Characterization of the Different Spectral Forms of Glutamate 1-Semialdehyde Aminotransferase by Mass Spectrometry†

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ABSTRACT: Glutamate 1-semialdehyde aminotransferase produces δ-aminolevulinate for the synthesis of chlorophyll, heme, and other tetrapyrrole pigments. The native enzyme from *Synechococcus* is pale yellow and has absorption maxima at 338 and 418 nm from vitamin B_6. Yellow, colorless, and pink forms of the protein are obtained by treatment with 4,5-dioxovalerate, 4,5-diaminovalerate, and acetylenic GABA, respectively. Compared to the native enzyme, the 418 nm absorption maximum in the yellow enzyme is enhanced and the 338 nm maximum reduced while the colorless enzyme has a heightened maximum at 338 nm and a barely detectable peak at 418 nm. The pink enzyme has an absorption maximum at 560 nm. When the native and colorless enzymes are repeatedly diluted in 0.5 M Na_2HPO_4, pH 7.0, and reconcentrated, pyridoxamine 5′-phosphate is released and the 338 nm maximum lost. Thus the 338 nm absorption maximum is associated with noncovalently bound pyridoxamine 5′-phosphate. $NaBH_4$ reduction proved that the absorbance at 418 nm is from pyridoxal 5′-phosphate cofactor bound by a Schiff base to the protein. When the native, colorless, and yellow enzymes were subjected to electrospray ionization mass spectrometry, the B_6 cofactor dissociated from the protein and gave a molecular weight of 46 401−46 418. Acetylenic GABA and $NaBH_4$ were used for protein modification, and they reacted with the native and yellow enzymes but had no effect on the colorless enzyme. Pyridoxal 5′-phosphate bound covalently to the protein after $NaBH_4$ reduction. Acetylenic GABA attached covalently to the enzyme produced an additional mass peak, 123−126 mass units higher, in the electrospray ionization spectrum. Tryptic peptide analysis showed no disulfide bonds in glutamate 1-semialdehyde aminotransferase and that Lys^{276} is the binding site for both acetylenic GABA and pyridoxal 5′-phosphate. A likely mechanism is suggested for covalent binding of acetylenic GABA to Lys^{276}.

Glutamate 1-semialdehyde aminotransferase (GSA-AT)[1] catalyzes the formation of δ-aminolevulinate, and it is the last enzyme in the $tRNA^{Glu}$-mediated pathway that produces δ-aminolevulinate for the synthesis of chlorophyll, heme, and other tetrapyrrole pigments (Kannangara *et al.*, 1994). This enzyme is found in plants (Kannangara & Gough, 1978; Nair *et al.*, 1991; Hofgen *et al.*, 1994), in algae (Wang *et al.*, 1984; Weinstein *et al.*, 1987; Mayer *et al.*, 1987; Breu, 1988; Houghton *et al.*, 1989; Jahn *et al.*, 1991), and in a number of bacteria (Friedmann *et al.*, 1987; Bull *et al.*, 1990; Ilag & Jahn 1992).

Purified GSA-AT (the native enzyme) is pale yellow with absorption maximas at 280, 338, and 418 nm (Smith *et al.*, 1991a,b). When it is exposed to 4,5-diaminovalerate, absorption at 338 nm increases and disappears at 418 nm. This form is the "colorless enzyme". When treated with 4,5-dioxovalerate, native enzyme turns bright yellow (absorption decreases at 338 nm and increases at 418 nm). This form is the "yellow enzyme". The yellow enzyme is sensitive to acetylenic GABA and $NaBH_4$. The colorless enzyme is insensitive to these compounds. When the yellow enzyme is treated with $NaBH_4$, absorption disappears at 418 nm and increases at 338 nm. The yellow enzyme reacts with acetylenic GABA, giving a new absorption maximum at 560 nm. This form is the "pink enzyme" (Kannangara *et al.*, 1994).

In this paper we present conclusive proof that in GSA-AT absorption at 338 nm is due to pyridoxamine phosphate, while absorption at 418 nm is due to pyridoxal phosphate bound to Lys^{272} by a Schiff base. In addition, a mechanism for inactivation by acetylenic GABA is presented.

MATERIALS AND METHODS

Purification of Glutamate 1-Semialdehyde Aminotransferase. The *Synechococccus* enzyme was expressed in *Escherichia coli* strain SG 13009 and purified essentially as described by Grimm *et al.* (1991) using, additionally, 1 mM pyridoxine during expression. Purified protein was trans-

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[1] Abbreviations: GSA-AT, glutamate 1-semialdehyde aminotransferase; AAT, aspartate aminotransferase; GABA, γ-aminobutyric acid; ESI, electrospray ionization; ESIMS, electrospray ionization mass spectrometry; LC/MS, on-line liquid chromatography/mass spectrometry.

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ferred into ultrapure (MilliQ) water using Sephadex G-25 columns, frozen in liquid N_2, lyophilized, and stored at −20 °C.

Preparation of Different Spectral Forms of Glutamate 1-Semialdehyde Aminotransferase and Modification of the Enzyme with Gabaculine and Acetylenic GABA. Two milligrams of lyophilized native enzyme was dissolved in 0.5 mL of BisTris buffer containing 0.1 M [bis(2-hydroxyethyl)imido]tris(hydroxymethyl)methane, pH 7.0, and 1 mM dithiothreitol and treated with either with 4,5-diaminovalerate (0.1 mM) or 4,5-dioxovalerate (1 mM) on ice to yield the colorless and yellow enzymes, respectively. Pink enzyme was obtained by treatment of the native enzyme with 1 mM acetylenic GABA on ice. The reactions were monitored spectrophotometrically. After 10 min the protein samples were transferred into water using Nap5 columns and lyophilized.

Modification of Glutamate 1-Semialdehyde Aminotransferase with $NaBH_4$. From a 2 M stock solution in BisTris buffer, $NaBH_4$ was added to an enzyme solution (4 mg mL^{-1}) also in BisTris buffer, pH 7.0, to give a final concentration of 100 mM $NaBH_4$. The samples were left to react on ice for 30 min and then gel-filtered into water using Nap5 columns and lyophilized.

Apoglutamate 1-Semialdehyde Aminotransferase. GSA-AT treated with 4,5-diaminovalerate was diluted to 10 mL with 0.5 M Na_2HPO_4 buffer, pH 7.0, and concentrated to 0.5−1.0 mL by ultrafiltration in an Amicon Centriprep-10 concentrator. The filtrate was saved, and the concentrated protein was rediluted with fresh 0.5 M Na_2HPO_4 buffer, pH 7.0 (10 mL), and reconcentrated. This procedure was repeated six times. Thereafter, the protein was gel-filtered into water and lyophilized.

Electrospray Ionization Mass Spectrometry (ESIMS). ESI mass spectrometry of intact GSA-AT was performed on a Vestec Model 201 single quadrupole mass spectrometer (Vestec Corp.). Lyophilized protein samples were dissolved in 1% acetic acid and 50% methanol to 5−10 pmol μL^{-1}, and 10 μL was infused by a syringe pump (Orion Research) at a flow rate of 0.3 μL min^{-1}. Full-scan spectra were acquired in the positive ion mode using a Technivent Vector 2 data system (Technivent, St. Louis, MO). The instrument was calibrated using the multiple charged ions of horse skeletal muscle myoglobin (Sigma, St. Louis, MO).

On-Line Liquid Chromatography/Mass Spectrometry of Peptides (LC/MS). Protein samples (0.8 nmol) were dissolved in 50 mM $NaHCO_3$ and 0.1 M urea, pH 7.8, and incubated with trypsin for 4 h at 37 °C. The resulting peptides were separated by reverse-phase HPLC (model 140B separation system, Applied Biosystems) on a Nucleosil-300, C_{18} column (2 × 100 mm) using an acetonitrile gradient in the presence of trifluoroacetic acid at 42 °C and a flow rate of 200 μL min^{-1}. The eluate from the column was split, and a fraction (5%) was fed directly to ESIMS (model TSQ700 mass spectrometer, Finnigan Mat) while the ultraviolet absorption (model 759A, Applied Biosystems) was measured with the rest. The electrospray ionization process was assisted by a coaxial N_2 flow and a sheath liquid flow of 2-methoxyethanol, 2-propanol, and formic acid, 66:32:1 (v/v/v), at a flow rate of 2 μL min^{-1}. The mass analyzer was set to scan from a *m/z* of 400−1900 in 4 s. Spectra were recorded in the positive ion centroiding mode (five samples per mass unit). The instrument was calibrated using the multiple charged ions of horse skeletal muscle myoglobin and the tetrapeptide MRFA.

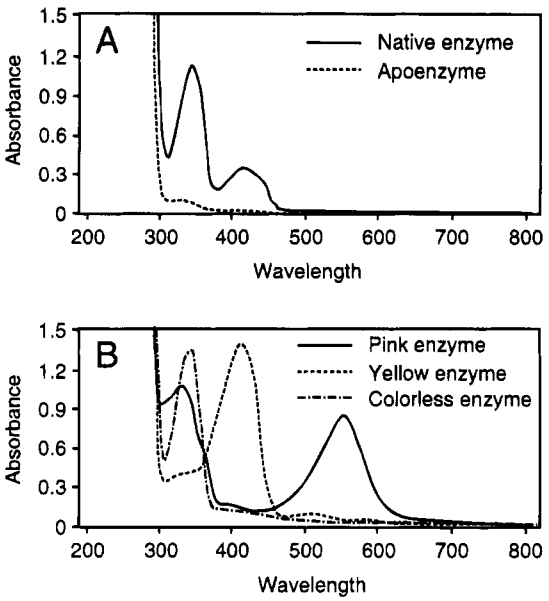

FIGURE 1: Absorption spectra of various forms of glutamate 1-semialdehyde aminotransferase.

Other Methods. The GSA-AT activity was determined using 100 μM substrate and 1−5 μg of enzyme in the assays (Smith *et al.*, 1991a). Spectral changes were analyzed using a Hewlett-Packard 8452A diode array spectrophotometer. Pyridoxamine 5′-phosphate concentrations were determined spectrophotometrically, using a standard curve prepared from known amounts of pyridoxamine 5′-phosphate dissolved in 0.5 M Na_2HPO_4 buffer, pH 7.0. Protein determinations were made by weighing, using a microbalance.

RESULTS AND DISCUSSION

Synechococccus GSA-AT produced in *E. coli* (native enzyme) has absorption maxima at 280, 338, and 418 nm (Figure 1A) and produced δ-aminolevulinate from glutamate 1-semialdehyde at the rate of 745 nmol mg^{-1} min^{-1}. Addition of 5−100 μM pyridoxamine 5′-phosphate to the reaction mixtures had no effect on the activity of this enzyme, which was induced in the presence of 1 mM pyridoxine. The yield of GSA-AT was approximately 10 mg per g wet weight of cells. No significant change in the yield was observed when *E. coli* cells were grown in the absence of pyridoxine. However, the protein purified from cells grown in the absence of pyridoxine produced δ-aminolevulinate at the rate of 570 nmol mg^{-1} min^{-1}, and this activity was stimulated 20−30% by the addition of 25 μM pyridoxamine 5′-phosphate. It is concluded that a significant fraction of the GSA-AT produced in the absence of added pyridoxine is in the apoprotein form and addition of pyridoxine to the culture medium eliminates this problem. The transfer of GSA-AT to water and lyophilization had no injurious effect, as judged by both the specific enzymatic activity and absorption spectra.

When native and colorless enzymes were concentrated by ultrafiltration after dilution with 0.5 M Na_2HPO_4 buffer, pH 7.0, absorption declined at 338 nm. The absorption spectrum of the filtrate showed that free pyridoxamine 5′-phosphate was released into the buffer. Starting from the colorless

enzyme and repeatedly diluting with fresh 0.5 M Na_2HPO_4 buffer and concentrating resulted in apo-GSA-AT with a barely detectable peak at 338 nm (Figure 1A). Six cycles of dilution and ultrafiltration released 350 nmol of pyridoxamine 5′-phosphate from 25.5 mg (547 nmol) of colorless enzyme. This corresponds to a cofactor release of 64% and is higher than that obtained for the enzyme purified from pea leaves (Nair *et al.*, 1991). Dilution in Na_2HPO_4 buffer and concentration did not affect absorption at 418 nm in either the native or yellow enzyme. The absorption at 560 nm in the pink form of the enzyme was also not affected by the phosphate buffer treatment. It is concluded that the absorption maximum of GSA-AT at 338 nm is due to noncovalently bound pyridoxamine 5′-phosphate.

Figure 1B shows the absorption spectra of the colorless, yellow, and pink forms of GSA-AT prepared by treatment with 4,5-diaminovalerate, 4,5-dioxovalerate, and acetylenic GABA, respectively. The rates of δ-aminolevulinate synthesis of these preparations in nmol (mg of protein)$^{-1}$ min^{-1} were colorless enzyme, 746; yellow enzyme, 744; and pink enzyme, 4. The absorption at 560 nm in the pink enzyme declined slowly and after 24 h reached 50% of its original value at room temperature. Incubating the pink enzyme at 50 °C resulted in a rapid decline in absorbance at 560 nm. No effect was observed either in the specific enzyme activity or in the absorption spectrum, when the colorless enzyme was incubated with 100 mM acetylenic GABA at 0 °C for 10 min and assayed after removal of the reagent.

Native and yellow forms of glutamate 1-semialdehyde aminotransferase react with $NaBH_4$, and after few seconds their absorption at 418 nm disappears. In contrast, the colorless form is not affected by $NaBH_4$. The rates of δ-aminolevulinate synthesis observed for these proteins after removal of $NaBH_4$ in nmol (mg of protein)$^{-1}$ min^{-1} were $NaBH_4$-treated native enzyme, 694; $NaBH_4$-treated yellow enzyme, 90; and $NaBH_4$-treated colorless enzyme, 740 (Table 1). The differential $NaBH_4$ inactivation indicates that various spectral forms of the enzyme differ in the ratio of pyridoxamine 5′-phosphate/pyridoxal 5′-phosphate-carrying protein molecules. It is concluded that absorption at 418 nm in GSA-AT is from pyridoxal 5′-phosphate bound by a Schiff base to the protein. Yellow GSA-AT turned colorless on exposure to acetic acid (5%) as result of hydrolysis of the Schiff base. Furthermore, on acidification both pyridoxamine and pyridoxal 5′-phosphate dissociated from the protein and could easily be separated from the protein by ultrafiltration and analyzed. Figure 2 gives the absorption spectra of the different forms of B_6 released from various forms of GSA-AT. By comparison with authentic compounds, the cofactors released from the colorless enzyme and yellow enzyme were identified as pyridoxamine and pyridoxal 5′-phosphate, respectively. On the same basis freshly prepared pink enzyme released pyridoxal phosphate, while the native enzyme and the pink enzyme incubated at 50 °C for 2 min released both pyridoxamine and pyridoxal 5′-phosphate on acidification. After removal of the cofactor by acidification, the protein showed only absorption at 280 nm. These observations verify the previous suggestion that absorption at 338 and 418 nm in GSA-AT results from pyridoxamine and pyridoxal 5′-phosphate, respectively.

Since the pyridoxamine 5′-phosphate and the Schiff base linked pyridoxal 5′-phosphate dissociate in acetic acid

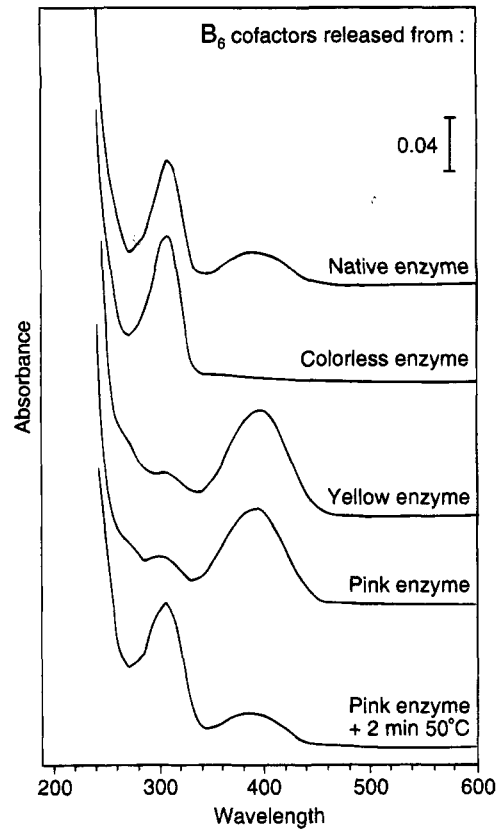

FIGURE 2: Absorption spectra of the vitamin B_6 cofactor released from different spectral forms of GSA-AT by acid. To GSA-AT (2−5 mg of protein) in 0.5 mL of water was added 10 mL of 5% acetic acid. The solution was concentrated to 1 mL in an Amicon Centriprep-10 concentrator, and the filtrate was carefully adjusted to pH 12 with 10 N NaOH and the spectrum determined.

solution, the mass spectra of the native enzyme and yellow enzyme are identical to that obtained from the apoenzyme. ESI mass spectra of the pink and the $NaBH_4$-treated yellow enzymes gave a set of signals in addition to that from the apoenzyme due to the presence of a component with a higher molecular mass (Figure 3D−F). The molecular weights of the protein ions determined from these and other spectra are summarized in Table 1.

Compared to native protein, the protein expressed by the synechoccocal *gsa* gene in the pDS56/RB11 vector is reported to have an N-terminal extension (Grimm *et al.*, 1991). This was determined by N-terminal sequencing to be M-R-G-S-. From the amino acid residues deduced from the cDNA sequence together with the N-terminal extension and assuming no disulfide bonds, the calculated M_r of the apoprotein is 46 401.8 and agrees well with the experimentally determined M_r.

Sodium borohydride treatment of both the native and yellow enzymes resulted in two mass peaks. The ones at 46 402 and 46 406 correspond to the apoenzyme. The others at 46 632 and 46 636 have a mass increase of 230 units, corresponding to one pyridoxal 5′-phosphate bound per protein molecule. The 46 632 peak, observed in the $NaBH_4$-treated native enzyme, was a minor peak compared to the mass peak at 46 402. On the other hand, with the $NaBH_4$-treated yellow enzyme, the peak at 46 636 was larger than the one at 46 406 (Figure 3E). From these results it is concluded that pyridoxal 5′-phosphate is bound by an

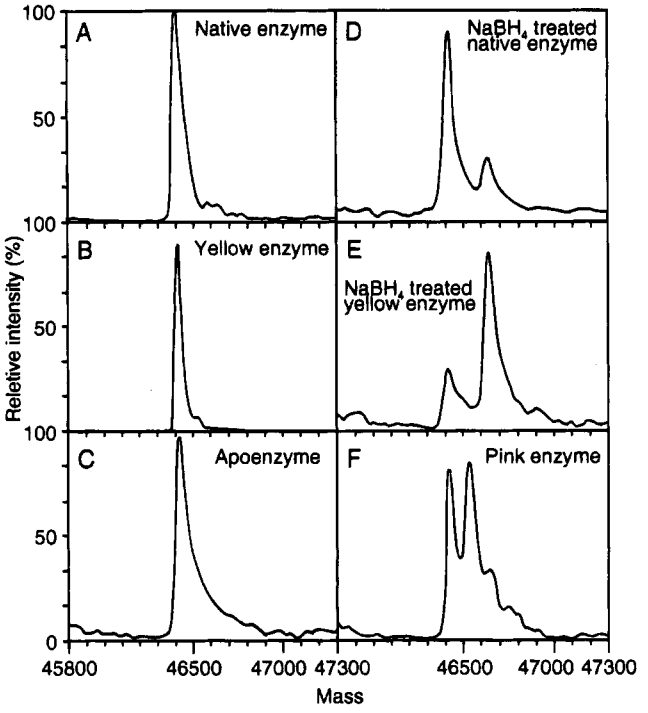

FIGURE 3: Electrospray ionization mass spectra of glutamate 1-semialdehyde aminotransferase. Panels: A, native enzyme; B, yellow enzyme; C, apoenzyme; D, $NaBH_4$-treated native enzyme; E, $NaBH_4$-treated yellow enzyme; F, acetylenic GABA-treated yellow enzyme (pink enzyme).

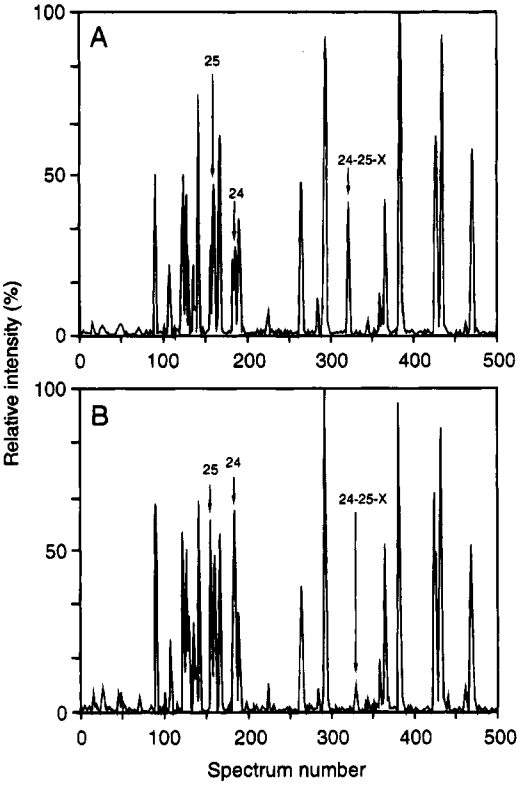

FIGURE 4: On-line liquid chromatography/electrospray ionization mass chromatograms of peptides derived from tryptic digestion of glutamate 1-semialdehyde aminotransferase. Panels: A, $NaBH_4$-treated yellow enzyme; B, acetylenic GABA-treated yellow enzyme. The peaks are marked with the peptide numbers identified from the underlying mass spectra in accordance to Table 2.

unstable Schiff base linkage in the native and yellow enzymes and that $NaBH_4$ reduces the Schiff base, covalently attaching the cofactor to the protein.

In the ESI spectrum of the pink enzyme there is, in addition to a peak at 46 411 ascribed to the apoenzyme, a peak 126 mass units higher (Figure 3F and Table 1). It is concluded that this mass peak is from protein molecules each carrying a single covalently bound molecule of acetylenic GABA. Relative to the apoenzyme peak, the peak at 46 527 was minor in the spectrum of the acetylenic GABA modified, native enzyme.

Previous studies with site-directed mutagenesis have indicated that Lys^{272} (Lys^{276} of the N-terminally extended protein) is the pyridoxal 5′-phosphate binding site (Grimm *et al.*, 1992). To further investigate the binding site of acetylenic GABA and the pyridoxal cofactor on the enzyme, equal amounts of the pink and the $NaBH_4$-reduced yellow enzymes were digested with trypsin, and the peptides were separated and analyzed by on-line liquid chromatography/mass spectrometry. Thirty-seven tryptic peptides are expected from the amino acid sequence deduced from the cDNA for glutamate 1-semialdehyde aminotransferase. Lys^{276} is within the tryptic peptide T24. Modifying it by covalent linkage is expected to prevent the tryptic cleavage at this position, and a new peptide is expected corresponding to T24−25. This will have a molecular mass of T24−25 + the mass of the binding group. As shown in Figure 4 and Table 2, all peptides above mass 400 were detected, providing a good sequence coverage. The N-terminal region was confirmed by detecting the T2 peptide with an M_r of 908.5, corresponding to the peptide ^{2}G-S-V-T-S-S-P-F-K^{11}. Because all five peptides containing cysteine, namely, T10,

Table 1: Absorption Maxima, Enzymatic Activity, and M_r of the Different Forms of Glutamate 1-Semialdehyde Aminotransferase

sample	absorption maximum (nm)	enzymatic activity[a]	M_r[b]	mass diff
native enzyme	280, 338, 418	745	46401 ± 2	
apoenzyme	280	0	46403 ± 4	
GSA-AT treated with				
4,5-dioxovalerate (yellow enzyme)	280, 418	746	46410 ± 3	
4,5-diaminovalerate (colorless enzyme)	280, 338	744	46418 ± 5	
$NaBH_4$, (a) native enzyme	280, 338	694	46402 ± 3 (46632 ± 4)	230
$NaBH_4$, (b) yellow enzyme	280, 338	90	46406 ± 3, 46636 ± 2	230
$NaBH_4$, (c) colorless enzyme	280, 338	740	nd	
acetylenic GABA, (a) native enzyme	280, 338 (560)	582	46401 ± 2 (46527 ± 3)	126
acetylenic GABA, (b) yellow enzyme[c]	280, 560	4	46411 ± 3, 46534 ± 3	123
acetylenic GABA, (c) colorless enzyme	280, 338	743	nd	

[a] The rate of δ-aminolevulinate synthesis in nmol (mg of protein)$^{-1}$ min^{-1}. [b] The M_r is determined by electrospray ionization mass spectrometry. [c] Acetylenic GABA-treated yellow enzyme = pink enzyme. nd, not determined. The M_r's are calculated from the spectra as an average of 10−25 peaks.

Table 2: On-Line Liquid Chromatography/Electrospray Ionization Mass Spectrometry of the Peptides Derived from Tryptic Digestion of the Yellow Form of Glutamate 1-Semialdehyde Aminotransferase Modified with $NaBH_4$ and Acetylenic GABA[a]

			treated with			
			(a) $NaBH_4$		(b) acetylenic GABA	
peptide no.	sequence	M_r calcd	M_r[b]	spectrum no.	M_r[b]	spectrum no.
1	1–2	305.4				
2	3–11	909.0	908.5	108–110	908.5	107–111
3	12–14	360.5				
4	15–24	1079.2	1078.9	122–124	1079.2	122–126
5	25–35	1099.3	1098.9	122–124	1098.8	122–126
6	36–38	364.4				
7	39–49	1174.3	1173.9	141–146	1174.2	141–145
8	50–51	245.3				
9	52–62	1281.3	1281.2	127–129	1280.6	127–129
10	63–87	2740.1	2740.0	261–264	2738.2	262–268
11	88–92	576.7	576.3	46–54	574.9	46–54
12	93–121	3020.5	3020.6	468–472	3019.0	467–474
13	122–135	1497.8	1497.2	189–192	1496.9	189–193
14	136–138	418.6	418.2	46–54	418.9	46–54
15	139–143	566.6	566.5	15–20	566.3	15–19
16	144–145	261.3				
17	146–148	372.5				
18	149–163	1754.0	1753.9	182–183	1753.6	181–183
19	164–181	1622.8	1623.0	166–170	1622.0	165–170
20	182–199	1909.1	1908.8	166–170	1909.2	165–170
21	200–235	3683.2	3681.6	432–435	3682.2	431–437
22	236–255	2335.7	2334.6	425–428	2334.0	424–430
23	256–264	964.1	963.7	90–93	963.6	90–93
24	265–276	1248.4	1247.7	182–187	1248.4	184–188
25	277–290	1258.5	1258.2	156–157	1258.1	155–158
26	291–291	174.2				
27	292–321	3031.6	3031.1	291–294	3031.0	292–297
28	322–327	743.9	744.7	159–163	744.6	159–164
29	328–340	1555.7	1556.3	159–163	1555.6	159–164
30	341–341	174.2				
31	342–382	4273.7	4273.4	364–369	4271.7	364–370
32	383–383	146.2				
33	384–388	589.6	588.1	110–111	589.5	107–111
34	389–391	408.5	408.4	26–34	408.1	25–34
35	392–394	458.5	458.4	26–34	458.2	25–34
36	395–430	3825.2	3822.3	381–389	3824.4	381–390
37	431–436	620.8	620.3	136–137	620.5	135–137
24–25–X[c]	265–290		2719.4	319–322	2616.3	328–331

[a] The standard deviations of the mass determinations were below 0.2 mass unit. [b] The M_r is determined from the selected and averaged mass spectra (spectrum number) extracted from the liquid chromatography/electrospray ionization mass chromatograms shown in Figure 5. The mass analyzer was set to scan from *m/z* 400 to *m/z* 1900. Thus, peptides below *m/z* 400 were not detected. [c] Modified peptide.

T12, T13, T18, and T31, were found in the tryptic digests, it is concluded that there are no disulfide bonds in glutamate 1-semialdehyde aminotransferase. Except for T24, T25, and the additional T24–25–X peptide, the peptide pattern obtained from the pink enzyme was identical to that obtained from the $NaBH_4$-reduced yellow enzyme (Figure 4). The M_r of the peptide T24–25 is 1248.4 + 1258.5 − 18.0 = 2488.9. The T24–25–X peptide obtained from the digest of the $NaBH_4$-reduced yellow enzyme has an M_r of 2719.4 due to covalently bound pyridoxal 5′-phosphate of theoretical mass 230.14. An M_r of 2616.3 was observed for the T24–25–X peptide obtained from the pink enzyme during on-line liquid chromatography/mass spectrometry. This value was confirmed by isolating this peptide and subjecting it to ESIMS (2616.6 by ESMIS). Thus the observed mass for the T24–25–X peptide from the pink enzyme is in close agreement with that calculated (T24–25 + $C_6H_6O_3$ = 2615.0). It is therefore evident that both acetylenic GABA and pyridoxal 5′-phosphate are present covalently bound to Lys^{276} after modification. Observed intensities both in UV light absorption and in ESI signal (Figure 4) for T24 and T25 from the $NaBH_4$-treated yellow enzyme were higher than for the T24 and T25 peptides from the pink enzyme. Accordingly, the intensity was high for the T24–25 + 230.5 peptide, which is found only in the tryptic digest of the $NaBH_4$-treated yellow enzyme, and it was low for the T24–25 + 127.5 peptide, present only in the digest of the pink enzyme. These intensities are in accordance with the relative intensity of the modified forms of GSA-AT observed in the ESI spectra of intact GSA-AT.

In the native and yellow forms of GSA-AT, as with other vitamin B_6 enzymes, the aldehyde group of pyridoxal 5′-phosphate forms a Schiff base with ϵ-NH_2 group of Lys^{276}. Reduction of the Schiff base with $NaBH_4$ results in covalent binding of the cofactor. Acetylenic GABA, by covalent binding, irreversibly inactivates glutamic acid decarboxylase (Jung *et al.*, 1978) and ornithine aminotransferase (De Biase *et al.*, 1991). However, unlike with GSA-AT, the inhibitor binding to these two enzymes does not produce higher absorbance maxima at longer wavelength. The likely reactions of acetylenic GABA with GSA-AT leading to covalent binding are given in Figure 5. Acetylenic GABA

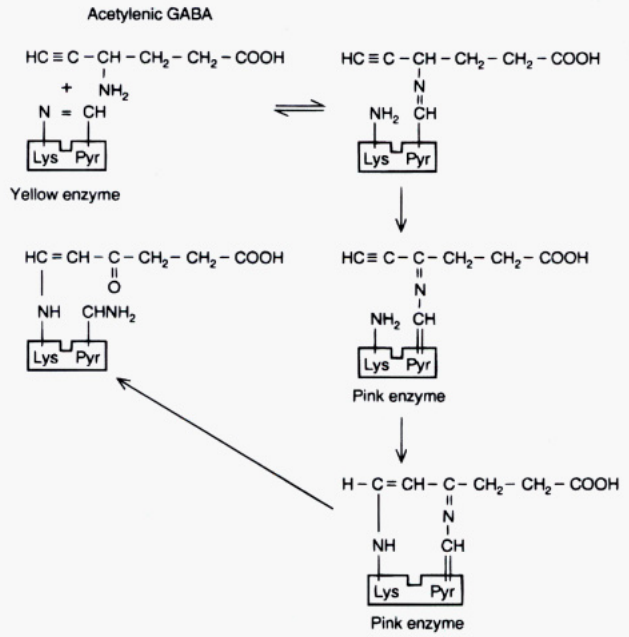

FIGURE 5: Likely mechanism for covalent binding of acetylenic GABA to glutamate 1-semialdehyde aminotransferase.

could attack the yellow enzyme, forming a new Schiff base between the pyridoxal aldehyde and the amino group of acetylenic GABA. A prototropic shift leading to tautomerization would result in the quinonoid structure in the pyridoxal ring and turn the protein pink. Since the double bonds are now conjugated with the triple bond of acetylenic GABA, the quinonoid structure produced here would be considerably more stable than that encountered previously with glutamate 1-semialdehyde (Smith *et al.*, 1991a). The ϵ-NH_2 group of Lys^{276} probably attacks the C6 of acetylenic GABA, establishing a covalent linkage. We think that the decline of the 560 nm absorption maximum of the pink enzyme, especially when it is heated to 50 °C for 2 min, is due to a hydrolysis of the unsaturated bond between the C4 of the acetylenic GABA backbone and its N, releasing pyridoxamine 5′-phosphate and leaving 4-ketohex-5-enoic acid covalently attached to Lys^{276}.

The crystal structures of several aminotransferases have been resolved, and their pyridoxal 5′-phosphate containing pockets show significant structural similarity (Jansonius *et al.*, 1994). In order to investigate the structural features in GSA-AT that lead to the pink, yellow, and colorless forms, computer models were made on the basis of the crystal structure of aspartate aminotransferase (AAT) (McPhalen *et al.*, 1992; Brookhaven Protein Data Bank Accession No. 7aat). The amino acids for the vitamin B_6 binding pocket of AAT were identified on the computer. These amino acids were replaced by the corresponding ones of GSA-AT using the programs Insight II version 2.3.0 and Discover version 3.1 (Biosym). We envisage that in the colorless form of GSA-AT the pyridoxamine 5′-phosphate is held in place only by hydrogen bonds while in the yellow form it is additionally bound to Lys^{276} by a Schiff base (Figure 6). In the color-stable pink form of GSA-AT, we think that acetylenic GABA is bound to pyridoxal 5′-phosphate by a Schiff base and to Lys^{276} by a covalent bond as shown in Figure 6. When acetylenic GABA binds to Lys^{276} and pyridoxal aldehyde, the molecule modeling program predicts that the phosphate side group of the cofactor is displaced too far for it to hydrogen bound to Arg^{287}. However, the carboxylic group of acetylenic GABA is close enough to Arg^{287} to be hydrogen bonded while the phosphate group still might form a hydrogen bond to Thr^{138}. Furthermore, the model predicts that the pyridoxal ring is displaced in the pink enzyme compared to the other forms, but the positively charged nitrogen can still form a hydrogen bond or salt bridge to Asp^{247}, as reported for the corresponding Asp^{222} in aspartate aminotransferase (John, 1995) and thereby stabilizing the whole complex.

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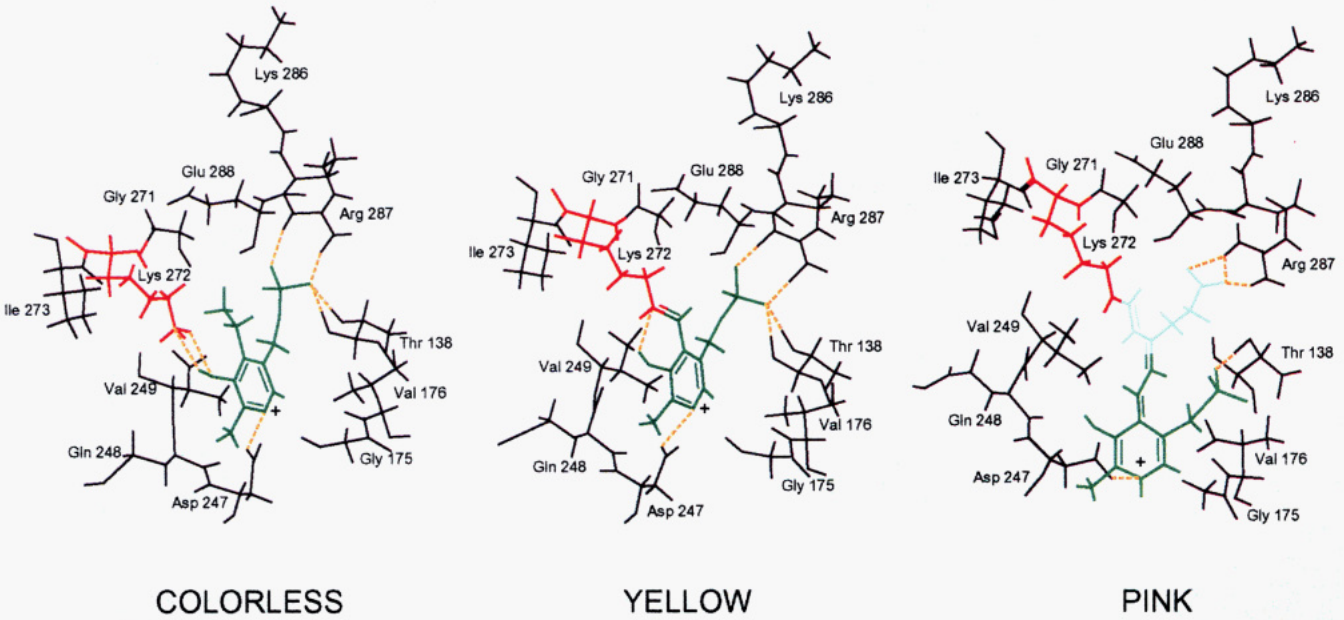

FIGURE 6: Models for the vitamin B_6 containing pockets of colorless, yellow, and pink forms of glutamate 1-semialdehyde aminotransferase. For numbering, the four extra amino acid residues at the N-terminus are not considered. Vitamin B_6, Lys^{272} (Lys^{276} of recombinant protein), and acetylenic GABA are drawn in green, red, and blue, respectively. The nitrogen in the pyridoxal 5′-phosphate is marked (+). Possible hydrogen bonds are indicated with orange dotted lines.

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