Lysine-313 of 5-Aminolevulinate Synthase Acts as a General Base during Formation of the Quinonoid Reaction Intermediates†

Gregory A. Hunter‡ and Gloria C. Ferreira*,‡,§

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

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ABSTRACT: 5-Aminolevulinate synthase catalyzes the condensation of glycine and succinyl-CoA to form CoA, carbon dioxide, and 5-aminolevulinate. This represents the first committed step of heme biosynthesis in animals and some bacteria. Lysine 313 (K313) of mature murine erythroid 5-aminolevulinate synthase forms a Schiff base linkage to the pyridoxal 5′-phosphate cofactor. In the presence of glycine and succinyl-CoA, a quinonoid intermediate absorption is transiently observed in the visible spectrum of purified murine erythroid ALAS. Mutant enzymes with K313 replaced by glycine, histidine, or arginine exhibit no spectral evidence of quinonoid intermediate formation in the presence of glycine and succinyl-CoA. The wild-type 5-aminolevulinate synthase additionally forms a stable quinonoid intermediate in the presence of the product, 5-aminolevulinate. Only conservative mutation of K313 to histidine or arginine produces a variant that forms a quinonoid intermediate with 5-aminolevulinate. The quinonoid intermediate absorption of these mutants is markedly less than that of the wild-type enzyme, however. Whereas the wild-type enzyme catalyzes loss of tritium from [2-$^3H_2$]-glycine, mutation of K313 to glycine results in loss of this activity. Titration of the quinonoid intermediate formed upon binding of 5-aminolevulinate to the wild-type enzyme indicated that the quinonoid intermediate forms by transfer of a single proton with a pK of 8.1 ± 0.1. Conservative mutation of K313 to histidine raises this value to 8.6 ± 0.1. We propose that K313 acts as a general base catalyst to effect quinonoid intermediate formation during the 5-aminolevulinate synthase catalytic cycle.

5-Aminolevulinate synthase (ALAS[1]; EC 2.3.1.37) catalyzes the first committed step of heme biosynthesis in nonplant eucaryotes and the alpha subdivision of purple bacteria, which is the condensation of glycine and succinyl-CoA to produce CoA, carbon dioxide, and ALA, the universal precursor to tetrapyrroles in living systems (*1*, *2*).

$$\text{Glycine} + \text{Succinyl-CoA} \leftrightarrow \text{CoA} + CO_2 + \text{ALA} \quad (1)$$

Synthesized in the cytosol as a precursor protein prior to transport into the mitochondrial matrix and removal of the presequence, the mature ALAS enzyme resides at the periphery of the inner mitochondrial membrane (*3*). Animals harbor two distinct ALAS genes, ALAS1 and ALAS2; while ALAS1 is a "housekeeping" gene expressed in all tissues, ALAS2 is expressed exclusively in differentiating erythrocytes (*1*). ALAS2 accounts for approximately 90% of the heme present in the human body at any given time. Defects in the ALAS2 gene are associated with X-linked sideroblastic anemia, a erythropoietic disorder (*4*).

ALAS requires PLP as an essential cofactor, and is evolutionarily related to transaminases, as well as other α-family PLP-dependent enzymes, which also catalyze covalency changes at the α-carbon of the amino acid substrate (*5*, *6*). All PLP-dependent enzymes that catalyze reactions involving amino acids can be said to share common mechanistic features (*7*, *8*). The cofactor is bound covalently to the enzyme through a Schiff base linkage to a lysine residue on the enzyme, forming a complex termed the "internal" aldimine, to distinguish it from the "external" aldimine formed between the PLP cofactor and the amino acid upon substrate binding. The first step following formation of the external aldimine is the specific, heterolytic cleavage of one of the bonds to the α-carbon of the substrate. The bond breaking process is facilitated by delocalization of the electrons from the bond through the conjugated system of the Schiff base and pyridinium ring. This stabilized carbanion is presumed to have the electronic structure of a quinonoid intermediate, where the electrons reside, in effect, primarily on the pyridinium ring nitrogen of the cofactor (*9*).

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* Correspondence should be directed to: Gloria C. Ferreira, Department of Biochemistry and Molecular Biology, College of Medicine, University of South Florida, 12901 Bruce B. Downs Blvd., Tampa, Florida 33612. Tel: (813)-974-5797. Fax: (813)-974-0504. E-mail: gferreir@com1.med.usf.edu.

‡ College of Medicine.

§ Institute for Biomolecular Science and H. Lee Moffitt Cancer Center and Research Institute.

[1] Abbreviations: ALAS, 5-aminolevulinate synthase; ALA, 5-aminolevulinate; DEAE, diethylaminoethyl; HEPES, (*N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethane sulfonic acid]); PLP, pyridoxal 5′-phosphate; BME, β-mercaptoethanol; EDTA, ethylenediaminetetraacetate.

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Because these intermediates are believed to be common to PLP-dependent enzymes, and PLP-dependent enzymes catalyze such a diverse array of fundamental biochemical reactions, the quinonoid reaction intermediates are of special enzymological interest (*9*−*15*).

The perfectly conserved active site lysine residue anchors the cofactor in the active site in the absence of substrate but can serve other functions as well (*16*−*19*). A model study of Schiff base formation reported that reaction of a PLP−Schiff base with an amine to give a new Schiff base proceeds more rapidly than reaction of free PLP with an amine to give the same product (*20*). This suggests that one role of the active site lysine may be to facilitate binding of the amino acid substrate, and release of the product, by allowing these reactions to occur through a transaldimination pathway. The role of the active site lysine residue in substrate binding has been examined in tryptophan synthase, *O*-acetylserine sulfhydrylase, and D-amino acid transaminase (*17*−*19*). The K87T active site mutant of tryptophan synthase (*17*), the K42A active site mutant of *O*-acetylserine sulfhydrylase (*18*), and the K145N active site mutant of D-amino acid aminotransferase (*19*) each bind substrates more slowly than the wild-type enzymes, indicating that the active site lysine does facilitate binding in these enzymes.

Following substrate binding the conserved lysine can also play a critical role in forming the common quinonoid intermediate. For many PLP-dependent enzymes, including transaminases, the first step following substrate binding is removal of the C-α proton of the amino acid substrate. In the case of aspartate aminotransferase it has clearly been shown that this reaction is catalyzed by the active site lysine, acting as a general base catalyst (*16*, *21*). The active site lysine residues of tryptophan synthase (*17*) and O-acetylserine sulfhydrylase (*18*) appear to perform a similar role in catalysis.

During the catalytic cycle of ALAS both the *pro-R* proton of glycine and the *pro-R* proton at C-5 of ALA are labilized (*22*). K313 of the mature murine erythroid ALAS has been identified as the residue that forms a Schiff base linkage with the PLP cofactor (*23*). Previously we reported that mutation of K313 to glycine, alanine, and histidine yields proteins that retain PLP-binding activity, although the cofactor is no longer bound to the protein covalently (*24*). The activity of these mutants, however, is at least 1000-fold lower than that of the wild-type enzyme, indicating a crucial role for this residue in catalysis. The mutants form external aldimines with glycine, demonstrating that K313 is not required for binding the amino acid substrate. Here we examine the possible role of K313 as a general base catalyst during the reaction cycle.

MATERIALS AND METHODS

Reagents. Restriction enzymes and T4 DNA ligase were from New England Biolabs and were utilized in accordance with the supplier's instructions. Sequenase and deoxy- and dideoxynucleotide triphosphates were purchased from United States Biochemicals. [α-^{35}S]dATP was from ICN. The oligonucleotide-directed mutagenesis kit was purchased from Amersham. The GeneClean II kit was a product of Bio 101 Inc. Sodium dodecyl sulfate−polyacrylamide electrophoresis reagents were obtained from Bio-Rad. The bicinchoninic acid protein determination kit was from Pierce Chemical Co. Ultrogel AcA 44 was from IBF Biotechnics. All other reagents were from Sigma Chemical Co. or Fisher Scientific and were the highest purity available.

Methods

Construction of K313R. The preparation of the K313G and K313H mutants has been described (*23*, *24*). K313 was mutated to arginine using the same protocol. Oligonucleotide-directed mutagenesis, using M13mp18 as the cloning vector, was carried out as described in the Amersham oligonucleotide-directed in vitro mutagenesis kit directions. The mutagenic primer was 5′-CCAAAGGCCCTGCCAAG-3′. The mutation was verified by sequencing according to the dideoxy chain termination method (*25*, *26*). The mutated ALAS DNA-encoding fragment was then subcloned into the ALAS expression plasmid, pGF23.

Overexpression, Purification, Storage, Handling, and Analysis of ALASs. Recombinant murine erythroid ALAS was overexpressed in *Escherichia coli* and purified as described by Ferreira and Dailey (*27*) with two modifications to the protein purification procedure. The pH of buffer A (20 mM potassium phosphate, pH 7.2, 5 mM BME, 1 mM EDTA, and 10% (v/v) glycerol) was increased to 7.5, and a 20−35% (by saturation) ammonium sulfate fractionation was utilized rather than the 0−40% (w/v) ammonium sulfate precipitation step. Following cell disruption and centrifugation, the magnetically stirred cytosoluble homogenate was raised to 20% saturation with respect to ammonium sulfate. This was accomplished by the dropwise addition of buffer A, pH 7.5, saturated with respect to ammonium sulfate at 4 °C. The solution was centrifuged at 25000*g* for 15 min and the supernatant brought up to 35% saturation with respect to ammonium sulfate as described above. Rapid precipitation was typically observed when the solution reached 30−33% ammonium sulfate saturation. After a 15 min centrifugation at 25000*g* the pellet was redissolved in a minimal volume of buffer A, pH 7.5, and applied to the AcA 44 gel filtration column, followed by the DEAE−Sephacel column as described (*27*). The purified enzyme was either dialyzed directly into 50 mM HEPES, pH 7.5, containing 10% v/v glycerol or concentrated by pressurized dialysis in an Amicon 8050 stir cell equipped with a YM30 membrane, and then dialyzed into the HEPES/glycerol buffer. Unless otherwise noted this buffer was used in each of the experiments reported here. The presence of glycerol was essential to keep the enzyme solubilized. The purified ALAS protein, typically at a concentration of 10−200 μM, was stored under liquid nitrogen in Nalgene 2.0 mL polypropylene cryovials when not in use, and thawed by incubating the cryovial in 23 °C tap water for 5−10 min. Purified ALAS was enzymatically stable under liquid nitrogen for up to a year, and at 4 °C for 3−4 days, after which it began to precipitate out of solution. Protein purity was determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and was never less than 95% (*28*). Enzymatic activity was determined using a continuous spectrophotometric assay (*29*). Protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as standard (*30*), and all protein concentrations are reported on the basis of a subunit molecular weight of 56 000 kDa.

UV/Vis Spectroscopy. UV/Vis spectra were recorded on a Shimadzu UV2100U UV/vis dual beam spectrophotometer

equipped with thermostatically controlled cell holders. Acquired spectra were exported as ASCII files and imported into the graphing program Sigma Plot for presentation.

Determination of Dissociation Constants for Glycine and ALA. Association of ALAS with glycine or ALA induces spectral changes in the PLP coenzyme (*12*, *27*). It is possible to determine dissociation constants for ligand binding by monitoring these changes (*12*). Equilibrium dissociation constants for the binding of glycine to the wild-type and mutant ALASs were determined at pH 7.5 and 30 °C by titrating the purified proteins with increasing concentrations of glycine and monitoring the increase in absorbance at 420 nm upon formation of the external aldimine. Binding of ALA to wild-type ALAS at pH 7.5 resulted in the formation of a new absorption at 510 nm, indicating the formation of a quinonoid intermediate (*9*). The dissociation constant for ALA from wild-type ALAS was thus evaluated at 510 nm. The complexes of K313G, K313H, and K313R ALAS with ALA were not converted to a quinonoid intermediate to any significant extent at pH 7.5. The dissociation constants for these mutants were therefore evaluated from the increase in absorbance at 420 nm. Typically, a 20−30 μM enzyme solution was titrated with small aliquots of concentrated ligand solution and the change in absorbance measured. Data were analyzed by nonlinear regression fitting to eq 2 to obtain values of K_D. In eq 2, A is the observed absorbance, and A_i and A_f are the fitted values of the initial and final absorbance, respectively. [L] is the ligand concentration, and [E] is the enzyme concentration. Determinations were made in triplicate, and the reported values represent the mean and standard error of measurement.

$$A = A_i + \left[\frac{(A_f - A_i)K_D + [L] + [E] - \sqrt{(K_D + [L] + [E])^2 - 4[L][E]}}{2[E]}\right] \quad (2)$$

ALAS-Catalyzed Loss of Tritium from Glycine. Labilization of the carbon−hydrogen bonds of glycine in the presence of wild-type and K313G ALASs was investigated using [2-$^3H_2$]-glycine as substrate. The buffer was 50 mM HEPES pH 8.10, 50 mM KCl, 10 mM $MgCl_2$, 25 mM sodium succinate, 1 mM coenzyme A, 1 μM [2-$^3H_2$]-glycine (5 μCi), and 1 μM [1-^{14}C]-glycine (0.5 μCi). [1-^{14}C]-Glycine was included as an internal standard to normalize the results. The final reaction volume was 130 μL. Time courses were run at 30 °C by incubating the solutions in 1.5 mL microcentrifuge tubes in a water bath. At various time points two microliters of each reaction mixture was spotted onto a silica thin-layer chromatography plate, and when all time points were collected, the plate was developed in methanol−acetone−aqueous ammonia (20:10:3, by volume). Nonradioactive glycine was also run in a control lane and stained with ninhydrin to ascertain the region corresponding to glycine. The radioactive glycine regions were excised and quantified by scintillation counting. The ^{3}H:^{14}C ratio as a function of time was recorded.

pH Titration of Quinonoid Intermediate Formation for Wild-Type and K313H ALASs. The pH dependence of quinonoid intermediate formation was investigated with ALA-saturated enzymes as described previously (*12*). Equation 3

$$Abs = ((Abs_{max} - Abs_{min})/(1 + 10^{(pK-pH)})) + Abs_{max} \quad (3)$$

was used to fit the titration curves where Abs is the observed absorbance at 510 nm, Abs_{max} and Abs_{min} are the theoretical maximal and minimal absorbances at 510 nm, and pK is the equivalence point for quinonoid intermediate formation.

RESULTS

K313 Mutants Do Not Form a Quinonoid Intermediate with Glycine and Succinyl-CoA. A previous study demonstrated that murine erythroid K313 ALAS mutants bind PLP noncovalently (*24*). The 425 nm absorption band of wild-type ALAS, which arises from the internal aldimine between K313 and the PLP cofactor, is replaced by a new absorption at 385−395 nm in the mutants, suggesting that the cofactor is bound as the free aldehyde. Binding of glycine to the mutants shifted the 385−395 nm absorption to 415 nm, indicating that formation of an external aldimine does occur despite the absence of an internal aldimine.

The visible spectrum of purified *Rhodopseudomonas spheroides* ALAS has been reported to form an additional absorption maxima at 510−520 nm in the presence of glycine alone (*31*). This absorption was increased by the addition of succinyl-CoA to the ALAS−glycine complex (*31*). As seen in Figure 1A wild-type murine erythroid ALAS only forms this absorption, which we ascribe to a quinonoid intermediate, in the presence of both glycine and succinyl-CoA. The quinonoid intermediate absorption with substrates was transient and could only be observed in the presence of excess succinyl-CoA. The transient nature of this intermediate made it necessary to record the spectrum immediately following the addition of succinyl-CoA to the glycine-saturated enzyme. While succinyl-CoA induced quinonoid intermediate formation in the wild-type ALAS−glycine complex, quinonoid intermediate formation was not observed under similar conditions with the K313G, K313H, or K313R mutants, suggesting that the reaction cycle is blocked at this step by mutation of K313.

The Conservative K313H and K313R Mutations Restore Partial Ability To Catalyze Quinonoid Intermediate Formation with ALA. As seen in Figure 2A, the wild-type ALAS also forms a quinonoid intermediate absorption in the presence of 20 mM ALA. This quinonoid intermediate arises from removal of the *pro-R* proton at C-5 of ALA (*22*). In contrast to the transient quinonoid intermediate formed in the presence of glycine and succinyl-CoA, the quinonoid intermediate formed in the presence of ALA is stable, the absorption spectrum being relatively unchanged even after one hour.[2] The spectrum in the presence of ALA represents a mixture of external aldimine and quinonoid intermediate, with the quinonoid intermediate form favored as the pH is increased (see Figure 4). In Figure 2B the spectral changes accompanying ALA binding to K313G ALAS are shown. The peak at 388 nm for the holoenzyme arises from the cofactor being bound to the enzyme as the free aldehyde (*24*). Upon addition of ALA to 20 mM this absorption is shifted to 415 nm, indicating the formation of an external

[2] G. A. Hunter, and G. C. Ferreira, unpublished observations.

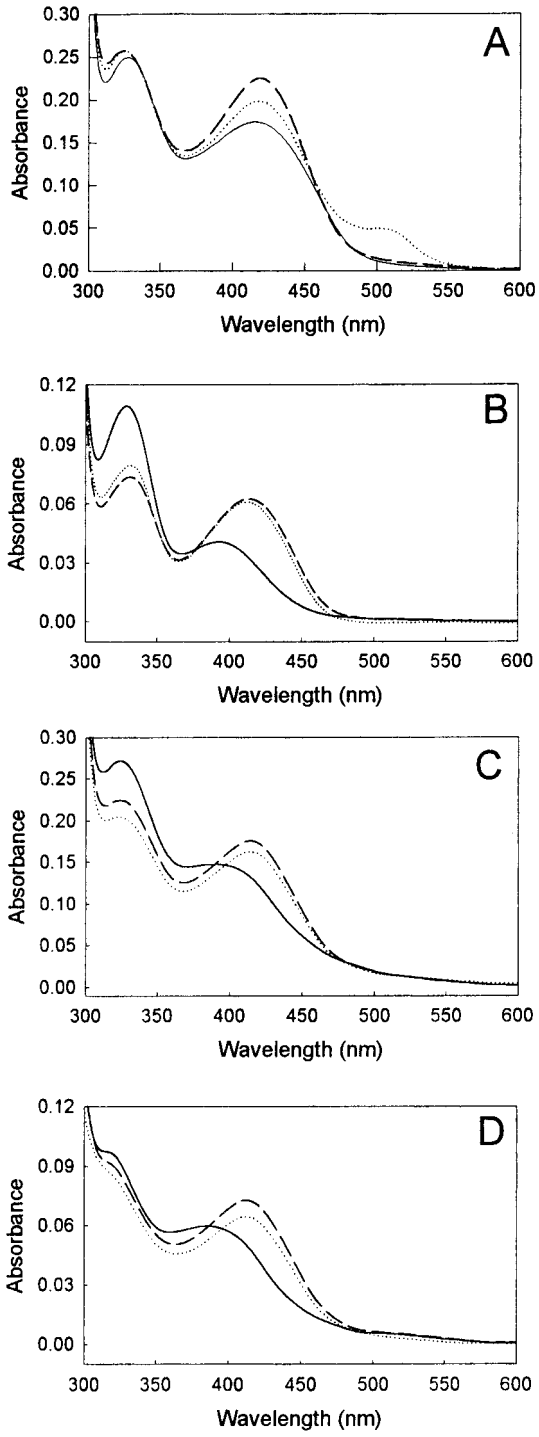

FIGURE 1: Changes in the cofactor absorption spectra of wild-type and K313 mutant ALASs upon addition of substrates. Spectra were acquired at 30 °C and pH 7.5: A, wild-type ALAS at 29 μM; B, K313G ALAS at 7 μM; C, K313H ALAS at 25 μM; and D, K313R ALAS at 9 μM. In each the solid line represents the holoenzyme, the dashed line represents the holoenzyme in the presence of 100 mM glycine, and the dotted line represents the holoenzyme in the presence of 100 mM glycine and 100 μM succinyl-CoA.

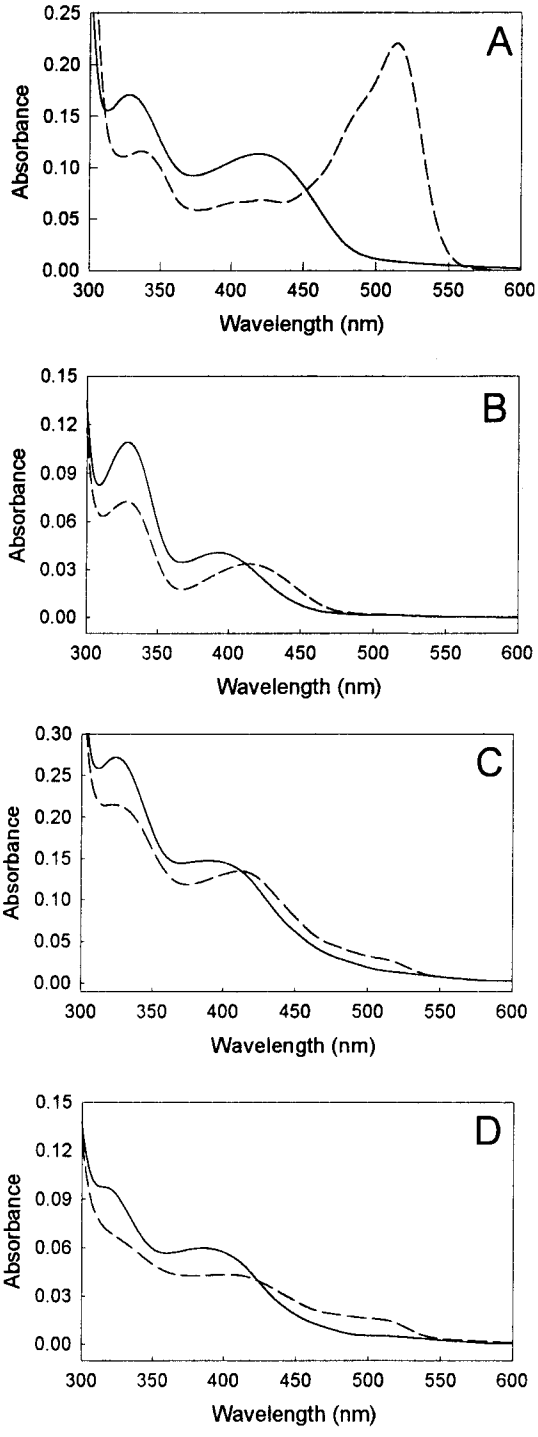

FIGURE 2: Changes in the absorption spectra of wild-type and K313 mutant ALASs upon addition of ALA. Spectra were acquired at 30 °C and pH 9.0: A, wild-type ALAS at 20 μM; B, K313G ALAS at 7 μM; C, K313H ALAS at 25 μM; and D, K313R ALAS at 9 μM. This spectrum had to be acquired immediately following the addition of ALA as the K313R mutant was unstable under these conditions, and precipitated in the cuvette within 2−3 min. In each case the solid line represents the holoenzyme, and the dashed line represents the holoenzyme in the presence of 20 mM ALA.

aldimine. Although the K313G mutant binds ALA, the absence of an absorption maximum at longer wavelengths indicates that the external aldimine is not converted to a quinonoid intermediate. Interestingly, conservative mutation of K313 to histidine or arginine partially restores the ability to form a quinonoid intermediate with ALA, as seen in Figure 2C,D, indicating that the reaction is base-catalyzed. The wild-type, K313G, and K313H ALAS complexes with ALA were stable at pH 9.0 and 30 °C. The K313R mutant complex with ALA was unstable under these conditions, however, and precipitated in the cuvette over the course of one to two minutes. The spectrum of the K313R-ALA complex was thus acquired immediately following the addition of ALA to the cuvette. This instability prevented reliable determination of

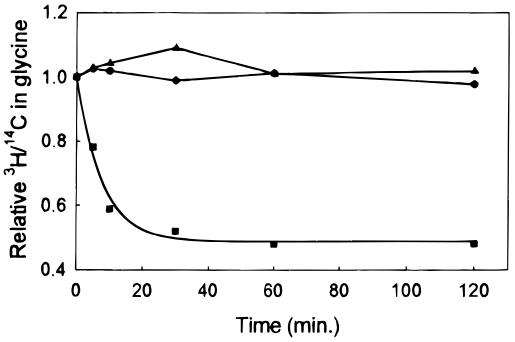

FIGURE 3: ALAS-catalyzed loss of tritium from glycine. [2-$^3H_2$]-Glycine was incubated at 30 °C in the presence of assay buffer (circles), 10 μM wild-type ALAS (squares), or 100 μM K313G ALAS (triangles).

the p*K* for quinonoid intermediate formation for this mutant.

K313 Catalyzes Loss of Tritium from [2-$^3H_2$]-Glycine. ALAS binds glycine such that the α-carbon of this substrate is recognized by the enzyme stereospecifically. The *pro-R* proton of glycine is selectively removed, and this position subsequently condenses with the thioester carbonyl of succinyl-CoA (*22*). By using extracts from *Rhodopseudomonas spheroides*, it has been reported that ALAS catalyzes the equilibration of the *pro-R* proton of glycine with solvent in the absence of the second substrate, succinyl-CoA (*32*). As seen in Figure 3, incubation of purified recombinant murine erythroid wild-type ALAS with [2-$^3H_2$]-glycine resulted in loss of half of the original tritium label within one hour. The data were fit to a single-exponential process by nonlinear regression analysis using the program Enzfitter and returned a rate constant of 0.13/min. We made the empirical observation that this reaction is significantly accelerated by the addition of coenzyme A to the assay buffer (not shown). When ALAS was not added to the incubation buffer, no decrease in the ratio of tritium to carbon-14 in glycine was observed. Significantly, mutation of K313 to glycine resulted in the loss of enzymatic capacity to catalyze abstraction of tritium from [2-$^3H_2$]-glycine, suggesting that K313 is involved in catalyzing the reaction.

Quinonoid Intermediate Formation with ALA Is pH-Dependent. When ALAS is saturated with ALA the complex exists as a mixture of external aldimine and quinonoid intermediate forms. In Figure 4A spectra of this complex were recorded as a function of pH. The quinonoid intermediate form was favored as the pH was increased. A sharp isosbestic point was not observed, perhaps indicating some microheterogeneity of the samples, or the occurrence of intersubunit interactions. A fit of the quinonoid intermediate absorbance to the pH demonstrated that the quinonoid intermediate was formed as a result of the loss of a single proton with an equivalence point at pH 8.1 ± 0.1 (Figure 4B).

It was also possible to perform a pH titration of the quinonoid intermediate formed upon complexation of K313H with ALA (Figure 4C,D). Two important differences from the wild-type enzyme were noted. The relative amplitude of the quinonoid intermediate absorption was smaller than that of the wild-type ALAS at every pH tested, and the equivalence point for quinonoid intermediate formation was increased to 8.6 ± 0.1. Each of these results indicates that the capacity of ALAS to form a quinonoid intermediate at physiological pH is significantly diminished by the K313H mutation.

Equilibrium Constants for ALASs. The dissociation constant for binding of glycine and ALA to wild-type ALAS and the K313 variants at pH 7.5 and 30 °C are recorded in Table 1. Mutation of K313 to glycine or histidine had no significant effect on glycine binding, while mutation to arginine doubled the dissociation constant. The dissociation constant for the wild-type ALAS−ALA complex was increased 100-fold by mutation of K313 to glycine or histidine and 150-fold by mutation to arginine. The K313H mutation increased the p*K* for quinonoid intermediate formation with ALA from 8.1 ± 0.1 to 8.6 ± 0.1.

DISCUSSION

In this communication we used site-directed mutagenesis of murine erythroid ALAS at K313 to test the hypothesis that this residue is involved in quinonoid intermediate formation. The spectroscopic and biochemical data presented indicate that K313 plays a critical role in the formation of the quinonoid intermediate by acting as a general base to remove the *pro-R* proton of glycine, and labilize the *pro-R* proton at C-5 of the product ALA.

Our experiments confirm that ALAS forms a quinonoid intermediate upon addition of succinyl-CoA to the glycine-saturated enzyme. This quinonoid intermediate arises from the loss of the *pro-R* proton of glycine, prior to condensation with succinyl-CoA. The presence of a lysine residue at position 313 appears to be essential to form this quinonoid intermediate, since the K313G, K313H, and K313R mutant spectra show no evidence of this intermediate. The data suggest that K313 is involved in catalyzing the formation of this quinonoid intermediate, and provide circumstantial evidence that K313 may be the catalytic base that directly removes the *pro-R* proton of glycine to form the quinonoid intermediate.

Wild-type ALAS also catalyzes labilization of the *pro-R* proton at C-5 of ALA (*33*). We have found that this partial reaction can be visualized spectroscopically by the appearance of a strong, stable quinonoid intermediate absorption at 510 nm upon exposure of the wild-type enzyme to ALA, as seen in Figure 2A. The K313G mutation results in a complete loss of formation of the quinonoid intermediate with ALA (Figure 2B). Interestingly, the conservative K313H and K313R mutations both markedly diminish quinonoid intermediate formation with ALA, but do not eliminate it completely (Figure 2C,D), strongly suggesting that formation of quinonoid intermediates in ALAS is general base-catalyzed. Since both the K313H and K313R mutants retain the capacity to form a quinonoid intermediate only with ALA, formation of this crucial reaction intermediate appears to occur more readily with ALA than with glycine plus succinyl-CoA. In contrast to the C-2 position of glycine, the C-5 position of ALA is labile due to the electron-withdrawing effect of the adjacent carbonyl group and the possibility of enol tautomerization. This intrinsic lability may account for the observed formation of a quinonoid intermediate with ALA, but not with glycine plus succinyl-CoA, in the K313H and K313R ALAS mutants. The degree to which enol tautomerization might contribute to stabilization of the quinonoid intermediate is unknown, however. Another pos-

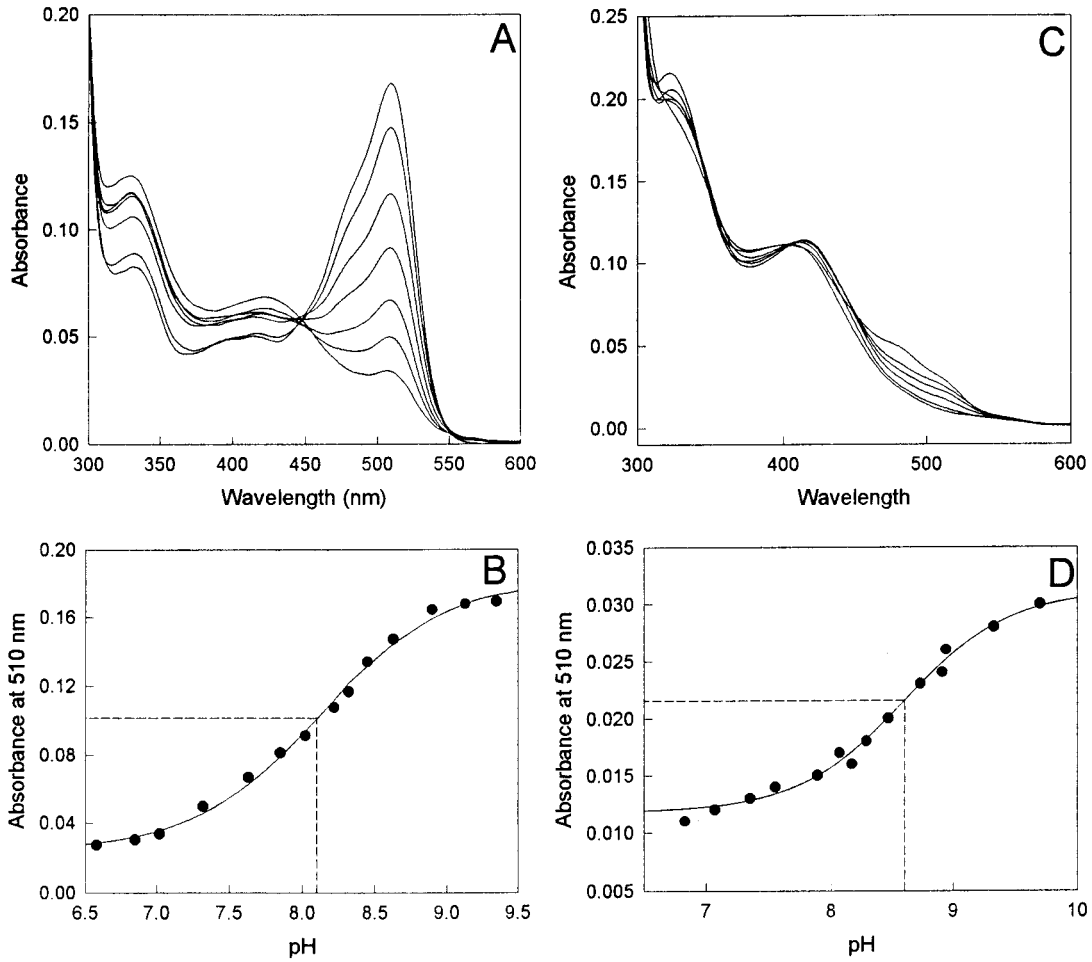

FIGURE 4: (A) Cofactor absorption spectra of 13 μM wild-type ALAS in the presence of 20 mM ALA at various pH values. From the lowest to highest absorbing spectrum at 510 nm the pH was 7.02, 7.32, 7.63, 8.02, 8.32, 8.63, and 9.13. (B) Titration curve of the observed absorbance at 510 nm as a function of pH. The best-fit line gives a pK of 8.1 $\pm$ 0.1. (C) Cofactor absorption spectra of 20 μM K313H ALAS in the presence of 20 mM ALA at various pH values. From the lowest to highest absorbing spectrum at 510 nm the pH was 6.83, 7.35, 8.29, 8.73, 8.94, and 9.70. (D) Titration curve of the observed absorbance at 510 nm as a function of pH. The best-fit curve gives a pK of 8.6 $\pm$ 0.1.

Table 1: Equilibrium Constants for ALAS Variants

ALAS	K_D^{Gly} (mM)	K_D^{ALA} (mM)	p$K^{Quinonoid}$
wild-type	10 $\pm$ 1	0.032 $\pm$ 0.002	8.1 $\pm$ 0.1
K313G	10 $\pm$ 3	3 $\pm$ 1	
K313H	14 $\pm$ 2	2.7 $\pm$ 0.4	8.6 $\pm$ 0.1
K313R	23 $\pm$ 2	5 $\pm$ 1	

sibility is that ALA is bound to the mutants more productively than glycine plus succinyl-CoA due to steric differences. For instance, glycine contains a carboxyl group at C-2 where none is present in the comparable C-5 position of ALA.

The data presented in Figure 3 support an earlier report that incubation of an ALAS-containing *Rhodopseudomonas spheroides* extract with [2-$^3H_2$]-glycine results in a 50% loss of the tritium label (*34*). In our hands, however, this reaction required the addition of coenzyme A, as in its absence the reaction was incomplete even after 6 h.[2] The loss of radioactivity from glycine is due to selective equilibration of the *pro-R* position with solvent, since the pro-S position remains radioloabeled (*34*). The data for wild-type murine erythroid ALAS fit well to a single-exponential process, as expected. The first-order rate constant for the process was 0.13/s. Significantly, labilization of tritium from glycine was not observed in the absence of wild-type ALAS or when the wild-type enzyme was replaced by the K313G variant. These results indicate that K313 is involved in labilizing the *pro-R* proton of glycine, and hence, in forming the quinonoid intermediate.

The titration data indicate that formation of the quinonoid intermediate with ALA occurs by transfer of a single proton. The pK for the transfer was raised from 8.1 $\pm$ 0.1 to 8.6 $\pm$ 0.1 by the K313H mutation. The proportion of enzyme in the quinonoid intermediate form at pH 7 is therefore lessened in the K313H mutant. The pK of histidine is significantly less than that of lysine, so it might have been expected that the K313H mutation would result in a lower pK for the formation of the quinonoid intermediate. The increase in pK with the K313H mutant suggests that the imidazole side chain is not ideally positioned to abstract the C-5 *pro-R* proton of ALA, or sterically hinders the formation of the quinonoid intermediate by a structural perturbation. In aspartate aminotransferase, replacement of the active site lysine by histidine (K258H) produces a mutant which catalyzes one-half of the ping−pong aminotransferase reaction cycle, but is 10^3−10^5 fold slower than the wild-type enzyme (*35*). The crystal structure of the mutant indicates that H258 is too distant from the α-carbon of the amino acid substrate to act as a general

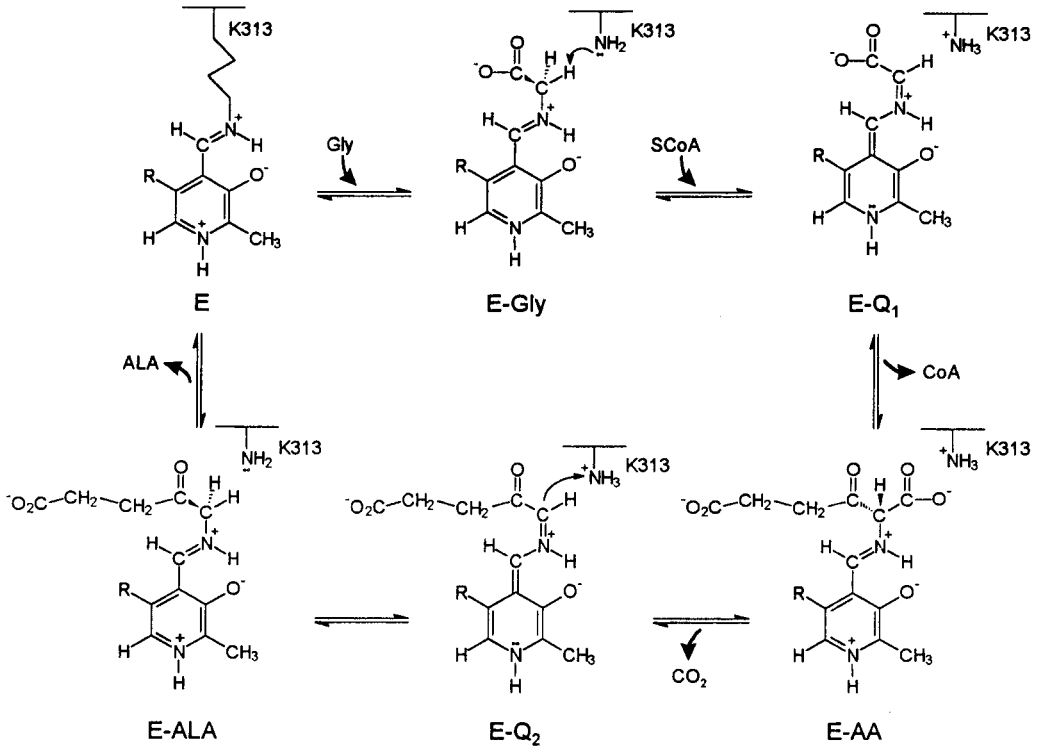

FIGURE 5: Model for the role of K313 in the ALAS catalytic cycle. ALAS contains PLP bound as an internal aldimine to K313 (E). Binding of glycine (E-Gly) and succinyl-CoA leads to the formation of a quinonoid intermediate (E-Q_1), in a reaction wherein K313 acts as a general base to remove the *pro-R* proton of glycine. The loss of CoA yields an aldimine to α-amino-*β*-ketoadipate (E-AA), which decarboxylates to form a second quinonoid intermediate (E-Q_2) and subsequently accepts a proton from K313 to form an aldimine to ALA (E-ALA). ALA is then released to regenerate (E).

base catalyst directly (*36*). It has been suggested that an intervening water molecule might mediate proton abstraction in this aspartate aminotransferase mutant (*36*). One possible explanation for the elevated p*K* for quinonoid intermediate formation in K313H ALAS is that a situation analogous to that in aspartate aminotransferase exists. In the absence of a crystal structure, however, other possibilities cannot be ruled out.

In contrast to the wild-type and K313H ALASs, the K313R ALAS mutant was unstable in the presence of 20 mM ALA at pH 9.0 and precipitated over the course of 2−3 min. We speculate that the process of ALA binding and formation of the quinonoid intermediate may involve some structural reorganization of the active site, and the bulky side chain of the K313R mutant could destabilize the ALA-bound conformer. Alternatively, the quinonoid intermediate formed by K313R ALAS may react with the guanidino side chain of the mutant residue, leading to enzymatic modification and precipitation.

The observation that the equivalence point for the formation of the quinonoid intermediate with ALA occurs at pH 8.1 ± 0.1 is intriguing, because of the possibility that this is the p*K* for an active site residue that acts as a general base catalyst to catalyze the formation of the quinonoid intermediate. On the basis of the evidence presented here the obvious candidate residue would seem to be lysine 313. Several lines of evidence support this postulate. Each of the K313 mutants is inactive (*24*), and the spectral data in Figure 1 suggest that the reaction cycle is blocked by the inability of the mutant proteins to remove the *pro-R* proton of glycine to form a quinonoid intermediate. This is supported by the data presented in Figure 3, where, in contrast to the wild-type enzyme, the K313G mutant did not catalyze the loss of tritium from tritiated glycine. Only the conservative K313H and K313R mutants retain partial capacity to form a quinonoid intermediate with ALA. Furthermore, K313 can reasonably be expected to be positioned near the hydrogen atom in question, and the analogous lysine residues in aspartate aminotransferase (*16*), tryptophan synthase (*17*), and *O*-acetylserine sulfhydrylase (*18*) have been shown to act as the general base catalyst for quinonoid intermediate formation in these PLP-dependent enzymes. The apparently low p*K* for a lysine residue might be explained by a low dielectric constant in the active site at the time of proton transfer. It is also noteworthy that the p*K* for the active site lysine of *O*-acetylserine sulfhydrylase has been reported to be 8.1 (*37*).

On the basis of the results presented here, we conclude that K313 of the mature murine erythroid ALAS plays a central role in catalyzing the formation of the quinonoid reaction intermediates (Figure 5). The simplest mechanism we can postulate to account for this is that K313 acts as a general base to labilize both the *pro-R* proton of glycine and the *pro-R* position at C-5 of ALA. During formation of ALA from glycine and succinyl-CoA, K313 would act as a general base catalyst to remove the *pro-R* proton of glycine, and as a general acid to donate a proton to the quinonoid intermediate formed from decarboxylation of the α-amino-*β*-ketoadipate intermediate.

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