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Mechanism-Based Inactivation of γ-Aminobutyric Acid Aminotransferase by 3-Amino-4-fluorobutanoic Acid

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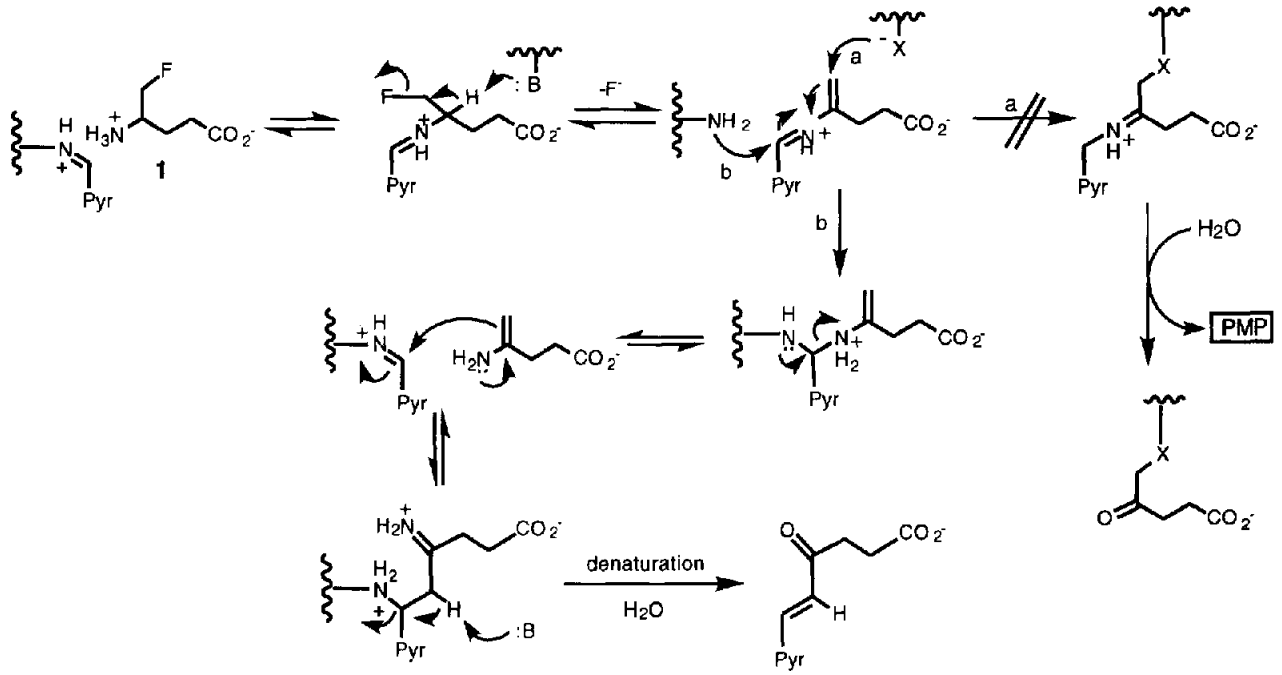
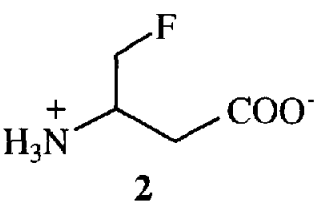
Abstract—The mechanism of inactivation of the pyridoxal 5′-phosphate (PLP)-dependent enzyme γ-aminobutyric acid (GABA) aminotransferase by 3-amino-4-fluorobutanoic acid (**2**) has been investigated. As in the case of the homologue, 4-amino-5-fluoropentanoic acid (**1**), 2 equiv of radiolabeled inactivator become covalently attached to the enzyme, and no transamination, as determined by the lack of conversion of [1-^{14}C] α-ketoglutarate into [1-^{14}C] glutamate during inactivation, was observed. In the case of **1**, the conclusion was that inactivation was completely the result of modification of the coenzyme and that there was no metabolic turnover; every enzyme molecule catalysed the conversion of one molecule of inactivator to the activated species, which inactivated the enzyme by an enamine mechanism. With **2**, however, 6.7 ± 0.7 equiv of fluoride ions were released during inactivation, and it took 7.6 ± 0.7 inactivator molecules to inactivate each enzyme dimer. Since no transamination was occurring, another metabolic event besides inactivation must result from the PLP form of the enzyme. Inactivation of GABA aminotransferase with [1,2-^{14}C]-**2** produced [^{14}C] acetoacetic acid (about 5.5 equiv) as the metabolite. The 1.93 ± 0.25 equiv of radioactivity covalently bound to the enzyme after inactivation with [1,2-^{14}C]-**2** and gel filtration were completely released by base treatment. HPLC analysis showed that three radioactive compounds, identified as **2**, the product of reaction of PLP with acetone (**3**), and the product of reaction of PLP with acetoacetate (**4**), were detected. The release of **3** and **4** and the prevention of release of radioactivity by treatment with sodium borohydride are consistent with the formation of covalent intermediates that have β-carbonyl-like character, such as **6** and/or **7** (Scheme 2). Inactivation of [^{3}H] PLP-reconstituted GABA aminotransferase with **2** followed by gel filtration then base denaturation released all of the radioactivity as a mixture of PLP, **3**, and **4**. Inactivation with [1,2-^{14}C]-**2** resulted in the release of 1.37 equiv of $^{14}CO_2$, which was shown to be the result of decarboxylation of the acetoacetate/**4** after release from the enzyme. These results are not consistent with a Michael addition mechanism (Scheme 3), but are consistent with inactivation by an enamine mechanism; release of the enamine five out of seven turnovers accounts for the formation of acetoacetate as the metabolite. To account for the detection of PLP and **2** after denaturation, it is suggested that a nonproductive formation of the Schiff base of PLP with **2** occurs in the second subunit of the enzyme; this complex is released and hydrolysed to PLP and **2** upon base denaturation. Copyright © 1996 Elsevier Science Ltd

Introduction

γ-Aminobutyric acid (GABA) aminotransferase is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that catalyses the conversion of the inhibitory neurotransmitter GABA and α-ketoglutarate to succinic semialdehyde and the excitatory neurotransmitter L-glutamic acid.[1] Since GABA is the product of metabolism of L-glutamic acid, a reaction catalysed by the PLP-dependent enzyme L-glutamic acid decarboxylase, it is apparent that GABA aminotransferase is important in the regulation of brain neurotransmission.[2] When brain levels of GABA diminish below a threshold concentration, convulsions can arise;[3] injection of GABA directly into the brain terminates the convulsion.[4] This suggests that GABA would be an effective anticonvulsant agent. Unfortunately, administration of GABA peripherally results in no anticonvulsant effect. This is because GABA, under normal circumstances, does not cross the blood–brain barrier. Alternative approaches have been taken to raise GABA levels; the approach that will be discussed here is one that uses a compound that can cross the blood–brain barrier, then once inside the brain, blocks the activity of GABA aminotransferase. Many compounds are now known to function by this approach, particularly mechanism-based inactivators[5] of GABA aminotransferase.[6] A mechanism-based inactivator is an unreactive compound that is converted by the target enzyme into a species that inactivates the enzyme prior to its release from the active site. One of the earlier series of compounds reported to function in vitro[7,8] and in vivo[9,10] as mechanism-based inactivators of GABA aminotransferase were the 4-amino-5-halopentanoic acids. The elimination–Michael addition inactivation mechanism originally hypothesized for these inactivators (Scheme 1, pathway a)[7,8] was shown to be incorrect and was modified, at least for 4-amino-5-fluoropentanoic acid (**1**), to an enamine inactivation mechanism (Scheme 1, pathway b).[11] Numerous mono- and difluorinated ω-amino acids were shown to be effective in vitro and in vivo inactivators of GABA aminotransferase and to cause dramatic increases in brain levels of GABA upon peripheral administration.[9,12] Several were shown to be time-dependent irreversible inactivators of purified GABA aminotransferase; 3-amino-4-fluorobutanoic acid was the most potent tested.[9] An elimination–Michael addition mechanism (Scheme 1, pathway a) was originally proposed as the relevant inactivation mechanism for this class of inactivators,[9] but later it was suggested



Scheme 1. Pyr represents the pyridine ring of PLP.



2

that inactivation could arise from both Michael addition and enamine mechanisms.[12] In this paper a detailed mechanistic study of the inactivation of GABA aminotransferase by 3-amino-4-fluorobutanoic acid [**2**; 3-(fluoromethyl)-β-alanine] is described, and it is shown that it inactivates GABA aminotransferase exclusively by an enamine mechanism.

Results

Synthesis of [1,2-$^{14}C_2$]-2

The doubly-labeled inactivator was synthesized by a modification of our earlier procedure,[13] because of the difficulties resulting from carrying out the earlier reactions on a small scale. Instead of using *t*-butyl [1,2-^{14}C] acetate in the synthesis,[13] benzyl [1,2-^{14}C] acetate was found to be easier to isolate and handle. [1,2-$^{14}C_2$]-3-Amino-4-fluorobutanoic acid was obtained in >99% radiopurity and with a specific radioactivity of 0.29 mCi/mmol.

Transamination of 2 by GABA aminotransferase

No [^{14}C] glutamate was produced during inactivation of GABA aminotransferase by **2** in the presence of [^{14}C] α-ketoglutarate, indicating that no transamination occurs. Further evidence against transamination is that inactivation of GABA aminotransferase by **2** goes to completion, even in the absence of α-ketoglutarate; no return of activity occurs upon addition of α-ketoglutarate to the inactivated enzyme.

Equivalents of radioactivity bound to GABA aminotransferase after inactivation with [1,2-$^{14}C_2$]-2 and gel filtration

Following inactivation and gel filtration, 1.93±0.25 equiv of radioactivity per enzyme dimer remained bound.

Equivalents of radioactivity bound to GABA aminotransferase after inactivation by [1,2-$^{14}C_2$]-2, gel filtration and acid precipitation

Acid denaturation resulted in 1.92 equiv of radioactivity remaining bound per dimer of enzyme.

Equivalents of radioactivity bound to GABA aminotransferase after inactivation by [1,2-$^{14}C_2$]-2, gel filtration, base denaturation, and acid precipitation

No radioactivity remained bound after base denaturation and acid precipitation.

Titration of GABA aminotransferase with 2

Complete inactivation of GABA aminotransferase by **2** required 7.6±0.7 equiv of inactivator (three experiments).

Release of fluoride ion during inactivation of GABA aminotransferase by 2

With the aid of a specific fluoride ion electrode, it was determined that 6.7 ± 0.3 F^- are released after complete inactivation of GABA aminotransferase by **2**.

Inactivation of [^{3}H] PLP-reconstituted GABA aminotransferase by 2

GABA aminotransferase, which was reconstituted with [^{3}H] PLP, was inactivated with **2**, the pH was raised, then the enzyme was precipitated with acid, and the supernatant was analysed by HPLC (Fig. 1). The major products (52% combined) eluted within minutes of the enamine adduct formed by inactivation of GABA aminotransferase by 4-amino-5-fluoropentanoic acid[11] and γ-vinyl GABA.[14] The peak of radioactivity at $t_R = 52$ min corresponds to the product of the aldol condensation of PLP and acetone (vide infra), and the $t_R = 56$ min peak corresponds to the product of the aldol condensation of PLP and acetoacetic acid (vide infra). PLP (38%) also was observed. The small peak of radioactivity at 20 min (3%) corresponds to PMP; however, it is within experimental error and often did not appear. Therefore, it is concluded that no PMP is released.

Equivalents of radioactivity bound to [^{3}H] PLP-reconstituted GABA aminotransferase after inactivation by 2, gel filtration, base denaturation, and acid precipitation

Base denaturation and acid precipitation resulted in complete release of the radioactivity from the enzyme.

Release of CO_2 during inactivation of GABA aminotransferase by [1,2-$^{14}C_2$]-2

GABA aminotransferase was inactivated with [1,2-$^{14}C_2$]-**2** in a closed container with a CO_2 trap. One hour after inactivation the protein was acid denatured and the dissolved CO_2 was trapped; 1.37 equiv of $^{14}CO_2$ was released. If the inactivated enzyme was treated with $NaBH_4$ for 1 h prior to acid denaturation, then 0.58 equiv of $^{14}CO_2$ was recovered. Treatment of the inactivated enzyme with hydroxide for an hour prior to acid denaturation resulted in the recovery of 1.70 equiv of $^{14}CO_2$.

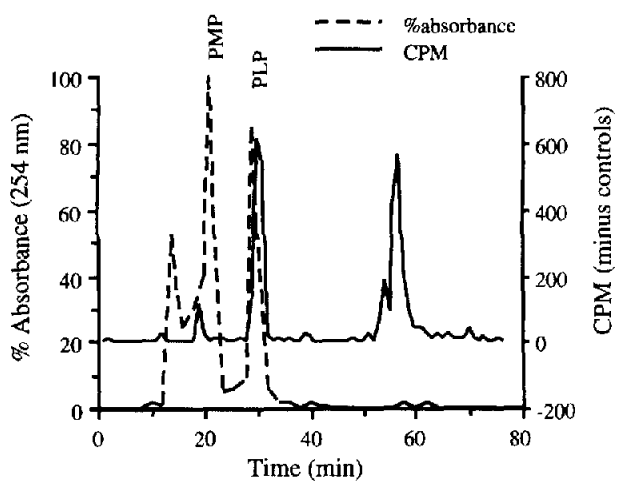


Figure 1. HPLC of the products released after inactivation of [^{3}H] PLP-reconstituted GABA aminotransferase by **2** followed by gel filtration and base denaturation. See Experimental for details.

Equivalents of radioactivity bound to GABA aminotransferase after inactivation with [1,2-$^{14}C_2$]-2 and incubation under different conditions

In the experiments described above for CO_2 release, the amount of radioactivity bound to the denatured protein was determined. Inactivation of GABA aminotransferase by [1,2-$^{14}C_2$]-**2** followed by denaturation with sulfuric acid resulted in 1.92 equiv of radioactivity bound per dimer. Incubation with sodium borohydride followed by sulfuric acid gave 2.50 equiv of radioactivity bound. Treatment with hydroxide followed by sulfuric acid resulted in only 0.035 equiv of radioactivity bound per dimer.

Small molecules produced during inactivation of GABA aminotransferase by [1,2-$^{14}C_2$]-2

The only metabolite produced was acetoacetic acid; excess [1,2-^{14}C]-**2** used in the experiment also can be seen (Fig. 2). On some occasions radioactive acetone also was detected. The amount of acetone produced, however, was variable; larger amounts were produced when the solution was not kept cold while the pH was adjusted for injection into the HPLC. In these cases, the amount of acetoacetic acid was correspondingly decreased, indicating that the acetone comes from the decomposition of acetoacetic acid into acetone and, presumably, carbon dioxide. Therefore, the metabolite generated by the enzyme reaction appears to be acetoacetic acid; acetone that is formed is a by-product of the work up procedure.

Small molecules released from GABA aminotransferase inactivated with [1,2-$^{14}C_2$]-2, gel filtered, then denatured

Following inactivation of GABA aminotransferase with [1,2-$^{14}C_2$]-**2**, gel filtration or ultrafiltration, then base

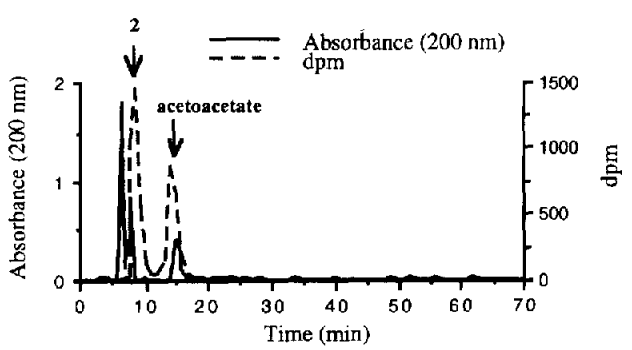


Figure 2. HPLC of the small molecules isolated by ultrafiltration after inactivation of GABA aminotransferase by [1,2-^{14}C]-**2**.

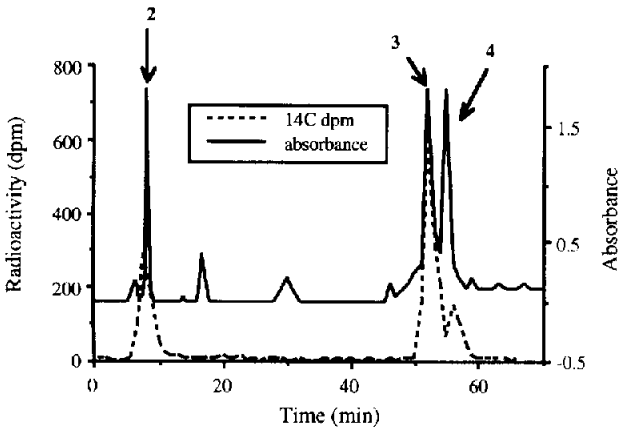


Figure 3. HPLC of the products released after inactivation of GABA aminotransferase by [1,2-^{14}C]-**2** followed by gel filtration and base denaturation. See Experimental for details.

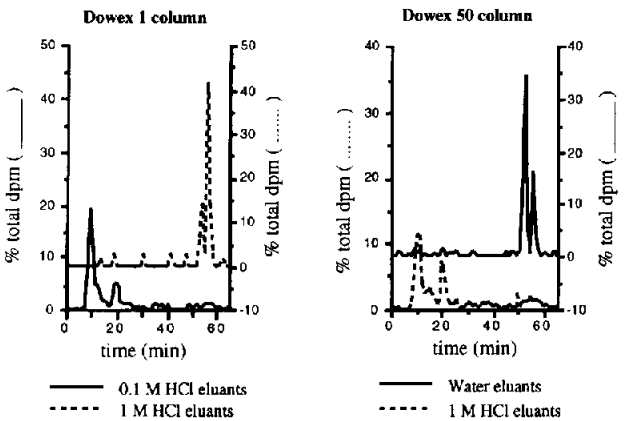


Figure 4. HPLC of the eluants from Dowex 1 and Dowex 50 columns of small molecules released in Figure 3. See Experimental for details.

and acid denaturation, three major radioactive products were observed by HPLC at $t_R = 8.5$, 52, and 56 min (Fig. 3). As described below, the peak at $t_R = 8.5$ min corresponds to **2**, the $t_R = 52$ min peak corresponds to the product of the aldol condensation of PLP and acetone, and the $t_R = 56$ min peak corresponds to the product of the aldol condensation of PLP and acetoacetic acid.

Ion exchange chromatography of small molecules released from GABA aminotransferase inactivated by [1,2-$^{14}C_2$]-2, gel filtered, then denatured

When the supernatant was run on Dowex 1 (anion exchanger) the $t_R = 8.5$ min peak washed off with dilute acid (0.1 M HCl) and the $t_R = 52$ and 56 min peaks stuck to Dowex 1 until washed off with 1 M HCl (Fig. 4). On Dowex 50 (cation exchanger), radioactivity eluted in both the water wash and the 1 M HCl wash. HPLC of these fractions showed that the $t_R = 52$ and 56 min peaks eluted in the water wash (not cationic) and the $t_R = 8.5$ peak was bound to Dowex 50 until washed off with 1 M HCl (cationic). These results are consistent with the $t_R = 8.5$ min peak being an amino acid and the $t_R = 52$ and 56 min peaks being modified PLP adducts. The peak at 20 min was from a degradation product, which did not appear in later experiments.

Synthesis of 5-[2-hydroxy-3-methyl-6-(phosphonoxymethyl)-4-pyridinyl]-2-oxo-3-butene (3) and 5-[2-hydroxy-3-methyl-6-(phosphonoxymethyl)-4-pyridinyl]-3-oxo-4-pentenoic acid (4)

Base-induced aldol condensation of PLP with acetone gave one detectable product by HPLC at about 52 min (Fig. 5), identified as 5-[2-hydroxy-3-methyl-6-(phosphonoxymethyl)-4-pyridinyl]-2-oxo-3-butene (**3**). Base-induced aldol condensation of PLP with acetoacetic acid gave two detectable products by HPLC, one at ~52 min, which corresponded to the aldol condensation product of PLP with acetone (**3**), and one at 56 min (Fig. 5), identified as 5-[2-hydroxy-3-methyl-6-(phosphonoxymethyl)-4-pyridinyl]-3-oxo-4-pentenoic acid (**4**), see Table 1.

Discussion

We had previously studied the mechanism of inactivation of GABA aminotransferase by 4-amino-5-fluoro-

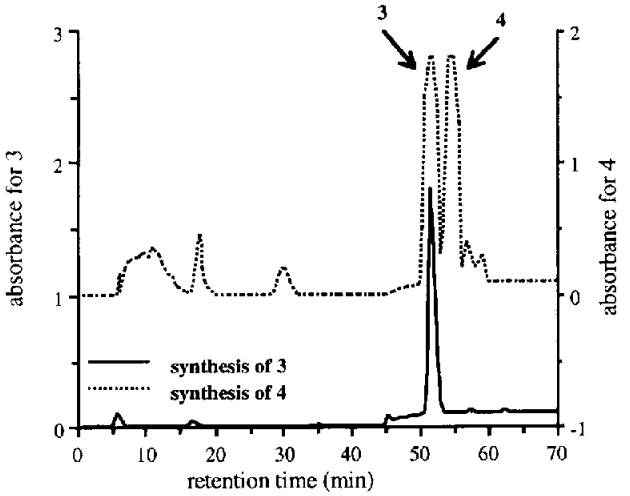
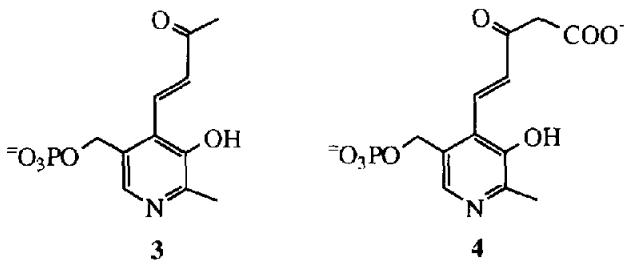


Figure 5. Comparison of the HPLC traces from the base-catalysed reaction of PLP with acetone (——) and PLP with acetoacetic acid (----). See Experimental for details.

Table 1. Summary of equivalents results after inactivation with **2**

Equiv ^{14}C-**2** bound after gel filtration	1.93 ± 0.25
Equiv ^{14}C-**2** bound after acid treatment	1.92
Equiv ^{14}C-**2** bound after base treatment	0
Equiv ^{14}C-**2** to titrate enzyme	7.6 ± 0.7
Equiv ^{3}H PLP bound after base treatment	0
Equiv F$^-$ released	6.7 ± 0.3
Equiv $^{14}CO_2$ released after acid treatment	1.37
Equiv $^{14}CO_2$ released after base treatment	1.70



pentanoic acid (**1**) and concluded that it proceeds by a single enamine mechanism (Scheme 1, pathway b).[11] In this case no transamination occurred and only 2 equiv of fluoride ions were released upon enzyme dimer inactivation. Two equiv of inactivator were covalently attached to the enzyme, but denaturation led to release of a modified coenzyme. The conclusion was that inactivation was completely the result of modification of the coenzyme and that there was no metabolic turnover; every enzyme molecule catalysed the conversion of one molecule of inactivator to the activated species, which inactivated the enzyme by an enamine mechanism. The partition ratio, the number of molecules of inactivator converted to product per inactivation event, then, was zero.

As in the case of **1**, compound **2** also could potentially inactivate GABA aminotransferase by either an enamine mechanism (Scheme 2) or a Michael addition mechanism (Scheme 3). Initial results with **2** suggested that the same course of events was occurring as in the case of **1**. Inactivation of GABA aminotransferase with [1,2-^{14}C]-**2** resulted in the covalent attachment of 1.93 ± 0.25 equiv of inactivator to the enzyme dimer

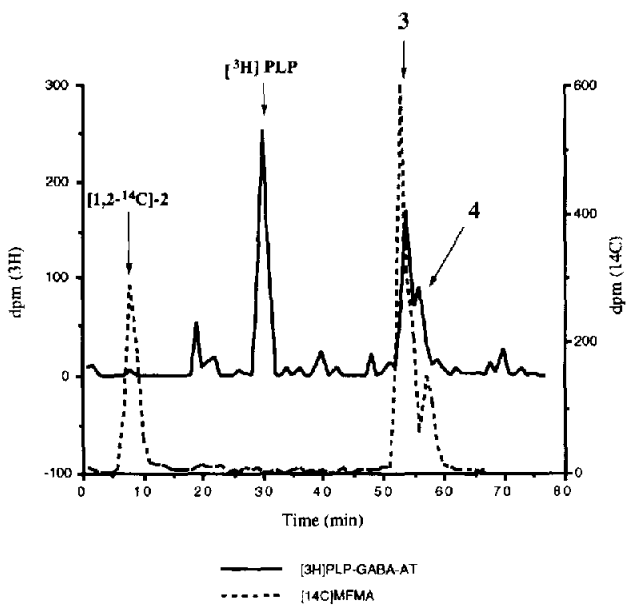


Figure 6. Comparison of the HPLC traces of the products released after inactivation of [^{3}H] PLP-Ereconstituted GABA aminotransferase by **2** (——) and of GABA aminotransferase by 1,2-^{14}C-**2** (----) followed by gel filtration and base denaturation. See Experimental for details.

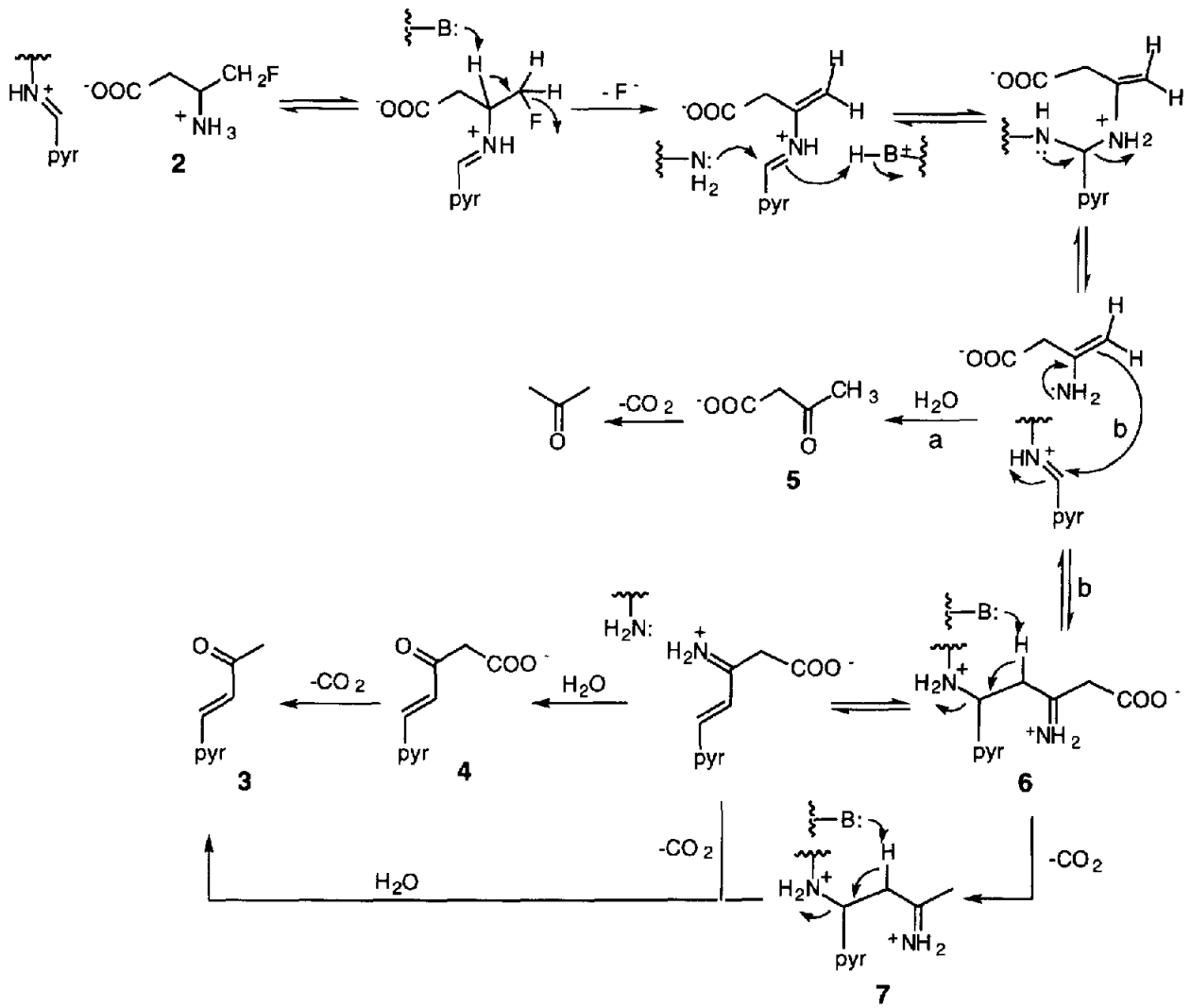
(after gel filtration), and no transamination, as determined by the lack of conversion of [1-^{14}C] α-ketoglutarate into any [1-^{14}C] glutamate during inactivation, was observed. However, the first indication that more was happening in the case of **2** than in the case of the higher homologue **1** was that 6.7 ± 0.7 equiv of fluoride ions were released during inactivation, and it took 7.6 ± 0.7 inactivator molecules to inactivate each enzyme dimer. Since no transamination was occurring, i.e., no conversion to PMP, another metabolic event besides inactivation must result from the PLP form of the enzyme. To determine what metabolites were being produced during inactivation, GABA aminotransferase was inactivated with [1,2-^{14}C]-**2**. The small molecules generated after enzyme inactivation were analysed by HPLC and only [^{14}C] acetoacetic acid (about 5.5 equiv) was detected (except, of course, for the excess inactivator; Fig. 2). Sometimes, depending upon the conditions of the experiment, acetone also was detected. In these cases, the amount of acetoacetic acid was correspondingly decreased, indicating that the acetone came from the decomposition of acetoacetic acid into acetone and, presumably, carbon dioxide. The sporatic formation of acetone, however, is evidence that decarboxylation occurs after release of acetoacetic acid rather than on the enzyme. These results are consistent with the 7.6 ± 0.7 molecules of **2** necessary to inactivate each dimeric enzyme molecule: about 5.5 molecules of **2** are turned over to acetoacetic acid and about two molecules of **2** inactivate the enzyme.

Following inactivation of GABA aminotransferase with [1,2-^{14}C]-**2**, the same amount of radioactivity (1.93 equiv) remained bound to the enzyme after gel filtration as with acid denaturation. However, if the enzyme is gel filtered then denatured in sodium hydroxide prior to acid denaturation of the protein, essentially no radioactivity remains bound to the enzyme. This suggests that acid denaturation may be sequestering the active site species, possibly as a result of rapid precipitation of the protein from solution, whereas in base the enzyme is denatured in a soluble form so anything reversibly bound to the active site is released into solution. The release of the radioactivity from the enzyme under basic conditions can be mostly prevented by treatment of the labeled enzyme with sodium borohydride in base. Under these conditions 1.7 equiv of radioactivity remained bound to the enzyme; presumably, a small amount of adduct release occurred prior to borohydride reduction of some carbonyl or imine, such as **6** or **7** (Scheme 2). All of the products from the Michael addition mechanism (**9**–**11**, Scheme 3) should be stable without sodium borohydride reduction, which is evidence against their existence. HPLC analysis of the species released from the radiolabeled enzyme following gel filtration and base treatment indicated the presence of three radioactive compounds, identified as **2**, **3**, and **4** (Fig. 3). The release of **3** and **4** and the prevention of release of radioactivity by treatment with sodium borohydride are consistent with the formation of intermediates **6**/**7** (Scheme 2). Since all of the radioactivity bound to the enzyme is released upon base denaturation, and the released products are **2**–**4**,

the Michael addition mechanisms shown in Scheme 3 can be excluded from further consideration.

The formation of acetone from the acetoacetic acid metabolite described above suggests that carbon dioxide is produced. Formation of CO_2 was monitored by base trapping during inactivation with [1,2-^{14}C]-**2**. When sulfuric acid was added directly to the inactivated enzyme solution, an average of 1.37 equiv of $^{14}CO_2$ were released. However, under these same conditions, 1.93 equiv of radioactivity remained bound to the enzyme. This suggests that CO_2 does not come from a covalent enzyme adduct (less than 1.93 equiv would be bound), but rather from the decarboxylation of metabolites (e.g., acetoacetic acid) released as turnover products or from the conversion of **4** to **3**. Control experiments showed that the CO_2 is not released from the labeled inactivator under similar conditions. Treatment of the enzyme with $NaBH_4$ prior to denaturation reduced the amount of CO_2 release to 0.58 equiv, which suggests that some decarboxylation occurred prior to reduction. The identification of acetone from the acetoacetic acid metabolite and the formation of **4** also support the notion that decarboxylation is occurring off the enzyme.

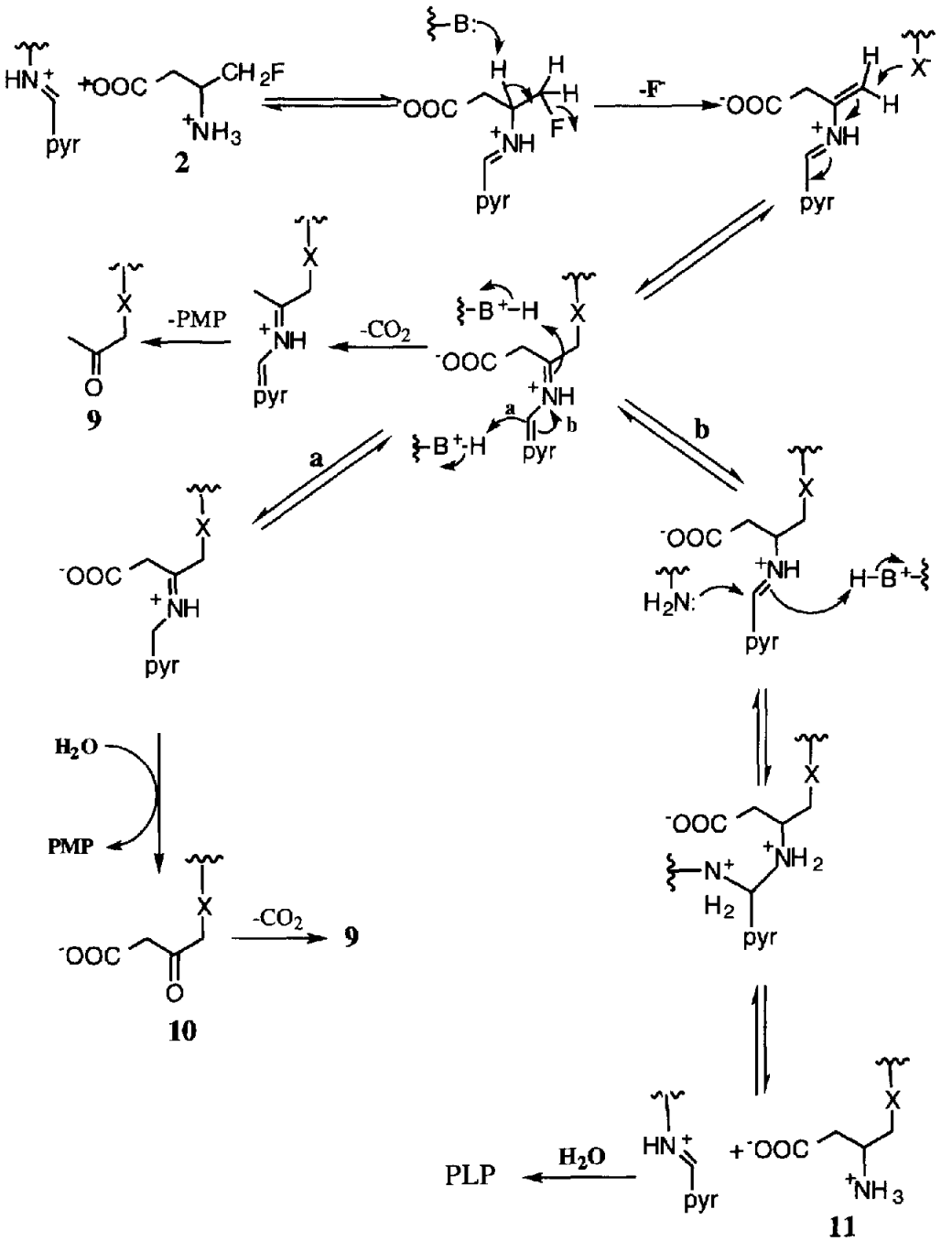
To characterize the species that are produced from the coenzyme during inactivation, [^{3}H] PLP-reconstituted GABA aminotransferase was inactivated with **2**. Denaturation by treatment with base followed by acid and HPLC analysis showed that three radioactive species were released, PLP, **3**, and **4** (Fig. 1). Formation of **3** and **4** corroborate the structures as being composed of both the coenzyme and the inactivator, since the same compounds are produced when [1,2-^{14}C]-**2** is used to inactivate GABA aminotransferase with unlabeled PLP. Figure 6 shows a comparison of the metabolites obtained from denaturation of [1,2-^{14}C]-**2** inactivated GABA aminotransferase and those obtained from denaturation of [^{3}H] PLP-reconstituted GABA aminotransferase that was inactivated with **2**. The release of PLP indicates that, at least, for part of the enzyme the PLP coenzyme is not altered. No radioactivity remained bound to the



Scheme 2. Pyr represents the pyridine ring of PLP.

protein after base denaturation. These results confirm those obtained above with radiolabeled inactivator and, again, exclude the Michael addition mechanisms.

Except for the release of PLP and **2** after denaturation of GABA aminotransferase inactivated by **2** (with radiolabels either in **2** or the PLP), all of these results are consistent with the enamine mechanism shown in Scheme 2. Initially, it was thought that the peak coeluting with **2** after gel filtration and denaturation was the result of excess inactivator that had not been successfully removed. However, multiple washings of the Centricon 30 retentate to remove all unbound radioactivity (as evidenced by the lack of radioactivity in the filtrate) failed to prevent the generation of **2** after denaturation, suggesting that the **2** was enzyme-bound after inactivation. Other possible metabolites that might elute at about the same retention time as **2** were considered. The mobility of the isolated metabolite on ion exchange chromatography was monitored. The compound was shown to be retained on Dowex 50 (cation resin), eluting with 1 N HCl and to be retained on Dowex 1 (anion resin), eluting with 0.1 N HCl. These results suggest that the compound is an amino acid or related. One possibility other than **2** is the hydrolysis product, 3-amino-4-hydroxybutanoic acid



Scheme 3.

(hydroxyl in place of fluoride). However, this compound and the corresponding lactone were shown to have shorter retention times on HPLC than **2**. TLC of the metabolite also confirmed it to be **2**, not the hydroxy analogue. How can the release of **2** and PLP by denaturation of the enzyme be rationalized? GABA aminotransferase is a homodimeric enzyme, but the subunits exhibit negative cooperativity.[15] One subunit may catalyse the enamine mechanism shown in Scheme 2, but formation of the Schiff base of **2** with PLP in the second subunit may become a dead-end product as a result of a conformational change occurring from the reaction in the first subunit toward the other subunit (negative cooperativity). Since β-proton removal is rate determining,[12] the Schiff base would be the dead-end product. This would account for release of PLP and **2** upon denaturation. A study of the inactivation of dimeric D-amino acid aminotransaminase by alanine[16] is an example of an inactivation of a homodimeric PLP aminotransferase in which inactivation occurs only at one active site, despite the fact that both subunits are catalytically competent. Inactivation of one subunit inactivates the entire enzyme molecule. With the aid of fluorescence studies, it was concluded that inactivation in one active site causes a conformational change that renders the second catalytic site inactive. Conformational changes in GABA aminotransferase also are well known.[17] Formation of a Schiff base dead-end product also would account for the observation that one less fluoride ion is released (6.7 ± 0.3) than the number of molecules of **2** that are required to inactivate the enzyme completely (7.6 ± 0.7). The 6.7 ± 0.3 equiv of fluoride ions can be accounted for by the 5.5 equiv of acetoacetic acid turnover product formed plus only 1 equiv of fluoride ion that is released from **2** leading to inactivation; the other molecule of **2** that is bound to GABA aminotransferase, presumably in the second subunit, retains its fluoride ion.

The mechanism shown in Scheme 2 accounts for the loss of fluoride ions, the production of acetoacetic acid (**5**) as a metabolite, the loss of CO_2 from it to give acetone, and the formation of **3** and **4**; base denaturation would release **3** and **4**. Sodium borohydride would reduce the ketone in acetoacetic acid, thereby preventing decarboxylation, and would reduce the imine of **6**, leading to retention of the radioactive adduct, even after base denaturation.

The enamine condensation with enzyme-bound PLP is, apparently, a very sensitive reaction. With 4-amino-5-fluoropentanoic acid (**1**) the enamine is trapped exclusively by the enzyme-bound PLP, leading to a partition ratio of zero.[11] The enamine with one less methylene, namely, the one generated from **2**, is not nearly as efficient at reacting with the enzyme-bound PLP and is released into solution, becoming hydrolysed to acetoacetic acid, about five out of seven turnovers. All of the inactivation of GABA aminotransferase by **2** is the result of an enamine mechanism, leading to **6** (Scheme 2); denaturation releases **4**, which can undergo nonenzymatic decarboxylation to **3**.

Experimental

General procedures

Proton NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. Chemical shifts are reported as δ values in ppm downfield from tetramethylsilane (TMS) in $CDCl_3$ or from 3-(trimethylsilyl) propionic acid in D_2O. Coupling constants are reported in Hz. Melting points were determined on a Fisher–Johns mp apparatus and are uncorrected. TLC was run on Whatman PE SIL/UV silica gel plates with UV indicator. Amines were visualized on TLC plates developed by heating the plate dipped in ninhydrin solution in *n*-butanol. Other compounds were visualized with either I_2 or phosphomolybdic acid in ethanol developed by heating. Centrifugation was done in a Beckman J-21C centrifuge, a Beckman Microfuge B, or an IEC clinical tabletop centrifuge. pH measurements were carried out with an Orion 720A pH meter or with a Ross 8301 combination pH semi-micro electrode. Fluoride ion measurements were done using an Orion 96–09 combination fluoride electrode with an Orion 701-A pH meter and was calibrated with sodium fluoride (0.1 M, Orion 94–09–06). Absorption spectra were recorded on a Beckman DU-40 spectrophotometer. Enzyme activity was measured with a Perkin–Elmer Lambda 1 spectrophotometer. Silica gel column chromatography was done on silica gel 60H (TLC grade silica). Ion-exchange chromatography was performed on either Dowex 50 X-8 cation exchange resin (200–400) or Dowex AG-1 anion exchange resin prewashed with 1 M HCl, 1 M KOH, and water before use. High-performance liquid chromatography (HPLC) was performed on either a Beckman Model 330 HPLC with a Beckman 421A controller, Beckman 110B delivery systems, and Beckman Model 153 UV detector at 254 nm or a Beckman System Gold HPLC with a model 166 detector and 125P solvent system. An Alltech Econosil C18 10μ column was used for HPLC separations. Radioactivity was measured on either a Beckman LS-3100 liquid scintillation counter using 10 mL of Fisher Scintisafe 30% LSC cocktail or a Packard Tri-Carb 2100TR liquid scintillation analyser using 10 mL of Packard Ultima Gold LSC cocktail.

Reagents

Unlabeled (*R,S*)-3-amino-4-fluorobutanoic acid was received as a gift from Merrell Dow Laboratories (MDL 72134A-02) or was synthesized as previously described.[4] 3-Amino-4-hydroxybutanoic acid and its corresponding lactone were synthesized by the reported procedures.[18] THF and diethyl ether were distilled under nitrogen from sodium metal. In-house distilled water was further purified by passage through a deionizer before use. Sodium boro, [^{3}H] hydride and [5-^{14}C] 2-keto glutaric acid were bought from Amersham. [1,2-^{14}C] Acetic acid, sodium salt (equal amount of ^{14}C at C_1 and C_2), was purchased from American Radiolabeled Chemicals. PLP, PMP, bovine serum albumin, β-mercaptoethanol, α-ketoglutarate, $NADP^+$, GABA, and potassium pyrophosphate were

purchased from Sigma Chemical Co. Mono- and dibasic potassium phosphate, HPLC grade water, and methanol were acquired from Fisher Chemical Co. GABAse was purchased from Boehringer Mannheim. Dowex 50 and Dowex 1 resins were purchased from Bio-Rad Laboratories. All other reagents were purchased from Aldrich Chemical Co.

(*R*,*S*) [1,2-^{14}C]-2. A solution of sulfuric acid (0.33% v/v in ether, 300 mL) was added to solid [1,2-^{14}C] acetic acid, sodium salt (1 mCi). The flask was capped and the solution was vortexed before being left to stir at room temperature for 15 h. The ether solution was then syringed into a clean flask containing 4 mL of freshly distilled ether. The original flask, containing the white sodium sulfate precipitate, was rinsed with 2×400 μL of freshly distilled ether and the rinses were added to the reaction flask. A 50 μL aliquot was counted for radioactivity to be sure that the conversion to acetic acid had occurred and that the compound was successfully transferred. Solid DCC (250 mg, 1.2 mmol) was added to the flask containing the radioactive acetic acid/ether solution and the solution was stirred for 10 min before the addition of benzyl alcohol (114 μL, 1.1 mmol) and dimethylaminopyridine (18 mg, 0.15 mmol). This solution was allowed to stir at room temperature for 30 min before the addition of unlabeled glacial acetic acid (57 μL, 1.0 mmol). The flask was capped with a septum and left stirring under N_2 at room temperature for 12 h. The heterogeneous reaction solution was then filtered to remove the dicyclohexylurea that had formed. The solid was rinsed with cold doubly distilled, deionized water and finally with cold ether. The water and organic layers were separated. The organic layer was washed with 3×10 mL of saturated sodium bicarbonate solution; then was dried ($MgSO_4$). Solvent was removed by rotary evaporation to give crude [1,2-^{14}C] benzyl acetate; TLC (hexane:ethyl acetate, 4:1); $R_f = 0.53$. The crude [1,2-^{14}C] benzyl acetate was purified on a silica gel column (15 g, 2.5 cm × 9.0 cm) using 9:1 hexane:ethyl acetate. Aliquots of 10 μL were taken from each fraction and counted for radioactivity. Fractions were analysed by silica gel TLC (hexane:ethyl acetate 4:1) and one radioactive spot coeluted with benzyl acetate ($R_f = 0.53$). The product-containing fractions were combined and the solvent was removed by rotary evaporation yielding [1,2-^{14}C] benzyl acetate (103.6 mg, 69%). The formation of the enamine product followed the synthesis described by Mathew et al.,[4] except for several modifications. Lithium bis(trimethylsilyl)amide was prepared in situ under N_2. *N*-Butyllithium (408 μL, 1.02 mmol) was added to 5 mL of freshly distilled THF. The reaction temperature was lowered to −30 °C and 1,1,1,3,3,3-hexamethyldisilazane (215 μL, 1.02 mmol) was syringed into the reaction solution. The solution was allowed to stir at −30 °C for 30 min, after which time the reaction temperature was lowered to −78 °C. A solution of lower specific activity [1,2-^{14}C] benzyl acetate was prepared by dissolving unlabeled benzyl acetate (25 mg, 0.17 mmol) and [1,2-^{14}C] benzyl acetate (103.6 mg, 0.69 mmol) in 800 μL of freshly distilled THF. The benzyl acetate solution was vortexed then slowly syringed into the lithium bis(trimethylsilyl)amide solution. The flask that had contained the benzyl acetate solution was rinsed with 2×500 μL of THF and the rinses were added to the reaction solution. The solution was then left stirring at −78 °C for 2 h before being warmed to −40 °C. The reaction was maintained at this temperature for an additional 45 min then was quenched with 7 N acetic acid in THF (1 mL) before being allowed to warm to room temperature. About 3 mL of water was then added to the reaction solution to dissolve the white solid that had formed upon quenching. The organic layer was separated and the water layer was extracted with 3×10 mL of ether. The combined organic layers were washed with 2×10 mL of water and finally with 1×10 mL of saturated sodium chloride solution. The organic layer was dried over $MgSO_4$. The drying agent and solvent were removed at 25 °C.

The yellow oil (133 mg) obtained was used directly in the subsequent reaction. The oil was dissolved in 3 mL of methanol, then sodium cyanoborohydride (69 mg, 110 mmol) was added to the solution. The flask was cooled to 0 °C and a solution of 5 N HCl in methanol (0.61 mL, 2:3 coned HCl) was added. The solution was stirred at 0 °C for 30 min and then was allowed to warm slowly to room temperature where it was left to stir for 12 h. The solid was removed by rotary evaporation to yield a white paste. This paste was dissolved in water (5 mL), then extracted with ethyl acetate (3×5 mL). The water solution was basified with 1 M NaOH to pH 9.5, and was then extracted with ethyl acetate (3×5 mL). Both the water layer and the second ethyl acetate fractions were worked up separately. The second ethyl acetate solution was dried ($MgSO_4$) before removing the solvent by rotary evaporation to yield benzyl 3-amino-4-fluorobutanoate as a pale-yellow oil. TLC in butanol:acetic acid:water, 3:1:1 gave one radioactive, ninhydrin positive spot, $R_f = 0.72$, which coeluted with a cold sample of the ester. To this, 5 N HCl (2 mL) was added, and the solution was left to stir at room temperature for 48 h. The acidic solution was then dried by rotary evaporation and the white solid was washed several times with water, which was subsequently removed by rotary evaporation. The resultant solid was recrystallized from ethanol–ethyl acetate to yield off white crystals; mp 150–152 °C, $R_f = 0.37$ (butanol:acetic acid:water 3:1:1).

The water was removed from the water layer by rotary evaporation, the residue was dissolved in water and basified to pH 9.5, and was then applied to a Dowex 1 column (hydroxide form, 0.7 × 10 cm). The column was washed with ~50 mL of water to remove all sodium ions. The product was eluted with a 1 M HCl solution. Radioactive fractions were combined, and solvent was removed by rotary evaporation. The remaining solid was evaporated several times from water to remove any excess acid, and the product was recrystallized in ethanol–ethyl acetate to yield off white crystals. A combined yield of [1,2-^{14}C]-**2** from both the hydrolysis of the benzyl ester and from the water layer was 37.2

mg (24%); mp 149–150 °C, R_f=0.37 (butanol:acetic acid:water 3:1:1).

Specific radioactivity of [1,2-^{14}C]-2. [1,2-^{14}C]-**2** (1.46 mg, 9.3×10^{-3} mmol) was dissolved in water (200 μL). Three aliquots (5 μL each) were taken and added to separate scintillation vials containing 10 mL of scintillation cocktail. The samples were each counted twice and the average dpm was calculated. The specific activity was 0.29 mCi/mmol.

Radiopurity of [1,2-^{14}C]-2. The above solution of [1,2-^{14}C]-**2** (46.33 mM) was spotted on half of a 20 cm long silica gel plate. A nonradioactive solution of **2** was spotted on the second half of the plate. The plate was eluted in a solution of butanol:acetic acid:water (3:1:1) until the solvent front had traveled at least 15 cm, then the plate was allowed to dry thoroughly before the radioactive side of the plate was divided into 17 fractions (1 cm each), which were scraped into separate scintillation vials. Water (1 mL) was added to each silica gel-containing vial to dissolve any compound adsorbed on the gel. The vials were gently shaken and allowed to sit for 4 h before scintillation fluid was added, and the samples were counted. The radiopurity was >99%.

Synthesis of [4-^{3}H] pyridoxal 5′ phosphate. [4-^{3}H] Pyridoxal-5′-phosphate was synthesized by a variation in the method of Stock et al.[19] The product was then purified by Dowex 50 cation exchange chromatography.[20] Directly before use, a sample was purified by reversed phase HPLC on an octadecylsilyl (C-18) semipreparative column eluting with water containing 0.1% TFA. The concentration of the [^{3}H] PLP-containing eluate was calculated by measuring the absorbance at 388 and 330 nm and using the extinction coefficients (4900 M^{-1} and 2500 M^{-1}, respectively) reported by Peterson and Sober.[21] Six aliquots (two each of 10 μL of concentrated PLP solution, 10:1 dilution, and 100:1 dilution) were added to 10 mL of scintillation cocktail and counted for radioactivity.

Synthesis of 4-[2-hydroxy-3-methyl-6-(phosphonooxymethyl)-4-pyridinyl]-2-oxo-3-butene (3). The adduct of PLP with acetone was prepared using a modified version of the procedure of Schnackerz et al.[22] PLP (27.5 mg, 0.1 mmol) was dissolved in 6 mL of 0.5 N potassium hydroxide (pH 13). Acetone (76 μL, 1 mmol) was added to the solution. The mixture was stirred for 24 h protected from light before it was titrated to pH 7.0 with concentrated perchloric acid. The white precipitate that formed was removed by centrifugation. The solution was extracted with ethyl acetate (3×5 mL) and the aqueous solutions were cooled and centrifuged again to remove more $KClO_4$. The solvent was removed by lyophilization. Recrystallization attempts were unsuccessful. The product gave one peak on HPLC (A=67 mM KPP_i, pH 2.6; B=methanol) with an elution program of 100% solvent A for 30 min (0.5 mL/ min) then increasing the flow rate to 1 mL/min over the next 5 min. A gradient from 100% A to 85% A/15% B was run over 15 min. The 15% B mixture was then run for additional 20 min. Solvent evaporation gave an orange–yellow solid; ^{1}H NMR (D_2O): δ 7.68 (d, 1 H, J=16 Hz), δ 7.58 (s, 1 H), δ 7.34 (d, 1 H, J=16 Hz), δ 4.87 (d, 2 H, J=16 Hz), δ 4.72 (s, HOD), δ 2.46 (s, 3 H), δ 2.38 (s, 3 H); ^{1}H NMR (DMSO-d_6): δ 7.71 (s, 2 H), 7.12 (s, 1 H), 4.56 (d, 2 H), 3.7 (bd, 1 H), 2.22 (s, 2 H), 2.17 (s, 2 H), 2.09 (s, 2 H); ^{13}C NMR (D_2O): δ 206.9, 165.3, 146.8, 139.3, 136.8, 134.7, 134.2, 122.8, 63.2, 28.4, 18.5.

A sample was purified on Dowex 50W (X8) by elution with water. The fractions were lyophilized to dryness, and the light yellow residue was analysed by HRMS; HRMS (FAB^-): calcd for $C_{11}H_{15}NO_6P$ (MH^+) m/z 288.0638, found m/z 288.0599.

Synthesis of 5-[2-hydroxy-3-methyl-6-(phosphonooxymethyl)-4-pyridinyl]-3-oxo-4-pentenoic acid (4). The adduct of PLP with acetoacetic acid was prepared exactly as described above except that lithium acetoacetate (54 mg, 0.5 mmol) was used in place of the acetone, and the solution was acidified with HCl before being extracted with ethyl acetate. The product obtained gave two peaks by HPLC on the system described above. Attempts were made to isolate this product, but some of the decarboxylated product (synthesized above) was always present in solution.

Enzymes and assays

GABA aminotransferase was isolated from pig brains deep frozen immediately after excision.[15] This enzyme, showing one band on $NaDodSO_4$-gel electrophoresis at pH 7.0 by Coomassie stain,[23] had a specific activity of 4.89 units/mg of protein. One unit is defined as the amount of enzyme that catalyses the transamination of 1 μmol of GABA/min at 25 °C. Enzyme activity was measured using a modification[6] of the coupled assay developed by Scott and Jakoby.[24] The final concentrations in the assay solution were 11 mM GABA, 5.3 mM αKG, 1.1 mM NADP, 5 mM β-mercaptoethanol, and excess succinic semialdehyde dehydrogenase (SSDH) in 50 mM potassium pyrophosphate buffer, pH 8.5. The enzyme activity was determined by measuring the change of absorbance at 340 nm caused by the production of NADPH. Succinic semialdehyde dehydrogenase (SSDH) was isolated from GABAse, a mixture of SSDH and GABA aminotransferase, by inactivation of the GABA aminotransferase with gabaculine with subsequent dialysis to remove excess inactivator as described previously.[25] Protein assays were carried out using bovine serum albumin (BSA) and Pierce Coomassie protein assay reagent for standard curves. Any aqueous solutions were prepared using distilled deionized water.

Preparation of [^{3}H] PLP GABA aminotransferase. *Apo*-GABA aminotransferase was prepared by the method of Churchich and Moses[15] with the following modifications: GABA aminotransferase (1.23×10^{-5} mmol) in potassium phosphate buffer (100 mM, pH

7.4) containing 0.25 mM of β-mercaptoethanol was incubated with GABA (11.45 mg, 0.11 mmol) in a total volume of 1.0 mL for 0.5 h at room temperature. The pH of the solution was lowered to pH 5.3 through the addition of 1 M monobasic potassium phosphate (1400 μL). The solution was then allowed to incubate at 4 °C for 90 min, then was dialysed against 2 L of potassium phosphate buffer (100 mM, pH 7.4), containing 0.25 mM of β-mercaptoethanol, for 4 h at 4 °C. [^{3}H] PLP (2.1×10^{-4} mmol) was added to the *apo*-enzyme solution and the sample was incubated at room temperature for 10 h, until the activity increased no further. The sample was then dialysed against 4×2 L potassium phosphate buffer (100 mM, pH 7.4, 0.25 mM β-mercaptoethanol) to remove excess [^{3}H] PLP. The protein concentration was determined using Pierce BCA protein assay reagent with bovine serum albumin as the standard. Aliquots (10 μL) of the protein were counted to determine radioactivity.

Inactivation and denaturation of [^{3}H] PLP GABA aminotransferase with 2. [^{3}H] PLP GABA aminotransferase (300 μL; 0.09 mg) was incubated in the dark at 25 °C in 100 mM potassium phosphate buffer (pH 7.4) containing 0.5 mM α-ketoglutarate, 0.5 mM β-mercaptoethanol, and 1.0 mM **2** in a total volume of 320 μL. Two controls were identical except that **2** was replaced with buffer or **1**, respectively. A third control was performed using GABA instead of α-ketoglutarate in a solution containing buffer instead of inactivator. Upon complete inactivation of the enzyme, small molecules were removed from the solution by dialysis against 700 mL of water for 4 h. The pH of each solution was adjusted to 11–12 with 1 M KOH. Samples were then left to incubate in the dark at room temperature for 1 h, then solid trichloroacetic acid (TCA) was added to each sample to make the solutions 10% in TCA. After incubating at room temperature, protected from light (10 min) the denatured enzyme solutions were microcentrifuged for 5 min to pellet any precipitated enzyme. The supernatants were removed and added to labeled eppendorf tubes. The pellets were washed with 60 μL of a 10% TCA solution, vortexed, and microcentrifuged for an additional 5 min. The rinses were added to the supernatants. This procedure was repeated a total of three times to remove any unbound radioactivity from the pellets.

Determination of equivalents of [^{3}H] bound to GABA aminotransferase after base denaturation and acid precipitation of [^{3}H] PLP GABA aminotransferase inactivated with 2. The washed pellets from the TCA precipitations described above were redissolved in 2 M KOH (100 μL). The redissolved pellets were transferred to scintillation vials and the Eppendorf tubes that had originally contained the pellets were rinsed with an additional 50 μL of 2 M KOH. The rinses were added to the scintillation vials. Scintillation fluid (10 mL) was added to each vial and the samples were counted for radioactivity.

HPLC analysis of tritiated products released from the 2-inactivated [^{3}H] PLP GABA aminotransferase after denaturation. Tritiated products released from the denatured [^{3}H] PLP GABA aminotransferase were analysed by HPLC with detection at 254 nm. Standard solutions of PLP and PMP were prepared using the same base/acid treatment used to denature and precipitate the enzyme of the inactivation solutions. The PLP and PMP standards (25 μL) were added to the supernatant solutions from the denaturation of the enzyme. An aliquot (75 μL) of a sample/standard solution mixture was injected into the HPLC system. Solvent A was 67 mM monobasic potassium phosphate titrated to pH 2.6 with phosphoric acid. Solvent B was methanol. The elution program used was as follows: Solvent A (0.5 mL/min) for 30 min and then the flow rate was increased to 1 mL/min over 5 min. After 35 min, the percentage of solvent B was increased to 15% over 15 min and then maintained there for an additional 60 min. Fractions were collected every min for 110 min. Scintillation fluid was added to each fraction and the samples were counted for radioactivity.

Release of fluoride ions during inactivation of GABA aminotransferase with 2. GABA aminotransferase (100 mL, 0.17 mg, 1.56×10^{-6} mmol) was incubated with **2** (1 mM), α-ketoglutarate (2 mM), and β-mercaptoethanol (0.5 mM) in potassium phosphate buffer (50 mM, pH 7.5) in a total volume of 200 mL at room temperature in the dark for at least 6 h, until the enzyme had less than 1% of its original activity. Two different controls were carried out: one contained inactivator but no enzyme, and the other contained enzyme but no inactivator. A standard fluoride ion concentration curve was constructed by plotting the relative voltage of standard sodium fluoride solutions against the log of the fluoride ion concentration (log [F^-]). The relative voltage of solutions containing various amounts of standard sodium fluoride solution (1×10^{-6} M to 9.1×10^{-6} M) in a 1:1 mixture of 50 mM potassium phosphate (pH 7.5) and low level total ionic strength adjusting buffer (58 g sodium chloride, 57 mL of acetic acid, 4 g EDTA in 1 L water adjusted to pH 5.25) were measured. The inactivated solutions and controls were tested for fluoride ion release by measuring the relative voltage of these solutions in a 1:1 potassium phosphate and low level total ionic strength adjusting buffer mixture. The concentration of fluoride ions was determined using the standard curve obtained. The control readings were subtracted from the experimental fluoride ion concentrations to determine the amount of fluoride released during inactivation.

Transamination events per inactivation of GABA aminotransferase with 2. The number of transamination events that occur per inactivation event was determined for the inactivation of GABA aminotransferase (25 μL, 0.04 mg, 3.9×10^{-7} mmol) in 100 mM potassium phosphate (pH 7.4) with **2** (1 mM) in the presence of ^{14}C α-ketoglutarate (5 mM, 0.062 μCi) and β-mercaptoethanol (0.5 mM) incubated in a total

volume of 200 μL at room temperature in the dark. Controls were run either without enzyme or without inactivator. After complete inactivation of the enzyme, the sample was acidified using a 20% TCA solution (v/v) to make the final concentration of TCA 9%. After being vortexed, the solutions were applied to prewashed Dowex 50 columns (0.5 × 6.0 cm). The columns were eluted with 6 mL of water, followed by 1 mL of 2 M ammonium hydroxide, and then 7 mL of 2 M ammonium hydroxide. Each washing of the column was collected separately. Scintillation fluid was added to each fraction and the samples were counted for radioactivity.

Titration of GABA aminotransferase with 2. GABA aminotransferase (25 μL, 0.04 mg, 3.9×10^{-7} mmol) in 100 mM was incubated with 1–10 equiv of **2** in the presence of α-ketoglutarate (25 μM) and β-mercaptoethanol (0.5 mM) in a total volume of 100 μL. Periodically, 5 μL aliquots of the inactivation solution were tested for activity. The activity remaining in the enzyme solution was followed for at least 40 h or until no activity remained.

Inactivation of GABA aminotransferase by 2 with and without α-ketoglutarate. GABA aminotransferase (25 μL, 0.04 mg, 3.9×10^{-7} mmol) was incubated with 5 or 6.5 equiv of inactivator in the presence of β-mercaptoethanol (0.5 mM) in a total volume of 100 μL either with or without α-ketoglutarate (10 μL, 0.32 mM). The solutions were periodically tested for activity. When the solutions showed no additional loss of activity, α-ketoglutarate (10 μL, 0.32 mM) was added to the samples previously devoid of this compound and buffer (10 μL) was added to samples already containing α-ketoglutarate. Aliquots (5 μL) were taken to be assayed for activity immediately upon addition of these compounds and again after 30 min of incubation.

Inactivation of GABA aminotransferase with [1,2-^{14}C]-2. In a typical experiment, GABA aminotransferase (250 μL, 0.25 mg) was incubated in the dark at 25 °C in a total volume of 280 μL of 100 mM potassium phosphate buffer (pH 7.4) containing a final concentration of 2.4 mM α-ketoglutarate, 2.0 mM β-mercaptoethanol, and 0.33 mM **2**. Aliquots (2 μL) were periodically taken to assess remaining activity.

Removal of unbound small molecules from the GABA aminotransferase solution. When the enzyme was less than 1% active, the small molecules were removed from the inactivation solution by one of two methods. In some cases, the solution was applied to a Sephadex G-50 column and eluted by the Penefsky spin method[26] using 100 μL of 100 mM potassium phosphate to rinse the column. The eluted enzyme was then used in subsequent experiments. When the small molecules needed to be collected, the enzyme was separated by filtration through a Centricon 30 ultrafiltration concentrator centrifuged at 3000 rpm for 10 min using a JA-20 rotor. The enzyme was rinsed with 4 × 200 mL of buffer or water to assure the removal of all small particles. The enzyme was then recovered in 2 × 150 mL buffer or water. The filtrate was used in experiments to elucidate turnover product.

Equivalents of [1,2-^{14}C]-2 bound to GABA aminotransferase after inactivation. GABA aminotransferase was inactivated, and the small molecules were removed as described under *Inactivation of GABA aminotransferase with [1,2-^{14}C]-2* and Removal of unbound small molecules from the GABA aminotransferase solution. The protein concentration was determined by Coomassie assay using BSA as the protein standard. Aliquots of 100 μL were counted for radioactivity. The number of equiv of inactivator bound to the enzyme was calculated using the ratio of inactivator to enzyme present in the sample based on the specific radioactivity of the inactivator used.

Denaturation of GABA aminotransferase labeled with [1,2-^{14}C]-2. After small molecules were removed from the inactivated enzyme as described under *Removal of unbound small molecules from the GABA aminotransferase solution* and the protein concentration determined by Coomassie assay, a portion of the sample was base denatured, and the enzyme was precipitated by acid addition. The pH of each solution was adjusted to 11–12 with 1 M KOH. Samples were left to incubate in the dark at room temperature for 1 h, then trifluoroacetic acid (TFA) was added to each sample to make the solutions 10% in TFA. After being incubated at room temperature, protected from light for an additional 10 min, the denatured enzyme solutions were microcentrifuged for 5 min to pellet any precipitated enzyme. The supernatants were removed and added to labeled eppendorf tubes. These solutions were saved for future experiments. The pellets were washed with 60 μL of a 10% TFA solution, vortexed, and microcentrifuged for an additional 5 min. The rinses were added to the supernatants. This procedure was repeated a total of three times to remove any unbound radioactivity from the pellets.

Determination of equivalents of [1,2-^{14}C]-2 bound to GABA aminotransferase after base denaturation and acid precipitation. The washed pellets from the TFA precipitations were redissolved in 2 M KOH (100 μL). The redissolved pellets were transferred to scintillation vials and the Eppendorf tubes that had originally contained the pellets were rinsed with an additional 50 μL of 2 M KOH. The rinses were added to the scintillation vials. Scintillation fluid (10 mL) was added to each vial, which were counted for radioactivity.

Release of $^{14}CO_2$ from GABA aminotransferase inactivated with [1,2-^{14}C]-2 and acid precipitated. GABA aminotransferase was inactivated as described in *Inactivation of GABA aminotransferase with [1,2-^{14}C]-2* except that the solution was incubated in a closed system in a scintillation vial capped with a septum fitted with a plastic well containing 150 μL of 2 M potassium hydroxide. The vials were gently shaken until less than 1% of the original enzyme activity

remained in a parallel experiment run in an open vial. To treat this sample the same as other experimental samples, after the enzyme was completely inactivated, 20 μL of 100 mM potassium phosphate buffer (pH 7.4) was carefully added to the solution via syringe. The solution was incubated for an additional 1 h while being shaken gently before the addition of sulfuric acid to make the solution 10% in acid. After the addition of the acid, the solution was shaken for an additional 1 h to allow for the complete release and base absorption of any $^{14}CO_2$ generated. The septum was gently removed from the vial, and a 100 μL aliquot of the KOH solution in the well was taken to be counted for the presence of radioactivity. Controls were treated identically except that no enzyme was added to the solution. The amount of $^{14}CO_2$ released was calculated using 0.5 times the specific radioactivity of the inactivator (the inactivator is labeled equally at C_1 and C_2) to determine the amount of $^{14}CO_2$ dissolved in the base solution. The ratio of this number with the amount of enzyme in the inactivation solution gave the number of equiv of $^{14}CO_2$ released.

Release of $^{14}CO_2$ from GABA aminotransferase inactivated with [1,2-^{14}C]-2 with base denaturation followed by acid precipitation. The enzyme solution was treated exactly as described above except that 20 μL of 1 M potassium hydroxide was added to the solution after complete inactivation of the enzyme instead of the buffer solution.

Release of $^{14}CO_2$ from GABA aminotransferase inactivated with [1,2-^{14}C]-2 denatured with KOH with simultaneous $NaBH_4$ reduction followed by acid precipitation. The enzyme was treated the same as described in *Release of $^{14}CO_2$ from GABA aminotransferase inactivated with [1,2-^{14}C]-2* and acid precipitated, except that 20 μL of $NaBH_4$ (66 mM) in 1 M potassium hydroxide was added instead of the buffer solution after total inactivation of the enzyme.

Determination of equivalents of ^{14}C bound to GABA aminotransferase inactivated with [1,2-^{14}C]-2 and treated with acid, base and acid, or $NaBH_4$ in base and acid. The enzyme precipitated from the solutions in the $^{14}CO_2$ release experiments was pelleted by microcentrifugation. The supernatant was removed to be analysed for enzyme products. The pellets were washed with 100 μL of a 10% sulfuric acid solution, vortexed, and recentrifuged. The rinses were added to the supernatants from the first pelleting. This process was repeated a total of three times for each sample. The pellets, now devoid of unbound small molecules, were redissolved in 200 μL of 2 M potassium hydroxide solution and transferred to scintillation vials. The eppendorf tubes originally containing the pellets were rinsed twice more with 50 μL of 2 M KOH. These rinses were added to the solutions containing the redissolved pellets. These solutions were counted for radioactivity and the number of equivalents bound to the enzyme was determined based on the specific radioactivity of the inactivator.

HPLC analysis of standard compounds: acetoacetic acid (AAA), acetone, 3-amino-4-hydroxybutanoic acid (AHBA), AHBA lactone, 2, 3, and 4. Each of the standard compounds was dissolved in 100 mM potassium phosphate buffer which was adjusted to pH 6.5 after the addition of the compound. Aliquots (10 μL) of each compound were injected into the HPLC and eluted with the program described under *HPLC analysis of tritiated products released from the inactivated ^{3}H PLP GABA aminotransferase upon denaturation*, and monitored at 200 nm. Retention times were determined for each compound. $t_R^{\text{lactone}} = 6.5$, $t_R^{\text{AHBA}} = 7.5$, $t_R^{2} = 8.5$, $t_R^{\text{AAA}} = 16$, $t_R^{\text{acetone}} = 22$, $t_R^{3} = 52.5$, $t_R^{4} = 56$ min.

HPLC analysis of ^{14}C labeled metabolites isolated from GABA aminotransferase inactivated with [1,2-^{14}C]-2 and treated with acid, base and acid, $NaBH_4$ and acid, or $NaBH_4$ in base and acid without removal of excess inactivator and turnover product. The supernatants isolated from enzyme under *Determination of equivalents of ^{14}C bound to GABA aminotransferase inactivated with [1,2-^{14}C]-2* and treated with acid, base and acid, $NaBH_4$ and acid, or $NaBH_4$ in base and acid were adjusted to pH 6.5 with potassium hydroxide. These solutions were analysed by HPLC using the elution program described under *HPLC analysis of tritiated products released from the inactivated [^{3}H] PLP GABA aminotransferase upon denaturation*. The absorbance was monitored at 200 nm. Fractions were collected every min for 70 min. Scintillation fluid was then added to each fraction. The samples were counted for radioactivity by liquid scintillation counting.

HPLC analysis of ^{14}C labeled metabolites formed during the inactivation of GABA aminotransferase with [1,2-^{14}C]-2. The small molecules isolated by Centricon 30 ultrafiltration as described in *Removal of unbound small molecules from the GABA aminotransferase solution* were analysed by HPLC using the same elution profile described under *HPLC analysis of tritiated products released from the inactivated [^{3}H] PLP GABA aminotransferase upon denaturation*. The filtrate solution, with acetone and acetoacetic acid added, was adjusted to pH 6.5 then injected into the HPLC. The elution was monitored at 200 nm, and the eluant was collected every 0.5 min for the first 30 min and every min for the remaining time. Scintillation fluid was added to each fraction before being counted for radioactivity.

TLC analysis of ^{14}C labeled metabolites formed during the inactivation of GABA aminotransferase with [1,2-^{14}C]-2. About 10 μL of the filtrate solution isolated by Centricon 30 ultrafiltration under *Removal of unbound small molecules from the GABA aminotransferase solution* was spotted 2.0 cm from the bottom of a 20 cm silica gel TLC plate. A solution containing each of the HPLC standards also was spotted on the plate. The plate was eluted in the dark with methanol containing 0.5% ammonium hydroxide. The standards were located by UV visualization. The half of the plate containing the radioactively labeled excess inactivator

and metabolites was cut into 0.5 cm fractions which were scraped from the plate. The scrapings were put into individually labeled scintillation vials and dissolved in 1 mL of water. The silica gel was soaked for about 2 h then diluted with 10 mL of scintillation fluid and counted for radioactivity. The half containing the standards was then developed using a ninhydrin stain; R_f[3] and **4** = 0.07, $R_f^{\text{lactone}} = 0.41$, R_f[2] = 0.78, $R_f^{\text{AAA}} = 0.84$.

HPLC analysis of ^{14}C labeled products released upon gel filtration, base denaturation, and acid precipitation of GABA aminotransferase inactivated with [1,2-^{14}C]-2. Denatured enzyme was centrifuged to separate precipitated enzyme from released products in the supernatant as described under *Denaturation of GABA aminotransferase labeled with [1,2-^{14}C]-2*. The supernatant solution was adjusted to pH 6.5 with potassium hydroxide while being cooled in an ice bath. Unlabeled standards (**2**, acetoacetate, PLP, **3**, and **4**) were added to the solution and monitored for absorbance at 200 nm. This solution was injected into the HPLC under the same conditions used for the elution of tritiated products as described under *HPLC analysis of tritiated products released from the inactivated ^{3}H PLP GABA aminotransferase upon denaturation*, except that absorbance was monitored at 200 nm. Fractions were collected in 0.5 min fractions for the first 30 min and from 50–60 min; 1 min fractions from 30–50 min and 60–70 min were collected. Scintillation fluid (10 mL) was added to each fraction and they were counted for the presence of radioactivity.

Separation by Dowex and HPLC analysis of products released from GABA aminotransferase after inactivation with [1,2-^{14}C]-2 and denaturation with KOH with TFA precipitation. Supernatant solutions saved after the base denaturation and acid precipitation of GABA aminotransferase processed as described under *Denaturation of GABA aminotransferase labeled with [1,2-^{14}C]-2* were applied to either a prewashed 0.5 × 7 cm Dowex 50 column or a prewashed 0.7 × 10 cm Dowex 1 column. The columns were washed with 11 mL of water, and 1 mL fractions were collected. The columns were subsequently eluted with 3 mL each of 0.1, 0.2, 0.3, 0.4, 0.6, 0.8 M HCl, and finally with 6 mL of 1.0 M HCl. Aliquots of 120 μL (Dowex 50) or 150 μL (Dowex 1) were counted by liquid scintillation counting. Fractions containing radioactivity were combined and the pH of the solutions were adjusted to 6.5. Each solution was injected into the HPLC and eluted using the same program described under *HPLC analysis of tritiated products released from the inactivated [^{3}H] PLP GABA aminotransferase upon denaturation*. Absorbance was monitored at 200 nm.

TLC analysis of ^{14}C labeled metabolites released upon denaturation of GABA aminotransferase inactivated with [1,2-^{14}C]-2. An aliquot (20 μL) of the supernatant saved after denaturation and precipitation of inactivated GABA aminotransferase as described under *Denaturation of GABA aminotransferase labeled with [1,2-^{14}C]-2* was spotted 2.0 cm from the bottom of one-half of a silica gel plate. The plate was then treated in the same manner as that under *TLC analysis of ^{14}C labeled metabolites formed during the inactivation of GABA aminotransferase with [1,2-^{14}C]-2*.

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