Stereochemistry of the Reactions of Glutamate-1-semialdehyde Aminomutase with 4,5-Diaminovalerate*

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Conversion of glutamate 1-semialdehyde to the tetrapyrrole precursor, 5-aminolevulinate, takes place in an aminomutase-catalyzed reaction involving transformations at both the non-chiral C5 and the chiral C4 of the intermediate 4,5-diaminovalerate. Presented with racemic diaminovalerate and an excess of succinic semialdehyde, the enzyme catalyzes a transamination in which only the L-enantiomer is consumed. Simultaneously, equimolar 4-aminobutyrate and aminolevulinate are formed. The enzyme is also shown to transaminate aminolevulinate and 4-aminohexenoate to L-diaminovalerate as the exclusive amino product. The interaction of the enzyme with pure D- and L-enantiomers of diaminovalerate prepared by these reactions is described. Transamination of L-diaminovalerate yielded aminolevulinate quantitatively showing that reaction at the C5 amine does not occur significantly. A much slower transamination reaction was catalyzed with D-diaminovalerate as substrate. One product of this reaction, 4-aminobutyrate, was formed in the amount equal to that of the diaminovalerate consumed. Glutamate semialdehyde was deduced to be the other primary product and was also measured in significant amounts when a high concentration of the enzyme in its pyridoxal form was reacted with D-diaminovalerate in a single turnover. Single turnover reactions showed that both enantiomers of diaminovalerate converted the enzyme from its 420-nm absorbing pyridoxaldimine form to the 330-nm absorbing pyridoxamine via rapidly formed intermediates with different absorption spectra. The intermediate formed with L-DAVA (λ_{max} = 420 nm) was deduced to be the protonated external aldimine with the 4-amino group. The intermediate formed with D-DAVA (λ_{max} = 390 nm) was deduced to be the unprotonated external aldimine with the 5-amino group.

Plants and bacteria obtain the aminolevulinate (ALA)[1] from which they synthesize porphyrins by isomerizing L-glutamate 1-semialdehyde (GSA) (1). The reaction is catalyzed by GSA-aminomutase, a vitamin B6-dependent enzyme that because it is not present in animals is considered to be a potential target for herbicides. The three-dimensional structure of the enzyme (2) shows it to be a member of the aspartate aminotransferase superfamily, and it is related closely to the ω-aminotransferases that act on ornithine and 4-aminobutyrate (GABA). The mechanism of the enzyme is similar to that of classical transamination with the exception that exchange of amino and oxo groups occurs within the same molecule (3, 4). The reaction begins and ends with the enzyme cofactor in the pyridoxamine phosphate form (Fig. 1, E_M). Central to the overall reaction is a series of complexes in which the intermediate, 4,5-diaminovalerate (DAVA), is bound to a form of the enzyme (E_L) that has the cofactor as an aldimine of pyridoxal 5′-phosphate either with Lys-272 of the enzyme (IV and IVa) or with one of the two amino groups of DAVA (III and IIIa). Furthermore, although dissociation of DAVA to give substrate-free E_L seems unnecessary, there is no doubt that it occurs to a significant extent *in vitro* (5). Thus, free E_L, which cannot react productively with GSA, is present at significant concentrations under the conditions used to assay the enzyme. For this reason, added DAVA significantly increases the rate of the reaction by keeping the E_L form saturated with this intermediate.

DAVA is asymmetric at C4 but because the separate enantiomers are not available commercially, the racemic mixture has been used in almost all of the previous investigations. However, Friedmann *et al.* (6) having synthesized the pure enantiomers from the appropriate enantiomer of 2-pyrrolidone 5-carboxylate concluded that the enzyme lacked strict stereospecificity because both forms of DAVA were capable of accelerating the overall reaction. If the observation that the catalytic reaction is activated by either stereoisomer is truly because of a lack of stereospecificity, it is unusual because other members of this family display high stereospecificity toward their chiral substrates. It is also in conflict with the observation that the enzyme shows absolute stereospecificity for L-4-aminohexenoate, a close structural analogue of DAVA (7). In this paper, we report the results of experiments aimed at quantifying and explaining the reactions of the enzyme with L- and D-enantiomers of DAVA. To facilitate the work, we developed methods for the enzymic preparation of both enantiomers.

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[1] The abbreviations used are: ALA, 4-ALA; GSA, L-glutamate 1-semialdehyde; DAVA, 4,5-diaminovalerate; GABA, 4-aminobutyrate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; E_L, GSA-aminomutase with cofactor bound as an aldimine of PLP with Lys-272; E_M, GSA-aminomutase with cofactor bound as PMP; HPLC, high pressure liquid chromatography; SSA, succinic semialdehyde; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; DOPA, dihydroxyphenylalanine.

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FIG. 1. **Mechanism of reaction catalyzed by GSA aminomutase.** *Complexes I* and *Ia* are Michaelis-Menten complexes of E_M with GSA or ALA. *Complex II* is the enzyme-bound aldimine of PMP with GSA, and *complex IIa* is the enzyme-bound ketimine of PMP with ALA. These forms have absorbance maxima at ~340 nm. *Complexes III* and *IIIa* are enzyme-bound aldimines of PLP with the 5- and 4-amino groups of DAVA. *Complexes IV* and *IVa* are Michaelis-Menten complexes of E_L with DAVA. *Complexes III*, *IIIa*, *IV*, and *IVa* have absorbance maxima at ~420 nm.
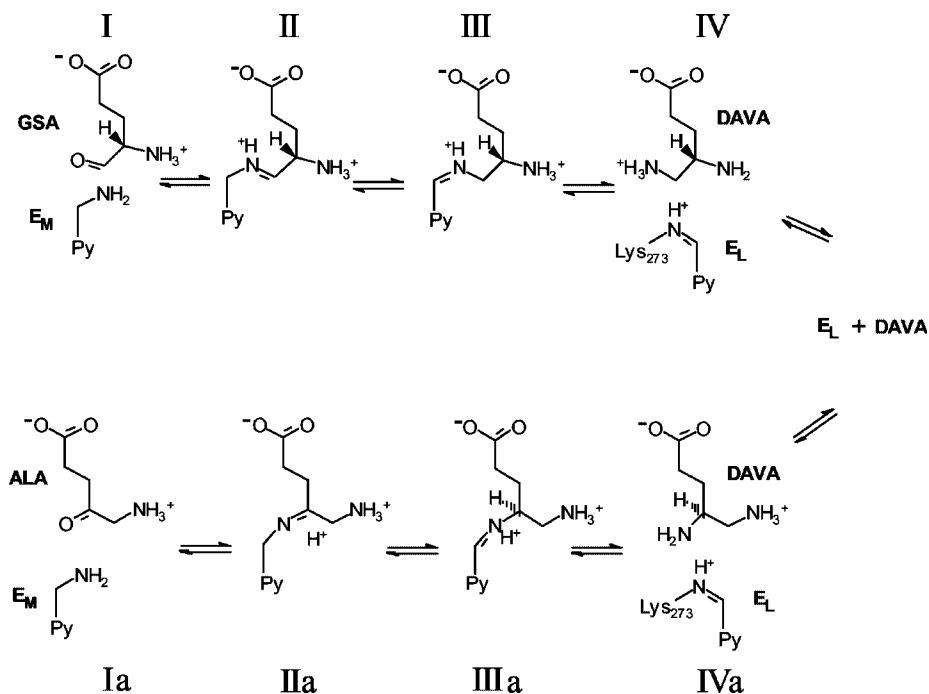
EXPERIMENTAL PROCEDURES

Materials—GSA aminomutase was expressed and purified as described previously (8). The L-4-aminohex-5-enoate from which L-GSA was prepared by ozonolysis (9) was a gift from Hoechst Marion Roussel (Cincinnati, OH). All of the other reagents were from Sigma.

Synthesis of L-DAVA—ALA (30 mM) and racemic 4-aminohexenoate (60 mM) were maintained at 30 °C in the presence of GSA-aminomutase (150 μM) for 4 h in Na-Tricine (0.1 M), pH 7.9. The solution (4.3 ml) was centrifuged through a Centricon 10-kDa filter, and the filtrate was brought to pH 6.0 by adding HCl and chromatographed on Dowex 50 (column 4 × 1 cm, 200–400 mesh, 8% cross-linked, H$^+$ form), equilibrated with ammonium phosphate (0.2 M, pH 6.0). The column was eluted with the same ammonium phosphate solution. Fractions (1 ml) were collected and monitored by thin layer chromatography on silica gel plates, developed in butan-1-ol/acetic acid/water (3:1:1), and detected with ninhydrin. ALA and aminohexenoate eluted together in the first five fractions. DAVA eluted in fractions 17–25. Ammonium phosphate was removed from the combined fractions on the same Dowex 50 column (H$^+$ form, equilibrated with water). After washing with water until the acidic eluate became neutral, the column was eluted with 0.5 M NH$_4$OH. DAVA eluted from 9 to 13 ml. The solution was lyophilized, taken up in water, and dried *in vacuo* over P$_2$O$_5$ to give 2 mg of diaminovalerate that migrated as a single ninhydrin-positive spot. This is a yield of 15%. The NMR spectrum was the same as that of authentic racemic DAVA. Enantiomeric purity was checked by HPLC using Marfey's reagent (see below).

Preparation of D-DAVA—DL-DAVA (γ-ornithine bought from Sigma, 30 mM) was reacted with succinic semialdehyde (100 mM) in the presence of GSA-aminomutase (90 μM) for 4 h at 30 °C in Tricine/HCl (0.1 M), pH 7.9. The solution was purified as described for the synthesis of L-DAVA. Enantiomeric purity was checked by HPLC using Marfey's reagent (see below).

Conversion of the Native Enzyme into the PLP and PMP Form—Complete conversion of the enzyme into the PLP or PMP form was achieved using succinic semialdehyde (SSA) and racemic 4, 5-diaminovalerate (DAVA), respectively, and the gel filtration method of Dixon and Severin (10). SSA (20 mM) or DAVA (5 mM) was added to a solution (1 ml) of enzyme (60–300 μM) in 0.1 M Na-Tricine buffer, pH 7.9. SSA or DAVA in the same buffer (1 ml, same concentrations) was loaded on a G-25 Sephadex column (45 × 1 cm) and allowed to drain in before loading the enzyme. The column was equilibrated and eluted with 0.1 M Na-Tricine, pH 7.9. The high molecular mass fraction was collected in the void volume well separated from small molecules.

Measurement of Enzyme Concentration—The molar concentration of enzyme subunits in the PLP form of the enzyme was determined from the absorbance of PLP released after treatment in 0.1 M NaOH using ϵ_{388} = 6550 cm^{-1} M^{-1} (11). Extinction coefficients of the PLP form at 418 nm (8270 M^{-1} cm^{-1}), 342 nm (2460 M^{-1} cm^{-1}), and 278 nm
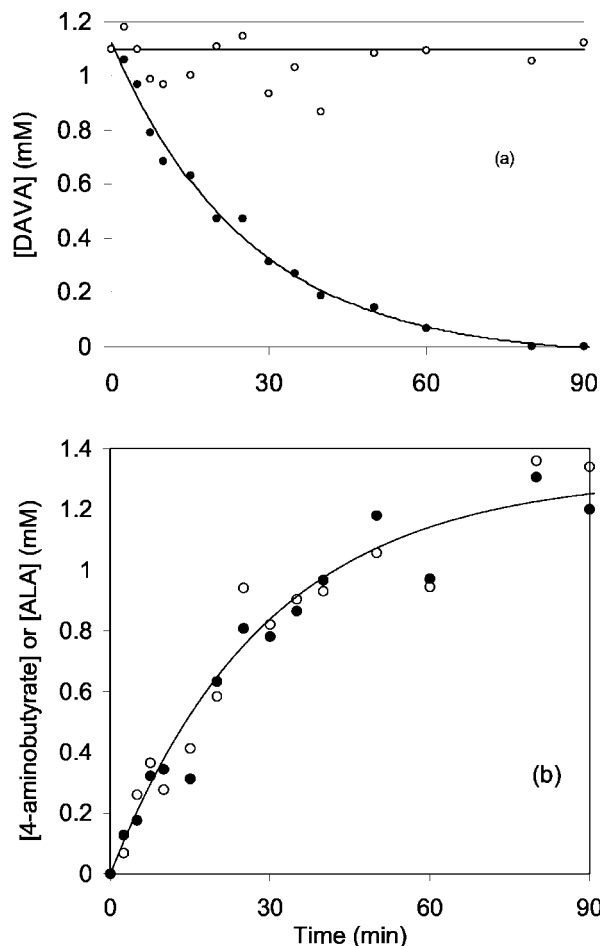

FIG. 2. **Enantioselectivity of transamination of DL-DAVA with succinic semialdehyde.** DL-DAVA (2 mM) was reacted with succinic semialdehyde (10 mM) in presence of GSAM (0.1 μM) at 37 °C in 0.1 M Tricine, pH 7.9. The concentrations of reactants and products were measured by HPLC. *Panel a* shows concentrations of L-DAVA (●) and D-DAVA (○). The continuous line through the L-DAVA data points is that predicted for an exponential process with a rate constant of 0.038 min^{-1}. *Panel b* shows concentrations of aminolevulinate (●) and 4-aminobutyrate (○).
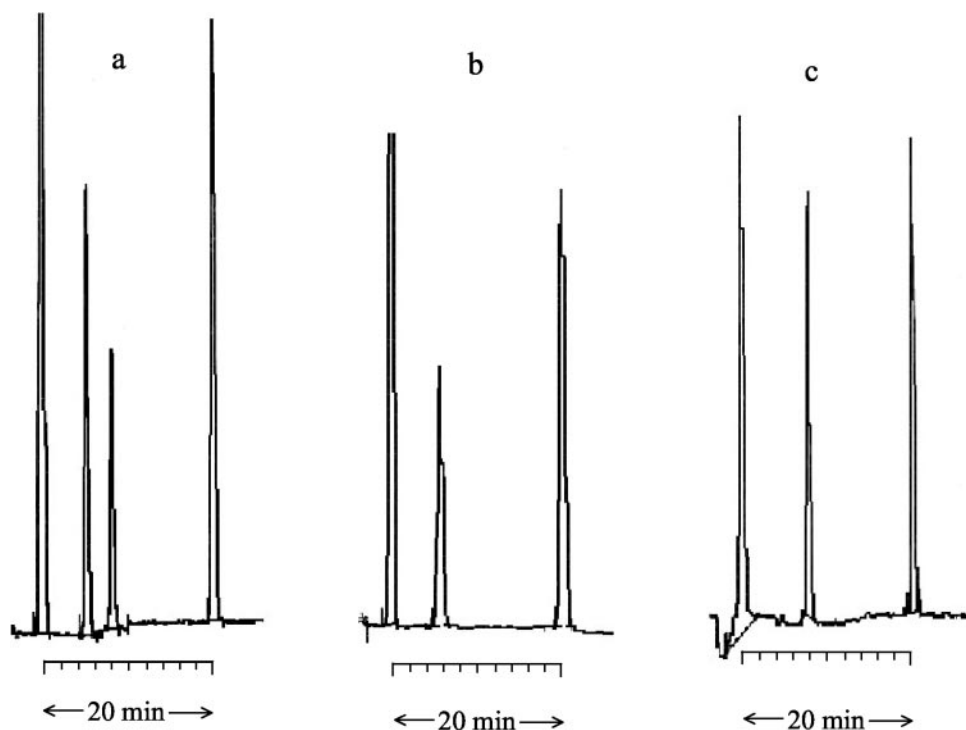
FIG. 3. **HPLC analysis of enantiomers of DAVA.** Portions of the chromatograms obtained from samples of DAVA after reactions with Marfey's reagent: racemic DAVA (*a*), material obtained from transamination between aminohexenoate (*b*), and ALA material remaining after treatment of DL-DAVA with succinic semialdehyde and GSA aminomutase (*c*). In all of the chromatograms, the first peak shown is because of the reagent and the last peak is from the internal standard D-leucine.
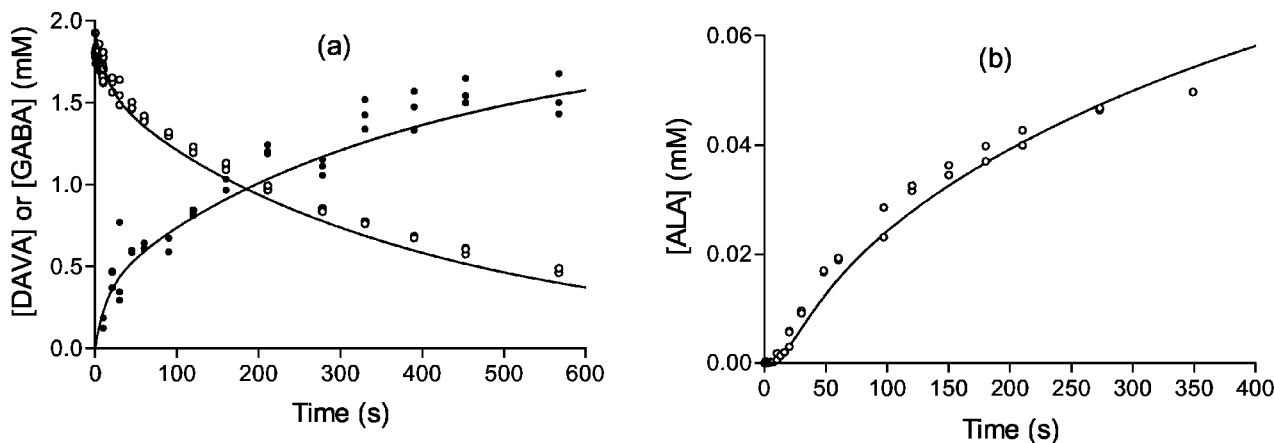

FIG. 4. **GSAM-catalyzed reaction of D-DAVA with succinic semialdehyde.** D-DAVA (2 mM) and succinic semialdehyde (10 mM) were treated with GSAM (10 μM). *a*, [D-DAVA] ($\bigcirc$) and [GABA] ($\bullet$). Reaction conditions are as in Fig. 2. All three sets of data were globally fitted to Scheme 1 by non-linear regression analysis using the relevant differential equations. Values returned by the analysis were $k_{cat(f)} = 6.7$ min^{-1}, $K_{DAVA} = 0.62$ mM, $K_{SSA} = 9.3$ mM, $k_{cat(b)} = 19$ min^{-1}, $K_{GSA} = 0.97$ mM, $K_{GABA} = 2$ mM, and $k_2 = 0.08$ mM^{-1} min^{-1}.

($43,894$ M^{-1} cm^{-1}) were derived from the absorption spectrum of the enzyme.

Assay of Compounds Involved in Reactions—Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alaninamide) and HPLC separation were used to quantify D-DAVA, L-DAVA, GABA, and ALA in the reaction of racemic DAVA with SSA (12, 20). The reaction was carried out at 37 °C in 0.1 M Tricine, pH 7.9, with DL-DAVA (2 mM), SSA (10 mM), and enzyme (0.1 μM). Aliquots of the reaction (2.5 μl) were taken at intervals and mixed with Marfey's reagent (1 mg/ml in acetone:acetonitrile 2:1, 12 μl), acetonitrile:triethylamine 3:2 (12 μl), and D-Leu (1 mM, 2.5 μl) as internal standard. Derivatives were separated by HPLC on a C18 Spherisorb column (250 × 4.6 mm) using a gradient from 85 to 50% of solvent A in 112 min. Solvent A was 0.2% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile:propan-2-ol 4:1. The flow rate during separations was 0.2 ml/min, and derivatives were detected by absorbance at 340 nm.

During the transamination between D-DAVA and SSA, concentrations of D-DAVA and GABA were measured photometrically after reac-

tion with *O*-phthaldialdehyde. Aliquots (30 μl) of the reaction mixture were mixed with an equal volume of phthalaldehyde reagent (60 mM phthalaldehyde and 230 mM 2-mercaptoethanol in 0.1 M NaHCO$_3$, pH 10). After 5 min, the sample was diluted in 0.1 M Na-Tricine, pH 7.9, and its absorption spectrum was recorded. Concentrations of D-DAVA and GABA were calculated from absorbance at 340 and 452 nm, using values of $\epsilon_{340} = 4960$ M^{-1} cm^{-1} and $\epsilon_{452} = 8700$ M^{-1} cm^{-1} for D-DAVA and $\epsilon_{340} = 3980$ M^{-1} cm^{-1} for GABA.

In the single turnover conversion of D-DAVA to D-GSA, the concentration of D-GSA was measured by reaction with phthalaldehyde and HPLC separation. The reaction was started by adding D-DAVA (2 mM) to the enzyme (150 μM) in 0.1 M Na-Tricine, pH 7.9, at 37 °C and stopped at intervals by mixing 35 μl of the reaction solution with 7 μl of HClO$_4$, 25% v/v. After centrifugation, KOH (8.25 μl, 4 M) was mixed with 30 μl of the supernatant. After further centrifugation, the supernatant (30 μl) was mixed with an equal volume of 60 mM phthalaldehyde and 230 mM 2-mercaptoethanol in 0.1 M NaHCO$_3$, pH 10. After 1 min, the mixture was separated isocratically by HPLC on C18 Spherisorb (250 ×

4.6 mm) in 40% v/v methanol, 0.05 M KH$_2$PO$_4$. L-Alanine was used as internal standard.

Stopped-flow and Data Analyses—An SF-61 stopped-flow spectrophotometer fitted with either an MG-6000 rapid scanning diode array detector or with an MG-60 photomultiplier (Hi-Tech, Salisbury, United Kingdom) was used to obtain absorption spectra or to measure absorbance at single wavelengths within 2 ms of mixing. Curve-fitting and statistical analyses were performed using the data manipulation software, Scientist (Micromath, Salt Lake City, UT). The absorbance spectra of individual component chromophores in complex absorption spectra were determined by best fitting the data to the sum of the required number of log normal curves (13). Equations used in fitting kinetic data were Equation 1 for time courses that fitted the sum of two exponentials and Equation 2 for Scheme 2.

$$A_t = A_0 e^{-k_1 t} + B_0 e^{-k_2 t} + c \qquad \text{(Eq. 1)}$$

$$k_{\text{obs}} = k_f \times \frac{[D]}{[D] + K_d} + k_b \qquad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

Reaction of Racemic DAVA with Succinic Semialdehyde—Succinic semialdehyde is known to convert the E$_M$ form of the enzyme to the E$_L$ form (7). It is also known that treatment with racemic DAVA rapidly converts the E$_L$ form of the enzyme to E$_M$ (4). Thus, the enzyme should catalyze coupled half-transamination reactions of succinic semialdehyde and DAVA to 4-aminobutyrate and one or both of the oxo compounds, namely the substrate GSA and the product ALA. Fig. 2 shows the results of an experiment in which succinic semialdehyde was reacted with racemic DAVA in the presence of the enzyme (0.1 μM). Within the error of the analysis, one of the enantiomers of DAVA is not consumed in the reaction, whereas the other enantiomer is completely exhausted and equimolar amounts of 4-aminobutyrate and ALA are formed. We have used this reaction to prepare one form of enantiomerically pure DAVA as described under "Experimental Procedures." HPLC analysis using Marfey's reagent showed the compound to be enantiomerically pure (Fig. 3c). These observations show that, in the presence of both enantiomers, the enzyme discriminates strongly, perhaps completely, in favor of one enantiomer of DAVA. The fact that the amount of ALA formed equals the amount of GABA shows that little, if any, of the DAVA is converted to GSA.

Synthesis of DAVA from 4-Aminohexenoate and 4-Aminolevulinate—It is known that L-aminohexenoate rapidly converts the E$_L$ form to E$_M$ and that D-aminohexenoate is completely unreactive (7). It is also known that the second half of the natural reaction with GSA in which DAVA is converted to ALA is reversible (14). Thus, the enzyme would be expected to catalyze a transamination reaction between ALA and the L-enantiomer of racemic aminohexenoate to make DAVA and 4-oxohexenoate. This expectation was confirmed by an experiment in which DL-4-aminohexenoate (10 mM) was mixed with ALA (10 mM) and GSA aminomutase (250 μM) in Tricine (0.1 M), pH 7.9 at 30 °C for 4 h, the course of the reaction being monitored by thin layer chromatography. A ninhydrin-positive spot that co-migrated with authentic DAVA increased in intensity for ~3 h and remained unchanged thereafter. Although at this point spots corresponding to ALA and aminohexenoate were also still present, the intensity of the spot in the DAVA position indicated substantial (>30%) conversion. Preparation of enzymically synthesized DAVA was routinely conducted under these conditions with the exception that the concentrations of aminohexenoate and ALA were increased to 50 and 25 mM, respectively. The NMR spectrum of the synthesized material was the same as that of authentic racemic DAVA. Analysis of the purified DAVA product by HPLC using Marfey's reagent (Fig. 3b) showed that it was enantiomerically pure and of the opposite
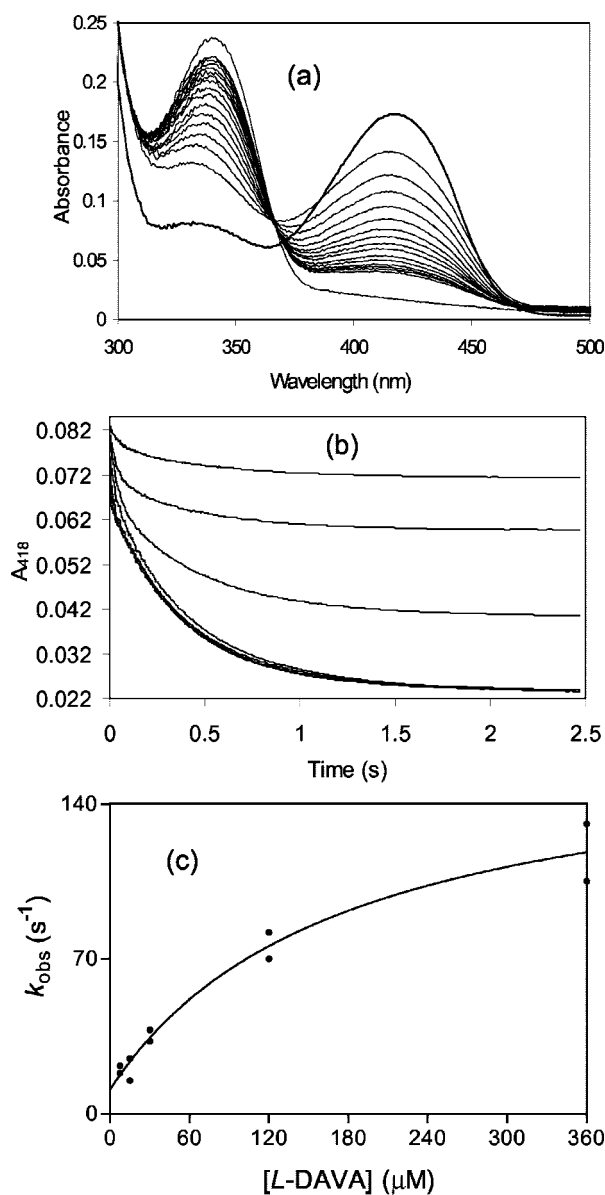
FIG. 5. **Reaction of L-DAVA with E$_L$ form of GSA aminomutase.** *a*, the spectrum shown in *boldface* is that of the E$_L$ form of the enzyme (25 μM) mixed with buffer solution containing no DAVA. The spectrum immediately below is the first captured (3 ms after mixing, L-DAVA = 2 mM). The next 16 spectra were taken at 75-ms intervals, and the final spectrum shown was that taken at 4.8 s. *b*, the course of the reaction resulting from reacting E$_L$ (10 μM) with different concentrations of L-DAVA measured using photomultiplier detection at single wavelength (418 nm). The reaction showing the smallest amplitude was conducted with L-[DAVA] = 1.9 μM. L-[DAVA] in successive reactions was 3.8, 7.6, 15, 30, 60, and 360 μM. *c*, reactions were was analyzed as the sum of two exponentials (see Equation 1). Values returned for the rate constants were fit to a hyperbola (see Equation 2).

chirality to the form that remained when the racemic DAVA was reacted with succinic semialdehyde in the experiments described under "Experimental Procedures." These results show that the enzyme adds a proton to C4 of ALA with complete stereospecificity.

In vivo, DAVA arises from L-glutamate via two reactions, neither of which involves the chiral carbon C4. Furthermore, the enzyme shows absolute stereospecificity for the L-enantiomer of the close structural analogue aminohexenoate (7). Thus, we conclude that the enantiomer of DAVA that is converted to ALA in the reaction of racemic DAVA with succinic semialdehyde is that with L-configuration and that the residual
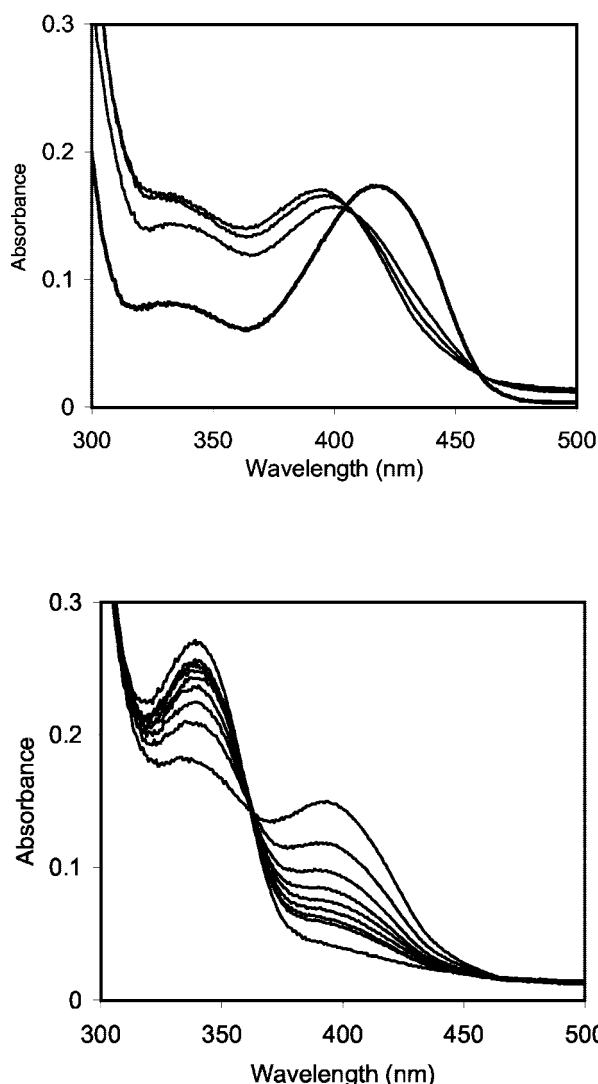
FIG. 6. **Reaction of D-DAVA with E$_L$ form of GSA aminomutase D-[DAVA] (2 mM).** *A*, the spectrum with highest absorbance at 420 nm is that observed when GSA aminomutase (25 μM) was mixed with buffer solution containing no DAVA. The remaining spectra were taken 5, 75, and 600 ms after mixing. *B*, spectra taken at 3-s intervals beginning 0.6 s after mixing. The spectrum with lowest absorbance at 390 nm was taken 96 s after mixing.

FIG. 7. **Deconvolution of an absorption spectrum from the single turnover between D-DAVA and E$_L$.** The experimentally determined spectrum (*open circles*) is that taken 0.6 s after mixing D-DAVA with E$_L$ (Fig. 6). Absorbances at 250 points between 300 and 500 nm were analyzed. To allow the quality of fit to be judged, only one in four of these points is included in the figure and a constant value of 0.01 has been added to both the experimental and fitted spectra but not to the spectra of the components.

DAVA is the D-enantiomer. Similarly, we conclude that the product of the reaction between aminohexenoate and ALA is L-DAVA.

GSA Aminomutase-catalyzed Reaction of D-DAVA with Succinic Semialdehyde—When GSA aminomutase (10 μM) was added to a solution of D-DAVA (2 mM) and succinic semialdehyde (10 mM), a reaction occurred in which D-DAVA was consumed and an equal amount of 4-aminobutyrate was formed (Fig. 4). The data did not fit well to a single exponential because of an initial faster phase accounting for ~15% of the total reaction. A little ALA was also formed, its amount being ~3% of the D-DAVA consumed. The biphasic nature of these curves demonstrates that the reaction cannot be described by a one-step conversion of substrate to products. It is also clear that the reaction goes to completion. The distinct lag in the production of ALA indicates that this compound is not formed directly but only as a result of a further reaction of one of the primary products. It is known that ALA is among the degradation products formed when GSA is brought from acid to neutral pH (15). Thus, the ALA detected in this experiment is likely to have been formed from the GSA that results from transamina-
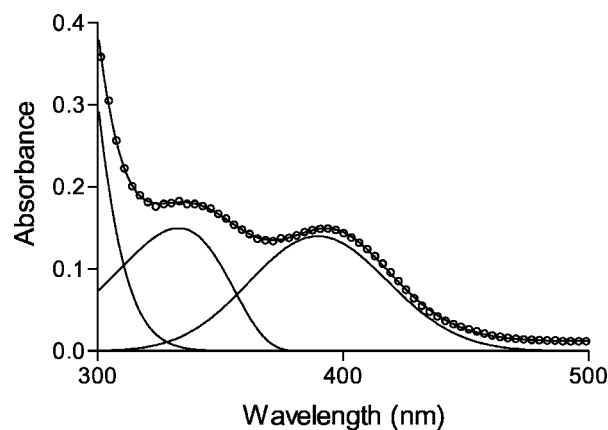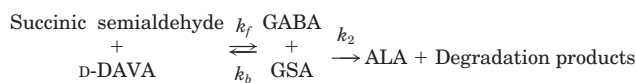
tion of the 5-amino group of D-DAVA. It seems most probable that the initial faster phase is because of an approach to equilibrium of the first step in the reaction scheme below (Scheme 1). In this proposal, completion of the reaction is made thermodynamically favorable by the conversion of GSA to multiple products including ALA. A global non-linear regression best fit of all of the three sets of data to the mechanism shown in Scheme 1 together with the values returned for the constants is shown in Fig. 4.

$$\begin{array}{c} \text{Succinic semialdehyde} \\ + \\ \text{D-DAVA} \end{array} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \begin{array}{c} \text{GABA} \\ + \\ \text{GSA} \end{array} \overset{k_2}{\longrightarrow} \text{ALA + Degradation products}$$
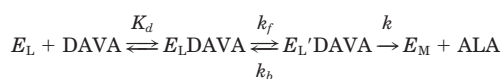
SCHEME 1

The number of parameters used to achieve the fit is large, and the values returned are accompanied by large standard deviations. Nevertheless, it is clear that the enzyme catalyzes a slow reaction of the D-enantiomer of DAVA. In earlier experiments aimed at characterizing the instability of GSA (9), it was shown that this compound decays according to a second-order process consistent with a reaction in which two molecules combine to make a six-membered ring containing two imine bonds. The value of the second-order rate constant determined in those experiments, which were conducted in the absence of other aldehydes or amines, was 2.3 mM^{-1} s^{-1}. This is too high to be consistent with the long lag in ALA formation seen in Fig. 4a. We suggest that the much lower value returned for k_2 in the present experiments is because of reversible formation of imines between the primary product GSA and both DAVA and SSA. Thus, the concentration of GSA is lowered and the bimolecular reaction that it undergoes is slower. In similar experiments, we made several attempts to measure GSA itself but failed, possibly because of reactions this compound underwent with the high concentrations of DAVA and SSA present. To reduce these complications, we reacted D-DAVA with a larger concentration of E$_L$ (150 μM) in the absence of SSA for 30 s. HPLC analysis showed the presence of 40 μM GSA.

Single Turnover Reactions of the Enantiomers of DAVA with E$_L$ Form of GSA Aminomutase—The enzyme was converted completely to the E$_L$ form and reacted separately with each of the enantiomers of DAVA using stopped-flow mixing with diode array recording of the resulting changes in the spectrum of the cofactor. Both enantiomers reacted, but significant differences

were observed both in the rates of reaction and in the spectra produced. With L-DAVA (360 μM), a significant fall in A_{420} and a corresponding increase at 340 nm were complete within the dead time (3 ms approximately with the instrument in diode array mode). Thereafter, A_{420} decreased and A_{335} increased exponentially with $k_{obs} = 2$ s^{-1} (Fig. 5a).

In a separate experiment, the reactions of different concentrations of L-DAVA with E_L were observed using photomultiplier detection at 418 nm so that more of the initial rapid reaction could be detected (Fig. 5b). All of the data fitted well to an equation describing the sum of two exponential processes. The rate constant describing the faster reaction showed hyperbolic dependence on L-DAVA concentration fitting Equation 2 with $k_f = 159 \pm 24$ s^{-1}, $k_b = 11 \pm 6$ s^{-1}, and $K_d = 174 \pm 80$ μM (Fig. 5c). We ascribe these constants to the first two steps in Scheme 2. The rate constant for the slower reaction changed very little over the whole concentration range even at DAVA concentrations that were lower than that of the enzyme itself. The mean value for 14 measurements at concentrations in the range 1.9–360 μM was 2.31 $\pm$ 0.18 s^{-1}. We ascribe this constant to k, which characterizes the final step in Scheme 2.

$$E_L + DAVA \underset{k_b}{\overset{K_d}{\rightleftharpoons}} E_L DAVA \overset{k_f}{\rightleftharpoons} E_L'DAVA \overset{k}{\rightarrow} E_M + ALA$$

SCHEME 2

We propose that the overall reaction observed in these single-turnover experiments with L-DAVA is the complete conversion of E_L to E_M and the accompanying formation of an equal concentration of ALA. We deduce that the amount of GSA formed is insignificant because our earlier experiments showed ALA to be the only oxo product of the multiple turnover reaction between L-DAVA and SSA. Thus, we propose that the reactions observed comprise the complexes Ia to IVa in Fig. 1. If the rapid phase is formation of the external aldimine IIIa from IVa (the internal aldimine in the Michaelis-Menten complex), IIIa must have a lower ϵ_{418} and higher ϵ_{340} than IVa. This would indicate an increased proportion of the 340-nm absorbing enolimine tautomer, suggesting a less polar environment. This explanation requires that the slow step ($k = 2.3$ s^{-1}) is the conversion of IIIa to IIa and that dissociation of ALA is fast. An alternative explanation is that the fast phase converts IVa to a pseudo-equilibrium mixture of IIa and IIIa with IIIa predominating. In this explanation, the slow step would be a dissociation of ALA. Scheme 2 illustrates the latter proposal. The observations that conversion to the 340-nm absorbing species is complete at all of the concentrations of L-DAVA greater than that of the enzyme show that the equilibrium is greatly in favor of species Ia and IIa.

With D-DAVA, an initial rapid reaction, more than half of which was lost in the dead time, showed decreasing absorbance at 420 nm and increasing absorbance with a maximum at 390 nm (Fig. 6). Thereafter, a slower reaction occurred in which the 390-nm chromophore was converted to one absorbing maximally at 340 nm. Repetition of the experiments at single wavelengths of 430 and 386 nm where the absorbance changes were greatest showed the initial fast reaction to be a single exponential process. The same k_{obs} (measured at 430 nm as 170 ± 2 s^{-1}) provided a good fit for the data at both wavelengths. The slow reaction also fitted well to a single exponential process with $k_{obs} = 0.163 \pm 0.002$ s^{-1} both at 430 and 340 nm.

The slow reaction in which A_{340} increased exponentially is clearly attributed to the formation of E_M. The rate constant for this process is similar to the value of k_{cat} estimated for catalysis

of the reaction in which D-DAVA and SSA are converted to GABA and products formed spontaneously from GSA. Deconvolution of the spectrum taken 0.6 s after mixing showed only two components with absorbance maxima above 300 nm and at 332 and 395 nm, respectively (Fig. 7). Thus, the 395-nm chromophore is converted stoichiometrically into E_M and GSA in the slow phase of the reaction. Although an absorption maximum at 395 nm is unusual for an intermediate in a PLP enzyme-catalyzed reaction, chromophores absorbing maximally between 385 and 400 nm have been observed during the decarboxylation of DOPA by DOPA decarboxylase (16–18) and in the unliganded Y225F mutant of aspartate aminotransferase at alkaline pH. In this latter example, strong evidence was presented to show that the chromophore ($\lambda_{max} = 386$ nm) is the unprotonated internal aldimine formed between PLP and Lys-258 (19). The 380-nm absorbing chromophore that occurs as a major intermediate in the decarboxylation of DOPA was also deduced to be an external aldimine with DOPA (16). It has been suggested that it is both the blue-shifted protonated imine (17) and the red-shifted unprotonated imine (18). Although it is very probable that the 395-nm chromophore that occurs in the GSA-aminomutase reaction is the internal aldimine with the 5-amino group of D-DAVA, in the absence of additional evidence, we cannot determine its protonation state or the reason for the spectral shift. However, the inductive effect of the DAVA carboxylate would be less on the external aldimine made with the 5′-amino group, indicating a lower pK and providing some support for identifying the chromophore as the unprotonated species.

In conclusion, GSA-aminomutase shows complete stereospecificity in the reactions it conducts at C4 of its substrates. It seems very probable that reactions at the non-chiral C5 are also stereospecific. The ability of both D- and L-DAVA to increase the rate of conversion of GSA to ALA is not because of a lack of stereospecificity but probably because of a stereospecific abstraction of one of the protons on C5.

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