Mechanisms of Inactivation of γ-Aminobutyric Acid Aminotransferase by 4-Amino-5-fluoro-5-hexenoic Acid

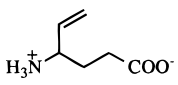
Richard B. Silverman,* Katherine A. Bichler, and Andrew J. Leon

Contribution from the Department of Chemistry and the Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208-3113

Received August 14, 1995[⊗]

Abstract: An investigation of the mechanisms of inactivation of the pyridoxal 5′-phosphate (PLP)-dependent pig brain γ-aminobutyric acid (GABA) aminotransferase by 4-amino-5-fluoro-5-hexenoic acid (**2**), a monofluorinated analogue of the anticonvulsant drug vigabatrin, is described. Inactivation of [^{3}H]PLP-reconstituted GABA aminotransferase with **2** followed by denaturation released the coenzyme in two forms, one as PLP and the other in a modified form in the ratio 7:3. All enzyme activity was lost upon inactivation by **2**, but about 30% of the activity returned upon incubation with PLP, consistent with the formation and release of 30% of the coenzyme in a modified form, as noted above. Inactivation of GABA aminotransferase with [2-^{3}H]-**2** followed by gel filtration resulted in the attachment of 0.7 equiv of tritium to the enzyme, even though complete inactivation occurred. This also is consistent with the above results that about 30% of inactivation is the result of release of a modified coenzyme, leaving 30% of the enzyme as its apoenzyme form. Isolation and mass spectral analysis of the modified coenzyme gave peaks consistent with a modified coenzyme formed from a reaction with the inactivator (**27**). Denaturation of the enzyme containing 0.7 equiv of radioactivity from the above experiment led to release of 0.2−0.3 equiv of the radioactivity as γ-acetyl-GABA (**20**). Treatment of the denatured enzyme with sodium periodate generated 0.2−0.25 equiv of succinic acid, leaving 0.15 equiv of radioactivity still covalently bound to the enzyme. Analysis of amine metabolites shows the formation of 0.5 equiv of **20**. Analysis of the nonamine metabolites resulted in the identification of 1 equiv of 4-oxo-5-hexenoic acid (**24**). After inactivation, 2.6 ± 0.1 equiv of fluoride ions was detected, consistent with the loss of 1 fluoride ion to produce inactivation, 1 fluoride ion to generate the 4-oxo-5-hexenoic acid, and 0.5 fluoride ion released in the production of γ-acetyl-GABA. Normal transamination also occurs; 6.3 ± 0.6 transamination events occurred during inactivation, as measured by the conversion of [^{14}C]-α-ketoglutarate to [^{14}C]glutamate. These results indicate that there are, at least, three different inactivation mechanisms in effect (Schemes 4-6). All of these mechanisms begin with Schiff base formation between **2** and the active site PLP followed by removal of the γ-proton and elimination of the fluoride ion. It is from this conjugated allene intermediate (**17**) that all of the inactivation pathways and metabolites result, except for the normal transamination product. The partition ratio, the amount of inactivator converted to a product per inactivation event, is about 8; 6.5 transaminations, 0.5 conversion to **20**, and 1.0 conversion to **24** per 1.0 inactivation event.

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter throughout the central nervous system.[1] When the concentration of GABA falls below a threshold level in the brain, convulsions begin.[2] These convulsions can be stopped by injecting GABA directly into the brain,[3] but this is not a practical means of controlling seizures. When oral or intravenous injection is attempted, however, a problem arises because GABA does not cross the blood−brain barrier and, therefore, is not an effective anticonvulsant agent.[4] One approach successfully used to raise GABA levels in the brain is the administration of a compound that crosses the blood−brain barrier and inhibits the enzyme responsible for GABA catabolism, namely, the pyridoxal 5′-phosphate (PLP)-dependent enzyme GABA aminotransferase.[5] The epilepsy drug, γ-vinyl-GABA (**1**, vigabatrin),[6] was designed to be converted by GABA



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aminotransferase in the brain into a species that irreversibly inhibited that same enzyme.[7] These types of irreversible enzyme inhibitors are known as mechanism-based enzyme inactivators.[8]

A detailed study of the mechanism of inactivation of GABA aminotransferase by γ-vinyl-GABA revealed that it functioned by two separate inactivation pathways, one leading to covalent attachment to the protein (70−75%) and the other leading to covalent attachment to the active site PLP (25−30%).[9] Because of the success of this inactivator as a drug, a series of fluorinated analogues of γ-vinyl-GABA were designed, synthesized, and

* Author to whom correspondence should be addressed [telephone (847) 491-5653; fax (847) 491-7713; e-mail Agman@chem.nwu.edu].

⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1996.

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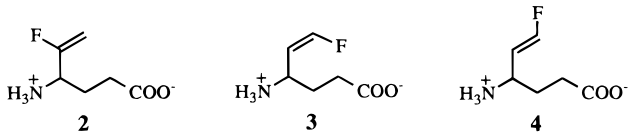
tested as mechanism-based inactivators.[10] Two of the analogues were monofluorinated, namely, 4-amino-5-fluoro-5-hexenoic acid (**2**) and (*Z*)-4-amino-6-fluoro-5-hexenoic acid (**3**). Despite



the minimal difference in the structures of these compounds, they showed vastly different inhibitory properties with GABA aminotransferase. On the basis of one-concentration assays of inactivation,[10] it appeared that whereas **3** was about half as potent as **1**, compound **2** was 1/50th or less as potent as **1**. We have now synthesized the diastereomer of **3**, namely, (*E*)-4-amino-6-fluoro-5-hexenoic acid (**4**), and have found it to be comparable in inactivation potency to **3**. There are many different inactivation mechanisms that can be proposed for these inactivators. In this paper we present our results on the mechanisms of inactivation of GABA aminotransferase by **2**, and in the subsequent paper we show that compound **3** and its isomer (**4**) proceed by inactivation pathways completely different from those of **2**, and, in fact, the inactivation mechanisms of the isomers also are different from each other.

Results

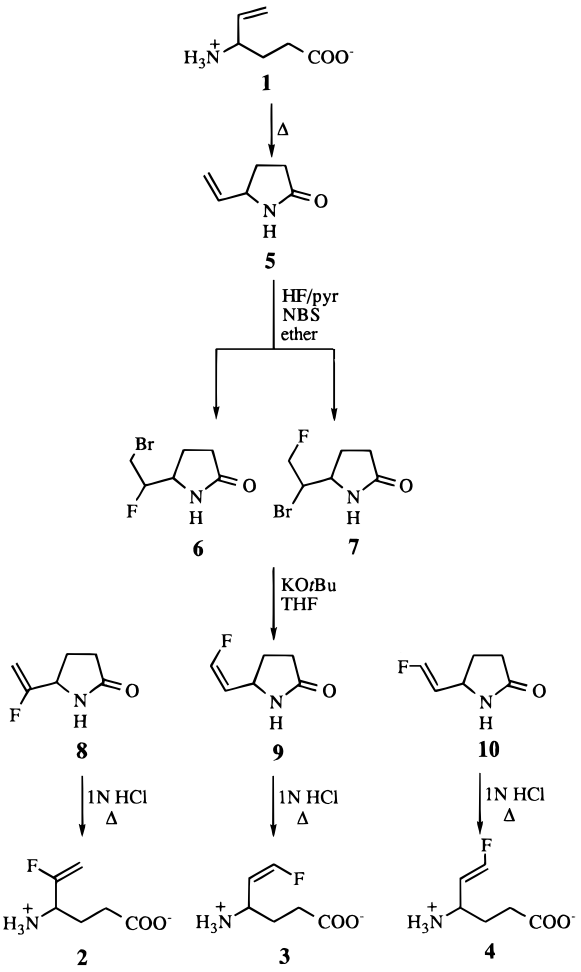
Syntheses of 4-Amino-5-fluoro-5-hexenoic Acid (2), (*Z*)-4-Amino-6-fluoro-5-hexenoic acid (3), and (*E*)-4-Amino-6-fluoro-5-hexenoic acid (4). Our synthetic route to compounds **2**−**4** is a shortened modification of that described by Kolb et al.[10] and is shown in Scheme 1. Kolb and co-workers synthesized 5-vinyl-2-pyrrolidinone (**5**, Scheme 1) by an eight-step reaction sequence; our synthesis was initiated by thermal neat cyclization of γ-vinyl-GABA, obtained as a generous gift from Marion Merrell Dow Research Institute, to the corresponding lactam **5**. The remainder of the synthesis followed that of Kolb et al.[10] with minor modifications. However, upon potassium *tert*-butoxide-induced elimination of HBr, a third isomer not reported by Kolb et al.,[10] (*E*)-(5-fluorovinyl)-2-pyrrolidinone (**10**), was isolated; instead of this isomer, Kolb et al.[10] report the isolation of the double-elimination product, namely, 5-ethynyl-2-pyrrolidinone. Compound **10** is the precursor to (*E*)-4-amino-6-fluoro-5-hexenoic acid (**4**), whose inactivation mechanism is discussed with that of **3** in the subsequent paper.

Synthesis of [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid. The incorporation of tritium into **2** is depicted in Scheme 2. Whereas treatment of **8** with a strong base, such as LDA, caused a large amount of elimination to the corresponding ethynyl lactam, it was found that an equilibrium reaction containing **8**, 3 equiv of a milder base (potassium *tert*-butoxide), and 2 equiv of ^3H_2O, allowed to react for several days, effectively incorporated the tritium label with no detectable elimination of HF.

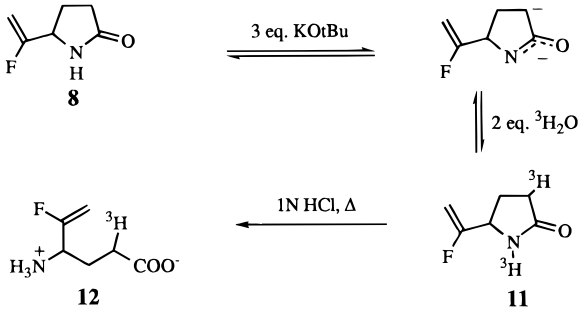
Kinetics of Inactivation of GABA Aminotransferase with 2. The values for K_I and k_{inact}, 9.1 mM and 0.04 min^{-1}, respectively, were determined by a Kitz and Wilson plot[11] (Figure 1).

Inactivation of [^{3}H]PLP-Reconstituted GABA Aminotransferase by 2 with Removal of Excess Inactivator. After control

Scheme 1. Synthesis of Monofluorovinyl GABAs



Scheme 2. Incorporation of Tritium into **2**



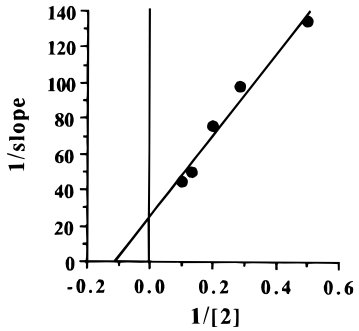


Figure 1. Kitz and Wilson[11] plot for inactivation of GABA aminotransferase by **2**.

radioactivity was subtracted, the **2**-inactivated [^{3}H]PLP-reconstituted GABA aminotransferase released 93% of its cofactor as [^{3}H]PLP. About 6% of the radiolabel remained

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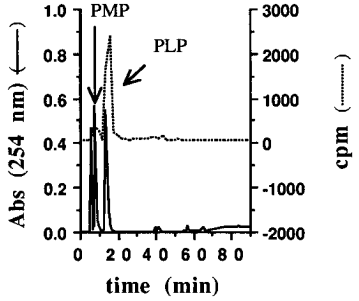


Figure 2. HPLC trace of the inactivation of [^{3}H]PLP-reconstituted GABA aminotransferase by **2** after removal of excess inactivator and denaturation (see Experimental Section for details).

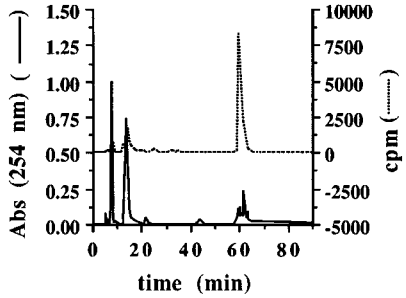


Figure 3. HPLC trace of the inactivation of [^{3}H]PLP-reconstituted GABA aminotransferase by **2** following denaturation without removal of excess inactivator (see Experimental Section for details).

bound to the enzyme and about 1% was released as PMP. The HPLC chromatogram and radioactivity elution are plotted in Figure 2.

Inactivation of [^{3}H]PLP-Reconstituted GABA Aminotransferase by 2 without Removal of Excess Inactivator. It was found that without removal of the excess inactivator a Schiff base complex between the inactivator and PLP formed; under the given elution conditions, the retention time was about 60 min. After control radioactivity was subtracted, the **2**-inactivated enzyme released 93% of its cofactor as a combination of the compounds with peaks at 13 min (PLP) and at 60 min, although the amount of radioactivity in the 60 min peak was at a higher proportion to that in the peak at 13 min than is usually seen in the inactivations with other inactivators under the same conditions (3:1 vs 2:1), indicating the possible presence of another species that elutes at about 60 min (see Identification of the Modified Coenzyme Formed during Inactivation of GABA Aminotransferase by **2**). Three percent of the remaining radioactivity coeluted with PMP and 4% remained bound to the enzyme, as ascertained by counting the radioactivity remaining in the pellet. The HPLC spectrum and radioactivity elutions are shown in Figure 3.

Partial Reactivation of 2-Inactivated GABA Aminotransferase by PLP. GABA aminotransferase was inactivated with **2**. After the excess inactivator was removed, the enzyme had 4% activity. Incubation with PLP resulted in the return of activity of GABA aminotransferase to 32% of the original activity, indicating that about 33% (normalized to account for the 17% loss of enzyme activity in the control over that time period) of enzyme inactivation results from release of a modified coenzyme.

Inactivation of GABA Aminotransferase with 50, 100, and 150 Equivalents of 2. GABA aminotransferase was inactivated with 50, 100, and 150 equiv of **2** to determine whether complete inactivation could be achieved with a concentration of **2** significantly under the K_I of the system. This was desirable because in the experiment in which the amine metabolites are analyzed, excess inactivator cannot be removed. The amine metabolites are analyzed by HPLC of the corresponding dansyl derivatives. Although dansylated **2** and dansylated γ-acetyl-GABA elute by HPLC about 5 min apart under the conditions developed for analysis of these dansylated amines, if the sample has a large excess of inactivator, it is difficult to detect the dansylated γ-acetyl-GABA. Incubation with 50 equiv of **2** inactivates the enzyme 80%. Increasing to 100 equiv improves the inactivation to only 90%, and 150 equiv of inactivator brings the enzyme to 95% inactivated. The sample with 150 equiv is about 1.8 mM **2**. Slightly more concentrated inactivator was used for the upcoming experiments (2.0 mM) to facilitate inactivation without having to use such an excess that the analyses become difficult.

Equivalents of [^{3}H]-2 Bound to GABA Aminotransferase after Inactivation. Inactivation of GABA aminotransferase by [^{3}H]-**2** resulted in 0.7 equiv of inactivator remaining bound to the enzyme following exhaustive dialysis.

Identification of the Modified Coenzyme Formed during Inactivation of GABA Aminotransferase by 2. GABA aminotransferase was inactivated with [^{3}H]-**2** and gel filtered, and excess inactivator was removed by Dowex 50 ion exchange. The nonamine eluant was analyzed by HPLC, and two radioactive peaks were observed, one at about 60 min and one at about 50 min (see Analysis of Nonamine Metabolites Formed during Inactivation of GABA Aminotransferase with [^{3}H]-**2**). The peak at about 60 min contained 0.3 equiv of tritium. The experiment was repeated with nonradiolabeled inactivator, and the fractions eluting from the HPLC at 58−60, 60−62, and 62−64 min were isolated. After lyophilization, FAB^+ (Figure 4) and FAB^- mass spectra were obtained. The major component of the 60−62 min fraction had a mass of 375 Da (FAB^+ gave 376 and FAB^- gave 374; another major peak was at 281 in the FAB^+ spectrum and at 279 in the FAB^- spectrum, the fragment after loss of the phosphate group), consistent with structure **27**.

Equivalents of [^{3}H]-2 Bound to GABA Aminotransferase after Inactivation and Denaturation with Urea. Analysis of urea-denatured [^{3}H]-**2**-inactivated GABA aminotransferase showed that only 0.35 ± 0.05 equiv of labeled inactivator remained bound to the enzyme. This indicates that denaturation of the enzyme with urea releases approximately half of the label that was bound to the enzyme after dialysis.

Analysis of Amine Products Released from GABA Aminotransferase after Inactivation with [^{3}H]-2 and Denaturation with Trichloroacetic Acid. Approximately 0.2−0.3 equiv (three experiments) of radioactivity, based on the specific activity of the inactivator, was released as γ-acetyl-GABA (**20**) from the [^{3}H]-**2** inactivated GABA aminotransferase following trichloroacetic acid denaturation and dansylation (Figure 5).

Sodium Periodate Oxidation of the Stable Adduct Formed after [^{3}H]-2 Inactivation and Urea Denaturation of GABA Aminotransferase. Sodium periodate oxidation of the stable adduct bound to GABA aminotransferase after [^{3}H]-**2** inactivation and denaturation resulted in the release of 0.2−0.25 equiv (three experiments) of tritium as succinic acid; about 0.15 equiv of tritium remained attached to the enzyme (Figure 6). A control, which contained denatured enzyme not oxidized with sodium periodate, retained all of the radioactivity bound to the enzyme directly following the urea denaturation.

Equivalents of [^{3}H]-2 Bound to GABA Aminotransferase after Inactivation and Denaturation with Trichloroacetic Acid. After trichloroacetic acid denaturation of [^{3}H]-**2**-inactivated GABA aminotransferase, 0.2 equiv of the inactivator remained bound to the enzyme.

Analysis of Amine Metabolites Formed during Inactivation of GABA Aminotransferase with [^{3}H]-2. The HPLC

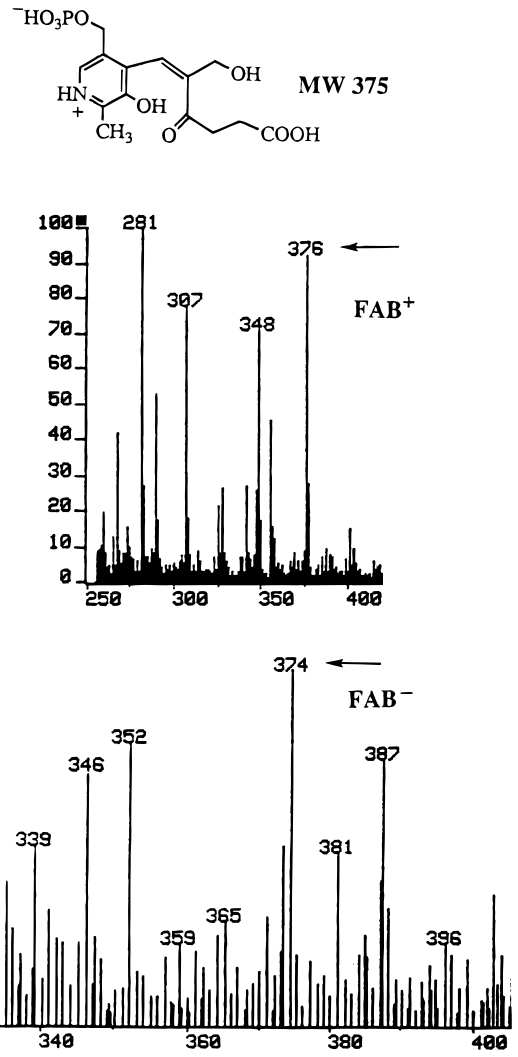


Figure 4. FAB^+ and FAB^- mass spectra of the nonamine eluting on the HPLC at about 60 min (see Experimental Section for details).

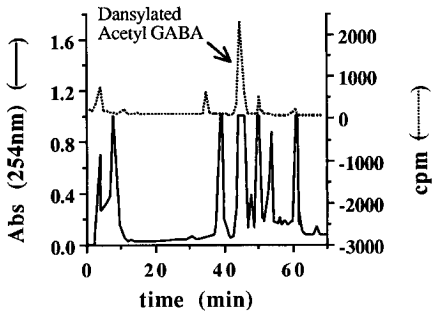


Figure 5. HPLC analysis of dansylated amine metabolites released from the adduct formed by inactivation of GABA aminotransferase by [^{3}H]-**2** (see Experimental Section for details).

spectrum of the amine metabolites released during GABA aminotransferase inactivation with **2** is shown in Figure 7. Because a large amount of [^{3}H]-**2** was necessary for complete inactivation of GABA aminotransferase, the peaks of dansylated **2** were removed from the plot for the amine metabolites to be readily visible. From the amount of radioactivity in the γ-acetyl-GABA peak, it was determined that 0.5 equiv of inactivator was converted to γ-acetyl-GABA during inactivation.

Analysis of Nonamine Metabolites Formed during Inactivation of GABA Aminotransferase with [^{3}H]-2. Analysis was done using the same HPLC conditions used to detect the cofactors released after inactivation of [^{3}H]PLP-reconstituted

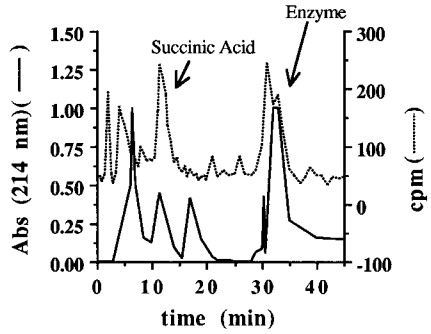


Figure 6. HPLC analysis of the sodium periodate oxidation of the stable adduct formed after [^{3}H]-**2** inactivation and urea denaturation of GABA aminotransferase.

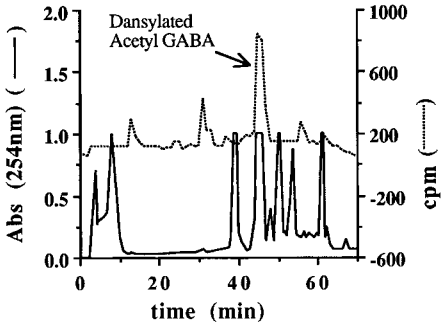


Figure 7. HPLC analysis of dansylated amine metabolites formed by turnover during inactivation of GABA aminotransferase with [^{3}H]-**2**.

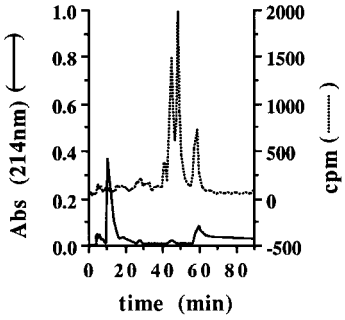


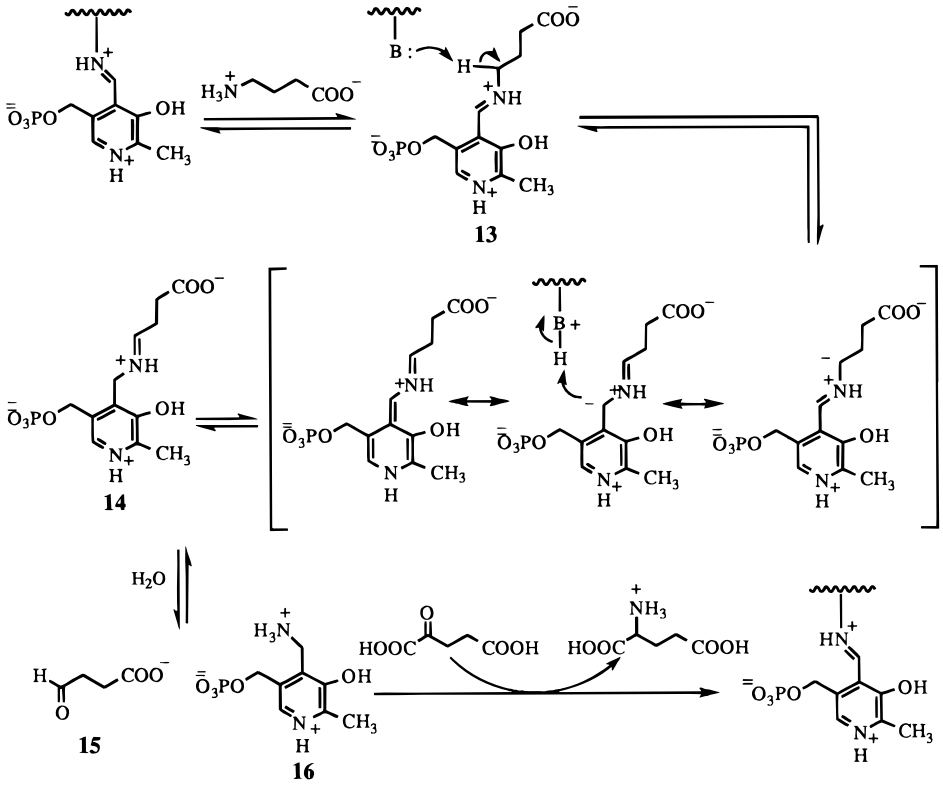
Figure 8. HPLC analysis of nonamine metabolites formed during inactivation of GABA aminotransferase with [^{3}H]-**2**.

GABA aminotransferase by **2** in which excess small molecules were not removed (see Identification of the Modified Coenzyme Formed during Inactivation of GABA Aminotransferase by **2**). A peak corresponding to 0.3 equiv of tritium, eluting at about 60 min, was detected (Figure 8), as was observed in the corresponding experiment with [^{3}H]PLP-reconstituted enzyme (Inactivation of [^{3}H]PLP-Reconstituted GABA Aminotransferase by **2** without Removal of Excess Inactivator). This confirms that the peak at about 60 min is a product containing part or all of PLP and **2** (such as **27**).

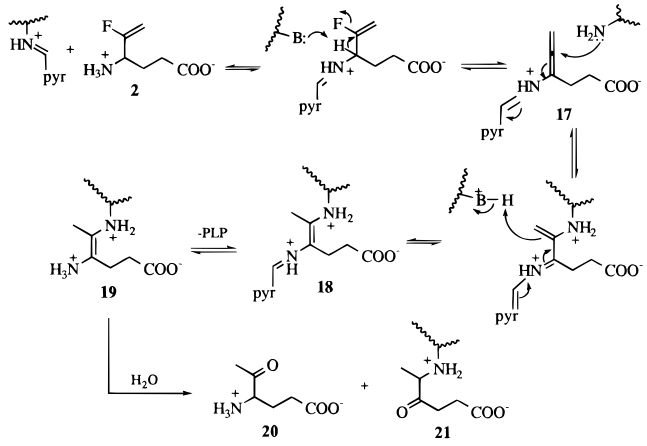
The radioactive peak eluting at approximately 50 min (Figure 8) was shown to comigrate on reversed-phase HPLC with 4-oxo-7-thio-9-hydroxynonanoic acid (the product of Michael addition of β-mercaptoethanol to **24**), synthesized as a standard by incubating 4-oxo-5-hexenoic acid and β-mercaptoethanol in 100 mM sodium phosphate buffer, pH 6.0. High-resolution FAB mass spectral analysis of the standard gave a peak with *m*/*z* of 205.0273, corresponding to 4-oxo-7-thio-9-hydroxynonanoic acid (*m*/*z* 205.0534).

Fluoride Ion Release during Inactivation of GABA Aminotransferase with 2. Inactivation of GABA aminotransferase by **2** was monitored for fluoride ion release. Because of the sensitivity of the system to pH fluctuations, the pH values of

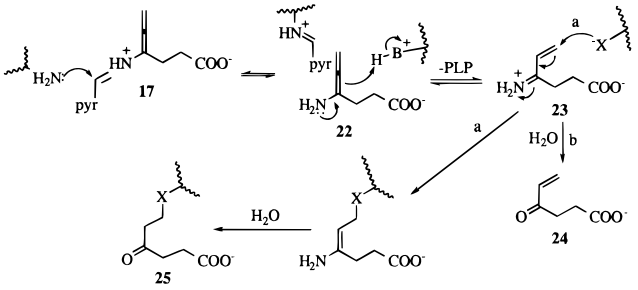
Scheme 3. Catalytic Mechanism for GABA Aminotransferase



Scheme 4. First Potential Mechanism of Inactivation of GABA Aminotransferase by **2**



Scheme 5. Second Potential Mechanism of Inactivation of GABA Aminotransferase by **2**



the inactivated samples and controls were carefully monitored. After correction for the controls, it was found that 2.6 ± 0.1 fluoride ions were released per inactivation event.

Transamination Events per Inactivation of GABA Aminotransferase with 2. GABA aminotransferase, which had been inactivated by **2** in the presence of [5-^{14}C]-α-ketoglutarate, produced 6.3 ± 0.6 equiv of [5-^{14}C]glutamate per inactivation event, which represents the number of transaminations per inactivation event.

Discussion

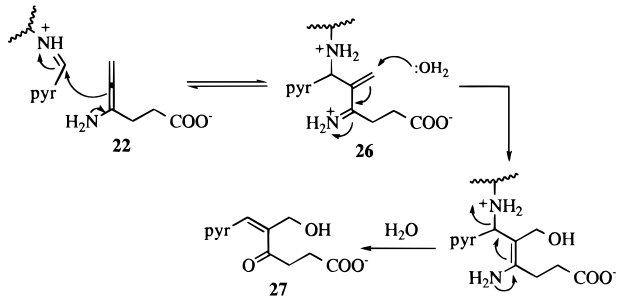
The mechanism of GABA aminotransferase is typical of PLP-dependent aminotransferases (Scheme 3). In the first half-reaction, the lysine-bound PLP undergoes transimination with GABA to give the PLP−GABA Schiff base (**13**). Removal of the γ-proton of substrate followed by reprotonation on the coenzyme gives **14**. Hydrolysis produces succinic semialdehyde (**15**) and pyridoxamine 5′-phosphate (PMP) (**16**), which is converted back to PLP in a second half-reaction involving the concomitant conversion of α-ketoglutarate to glutamate.

With this mechanism in mind, there are several mechanisms that could be written for the inactivation of GABA aminotransferase by **2** (Schemes 4−7 show four possibilities). All of these mechanisms are initiated by the normal catalytic mechanism, namely, Schiff base formation with the PLP followed by γ-proton removal. As shown in Scheme 4, following γ-proton removal is β-elimination of the fluoride ion to give the allene **17**. Nucleophilic addition of an active site lysine residue to the conjugated allene followed by isomerization would give covalent adduct **18**. This is the same adduct that was suggested previously[12] to be the adduct generated from inactivation of GABA aminotransferase by γ-ethynyl-GABA. Upon denaturation of **18**, PLP would be liberated and adduct **19** would be formed. Hydrolysis of **19** would give γ-acetyl-GABA (**20**) and/or covalent adduct **21**. Both of these products were identified as the products of γ-ethynyl-GABA inactivation of GABA aminotransferase.[12]

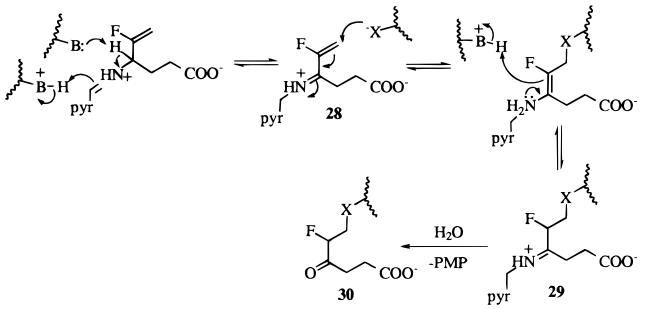
A second mechanism (Scheme 5) is the same as Scheme 4 up to **17**. Instead of nucleophilic addition of the active site

(12) Burke, J. R.; Silverman, R. B. *J. Am Chem. Soc.* **1991**, *113*, 9329−9340.

Scheme 6. Third Potential Mechanism of Inactivation of GABA Aminotransferase by **2**



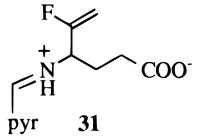
Scheme 7. Fourth Potential Mechanism of Inactivation of GABA Aminotransferase by **2**



lysine to the allene, transimination occurs, leading to enzyme-bound PLP and the alleneamine **22**, which is protonated to the α,β-unsaturated imine **23**. Release of **23** would give 4-oxo-5-hexenoic acid (**24**); nucleophilic addition of an active site residue followed by hydrolysis would lead to covalent adduct **25**. A small amount of this pathway may have occurred in the case of γ-ethynyl-GABA inactivation of GABA aminotransferase.[12]

An alternative to protonation of **22** in Scheme 5 is alleneamine addition to the enzyme-bound PLP, giving **26** (Scheme 6). Addition of water to **26** gives the modified coenzyme **27**. No evidence was found for the formation of this adduct when γ-ethynyl-GABA was the inactivator. A fourth mechanism (Scheme 7) does not involve elimination of fluoride ion at all. Isomerization, as with GABA, gives a potent Michael acceptor (**28**); active site nucleophilic attack would give **29**. Hydrolysis produces PMP and covalent adduct **30**, without loss of fluoride ion. Table 1 summarizes the differences in the results expected for the four mechanisms.

To determine the fate of the coenzyme after inactivation, GABA aminotransferase was reconstituted with [^{3}H]PLP and inactivated with **2**. Following gel filtration and denaturation, HPLC of the supernatant showed that essentially all of the released radioactivity (93% of the total radioactivity) comigrated with [^{3}H]PLP (1% with PMP) (Figure 2); 6% of the radioactivity remained bound to the enzyme. The fact that essentially no PMP is formed by denaturation eliminates the fourth mechanism (Scheme 7). If gel filtration is omitted prior to denaturation, however, almost all of the radioactivity migrates with three peaks; PLP (13 min), the Schiff base of **2** and PLP (**31**) (60 min), and an unknown modified coenzyme (about 60−64 min) (Figure 3). The Schiff base **31** arises when the active site PLP



is released upon denaturation in the presence of the inactivator, which is present because of the omission of the gel filtration step. Compound **31** was easily prepared by the addition of **2** to PLP under the conditions of this experiment. It appears, then, that, although gel filtration does not remove PLP, it releases some modified coenzyme. This suggests that part of the cause for inactivation is simply a function of apoenzyme formation, resulting from release of a modified coenzyme. In fact, following inactivation and gel filtration, 33% of the enzyme activity could be regenerated by incubation with PLP, consistent with the formation of about 0.3 equiv of apoenzyme. The identity of the modified coenzyme is discussed below.

The next phase of the investigation was concerned with the fate of the inactivator after inactivation. Dialysis or gel filtration of GABA aminotransferase that was inactivated with [2-^{3}H]-**2** resulted in no return of enzyme activity, yet only 0.7 equiv of radioactivity was tightly bound to the protein. This is consistent with the formation and release of a modified coenzyme that accounts for the loss of activity of about 0.3 equiv of the enzyme. The regain of about 33% of the enzyme activity upon incubation of the inactive enzyme with PLP is consistent with the attachment of only 0.7 equiv of inactivator to the enzyme, even though complete loss of enzyme activity occurs. Since the release of 0.3 equiv of tritium results regardless of whether [^{3}H]PLP-reconstituted enzyme or [2-^{3}H]-**2** is used suggests that the modified coenzyme is some type of an adduct between PLP and **2**. Isolation and HPLC analysis of the nonamines gave peaks at approximately 50 min (1 equiv), shown to be the β-mercaptoethanol Michael addition product to 4-oxo-5-hexenoic acid (**24**, Scheme 5; see below), and at about 60 min (0.3 equiv). The 0.3 equiv of radioactivity was shown to correspond to the released modified coenzyme by repeating the experiment with unlabeled **2** and isolating the HPLC fractions between 60 and 64 min. Both FAB^+ and FAB^- mass spectrometries were consistent with structure **27** (Scheme 6), lending support to the notion that this modified coenzyme results in inactivation of about 30% of the enzyme molecules. The mechanism shown in Scheme 6 is closely related to the mechanism proposed for the formation of the modified coenzyme when γ-vinyl-GABA inactivates GABA aminotransferase (**32**, Scheme 8).[9] The only difference in the two modified coenzymes (**27** vs **32**) is that **27** has an additional hydroxyl group, as expected, since **2** is one oxidation state higher than **1**.

When GABA aminotransferase, which was inactivated with [2-^{3}H]-**2**, was dialyzed and then acid denatured, only about half of the 0.7 equiv of radioactivity originally bound to the enzyme remained bound. This suggests that at least three different pathways are important to inactivation: one that gives **27** (Scheme 6), one that forms a covalent adduct to the enzyme that is labile to acid denaturation, and one that forms an adduct that is stable to acid denaturation, all in roughly the same amounts.

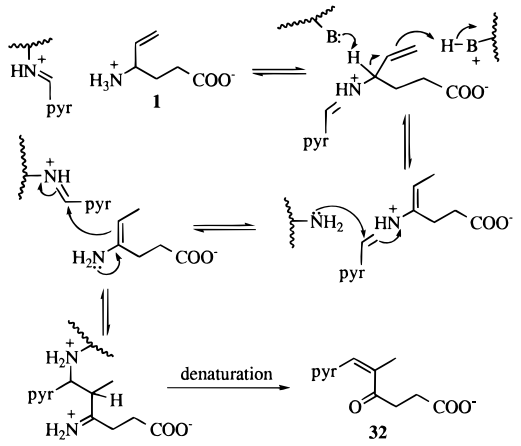
According to the mechanism in Scheme 4, denaturation of the inactivated enzyme should generate γ-acetyl-GABA (**20**). To detect this amino acid, dansylation was carried out prior to HPLC analysis, which showed the formation of 0.2−0.3 equiv of **20** produced upon acid denaturation (Figure 5). Scheme 4 also predicts that, concomitant with the formation of **20**, a stable covalent adduct (**21**) should be formed. Sodium periodate treatment of the labeled covalent adduct obtained after **2** inactivation and gel filtration produced 0.2−0.25 equiv of succinic acid (Figure 6). This result is consistent with the predicted α-amino ketone structure of **21**; sodium periodate is known to oxidize α-amino ketones to carboxylic acids.[13] Since

(13) Moehrle, H.; Haug, W.; Federolf, E. *Arch. Pharm. (Weinheim)* **1973**, *306*, 44−54.

Table 1. Expected Differences in the Inactivation Mechanisms

mechanism	fluoride release	cofactor release	unstable adduct release
β-elimination−Michael addition (Scheme 4)	yes	PLP	γ-acetyl-GABA
β-elimination, then stable adduct (Scheme 5)	yes	PLP	no
cofactor−inactivator complex (Scheme 6)	yes	modified cofactor	release of modified cofactor
isomerization/Michael addition (Scheme 7)	no	PMP	no

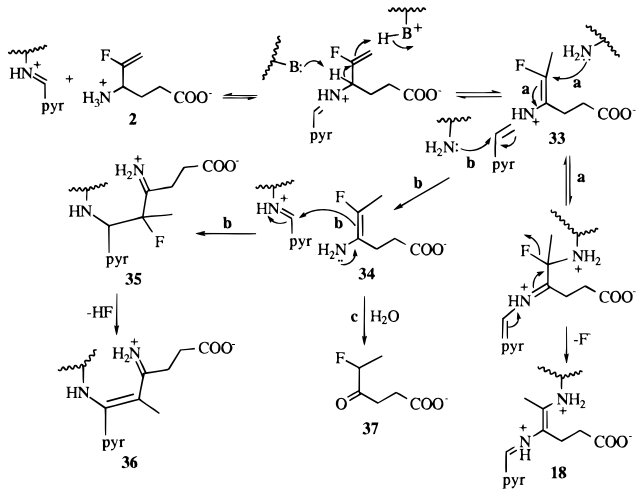
Scheme 8. Proposed Mechanism for the Formation of a Modified Coenzyme upon Inactivation of GABA Aminotransferase by γ-Vinyl-GABA[9]



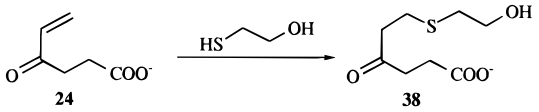
sodium periodate does not oxidize α-keto ethers, α-keto thioethers, or α-keto esters, the amino acid residue attached to the inactivator cannot be serine, threonine, cysteine, aspartate, or glutamate;[14] the only amino acid adduct that is consistent with the formation of succinic acid is one with a lysine residue. The same covalent adduct was suggested as the product of inactivation of GABA aminotransferase by γ-ethynyl-GABA,[12] and sodium periodate oxidation released succinic acid in that case as well. As shown in Figure 6, however, not all of the radioactivity is released by treatment with sodium periodate. Approximately 15% of the radioactivity remains bound. This stable adduct could be the result of addition of another amino acid to the conjugated allene (**17**, Scheme 4) or the product of Michael addition of the lysine residue to **23** (Scheme 5) to give adduct **25**; β-amino ketones are stable to periodate oxidation.[12,13]

The inactivation mechanisms shown in Schemes 4−6 all begin with the elimination of HF. There are many examples of elimination of HCl or HBr from halovinyl compounds to give allenes,[15] but we were unable to find an example of a fluorovinyl analogue undergoing elimination of HF to the allene.[16] The enzyme-catalyzed elimination of HF from (1-fluorovinyl)glycine was proposed as its mechanism of inactivation of alanine racemase[17] and tryptophan synthase;[18] however, no evidence to support elimination of HF was offered. An alternative mechanism, shown in Scheme 9 (pathway a), was suggested in a footnote to a paper.[16,18] Although this mechanism would eventually lead to the elimination of fluoride ion and produce **18**, as in the case of Scheme 4, it cannot rationalize any result that requires an unbound intermediate. For example, the mechanisms shown in Schemes 5 and 6 require the formation

Scheme 9. Potential Mechanism of Inactivation of GABA Aminotransferase by **2** without Initial Elimination of HF



Scheme 10. Reaction of β-Mercaptoethanol with **24**



of allene **22** to produce the observed products **24** and **27**, respectively. These cannot be generated by any of the pathways shown in Scheme 9. Furthermore, two additional products should have resulted if Scheme 9 were relevant. Scheme 9, pathway b, modeled after the mechanism in Scheme 6, would give a stable ternary adduct (**35**); elimination of HF in base would give adduct **36**. Product **27** (Scheme 6), however, was obtained just by gel filtration at neutral pH. Adducts **35** and **36** would be stable under those conditions, and no **27** would be observed. Furthermore, metabolite **24** would not be generated by the mechanism shown in Scheme 9. Although **37** (Scheme 9) was not synthesized as a standard, the following evidence suggests that it is not a metabolite. The metabolite suspected to be **24**, in the presence of β-mercaptoethanol, was converted into the β-mercaptoethanol adduct **38** (Scheme 10). As described under Results, β-mercaptoethanol adduct **38** is the 50 min peak in Figure 8. This is consistent with the formation of **24**, not **37**; compound **37** would not be expected to react with β-mercaptoethanol to give **38**. These results suggest that the mechanism shown in Scheme 9 is not important in the inactivation of GABA aminotransferase by **2**.

To characterize the action of GABA aminotransferase on [^{3}H]-**2** fully, the metabolites generated during inactivation were isolated and characterized. With regard to amine metabolites, dansylation of the amine-containing small molecules isolated by gel filtration and Dowex 50 purification produced one major radioactive peak (and three small peaks). The major peak, determined to contain 0.5 equiv of radioactivity, was identified by HPLC as (dansylated) γ-acetyl-GABA (Figure 7). This could be the product of water addition to the conjugated allene (Scheme 11).

As noted above, the nonamine fraction of metabolites obtained by treatment of GABA aminotransferase with [^{3}H]-**2** gave a

(14) (a) March, J. *Advanced Organic Chemistry*, 3rd ed.; Wiley: New York, 1985; p 1063. (b) Perlin, A. S. In *Oxidation*; Augustine, R. L., Ed.; Dekker: New York, 1969; Vol. 1, pp 189−212. (c) Jackson, E. L. *Org. React.* **1944**, *2*, 341−375. (d) Fatiadi, A. J. In *Synthetic Reagents*, Pizey, J. S., Ed.; Halsted Press: New York, 1981; Vol. 4, p 227.

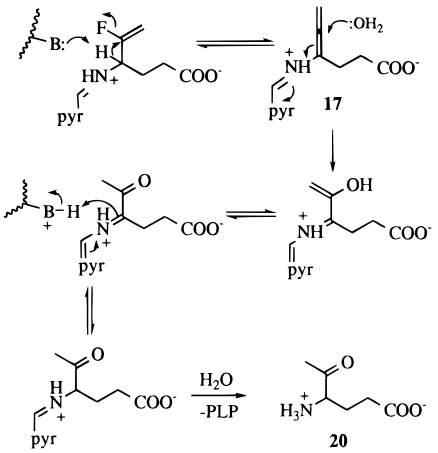
(15) Taylor, D. R. *Chem. Rev.* **1967**, *67*, 317−359.

(16) We thank a reviewer for pointing this out to us.

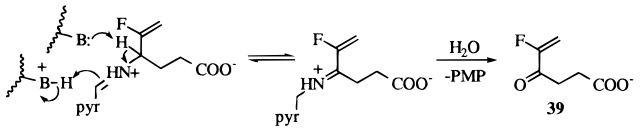
(17) Thornberry, N. A.; Bull, H. G.; Taub, D.; Wilson, K. E.; Gimenez-Gallego, G.; Rosegay, A.; Soderman, D. D.; Patchett, A. A. *J. Biol. Chem.* **1991**, *266*, 21657−21665.

(18) Xu, Y.; Abeles, R. H. *Biochemistry* **1993**, *32*, 806−811.

Scheme 11. Proposed Mechanism for the Formation of γ-Acetyl-GABA during Turnover of **2**



Scheme 12. Transamination of **2**



peak in the HPLC corresponding to the β-mercaptoethanol Michael addition product of 4-oxo-5-hexenoic acid (**38**) equal to about 1 equiv of radioactivity (Scheme 5, pathway b produces **24**, which is converted into **38** by the reaction with β-mercaptoethanol, as shown in Scheme 10).

The inactivation mechanisms in Schemes 4−6 are initiated by a β-elimination of fluoride ion from **2**. Consequently, the release of fluoride ion was measured with a specific fluoride ion electrode. After complete inactivation, 2.6 ± 0.1 fluoride ions were detected in solution. This is consistent with the results obtained: 1.0 equiv of F^- released for inactivation, 0.5 equiv of F^- to generate the γ-acetyl-GABA (**20**) metabolite, and 1.0 equiv of F^- to generate the 4-oxo-5-hexenoic acid (**24**) nonamine metabolite.

Finally, the amount of transamination (normal turnover by azallylic isomerization into the pyridine ring of the coenzyme) of **2** by GABA aminotransferase, presumably to generate 5-fluoro-4-oxo-5-hexenoic acid (**39**) and PMP (Scheme 12), was determined by incubation of the enzyme with **2** in the presence of [^{14}C]-α-ketoglutarate. If PMP is formed, then the second half-reaction of the enzyme will catalyze the conversion of the PMP back to PLP, concomitant with the conversion of the [^{14}C]-α-ketoglutarate to [^{14}C]glutamate. Transamination does not lead to fluoride ion release. [^{14}C]Glutamate (6−7 equiv) was produced, indicating that normal turnover (azallylic isomerization) occurs six to seven times for every two to three times fluoride ion is released (inactivation and metabolite formation all eliminate fluoride ion).

Conclusion

Inactivation of GABA aminotransferase is a multimechanism process; three of the five mechanisms described above (Schemes 4−6) appear to be involved. Appoximately 50% of the total inactivation pathways appear to occur by the mechanism (or a related one) shown in Scheme 4 (**18**). Upon denaturation, **18** decomposes, with release of PLP, to equal amounts of **20** and **21**. About 30% of the total inactivation results from the mechanism (or a related one) depicted in Scheme 6. In this case the coenzyme is modified to **27**, which is loosely bound to the active site, and is released spontaneously, thereby leaving 30% of the enzyme as the apoenzyme. The last 20% or so of the total inactivation comes from a mechanism like that shown in Scheme 5, producing **25**. Because of the stability of this adduct, it is likely that the amino acid to which it is attached is a lysine residue at the active site. In addition to these inactivation pathways, there are several other routes that do not produce inactivated enzyme. Hydrolysis of the purported conjugated allene intermediate (**17**, Scheme 11) appears to occur to produce 0.5 equiv of γ-acetyl-GABA (**20**) (in addition to the 0.2−0.3 equiv of **20** that was generated upon denaturation of **18**). Release and hydrolysis of intermediate **23** (Scheme 5) could be responsible for the formation of 1.0 equiv of **24**, in addition to the 0.1−0.2 equiv of **23** that, presumably, was trapped by the enzyme to produce **25**. Finally, normal transamination generates about 6.5 equiv of product. Therefore, the partition ratio, the amount of inactivator converted to a product per inactivation event, is about 8; 6.5 transaminations, 0.5 conversions to **20**, and 1.0 conversion to **24** per 1.0 inactivation event.

Experimental Section

Analytical Methods. Optical spectra and GABA aminotransferase assays were recorded on either a Perkin-Elmer Lambda 1 or a Beckman DU-40 UV−vis spectrophotometer. ^{1}H NMR spectra were recorded on either a Varian XL-400 400 MHz or a Gemini 300 MHz spectrometer. Chemical shifts are reported as δ values in parts per million downfield from tetramethylsilane (TMS) in $CDCl_3$ or from 3-(trimethylsilyl)propionic acid in D_2O. ^{19}F NMR spectra were recorded on a Gemini 300 MHz spectrometer; chemical shifts are reported as δ values upfield from $CFCl_3$. Coupling constants are reported in hertz. An Orion Research Model 720A pH meter was used with a general combination electrode for pH measurements and with an Orion Research Model 96-09-00 combination fluoride electrode for fluoride ion concentration measurements. For centrifugations, a Beckman Model J-21C centrifuge was used with either a JA-10 or JA-20 rotor, and a Beckman Microfuge B was used for microcentrifugations. Column chromatography was done with either silica gel 60 (230−400 mesh) or silica gel 60H (TLC grade silica), both from Merck. Thin-layer chromatography was performed using Whatman PE SIL/UV silica gel plates with UV indicator. Amines were visualized on TLC plates by dipping the plate in a solution of ninhydrin in *n*-butanol and then heating. Other compounds were visualized with I_2 or phosphomolybdic acid in ethanol followed again by heat. Radioactivity was measured by liquid scintillation counting using a Beckman LS-3133T counter and Fisher ScintiSafe scintillation cocktail. [^{14}C]Toluene (4×10^5 dpm/mL) and [^{3}H]toluene (2.22×10^6 dpm/mL, corrected for first-order decay) from New England Nuclear were used as external standards. Radiopurities of radioactive compounds were assessed by running TLC against a standard compound on a 10 cm plate, scraping the silica off in 5 mm sections, and scintillation counting. HPLC was performed using Beckman 110B pumps in series with a Beckman 163 variable wavelength detector or a Beckman Gold system with a 125P solvent delivery module and 166 variable wavelength UV detector fitted with an analytical flow cell. Microcentrifugation was carried out with a Beckman Microfuge B. Mass spectral analyses were performed by the Analytical Services Laboratory at the Department of Chemistry, Northwestern University, on a Model VG70-50SE high-resolution mass spectrometer and integrated data system from VG Analytical Instruments equipped with both electron impact (EI) and liquid secondary ion mass spectrometry ion sources (LSIMS). Elemental analyses were performed by Oneida Research Services in Whitesboro, NY.

Reagents. The synthesis of 4-amino-5-oxohexanoic acid hydrochloride (γ-acetyl-GABA) was previously reported.[19] 4-Oxo-5-hexenoic acid was synthesized using a modification of the method of Condon et al.[20] 4-Amino-5-hexenoic acid (γ-vinyl-GABA) was obtained as a generous gift from Marion Merrell Dow Research Institute. Dansyl chloride, sodium periodate, potassium carbonate, 1.0

(19) Bullerwell, R. A. F.; Lawson, A.; Morley, H. V. *J. Chem. Soc.* **1954**, 3283−3287.

M potassium *tert*-butoxide in THF, *N*-bromosuccinimide, and HF/pyridine were purchased from Aldrich. Sodium sulfate, phosphoric acid, potassium phosphate, and sodium phosphate were acquired from Fisher. γ-Aminobutyric acid, α-ketoglutarate, β-mercaptoethanol, $NADP^+$, gabaculine, bovine serum albumin (BSA), pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, and potassium pyrophosphate from Sigma were used. Ultrapure urea was a product of ICN Biomedicals. [5-^{14}C]-α-Ketoglutarate and [^{3}H]sodium borohydride were obtained from Amersham. [^{3}H]Water (5 C_i/mL) was a product of New England Nuclear. Dowex 50 and Dowex 1 resins from Bio-Rad Laboratories were utilized. GABAse was purchased from Boehringer Mannheim. HPLC grade methanol was obtained from Mallinckrodt and was filtered and degassed before use. Doubly distilled deionized water was also filtered and degassed before use as a HPLC solvent. Tetrahydrofuran and ether were distilled over sodium with benzophenone ketyl indicator under nitrogen directly before use.

5-Vinyl-2-pyrrolidinone (5). 4-Amino-5-hexenoic acid (**1**, 4.5 g, 34.8 mmol) was heated under argon in a pressure tube until it changed from a white solid to a brown oil (180 °C). This oil was taken up in chloroform and purified on a silica gel column (230−400 mesh, 5 g) using ethyl acetate as solvent. Fractions containing a compound with an R_f value of 0.30 (ethyl acetate) were combined, and the solvent was evaporated, yielding 3.66 g (95%) of 5-vinyl-2-pyrrolidinone (**5**) as a clear colorless oil: ^{1}H NMR ($CDCl_3$) δ 7.55 (s, 1 H, CONH), 5.75 (ddd, 1 H, C*H*CH$_2$, J_{HHcis} = 10.3 Hz, $J_{HHtrans}$ = 17 Hz, J_{HH} = 6.7 Hz), 5.17 (dd, 1 H, CHC*H*$_2$, J_{HHgem} = 1.0 Hz, $J_{HHtrans}$ = 17 Hz), 5.05 (dd, 1 H, CHC*H*$_2$, J_{HHgem} = 1.0 Hz, J_{HHcis} = 10.3 Hz), 4.10 (m, 1 H, CHN), 2.25 (m, 3 H, CH*H*C*H*$_2$CO), 1.75 (m, 1 H, C*H*HCH$_2$CO).

5-(1(or 2)-Bromo-2(or 1)-fluoroethyl)-2-pyrrolidinone (6 or 7). *N*-Bromosuccinimide (6.8 g, 38.2 mmol) was dissolved under nitrogen in 30 mL of ether in a 50 mL plastic centrifuge tube equipped with a septum and a line to N_2. To this stirred solution was added 33 mL of HF/pyridine (70%, 1.16 mol). The mixture was stirred in an ice bath for 15 min, at which time 3.66 g (32.9 mmol) of **5** in 3 mL of ether was added slowly. The reaction was allowed to warm to room temperature over 30 min. After being stirred at room temperature for 2 h, the mixture was poured into ice−water containing K_2CO_3 (79.8 g, 0.578 mol). The reaction mixture was extracted four times, each with 300 mL of ether, and then the water layer was subjected to continuous extraction with ether for 6 h. The combined ether layers from these extractions were dried over Na_2SO_4. The ether was evaporated on a rotary evaporator and the remaining yellow oil was chromatographed on a 15 g silica gel column (230−400 mesh) to separate the succinimide. The product (3.30 g, 15.7 mmol, 48% yield) was obtained as a 1:3 mixture of 5-(2-bromo-1-fluoroethyl)-2-pyrrolidinone (**6**) and 5-(1-bromo-2-fluoroethyl)-2-pyrrolidinone (**7**):[10] ^{1}H NMR ($CDCl_3$) δ 4.65 (m, 2 H from **7**), 4.1 (m, 2 H from **6**, 2 H from **7**), 3.5 (m, 2 H from **6**), 2.4 (m, 3 H from **6**, 3 H from **7**), 2.0 (m, 1 H from **6**, 1 H from **7**).

5-(1-Fluorovinyl)-2-pyrrolidinone (8). The mixture of **6** and **7** (3.30 g, 15.7 mmol) in 20 mL of THF was cooled to −78 °C in a dry ice/acetone bath, and 63 mL of 1.0 M potassium *tert*-butoxide in THF was added dropwise. The reaction was allowed to warm to −30 °C and was kept between −30 and −20 °C for 2 h. It was then cooled back down to −78 °C, and 2.7 mL of acetic acid was added followed by 15 mL of ether. The precipitate was removed by filtration and washed with ether. Solvents were evaporated, leaving 1.93 g (95% yield) of a 2:5:1 mixture of 5-(1-fluorovinyl)-2-pyrrolidinone (**8**), (*Z*)-5-(fluorovinyl)-2-pyrrolidinone (**9**), and (*E*)-5-(fluorovinyl)-2-pyrrolidinone (**10**). The mixture was column chromatographed using 15 g of TLC grade silica gel and a 5:1 ethyl acetate/hexane solvent system. The first compound to elute off the column, with an $R_f \cong 0.30$, was **8**. Several of these columns were necessary, with 100 mg of the mixture of (fluorovinyl)pyrrolidinones applied each time, and then repeat columns were run on the fractions that contained mixtures of products. The three compounds have R_f values very close to one another, and analysis of the column fractions was done by removing the solvent by rotary evaporation and checking for purity by NMR. At the end of the chromatography, 132 mg of pure **8**,[10] a light yellow oil, was obtained (27% yield): ^{1}H NMR ($CDCl_3$) δ 4.71 (dd, 1 H, cis CFCH$_2$, J_{HH} = 3.5, J_{HF} = 16), 4.54 (dd, 1 H, trans CFCH$_2$, J_{HH} = 3.5, J_{HF} = 48), 4.2 (m, 1 H, CHN), 2.4 (m, 4 H, CH_2CH_2); ^{19}F NMR ($CDCl_3$) δ −111 (ddd, 1 F, J_{HF} = 16, 48, 16); high-resolution electron impact mass spectrometry calcd for C_6H_8FNO 129.0590, found 129.0593. Isolation of **9** and **10** is described in the subsequent paper.

After 4 months of storage in a desiccator, the 132 mg of **8** was taken up in ethyl acetate. A solid precipitated from the solution which was filtered, leaving, after rotary evaporation of solvent, 95 mg of a yellow oil, which was further purified by column chromatography with 15 g of TLC grade silica gel and a 5:1 ethyl acetate/hexane solvent system, giving **8** (58 mg) as a light yellow oil.

4-Amino-5-fluoro-5-hexenoic Acid (2). Compound **8** (79 mg, 0.61 mmol) was heated in 1 N HCl to 85 °C for 19 h, and then the water was evaporated. The residue was dissolved in water, and the water was evaporated. This dilution/evaporation process was repeated twice more. The resulting brown semisolid was triturated with ether, leaving 83 mg of a light brown powder. Attempts to recrystallize this solid with 2-propanol/ether met with no success. The solid remaining after recrystallization attempts was purified on a 5 × 70 mm Dowex 50 column, using 1 N NH_4OH as eluent. Ninhydrin positive fractions were combined and lyophilized, leaving 29 mg of a light brown solid[10] (33% yield): ^{1}H NMR (D_2O) δ 5.04 (dd, 1 H, cis CH$_2$CF, J_{HF} = 17.3, J_{HH} = 4), 4.87 (dd, 1 H, trans CH$_2$CF, J_{HF} = 50, J_{HH} = 4), 4.07 (dt, 1 H, NCHCF, J_{HF} = 23, J_{HH} = 8), 2.52 (m, 2 H, CH_2COOH), 2.10 (m, 2 H, $NCHCH_2$); ^{19}F NMR (D_2O) δ −114 (ddd, J_{HF} = 50, 23, 17); high-resolution electron impact mass spectrometry calcd for $C_6H_{11}FNO_2$ 147.0696, found 147.0699.

[^{3}H]-5-(1-Fluorovinyl)-2-pyrrolidinone. To 47.5 mg of 5-(1-fluorovinyl)-2-pyrrolidinone (**8**) in THF under nitrogen was added 17 μL of ^3H_2O (5 Ci/mL, corrected for decay, 2 equiv). Potassium *tert*-butoxide in THF (1.1 mL, 1.0 M, 3 equiv) was added, which turned the reaction solution cloudy yellow. The reaction mixture was allowed to stir for 8 days, and then another 17 μL of ^3H_2O was added. The solution was stirred for another hour, and then it was quenched with 3 drops of glacial acetic acid. The solid was removed by filtration and rinsed with THF (1 mL). Solvent was evaporated by bulb-to-bulb distillation in a closed system. The remaining semisolid was dissolved in water and evaporated by bulb-to-bulb distillation two times. The result was 7.1 mg of **11** as a gold solid in a 15% yield. TLC on silica gel utilizing ethyl acetate as the mobile phase showed that the radioactivity comigrated with 5-(1-fluorovinyl)-2-pyrrolidinone.

[^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid (12). [^{3}H]-5-(1-Fluorovinyl)-2-pyrrolidinone (7.1 mg, 0.055 mmol, **11**) was dissolved in 5 mL of 1 N HCl and was stirred at 84 °C for 17 h. The residue was dissolved in 2 mL of water and the water was evaporated. Additional water was added and evaporation was repeated. The product was then dissolved in 1 mL of water, 1 mL of benzene was added, and the solvent was evaporated again. The resulting brown semisolid was purified on a 5 × 70 mm Dowex 50 column, eluting with 1 N NH_4OH. Fractions were counted, and those containing radioactivity were combined and evaporated, leaving 4.4 mg of an off-white solid (**12**, 54% yield). The compound was found to have a specific activity of 9.7 mCi/mmol and a radiopurity of 97%. The radioactivity comigrates with 4-amino-5-fluoro-5-hexenoic acid on silica gel with a 3:1:1 *n*-BuOH/HOAc/H_2O solvent system. Some unlabeled **2** was added to increase the amount of product and to lower the specific activity. This mixture was dissolved in water and run over a 5 × 70 mm Dowex 50 column, resulting in [^{3}H]-**2** (**12**), which is 97% radiopure and has a specific activity of 3.3 mCi/mmol.

[7-^{3}H]Pyridoxal 5′-phosphate. Tritiated pyridoxal 5′-phosphate ([^{3}H]PLP) was synthesized using a variation on the method of Stock et al.[21] PLP (140 mg) was dissolved in 1.8 mL of water and 20 drops of 1 M sodium hydroxide. The solution was protected from light and cooled to 0 °C. Sodium [^{3}H]borohydride (100 mCi, 11.6 Ci/mmol) was dissolved in 450 μL of a 0.3 M solution of sodium borohydride in 0.1 M NaOH. This was added to the cold PLP solution and was stirred for 20 min. Another 2.5 mg of unlabeled sodium borohydride was added in an attempt to remove all of the yellow color from the solution. This solution was then stirred at 0 °C for another 20 min. The solution

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remained very light yellow, however, and the reaction was quenched with 120 μL of concentrated HCl.

At this point, 74 mg of manganese dioxide, which had been purified and dried as described by Silverman and Invergo,[22] was added. The solution was stirred at room temperature for about 2 h with no visible change. The solution was divided into two centrifuge tubes and brought to pH 8 with 1 M NaOH. After centrifugation with a table-top centrifuge, the pellet was dark brown and the supernatant dark yellow. The supernatant was collected, and the pellet was rinsed six times with water. All of the supernatants were combined, and the solvent was removed by bulb-to-bulb distillation in a closed system.

The [^{3}H]PLP was purified over a Dowex 1 column that had been cleaned by washing with 5 M acetic acid, 1 M NaOH, and 5 M acetic acid once more. Between each of these washes, the column was washed with water until the eluate was neutral. Finally, the column was equilibrated in 4 M sodium acetate. The [^{3}H]PLP solution was applied to the column and was eluted with 10 mL of water, followed by a gradient of 250 mL of water and 250 mL of 5 M acetic acid. A peristaltic pump was attached to the column and run at 1.4 mL/min while 10 min fractions were collected. The fractions that were radioactive and yellow were combined, and the solvent was removed by lyophilization.

Just before use, the [^{3}H]PLP was purified using three HPLC systems. All utilized a C_{18} semipreparative HPLC column with solvent flowing at 1.0 mL/min. Detection was at 254 nm. The first purification was done using H_2O/0.1% TFA as the mobile phase. Five milliliter fractions were collected; those containing radioactivity and coeluting with authentic PLP were combined, and the solvent was removed by lyophilization. The second column was run with 100 mM potassium phosphate, pH 7.0, as the mobile phase. Fractions of 1.0 mL were collected, and those coeluting with unlabeled PLP and containing radioactivity were combined and lyophilized. Finally, desalting was carried out with H_2O/0.1% TFA as eluant. This purified [^{3}H]PLP was then used immediately in the reconstitution of apoGABA aminotransferase (vide infra).

Enzymes and Assays. GABA aminotransferase was isolated from pig brains as described by Churchich and Moses.[23] Succinic semialdehyde dehydrogenase (SSDH) was isolated from GABAse, a commercially available mixture of SSDH and GABA aminotransferase, by inactivation of the GABA aminotransferase with gabaculine as described previously.[24] Protein assays were carried out using BSA and Pierce Coomassie protein assay reagent for standard curves. All buffers and solutions were prepared in distilled deionized water. GABA aminotransferase activity assays were carried out using a modification of the coupled assay developed by Scott and Jakoby.[25] The assay solution contained 11 mM GABA, 5.3 mM α-ketoglutarate, 1.1 mM $NADP^+$, and 5 mM β-mercaptoethanol in 50 mM potassium pyrophosphate, pH 8.5. Excess SSDH was used for the assays. Using this assay, the change in absorbance at 340 nm indicates production of NADPH which is directly proportional to the activity of GABA aminotransferase.

Kinetics of Inactivation of GABA Aminotransferase by 2. GABA aminotransferase (15 μL, 0.23 nmol) was diluted 10-fold by addition to 135 μL of solutions containing various concentrations of **2**: 1.0, 2.0, 3.5, 5.0, 7.5, and 10.0 mM. The solutions were incubated in a water bath at 25 °C, and assays were performed at various time points until the sample was less than 30% active. The log(% activity) was plotted against time for each inactivator concentration; then the inverse slopes from these plots were plotted against the inverse of the concentration. Using a Kitz and Wilson[11] plot of this sort, the K_I and k_{inact} values were extrapolated.

Formation of apoGABA Aminotransferase. GABA aminotransferase was incubated with 120 mM GABA in 100 mM potassium phosphate, pH 7.4, at room temperature and protected from light for 30 min. Monobasic potassium phosphate was added to a concentration of 0.5 M. This solution was dialyzed against 2 L of 0.5 M KH_2PO_4 for 2 h. An assay showed less than 1% activity remaining. The enzyme solution was then dialyzed against 4 L of 100 mM potassium phosphate, pH 7.0, for 4 h to remove the PLP that had been released from the active site of the enzyme.

Reconstitution of apoGABA Aminotransferase with [^{3}H]PLP. The apoGABA aminotransferase prepared as described above was incubated with a 30-fold excess of [^{3}H]PLP until maximum activity returned. Assays were performed every hour to monitor the return of activity, which generally took about 4 h. About 60% of the activity returned. The reconstituted enzyme was isolated from the excess [^{3}H]-PLP by running 250 μL portions over Sephadex G-50 using the Penefsky spin method[26] in a 3 cm^3 syringe fitted with a polyethylene frit and spun in a table-top centrifuge at mach 3 for 1.5 min. The columns were rinsed with 100 μL of 100 mM potassium phosphate at pH 7.4. All of the enzyme samples and rinses were combined and dialyzed against 4 L of 100 mM potassium phosphate, pH 7.4, overnight and used in experiments the next day to avoid decomposition of the [^{3}H]PLP.

Inactivation of [^{3}H]PLP-Reconstituted GABA Aminotransferase by 2 with Removal of Excess Inactivator. GABA aminotransferase, which had been reconstituted with [^{3}H]PLP, was incubated at 25 °C and protected from light in 100 mM potassium phosphate containing 9.0 mM **2**, 5.0 mM α-ketoglutarate, and 5 mM β-mercaptoethanol at pH 7.4. A control was run with the same concentrations of each reagent, excluding inactivator. After the enzyme was greater than 95% inactivated, excess small molecules were removed by running the solutions over Sephadex G-50 using the Penefsky spin method. The pH of each solution was adjusted to 11−12 using 1 M KOH. These were incubated at room temperature for 1 h and then added to enough TFA to quench the base and make a 10% v/v TFA solution. After being allowed to stand at room temperature for 10 min, the denatured enzyme solutions were microcentrifuged for 5 min. The supernatants were collected individually, 75 μL of 10% TFA was added, and the solution was microcentrifuged for another 5 min to rinse the pellet. This was repeated three times, and the rinses were added to the supernatants, which were freeze-dried. Analysis of the cofactors was carried out by dissolving the resulting solid in 100 μL of water and adding 20 μL of a PLP/PMP standard, which had also undergone the basification and acidification steps, and then injecting the samples onto a C_{18} reversed-phase HPLC column. The mobile phase was H_2O with 0.1% TFA flowing at 0.5 mL/min for 15 min. The flow rate was increased to 1.0 mL/min from 15 to 20 min, and then a solvent gradient into 100% methanol was run over the next 60 min. Under these conditions, PLP elutes at 13 min and PMP at 7.5 min. Fractions were collected every minute, and the elution of radioactivity was followed by liquid scintillation counting.

Inactivation of [^{3}H]PLP Reconstituted GABA Aminotransferase by 2 without Removal of Excess Inactivator. The same experiment described above was run with no attempt to remove excess small molecules. In this experiment, both the inactivation and the control were run as above; however, a second control was run with 40 mM GABA containing no inactivator or α-ketoglutarate. The first control should release the cofactor as PLP, while the second should release PMP. When the enzyme in the inactivator solution was less than 5% active, the solutions were immediately adjusted to pH 11−12. Purification over Sephadex was omitted. The rest of the experiment was identical to that described above. HPLC analysis was done using water containing 0.1% TFA flowing at 0.5 mL/min for 20 min. The flow rate was changed to 1.0 mL/min over the next 5 min; then a 15 min gradient to 15% methanol was run. Elution at 15% methanol was continued for 10 min, and then the column was washed with methanol by changing the solvent to 100% methanol over 2 min. Fractions were collected for liquid scintillation counting every minute for 90 min.

Partial Reactivation of 2-Inactivated GABA Aminotransferase by PLP. GABA aminotransferase (2.1 mg) was incubated with **2** (2 mM) in 2.03 mL of 100 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM α-ketoglutarate and 7.0 mM β-mercaptoethanol at 25 °C in the dark. A control containing no inactivator also was prepared. The sample containing **2** had only 10% of the original activity remaining after 4 h and 4% after 22 h. The control containing GABA aminotransferase with no **2** retained 100% of its original activity over 4 h and 83% after 22 h. The small molecules were separated from

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enzyme by the Penefsky spin method.[26] The effluents were collected and assayed for activity. The inactivation sample contained 4% of its original activity, while the control remained at 83% its original activity (on the basis of 83% activity in the control, the inactivated sample was normalized to 5% active). The effluent of the inactivation sample was then made 1.0 mM in PLP by addition of solid PLP, and the sample was incubated in the dark for 30 min at ambient temperature.

Schiff Base Adduct Formed between PLP and 2. A 50 μL solution containing 20 mM PLP and 100 mM **2** in 100 mM potassium phosphate, pH 7.4, was incubated at room temperature for 1 h. The pH was adjusted to 11−12 with 1 M KOH (5 μL), and the solution was incubated for another hour at room temperature protected from light. Enough TFA to quench the base and form a 10% v/v TFA solution was added (5.5 μL). The solution was microcentrifuged for 5 min, and then the solvent was removed by lyophilization. The resulting solid was dissolved in 100 μL of H_2O with 0.1% TFA and injected onto the HPLC with a H_2O/0.1% TFA solvent system, flow rate 0.5 mL/min. After 20 min, the flow rate was changed to 1.0 mL/min over 5 min; then a gradient to 15% methanol was run over the next 15 min. The column was eluted with 15% methanol for another 10 min, and then the solvent was changed to 100% methanol over 2 min and the analysis continued for another 40 min.

Inactivation of GABA Aminotransferase with 50, 100, and 150 Equivalents of 2. Solutions were made containing 77 μL of concentrated GABA aminotransferase (1.16 nmol) and 18 μL of a solution of **2** (3.3, 6.6, or 10 mM) in 100 mM potassium phosphate containing 0.5 mM α-ketoglutarate and 5 mM β-mercaptoethanol, pH 7.4. The solutions contained 50, 100, and 150 equiv of **2**, respectively. These solutions were monitored for loss of GABA aminotransferase activity at various time points.

Inactivation of GABA Aminotransferase by [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid. In a typical experiment, GABA aminotransferase was incubated with 2.5 mM [^{3}H]-**2**, 0.5 mM α-ketoglutarate, and 5 mM β-mercaptoethanol in 100 mM potassium phosphate for 21 h. After the enzyme was less than 3% active, it was dialyzed four times against 700 mL of 100 mM potassium phosphate, pH 7.4, at which point no additional radioactivity was removed from the enzyme solution as monitored by liquid scintillation counting of 5 mL of each dialysate.

Equivalents of [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid Bound to GABA Aminotransferase after Inactivation. GABA aminotransferase was inactivated and dialyzed as described under *I*nactivation of GABA Aminotransferase by [3H]-4-Amino-5-fluoro-5-hexenoic Acid. A 100 μL aliquot of this sample was removed and analyzed for equivalents of inactivator bound to GABA aminotransferase after inactivation. The protein concentrations of 30 μL of the inactivated enzyme sample and BSA standards were determined in 100 mM potassium phosphate, pH 7.4. The sample remaining after the protein assay (about 65 μL of the inactivated enzyme solution) was counted. Equivalents of inactivator bound to the enzyme were calculated as the ratio of millimoles of inactivator in the sample (determined by scintillation counting and the specific activity of the inactivator) to the millimoles of enzyme in the sample (determined by the protein assay).

HPLC and Mass Spectrometric Analysis of the Modified Coenzyme (27) Formed during 2 Inactivation of GABA Aminotransferase. GABA aminotransferase (0.32 mg) was inactivated with **2** (2 mM) in a total volume of 303 μL of 100 mM potassium phosphate buffer, pH 7.4, containing 0.5 mM α-ketoglutarate and 7.0 mM β-mercaptoethanol at 25 °C in the dark along with a control containing no inactivator. The enzyme samples were placed into Centricon 10 microconcentrators and were centrifuged to dryness. The effluents were applied to eight prewashed 5 × 70 mm Dowex 50W-X8 columns. Each column was eluted with 5 mL of deionized water, and the effluents were collected, frozen, and lyophilized. The resulting residue was taken up in 200 μL of deionized water with 0.1% TFA and injected onto an Alltech Econosil C_{18} column (4.6 × 250 mm, 10 μm). The sample was eluted with a 0.1% TFA in water/methanol gradient (0% MeOH for 20 min followed by an increase to 15% MeOH over the next 15 min, elution at 15% methanol was continued for 10 min, and then 100% MeOH over the next 2 min) at a flow rate of 0.5 mL/min for the first 20 min and then 1.0 mL/min for the duration of the run with detection at 214 nm. Fractions were collected every 2 min and then were stored at −78 °C until ready for analysis by mass spectrometry. Prior to mass spectral analysis, the 62−64 min fraction was lyophilized to dryness and then was taken up in 30 μL of 0.1% TFA in water and lyophilized to dryness again. The fraction from 62−64 min, which during previous experiments with [^{3}H]-**2** contained substantial amounts of radioactivity, was analyzed by mass spectrometry using both FAB^+ and FAB^- ionization.

Equivalents of [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid Bound to GABA Aminotransferase after Inactivation and Denaturation with Urea. GABA aminotransferase was inactivated as described under Inactivation of GABA Aminotransferase by [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid. A 450 μL portion of the inactivated enzyme sample was dialyzed two times against 1 L of 10 mM sodium phosphate with 6 M urea, pH 7.0, for 4 h each time. A protein assay using BSA standards in 10 mM sodium phosphate and 6 M urea, pH 7.0, was run on the final solution, and 65 μL was counted by liquid scintillation counting. Equivalents of inactivator remaining bound to the enzyme were determined by the ratio of millimoles of radioactivity present from the specific activity of the inactivator to the millimoles of active enzyme.

Analysis of Amine Products Released from GABA Aminotransferase after Inactivation with [^{3}H]-2 and Denaturation with TCA. GABA aminotransferase was inactivated and dialyzed as described under Inactivation of GABA Aminotransferase by [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid. A 500 μL sample of the enzyme inactivator solution was added to 50 mg of trichloroacetic acid, making a 10% (w/w) solution. This was incubated at 25 °C for 5 min and was then microcentrifuged. The pellet was rinsed twice with 500 μL of water and all of the supernatants were combined. The supernatants were diluted to 15 mL using water and were applied to a prewashed 5 × 70 mm Dowex 50 column. Three milliliters of water was used to wash the column after all of the solution was applied, and the column was then eluted with 1.5 N HCl. Fractions of 2 mL were collected. A 20 μL portion of each fraction was counted by liquid scintillation counting, and those containing radioactivity were combined, frozen, and lyophilized. The resulting solid was applied to a second Dowex 50 column which was eluted with 0.25 M HCl. Once again the fractions containing radioactivity were combined and freeze-dried. This second Dowex 50 column was necessary because of contamination of the products with sodium chloride from the buffer and the hydrochloric acid. Sodium chloride in the sample does not allow the two layers in the next step to mix, thus seriously hindering the desired reaction.

The amine products released upon TCA denaturation were taken up in 125 μL of 40 mM Li_2CO_3. A 20 μL sample of this solution was counted, and 100 μL was added to a micro-test tube containing 6 μL of 20 mM γ-acetyl-GABA (**20**). A 100 μL sample of 20 mM dansyl chloride in acetonitrile was added, and the mixture was stirred at room temperature for 1 h, protected from light. The entire reaction was then injected onto the HPLC equipped with a C_{18} column. The detector was set at 254 nm and the flow rate at 1.0 mL/min using solvent A (80% H_2O/20% methanol containing 0.6% acetic acid and 0.03% triethylamine). After 2 min, a 60 min gradient into solvent B (100% methanol) was begun. Fractions were collected every minute for 70 min and counted by liquid scintillation counting.

Sodium Periodate Oxidation of the Stable Adduct after [^{3}H]-2 Inactivation and Urea Denaturation of GABA Aminotransferase. GABA aminotransferase was inactivated as described under Inactivation of GABA Aminotransferase by [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid. A 450 μL portion of the inactivated enzyme sample was dialyzed two times against 1 L of 10 mM sodium phosphate with 6 M urea, pH 7.0, for 4 h each time. To 2.4 mg of sodium periodate was added 240 μL of the inactivated enzyme solution, making a 50 mM sodium periodate solution. This solution was stirred gently at room temperature protected from light for 72 h. A control also was run in which 200 μL of the urea-denatured sample was incubated at room temperature protected from light for 72 h. Saturated succinic acid (15 μL) was added to the mixtures, and they were analyzed by HPLC using a C_{18} column. Water with 0.1% TFA was the mobile phase, and the flow rate was 1.0 mL/min. The detector was set to 214 nm. Fractions were collected every 30 s for 15 min and then every 2 min for another 26 min. The fractions were counted by liquid scintillation counting.

Equivalents of Radioactivity Bound to GABA Aminotransferase after Inactivation with [^{3}H]-2, Denaturation with Urea, and Oxida-

tion with Sodium Periodate. GABA aminotransferase was inactivated and denatured as described under Sodium Periodate Oxidation of the Stable Adduct after [^{3}H]-2 Inactivation and Urea Denaturation of GABA Aminotransferase. Approximately half of this solution was oxidized with 50 mM sodium periodate. This solution was stirred gently at room temperature protected from light for 120 h. A control also was run in which 200 μL of the urea-denatured sample was incubated at room temperature protected from light for 120 h. Each of these samples was dialyzed for 4 h against 1 L of 100 mM potassium phosphate at pH 7.4 containing 1 mM β-mercaptoethanol. Protein assays were performed on each sample using BSA standards in 100 mM potassium phosphate, pH 7.4. The remainder of the solutions were counted by liquid scintillation counting. Equivalents of inactivator remaining bound to the enzyme were calculated by dividing the millimoles of radioactivity, determined from the specific activity of the inactivator, by the millimoles of active enzyme used.

Equivalents of [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid Bound to GABA Aminotransferase after Inactivation and Denaturation with Trichloroacetic Acid. GABA aminotransferase was inactivated as described under Inactivation of GABA Aminotransferase by [^{3}H]-4-Amino-5-fluoro-5-hexenoic Acid. About 500 μL of the inactivated enzyme sample was added to 50 mg of trichloroacetic acid (TCA). This solution was mixed well and then incubated at room temperature for 5 min. The enzyme which had fallen out of solution was removed by microcentrifugation. The supernatant was separated and the pellet rinsed three times with water, followed by microcentrifugation each time. A 500 μL aliquot of 2 M potassium hydroxide was added to each pellet. This was mixed several times on a vortex stirrer and was allowed to dissolve overnight. An equivalents bound determination was done by first performing a protein assay using BSA standards in 2 M KOH and then counting the remaining 450 μL of enzyme solution by liquid scintillation counting. Equivalents of inactivator remaining bound to the enzyme were determined by the ratio of millimoles of radioactivity, determined from the specific activity of the inactivator, to the millimoles of active enzyme used.

Formation of Amine Metabolites after Inactivation of GABA Aminotransferase with [^{3}H]-2 and Denaturation. One milliliter of GABA aminotransferase (15 nmol) was inactivated as described directly above. Part of this solution (100 μL, 1.4 nmol) was applied to a Centricon 30 and was centrifuged using a JA-20 rotor at 5000 rpm for 5 min. The centrifugation was repeated after the addition of 200 μL of water. This was done a total of three times. The final spin was for 10 min to get all of the liquid through the membrane.

The filtrate was applied to a prewashed Dowex 50 column. The column was washed with 5.5 mL of water and then eluted with 10 mL of 1.5 N HCl. The acidic elution solution was freeze-dried.

The amine metabolites were analyzed by dissolving the solid in 125 μL of 40 mM Li_2CO_3. Liquid scintillation counting was performed on 20 μL of this solution, and 100 μL was added to a micro-test tube containing 6 μL of 20 mM γ-acetyl-GABA. A 100 μL portion of 20 mM dansyl chloride in acetonitrile was added, and the mixture was stirred at room temperature protected from light for 1 h. The entire reaction was then injected into the HPLC equipped with a C_{18} column. The flow rate was set at 1.0 mL/min with a solvent system consisting of 80% H_2O/20% methanol containing 0.6% acetic acid and 0.03% triethylamine. After 2 min, a 60 min gradient to 100% methanol was begun. Detection was at 254 nm. Fractions were collected every min for 70 min and counted by liquid scintillation counting.

Analysis of Nonamine Metabolites Formed during Inactivation of GABA Aminotransferase with [^{3}H]-2. GABA aminotransferase (200 μL, 2.2 nmol) was incubated for 6 h in 100 mM potassium phosphate, pH 7.4, containing 9 mM [^{3}H]-**2**, 5 mM α-ketoglutarate, 5 mM β-mercaptoethanol, and 3 mM 4-oxo-5-hexenoic acid. A 125 μL portion of this solution was applied to a Centricon 30 and centrifuged using a JA-20 rotor at 5000 rpm for 5 min. To rinse the membrane, 75 μL of water was then added and the centrifugation repeated. This second spin was for 10 min to get all of the liquid through the membrane.

The filtrate was applied to a prewashed 5 $\times$ 70 mm Dowex 50 column. The column was washed with 20 $\times$ 100 μL portions of water which were collected; 5 μL of each fraction was counted by liquid scintillation counting. The two fractions containing the greatest amount of radioactivity were analyzed by HPLC using a C_{18} column with 90% 100 mM sodium phosphate, pH 6.0, and 10% methanol flowing at 1.0 mL/min with detection at 254 nm. Fractions were collected every minute. Another analysis of nonamine metabolites was carried out as described above with no 4-oxo-5-hexenoic acid added. This analysis was done using a C_{18} column with a H_2O/0.1% TFA solvent system flowing at 0.5 mL/min for 20 min. The flow rate was changed to 1.0 mL/min over the next 5 min, and then a 15 min gradient to 15% methanol was run. The column was eluted with 15% methanol for 10 min and then was rinsed with methanol by changing the solvent to 100% methanol over 2 min. Fractions were collected for liquid scintillation counting every minute for 90 min.

Analysis of Fluoride Ion Release during Inactivation of GABA Aminotransferase by 2. Two samples containing 100 μL of GABA aminotransferase and 9.0 mM **2**, 3.5 mM α-ketoglutarate, 5 mM β-mercaptoethanol, and 100 mM potassium phosphate, pH 7.4, were incubated at room temperature protected from light. Two controls containing all but inactivator and two controls containing all but enzyme were incubated as well. After 20 h, the inactivation samples were less than 2% active.

A standard curve was constructed by measuring the relative millivolts of a variety of sodium fluoride solutions ranging from 1×10^{-6} to 2×10^{-5} M in a 1:1 mixture of 100 mM potassium phosphate, pH 7.4, and a total ionic strength adjustment buffer (TISAB; made from 5.8 mg of sodium chloride and 5.7 mL of acetic acid in 100 mL of water, adjusted to pH 5.2). The millivolt reading was plotted against log-[NaF] and a second-order fit was obtained.

The relative millivolts of the inactivated enzyme solution and controls, also in a 1:1 solution of 100 mM potassium phosphate and TISAB, were read. Using the formula from the second-order plot obtained from the standard curve described above, the millivolt reading was converted to the concentration of fluoride ions in the sample. The control readings were subtracted out of the inactivated sample reading, and the concentration of fluoride ions was divided by the concentration of GABA aminotransferase to find the number of fluoride ions released per inactivation event.

Transamination Events per Inactivation of GABA Aminotransferase with 2. The number of turnovers, or transamination events, of GABA aminotransferase during the inactivation by **2** was determined by incubating GABA aminotransferase (0.4 nmol) with 9.0 mM **2**, 10 mM [^{14}C]-α-ketoglutarate (57 mCi/mmol diluted to 1.4 mCi/mmol with cold α-ketoglutarate), and 5 mM β-mercaptoethanol. Controls were run containing all but enzyme, two containing everything except inactivator, and two containing nothing but the labeled α-ketoglutarate in buffer. After being incubated at room temperature and protected from light for 20 h, the inactivation sample was less than 2% active. Each sample was denatured by adding enough 20% TCA to make an 8% solution. These were mixed well and then applied to prewashed 5 $\times$ 70 mm Dowex 50 columns. The columns were washed with 6 mL of water and then eluted with 0.5 mL of 2 M NH_4OH, followed by 5.0 mL of 2 M NH_4OH and then another 2.0 mL of 2 M NH_4OH to be certain that all of the radioactivity had eluted from the column. Each of these four solutions for each sample was collected separately in scintillation vials and counted by liquid scintillation counting. The number of counts eluting with the ammonium hydroxide solution is indicative of the amount of [^{14}C]glutamate present in the sample. By subtraction of the controls from the inactivated sample and conversion of the number of counts to millicuries then to millimoles based on the specific activity of the original [^{14}C]-α-ketoglutarate, the millimoles of glutamate formed during the inactivation can be calculated. The ratio of millimoles of glutamate produced to millimoles of active enzyme used in the sample is the number of transaminations per inactivation event.

Acknowledgment. We are grateful to the National Institutes of Health (NS15703) for financial support of this research. We thank Dr. Cynthia Burnell-Curty for synthesizing the 4-oxo-5-hexenoic acid standard and Marion Merrell Dow for a generous gift of 4-amino-5-hexenoic acid.

JA9527885