Transient Kinetic Studies Support Refinements to the Chemical and Kinetic Mechanisms of Aminolevulinate Synthase*‡§

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5-Aminolevulinate synthase catalyzes the pyridoxal 5'-phosphate-dependent condensation of glycine and succinyl-CoA to produce carbon dioxide, CoA, and 5-aminolevulinate, in a reaction cycle involving the mechanistically unusual successive cleavage of two amino acid substrate α-carbon bonds. Single and multiple turnover rapid scanning stopped-flow experiments have been conducted from pH 6.8–9.2 and 5–35 °C, and the results, interpreted within the framework of the recently solved crystal structures, allow refined characterization of the central kinetic and chemical steps of the reaction cycle. Quinonoid intermediate formation occurs with an apparent pKₐ of 7.7 ± 0.1, which is assigned to His-207 acid-catalyzed decarboxylation of the α-amino-β-ketoacid intermediate to form an enol that is in rapid equilibrium with the 5-aminolevulinate-bound quinoid species. Quinonoid intermediate decay occurs in two kinetic steps, the first of which is acid-catalyzed with a pKₐ of 8.1 ± 0.1, and is assigned to protonation of the enol by Lys-313 to generate the product-bound external aldimine. The second step of quinonoid decay defines k_cat and is relatively pH-independent and is assigned to opening of the active site loop to allow ALA dissociation. The data support important refinements to both the chemical and kinetic mechanisms and indicate that 5-aminolevulinate synthase operates under the stereoelectronic control predicted by Dunathan’s hypothesis.

5-Aminolevulinate synthase (ALAS)‡ is a homodimeric pyridoxal 5'-phosphate (PLP)-dependent enzyme that is evolutionarily related to transaminases and catalyzes the first committed step of tetrapyrrole synthesis in non-plant eukaryotes, as well as the α-subclass of purple bacteria (1–3). Many organisms, including animals and some bacteria, are known to encode two genetically distinct ALAS genes. In animals one of these genes is expressed exclusively in developing erythrocytes, and mutations in the human erythroid-specific ALAS are correlated with hereditary X-linked sideroblastic anemia, a blood disorder characterized by iron-overloaded, heme-deficient red cells (4).

PLP-dependent enzymes catalyze a wide variety of reactions, including transaminations, decarboxylations, racemizations, and retro-aldo cleavages (5, 6). In the vast majority of cases the biochemical versatility of PLP can be rationalized in terms of a single property of the cofactor, the potential to act as an electron sink, and stabilize negative charge at the α-carbon of the substrate amino acid. Electrons from cleaved bonds of the covalently bound substrate can delocalize into the conjugated pyridine ring system to form quinonoid intermediates, which are often sufficiently stable to be spectroscopically observable and are characterized by strong absorption maxima of ~500 nm. These and other changes in the spectroscopic properties of the PLP cofactor during partial or complete reaction cycles can provide important insights into the chemical and kinetic properties of these enzymes.

The generally accepted chemical mechanism of ALAS is outlined in Scheme 1 (7–9). This reaction mechanism appears unnecessarily complex, because decarboxylation of glycine followed by condensation with succinyl-CoA to generate ALA directly would represent a more straightforward synthesis. This possibility is negated, however, by experimental data indicating that the initial and final steps of the chemical mechanism involve proton transfers from and to the amino acid α-carbon, in analogy to aminotransferases (9–11). The existence of the α-amino-β-ketoacid intermediate (IV) has not been definitively confirmed, but the occurrence of the corresponding intermediate in both amino-oxononanoate synthase (12) and ketobutyrate ligase (13), enzymes that are structurally and mechanistically homologous with ALAS, suggest it is on the ALAS reaction pathway.

Notwithstanding this supporting evidence, the mechanistically unusual sequential cleavage of not one but two bonds to the amino acid substrate α-carbon is another factor casting doubt on the accuracy and sufficiency of Scheme 1. Cleavage of two α-carbon bonds is particularly difficult to reconcile with the stereoelectronic control hypothesis explaining the remarkable reaction-type specificity of PLP-dependent enzymes (6, 14). Based on earlier work by Corey (15), Dunathan proposed
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The reaction pathway involves the PLP-dependent and mechanistically unusual sequential cleavage of two of the amino acid substrate α-carbon bonds (9, 11). As drawn here each of these steps leads to a quinonoid intermediate wherein the cofactor is directly utilized as an electron sink to catalyze bond cleavage. The crystal structure indicates that the pro-R proton bond of glycine cleaved in steps (II) and (III) is aligned perpendicular to the plane of the cofactor ring, in agreement with Dunathan’s hypothesis regarding the stereoelectronic control of electron sink functionality (14), but the bond cleaved in steps (II) and (III) is aligned perpendicular to the plane of the cofactor ring, as has been suggested for amino-oxononanoate synthase (18), or ALAS may represent a notable exception to Dunathan’s hypothesis. Resolution of these ambiguities would provide insights not only into the ALAS catalytic mechanism, but also the scope and stringency of Dunathan’s stereoelectronic control hypothesis.

A model for catalysis by ALAS involving interconversion between “open” and “closed” conformations has been proposed based on kinetic data (19). Specifically, it has been suggested that a portion of the intrinsic binding energy for the second substrate, succinyl-CoA, is sacrificed to shift the equilibrium toward the closed conformation where catalysis occurs, and turnover is limited by reversion to the open conformation, which is associated with release of ALA from the enzyme. The recently solved crystal structures of the Rhodobacter capsulatus ALAS holoenzyme, along with structures in which glycine or succinyl-CoA are bound at the active site, appear to be remarkably consistent with this kinetically derived model (20). The active site is located at the bottom of a channel at the interface between the subunits, and a short stretch of highly conserved amino acids near the C terminus closes over the active site in the substrate-bound structures but adopts a more open conformation in one subunit of the holoenzyme structure. The structures suggest that this “loop” closes over the active site and locks succinyl-CoA in the proper juxtaposition for catalysis and, subsequently, opens to allow ALA dissociation. The succinyl-CoA-bound structure indicates that molecular recognition of this substrate includes interactions that bridge the apposing enzyme domains and apparently stabilize the closed conformation.

The ALAS active site contains distinct features that should help further clarify the catalytic mechanism. The PLP cofactor is oriented such that the plane of the pyridinium ring is nearly perpendicular to the active site channel, and the pro-R proton of bound glycine faces down toward the protein core perpendicular to the plane of the cofactor ring and toward the side chain of the catalytic lysine residue, whereas the carboxyl group is oriented ~30° out of the plane of the cofactor ring. The side of the cofactor ring facing the active site channel is not entirely solvent-exposed, because it is overlaid by a histidine residue that forms part of a conserved network of hydrogen bonds that bridge the pyridinium ring nitrogen of the cofactor through four amino acid side chains to the thioester carbonyl of succinyl-CoA (Fig. 1). This carbonyl is conserved in the product ALA and appears to be required to form a quinonoid intermediate, based on the absence of a quinonoid upon binding of aminopentanoate, an ALA analog in which the carbonyl carbon is saturated (21). The observation that these two potential electron sinks are bridged by a conserved hydrogen bonding network suggests the possibility of a functional connectivity.
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Overexpression, Purification, Storage, Handling, and Analysis of mALAS-2—The overexpression, purification, storage, handling, and analysis of mALAS-2 were conducted as described previously (19). Protein concentrations were determined by the bicinchoninic acid method using bovine serum albumin as the standard, from which the extinction coefficient for the purified enzyme was estimated as 46,000 liter mol⁻¹ cm⁻¹ at 278 nm. Reported enzyme concentrations are based on a monomeric molecular mass of 56,523 kDa, as calculated from the primary amino acid sequence (22).

Stopped-flow Spectroscopy—Rapid scanning stopped-flow kinetic measurements were conducted using an OLIS model RSM-1000 stopped-flow spectrophotometer. The dead time of this instrument is ~2 ms, and the observation chamber optical path length is 4.0 mm. Scans covering the wavelength region 320–550 nm were acquired at a rate of 1000 scans per second. For reactions longer than 3–4 s, the collected scans were averaged to yield either 62.5 or 31.25 scans per second to condense the resulting data files to a size suitable for global fit analyses. In all cases the resulting data files contained 3000–4000 total spectral scans. An external water bath was utilized to control the temperature of the syringes and observation chamber, and the temperature at the syringes and observation chamber was determined with a Fisherbrand −20 to 110 °C thermometer. The concentrations of reactants loaded into the syringes were always 2-fold greater than that reported, such that the reported concentrations represent the final concentrations present in the stopped-flow cell compartment after mixing.

Observed rate constants were determined by Robust Global Fitting of the acquired spectral data, using the single value decomposition software provided by OLIS, Inc. (17, 18). Quality of fits was judged by analysis of the calculated residuals, and the simplest mechanism adequate to accurately describe the experimental data was used. Single turnover data were modeled using a three-kinetic-step mechanism as described by Reaction 1, whereas multiple turnover data were modeled using a two-step kinetic mechanism as described by Reaction 2.

\[
\begin{align*}
A & \overset{k_{1 \text{obs}}}{\longrightarrow} B \overset{k_{2 \text{obs}}}{\longrightarrow} C \overset{k_{3 \text{obs}}}{\longrightarrow} D \\
\text{REACTION 1} \\
& \overset{k_{1 \text{obs}}}{\longrightarrow} B \overset{k_{2 \text{obs}}}{\longrightarrow} C \\
\text{REACTION 2}
\end{align*}
\]

For each set of experimental conditions the observed rate constants were determined from three or more replicate experiments, and the reported values represent the average and standard error of measurement for each experimental condition.

Estimation of both forward and reverse rate constants was accomplished by modeling single wavelength kinetic traces at 510 nm with KinTekSim kinetic simulation software (23). The eight interior rate constants were allowed to float, while the \( K_D \) values were held constant as determined separately (19).

EXPERIMENTAL PROCEDURES

Reagents—The following reagents were from Sigma: DEAE-Sephalc, β-mercaptoethanol, PLP, bovine serum albumin, α-ketoglutarate dehydrogenase, α-ketoglutarate, NAD⁺, thiamin pyrophosphate, succinyl-CoA, ALA-hydrochloride, HEPES free acid, and the bicinchoninic acid protein determination kit. Glycerol, mono- and dibasic potassium phosphate, disodium EDTA dihydrate, ammonium sulfate, magnesium chloride hexahydrate, glycine, [2-²H₂]glycine, and sodium hydroxide were purchased from Fisher. Ultrogel AcA-44 was from IBF Biotechnics. SDS-PAGE reagents were purchased from Bio-Rad.
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Single Turnover pH Variation Experiments—Single turnover stopped-flow pH studies were conducted at 30 °C under a constant set of background conditions with the buffer and pH as the only variables. One syringe was filled with a solution containing 100 μM purified mALAS-2, 260 mM glycine, 10% (v/v) glycerol, and 100 mM buffer, whereas the other syringe contained 20 μM succinyl-CoA, 10% (v/v) glycerol, and 100 mM buffer. The buffers utilized were: MES, pH 6.0–6.5; MOPS, pH 6.6–7.0; HEPES, pH 7.1–7.8; TAPS, pH 7.9–8.5; and AMPSO, pH 8.6–9.2. These conditions yielded a solution of 100 μM mALAS-2-glycerine complex reacting with an equivalent volume of a solution containing 20 μM succinyl-CoA such that the final concentrations in the observation chamber were 50 μM mALAS-2-glycerine complex, 10 μM succinyl-CoA, 10% (v/v) glycerol, and 100 mM buffer. The presence of glycerol was essential to maintain enzyme solubility at lower pH values.

Multiple Turnover pH Variation Experiments—Multiple turnover stopped-flow pH studies were conducted at 20 °C. Again, the only variables were the buffer and pH. One of the syringes was loaded with a solution containing 80 μM mALAS-2, 260 mM glycine, 10% (v/v) glycerol, and 100 mM buffer, whereas the other syringe contained 200 μM succinyl-CoA, 10% (v/v) glycerol, and 100 mM buffer. Upon mixing equivalent volumes from each of the two syringes the final concentrations of enzyme and substrates in the observation chamber were halved such that the reaction observed was between 40 μM mALAS-2–glycerine complex and 100 μM succinyl-CoA. The buffers and pH ranges were the same as described for the single turnover experiments.

For both single and multiple turnover experiments the shots from each set of conditions were collected into a clean waste syringe, and the final pH was verified by direct reading using an Accumet AR15 pH meter. The syringes and observation chamber were flushed with 3–4 syringe volumes of purified water between each set of experimental conditions to remove residual buffer between experiments. pH determinations were conducted at the same temperature as that used in the stopped-flow experiments. Apparent pKₐ values for quinonoid intermediate formation and the first step of quinonoid intermediate decay were determined by fitting the observed rate constants as a function of pH to Equation 1 using SigmaPlot graphing software,

\[
\ln(k_{\text{obs}}) = \frac{A}{(1 + 10^{pH-pK_a})} + B \quad (\text{Eq. 1})
\]

where \(k_{\text{obs}}\) is the observed rate constant, \(A\) is the theoretical difference in the observed rate constants associated with the protonated and deprotonated species, \(pK_a\) is the pH at which the ionizing group is 50% protonated, and \(B\) is the rate of reaction when the ionizing group is 100% deprotonated.

Single Turnover Temperature Variation Studies—Single turnover temperature variation studies were conducted using conditions identical to those described for the single turnover pH studies, with the pH held constant at 8.1 and the temperature varied from 5 to 35 °C in 5 °C increments. A pH of 8.1 was considered to represent a reasonable compromise between the low signal amplitudes observed at lower pH values and the extended reaction times required at higher pH values, as seen in Fig. 3. The formation of condensate on the stopped-flow cell at low temperatures was prevented by purging the observation chamber with nitrogen during the experiments.

Thermodynamic Activation Parameters—Temperature dependence data were fit to the Arrhenius equation (Equation 2),

\[
\ln(k_{\text{obs}}) = \frac{-E_a}{R} \frac{1}{T} + \ln(A) \quad (\text{Eq. 2})
\]

where \(E_a\) is the activation energy, \(R\) is the universal gas constant, \(T\) is the absolute temperature, and \(A\) is the frequency factor. Activation energies were calculated by multiplying the slopes of the Arrhenius plots by the negative reciprocal of the gas constant. Enthalpies of activation were then obtained using the activation energy and Equation 3, where \(\Delta H^\ddagger\) is the enthalpy of activation.

\[
\Delta H^\ddagger = E_a - RT \quad (\text{Eq. 3})
\]

Observed free energies of activation were determined separately at 30 °C and pH 7.5 using Equation 4, which relates the free energy of activation to the observed rate constant.

\[
\Delta G^\ddagger = -RT \ln(k_{\text{obs}}/k_aT) \quad (\text{Eq. 4})
\]

Where \(\Delta G^\ddagger\) is the observed free energy of activation, \(k_a\) is Boltzmann’s constant, and \(h\) is Planck’s constant. Observed entropies of activation were estimated using Equation 5 and the derived free energies and enthalpies of activation as described above.

\[
\Delta S^\ddagger = -\frac{\Delta G^\ddagger - \Delta H^\ddagger}{T} \quad (\text{Eq. 5})
\]

Kinetic Isotope Effects with Dideuteroglycine—Single turnover studies to determine the kinetic isotope effects resulting from substitution of glycine with dideuteroglycine were conducted at pH 7.5 and 30 °C. One syringe contained 200 μM enzyme and 320 mM glycine (or dideuteroglycine) in 50 mM HEPES, pH 7.5, and 10% glycerol, whereas the other syringe contained 40 μM succinyl-CoA in the same buffer. Glycine or dideuteroglycine was added last, and the experiments were conducted immediately thereafter.

Structural Analyses—The protein data base files 2BWN, 2BWO, and 2BWP, corresponding to the holoenzyme, succinyl-CoA bound, and glycine-bound R. capsulatus ALAS crystal structures, were used as models for the mALAS-2 catalytic core (20). Superpositioning of crystal structures and hydrogen bond determinations were accomplished using Deepview/Swiss-PdbViewer software (24, 25). Modeling of the α-amino-β-ketoadiolate intermediate into the active site was accomplished using PyMOL software and drawing the α-amino-β-ketoadiolate intermediate such that the hydrogen bonds to the succinyl moiety of succinyl-CoA were preserved in the corresponding atoms of the intermediate.

4 W. L. DeLano (2002) PyMOL, DeLano Scientific, San Carlos, CA.
RESULTS

The major spectral change observed during single turnover stopped-flow absorbance studies was the formation and decay of a quinonoid intermediate with an absorbance maximum of \(\sim 510\) nm. During early pilot experiments a variety of kinetic mechanisms were examined as prospective models for fitting the global data, with the goal of ascertaining the simplest kinetic mechanism that accurately described the experimental data. The single turnover kinetic profiles were found to be best described by the three-step sequential mechanism represented by Reaction 1. The three reaction steps were visually observable in single wavelength traces of quinonoid intermediate formation and decay, as illustrated in Fig. 2, which is from a representative single turnover experiment at pH 7.5 and 30 °C. In Fig. 2A, the time course at 510 nm was overlaid with the global fit of the spectral data at the same wavelength using Reaction 1. The reaction was observed to involve a single kinetic step of quinonoid intermediate formation followed by two decay steps. Other early experiments indicated the rate and amplitude of the second step of quinonoid intermediate decay was dependent upon glycine concentration, a result that was interpreted as a reflection of competitive binding between the ALA produced during the single turnover, which binds relatively tightly and forms a quinonoid intermediate, and glycine, which does not bind tightly and does not form a quinonoid intermediate. To ensure that the second step of quinonoid intermediate decay accurately approximated the rate of ALA release from the enzyme, excess glycine was essential to trap the enzyme in the glycine-bound form at the reaction end point. In the experiments reported here the concentration of glycine was maintained at seven or more times the spectrophotometrically determined dissociation constant, whereas the concentration of ALA formed was never more than approximately one-half the dissociation constant.

The calculated spectra for each of the four kinetic species from the global fitting are graphed in Fig. 2B. The starting and ending spectra were similar, whereas the two intermediary spectra, corresponding to the two kinetic intermediates, differed primarily in the magnitude of quinonoid absorbance. The global fit indicated that the quinonoid intermediate arose from the 420 nm peak with an isosbestic point at \(442\) nm. A video of the time course entitled “mALAS-2 single turnover” is provided as supplemental data.

The first 5 s of the modeled time courses for each of the four species indicate that the two intermediates were fully formed within 250 and 1600 ms, respectively. The observed rate constants for the three steps of the reaction were \(5.3 \pm 0.6/s, 2.8 \pm 0.3/s,\) and \(0.074 \pm 0.005/s.\)

The pH dependence of the single turnover reaction kinetics at 30 °C was investigated. These studies indicated that pH changes had substantial effects on the observed rate constants for quinonoid intermediate formation and the first step of quinonoid decay, whereas the second step of quinonoid decay was relatively pH-independent over the range tested (Fig. 3). In Fig. 3A representative 510 nm single-wavelength kinetic traces were extracted and overlaid with the best global fit to the spectral data at this wavelength. The observed rate constants for all three kinetic steps increased as the pH was lowered, although only the first step of quinonoid intermediate decay could be accurately described by Equation 1 for an acid-catalyzed reac-

![FIGURE 2: mALAS-2 single turnover experiment and global fitting analysis.](image-url)
tion involving transfer of a single proton. This set of experiments suggested quinonoid intermediate formation was also acid-catalyzed, but the small proportion of data points associated with this kinetic phase, particularly at lower pH values where the signal amplitude was also low, led to large standard errors. Additionally, it was not possible to estimate an acid-catalyzed end point for quinonoid intermediate formation, due to increasing instability of the enzyme as the pH was dropped below 7.0. The single turnover pH dependence of the three phases are plotted in Fig. 3. A fit of the first step of quinonoid decay to Equation 1 resulted in an apparent kinetic pKₐ of 8.1 ± 0.1. This value was virtually identical to the previously determined apparent spectroscopic pKₐ for Lys-313-catalyzed abstraction of the C-4 proton of bound ALA to form a quinonoid intermediate (11).

The difficulties associated with accurate determination of the pH dependence of quinonoid formation were overcome by switching to multiple turnover conditions with excess succinyl-CoA at 20 °C. Under multiple turnover conditions quinonoid intermediate formation is followed by a single step representing decay into the steady state (19), and these two steps were completed within ~2–4 s for the experiments reported here. The shorter time courses resulted in quinonoid intermediate formation constituting a much larger proportion of the total data collected, and along with the larger signal amplitudes observed under saturating conditions, facilitated increased precision of rate determinations for this reaction. It was also reasoned that by conducting the reactions at a lower temperature, the apparent pKₐ might increase, in accordance with the Van’t Hoff equation, sufficiently to allow the titration endpoints to be resolved within the pH range of ~6.5–9.0.

The multiple turnover pH dependence of quinonoid formation at 20 °C is plotted in Fig. 4. The best fit of the data to Equation 1 indicated that quinonoid formation occurred in an acid-catalyzed reaction involving a single proton with an apparent pKₐ of 7.7 ± 0.1.

The temperature dependence of the three steps of quinonoid intermediate formation and decay were characterized over the range 278–308 K with the results summarized in Fig. 5 and Table 1. In Fig. 5 (A and B) representative traces from the spectral data are overlaid with the global fit to Reaction 1, illustrating the effect of temperature on the observed time courses. The total reaction time was increased from 30 to 1800 s as the temperature was decreased to fit all three kinetic steps. The observed rate constants for the three reaction steps were used to construct Arrhenius plots in Fig. 5C, from which the thermodynamic activation parameters were derived.

**DISCUSSION**

ALAS catalyzes the PLP-dependent Claisen-type condensation of glycine with succinyl-CoA to produce CoA, carbon dioxide, and ALA. During the course of the catalytic cycle the C-α glycine bonds to both the pro-R proton and the carboxyl group are sequentially cleaved. This led to the proposal that the
enzyme utilizes the cofactor to form quinonoid intermediates at two distinct points along the reaction pathway (19). However, reconciliation of the stereochemistry required by Scheme 1 and the spatial arrangement of the active site with Dunathan’s hypothesis (14) governing electron delocalization into the PLP ring to form a quinonoid intermediate is neither straightforward nor mechanistically intuitive. Despite the data supporting occurrence of two discrete quinonoid intermediates during the reaction cycle, the only quinonoid species definitively known to be spectroscopically observable is in the presence of the product, ALA (11). In the presence of glycine alone the enzyme catalyzes slow exchange of the pro-

\[ R ^ { - } \text{proton} \] with solvent, but the steady-state level of quinonoid intermediate presumably formed during this partial reaction is not discernible spectroscopically. During transient kinetic experiments the quinonoid intermediate is fully formed only upon addition of the second substrate to the enzyme-glycine complex. The lack of a steady-state KIE on maximal velocity when dideuteroglycine is substituted for glycine suggests that the formation of this first quinonoid intermediate is kinetically insignificant. In this study we report the pH and temperature dependence of the transient steps of quinonoid intermediate formation and decay as an approach toward a better understanding of the chemistry and kinetics involved in transformation of substrates into products, and to contribute to the resolution of the uncertainties noted above. The results of these studies, interpreted within the context of the recently solved crystal structures, allow conclusions to be drawn that support important refinements to both the chemical and kinetic mechanisms of ALAS.

Specifically, we propose that the chemical and kinetic mechanisms be refined as follows (summarized in Figs. 6–8 and Scheme 2).

Refinement 1—Quinonoid formation observed during transient kinetic experiments does not reflect loss of the pro-

\[ R ^ { - } \text{proton} \] of glycine to form structure (III) but is instead indicative of formation of the ALA-bound quinonoid intermediate (VII), which forms via the enol (VI) in a reaction in which His-207 acts as an acid catalyst by donating a proton to the \[ \beta \text{-keto carbonyl} \] oxygen of the \[ \alpha \text{-amino-} \beta \text{-keto adipate intermediate (V).} \] The apparent \( pK_a \) of 7.7 ± 0.1 at 20 °C is assigned to the equivalence point for the proton transfer and indicates that the rate-limiting step following substrate binding and leading up to quinonoid intermediate formation is decarboxylation. The chemical steps leading up to the decarboxylation are proposed to be rapid and therefore kinetically insignificant and drop out of the minimal kinetic mechanism described in Fig. 7. If quinonoid intermediate formation were reflective of abstraction of the pro-

\[ R ^ { - } \text{proton} \] of PLP-bound glycine by the active site lysine, as we had previ-

\[ ^{5} \text{J. Zhang and G. C. Ferreira, unpublished observations.} \]
ously proposed, the reaction would be expected to be base-catalyzed and exhibit a KIE upon substitution of glycine with dideuteroglycine. The experiments reported here are not consistent with this possibility, because the process of quinonoid intermediate formation is acid-catalyzed and no KIE is observed. The fact that no KIE is observed might be explained by proposing that something other than cleavage of the pro-\(R\) proton of glycine, such as a conformational change of the enzyme, limits the quinonoid intermediate (III) formation rate, but acid-catalyzed formation of this quinonoid intermediate is not easily rationalized in terms of the chemistry involved or the active site structure. It might be contemplated that the observed \(\text{pK}_a\) is assignable to protonation of the cofactor pyridinium ring nitrogen by Asp-279, which facilitates quinonoid formation, but pH titration data for the ALA-bound enzyme indicates the pyridinium ring nitrogen remains protonated at pH values \(>9.0\), excluding this possibility (27). The crystal structure does predict donation of a hydrogen bond by the side chain of His-207 to the thioester carbonyl of succinyl-CoA. This interaction indicates that His-207 is in excellent position to stabilize the developing negative charge in the tetrahedral intermediate (IV), act as an acid catalyst for quinonoid formation by donating a proton to the \(\beta\)-keto group during decarboxylation (V), and then abstract this proton during formation of the quinonoid intermediate (VII) or the ALA external aldimine (VIII). The possibility that His-207 forms part of a conserved hydrogen bond network connecting the pyridinium ring nitro-
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directly into the ring system. In the enol (VI) the alkene bond is predicted to be in the proper stereo-electronic orientation for delocalization of electrons into the ring system to form the quinonoid intermediate. Presumably it would be the electrons in the sigma orbital of the alkene bond that preferentially delocalize into the ring, and not the pi orbital electrons, because the former are best aligned for orbital overlap with the pi orbitals of the conjugated ring system during the orbital rehybridization that must occur in the transient transition state leading to the quinonoid.

Refinement 2—The enol (VI) and quinonoid intermediate (VII) are proposed to be in rapid equilibrium and interconvert faster than decarboxylation of (V) and Lys-313-catalyzed tautomerization of the enol intermediate to yield the ALA-bound external aldimine (VIII). The enol and quinonoid intermediates are not proposed to be alternate resonance forms; however, because the alkene bond is not expected to lie in the plane of the conjugated ring system.

Refinement 3—The first step of quinonoid intermediate decay observed during single turnover stopped-flow experiments corresponds to protonation of the enol (VI) by Lys-313 to form the ALA-bound external aldimine (VIII). Two key observations support this interpretation; the apparent kinetic $pK_a$ of 8.1 ± 0.1 for this transient reaction is identical to the spectroscopic $pK_a$ determined for Lys-313-dependent quinonoid intermediate formation with ALA in the reverse reaction direction (11), and protonation at the $\alpha$-carbon would necessarily result in loss of quinonoid absorbance. Although substitution of dideuteroglycine for glycine has no effect on quinonoid intermediate formation, a small but significant isotope effect for the first step of quinonoid intermediate decay is observed. This unexpected result suggests that the pro-$R$ proton removed from glycine by Lys-313 earlier in the reaction sequence may be, at least in a significant proportion of turnovers, the same proton returned to the $\alpha$-carbon by Lys-313 during this reaction step. It has been reported that this proton equilibrates with water during the reaction (9), but the necessity of chemically trapping the proton in the product, conservation of the corresponding proton of serine by the closely related enzyme serine palmitoyltransferase during sphingosine biosynthesis (29), and the KIE observed here, leave the possibility of conservative proton transfer open to future consideration. It is clear that the proton in question can exchange with solvent in both the amino acid-bound substrate and product forms (10, 11). Thus, if conservative proton transfer occurs it would have to be concluded that the active site lysine is excluded from solvent only in the Michaelis and intermediate complexes. Quinonoid intermediate formation in the reverse reaction is not observed when ALA is replaced by 5-aminopentanoic acid, a structural analog that lacks the carbonyl group but does bind at the active site to form an external aldimine (21). The apparent requirement for the carbonyl group during quinonoid intermediate formation with ALA might be explained if it is the enol (VI) that is initially formed and then converted to the quinonoid intermediate (VII). This would necessarily imply, by the princi-
ple of microscopic reversibility, that quinonoid intermediate decay in the forward reaction proceeds via donation of a proton to the α-carbon of the enol, as proposed in Fig. 6. Enol tautomerization, rather than direct protonation of the quinonoid intermediate, is also more consistent with Dunathan’s hypothesis, because the crystal structures imply the stereoelectronic arrangement of the quinonoid intermediate is that shown in Scheme 2, wherein the succinyl group is bound in the Dunathan orientation, precluding proton transfer at the position 180° opposite.

Refinement 4—The second step of quinonoid intermediate decay observed during the single turnover experiments is most likely indicative of opening of the active site lid to allow ALA dissociation. This step closely corresponds to \( k_{\text{cat}} \), is relatively pH-independent, and is not readily assignable to a chemical step. It could conceivably be assigned to the Lys-313-catalyzed transimination reaction required for ALA release, but this reaction, which is common in PLP-dependent enzymes, is typically orders of magnitude faster than the rate of 0.12/s reported in Fig. 7. The relatively large and positive entropy of activation for this reaction (as well as for quinonoid intermediate formation) suggest a high degree of structural organization that is relieved in the transition state. This, together with the high enthalpy of activation, is consistent with the possibility of some structural rearrangement. Additionally, the crystal structures reveal the closure of a conserved C-terminal loop over the active site in the substrate-bound structures, which presumably must revert to the more open conformation observed in the holoenzyme in order for ALA to dissociate. This reaction step, and by extension the entire catalytic cycle, is therefore proposed to be rate-limited by opening of the active site loop, in accordance with the catalytic model proposed previously (19). The observation that this step, which follows protonation of ALA, still involves the presence of some quinonoid absorbance is interpreted as indicating that at this point in the single turnover reaction sequence an equilibrium between the quinonoid and the subsequent reaction steps is established, wherein the slow step of quinonoid decay reflects partitioning among these intermediates while the active site lid slowly opens and ALA dissociates. This interpretation is supported by the resolved rate constants in Fig. 7, which indicate that conversion of EAL1 back to EQ occurs more rapidly than conversion of EAL1 to EAL2. Finally, we note that a rate-determining conformational change would provide a straightforward biochemical means for allosteric regulation of enzyme activity, a possibility that deserves further investigation.

Refinement 5—The minimal kinetic mechanism involves ordered binding of the substrates followed by formation of the enol–quinonoid, protonation of the enol, opening of the active site loop, and ALA dissociation. The relatively rapid steps between substrate binding and quinonoid intermediate formation are kinetically insignificant and drop out of the minimal kinetic mechanism. Additionally, the kinetic mechanism includes a step subsequent to ALA formation and assigned to a conformational change, which does not appear in the chemical mechanism.

These refinements are consistent not only with the data reported here, but also with previously reported data (11, 19, 21, 27) and the recently solved crystal structures (20). Furthermore, these refinements indicate ALAS catalyzes sequential cleavage of two substrate amino acid α-carbon bonds without violating Dunathan’s hypothesis (14, 30). Indeed, these refinements provide support for Dunathan’s hypothesis. It should also be noted that the proposed chemical mechanism for ALAS is nearly synonymous with that recently proposed for the structurally and mechanistically homologous enzyme amino-oxo-nonanoate synthase (18). Clearly, however, other interpretations are possible, and the refinements proposed here should be considered only as the general framework for a working model suitable for future study and critical analyses.

The refined mechanisms might also contribute to our understanding of the evolutionary history and trajectory of ALAS. ALAS is evolutionarily related to transaminases (1, 3), and this relatedness appears to be reflected in the chemical mechanism. The first and last steps of both ALAS and aminotransferase reaction cycles involve proton transfers from and to the cofactor-bound amino acid, in reactions catalyzed by the active site lysine. It is plausible that early in the evolution of ALAS the enzyme catalyzed formation of α-amino-β-ketoacid, which was released from the enzyme and spontaneously decarboxylated in solution to yield ALA. In this scenario the enzyme would have subsequently evolved the capacity to catalyze the decarboxylation reaction at the active site, which, due to stereoelectronic considerations, was directed down the pathway toward decarboxylation of a β-ketoacid rather than decarboxylation to yield a quinonoid intermediate directly. If ALAS had evolved from decarboxylases rather than transaminases, the reaction mechanism would have been considerably less complex, because decarboxylation of bound glycine followed by condensation with succinyl-CoA would yield ALA directly. These observations speak to the complexity and serendipity of the evolutionary process.

In summary, systematic transient kinetic studies such as those presented here represent powerful tools for the elucidation of enzyme mechanisms by facilitating the detailed characterization and assignment of multiple transient kinetic steps. The capacity to produce large quantities of enzymes using recombinant techniques should allow these experimental approaches to become more widely utilized and add substantially to our understanding of enzyme chemistry, mechanism, and evolution. This report has recorded the results of single and multiple turnover transient kinetic studies of ALAS, as functions of pH or temperature. The results of these experiments support important refinements to the chemical and minimal kinetic mechanisms, facilitate assignment of observed kinetic steps to discrete chemical processes, and provide excellent support for the general applicability of Dunathan’s hypothesis. Most importantly, these refinements provide an experimentally based working model amenable to future studies.

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