Glutamate 1-Semialdehyde Aminotransferase: Anomalous Enantiomeric Reaction and Enzyme Mechanism†

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ABSTRACT: Glutamate 1-semialdehyde aminotransferase (GSA-AT) catalyzes near 50% conversion of the racemic mixture of GSA to 5-aminolevulinate (ALA), indicating quantitative use of the L-glutamate-derived natural (*S*)-enantiomer as substrate. This enzymic reaction has been extensively studied with (*R,S*)-GSA because it is readily purified in high yields following ozonolysis of racemic 4-vinyl-4-aminobutyric acid. However upon addition of (*R,S*)-GSA, GSA-aminotransferase is converted to the pyridoxal-P or internal aldimine form (418 nm) and not rapidly cycled back to the original pyridoxamine-P, as predicted by the rate of product (ALA) accumulation. Addition of the putative intermediate, (*R,S*)-4,5-diaminovalerate (DAVA), eliminates this rapid conversion of the enzyme by (*R,S*)-GSA to the internal aldimine and stimulates initial rates of ALA synthesis (2–3-fold) and results in corresponding increases in apparent equilibrium concentrations of ALA. These results indicate that DAVA is rate limiting and suggest anomalous reactivity of (*R*)-GSA. Steady-state and spectral kinetic experiments with individual purified enantiomers confirm anomalous reactivity of (*R*)-GSA: in the case of (*S*)-GSA, spectral changes are lesser in amplitude and at least 1 or 2 orders of magnitude more rapid. Only (*S*)-GSA yielded significant amounts of ALA. Since (*R*)-GSA is an apparent substrate in the first half-reaction, the resulting (*R*)-DAVA is either inactive or a poor substrate in the second half-reaction.

Glutamate 1-semialdehyde aminotransferase (GSA-AT,[1] EC 5.4.3.8) is a vitamin B_6 requiring enzyme (Grimm et al., 1991; Nair et al., 1991; Jahn et al., 1991) which catalyzes the net transfer of the C_2 amino group of glutamate 1-semialdehyde (GSA) to the C_1 position, giving 5-aminolevulinate (ALA) (Beale & Weinstein, 1990; Kannangara et al., 1988; Castelfranco & Beale, 1983). Structural (Grimm, 1990; Elliott et al., 1990), spectrophotometric (Grimm et al., 1991; Smith et al., 1991a), and steady-state kinetic analyses (Smith et al., 1991b) intimate a mechanism for this amino group transfer which is comparable to that of aspartate aminotransferase (Kirsch et al., 1984; Arnone et al., 1985; Jansonius et al., 1987). This principal catalytic event involves aldimine–ketimine tautomerization, which is probably mediated by a single acid/base group (Gehring et al., 1977). This prototropic rearrangement is stereospecific and occurs from the *si* side of the putative quinonoid intermediate (Dunathan et al., 1968). Protonation from the opposite side (*re*) gives rise to the corresponding (*R*)-amino acid. In the case of aspartate aminotransferase, the enantiomeric error frequency is about 1.5×10^{-7} (Kochhar & Christen, 1988).

GSA-AT-bound coenzyme exhibits spectral changes, which report specific events with respect to various substrates (Grimm et al., 1991). Such spectral changes provide a means for comparing reactions with various enzymes, coenzyme forms (pyridoxal-P or pyridoxamine-P), and substrates or substrate analogues. These spectral changes are amenable to kinetic analyses, which in combination with steady-state kinetic data provide convincing evidence that DAVA is the intermediate in a ping-pong bi-bi mechanism (see Scheme I) in which GSA is converted to ALA by GSA-AT in bacteria and higher plants (Smith et al., 1991a,b; Berry-Lowe et al., 1992). The mechanistic similarities are reflected in multiple sequence alignment analyses (PILEUP; Genetic computer group, Madison, WI) in which numerous invariant amino acids are conserved in a representative group of enzymes, including GSA- and aspartate-aminotransferases (Grimm & Jäger, 1992).

In this communication, we present evidence for the anomalous enantiomeric reaction of *Synechococcus* GSA-AT with the (*R*) enantiomer of GSA. This anomalous reaction gives rise to spectral characteristics which may distort interpretation of kinetic data, from a mechanistic point of view.

EXPERIMENTAL PROCEDURES

Chemicals. (*R,S*)-, (*R*)-, and (*S*)-GSA were synthesized from (*R,S*)-, (*R*)-, (*S*)-4-vinyl-4-aminobutyric acid by reductive ozonolysis and purified as previously described (Gough et al., 1989). DOVA was kindly provided by Dieter Dornemann, Phillips University, Marburg, Germany. Gabaculine (3-amino-2,3-dihydrobenzoic acid hydrochloride) and ALA were obtained from Sigma Chemical Co. 4,5-Diaminovaleric acid was synthesized from (*R,S*)-allylacetic acid (Sigma Chemical Co.) as previously described (Smith et al., 1991b).

GSA-aminotransferase. Synechococcus GSA-AT was purified to near homogeneity from lysates of transformed *Escherichia coli*, as previously described (Grimm et al., 1991). Quantitative conversion of GSA-AT to either the pyridoxamine-P or pyridoxal-P form is described in Smith et al. (1991a).

Enzyme Assays for GSA-aminotransferase. Enzymic activities of GSA-AT were determined by measurements of

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[1] Abbreviations: ALA, 5-aminolevulinate; GSA, glutamate 1-semialdehyde; GSA-AT, glutamate 1-semialdehyde aminotransferase; DAVA, 4,5-diaminovalerate.

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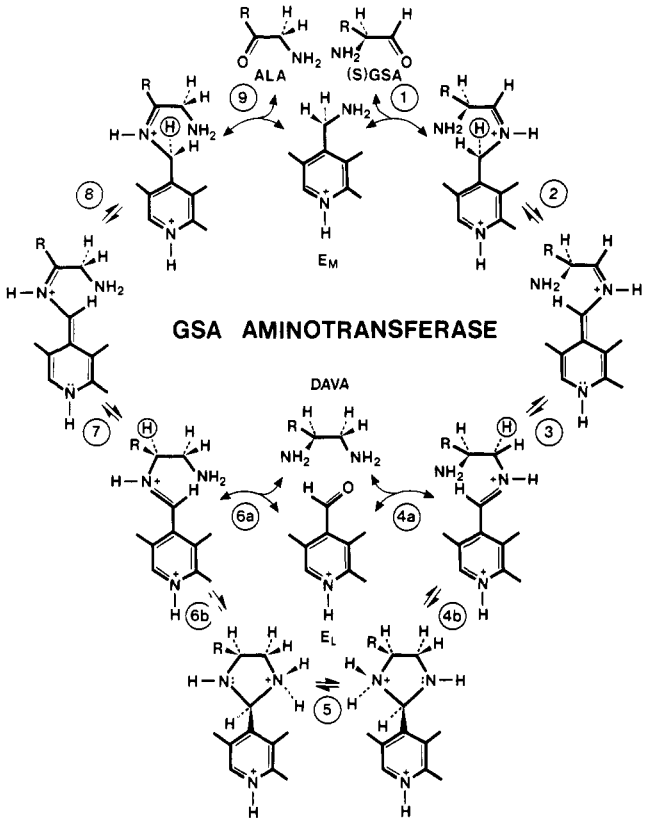
Scheme I: Stereospecificity of GSA-Aminotransferase in the Mechanism Proposed for the Enzymic Conversion of (*S*)-GSA to ALA[a] (Smith et al., 1991a,b)



[a] This reaction is initiated with GSA and the pyridoxamine-P form (338 nm) of the enzyme (E_M), resulting in the formation of the C_1 aldimine of (*S*)-GSA (step 1). Abstraction of a C_4' methylene hydrogen from the *si* face (step 2) gives the corresponding quinonoid intermediate, which upon proton transfer to C_1 is converted to the external aldimine of enzyme-bound pyridoxal-P (step 3). This intermediate undergoes transaldimination by the C_6 amino group of the active site Lys272 (step 4a) or by the C_2 amino group of GSA (step 4b), giving (*S*)-DAVA and the pyridoxal coenzyme form of GSA-AT (E_L) or the corresponding cyclic imidazolidine adduct of pyridoxal-P, respectively. The second half-reaction (steps 6–9) is similar except that the C_2 chiral carbon rather than the C_1 carbonyl of GSA is directly involved in amino group transfer, and the steps are in reverse order. Prototropic rearrangement (from C_2 of GSA or C_4 of DAVA to the C_4' methylene, steps 7 and 8) is also from the *si* side of the original quinonoid intermediate. In the case of the enantiomeric reaction with GSA, the configuration about C_2 is opposite to that shown in the scheme. Nevertheless, this carbon is not directly involved in the first half-reaction (steps 1–4). As a consequence, it is more or less comparable to that of (*S*)-GSA (as judged by a spectrophotometric shift from 338 to 418 nm; Figure 1); however, the expected product is DAVA. In the second half-reaction (steps 6–9), the C_2 chiral center is directly involved in amino group transfer. Because the C_4 hydrogen of DAVA is on the *re* side of the original quinonoid intermediate (step 6), prototropic rearrangement is very unlikely. By analogy with aspartate aminotransferase, proton abstraction from this side apparently does not occur at appreciable rates (Kochhar & Christen, 1988). R = $-CH_2CH_2COOH$.

initial rates of ALA synthesis and by spectrophotometric kinetic analyses as previously described (Smith et al., 1991a,b). Assays (0.5 mL) were in BisTris (0.1 M, pH 7.0) or MOPS (0.1 M, pH 6.7) at 28 °C, 3 min, with concentrations of GSA as indicated. Reactions were terminated by addition of one-fifth volume of hot (100 °C) ethyl acetoacetate. After heating (100 °C, 10 min), addition of one volume of Ehrlich's reagent, and centrifugation (13000*g*, 5 min), the ALA pyrrole concentration was determined spectrophotometrically (553 nm) using a molar extinction coefficient of 7.2×10^4 M^{-1} cm^{-1} (Mauzerall & Granick, 1956). The coupled enzyme assay for uroporphyrinogen synthesis was performed as described in Gough et al. (1989).

Extinction Coefficients of GSA-aminotransferase. Concentrations of GSA-AT were determined colorimetrically (Bradford, 1976) or from molar extinction coefficients (278 nm = 3.5×10^4 M^{-1} cm^{-1}) as previously described (Smith et al., 1991b).

RESULTS

GSA-Induced Spectral Changes of GSA-aminotransferase. Purified *Synechococcus* GSA-AT has absorbance maxima at approximately 278, 338, and 418 nm, characteristic of other vitamin B_6 containing enzymes (Grimm et al., 1991). By analogy with aspartate aminotransferase these maxima correspond to protein, in part to bound pyridoxamine-P, and to the protonated form of bound pyridoxal-P, respectively. Purified GSA-AT is primarily in the pyridoxamine form. Upon addition of (*R,S*)-GSA, 5-aminolevulinate (ALA) is rapidly synthesized, presumably via the intermediate 4,5-diaminovalerate (DAVA) or the corresponding cyclic imidazolidine. A minimal mechanism for this reaction pathway is summarized in Scheme I. Steady-state and spectral kinetic data are in agreement with this scheme (Smith et al., 1991a,b).

However, the observation that GSA-AT was converted to the pyridoxal-P or internal aldimine form (418 nm) (Smith et al., 1991a) upon addition of (*R,S*)-GSA was quite unexpected. The time course of this conversion is shown in Figure 1A, where a corresponding decrease in the pyridoxamine-P form (338 nm) is also seen. The resulting spectral ratio (418/338), which indicates a preponderance of the pyridoxal-P form (418 nm) of the enzyme, was not significantly altered by Sephadex G-50 column chromatography. This suggests that coenzyme-bound substrate or product intermediates (Velick & Vavra, 1962), such as the external aldimine resulting from GSA addition, probably do not contribute significantly to this spectral pseudoequilibrium and that the internal aldimine of GSA-AT is likely the major product.

Influence of DAVA on GSA-Induced Spectral Changes of GSA-aminotransferase. Accumulation of the internal aldimine (418 nm) upon addition of (*R,S*)-GSA indicates that the second half-reaction is limiting (Scheme I, reactions 6–9). Had the reaction gone to completion, the pyridoxamine-P coenzyme form (338 nm) should have been regenerated. In agreement with this hypothesis that the second half-reaction is rate limiting, stimulation (2–3-fold) of the initial rate or accumulation of ALA is observed upon addition of (*R,S*)-DAVA. Steady-state kinetic analysis of this stimulation of ALA synthesis at various fixed concentrations of DAVA gave a series of parallel lines in Lineweaver–Burke linear transformations, typical of ping-pong bi-bi kinetics upon addition of second substrate (Smith et al., 1991b). Spectral changes induced by (*R,S*)-GSA are also significantly altered upon addition of DAVA to high concentrations (10 μM) of GSA-AT (compare panels A and B of Figure 1). Whereas in the absence of DAVA the conversion of the enzyme to the pyridoxal form (418 nm) by (*R,S*)-GSA begins immediately, in the presence of substoichiometric amounts of (*R,S*)-DAVA a lag (about 120 s) is observed before this spectral transition occurs. Upon addition of a second aliquot of DAVA, GSA-AT rapidly (1–10 s) cycles back to the pyridoxamine-P form (338 nm) (Figure 1C). However, after a similar lag period (1–2 min) the aldimine form of the enzyme was again regenerated, although not quite as rapidly or to the same extent, indicating substrate depletion. This process could be repeated several times, depending on the amount of (*R,S*)-DAVA

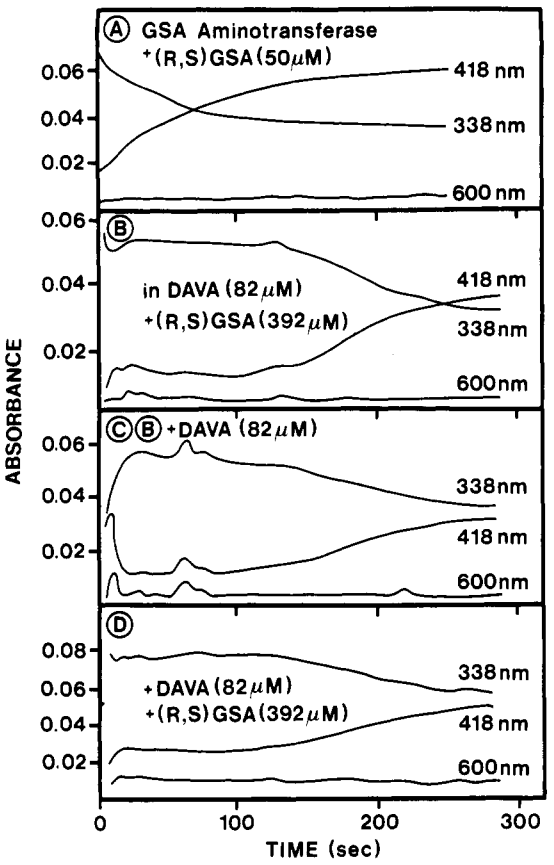


FIGURE 1: Time courses of interconversion between the pyridoxamine-P (338 nm) and pyridoxal-P (418 nm) coenzyme forms of GSA-aminotransferase (10 μM) upon addition of GSA and DAVA. (A) Conversion of purified GSA-AT to the pyridoxal-P form (418 nm) upon addition of (*R,S*)-GSA (50 μM). (B) Conversion of the DAVA-elicited pyridoxamine form of GSA-AT to the pyridoxal-P form (418 nm) upon addition of GSA (392 μM). (C) Cycling of GSA-AT between the pyridoxal (418 nm) and pyridoxamine (338 nm) forms upon addition of DAVA (82 μM) at the end of the reaction shown in (B). (D) Conversion of GSA-AT to the pyridoxal-P form (418 nm) upon addition of (*R,S*)-GSA (392 μM), after preincubation (300 s) with DAVA (82 μM). Background spectral changes (600 nm) are shown for comparison.

added, larger additions correlated with longer lag periods before the enzyme was cycled to the pyridoxal-P form by (*R,S*)-GSA. Preincubation of GSA-AT with DAVA, before GSA addition, did not significantly alter the delay in the conversion of the enzyme to the aldimine form (Figure 1D), indicating that both enzyme and substrate (GSA) were required in this DAVA-induced coenzyme cycling. Such coenzyme cycling upon successive additions of the intermediate or second substrate, DAVA, to reactions containing the first substrate, GSA, indicates amino group transfer and accumulation of the second product ALA.

Course of ALA Accumulation during GSA- and DAVA-Induced Spectral Changes. As previously shown (Smith et al., 1991a), ALA synthesis or accumulation, in the absence of DAVA, attains a maximum stable value before significant spectral changes occur. These maxima or near equilibrium concentrations of ALA were directly proportional with and approximately equal to about half the initial amount of (*R,S*)-GSA added, indicating near stoichiometric conversion of the natural (*S*) enantiomer to ALA. Whether or not ALA accumulated beyond this apparent maximum, during DAVA-induced coenzyme cycling, was tested in new experiments using conditions typical for spectral kinetic experiments, i.e., high level concentrations of GSA-aminotransferase (Figure 2A). Addition of various fixed, substoichiometric amounts

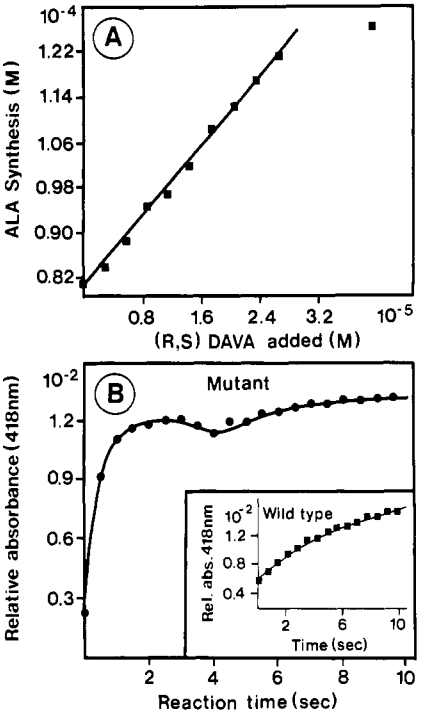


FIGURE 2: Interaction of GSA-aminotransferase with GSA. (A) Synthesis of ALA upon addition of various fixed amounts of (*R,S*)-DAA. ALA synthesis was initially induced by (*R,S*)-GSA (260 μM) in the presence of substrate-level concentrations of the enzyme (8 μM). After near maximum ALA synthesis and spectral increases (418 nm) had been obtained, various amounts of (*R,S*)-DAVA were added. After each addition, an aliquot (20 μL) of the reaction mixture was removed and allowed to equilibrate (30 min), and the resulting total accumulation of ALA was determined. (B) Conversion of mutant GSA-AT to the pyridoxal-P form upon addition of GSA. A gabaculine-resistant mutant of GSA-AT was purified from transformed *E. coli* (Grimm et al., 1991). Spectral changes associated with the aldimine form (418 nm) of the enzyme (3.3 μM), upon addition of (*R,S*)-GSA (387 μM), are shown as a function of time. A corresponding decrease in the pyridoxamine-P form (338 nm) was obtained (data not shown). Inset: Wild-type enzyme (8.0 μM), GSA (50 μM).

of (*R,S*)-DAVA resulted in corresponding linear increases in ALA synthesis or accumulation. Such increases were beyond pseudoequilibrium values initially obtained before DAVA addition (*y* intercept where DAVA = 0, about 0.81×10^{-4} M ALA).

A mutant form of GSA-AT, isolated from *Synechococcus* exposed to increasing concentrations of the suicide inhibitor gabaculine (Bull et al., 1991), has a k_{cat} for GSA which is about one-sixth that of the wild-type enzyme (Smith & Grimm, 1992) and gives a bimodal absorbance change (338–418 nm) upon addition of (*R,S*)-GSA (Figure 2B). This indicates two qualitatively similar reactions occurring at different rates, one corresponding to the utilization of (*S*)-GSA and the other to the (*R*)-GSA substrate.

Spectral and Steady-State Kinetics with Purified Enantiomers of GSA. Spectral changes were also observed upon addition of purified enantiomers of GSA to high-level concentrations (20 μM) of GSA aminotransferase (Figure 3). Such changes induced upon addition of equal amounts of the racemate or each of the individual enantiomers were qualitatively similar and were characterized by the spectral shift of the purified recombinant enzyme from the absorption maximum of the pyridoxamine to that of the pyridoxal-P form (Figure 3, 338–418 nm, respectively). Whereas (*R,S*)- and (*R*)-GSA were quantitatively similar, spectral changes induced upon addition of (*S*)-GSA were much less in amplitude and

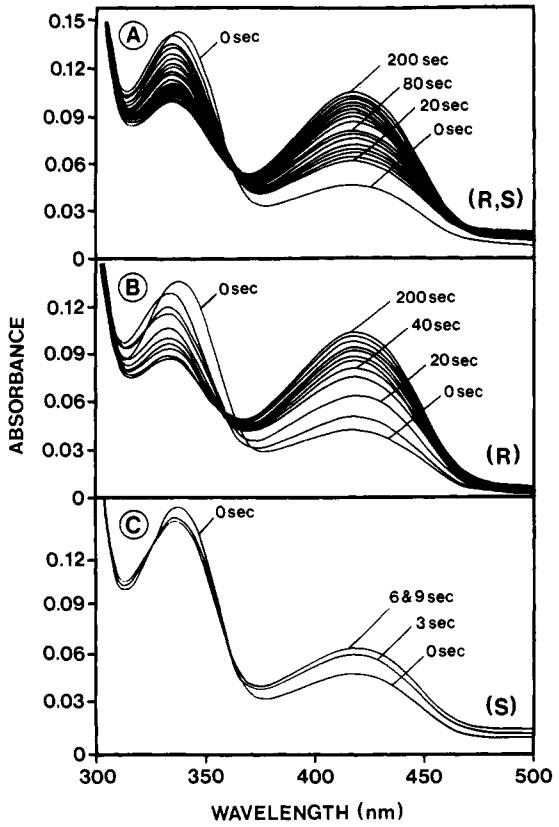


FIGURE 3: Spectral changes of GSA-aminotransferase, induced upon addition of enantiomers of GSA. Spectral changes are associated with the conversion of GSA-AT to the pyridoxal-P form (418 nm). Reactions contained enzyme (26 μM) and GSA (200 μM) in 0.15 M MOPS, pH 6.7, at 26 °C. (A) (*R,S*)-GSA at 10-s intervals. (B) (*R*)-GSA at 10-s intervals. (C) (*S*)-GSA at 3-s intervals.

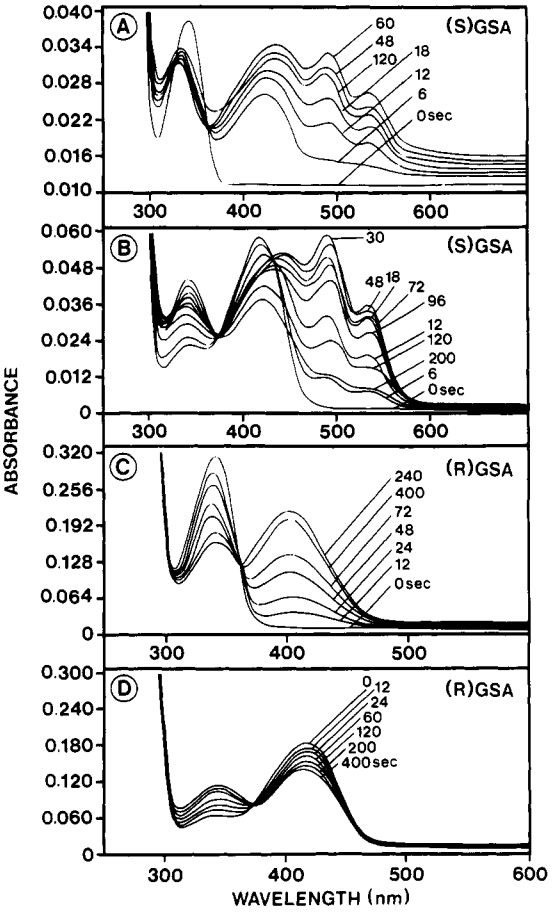


FIGURE 4: Spectral changes of the pyridoxamine-P (panels A and C) and pyridoxal-P form (panels B and D) of GSA aminotransferase induced upon addition of the individual enantiomers (*S*)- and (*R*)-GSA. The reaction contained enzyme at concentrations of 5 μM (A), 15 μM (B), or 50 μM (C, D) plus (*R*)- or (*S*)-GSA (200 μM) in 0.1 M BisTris, pH 7.0 at 20 °C. In panel B there are two isosbestic points: 375 and 432 nm. The latter is only seen during the initial conversion of the pyridoxal-P to the pyridoxamine-P form (0–18 s), whereafter the spectral changes are comparable to those in panel A.

much more rapid. Maximum increases (ΔA_{418nm} about 0.060) in the pyridoxal-P form of GSA-AT occurred in about 200 s with (*R,S*)- and (*R*)-GSA and in about 6 s or less (ΔA_{418nm} about 0.016) with (*S*)-GSA (compare panels A, B, and C of Figure 3). In the case of (*R,S*)-GSA, this conversion was biphasic. The initial change (ΔA_{418nm} about 0.015) was very rapid (<10 s) and was followed by a much slower conversion of the enzyme to the pyridoxal-P form. This initial burst, not observed with the (*R*) enantiomer, was similar in amplitude to that observed with (*S*)-GSA and indicates rapid reaction of this enantiomer in the racemic mixture. The spectral shift of the pyridoxamine-P form (338 nm) of GSA-AT to shorter wavelengths (335 nm), observed between 0 and 10 s with (*R,S*)- and (*R*)-GSA and between 0 and 3 s with (*S*)-GSA, probably indicates rapid conversion of the coenzyme to the ketimine form, upon addition of GSA. An isosbestic point between this ketimine and the corresponding aldimine occurs at about 360 nm.

Whereas spectral changes induced with (*R*)- and (*S*)-GSA under initial reaction conditions (Figure 3) are comparatively simple, similar data obtained over extended periods are more complex and apparently reflect competition between thermodynamic and kinetic forces, in the presence of product, as equilibrium is approached. Addition of (*S*)-GSA to the pyridoxamine-P form of GSA-AT induces an immediate biphasic conversion of the enzyme to the pyridoxal-P form (338–418 nm) with an isosbestic point at about 364 nm (Figure 4A). The initial rapid phase of this reaction (0.6 s) is comparable with Figure 3C and is the period during which ALA is synthesized (Smith et al., 1991a). The slower second phase (>6 s) is characterized by the simultaneous, transient appearance of quinonoid or other intermediates (490 and 530 nm), and ALA concentration remains more or less constant.

Spectral changes induced upon addition of (*S*)-GSA to the pyridoxal-P form of the enzyme resulted in a relatively slower conversion in the opposite direction (Figure 4B, 418–338 nm), similar to those previously observed with (*R,S*)-GSA, (Smith et al., 1991a). These spectra are characterized by the initial conversion of the aldimine (418 nm) to quinonoid or other intermediates (490 and 530 nm) with isosbestic points at about 432 nm. At later stages, the spectra are reminiscent of those obtained with the pyridoxamine-P form (Figure 4A), except that the single isosbestic point is at a somewhat longer wavelength (about 375 nm).

Addition of (*R*)-GSA to the pyridoxamine-P (Figure 4C) or pyridoxal-P (Figure 4D) forms of GSA-AT resulted in relatively less complex spectral changes, typical of half-reactions in which intermediates (DAVA) are not subsequently converted to product (ALA). These two enzyme forms were converted to the pyridoxal-P (338–418 nm) and the pyridoxamine-P (418–338 nm) forms, respectively. Isosbestic points (about 363 and 374 nm) were practically identical with (*S*)-GSA counterparts in panels A and B of Figure 4, respectively. However, spectral changes were noticeably slower than those obtained with (*S*)-GSA, particularly in the case of the reaction with the pyridoxal coenzyme form, in which the chiral carbon (C2) is directly involved in amino

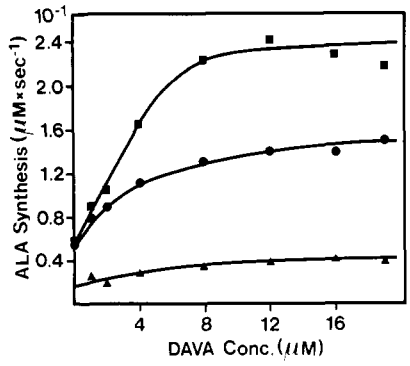


FIGURE 5: Stimulation of the rate of ALA synthesis by DAVA. Curves: top, (S)-GSA (79 μM, 2.5 min); middle, (R,S)-GSA (79 μM, 2.5 min); bottom, (R)-GSA (260 μM, 7.5 min). Reactions were carried out in MOPS buffer (0.1 M, pH 6.7, 28 °C).

group transfer (Figure 4D). (R)-GSA did not induce significant accumulation of quinonoid or other intermediates.

Interpretation of these results requires an awareness of the fact that enzyme concentrations in these spectral kinetic experiments are 1–2 orders of magnitude larger than in typical steady-state experiments. As a consequence, maximum conversion of (S)-GSA to product (ALA) is rapid (<6 s). No ALA synthesis was observed with the unnatural (R) enantiomer.

Under steady-state conditions, ALA is rapidly synthesized from either the racemic mixture or (S)-GSA, while (R)-GSA by comparison is very much less reactive and difficult to quantitate because of interference by the nonenzymic reaction (Smith et al., 1991b). As mentioned above, the enzymic conversion of GSA to ALA is stimulated by DAVA. This is particularly noticeable with reactions containing (R,S)- or (S)-GSA (Figure 5). DAVA stimulation of the rate of ALA synthesis by the purified (S) enantiomer is about twice that of the racemic mixture. This is probably due to the fact that the natural enantiomer is half as concentrated in the racemate. The rate of ALA synthesis from the unnatural (R) enantiomer is much smaller, approximately one-third, even with about 3 times higher GSA concentrations and 3-fold longer reaction times. However, this greatly reduced reaction with the unnatural isomer also shows stimulation with DAVA.

In a coupled enzyme assay containing barley GSA-AT, ALA-dehydrase, and PBG-deaminase, (S)-GSA is about 10 times more efficiently converted into uroporphyrinogen than (R)-GSA (data not shown).

DISCUSSION

A minimal mechanism for the enzymic conversion (S)-GSA to ALA is illustrated in Scheme I. According to this scheme, the pyridoxamine form of GSA-AT forms a Schiff base with the C_1 carbonyl of GSA (step 1). This is followed by the principal event of transamination which involves the stereospecific prototropic shift from the *si* side of the C_4' methylene to the C_1 aldimine of GSA (steps 2 and 3). Transaldimination of the resulting intermediate (C_5 aldimine of DAVA) leads to the release of DAVA (step 4a) or to the formation of the corresponding cyclic imidazolidine of DAVA (step 4b). The C_4 amino group of DAVA is subsequently involved in aldimine formation (steps 6a and 6b). This C_4 aldimine then undergoes stereospecific prototropic rearrangement (steps 7 and 8) to form the ketimine of ALA, which in turn is released by hydrolysis (step 9). According to this proposal, bound cofactor cycles from the pyridoxamine-P (E_M) to the pyridoxal-P (E_L), and then back to the original pyridoxamine-P form (E_M).

The fact that the aldimine form (418 nm) of GSA-AT accumulates upon addition of (R,S)-GSA (Figure 1A) and is not rapidly recycled back to the pyridoxamine form was an enigma. Near quantitative recovery of this aldimine after Sephadex G-50 gel filtration implicates the internal aldimine, rather than unstable external aldimines, involving coenzyme-bound substrate or product intermediates.

From the present data, it is concluded that the enigma is due to anomalous reactivity of the unnatural (R) enantiomer of GSA as follows: (1) Upon repeated additions of substoichiometric amounts of the intermediate or amino donor (R,S)-DAVA to assays containing (R,S)-GSA, the enzyme was transiently converted to the pyridoxamine-P form (panels B and C of Figure 1), and proportional increases in ALA synthesis or accumulation were observed (Figure 2A). (2) (R,S)-GSA-induced conversion of GSA-AT to the pyridoxal-P form reflects the rapid reaction of (S)-GSA followed by the slower reaction of the unnatural (R) enantiomer (Figures 3, 0–10 s and 10–200 s, respectively). (3) Maximum levels of ALA accumulation, in the absence of added DAVA, are rapidly obtained (<10 s, data not shown) and correlate with reaction of (S)-GSA, whereas the conversion of the enzyme to the pyridoxal form upon addition of (R,S)- and (R)-GSA was gradual.

The apparent anomalous enantiomeric reactivity of (R)-GSA can be rationalized on the basis of the following considerations: (1) The first half-reaction involves the initial transfer of an amino group to the nonchiral C_1 carbonyl of GSA. Apparently, both (R) and (S) enantiomers bind to the catalytic site and form the corresponding coenzyme–ketimine (Figure 3; 3-nm spectral shift from about 338 to about 335 nm, 0–10 s), undergo tautomeric rearrangement (Figure 3; ketimine–aldimine spectral shift, 338–418 nm), and are subsequently converted to the corresponding intermediates of DAVA and released (as suggested by the stability of the resulting aldimine coenzyme form (418 nm) to Sephadex G-50 chromatography, data not shown) (Scheme I, steps 1–4). This sequence of events involves an achiral carbon (C_1 of GSA). (2) Transfer of the C_4 amino group from the resulting intermediates, (R)- and (S)-DAVA in the second half-reaction, directly involves the chiral center (Scheme I, steps 6–9). The substrate specificity of the (S) enantiomer is about 2 orders of magnitude (or more) greater than that of the (R) enantiomer (Smith et al., 1991a). By analogy with aspartate aminotransferase, this increased specificity probably results from the orientation of the C_4 hydrogen of DAVA. In the case of (S)-DAVA, this C_4 hydrogen is on the *si* side of the original quinoid intermediate, as shown in Scheme I (step 6), while in the case of (R)-DAVA it is on the *re* side.

(S)-GSA is nearly quantitatively converted to ALA during the initial burst of spectral activity (Figure 3; compare (R,S)- and (S)-GSA, 0–10 and 0.3 s, respectively). Conversion of the remaining (R) enantiomer (reaction 1 below) is limited by the relative intensity of (R)-DAVA (reaction 2):

$$(R)\text{-GSA} + E_M = (R)\text{-DAVA} + E_L \quad (1)$$

$$(R)\text{-DAVA} + E_L = \text{ALA} + E_M \quad (2)$$

$$(S)\text{-DAVA} + E_L = \text{ALA} + E_M \quad (3)$$

$$(R)\text{-GSA} + (S)\text{-DAVA} = \text{ALA} + (R)\text{-DAVA} \quad (4)$$

In the presence of (S)-DAVA, the apparent net conversion of (R)-GSA to ALA is observed (reaction 4, or sum of reactions

1 and 3). This explanation is consistent with results obtained with enantiomeric substrates of aspartate aminotransferase (Kochhar & Christen, 1988).

Although interpretation of initial spectral changes induced upon addition of GSA (Figure 3) is straightforward, subsequent longer term changes observed as spectral equilibrium is approached (Figure 4) are more complex. Upon prolonged incubation of (*R,S*)- and (*R*)-GSA with the pyridoxamine-P form of the enzyme, the resulting internal aldimine form (418 nm) reverts very slowly (few hours) to the pyridoxamine-P form (338 nm). Similar results were observed with the (*S*) enantiomer, but this transition is more rapid.

The initial accumulation of quinonoid or other intermediates (490, 530 nm) during early stages of the reaction of (*S*)-GSA with the pyridoxal form of GSA-AT (Figure 4B), and the corresponding isosbestic points (432 and 375 nm) with aldimine and ketimine coenzyme forms, reflects the potential for additional resonance structures of β-aldehydes and ketones as previously suggested (Smith et al., 1991b). The absence of the aldimine/quinonoid isosbestic point upon reaction with the pyridoxamine-P form of the enzyme (Figure 4A) indicates a considerably more complex scheme of events.

Interpretation of such long-term spectral changes induced with (*S*)-GSA are complex due to product interactions. ALA reacts with both the pyridoxamine (K_m = 390 μM) and the pyridoxal (K_m = 11 μM) coenzyme forms of GSA-AT. However, only the former results in prototropic rearrangement or interconversion between the ketimine and aldimine (Smith et al., 1991a). Thus, longer term transient spectral changes induced upon GSA addition reflect interconversions among various substrate– and product–coenzyme complexes as equilibrium is approached with respect to kinetic and thermodynamically driven pseudoequilibria. Aspartate aminotransferase racemizes dicarboxylic amino acid substrates in the presence of their cognate oxo acids (Kochhar & Christen, 1988, 1992). It cannot be excluded that racemization of GSA by GSA-AT is involved in the long-term spectral changes of the enzyme. The mechanism involved in the conversion of GSA to ALA is of considerable interest. Scheme I illustrates a nonprocessive reaction in which DAVA is released by transaldimination (step 4a) and a processive reaction in which the cyclic imidazolidine is formed (step 4b). Stimulation of initial rates of ALA synthesis by (*R,S*)-DAVA, in reactions containing (*R,S*)-GSA (Smith et al., 1991b), could be a kinetic artifact resulting from the anomalous relativity of (*R*)-GSA and the relative inactivity of the expected intermediate (*R*)-DAVA (reactions 1 and 2 above). However, DAVA stimulation of ALA synthesis from (*R,S*)-GSA clearly reflects the major contribution of the (*S*) enantiomer in comparison to that of the relatively inactive (*R*) enantiomer (Figure 5). This indicates that a processive intramolecular transaldimination resulting in the formation of the cyclic imidazolidine (Scheme I, step 4b) is unlikely from a mechanistic point of view. The nonprocessive reaction (Scheme I, step 4a) is more consistent with reported ping-pong bi-bi kinetics (Smith et al., 1991b).

The observed inhibition of ALA synthesis by gabaculine (GAB) (Smith & Grimm, 1992; Grimm et al., 1991), using GSA-AT that is primarily in the pyridoxamine form, is also consistent with a nonprocessive ping-pong bi-bi reaction mechanism. GAB only reacts with pyridoxal-P form of the enzyme, which would not be generated in a processive reaction (Scheme I, step 4b). Of course one cannot exclude the possibility that gabaculine inactivation also results in part from external transaldimination.

In summary, the accumulation of the putative internal aldimine (418 nm) of GSA-AT upon addition of (*R,S*)-GSA is very likely a consequence of the anomalous first half-reaction of (*R*)-GSA. This reaction probably results in the formation of (*R*)-DAVA, which in turn is a very poor substrate in the second half-reaction leading to ALA synthesis.

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