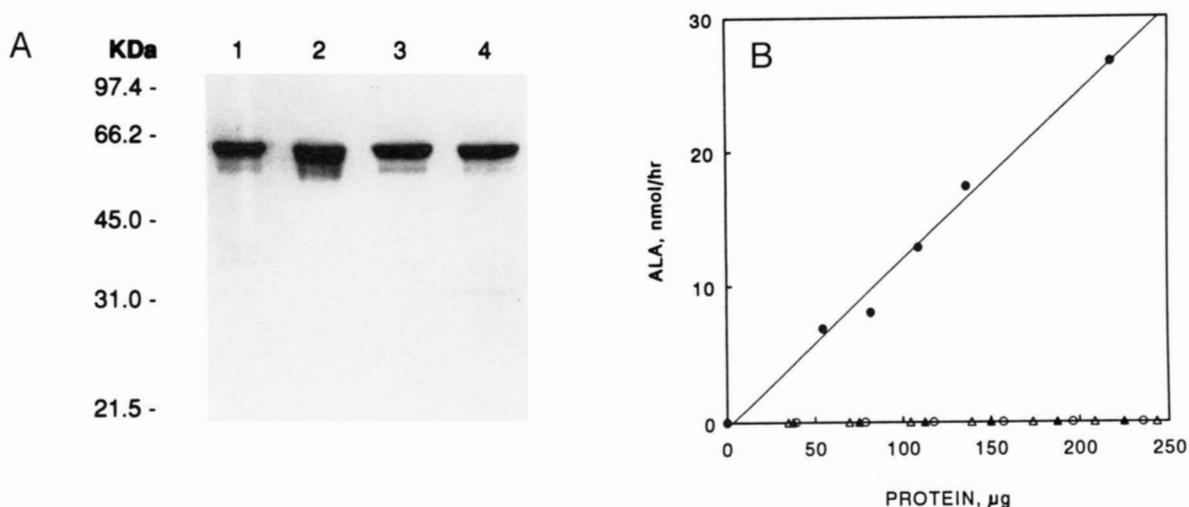


Fig. 1. **A:** Recombinant ALAS proteins (purified from overproducing bacterial strains). Proteins were separated by SDS-PAGE and detected by staining with fast stain (see the Materials and methods). Lane 1, ALAS-WT (i.e., wild-type ALAS); lane 2, K313A (i.e., ALAS with lysine-313 mutated into alanine); lane 3, K313H (i.e., ALAS with lysine-313 mutated into histidine); lane 4, K313G (i.e., ALAS with lysine-313 mutated into glycine). Molecular masses are indicated on the left. **B:** ALAS activity of wild-type and ALAS K313-directed mutant forms. ●, ALAS-WT; ▲, K313A; △, K313H; ○, K313G.

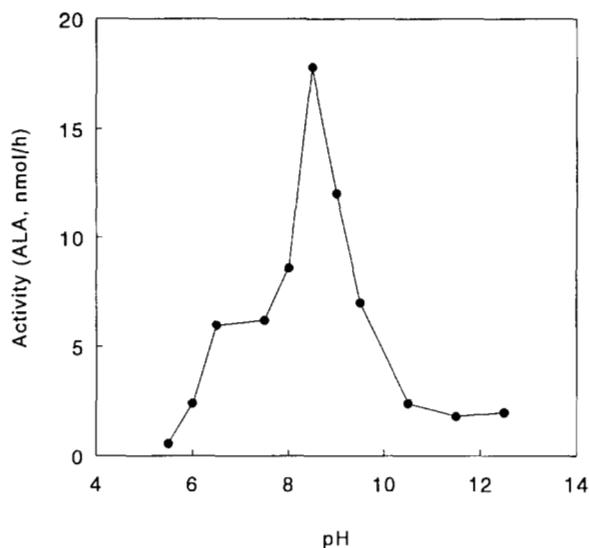


Fig. 2. pH dependence of ALAS activity. ALAS assays were done in the following buffers at a concentration of 60 mM: MES, pH 5.5–6.5; Tris-HCl, pH 7.0–8.5; CHES, pH 9.0–12.5 with 100 mM glycine and 100 μ M succinyl-CoA.

CD spectra

To verify that no gross changes in secondary structure were introduced in the K313-directed mutants, CD spectra (200–270 nm) of the wild-type and mutant enzymes were compared. The spectra of ALAS-WT, K313A, K313H, and K313G did not differ significantly in the far UV region (Fig. 3A). These results suggest that no significant change in the overall conformation of the enzyme protein was introduced by mutation of Lys-313 to Ala, His, or Gly. It is likely, however, as has been shown with the K258A mutant of *E. coli* aspartate aminotransferase (Smith et al. 1989), that small conformational changes would be confined to the region immediately surrounding the site of the mutation. In the visible region (300–500 nm), ALAS-WT shows a strong positive CD maximum, typical of many PLP-dependent enzymes, with the maximum rotation at 412 nm, corresponding to the absorption maximum of aldimine-bound PLP. In contrast, K313A, K313H, and K313G show no measurable CD at 412 nm (Fig. 3B).

UV-visible spectra

The spectra of the K-313 ALAS mutant enzymes indicate that they contain PLP, although the 428-nm peak, characteristic of the ALAS-WT (Ferreira & Dailey, 1993), was shifted to a shorter wavelength (~392 nm) (Fig. 4A). The absorbance maxima at 428 and 392 nm for the wild-type and mutant enzymes, respectively, correspond to the internal aldimine in the wild-type enzyme (Ferreira & Dailey, 1993) and the free aldehyde form in the mutant enzymes (Fig. 4A). Of interest, there is an absorption maximum at 412 nm upon the addition of the glycine substrate as with the wild-type enzyme (Fig. 4B). This suggests that an external aldimine can be formed between the substrate and the PLP cofactor both in the wild-type ALAS and K313-ALAS mutant enzymes. In contrast, however, the addition of succinyl-CoA to the PLP·glycine·enzyme complex leads to the appear-

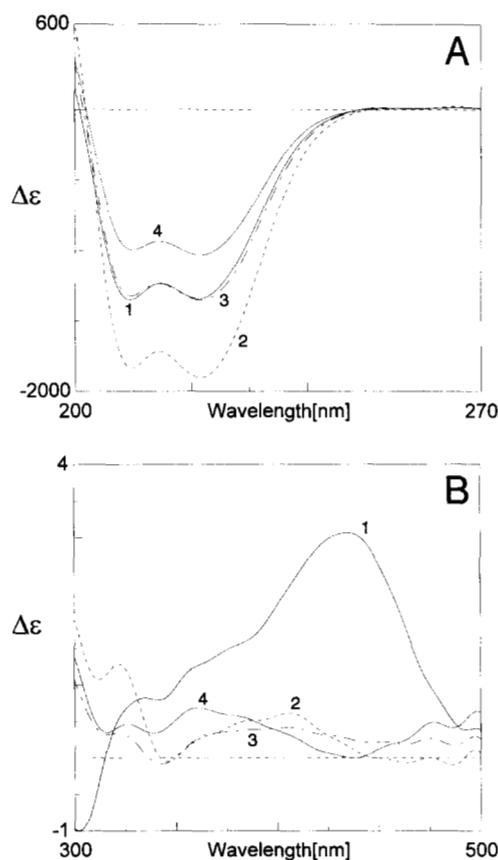


Fig. 3. **A:** CD spectra of ALAS enzymes in the far UV region. ALAS-WT (curve 1); K313A (curve 2); K313H (curve 3); K313G (curve 4). Spectra were recorded at 10 μ M protein (for ALAS-WT and K313H), at 19 μ M protein (for K313A), and at 6 μ M protein (for K313G) in 0.02 M K_2HPO_4 , 20 μ M PLP, pH 7.2, as described in the Materials and methods. (Differences in ϵ reflect differences in protein concentration.) **B:** CD spectra of PLP at the active site of ALAS enzymes. ALAS-WT (curve 1); K313A (curve 2); K313H (curve 3); K313G (curve 4). Spectra were recorded at 46.5 μ M protein in 0.02 M K_2HPO_4 , 20 μ M PLP, pH 7.2, as described in the Materials and methods.

ance of a second absorbance maximum (at 510 nm) with the wild-type but not with the K313 mutant enzymes. Significantly, the 510-nm absorbance maximum decreases with the progress of the reaction (G.A. Hunter & G.C. Ferreira, unpubl. results). The purified K313 mutants were immunochemically indistinguishable from ALAS-WT when examined by immunoblot analysis (Fig. 4D). However, polyclonal antibodies raised against PLP recognized ALAS-WT but not the K313-directed ALAS mutants. Upon reduction of the Schiff base linkage, the PLP cofactor remained covalently bound to ALAS-WT, even during SDS-PAGE (Kittler et al., 1986). Because the K313 ALAS mutants did not form a Schiff base linkage with PLP, the cofactor was not covalently bound to the enzyme and was dissociated from the protein during SDS-PAGE (Fig. 4C).

Discussion

In ALAS, the PLP cofactor is bound to an active-site lysyl residue through a Schiff base linkage, as has been reported for

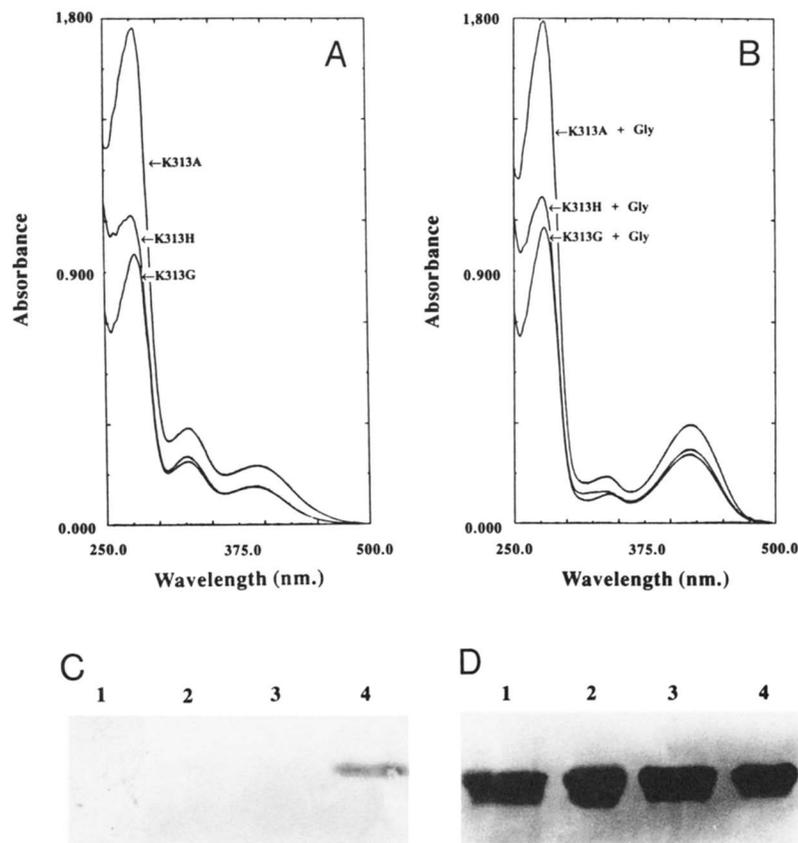


Fig. 4. **A:** Absorption spectra of ALAS K313-directed mutant forms. K313A (28.0 μ M); K313H (25.3 μ M); K313G (24.8 μ M). **B:** Absorption spectra of ALAS K313-directed mutant forms after addition of the glycine substrate. K313A (28.0 μ M); K313H (25.3 μ M); K313G (24.8 μ M). Both sample and reference cuvettes contained 20 mM potassium phosphate buffer, pH 7.2, 20 μ M PLP, and 100 mM glycine. **C:** Immunoblot showing that polyclonal antibodies raised against PLP only recognize ALAS-WT. 1, K313G (approximately 6 μ g); 2, K313A (approximately 6 μ g); 3, K313H (approximately 6 μ g); 4, ALAS-WT (approximately 6 μ g). **D:** Immunoblot showing that polyclonal antibodies raised against recombinant ALAS cross-react with K313-directed ALAS mutants. 1, ALAS-WT (approximately 6 μ g); 2, K313A (approximately 6 μ g); 3, K313H (approximately 6 μ g); 4, K313G (approximately 6 μ g). Protein samples were reduced with sodium borohydride and only then were heated for 3 min at 95 $^{\circ}$ C in SDS-mercaptoethanol loading buffer (see the Materials and methods).

all other PLP-dependent enzymes. Recently, the ALAS-E ϵ -N-pyridoxyllysine was isolated and sequenced, thus establishing that the PLP cofactor is bound as a Schiff base linkage to the Lys-313 ϵ -amino group (Ferreira et al., 1993). The initial step in the ALAS-catalyzed reaction 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 other words, the linkage between PLP and the enzyme, which is also referred as an internal aldimine, facilitates the transfer of the cofactor to the substrate to form an external aldimine. In this report, we demonstrate that K313-directed mutants (K313A, K313H, and K313G), which are virtually inactive, can bind, although noncovalently, PLP and glycine to produce external aldimines.

Lysine residues involved in internal aldimines in PLP-dependent enzymes have been proposed to have a multitude of roles (Cordes & Jencks, 1962; Toney & Kirsch, 1989; Nishimura et al., 1991; Lu et al., 1993). They have been shown to increase the reactivity of the PLP 4'-aldehyde group, facilitating the formation of an external aldimine between the substrate and the PLP cofactor (Cordes & Jencks, 1962). In addition, lysine residues have also been shown to be involved in catalysis (Toney & Kirsch, 1989; Lu et al., 1993), in formation of enzyme-substrate intermediates and product release (Lu et al., 1993), and in cofactor binding (Nishimura et al., 1991; Ferreira et al., 1993; Lu et al., 1993).

Purified K313-directed ALAS mutants (e.g., K313A, K313H, and K313G), which are immunologically indistinguishable from ALAS-WT, are virtually inactive under the standard ALAS as-

say conditions (Figs. 1B, 4C). The introduced site-directed mutations did not cause a significant conformational change as verified by CD (Fig. 3A). However, the K313 mutants, although they do not contain an internal aldimine (Figs. 3B, 4A), still bind the PLP cofactor. The two absorbance maxima for the K313 mutant enzymes at 392 nm and 330 nm probably are from the free aldehyde form of enzyme-bound PLP and its hydrated form or some other type of adduct, as previously suggested for a mutant form of D-amino acid transaminase, in which the active-site Lys residue was replaced by Asn (Yoshimura et al., 1992). Upon the addition of glycine, the 388 nm absorbance maximum is shifted to 412 nm, corresponding to the formation of an external aldimine between the PLP cofactor and the glycine substrate. When succinyl-CoA is added to the glycine·PLP·enzyme complex, an additional absorbance maximum is observed with only the wild-type ALAS. Further, this absorbance peak decreases with time, suggesting that different enzyme-substrate intermediates are formed and can be monitored during the reaction progress. If succinyl-CoA is added to the enzymes alone (i.e., either ALAS-WT or K313-directed mutants), the enzymes' spectra remain unchanged (G.A. Hunter & G.C. Ferreira, unpubl. results). In the ALAS-catalyzed reaction mechanism, a proton is removed from C-2 of the glycine·PLP·ALAS complex, yielding the carbanion to which the second substrate, succinyl-CoA, is condensed. This deprotonation step is most likely catalyzed by ALAS. One possible candidate for the catalytic base is the ϵ -NH₂ moiety of Lys-313. This situation, where the active-site Lys has a catalytic role, has been observed with other PLP-dependent enzymes (Toney & Kirsch, 1989; Nishi-

mura et al., 1991; Lu et al., 1993). For example, the ϵ -amino group of Lys-258 of L-aspartate aminotransferase (i.e., the active-site Lys) catalyzes the proton transfer in the tautomerization of the external aldimine to ketimine (Kirsch et al., 1984; Toney & Kirsch, 1989). Kirsch and coworkers (1990) demonstrated that primary amines functionally replace the PLP-binding Lys by catalyzing both the 1,3 prototropic shift and external aldimine hydrolysis reactions with inactive L-aspartate aminotransferase K258A. Interestingly, the substitution of ALAS K313 with histidine (K313H) yields an inactive enzyme, although at optimum pH the histidine residue is probably unprotonated. This suggests that the mutation affected the reactivity of the enzyme by either altering the conformation of the active site or altering the positioning of the PLP cofactor. However, the actual protonation state of the histidine residue in the active-site microenvironment is presently unknown.

In summary, although substitution of Lys-313 by Ala, His, and Gly did not prevent PLP binding or the formation of an external aldimine with glycine, the mutant enzymes were essentially inactive. ALAS has the PLP cofactor tightly bound to the protein through both noncovalent interactions and a covalent linkage with the ϵ -amino group of a lysine residue at the active site. Because K313A, K313H, and K313G, which are virtually inactive, can produce external aldimines, this suggests a catalytic role for Lys-313. Future experiments on ALAS-E will be directed at determining the functional role(s) of Lys-313.

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. [³⁵S]d α ATP was from ICN. The oligonucleotide-directed in vitro mutagenesis kit was purchased from Amersham. GeneClean II kit was a product of Bio 101 Inc. Acrylamide and gel reagents were purchased from Bio-Rad. Fast stain was from Zoon Research. The ProtoBlotR Western Blot AP system was from Promega. Nitrocellulose (0.45 μ m) and Affi-Gel Protein A agarose were from Bio-Rad. The biconinonic acid protein assay reagents were obtained from Pierce Chemical Co. DEAE-Sephacel was obtained from Sigma Chemical Co. and Ultrogel Aca 44 was from IBF Biotechnics Inc. All other chemicals were of the highest purity available.

Oligonucleotide-directed mutagenesis

Lysine-313, the amino acid involved in the Schiff base linkage of the PLP cofactor, was mutated to alanine, histidine, and glycine as previously described (Ferreira et al., 1993). 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 Lys-313 mutations 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, replacing the wild-type region (Ferreira & Dailey, 1993; Ferreira et al., 1993).

Purification of K313A, K313H, and K313G

Wild-type and mutant forms of ALAS were purified from extracts of a host *E. coli* overproducing strain as described for the wild-type enzyme in Ferreira and Dailey (1993). Briefly, DH5 α cells harboring the different expression plasmids (i.e., for K313A, K313H, K313G, and wild-type ALAS) were grown in low phosphate medium (MOPS) containing 100 μ g/mL ampicillin at 37 °C for 20 h. The alkaline phosphatase promoter, which controls the expression of ALAS and the ALAS K313-mutants, is induced by starvation in phosphate. Cells were then harvested by centrifugation. The purification procedure involves lysis of cells in a French press cell, precipitation of ALAS as well as of its mutant variants with ammonium sulfate, gel-filtration (Ultrogel Aca 44), and ion-exchange (DEAE-Sephacel) chromatographies.

Spectroscopic methods: CD and UV-visible spectra

Wild-type ALAS, K313A, K313H, and K313G were concentrated in an Amicon stirred cell with a YM10 membrane. Denatured protein was removed by centrifugation and the absorption spectra were determined on the supernatant solutions. CD spectra were recorded on a Jasco model 710 spectropolarimeter at 25 °C using a cuvette of a pathlength of 1 cm for 500–300 nm or 0.1 cm for 270–200 nm spectra. The observed rotation in degrees (θ_{obs}) was converted to molar ellipticity. Absorption spectra and difference absorption spectra were recorded using a Shimadzu UV 2100U spectrophotometer.

Preparation of antibodies and purification of IgG fractions

Polyclonal antibodies were raised against ALAS and against PLP. Two rabbits were immunized with purified ALAS apoenzyme and two with the pyridoxyl-BSA complex. Additional injections of the ALAS antigen were made 2 and 7 weeks after the first injection. The pyridoxyl-BSA complex was prepared by adding PLP (10 mM) to BSA (3 mg/mL) in a final volume reaction of 3 mL. After stirring for 15 min at 4 °C, small amounts of sodium borohydride were added and stirring was continued for 15–30 min. To determine the extension of the derivatization, absorbance at 330 nm was measured. The pyridoxyl-BSA complex was dialyzed extensively against 20 mM potassium phosphate, pH 7.2. After 1 month of pyridoxyl-lysine complex injections, the two rabbits were injected with a PLP-poly-L-lysine complex antigen. The PLP-poly-lysine antigen was prepared by adding PLP (3.5 mg/mL) to poly-lysine (0.85 mg/mL) in a final volume reaction of 30 mL. After stirring for 1 h at 4 °C, sodium borohydride (100 mg) was added very slowly, with constant stirring, until the PLP yellow color disappeared. The pyridoxyl-lysine complex was then extensively dialyzed against 4 L of distilled water. Preimmune sera were drawn before the first injection. The immunization and sera drawing procedures were carried out at Spring Valley Laboratories, Maryland. Antisera titers were performed every 3–4 weeks for 6 months, using standard enzyme-linked immunosorbent assay (Ausubel et al., 1992). To purify the IgG fractions of the ALAS and PLP polyclonal antibodies, Affi-gel protein A agarose (Bio-Rad) chromatography was performed as described in the manufacturer's instructions.

SDS-PAGE, immunoblot analysis, and protein determination

SDS-PAGE was performed as described by Laemmli (1970); 15% acrylamide and 1.5-mm-thick gels were used. Aliquots of the protein samples (10–15 μ L) were heated for 3 min at 95 °C in SDS-mercaptoethanol loading buffer (Laemmli, 1970) and the proteins were visualized upon fast-staining. For immunoblot analysis, SDS-PAGE was carried out as described above and the electrophoretic transfer to nitrocellulose filters according to Towbin et al. (1979) in a Bio-Rad mini Trans-Blot cell filled with 20% (v/v) methanol, 192 mM glycine, and 25 mM Tris. Antibodies were used at a dilution of 1:500. The blots were incubated with the antibodies for 1 h at room temperature. The binding of the antibodies to ALAS or to PLP on the nitrocellulose filters was detected using the ProtoBlotR AP (from Promega), as recommended by the manufacturer. Briefly, the incubation with the anti-IgG second antibody conjugated with alkaline phosphatase (1:5,000 dilution) was for 30 min and the “Western blue stabilized substrate for alkaline phosphatase” was used as provided by Promega. Protein concentrations were determined by the bicinchoninic acid assay, using BSA as the standard.

Enzyme assay and PLP determination

ALAS activity was determined according to the method of Lien and Beattie (1982). One unit of activity in any reaction corresponds to the formation of 1 nmol of ALA product in 60 min at 37 °C. PLP was determined by the fluorimetric method of Adams (1979).

Acknowledgments

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