The Contribution of a Conformationally Mobile, Active Site Loop to the Reaction Catalyzed by Glutamate Semialdehyde Aminomutase*

(Received for publication, March 11, 1999, and in revised form, November 8, 1999)

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The behavior of glutamate semialdehyde aminomutase, the enzyme that produces 4-aminolevulinate for tetrapyrrole synthesis in plants and bacteria, is markedly affected by the extent to which the central intermediate in the reaction, 4,5-diaminovalerate, is allowed to dissociate. The kinetic properties of the wild-type enzyme are compared with those of a mutant form in which a flexible loop, that reversibly plugs the entrance to the active site, has been deleted by site-directed mutagenesis. The deletion has three effects. The dissociation constant for diaminovalerate is increased approximately 100-fold. The catalytic efficiency of the enzyme, measured as \( k_{\text{cat}}/K_m \) in the presence of saturating concentrations of diaminovalerate, is lowered 30-fold to 2.1 \( \text{mmol}^{-1} \text{s}^{-1} \). During the course of the reaction, which begins with the enzyme in its pyridoxamine form, the mutant enzyme undergoes absorbance changes not seen with the wild-type enzyme under the same conditions. These are proposed to be due to abortive complex formation between the pyridoxal form of the enzyme (formed by dissociation of diaminovalerate) and glutamate semialdehyde itself.

Plants and bacteria obtain the aminolevulinate from which they synthesize porphyrins, by isomerizing l-glutamate-1-semialdehyde (GSA)1 (1). The reaction is catalyzed by GSA-aminomutase, a vitamin B6-dependent enzyme for which a high resolution structure has recently become available (2). Unusually for a dimeric protein composed of subunits having identical sequence, the enzyme shows significant asymmetry in the two subunits, a loop of polypeptide chain comprising residues 153–181 is structured as two short helices one of which completely fills the active site entrance. In the other subunit, the active site is accessible because the corresponding loop is disordered.

The enzyme is clearly related to the aminotransferases in both primary and tertiary structure, the resemblance to ornithine aminotransferase (3) being particularly strong. Despite the fact that the enzyme has only one substrate, the catalytic mechanism (Scheme 1) is also strongly reminiscent of that of the aminotransferases in that the cofactor shuttles between two forms (4, 5). In the initial reaction, involving the enzyme in its pyridoxamine 5′-phosphate form (\( E_{\text{p}} \)), an aldimine (Scheme 1, I) is formed between the aldehyde group of GSA and the amino group of the cofactor. Next, a proton is transferred from \( \text{C-4}^+ \) of the cofactor to \( \text{C-1} \) of the substrate to give an aldime (III) of the pyridoxal phosphate form of the enzyme (\( E_{\text{v}} \)) with the 5-amino group of 4, 5-diaminovalerate (DAVA). Further reaction requires formation of an aldime (V) with the 4-amino group of DAVA, and it is clear that, before this occurs, DAVA is released from the enzyme, at least to some extent (6), thereby leaving the cofactor partially as an internal aldime of pyridoxal phosphate (IV). At first sight, release of the intermediate DAVA would seem to be not only unnecessary but detrimental to the catalytic efficiency of the enzyme.

To assess the contribution of the flexible loop to the enzyme’s mechanism, we have prepared a mutant form (GSAMdel) in which the portion of the flexible segment that forms the helical active site plug (residues 159–172) has been deleted and replaced with a glycine residue. The study makes extensive use of stopped flow spectrophotometry of both wild-type and mutant enzymes because of the potentially informative changes in absorption spectrum that the cofactor undergoes when the enzyme reacts with substrate and with analogues. In the present paper, we base our comparisons of the reactions of the wild-type and mutant enzymes on a model that differs substantially from that recently published for the wild-type enzyme (7), and we support our interpretation with the input of additional data from quenched flow analysis of product formation.

EXPERIMENTAL PROCEDURES

Materials—Wild-type and deletion mutant forms of GSA-aminomutase were expressed and purified as described (8). Forms of the wild-type and deletion mutant enzymes containing six additional histidine residues at the carboxyl terminus were prepared and purified using the QIAexpress metal affinity chromatography system (Qiagen GmbH, Germany). The 1,4-aminohex-5-enoate from which L-glutamate 1-semialdehyde was prepared by ozonolysis (9) was a gift from Hoechst Marion Roussel (Cincinnati, OH).

Site-directed Mutagenesis—Site-directed mutagenesis was performed by polymerase chain reactions (10). The template was the plasmid pSAT 1.4 (8), which contains an EcoRI fragment of the Synchococcus (PCC 6301) genome. For the deletion mutant, two pairs of synthetic oligonucleotide primers were used to amplify the template DNA on either side of the section to be deleted and to introduce BamHI restriction sites at the end of each amplified fragment nearest the deletion. A 350-base pair fragment, upstream of the deletion and ter-
minating in Apal and BamH I restriction sites, was obtained with primers 5'-GATCCGGTGAAGAAGC-3' and 5'-CCGGGATCCACGAGA-
CATGGAC-3'. The 600-base pair fragment, downstream of the deletion and terminating in BamH I and Apal sites, was obtained with primers 5'-CCGAGATTCCGGGGAATATCCAGAAG-3' and 5'-CCTGTGGTA-
GAAGAAGC-3'. The digested fragments and the plasmid pSAT 1.4 (8), already cut with Apal and Sgf I were joined in a single ligation reaction. The mutant DNA clone was sequenced in both directions starting 50 nucleotides before each of the two restriction sites used in the recombinations (Apal and Sgf I). The wild-type clone was similarly sequenced in both directions. The only differences present were those intended. The protein encoded, GSAMdel, contained a single glycine residue
in both directions. The only differences present were those intended.

A form of the enzyme containing six additional histidine residues at
the carboxyl terminus was obtained by polymerase chain reaction mu-
tagenesis and insertion into the pQE60 plasmid (Qiagen GmbH). Two primers (5'-GAAGCTTGGCCGAGCGACCTGGC-3' and 5'-CATTGGGCTTCAGCCACGTACCCATTGGC-3') were designed to amplify the 350-base pair carboxyl-terminal section of the
coding region, to eliminate the stop codon, and to introduce a BgIII restriction site immediately after the last codon. The resulting polym-
erase chain reaction product was digested with BgIII and Sgf I restric-
tion enzymes and ligated to the pQ60 plasmid containing the remaining section of the hem L gene coding region. This procedure inserted DNA encoding arginine and serine, followed by six histidine residues, after
the carboxyl-terminal Leu 433 of the wild-type enzyme. A His 6-tagged
version of the GSAMdel enzyme was obtained by replacing the wild-
type Apal/Sgf I DNA fragment in the pQ60 vector with the correspond-
ning mutant fragment described above.

Expression and Purification—GSAMdel was expressed and purified as
the wild-type enzyme (8). Expression of His-tagged forms of the
enzyme was induced by adding 1 mM isopropyl-β-D-thiogalactoside.
After incubation at 37 °C with constant agitation for 20 h, cells were
harvested and suspended in 3 ml of buffer A (50 mM sodium phosphate
buffer, pH 8, containing 0.3 M NaCl and 0.1% Triton X-100). The cell
lysate was centrifuged, and the supernatant was loaded on a column (2.5 cm × 8 cm) containing 25 ml of nickel-nitrilotriacetic acid resin (Qiagen
GmbH), equilibrated with buffer A. The column was eluted successively
with buffer A (250 ml), buffer B containing 40 mM imidazole (250 ml), and a 400-ml linear gradient from 40 mM to 0.5 M imidazole in buffer A. Fractions containing the enzyme were detected by SDS-polyacrylamide
gel electrophoresis.

Stopped and Quenched Flow Analyses, Spectral and Data Analysis—
Stopped flow experiments were performed on an SF1 stopped flow spectrophotometer (Hi-Tech, Salisbury, United Kingdom). Product for-
mation during the period from 0.1 to 7 s was measured using a quenched flow apparatus (11). Most reactions were conducted at pH 7.9 in
0.1 M Tricine buffer. The substrate GSA, dissolved in 50 mM HCl (to
keep it stable) in one syringe, was mixed with an equal volume of
drug dissolved in 0.2 M Tricine, pH 7.9, to which NaOH had been
added to a final concentration of 50 mM (to neutralize the HCl) from
the other syringe. Quenched flow reactions were stopped with perchloric acid (final concentration of 1.5%). Aminolevulinate was quantified with
Ehrlich’s reagent (9). The molar concentration of enzyme subunits was
determined from the absorbance of pyridoxal 5'-phosphate released
after treatment in 0.1 M NaOH using εmax = 6550 cm−1 M−1 (12). Absorption
spectra were measured with a Hewlett-Packard model 8452 diode array spectrophotometer. CD spectra were recorded as the average of
three scans on a Jasco 710 spectropolarimeter equipped with a
DP 520 processor at 25 °C using a 2-mm quartz cell. Mass spectra were
determined using a Finnigan LCQ electrospray ionization mass spec-
 trometer and statistical analyses were performed using the data
manipulation software, Scientist (Micromath, Salt Lake City,
UT). Equations 1 and 2 were used for global fitting of increasing product concentration and the associated changes in cofactor absorbance.

\[
\frac{d\alpha_{418}}{dt} = k_{cat}\frac{[E]_0}[S_0] - [P]
\]

(Eq. 2)

The dependence of the steady-state velocity (measured in the absence
of added DAVA) on total enzyme concentration \([E]_0\) was fitted by combining
Equation 3 with the quadratic relationship (Equation 4) that predicts how the steady-state concentration of \([ES]\) in Scheme 4 increases nonlinearly as a function of total enzyme concentration \([E]_0\).

\[
\frac{1 + K_v}{[S]} [ES]^2 - \left[2 \left(1 + \frac{K_v}{[S]}\right)[E]_0 + K_v\right][ES] + [E]_0^2 = 0
\]

(Eq. 4)

Global fits of product formation and changes in cofactor absorbance
to Schemes 2 and 3 were made by numerical integration of the differential
equations governing all of the steps linked by arrows outside the brack-
etes. Species shown within brackets were treated as being at equilibrium.

RESULTS

Absorption, CD, and Mass Spectra—The cofactor absorption
spectrum of GSAMdel (Fig. 1) is very similar to that of the
wild-type enzyme, although a significant difference is discern-
able. The proportion of 340-nm absorbing chromophore, attrib-
utable to the enolimine tautomer of the internal aldimine of
Lys 773 with pyridoxal phosphate (13), is slightly lower in the
deletion mutant. When the mutant enzyme was treated with
diaminohexane, the 420-nm chromophore was converted com-
pletely to the 340-nm chromophore characteristic of the pyri-
doxamine form of the enzyme, thus demonstrating that all of
the mutant enzyme is still capable of this half-reaction. Simi-
larly, treatment with succinic semialdehyde restored the
420-nm absorbing form of the enzyme. The CD spectra of both
forms of the enzyme are also very similar. The slightly lower
molar ellipticity in the 220–240-nm region is consistent with
the expected minor loss of α-helix in the mutant. The level of
expression of the mutant enzyme (40 mg/liter of culture) was
expressed. It began with a short lag, reached its fastest rate at a
product concentration, which accounts for 80% of the reaction, occurs at a
rate far below that expected from $k_{\text{cat}}$ and $K_m$, which are $1 \text{ s}^{-1}$ and $16 \mu\text{M}$, respectively (4), and which predict that the reaction should be complete in less than 10 s. The amount of product formed when the reaction was complete was that expected from the amount of GSA present at the beginning, but the major inconsistency between the observed rate of product formation and that predicted by $k_{\text{cat}}$ and $K_m$ shows that the mechanism contains an additional feature. The fact that 20% of the substrate was converted to product at the rate expected, whereas the remainder was converted much more slowly, suggests that the substrate is present as two interconvertible forms and that only the minor form is acted on directly by the enzyme. This interpretation was supported by an experiment in which the amount of product formed in the fast process was shown to increase toward a limiting value (25%) as enzyme concentration was increased. A similar, biphasic reaction occurs during the malate dehydrogenase-catalyzed reduction of oxaloacetate, because the majority of the reaction is rate-limited by enol to keto isomerization of the unbound substrate (14). In this interpretation, the large fall in $A_{420}$ observed after approximately 1 s would be due to depletion of the proposed, minor, rapidly reacting component of the GSA solution rather than to a transient, pre-steady-state step in the enzyme-catalyzed reaction itself. We concluded that interpretation of the substrate concentration dependence of the kinetic properties of the enzyme under these conditions, where the concentrations of enzyme and reactive form of the substrate are similar, would be difficult. To avoid this complication when making comparisons with GSAMdel, we chose to characterize the kinetic behavior of the wild-type enzyme at the much lower concentration normally used in steady-state measurements (approximately $0.3 \mu\text{M}$) so that interconversion of substrate was not rate-limiting and substrate was not significantly depleted by formation of complexes with the enzyme. Changes in cofactor absorbance were measured continuously at 420 nm, and the aminolevulinate product was measured at intervals (a). $a$, the first 10 s of reaction; $b$, the complete reaction.

**FIG. 2.** Correlation of transient changes in cofactor absorbance with product formation at high concentration of wild-type GSA-aminomutase. The enzyme (14.5 μM) was mixed with GSA (105 μM) in both stopped flow and quenched flow instruments under identical conditions (25 °C, pH 7.9, in presence of 350 μM DAVA). Changes in cofactor absorbance were measured continuously at 420 nm, and the aminolevulinate product was measured at intervals (○). a, the first 10 s of reaction; b, the complete reaction.

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DAVA increases both $K_m$ and $k_{cat}$ to the same extent (approximately 3.5-fold) and has no significant effect on $k_{cat}/K_m$; its effect may be considered as relief of an uncompetitive inhibition that is intrinsic to the enzyme's mechanism. The most likely identity of the inhibitory process is dissociation of DAVA from one of the intermediate enzyme-DAVA complexes. At the end of the reaction, $A_{420}$ returned to its initial zero value when DAVA was added, but, in the absence of added DAVA, a small but significant 420-nm absorbing component was present. We believe this to be due to the presence of $E_L$ and $E_1D$ at equilibrium with $E_M$ and the product aminolevulinate (Scheme 2). This proposal is supported by the observation (7) that mixing $E_M$ with aminolevulinate produces a decrease in $A_{420}$ and a corresponding increase in $A_{420}$. We have also demonstrated by thin layer chromatography and NMR that DAVA is formed when aminolevulinate and $E_M$ are mixed.2

During the first few seconds of the reaction, a burst of product formation was evident in experiments with and without added DAVA (Fig. 3b). Approximately 1.5 $\mu$m product was produced in this burst, indicating that it is much too small to be due to exhaustion of the rapidly reacting form of the substrate. We consider that this burst is not due to the existence of a third form of the substrate, because the corresponding reaction profile seen with a mutant form of the enzyme (M248I) resistant to the fungal toxin gabaculine does not show a burst, although its steady state specific activity is the same as that of the wild-type enzyme.3 The wild-type enzyme (0.3 $\mu$m) must have undergone approximately five turnovers during the period of the burst. The maximum rate of product formation during the burst indicated a transient value for $k_{cat}$ of at least 12 s$^{-1}$. The rate constant ($\sim 4$ s$^{-1}$) governing the increase in $A_{420}$ during the first few seconds is significantly slower than 12 s$^{-1}$, demonstrating that the reaction responsible cannot be directly on the path of aminolevulinate production. Although the noise level at the start of the trace was too high to be confident, there was a possible lag at the start of the reaction, suggesting the existence of a preceding step. The existence of a faster, measurable process was confirmed by conducting measurements at 330 nm, where a rapid rise in absorbance was followed by a fall that parallels the rise at 420 nm (data not shown). The time scales of the rise in $A_{420}$ and fall in $A_{330}$ were noticeably similar to that of the burst in product formation. The continuous lines through the $A_{420}$ and product formation data of Fig. 3b are those of global best fit to Scheme 2 in which we propose that $E_L$, $E_1D_1$, and $E_1D_2$ are the only species that absorb significantly at 420 nm. Thus, we consider that the slowing of the rate of product formation, the increase in absorbance at 420 nm, and the fall in absorbance at 330 nm are all due to the same process, namely the conversion of $E_1$ to $E_1D_1$. In the absence of DAVA, the rise in $A_{420}$ is larger and occurs in two phases, the second of which we propose to be dissociation to free $E_L$ and DAVA. In the presence of DAVA, the faster phase is still present with the same amplitude and rate constant, but the slower phase entails only a small change in $A_{420}$, and in the reverse direction (due to conversion of $E_1D_1$ to $E_1D_2$).

The reactions in the pathway shown horizontally in Scheme 2 are those essential to the formation of aminolevulinate from GSA. The only reaction on this main path that can be measured by the stopped flow method is that which shows a rise at 330 nm linked to the lag in increase in $A_{420}$. The slower reactions, those that lead to an increase at 420 nm, are due to reactions not on the catalytic pathway but on the side reaction path, which slows the reaction rate and leads to the dissociation of DAVA.

Kinetic Properties of GSAMdel—The deletion mutant (GSAMdel) does not have detectable catalytic activity when it is measured at 0.3 $\mu$m in the standard assay. However, at higher concentration (14.5 $\mu$m), aminolevulinate was formed at a rate similar to that seen for the wild-type enzyme at 0.3 $\mu$m. We verified that this activity was due to GSAMdel itself and not to a minor contaminant of wild-type enzyme from the host, by measuring the activity of the His-tagged form of GSAMdel, which does not copurify with the wild-type enzyme and which

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2 R. Contestabile and R. A. John, unpublished results.
3 M. Markova and R. A. John, unpublished results.

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FIG. 3. Correlation of transient changes in cofactor absorbance with product formation at low concentration of wild-type GSA-aminomutase. The enzyme (0.3 $\mu$m) was mixed with GSA (115 $\mu$m) in the absence and presence of DAVA (350 $\mu$m) at pH 7.9 and 25 °C in both stopped flow and quenched flow instruments. Changes in cofactor absorbance were observed at 420 nm (continuous jagged lines) after mixing in the stopped flow instrument. Aminolevulinate product was measured at intervals after quenched flow mixing (in the presence of DAVA (●) and in the absence of DAVA (○)). a, reactions occurring over 1500 s. The smooth lines through both sets of experimental data are those predicted by a model based on numerical integration of the Michaelis-Menten relationship (Equations 1 and 2). The constants found and used to give the fits shown are given in Table I. b, reactions occurring over 15 s in the absence and presence of DAVA. The continuous smooth lines through all of the experimental data are those from global best fits based on Scheme 2 but simplified by assuming negligible substrate depletion and product formation over this time period. Only $E_1D_1$, $E_2D_2$, and $E_L$ were assumed to contribute significantly to $A_{420}$. The constants of best fit were as follows: $k_1 = 29$ s$^{-1}$, $k_2 = 2$ s$^{-1}$, $k_3 = 3.2$ s$^{-1}$, $k_{cat} = 0.84$ s$^{-1}$, $k_{cat} = 3.9$ s$^{-1}$, and $k_{cat} = 1.93$ s$^{-1}$ in the presence of DAVA and $k_1 = 17$ s$^{-1}$, $k_2 = 27$ s$^{-1}$, $k_3 = 10$ s$^{-1}$, $k_{cat} = 1.5$ s$^{-1}$, $k_{cat} = 0.67$ s$^{-1}$, and $k_{cat} = 0.34$ s$^{-1}$ in the absence of DAVA.  

| Enzyme form                  | $K_m$  | $k_{cat}$ | $k_{cat}/K_m$ |
|-----------------------------|--------|-----------|---------------|
| Wild type                   | 9.0    | 0.55      | 61            |
| Wild type plus DAVA         | 28.7   | 1.99      | 69            |
| GSAMdel plus DAVA           | 71     | 0.149     | 2.1           |

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TABLE I
Flexible Loop in Glutamate Semialdehyde Aminomutase

(a) changes in $A_{420}$ and increase in aminolevulinate were monitored when GSAMdel (14.5 $\mu$M) was reacted with GSA (115 $\mu$M) in the absence and presence of DAVA (2.5 mM). When analyzing the data obtained in the presence of DAVA, the $A_{420}$ profile (lower curve), after exclusion of the first 15 s, and the formation of aminolevulinate (solid line) were globally fitted to Equations 1 and 2. Constants of best fit (Table I) were used to generate the continuous line through the experimental data sets. The continuous line profile determined in the absence of DAVA (solid curve) was generated by fitting the data to $[P]$ in Equation 1, although, because the $A_{420}$ changes did not conform to Equation 1, the former fit has little analytical significance. b, effect of 2.5 mM DAVA on the first 10 s of reaction. The experimental data were analyzed in terms of a scheme in which the $A_{420}$ species is formed in the second of two consecutive, irreversible reactions and in which the rate of aminolevulinate production is proportional to the $A_{420}$ species. The global best fit of the four sets of data (continuous lines) gave rate constants of $k_1 = 43$ s$^{-1}$ and $k_2 = 0.82$ s$^{-1}$. The amplitude of the change in $A_{420}$ shows a hyperbolic dependence on DAVA concentration (Fig. 5, inset), with an apparent dissociation constant of 28 ± 7 $\mu$M.

In the presence of high concentrations of added DAVA (2.5 mM and above), the kinetic behavior of GSAMdel was relatively simple. Once the initial rapid increase in $A_{420}$ was complete (~5 s), both the subsequent decrease in $A_{420}$ and the accompanying increase in aminolevulinate concentration conformed closely to the Michaelis relationship, with $k_{\text{cat}}$ being decreased 13-fold and $K_m$ increased 2.5-fold relative to the wild-type enzyme (Fig. 4a, Table I). As with the wild-type enzyme, there was an exponential increase in $A_{420}$ during the first 5 s, but this was accompanied by a lag, rather than a burst, in aminolevulinate production (Fig. 4b). Another significant difference between the wild-type and mutant enzymes was that, when DAVA was not added, the initial exponential increase in $A_{420}$ was followed by a further slow increase, which continued until substrate was almost exhausted, whereupon $A_{420}$ decreased to a final value that was significantly higher than that at the start.

(Figs. 4a and 5). This slow increase in $A_{420}$ was not present at all in the wild-type enzyme reaction nor in the mutant reaction when DAVA was omitted (Fig. 5), and we propose that it is due to combination of the $E_L$ form of the enzyme with GSA to form an abortive complex (see below).

A possible explanation for the fact that initial increase in $A_{420}$ is accompanied by a lag rather than a burst in product formation is that high concentrations of 420-nm absorbing intermediates accumulate on the main path of the reaction catalyzed by GSAMdel because the mutation has selectively decreased the rate of breakdown of one of the aldmines (III or V in Scheme 1). However, it seems more likely that, in the mutant enzyme, a step (or steps) on the main path has slowed sufficiently for the 420-nm absorbing species in the side reaction leading to DAVA to be effectively in equilibrium with EX. In these circumstances, EX and both $E_L$-containing forms would behave as a single kinetic species (Scheme 3), which would increase exponentially during the establishment of a near steady state as observed. Adding a high concentration of DAVA (2.5 mM) decreased the amplitude of the $A_{420}$ transient by one-third without affecting the rate constant and approximately doubled the rate of ALA formation. This behavior is predicted by Scheme 3. The constants returned by a global best fit of all of the data shown in Fig. 4b to Scheme 3 were as follows: $k_1 = 0.78$ s$^{-1}$, $k_2 = 0.22$ s$^{-1}$, $K = 1.6$, and $K_d = 13$ $\mu$M. The imperfect fit to the $A_{420}$ data may be due to the presence of the additional slow phase, which is not included in Scheme 3.

The $A_{420}$ profiles determined at DAVA concentrations between 0 and 2.5 mM altered systematically between the extremes shown in Fig. 4, and analysis showed that the amplitude of the rapid rise in $A_{420}$ decreased with increasing [DAVA] (Fig. 5, inset), whereas the rate constant governing this process remained constant. The $A_{420}$ profiles also altered systematically when the initial concentration of GSA was increased (Fig. 5) and, in the presence of high DAVA concentrations, were as expected for a system conforming to the Michaelis relationship. In the absence of added DAVA, the slow rise in $A_{420}$ continued for longer before reversing in direction, consistent with the proposal that this phase is due to combination of the $E_L$ form of the enzyme with GSA to form an abortive complex.

**Dependence of Steady-state Rate on Enzyme Concentra-**

**FIG. 5. Dependence of absorbance changes on GSA and DAVA concentration.** $A_{420}$ was observed at three concentrations of GSA (60 $\mu$M (i), 120 $\mu$M (ii), and 240 $\mu$M (iii) in the presence of 10 mM DAVA (lower three lines) and in the absence of added DAVA (upper three lines). Enzyme concentration was 14.5 $\mu$M. Inset, dependence of amplitude of the rapid change in $A_{420}$ on DAVA concentration. The continuous line is a hyperbola predicted for reversible binding with an apparent dissociation constant of 28 ± 7 $\mu$M.
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FIG. 6. Effect of increasing enzyme concentration on steady-state rate. The rate of aminolevulinate production catalyzed by the wild type and the mutant enzymes was measured at increasing enzyme concentrations and at constant substrate concentration (125 μM) in the presence of 10 μM DAVA (●) and in the absence of DAVA (○). The data for GSAMdel are shown. The continuous line through the points in the presence of DAVA is that for a straight line described by Equation 3, while data collected in the absence of DAVA were best fitted to Equation 3. The two sets of data were analyzed on the basis of the relevant equations, in a global fit that used shared parameters. $K_{d}$, taken from stopped flow measurements, was fixed at 71 μM, and the best fit values found for the other parameters were $K_{m} = 33 ± 3 μM$ and $k_{cat} = 0.122 ± 0.002 s^{-1}$. Inset, the data obtained when using the wild-type enzyme were similarly analyzed. $K_{m}$ was fixed at 28.8 μM, and the best fit values for the other constants were estimated to be $K_{m} = 0.29 ± 0.07 μM$ and $k_{cat} = 1.22 ± 0.04 s^{-1}$.

DISCUSSION

Removal of the 159–172 loop reduces the efficacy of the enzyme for more than one reason. The loop can be seen to make a significant contribution to the catalytic process itself because, even at saturating concentrations of the central, dissociable intermediate DAVA, $k_{cat}/K_{m}$ is 30 times lower in the mutant than in the wild-type enzyme. Comparison of the dependence of the steady state velocities of reactions of wild-type and mutant enzymes indicates that the loop also decreases the dissociation constant of DAVA approximately 100-fold. Estimates of the combined effect of the contribution of the loop to the catalytic process and to dissociation of DAVA are complicated by the fact that the two effects are interdependent, because dissociation of DAVA increases both apparent $k_{cat}$ and apparent $K_{m}$. According to Scheme 4 the extent of DAVA dissociation depends on both GSA and enzyme concentrations. We calculate that, in the conditions used in the standard assay (0.3 μM enzyme and 125 μM GSA) and in the absence of exogenous DAVA, dissociation of DAVA from GSAMdel is 40 times greater than from the wild-type enzyme. Accordingly, the steady-state velocity of ALA production is 600 times lower than with the wild-type enzyme (40-fold from difference in extent of dissociation, 15-fold from difference in $k_{cat}$). At very high concentrations of enzyme and of both GSA and DAVA, we estimate that there would be only a 15-fold differ-
ence between the two activities, corresponding to the difference in \( k_{\text{cat}} \). However, in the absence of added DAVA and at very low concentrations of the enzymes, the difference approaches a limiting value of 1500-fold, due to the combined effects of the 15-fold difference in \( k_{\text{cat}} \) and the 100-fold difference in \( K_{M} \).

A further contribution of the loop to the catalytic process is that it prevents the process responsible for the 420-nm absorbing material that forms slowly when the mutant enzyme is reacted with GSA in the absence of added DAVA. The fact that inclusion of high concentrations of DAVA prevents this process from occurring suggests that it is a reaction of \( E_L \), that arises when DAVA dissociates. The further observation that formation of this chromophore stops abruptly when [GSA] falls to 0 is consistent with a hypothesis that the reaction is with GSA itself. The apparent absence of this process from the reaction catalyzed by the wild-type enzyme is not simply because \( E_L \) is formed less readily but also because GSA reacts less rapidly with the \( E_L \) form of the wild-type enzyme. We arrive at this conclusion because no process corresponding to the slow increase in \( \Delta A_{420} \) observed when the \( E_M \) form of GSAMdel is reacted with GSA is evident in the corresponding reaction with the wild-type enzyme, even under conditions in which the free \( E_L \) form is present in equivalent proportions (compare Figs. 3a and 4a). We propose that the reaction responsible for the slow rise in \( \Delta A_{420} \) that occurs with GSAMdel occurs because, after dissociation of DAVA, \( E_L \) forms an external aldime with the amino group of GSA (Scheme 5, Ia). This tautomerizes to the enol form, IIIa, via the 500-nm absorbing quinonoid structure, IIa. The extension of conjugation to the cofactor imine would be expected to result in a relatively stable external aldime with enol-GSA. We suggest that it is important that the enzyme avoid forming this stable inactive structure. This hypothesis would explain why the enzyme mechanism begins by reaction of the substrate oxo group with \( E_M \) rather than reaction of the amine with \( E_L \) and proceeds through dianimoxalate rather than dioxoalenate as intermediate.

In the reaction catalyzed by the wild-type enzyme, the initial increase at 420 nm is so much slower than aminolevulinate formation that the \( E_L \)-containing species that is formed cannot be on the reaction path. Furthermore, formation of this species is accompanied by a 5-fold slowing of the rate of aminolevulinate production. In Scheme 1, the only species that would absorb maximally near 420 nm are the aldimes III, IV, and V, and these must therefore be pseudoequilibrium components of the kinetic species shown as EX in Scheme 2. Because there is no obvious 420-nm absorbing species that is covalently different from these three aldimes, it is necessary to propose that \( E_D \) and \( E_D \) arise from conformational rather than covalent changes. The currently available data would be explained by a hypothesis in which the side reaction that leads to the parallel increase in \( A_{420} \) and fall in the rate of reaction is due to opening of the loop, while the enzyme is as \( E_L \) with DAVA bound as a noncovalent Michaelis complex. At the beginning and end of

the reaction, when the enzyme is in the \( E_M \) form, opening and closing must occur with a frequency at least equal to that of the value of \( k_{\text{cat}} \) estimated from the velocity before the reaction has slowed down (12 s\(^{-1}\)). Thus, the observations presented in this paper support a hypothesis in which the rate and extent of loop opening are influenced by the state of the cofactor, being rapid and extensive when the enzyme is in the \( E_M \) form to allow substrate to enter and product to leave but slower and less extensive in the \( E_L \) form to impede the dissociation of DAVA and abortive complex formation with GSA.

Sequence alignment (16) shows that this loop is present as an insert in only a small subfamily of B2-dependent enzymes that are closely related in sequence, structure, and function. The enzymes in this group include ornithine aminotransferase, for which an x-ray structure is available and in which the loop is also at the surface, where it forms a part of the wall of the active site near the entrance. The reason for the mobility of this part of the protein in GSA-aminomutase and the apparent immobility in ornithine aminotransferase appears to be replacement of the Gly/Ala-rich sequence AGSGVA in GSA-aminomutase by STDPTS in ornithine aminotransferase.

The mobile active site loop in GSA-aminomutase is similar in its disposition with respect to the active site to those described for other enzymes. In lactate dehydrogenase, binding of substrate induces the closure of the active site entrance by a 13-residue loop (17). The closure, which is rate-limiting, drives out water and provides a correctly positioned catalytic residue (Arg\(^{109}\), which polarizes the carbonyl of pyruvate). In triose phosphate isomerase, a 10-residue loop moving as a rigid, hinged lid is similarly induced by substrate binding to close the active site (18). Deletion of a small part of this loop made \( k_{\text{cat}} \) 10\(^5\)-fold lower and allowed the unstable enediol phosphate intermediate to be released. It was concluded that loop closure preferentially stabilizes this intermediate as well as the transition states on either side of it in the mechanism. Thus, in these examples, interactions between residues of the loop and the substrate make a major contribution to the catalytic mechanism such that excision of the loop or a small part of it results in massive loss of catalytic activity. The loop in GSA-aminomutase is of similar size to those of lactate dehydrogenase and triose phosphate isomerase, and it appears to undergo similar movements. However, it is clearly much less important to catalysis itself, since, if dissociation of the DAVA is prevented by inclusion of high concentrations of this intermediate, the enzyme is only 30-fold poorer as a catalyst. The consequences of dissociation of DAVA are less serious than the dissociation of the unstable enediol phosphate intermediate from triose phosphate isomerase, since DAVA is not unstable, and the direct effects of dissociation are small at high enzyme concentration. Perhaps in GSA-aminomutase, the main purpose of the loop is to prevent the formation of a stable abortive complex between GSA and the \( E_L \) form of the enzyme.

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