Gabaculine Resistance of *Synechococcus* Glutamate 1-Semialdehyde Aminotransferase†

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ABSTRACT: Glutamate 1-semialdehyde aminotransferase (GSA-AT) catalyzes the transfer of the C_2 amino group of glutamate 1-semialdehyde (GSA) to the C_1 position. Nucleic acid sequences encoding this enzyme from wild type and a gabaculine (GAB) resistant strain of *Synechococcus* have been cloned and overexpressed in *Escherichia coli*. Tolerance to GAB of the mutant GSA-AT resulted from a point mutation, Met-248–Ile, in the middle of the polypeptide chain accompanied by a deletion of three amino acids close to the NH_2 terminus but can also be effected by the point mutation alone. Purified enzymes from these two strains contain vitamin B_6 and use a typical ping-pong Bi-Bi mechanism, in which 4,5-diaminovalerate (DAVA) is a likely intermediate. The catalytic efficiency (k_{cat}/K_m) of wild-type GSA-AT for GSA is about 3 times larger than that of the mutant enzyme. Comparison of substrate specificities (k_{max}/K_m) for GSA and various analogues reveals that wild-type GSA-AT has values that are about 2–20 times larger than those of the mutant enzyme, except in the case of GAB for which the specificity is 2–3 orders of magnitude larger. These differences are attributed to impaired prototropic rearrangement and transaldimination by mutant GSA-AT. They lead to accumulation of quinonoid and other intermediates upon addition of various substrates such as ALA and DOVA, as well as to instability of their aldimines (418 nm) upon Sephadex gel filtration.

Glutamate 1-semialdehyde aminotransferase (GSA-AT;[1] EC 5.4.3.8) is the last of three enzymes involved in the conversion of glutamate to 5-aminolevulinate (ALA) (Beale & Weinstein, 1990; Kannangara, 1991; Castelfranco & Beale, 1983). This reaction sequence is of particular interest because of its involvement in the regulation of chlorophyll biosynthesis [reviewed by Beale and Weinstein (1990)]. On the basis of structural (Grimm, 1990; Elliott et al., 1990), spectrophotometric (Grimm et al., 1991b; Smith et al., 1991a), and steady-state kinetic analyses (Smith et al., 1991b), GSA-AT is related to other aminotransferases. It also requires vitamin B_6 (Grimm et al., 1991b; Nair et al., 1991; Jahn et al., 1991), and the enzymic mechanism by which GSA is converted to 5-aminolevulinate probably consists of two half-reactions, in which DAVA is a likely intermediate (Smith et al., 1991b). Upon interaction with various substrates, GSA-AT-bound coenzyme exhibits spectral changes (Grimm et al., 1991b) which report specific molecular events occurring at the catalytic site. These spectral changes are amenable to kinetic analyses and provide a means for comparing reactions of various enzymes, or coenzyme forms (pyridoxal or pyridoxamine), with different substrates or substrate analogues and for elucidation of potential enzyme mechanisms (Smith et al., 1991a,b). All GSA aminotransferases described to date show sensitivity to the neurotoxin gabaculine (GAB) (Grimm et al., 1991b; Jahn et al., 1991). This potent suicide inhibitor inactivates aminotransferases with widely different substrate specificities, but the enzymes appear to be related to enzymic exchange of β-protons of their normal substrates (Soper & Manning, 1982).

A gabaculine-tolerant mutant (GR6) has been isolated from *Synechococcus* (PCC 6301) (Bull et al., 1990), and both wild-type and the gabaculine-induced mutant genes have been sequenced. In comparison with the wild-type gene, the GAB-resistant mutant contains a deletion of nine nucleotides (positions 12–20) and a guanine to adenine substitution (position 743). This results in the loss of three amino acids (Ser-Pro-Phe) close to the N-terminal end and a substitution of Ile for Met-248 (Met-248–Ile). Wild-type and mutant genes of GSA-AT were expressed in *Escherichia coli*, and the resulting highly active enzyme was purified to near homogeneity (Grimm et al., 1991b). These purified enzymes provided the basis for comparison of steady-state and spectral kinetic characteristics and for elucidation of gabaculine resistance in *Synechococcus* GR6 described here.

Experimental Procedures

Chemicals. D,L-GSA was synthesized and purified as previously described (Gough et al., 1989). DOVA was a gift from Dieter Dörnemann, Phillips University, Marburg, Germany. ALA and D,L-GAB (3-amino-2,3-dihydrobenzoic acid hydrochloride) were obtained from Sigma Chemical Co. 4,5-Diaminovaleric acid was synthesized from D,L-allylacetic acid (Sigma Chemical Co.) (Smith et al., 1991b).

Purification of Recombinant GSA-AT. The region encoding GSA-AT of *Synechococcus* was inserted into the expression plasmid pDS 56/RBS II, *Sph*I (Bujard et al., 1981), expressed in *E. coli* SG 13009 (Gottesman et al., 1981), and then purified to near homogeneity (Grimm et al., 1991b).

Enzyme and Kinetic Assays for GSA-AT. GSA-AT enzyme activities were determined by measuring initial rates of ALA synthesis, and spectrophotometric and kinetic analyses were as previously described (Smith et al., 1991a,b). Assays (0.5 mL) were in Bistris (0.1 M, pH 7.0, 28 °C, 3 min) with

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[1] Abbreviations: ALA, 5-aminolevulinate; GSA, glutamate 1-semialdehyde; LEV, levulinate; GSA-AT, glutamate 1-semialdehyde aminotransferase; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; DAVA, 4,5-diaminovalerate; DOVA, 4,5-dioxovalerate; GAB, gabaculine.

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Table I: Molar Extinction Coefficients of Wild-Type GSA-AT

	278 nm (M^{-1} cm^{-1})	PMP form, 338 nm (M^{-1} cm^{-1})	PLP form, 418 nm (M^{-1} cm^{-1})
ϵ_M[a]	3.5×10^4	6.0×10^3	5.4×10^3
ϵ_M[b]	4.8×10^4	8.3×10^3	5.0×10^3

[a] Protein content was determined colorimetrically (Bradford, 1976) using a bovine serum albumin standard, and the deduced subunit mass was 46 kDa (Grimm et al., 1991b). Absorbance of the PMP (338-nm) form of GSA-AT was determined in Bistris, 0.1 M, pH 7.0, after saturation with coenzyme and treatment with DAVA (82 μM), followed by Sephadex G-50 column chromatography. Absorbance of the PLP form was determined in the same way after treatment with DOVA (6.0 mM). [b] Obtained by spectrophotometric titration of wild-type GSA-AT with DAVA; cf. Figure 1.

concentrations of GSA, DAVA, DOVA, ALA, and GAB as indicated. GSA-AT concentrations were about 0.3 and 8 μM in steady-state and spectral kinetic experiments, respectively. Reactions were terminated by addition of $^1/_5$ volume of hot (100 °C) ethyl acetoacetate. After heating (100 °C, 10 min), addition of 1 volume of Ehrlich's reagent, and centrifugation (13000g, 5 min), 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). Pseudo-first-order rate constants (k_{app}), at various fixed concentrations of substrate [S], were obtained directly from steady-state and spectrophotometric data using nonlinear regression analysis (Enzfitter software, Sigma Chemical Co.). Michaelis constants (K_m) and rate constants (k_{max}, or k_{cat}) were subsequently obtained with the same software, assuming ping-pong kinetics like that of aspartate aminotransferase (Cronin & Kirsch, 1988; Inoue et al., 1989), and the following relationship: $k_{app} = k_{max}[S]/(K_m + [S])$.

Extinction Coefficients of GSA-AT. GSA-AT protein concentrations were determined colorimetrically (Bradford, 1976), assuming a deduced subunit mass of 46.0 kDa (Grimm et al., 1991a). Molar extinction coefficients were then calculated from absorbance maxima (278, 338, and 418 nm). The 278-nm value was 3.5×10^4 M^{-1} cm^{-1} (Table I). This compares with that of aspartate aminotransferase [*E. coli*, 5.1×10^4 (Kuramitsu et al., 1987), and chicken mitochondria, 7.0×10^4 (Gehring et al., 1977)]. Similar values were also obtained by titration with DAVA (Figure 1). Because of difficulties encountered in obtaining quantitative yields of some coenzyme forms of the mutant protein (see Figure 4), their concentrations were calculated using extinction coefficients of wild-type counterparts.

Isolation of PMP and PLP Forms of GSA-AT. Purified GSA-AT overexpressed in *E. coli*, which is primarily in the PMP form (Grimm et al., 1991b), was converted quantitatively into this form by preincubation with PMP (13–130 μM depending on enzyme concentration, room temperature, 30 min) and then by incubation with excess DAVA, whereafter it was purified by Sephadex G-25 column chromatography. The PLP form was similarly prepared after treatment with coenzyme and DOVA or GSA.

Results

A gabaculine (GAB) resistant strain of *Synechococcus* (GR6) has been selected by its ability to grow in the presence of increasing concentrations of GAB (Bull et al., 1990). Prime targets for this antibiotic are vitamin B_6 containing enzymes, such as GSA aminotransferase, which in all organisms tested shows GAB sensitivity. Wild-type and mutant GSA-ATs were expressed in *E. coli*, purified, and compared on the basis of spectrophotometric and steady-state kinetics.

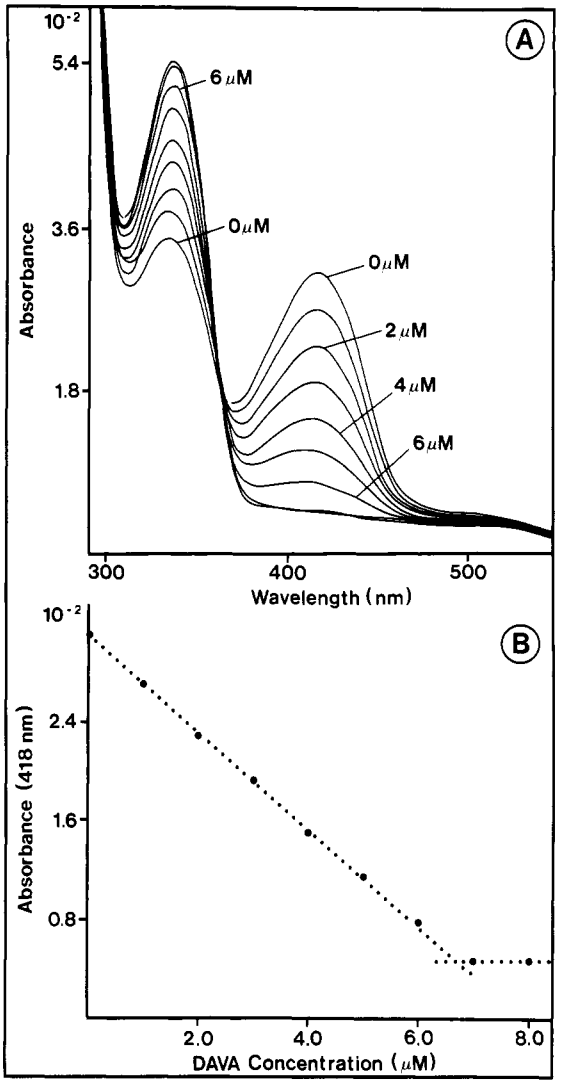


FIGURE 1: Spectrophotometric equilibrium titration of GSA aminotransferase with DAVA. The aldimine form of GSA-AT (8 μM, in 1.0 mL of 0.1 M Bistris, pH 7.0) was titrated with successive additions (1.0 μM) of DAVA. (A) Spectra were recorded after each addition after the absorbance (418 nm) reached a constant value (about 5 min). (B) Equilibrium absorbance values (418 nm) were plotted as a function of the initial DAVA concentration. The intersection of the extrapolated initial slope with the limiting absorbance gives the concentration of bound coenzyme.

Spectrophotometric Titration of GSA Aminotransferase. In order to normalize rate constants of pyridoxamine and pyridoxal coenzyme forms of wild-type and mutant GSA-ATs, it was necessary to determine their individual subunit concentrations. Purified enzyme is readily converted to the PLP or aldimine form with excess DOVA and back to the PMP form with DAVA. Such titrations with oxo and amino derivatives of GSA provide convincing evidence that ALA formation proceeds in two half-reactions similar to aspartate aminotransferase and that both coenzyme forms are required (Smith et al., 1991b). Equilibrium absorbance spectra obtained after each addition of DAVA to the putative internal aldimine form (418 nm) of GSA-AT are illustrated in Figure 1A. This conversion of wild-type GSA-AT to the PMP form (338 nm) with DAVA proceeds stoichiometrically even at submicromolar concentrations. Sephadex G-25 filtration of such equilibrium reaction mixtures does not significantly alter their spectra. Absorbance decreases of the aldimine form of GSA-AT (418 nm) are essentially linearly related to the amounts of DAVA added (Figure 1B). On the assumption

Scheme I

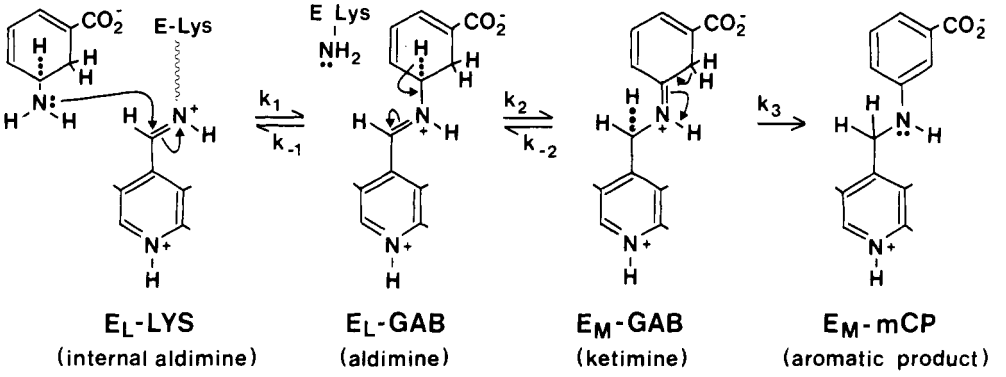


Table II: Comparison of Steady-State Kinetic Parameters of Wild-Type and GAB-Resistant GSA Aminotransferase[a]

	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
wild-type GSA-AT	4.6×10^{-5}	4.7×10^{-1}	1.0×10^{4}
mutant GSA-AT	2.2×10^{-5}	7.7×10^{-2}	3.5×10^{3}

[a] Values were derived from initial rates of ALA synthesis at various fixed concentrations of D,L-GSA by nonlinear regression analysis. K_m values are in terms of $^1/_2$ D,L-GSA concentration.

that substrate complexes do not accumulate (i.e., spectra are stable to Sephadex G-25 filtration), the intersection of the extrapolated initial slope with the limiting absorbance gives the concentration of bound coenzyme (Velick & Vavra, 1962), from which molar extinction coefficients were calculated (Table I). These coefficients were used to derive values for k_{cat} of various coenzyme forms of mutant and wild-type GSA-AT.

Steady-State Kinetics of Wild-Type and Mutant GSA Aminotransferase. Enzymic synthesis of ALA by wild-type and mutant GSA-ATs has been compared under steady-state conditions. Rates of ALA synthesis were determined at various fixed concentrations of GSA and K_m and k_{cat} calculated by nonlinear regression analysis assuming Michaelis–Menten kinetics. The catalytic efficiency (k_{cat}/K_m) of wild-type enzyme (1.0×10^4 M^{-1} s^{-1}) is only about 3 times larger than that of mutant GSA-AT (3.5×10^3 M^{-1} s^{-1}). This difference is due to about a 6-fold decrease in k_{cat} and a 2-fold decrease in K_m, indicating increased affinity of the mutant enzyme for the substrate (Table II).

Suicide Inactivation of GSA Aminotransferase with GAB. Upon reaction with the neurotoxin GAB, GSA-AT is completely and irreversibly inactivated as illustrated in Scheme I. Putative steps (n = 1, 2, or 3) of this inactivation are indicated by forward and reverse rate constants (k_n and k_{-n}, respectively). The first step involves transaldimination of the internal aldimine of GSA-AT (E_L–Lys, λ_{max} 418 nm) by GAB, presumably through a tetrahedral intermediate, and formation of the corresponding external aldimine of the pyridoxal coenzyme form and GAB (E_L–GAB, about 418 nm). The second step involves prototropic rearrangement, giving the corresponding ketimine of GAB and the pyridoxamine coenzyme form of GSA-AT (E_M–GAB, about 338 nm). In the last step a β-proton is abstracted, leading to irreversible aromatization and formation of the *m*-carboxyphenyl derivative of the pyridoxamine coenzyme form (E_M–mCP, 338 nm).

GAB sensitivity of GSA-AT is indicated by a decrease in initial rates of ALA synthesis, as shown for enzyme purified from the gabaculine-resistant mutant (GR6) and wild-type *Synechococcus* (Figure 2). Whereas a 50% decrease in initial rates of ALA synthesis by mutant enzyme required relatively

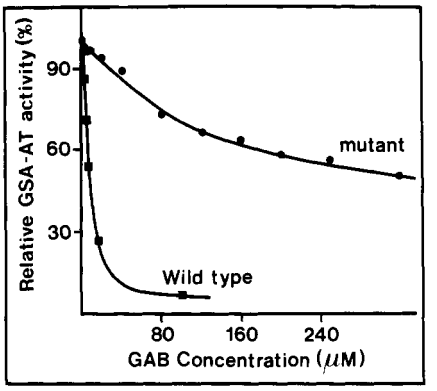


FIGURE 2: Inhibition of wild-type and mutant GSA aminotransferase by gabaculine. Initial rates of ALA synthesis were determined in the presence of various fixed concentrations of GAB and expressed in terms of the percent of the uninhibited reaction rate. In practice, GSA (90 μM) was rapidly mixed with a buffered (0.1 M Bistris, pH 7, 0 °C) enzyme (about 0.3 μM) solution, and aliquots were transferred to Eppendorf tubes containing various fixed amounts of GAB and immediately transferred to a 28 °C water bath. Reactions were terminated after 3 min by addition of hot (100 °C) ethyl acetoacetate.

high concentrations of GAB (>0.3 mM), wild-type GSA-AT was at least 100 times more sensitive (2.5 μM). Passing such enzymic reaction mixtures through Sephadex G-50 did not restore activity.

Spectral Changes of GSA Aminotransferase Induced by Gabaculine. GAB inactivation of GSA-AT can also be visualized spectrophotometrically, since coenzyme bound to GSA-AT is a chromogenic reporter of molecular events occurring at the catalytic site. Spectral changes associated with addition of GAB to wild-type enzyme are shown in Figure 3A. The rapid (about 30 s) decrease in the putative aldimine form of GSA-AT (418 nm) was accompanied by a corresponding increase at 338 nm, with a well-defined isosbestic point at about 365 nm. Such absorbance changes at several fixed concentrations of GAB followed pseudo-first-order kinetics (solid lines generated by nonlinear regression analysis, Enzfitter, Figure 3B). That these spectral changes are directly associated with enzyme inactivation, under steady-state conditions, is shown in Figure 3C. There is a linear decrease in enzyme activity with corresponding increases in equilibrium absorbance change (418 nm), induced by increasing concentrations of GAB.

Pseudo-first-order rate constants calculated from such spectral changes of wild-type and mutant GSA-ATs are dependent on GAB concentration. Data were evaluated by nonlinear regression analysis (Enzfitter) assuming the relationship $k_{app} = k_{max}[GAB]/(K_m + [GAB])$ and kinetic parameters listed in Table III. The substrate specificity (k_{max}/K_m) of wild-type enzyme for GAB (1.2×10^4 M^{-1} s^{-1})

Table III: Comparison of Spectral Kinetic Parameters of Wild-Type and GAB-Resistant Mutant of GSA Aminotransferase[a]

	K_m (μM)		k_{max} (s^{-1})		k_{max}/K_m (M^{-1} s^{-1})		
	wt	mu	wt	mu	wt	mu	k_{max}/K_m, wt/mu
GSA	110	220	(<0.27)[b]	0.27	(≫1200)[b]	1200	
DAVA	15	27	1.2	0.010	80000	3700	22
DOVA	140	8300	0.0092	0.05	66	6.0	11
ALA	390		0.45		1000–2000		
GAB	8.4	290	0.1	0.016	12000	55	218

[a] Apparent rate constants (k_{app}) were first determined from spectral changes (418 nm) induced by various fixed concentrations of each substrate or analogue (see Figure 3), assuming first-order kinetics. K_m and k_{max} were subsequently derived from the concentration dependence of such pseudo-first-order constants (k_{app}) by nonlinear regression analysis, assuming the following relationship: $k_{max} = k_{app}[A]/(K_m + [S])$. [b] The reaction of the L-enantiomer is too fast to be measured.

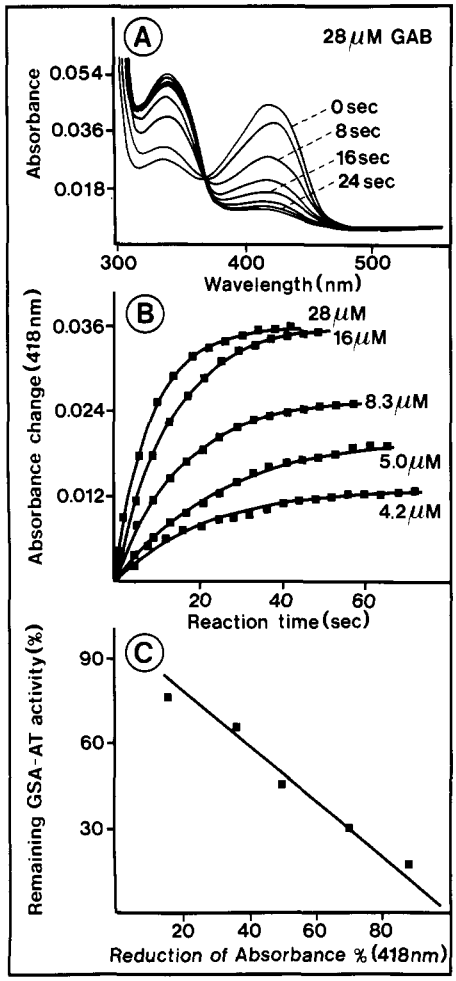


FIGURE 3: Spectral kinetics of GSA aminotransferase induced by GAB. Purified wild-type GSA-AT was converted to the aldimine form with excess DOVA, passed through Sephadex G-25, and treated with various fixed concentrations of GAB. (A) Time course of spectral changes of GSA-AT (5.2 μM) after addition of GAB (28 μM). (B) Dependence of spectral change rates of GSA-AT on GAB concentration. Spectral changes of GSA-AT (418 nm) were monitored as a function of time, at various fixed concentrations of GAB, as indicated. Data points are compared by nonlinear regression analysis (Enzfitter), assuming first-order kinetics. (C) Loss of enzymic activity (after 1 min) associated with decreases in the aldimine form (418 nm) of GSA-AT, induced by addition of various fixed concentrations of GAB.

is more than 200 times larger than that of the mutant enzyme (5.5×10^1 M^{-1} s^{-1}), consistent with steady-state data (compare with Figure 2).

Spectral Changes of GSA Aminotransferase Induced by GSA and Analogues. Spectral changes of wild-type and mutant GSA-AT, induced by various substrates and analogues (DAVA, DOVA, and ALA), have also been compared. Data were collected and kinetic parameters derived in much the same manner as detailed for GAB (Figure 3), and values are

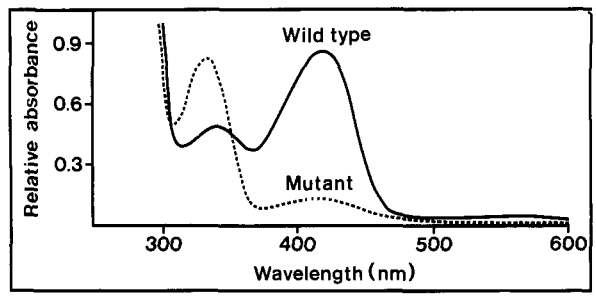


FIGURE 4: Spectral stability of GSA aminotransferase treated with excess DOVA: (solid line) wild-type enzyme before or after Sephadex G-25 chromatography; (dotted line) mutant enzyme after gel filtration. Before gel filtration the spectrum of mutant GSA-AT was similar to that of wild type.

listed in Table III. However, some spectral changes of the GAB-resistant mutant of GSA-AT, induced by addition of GSA, were clearly biphasic. In such situations kinetic parameters were derived from only initial (0–20-s) absorbance changes.

Aldimine Stability of Mutant GSA Aminotransferase. A characteristic feature of the GAB-resistant mutant of GSA-AT is the relative instability of putative aldimines (418 nm) generated upon addition of DOVA or ALA to purified enzyme. When such reactions are subjected to gel filtration, spectra revert to the original PMP form (Figure 4), even after prolonged incubation (60 min). This spectral instability indicates accumulation of enzyme–product intermediates (probably the external aldimine, 418 nm) and rate-limiting transaldimination. Another characteristic feature of mutant GSA-AT catalysis is the transient accumulation of putative quinonoids (490 and 530 nm), especially upon addition of DOVA (Figure 5A). In contrast, addition of GSA to the PMP form of the GAB-resistant GSA-AT followed by gel filtration generated a stable spectral shift to 418 nm.

Transaldimination of Mutant GSA-AT External Aldimines. Putative external aldimines of mutant GSA-AT, induced by DOVA or ALA, are reactive. Indeed the Sephadex G-25 unstable aldimine (418 nm), generated upon addition of ALA to the PMP form of GSA-AT, is converted to the aromatic coenzyme adduct (338 nm) upon addition of GAB. This conversion follows pseudo-first-order kinetics at both 338 and 418 nm (Figure 5B). Presumably GAB inactivation occurs by transaldimidation.

Discussion

In comparing wild-type and mutant GSA-AT by steady-state and spectral analyses, it was necessary to determine the concentration of relevant coenzyme forms and to assign molecular species to relevant chromophores. Although purified GSA-AT from *Synechococcus* is primarily in the PMP form (Grimm et al., 1991b), a significant fraction is in the PLP form. In addition, stimulation (2–3-fold) by added PMP indicates only partial coenzyme saturation. Therefore the

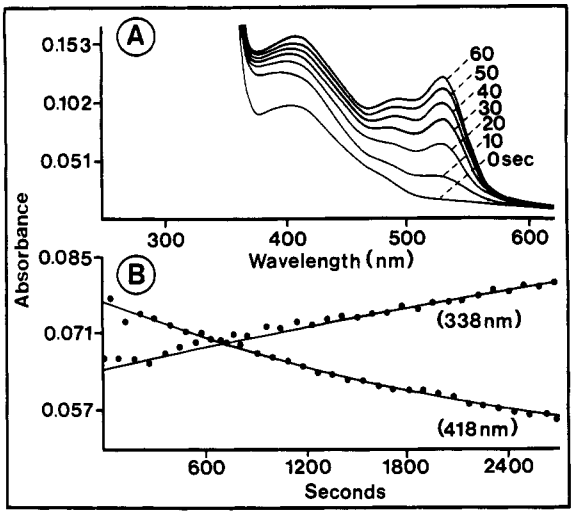
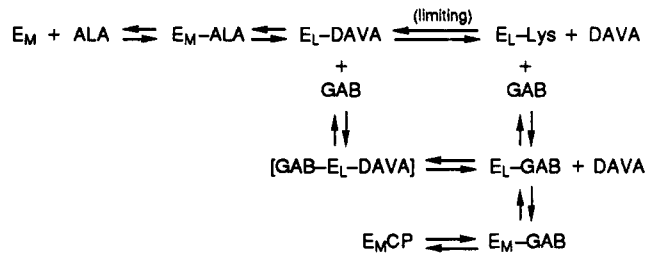


FIGURE 5: Reaction of mutant GSA aminotransferase with DOVA and ALA. (A) Spectral changes of mutant GSA-AT (117 μM) induced by addition of DOVA (4.8 mM). (B) Absorbance changes of the ALA aldimine (418 nm) of mutant GSA-AT, induced by addition of GAB. The PMP form of GSA-AT (18 μM) was initially exposed to excess ALA (4.4 mM). The resulting putative ALA external aldimine (which spontaneously reverts to the PMP form upon Sephadex G-25 gel filtration) was irreversibly converted to the aromatic adduct of the PMP form (338 nm) with GAB (0.93 mM).

extinction of relevant coenzyme forms of GSA-AT was used to obtain catalytic subunit concentrations. These concentrations were then used to calculate values for k_{cat}. The extinction coefficient of the putative aldimine form of wild-type GSA-AT, determined by spectral titrations with DAVA (Figure 1), is in reasonable agreement with that determined by standard colorimetric methods of total protein and reduced subunit mass (Table I). Correct assignment of molecular species to particular chromophores is essential to quantitative interpretation of special kinetic data. In general, such assignments were by analogy with spectral characteristics of aspartate aminotransferase (Velick & Vavra, 1962), except in the case of the absorbance maximum of the putative internal aldimine of GSA-AT, which is not pH dependent (Grimm et al., 1991b). However, the possibility must be considered that apparent equilibria are shifted by accumulation of enzyme–substrate or enzyme–product intermediates. Titration of GSA-AT with DAVA (Figure 1) and subsequent G-25 chromatography demonstrated that resulting spectra of the PMP form (338 nm) of the enzyme are not significantly altered by gel filtration. Similar results were obtained with DOVA titrations and the resulting PLP or aldimine form (418 nm) of wild-type enzyme (Figure 4, instability of mutant GSA-AT aldimines will be discussed later). As a first approximation, we have assumed that intermediates do not accumulate with wild-type enzyme. However, we cannot exclude this possibility because substrate or product complexes may occur in forms that are not spectrally distinguishable from those of the free enzyme–coenzyme complex.

GAB sensitivity is reflected in the substrate specificity of wild-type GSA-AT, which is 2–3 orders of magnitude greater than that of enzyme isolated from the GAB-tolerant strain (GR6) (Table III). Substrate specificities for DAVA and DOVA are also significantly larger (about 10–20-fold) for wild-type enzyme. The apparent decrease (ratio = 0.24) in substrate specificity for D,L-GSA is probably due to the reactivity of D-GSA, not L-GSA. Reaction with L-GSA is very rapid and difficult to detect by ordinary spectral methods. Enzymic synthesis of ALA from D,L-GSA is also very rapid and essentially complete (about 50% of D,L-GSA) before

Scheme II



significant absorbance changes are observed (Smith et al., 1991a). By contrast, spectral changes of mutant enzyme with D,L-GSA are slower and biphasic. This indicates that initial spectral changes observed with mutant enzyme are probably for the L-enantiomer. In preliminary reactions with impure L-GSA (Hoober et al., 1988), the substrate specificity of wild-type GSA-AT is at least 10 times larger (Smith et al., 1991a) than the apparent value of D,L-GSA shown in Table III.

Differences in catalytic specificities of wild-type and mutant enzymes are probably a reflection of unmistakable differences in catalysis by wild-type and mutant enzymes in the presence of GSA and various analogues. Unlike wild-type enzyme, the aldimines of mutant GSA-AT generated by addition of DOVA or ALA are unstable and revert back to the ketimine or PMP forms upon gel filtration (Figure 4). These unstable aldimines (418 nm) are probably external aldimines of DOVA or ALA, which undergo spontaneous tautomeric rearrangement (to the ketimine) and subsequent hydrolysis upon substrate removal by gel filtration. This suggests that transaldimination in the GAB-resistant GSA-AT is at least in part rate limiting. K_m and k_{max} values shown in Table III then do not represent intrinsic rate constants but combinations of rate constants involved in binding and various intramolecular rearrangements (Inoue et al., 1989; Velick & Vavra, 1962). Such intramolecular rearrangements are indicated by transient accumulation of covalent intermediates (480, 530 nm) with DOVA (Figure 5A). Intermediates also accumulate upon reaction of the aldimine form of wild-type enzyme with GSA (Smith et al., 1991a).

The conclusion that putative external aldimines of mutant GSA-AT accumulate raised the question concerning the reactivity of ALA-treated enzyme with GAB (Figure 5B), as shown in Scheme II. (E_M and E_L are the pyridoxamine and pyridoxal forms of GSA-AT, free and in complex with Lys-272 or various substrates or products, as shown; E_MCP is the inactive *m*-carboxyphenyl derivative formed from GAB.)

This scheme shows the initial reaction of ALA with the PMP form of GSA-AT (E_M), giving the corresponding ketimine (E_M–ALA), which upon prototropic rearrangement yields the coenzyme external aldimine of DAVA (E_L–DAVA), which is converted to the internal aldimine (E_L–Lys) plus DAVA by transaldimination. GAB is shown to react with either the external (E_L–DAVA) or internal (E_L–Lys) aldimine of GSA-AT. Since these aldimines have similar absorbance maxima, they are not spectrophotometrically distinguishable. On the basis of the related observation that ALA gives mixed, rather than competitive, inhibition of GSA-AT inactivation by GAB (Smith et al., 1991a), it would appear that ALA and perhaps GAB react with more than one enzyme form. However, at present there is no additional evidence for the formation of the ternary complex (GAB–E_L–DAVA) shown.

The fact that prolonged incubation of mutant enzyme with appropriate substrates does not give higher yields of stable internal aldimines, while stable internal aldimines of wild-type

enzyme are rapidly formed (Figure 4), suggests that accumulation of intermediates is not an equilibrium but a catalytic problem. Taken together, the accumulation of intermediates, the formation of aldimines which are unstable to gel filtration, and the fact that substrate specificities of mutant GSA-AT are in general lower than those of wild-type enzyme suggest that prototropic rearrangement and transaldimination are hindered in the GAB-resistant mutant. Experiments with deuterated GAB indicate that α-proton abstraction is at least in part rate limiting (Rando, 1977). The loss of enzymic activity in direct proportion to the conversion of the aldimine form of GSA-AT (418 nm) to the corresponding ketimine (338 nm) (Figure 3C) is consistent with these results. But this does not explain the large difference between mutant and wild-type enzymes for GAB. The catalytic efficiency of mutant GSA-AT is only 3 times lower for GSA (Table II), whereas the substrate specificity for GAB is 2–3 orders of magnitude lower (Table III).

The functional role of Met-248 and the three deleted amino acid residues, 5–7, remains elusive. It is interesting to note that sequence alignment of GSA-AT and aspartate aminotransferase revealed that Met-248 corresponds to Tyr-225 and residues 5–7, Ser-Pro-Phe, correspond to residues 4–6, Ser-Ile/Val-Phe, in various cytoplasmic aspartate aminotransferases (Grimm et al., 1991b). Tyr-225 is hydrogen bonded to the O(3′) of PLP to stabilize the unprotonated Schiff base with the active site lysyl residue of the enzyme at physiological pH, and the three amino acids belong to the functional part of the amino acid terminal arm of aspartate aminotransferase which is noncovalently associated via residues 3–11 with the coenzyme binding domain of the adjacent subunit (Jansonius & Vincent, 1987).

We have recently constructed two GSA-AT mutants which either lack the three residues near the amino-terminal end or contain Ile instead of Met at position 248. Comparison of the GAB sensitivity of these mutants indicated that the Met-248–Ile point mutation confers GAB resistance to GSA-AT (to be published later). The reason that the Met-248–Ile mutation decreases the substrate specificity of GAB in comparison to other substrates is probably related to (1) steric interference by alkyl groups around the β-carbon of Ile in comparison with β-hydrogens of Met and (2) the rigid planar structure of GAB in comparison with GSA and its analogues. In light of the fact that GAB sensitivity is purportedly related to enzymic exchange of β-protons of normal substrates (Soper & Manning, 1982), it would be of interest to compare such enzymic-dependent hydrogen-exchange activities of wild-type and mutant GSA-AT.

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