5-Aminolevulinate synthase (ALAS) is the first enzyme of the heme biosynthetic pathway in non-plant eukaryotes and the α-subclass of purple bacteria. The pyridoxal 5'-phosphate cofactor at the active site undergoes changes in absorptive properties during substrate binding and catalysis that have allowed us to study the kinetics of these reactions spectroscopically. Rapid scanning stopped-flow experiments of murine erythroid 5-aminolevulinate synthase demonstrate that reaction with glycine plus succinyl-CoA results in a pre-steady-state burst of quinonoid intermediate formation. Thus, a step following binding of substrates and initial quinonoid intermediate formation is rate-determining. The steady-state spectrum of the enzyme is similar to that formed in the presence of 5-aminolevulinate, suggesting that release of this product limits the overall rate. Reaction of either glycine or 5-aminolevulinate with ALAS is slow ($k_\text{cat} = 0.15 s^{-1}$) and approximates $k_\text{cat}$. The rate constant for reaction with glycine is increased at least 90-fold in the presence of succinyl-CoA and most likely represents a slow conformational change of the enzyme that is accelerated by succinyl-CoA. The slow rate of reaction of 5-aminolevulinate with ALAS is 5-aminolevulinate-independent, suggesting that it also represents a slow isomerization of the enzyme. Reaction of succinyl-CoA with the enzyme-glycine complex to form a quinonoid intermediate is a biphasic process and may be irreversible. Taken together, the data suggest that turnover is limited by release of 5-aminolevulinate or a conformational change associated with 5-aminolevulinate release.

5-Aminolevulinate synthase (ALAS)$^1$ (EC 2.3.1.37) catalyzes the condensation of glycine and succinyl-CoA to yield coenzyme A, carbon dioxide, and ALA (1). This reaction is the first step of heme biosynthesis in non-plant eukaryotes and the α-subclass of purple bacteria (2). ALAS utilizes PLP as an obligatory cofactor and is evolutionarily related to transaminases (3). Mammals encode two distinct ALAS isoforms, one of which is expressed only in developing erythrocytes (4). The erythroid-specific ALAS isoform accounts for approximately 90% of the heme in the body, and defects in the gene are associated with the erythropoietic disorder, X-linked sideroblastic anemia (5).

Current understanding of the ALAS catalytic mechanism is founded largely upon the results of radiolabeling studies conducted before the advent of molecular cloning technology and is summarized in Fig. 1 (6–9). Following formation of an external aldime with glycine (II), the pro-R proton of glycine is lost, transiently forming a quinonoid intermediate (III) in the presence of succinyl-CoA (10). Condensation with succinyl-CoA results in formation of an aldime to α-amino-β-ketoadipate (V). The glycine-derived carboxyl group is then lost (VI) and replaced by a proton to form an aldime to ALA (VII), which dissociates to regenerate the holoenzyme.

It had been considered in the literature that the carboxyl group of glycine might be lost before condensation with succinyl-CoA. This possibility was discounted when it was found that in the presence of glycine alone ALAS liberates the pro-R proton but does not catalyze decarboxylation. Another possible mechanism involved the dissociation of the α-amino-β-ketoadipate intermediate (V) from the enzyme, followed by spontaneous decarboxylation to yield ALA. However, the observation that the pro-S proton of glycine is found in the pro-S position of ALA indicates that the decarboxylation of the α-amino-β-ketoadipate intermediate must occur on the enzyme surface, because it had occurred free in solution the C-5 position of product ALA would be racemic.

Whereas the chemical mechanism of ALAS is well characterized, much less is known about the kinetic mechanism and the role the protein plays in catalyzing the reaction. ALAS exists as a homodimer in which the active site resides at the subunit interface (11). Steady-state kinetic studies demonstrate a sequential mechanism with glycine binding before succinyl-CoA and with ALA released last (10, 12). Turnover is slow, with a $k_\text{cat}$ of 0.17 s$^{-1}$ at pH 7.2 and 30 °C (13). Lysine 313 of murine erythroid ALAS forms a Schiff base with the PLP cofactor in the absence of amino acid substrate (14). This residue is not essential for the binding of glycine or ALA, but it is required for catalysis (15). A crystal structure of ALAS is not currently available, but the evolutionary relatedness to other, more extensively characterized PLP-dependent enzymes, and in particular aspartate aminotransferase, has provided a basis for homology modeling studies of ALAS structure and function (13, 16, 17). Arginine 439 has been shown to be important for the recognition and binding of the carboxyl group of the substrate glycine (16), and aspartate 279 has been identified as a crucial residue that enhances the electron withdrawing capacity of the PLP cofactor by stabilizing the protonated form of the cofactor ring nitrogen (13). Additionally, tyrosine 121 has been
implicated in binding the PLP cofactor (17). To understand more precisely the role(s) of these and other active site amino acids in the catalytic process, a quantitative assessment of the microscopic rate constants governing the wild-type reaction is a necessary prerequisite.

Overproduction of recombinant murine erythroid ALAS in Escherichia coli has allowed us to begin detailed studies of the reaction mechanism. Here we present the results of stopped-flow absorbance studies of the reactions of ALAS with glycine and ALA, as well as the initial chemical event of the reaction cycle, the removal of the pro-R proton of glycine in the presence of succinyl-CoA to form a quinonoid intermediate.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The following reagents were from Sigma: DEAE-Sepha-

FIG. 1. Putative ALAS reaction pathway. The structures marked III and VI denote the two points along the reaction pathway where the pyridoxal phosphate cofactor functions as an electron sink, forming quinonoid intermediates. Arrows drawn in structures II and V indicate the movement of electrons during formation of these putative intermediates. Structure I is drawn as the Schiff base linkage to lysine 313 in the holoenzyme (10). Because the protonation state of the PLP cofactor during catalysis is not known, all structures are drawn as the protonated form of the Schiff base for convenience only. The absolute stereochemistry of structures IV and V about the α-carbon is not known and may be the opposite of that depicted here.

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 Nal-
gene 2.0-ml polypropylene cryovials when not in use and thawed by incubating the cryovials in 23 °C tap water for 5–10 min. Protein purity was determined by sodium dodecyl sulfate-polyacrylamide gel electro-

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The resolved rate constants are reported with the standard error of measurement obtained from the fitting.

RESULTS

Reaction of ALAS with Substrates—The reaction of 60 μM murine erythroid ALAS with 100 mM glycine plus 100 μM succinyl-CoA was analyzed by rapid scanning stopped-flow spectroscopy at pH 7.5 and 30 °C (results shown in Fig. 2). Data were collected at a rate of 1000 scans/s and were averaged to yield 62 scans/s. The first event observed is the formation of a quinonoid intermediate absorption at 510 nm. The pre-steady-state burst of quinonoid intermediate formation reaches a plateau at 130 ms before it decays into the steady state (Fig. 2B). Upon exhaustion of succinyl-CoA, the limiting substrate, the steady-state quinonoid intermediate absorbance decays to a final value slightly greater than the starting absorbance. An expanded view of the first 3 s of the reaction is given in the inset. This portion of the time course could be described by a two-exponential process with rates of 12.6 and 1.83 s⁻¹ for quinonoid intermediate formation and decay into the steady state, respectively. The plot of the residual error associated with this fit had some structured deviation from ideality in the early data points, suggesting the time course might be better fit by a three-exponential process. This was not possible for this particular time course, however, because of an insufficient number of data points describing the process of quinonoid intermediate formation, but it was possible when the data were not averaged to give more data points in this region of the time course (see below).

Representative spectra from the 2,500 spectra collected are shown in Fig. 2C. Spectra corresponding to time 0 and 0.128 s are identical to those given in Fig. 2A and are shown here for comparative purposes only. The spectrum at 4 s corresponds to ALAS during steady-state catalysis and shows considerable quinonoid intermediate absorption. The spectrum at 40 s also indicates some residual quinonoid intermediate absorption because of the accumulation of ALA, which binds to ALAS some 300-fold more tightly than glycine at pH 7.5.²

Reaction of Glycine with ALAS—The reaction of glycine with ALAS can be analyzed by the increase in absorbance at 425 nm (15). The reaction of 65 μM murine erythroid ALAS with 100 mM glycine at pH 7.5 and 30 °C requires more than 30 s to reach equilibrium even at this saturating concentration of glycine (Fig. 3). The time course was best described by a biphasic process, with rates for the fast and slow phases of 1.16 ± 0.04 and 0.118 ± 0.001 s⁻¹, respectively. The small amplitude of the fast phase resulted in a low signal to noise ratio. It was not possible to determine reliably the effect of glycine concentration on the observed rate constant for this phase. It was possible, however, to determine the concentration dependence of the slow phase, as shown in Fig. 3B. The hyperbolic nature of the rate dependence verifies that the reaction of glycine with ALAS is a two-step process involving a kinetically significant inter-

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

The pre-steady-state spectra from the 2500 spectra collected during the reaction. Spectra shown are, sequentially from the lowest to highest absorbing at 510 nm, as follows: 16, 32, 48, 64, 80, 96, 112, and 128 ms. B, time course of the reaction at 510 nm. The inset is an expansion of the first 3 s of the time course. The collected data (open circles) were fit to a two-exponential equation with rates of 12.6 and 1.83 s⁻¹ for the fast and slow phases, respectively. The residual error (R.E.) for this fit is also given above the inset. C, selected spectra from the overall time course. The spectra shown are: ——, 0.000 s; — — —, 0.128 s; ••••, 4.000 s; and — — — — —, 40.000 s.
mediate. The data were fit to Equation 3 for a two-step process. The best fit of the points gave a value of $K_d = 544 \pm 69 \text{mM}$ for the initial binding, followed by $k_f = 0.028 \pm 0.006 \text{s}^{-1}$ and $k_r = 0.15 \pm 0.02 \text{s}^{-1}$, giving an overall binding $K_d = 8 \text{mM}$.

Time Course of Quinonoid Intermediate Formation with ALA—The interaction of ALA and ALAS includes the binding and formation of a stable quinonoid complex. The time dependence of this reaction is shown in Fig. 4A, which records the reaction of 2 mM ALA with 65 mM ALAS at pH 7.5 and 30 °C. No evidence of an initial increase in absorbance at 420 nm is observed prior to formation of the quinonoid intermediate, and instead the kinetics are dominated by quinonoid complex formation. A biphasic process describes the time course of the reaction at 510 nm, with observed rates for the fast and slow phases of 1.43 and 0.147 s$^{-1}$, respectively. The rate dependence on ALA concentration was also determined, as shown in Fig. 4B. The fast phase of reaction with ALA was concentration-independent with an average value of $0.15 \pm 0.02 \text{s}^{-1}$ over this saturating range of ALA concentration.
Pre-steady-state Reaction of ALAS

Fig. 5. Reaction of murine erythroid ALAS-glycine complex with succinyl-CoA. The residual errors associated with a two-exponential fit to the data given in C are shown in A. This fitting returned a Durbin-Watson ratio of 0.73. A better fit was obtained by fitting the time course data to an equation for a three-exponential process, as shown by the residuals for this fit (B), which returned a Durbin-Watson ratio of 1.16. In C, the time course for reaction of the ALAS-glycine complex with 400 μM succinyl-CoA at 510 nm (circles) is overlaid with points calculated from the fitting to Equation 1 for a three-exponential process with observed rate constants of 55, 18.4, and 3.16 s⁻¹. D, rates of reaction for the two phases of quinonoid intermediate formation as a function of succinyl-CoA concentration. The parameter values for the fitted lines are given under "Results."

Pre-steady-state Reaction of ALAS—
The pre-steady-state reaction of 25 μM murine erythroid ALAS-glycine complex (100 mM glycine) with 400 μM succinyl-CoA at pH 7.5 and 30 °C is depicted in Fig. 5. A fit of the data to a biphasic process left some structured deviation in the residuals (Fig. 5A). The Durbin-Watson ratio for this fit was less than 1, indicating a suboptimal fit. A satisfactory fit was obtained by fitting to a triphasic process (Fig. 5B) and in the fit to the experimental data points (Fig. 5C, solid line). The Durbin-Watson ratio for this fit was 1.16. Binding of succinyl-CoA and conversion of the ternary complex into a quinonoid intermediate thus occur in two kinetic steps, the first of which can be seen at 510 nm as a concave inflection with k_{obs} = 53 s⁻¹. The second, slower phase occurs with k_{obs} = 18 s⁻¹ and is followed by decay into the steady state.

At 25 μM ALAS-glycine, the three phases for reaction of succinyl-CoA with the ALAS-glycine complex were resolvable, and it was possible to determine the effect of succinyl-CoA on the observed rate constants down to 240 μM succinyl-CoA (Fig. 5D). To acquire pseudo-first order rate constants at lower succinyl-CoA concentrations, and thus investigate the possibility of saturation kinetic behavior for the slow phase, the ALAS-glycine concentration was lowered to 10 μM. At this concentration the fast phase was not resolved from the slow phase, and a rate constant could be computed only for the slow phase. The rate of the fast phase was linearly dependent on the concentration of succinyl-CoA, with a non-zero intercept, whereas the slow phase was saturable. These data are consistent with two-step reaction kinetics of the following type.

\[ \frac{k_1}{k_{-1}} = \frac{k_2}{k_{-2}} \]

\( k_1 \) is defined as the slope of the best fit line to the fast phase and equals 67,000 ± 4,000 M⁻¹ s⁻¹. The intercept of this line, 29 ± 4 s⁻¹, is equal to the sum of the other three rate constants, \( k_{-1}, k_{-2}, \) and \( k_{-3} \). A fit of the slow phase data to Equation 3 yields values of \( K_d = 110 ± 50 \mu M, k_2 = 21 ± 2 s^{-1}, \) and \( k_{-2} = 1.4 ± 0.5 s^{-1}. \) The maximal rate of the slow phase, equivalent to the sum of \( k_2 \) and \( k_{-2} \), differs from the intercept value of the fast phase by \( 7 ± 4 s^{-1}, \) yielding estimates of all four rate constants.

DISCUSSION

The pre-steady-state kinetics of murine erythroid ALAS have been here investigated for the first time. The PLP cofactor at the active site of the holoenzyme undergoes changes in absorptive properties during catalysis that have allowed us to characterize both the reactions of ALAS with glycine and ALA and the initial chemical event in the forward reaction, removal of the pro-R proton of glycine to form a quinonoid intermediate. In contrast to the quinonoid intermediate formed in the presence of ALA, this quinonoid intermediate is unstable and decays rapidly. The data support a model in which release of the product ALA, or a conformational change associated with the release of ALA, limits the turnover rate.

A pre-steady-state burst of quinonoid intermediate formation is observed when saturating concentrations of substrates are simultaneously reacted with ALAS. The observation of burst kinetics under these conditions indicates that a step following binding of substrates and formation of the initial quinonoid intermediate is rate-determining. The absorbance
spectrum of ALAS during steady-state catalysis is also informative, because in the steady state the largest proportion of intermediates at any given instance will be at the rate-determining step. The steady-state spectrum will thus be that of the rate-limiting step if one step is completely rate-limiting, or it will be the sum of more than one intermediate if multiple steps are partially rate-limiting. Because considerable quinonoid intermediate is observed during steady-state catalysis, and the formation and decay of the first quinonoid intermediate occur faster than $k_{cat}$, this quinonoid intermediate must correspond to the one formed in the presence of the product ALA. The data from this experiment thus indicate that the rate-determining step is associated with product release and suggest that it is associated with the release of ALA rather than CoA or carbon dioxide.

Steady-state product inhibition studies have demonstrated that the first step of the ALAS catalytic cycle is the binding of glycine (10, 12). In the absence of succinyl-CoA, glycine binding is a two-step process. These steps could represent an initial noncovalent interaction followed by slow conversion of the internal aldimine to the external aldimine or formation of the external aldimine followed by a slow conformational change of the enzyme, among other possibilities. Many PLP-dependent enzymes, including aspartate aminotransferase (22), serine hydroxymethyltransferase (23), and tryptophan synthase (24), undergo transitions from "open" to "closed" conformations upon amino acid binding. Differences in the two conformations have been described in detail for aspartate aminotransferase (22, 25–28). Closure of the enzyme around the substrate appears to function primarily to increase substrate specificity. Interestingly, ß-amino acid aminotransferase, a PLP-dependent enzyme that does not undergo significant conformational changes during reaction, has a broad substrate specificity (29, 30). ALAS, however, shows strict substrate specificity for glycine; no other naturally occurring amino acid has been found to act as a substrate (31). These considerations argue that ALAS probably does undergo some structural rearrangement during catalysis.

In the absence of succinyl-CoA, glycine reacts with ALAS in two kinetic steps. We postulate that these steps represent an initial binding step followed by a conformational change of the enzyme-glycine complex. The second step occurs with a rate constant of 0.15 s$^{-1}$, a rate that approximates $k_{cat}$, suggesting that it may limit turnover. When 100 μM glycine and 100 μM succinyl-CoA are added to ALAS simultaneously, however, the first observed event is formation of a quinonoid intermediate with an observed pseudo-first order rate constant of 12 s$^{-1}$. The rate constant of the second step of the reaction of glycine with ALAS in this experiment could be no less than 12 s$^{-1}$, 90-fold faster than in the absence of succinyl-CoA. If the slow step of the glycine binding reaction is a conformational change to a catalytically active configuration, one possible explanation for this phenomenon is that succinyl-CoA accelerates the rate at which this conformational change occurs. Another possibility is that succinyl-CoA acts as an allosteric effector for glycine binding by binding at some site distal to the active site.

Reaction of ALA with ALAS differs from reaction with glycine in that ALA is converted to a quinonoid intermediate, whereas glycine is bound as a stable external aldimine. ALA reacts to form a quinonoid intermediate in two phases. The fast phase is saturable with a rapid equilibrium $K_D$ of 700 ± 300 μM, $k_r = 1.2 ± 0.2$ s$^{-1}$ and $k_f = 0.6 ± 0.2$ s$^{-1}$. This is followed by a slow phase of 0.15 ± 0.02 s$^{-1}$ that is independent of the ALA concentration and identical to the rate at which glycine reacts in the absence of succinyl-CoA. The reaction between ALA and ALAS thus occurs in three kinetic steps and appears to be analogous to the reaction of glycine with ALAS with the addition of one step, which probably represents the process of quinonoid intermediate formation. The value of 0.6 ± 0.2 s$^{-1}$ for $k_f$ indicates that the release of ALA is at least partially rate-determining. The requirements for excess substrate, and enough enzyme to observe sufficient signal, precluded a more precise determination of this rate constant. If this reaction could be monitored by fluorescence emission, the greater sensitivity of fluorescence detection stopped flow would permit the use of lower ALAS concentrations, which would in turn permit the measurement of $k_{obs}$ at lower ALA concentrations. Fluorescence detection might also allow a more precise determination of the reverse rate constant for the second step of quinonoid intermediate formation, a reaction that may be irreversible. The kinetic data obtained in this study are summarized in Fig. 6.

The strict substrate specificity of ALAS for glycine is not observed with the second substrate, succinyl-CoA (31). The maximal rates of reaction of ALAS with glycine and a series of different CoA esters have been reported (32). Interestingly, the maximal rates for the various compounds are similar to succinyl-CoA and in some cases markedly higher. Identical rates of reaction of a series of related substrates are often considered evidence for the formation of a common structural intermediate that precedes the rate-determining step (33–36). A similar interpretation for ALAS is complicated by the observation that different CoA esters would necessarily give rise to different chemical intermediates and hence different products. A viable hypothesis would be that the various CoA esters each stimulate the same conformational change of the enzyme to a common conformer and the breakdown of this conformer to the initial state limits the rate of the overall reaction.

Thus, the possibility that the kinetics of ALAS catalysis are dominated by interconversion of the enzyme between alternate conformations has not been discounted. An oversimplified model for this would be that ALAS exists in two conformational states, an open conformation in which the substrates bind and a closed conformation induced by substrate binding. Conversion of the open conformation to the closed conformation upon binding of either glycine or ALA is slow. In the presence of both glycine and succinyl-CoA, the rate of the conformational change is appreciably accelerated. Because the ALA alone does not accelerate the conformational change, it appears that the impetus for lowering the energy barrier for going to the closed conformation is supplied from binding energy to the coenzyme A portion of succinyl-CoA. Thus, binding interactions between ALAS and the CoA portion of succinyl-CoA may drive a conformational change of ALAS toward the closed state wherein the condensation between glycine and succinyl-CoA occurs. CoA is then expelled, removing the energetic impetus for maintaining the closed conformation, and the enzyme slowly returns to the open conformation as ALA is released. In this model the rate-determining step is not simply the release of the product ALA from the enzyme but is a conformational change associated with release of ALA. This model could be tested in a number of ways. Fluorescence detection stopped-flow, circular dichroism,
and isotope exchange studies should each offer more insight into the reaction mechanism.

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