# An Aromatization Mechanism of Inactivation of γ-Aminobutyric Acid Aminotransferase for the Antibiotic L-Cycloserine

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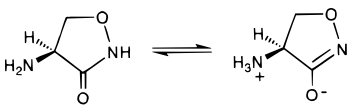
**Abstract:** The mechanism of inactivation of pig brain γ-aminobutyric acid (GABA) aminotransferase by the antibiotic L-cycloserine was investigated. L-Cycloserine is a time-dependent inactivator of GABA aminotransferase; no enzyme activity returns upon gel filtration or dialysis. Treatment of GABA aminotransferase with [$^{14}$C]-L-cycloserine, followed by rapid gel filtration, gives enzyme containing l.l equiv of radioactivity bound. Dialysis or denaturation by acid, base, or urea releases the radioactivity. Inactivation of [$^{3}$H]pyridoxal 5′-phosphate (PLP)-reconstituted GABA aminotransferase with L-cycloserine followed by dialysis or denaturation also leads to the release of radioactivity from the enzyme. Both the released [$^{14}$C]- and [$^{3}$H]-labeled adducts comigrate by HPLC, suggesting that the inactivation adduct is a condensation product of L-cycloserine with the PLP coenzyme. By HPLC comparison, it was shown that the radiolabeled adduct is not PLP, PMP, PLP oxime, or 4-[3-hydroxy-2-methyl-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid (**20**), the expected product of an enamine-type inactivation mechanism. On the basis of the stability of the released adduct to acid and base and its UV−visible spectrum, which has the appearance of a PMP analogue, a simple Schiff base between PLP and cycloserine also was excluded. HPLC of the cycloserine−coenzyme adduct had a retention time very similar to that of the gabaculine−coenzyme adduct. Electrospray ionization tandem mass spectrometry of the isolated cycloserine−coenzyme adduct is consistent with a structure that is one of the tautomeric forms of the Schiff base between PMP and oxidized cycloserine (**21**).

## Introduction

L-Cycloserine (**1**, Seromycin), an oral antibiotic used in the treatment of active



**1a** **1b**

pulmonary and extrapulmonary tuberculosis,[1] was isolated from various strains of *Streptomyces* in 1955 by four different groups.[2] In addition to its activity as a broad spectrum antibiotic,[1,2] it also exhibits the ability to increase the levels of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) in vivo[3−5] and has previously been reported to inhibit the pyridoxal 5′-phosphate (PLP)-dependent enzyme GABA aminotransferase (EC 2.6.1.19) in vitro.[3] Time-dependent inhibition of GABA aminotransferase from *E. coli*, cat brain, and monkey brain was reported for DL-cycloserine.[6] D-Cycloserine was found to inactivate D-amino acid aminotransferase from *B. sphaericus*; dialysis against PLP at pH 8.5 results in no return of enzyme activity, but at pH 6.5 complete activity returns upon dialysis against PLP.[7] D- and L-Cycloserine also are irreversible inhibitors of *Bacteriodes levii* and mouse brain 3-ketodihydrosphingosine synthetase, a PLP-dependent enzyme that catalyzes the condensation of palmitoyl-CoA and serine, the first step in the biosynthesis of sphingolipids.[8] Cycloserine also inhibits alanine racemase, D-Ala-D-Ala synthetase, and other aminotransferases and decarboxylases.[1] The recognition of cycloserine by these enzymes can be related to the fact that the p$K_a$ of the protonated amino group is 7.3 and that of the amido functionality is 4.4,[9] and, therefore, at neutral pH, the compound exists in a zwitterionic form (**1b**).

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§ Carried out all of the experiments except for the ones related to electrospray mass spectral analysis of the modified coenzyme.

⊥ Carried out the preparation, isolation, and purification of the modified coenzymes used for the electrospray mass spectral analysis.

|| Carried out the electrospray mass spectral analysis of the modified coenzymes.

(1) (a) *Physicians' Desk Reference*, 50th ed.; Medical Economics Co.: Des Moines, IA, 1996. (b) Epstein, I. G.; Nair, K. G. S.; Boyd, L. *J. Transactions of the 14th Conference on the Chemotherapy of Tuberculosis*; 1955; p 326.

(2) Neuhaus, F. C. In *Antibiotics I, Mechanism of Action;* Gottlieb, D., Shaw, P. D., Eds.; Springer-Verlag: New York, 1967; pp 40−83.

(3) Chung, S.; Johnson, M. S.; Gronenborn, A. M. *Epilepsia* **1984**, *25*, 353.

(4) Scotto, P.; Monaco, P.; Scardi, V.; Bonavita, V. *J. Neurochem.* **1964**, *10*, 831.

(5) Wood, J. D.; Peesker, S. J.; Gorecki, D. K. J.; Tsui, D. *Can. J. Physiol. Pharmacol.* **1978**, *56*, 62.

(6) Dann, O. T.; Carter, C. E. *Biochem. Pharmacol.* **1964**, *13*, 677.

(7) Soper, T. S.; Manning, J. M. *J. Biol. Chem.* **1981**, *256*, 4263.

(8) Sundaram, K. S.; Lev, M. *J. Neurochem.* **1984**, *42*, 577.

(9) Hidy, P. H.; Hodge, E. B.; Young, V. V.; Harned, R. L.; Brewer, G. A.; Phillips, W. F.; Runge, W. F.; Stavely, H. E.; Pohland, A.; Boaz, H.; Sullivan, H. R. *J. Am. Chem. Soc.* **1955**, *77*, 2345.

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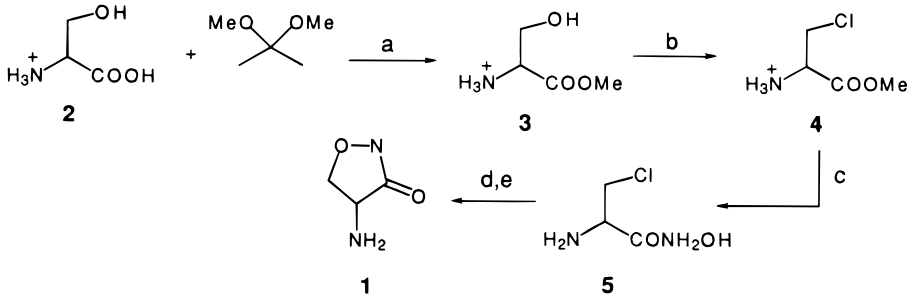
**Scheme 1.** Synthesis of L-[$^{14}$C]Cycloserine




[a] a, HCl; b; $PCl_5/CHCl_3$; c, $NH_2OH/NaOH$; d, Dowex 50; e, 0.2 M $NH_4OH$.

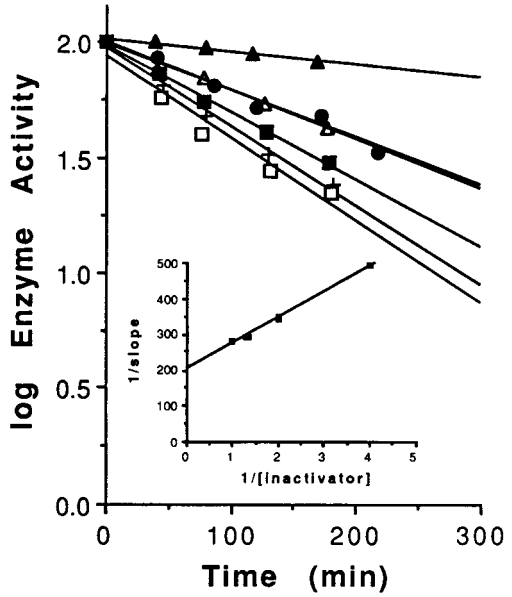



**Figure 1.** Time-dependent inactivation of GABA-AT by L-cycloserine. Log of remaining enzyme activity vs time for L-cycloserine at pH 8.5, 25 °C: 0.0 (▲), 0.25 (△), 0.50 (■), 0.75 (+), l.0 (□), 0.5 mM L-cycloserine, and 5 mM GABA (●). See Experimental Section for details. (Inset) Absolute value of the slopes from the lines in Figure l vs the inverse of the inactivator concentration.

The mechanism of inactivation of these PLP-dependent enzymes by cycloserine is unknown. Experiments described in this paper are directed at the elucidation of the mechanism of inactivation of GABA aminotransferase by cycloserine, but the results described here should be directly applicable to the mechanism of inactivation of other PLP-dependent enzymes by cycloserine.

## Results

**Synthesis of L-[U-$^{14}$C]Cycloserine.** L-[$^{14}$C]Cycloserine was synthesized starting from L-[U-$^{14}$C]serine (**2**, Scheme 1), which was converted to its methyl ester (**3**) with 2,2-dimethoxypropane followed by conversion to 3-chloroalanine methyl ester (**4**), then to 3-chloroalanine hydroxamate methyl ester (**5**), and finally cyclization to L-[U$^{14}$C]cycloserine (**1**).

**Time-Dependent Inactivation of GABA Aminotransferase by L-Cycloserine.** Inhibition of GABA aminotransferase by cycloserine was time-dependent (Figure 1); $K_I$ and $k_{inact}$ values were determined by the method of Kitz and Wilson[10] (Figure 1, inset) to be 360 $\mu$M and 0.005 min$^{-1}$, respectively. Substrate was shown to protect the enzyme from inactivation.

**Time-Dependent Inactivation of GABA Aminotransferase by L-Cycloserine at pH 9.25.** Inactivation of GABA aminotransferase at pH 9.25 was carried out with cycloserine. In 50 min the control (no inactivator) lost 14% of its enzyme activity, whereas the cycloserine enzyme solution was completely inactivated.

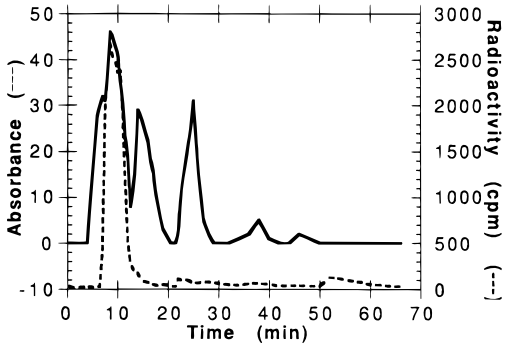



**Figure 2.** HPLC analysis of the product released by dialysis of [$^3$H]-PLP-reconstituted GABA aminotransferase inactivated by L-cycloserine. Radioactivity and absorbance are plotted versus retention time of the standards and [$^3$H]labeled metabolites. The absorbance peaks correspond to the standards: PMP (10 min), PLP (16 min), and PLP oxime (27 min). See Experimental Section for details.

**Determination of the Stoichiometry of L-[$^{14}$C]Cycloserine Bound to GABA Aminotransferase after Inactivation.** After GABA aminotransferase was inactivated with L-[$^{14}$C]-cycloserine and gel filtered, 1.12 equiv were bound covalently, suggesting that one molecule of L-cycloserine binds to one dimer of GABA aminotransferase.

**Determination of the Amount of L-[$^{14}$C]Cycloserine Covalently Bound to GABA Aminotransferase after Denaturation.** Following acid precipitation and base solubilization of the enzyme inactivated with L-[$^{14}$C]cycloserine, no radioactivity was detected attached to the protein. Likewise, when the labeled enzyme was treated with base or 8 M urea and was analyzed by HPLC, little or no radioactivity was detected in the methanol rinse that contained the protein. The enzyme also was separated from the pH 12 and 8 M urea solutions by removal of the metabolites by ultrafiltration; the protein left on the membrane had no radioactivity bound to it.

**Inactivation of [$^3$H]PLP-Reconstituted GABA Aminotransferase by L-Cycloserine.** The major metabolite formed from the inactivation of [$^3$H]PLP-reconstituted GABA aminotransferase by L-cycloserine followed by dialysis or denaturation of the enzyme had a retention time by HPLC similar to that of pyridoxamine 5′-phosphate (PMP) (Figure 2). The data in Figure 2 were from a microdialysis experiment.

**Inactivation of GABA Aminotransferase by L-[$^{14}$C]Cycloserine.** The metabolite formed from the inactivation of GABA aminotransferase by L-[$^{14}$C]cycloserine followed by dialysis or denaturation of the enzyme had a retention time by HPLC the same as the experiment with [$^3$H]PLP-reconstituted

(10) Kitz, R.; Wilson, I. B. *J. Biol. Chem.* **1962**, *237*, 3245.

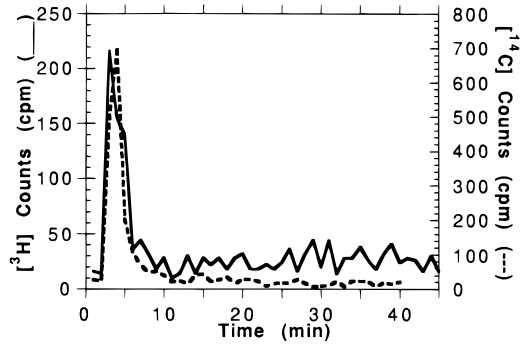



**Figure 3.** Comparison of the metabolites formed from inactivation of [$^3$H]PLP-reconstituted GABA aminotransferase with cycloserine or GABA aminotransferase with L-[$^{14}$C]cycloserine. See Experimental Section for details.

GABA aminotransferase and L-cycloserine (Figure 3). No radioactivity comigrated with 4-[3-hydroxy-2-methyl-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid (**20**) or with the oxime of PLP.

***o*-Phthalaldehyde (OPA) Derivatives of PMP, Cycloserine, and the Cycloserine Metabolite.** Whereas PMP and cycloserine elute at about 3 min with the buffer HPLC system, the corresponding OPA derivatives elute at 16 and 28 min, respectively. Under conditions that convert PMP and cycloserine into OPA derivatives, the radioactive metabolite formed from the inactivation of [$^3$H]PLP-reconstituted GABA aminotransferase by cycloserine or from inactivation of GABA aminotransferase by L-[$^{14}$C]cycloserine, followed by dialysis or denaturation of the enzyme, still eluted at about 3 min. When the metabolite was incubated at room temperature in 0.5 M HCl or at 100 °C in 0.1 M HCl, no change in the results of the experiment were observed; all of the radioactivity eluted at about 3 min. Therefore, the metabolite is not PMP or cycloserine and appears to be stable to acid and heat treatment.

**Phenyl Isothiocyanate (PITC) Derivatives of PMP, Cycloserine, and the Cycloserine Metabolite.** PITC derivatives of PMP and cycloserine have retention times of 12 and 14 min, respectively, when using the same HPLC conditions as above. Under conditions that convert PMP and cycloserine into PITC derivatives, the radioactive metabolite formed from the inactivation of [$^3$H]PLP-reconstituted GABA aminotransferase by cycloserine or from inactivation of GABA aminotransferase by L-[$^{14}$C]cycloserine, followed by dialysis or denaturation of the enzyme still eluted at 3 min. Addition of $NaBH_4$ to the inactivation solution also had no effect.

**Dialysis of L-Cycloserine-Inactivated GABA Aminotransferase.** The GABA aminotransferase solution inactivated with cycloserine returned to 57% of the activity of the dialyzed control in 2.5 h after the addition of PLP. When [$^3$H]PLP-reconstituted GABA aminotransferase was inactivated with cycloserine, 99% of the total radioactivity was removed by dialysis. When L-[$^{14}$C]cycloserine was used with GABA aminotransferase, 92% of the total radioactivity was initially removed, but the remaining radioactivity, which was released from the enzyme upon denaturation, had the same retention time as radioactivity from the dialysate; consequently, it appears that dialysis had not been allowed to proceed long enough in this experiment.

**UV−Visible Spectral Analysis of the Inactivation Product Formed from L-Cycloserine Inactivation of GABA Aminotransferase.** The inactivation of GABA aminotransferase by L-cycloserine was monitored by UV−visible spectroscopy and periodic activity measurements. As the inactivation proceeded, the enzyme absorbance maximum at 412 nm decreased and the peak at 332 nm increased (Figure 4). Denaturation of the enzyme at pH 12 (pH was lowered to 7.4 after an hour) or in 8 M urea yielded a product that had the same UV−visible absorbance profile as PMP, with a maximum at 332 nm.

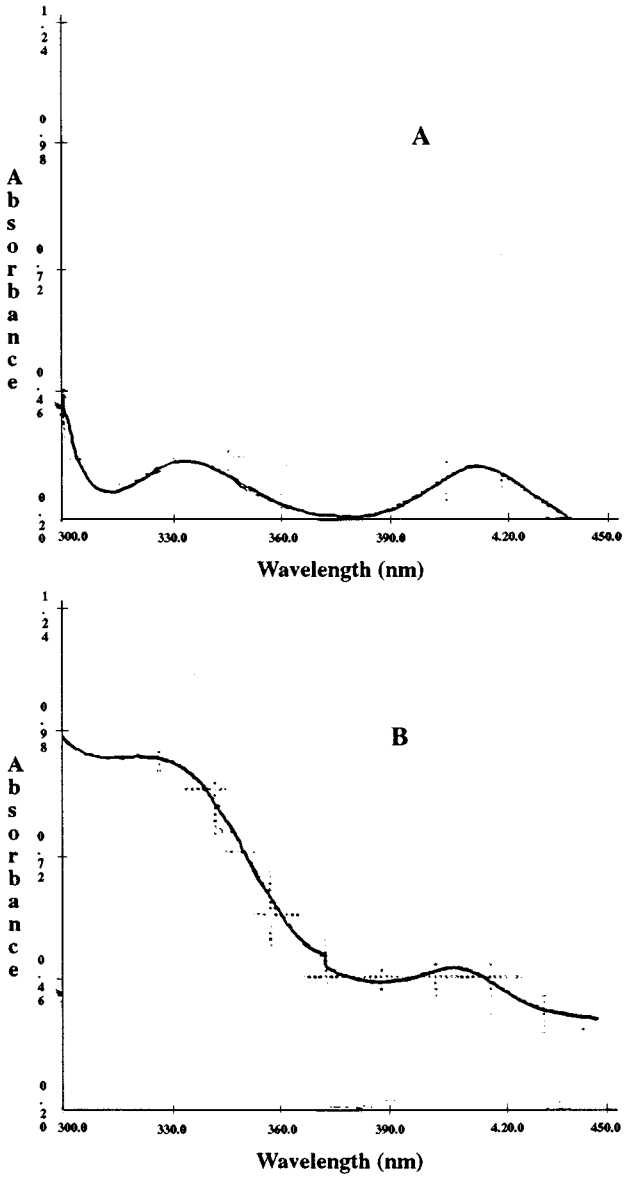



**Figure 4.** A, UV−visible spectrum of GABA aminotransferase. B, UV−visible spectrum of GABA aminotransferase inactivated by L-cycloserine. See Experimental Section for details.

**UV−Visible Spectral Analysis of the Inactivation Product Formed from Hydroxylamine Inactivation of GABA Aminotransferase.** When the inactivation of GABA aminotransferase by hydroxylamine was monitored by UV−visible spectroscopy, the same changes in the enzyme absorbance spectrum that occurred using cycloserine were observed, except that a shoulder grew into the 332 nm absorbance peak from 375 to 385 nm (Figure 5). Karpeiskii and co-workers[11] reported that oximes with PLP have an absorbance at 380 nm.

**Preparation of the Cycloserine−Coenzyme Adduct for Electrospray Mass Spectrometry.** To get cleaner product for electrospray mass spectral analysis, a new HPLC elusion system was developed that separated the cycloserine−coenzyme adduct from PMP and PLP. Figure 6 shows the separation when [$^3$H]-PLP-reconstituted enzyme was inactivated by cycloserine. The sample used for electrospray mass spectral analysis was carried

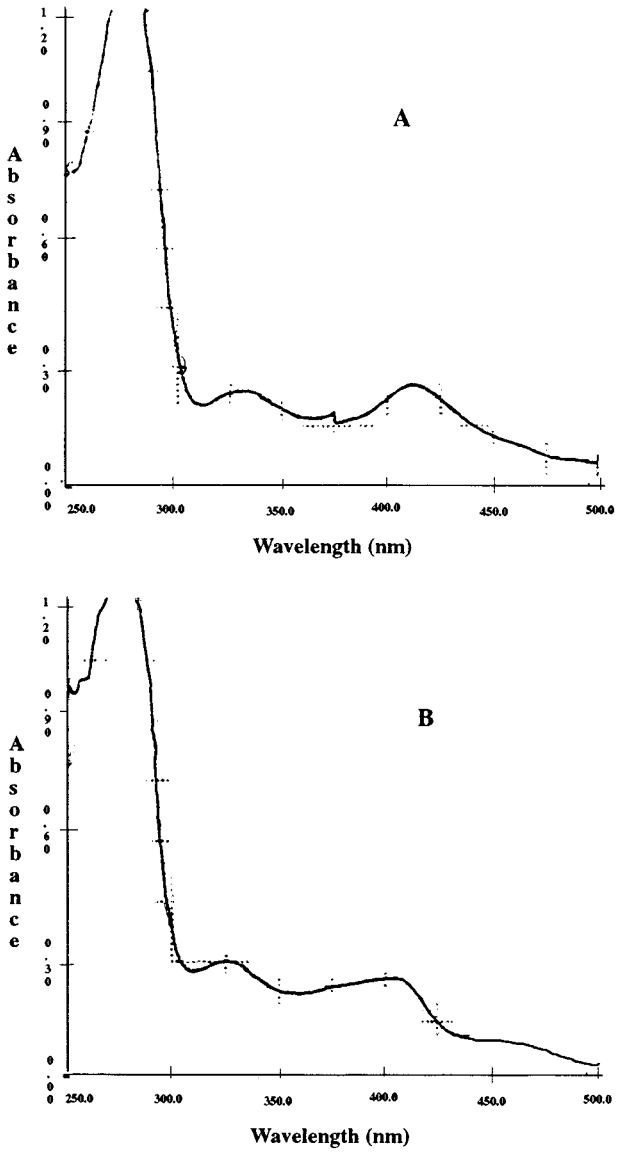



**Figure 5.** A, UV−visible spectrum of GABA aminotransferase. B, UV−visible spectrum of GABA aminotransferase inactivated by hydroxylamine. See Experimental Section for details.

out with unlabeled enzyme, and the fractions from 35 to 37 min were collected.

**Electrospray Ionization Tandem Mass Spectrometry of the Cycloserine−Coenzyme Adduct.** Electrospray ionization mass spectrometry (EIMS) of PLP and PMP showed the expected molecular ions ($[M + H]^+$); the next most prominent peak was $[M + H - 98]^+$, corresponding to the loss of the $H_3O_3PO^+$ ion from the parent molecule. Figure 7 shows the results for PLP. The peak at *m*/*z* 248 corresponds to the molecular ion ($[M + H]^+$), *m*/*z* 150 is the $[M + H - 98]^+$ fragment, and *m*/*z* 495 represents a dimer molecular ion peak. EIMS of the cycloserine−coenzyme adduct (**21**) (Figure 8A) shows an apparent mixture of compounds, although many of the peaks may be attributed to noise in the background as a result of the small quantity of the sample, which is estimated to be l pmol/$\mu$L. The expected molecular ion ($[M + H]^+$) is at *m*/*z* 332. Tandem mass spectrometry (MS/CID/MS) performed on the *m*/*z* peak (Figure 8B) shows the prominent daughter ion peak ($[M + H - 98]^+$) at *m*/*z* 234.

## Discussion

L-Cycloserine is a good time-dependent inactivator of purified pig brain GABA aminotransferase (Figure l), having $K_I$ and $k_{inact}$ values of 360 $\mu$M and 0.005 $min^{-l}$, respectively. No enzyme activity returns upon dialysis or gel filtration, suggesting that an irreversible inhibition occurs. Various mechanisms could account for this observation; two mechanisms for the inactivation of PLP-dependent enzymes with cycloserine have been proposed in the literature. Karpeiskii et al.[11] suggested the formation of an acylated enzyme when aspartate-glutamate aminotransferase was inactivated with cycloserine (Scheme 2). Initial Schiff base formation (**6**) is proceeded by enzyme acylation (**7**), followed by cyclization (**8**) and ring opening to **9**. The proposed mechanism was supported by a comparison of UV spectra of other known oximes of PLP to the UV spectrum of the inactivation adduct. Churchich[12] also studied the same inactivation using fluorescence spectrophotometry and proposed, instead, that inactivation arises from the release of Schiff base **6**; the enzyme is inactive without its coenzyme. Scheme 3 shows the mechanism proposed by Rando[13] for the inactivation of PLP-dependent alanine racemase with cycloserine. In this mechanism isomerization of Schiff base **12** (**6** in Scheme 2) leads to ketimine **13**, which is then attacked by an active site nucleophile to make the covalently bound adduct **14**.

Two other inactivation mechanisms, however, also should be considered. The mechanism of inactivation of several PLP-dependent enzymes by serine analogues with good leaving groups in place of the serine hydroxyl group, referred to as the enamine mechanism, was initially reported by Metzler and coworkers;[14] in Scheme 4 it is adapted for cycloserine. Eliminative ring opening of **12** to **17** followed by transfer of the PLP back on to the active site lysine residue gives enamine **18**. Attack of **18** at the lysine-bound PLP produces a stable adduct to the coenzyme, which is now covalently attached to the protein (**19**). A fifth possible inactivation mechanism is derived from the known mechanism of inactivation of GABA aminotransferase by gabaculine[15] and is adapted for cycloserine in Scheme 5. This mechanism is initiated by the usual Schiff base formation and isomerization to **13**. Instead of nucleophilic attack, however, this adduct is poised to undergo double tautomerization to give 4-{[3-hydroxy-2-methyl-5-(phosphooxymethyl)-4-pyridinyl]methylamino}-3-hydroxyisoxazole (**21b**), which should be relatively stable. It is not clear, however, which tautomer, **21a** or **21b**, is more stable.

Each of these mechanisms could be differentiated by determining where the cycloserine is attached and what is expected to occur upon denaturation of the inactivated enzyme. Both the Karpeiskii et al.[11] (Scheme 2) and Rando[13] (Scheme 3) mechanisms result in acylation of the enzyme (**9** and **14**, respectively), whereas the Churchich[12] mechanism produces the Schiff base of PLP and cycloserine (**6**), the enamine mechanism (Scheme 4) gives a ternary complex of the inactivator, the protein, and the coenzyme (**19**), and the aromatization mechanism (Scheme 5) forms a PMP analogue (**21**). Denaturation

(11) Karpeiskii, M. Ya.; Khomutov, R. M.; Severin, E. S.; Breusov, Yu. N. In *Chemical and Biological Aspects of Pyridoxal Catalysis. I. U. B. Symposium Series, Vol. 30*; Snell, E. E. et al., Eds.; Pergamon Press: New York, 1963; pp 323−331.
(12) Churchich, J. E. J. *Biol. Chem.* **1967**, *242*, 4414.
(13) Rando, R. R. *Biochem. Pharmacol.* **1975**, *24*, 1153.
(14) (a) Likos, J. J.; Ueno, H.; Feldhaus, R. W.; Metzler, D. E. *Biochemistry* **1982**, *21*, 4377. (b) Ueno, H.; Likos, J. J.; Metzler, D. E. *Biochemistry* **1982**, *21*, 4387.
(15) (a) Rando, R. R. *Biochemistry* **1977**, *16*, 4604 (b) Rando, R. R.; Bangerter, F. W. *J. Am. Chem. Soc.* **1977**, *99*, 5141.

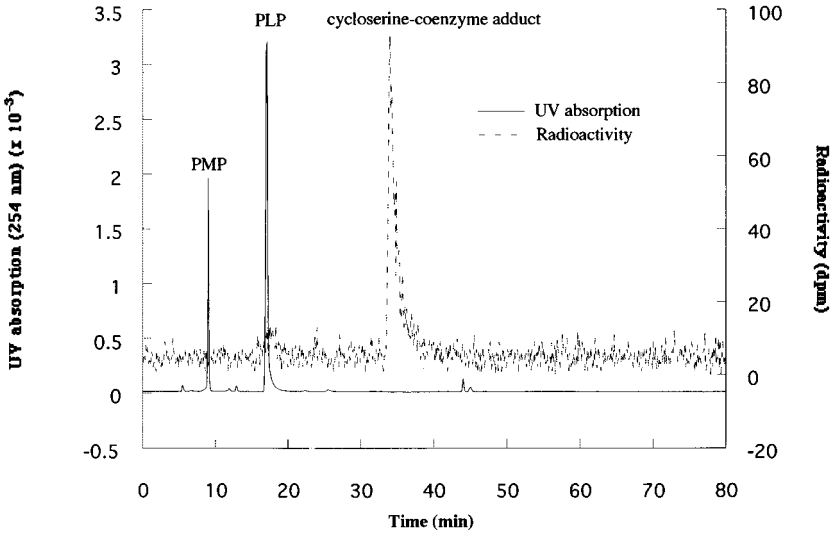



**Figure 6.** HPLC of cycloserine-[$^3$H]coenzyme adduct used in electrospray mass spectral studies. PMP and PLP are added as standards.

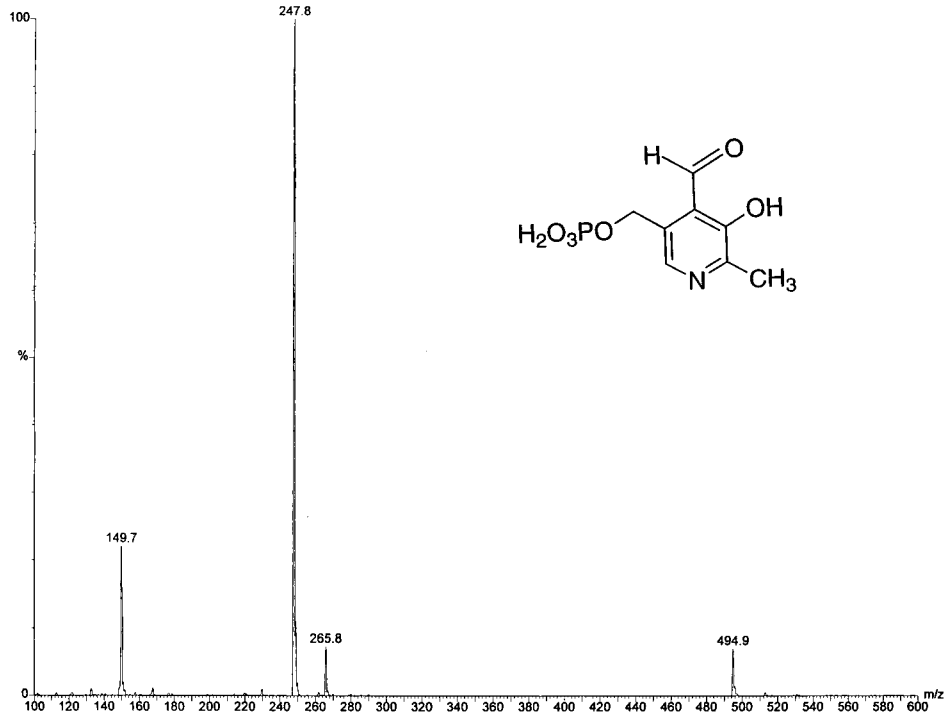



**Figure 7.** Electrospray mass spectrum of PLP.

of the acylated enzyme adducts would result in either release of PLP (Scheme 2) or PMP (Scheme 3) and formation of either a new acylated adduct (**10** or **15**) or hydrolysis to metabolites **11** or **16**. Denaturation of the enamine adduct would give 4-[3-hydroxy-2-methyl-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid (**20**), a compound previously reported by Schnackerz et al.,[16] and denaturation of the aromatization adduct just releases that adduct (**21**).

Another consideration is the fact that under acidic conditions, cycloserine can decompose to serine and hydroxylamine,[9] and the hydroxylamine could react with the PLP to give the oxime of PLP. Cycloserine is stable under basic conditions.[9]

To determine which, if any, of these inactivation mechanisms is most likely, inactivation and metabolite studies of GABA aminotransferase inactivated with L-[$^{14}$C]cycloserine were compared with studies using GABA aminotransferase reconstituted with [$^3$H]PLP and L-cycloserine. Inactivation of GABA aminotransferase with L-[$^{14}$C]cycloserine resulted in the attachment of 1.1 equiv of radioactivity per enzyme molecule after Penefsky spin gel filtration,[17] indicating a stoichiometric reaction. Surprisingly, dialysis led to complete release of radioactivity from the enzyme. Apparently, the extended time period required for dialysis results in efficient removal of the labeled moiety. Inactivation of [$^3$H]PLP-reconstituted GABA aminotransferase with L-cycloserine followed by dialysis also led to the complete release of radioactivity from the enzyme, suggesting that the product of inactivation of GABA aminotransferase with L-cycloserine is a covalent adduct with the coenzyme. Inactivation of [$^3$H]PLP-reconstituted GABA aminotransferase with L-cycloserine followed by gel filtration, denaturation, and HPLC

(16) Schnackerz, K. D.; Ehrlich, J. H.; Geisemann, W.; Reed, T. A. *Biochemistry* **1979**, *18*, 3557.

(17) (a) Penefsky, H. *J. Biol. Chem.* **1977**, 252, 2891; (b) Penefsky, H. *Methods Enzymol.* **1979**, *56*, 527.

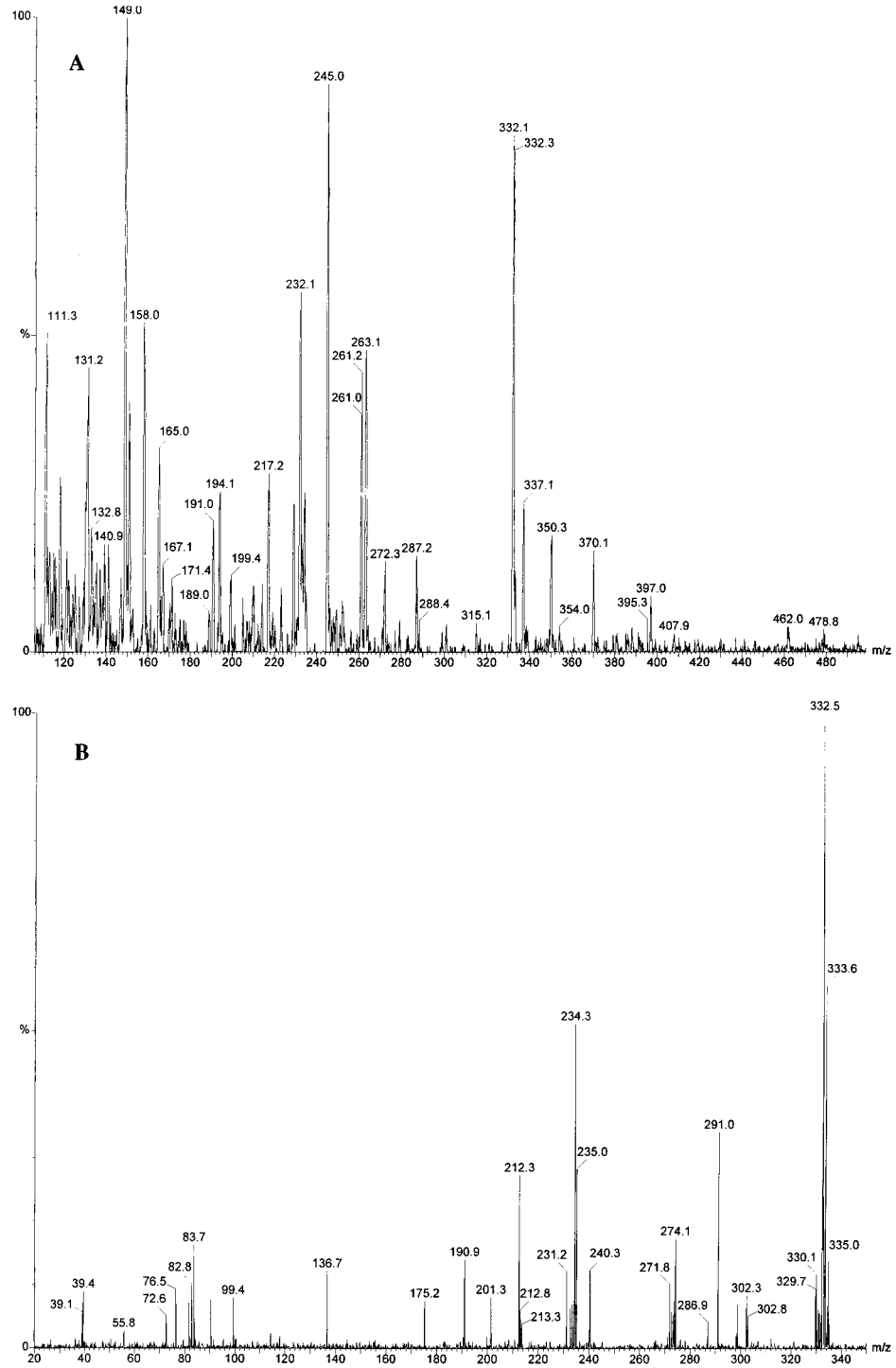


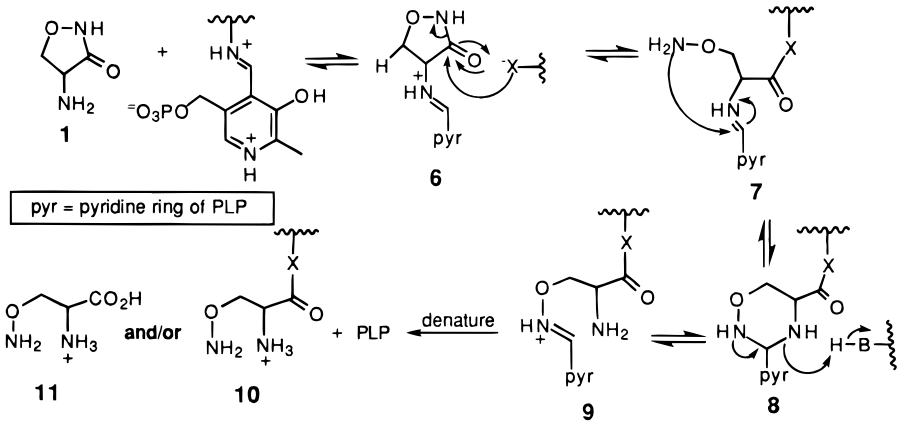

**Figure 8.** A. Electrospray ionization mass spectrum of the isolated cycloserine−coenzyme adduct. B. Tandem MS/CID/MS spectrum of the ion at *m*/*z* 332 from A. See Experimental Section for details.

gave one peak of radioactivity whose retention time was similar to that of PMP, but which did not correspond to that of PLP or the oxime of PLP (Figure 2). This peak of radioactivity corresponded to the peak of radioactivity obtained by dialysis or denaturation of GABA aminotransferase inactivated with L-[$^{14}$C]cycloserine (Figure 3), consistent with an adduct formed between cycloserine and the PLP coenzyme.

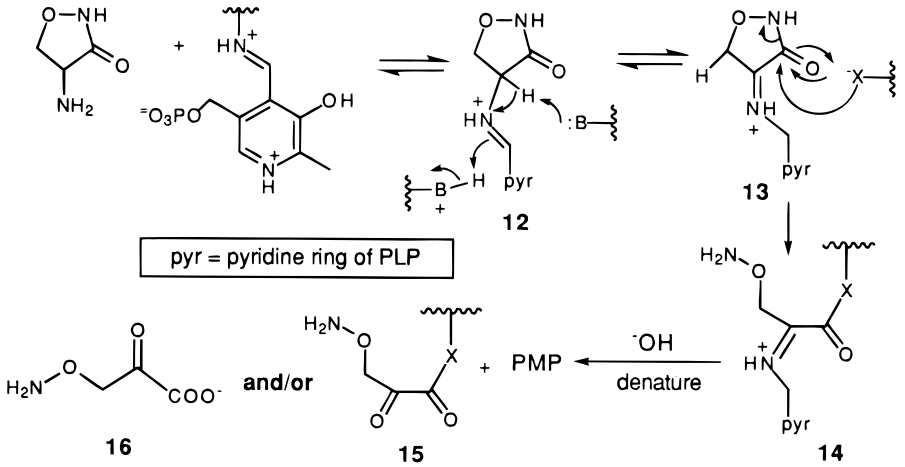
Karpeiskii and co-workers[11] proposed the formation of a covalently bound oxime derivative as the end product for the inactivation of aspartate-glutamate aminotransferase with D,L-cycloserine. Their proposed mechanism (Scheme 2) involves the formation of the aldimine between cycloserine and PLP, followed by the opening of the lactam by an active site nucleophile and subsequent rearrangement to the oxime. This conclusion was based on the following experiments. First, in the UV−visible spectrum of the inactivated enzyme, a shoulder at 380 nm was observed which is indicative of PLP oximes.[18,19] After elution of the inactivated enzyme from a Sephadex G25 column, less than 20% of the enzyme activity returned. In contrast, when enzyme that was inactivated with $\beta$-amino-hydroxyalanine was passed through a Sephadex G25 column, all of its activity was regained. After gel filtration, both inactivated enzyme solutions lost their peak at 380 nm, which was interpreted as the result of hydrolysis of the oxime and the

(18) Matsuo, Y.; Greenburg, D. M. *J. Biol. Chem.* **1958**, *230*, 561.
(19) Shukuya, R.; Schwert, G. W. *J. Biol. Chem.* **1960**, *235*, 1653.

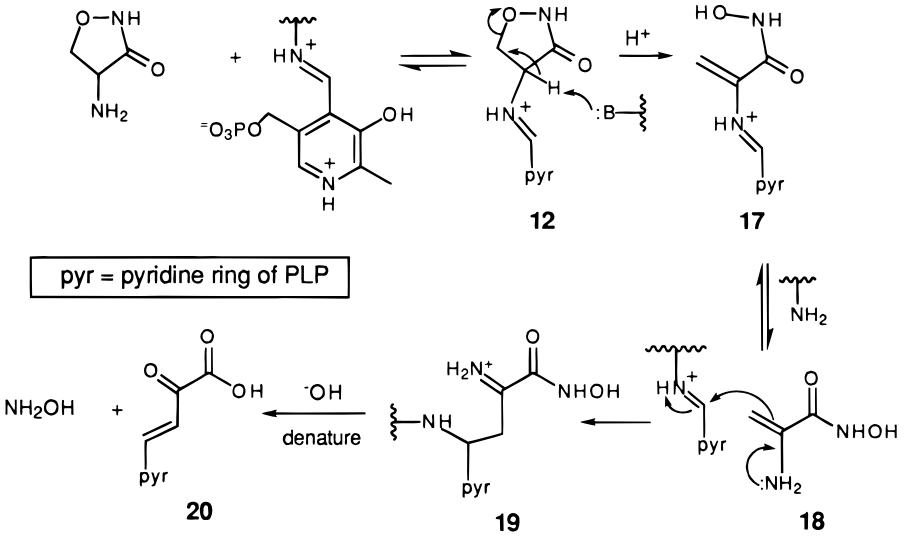
**Scheme 2.** Karpeiskii et al.[11] Proposed Mechanism of Inactivation of Asp-Glu Aminotransferase by Cycloserine




**Scheme 3.** Rando[13] Mechanism of Inactivation of Alanine Racemase by Cycloserine



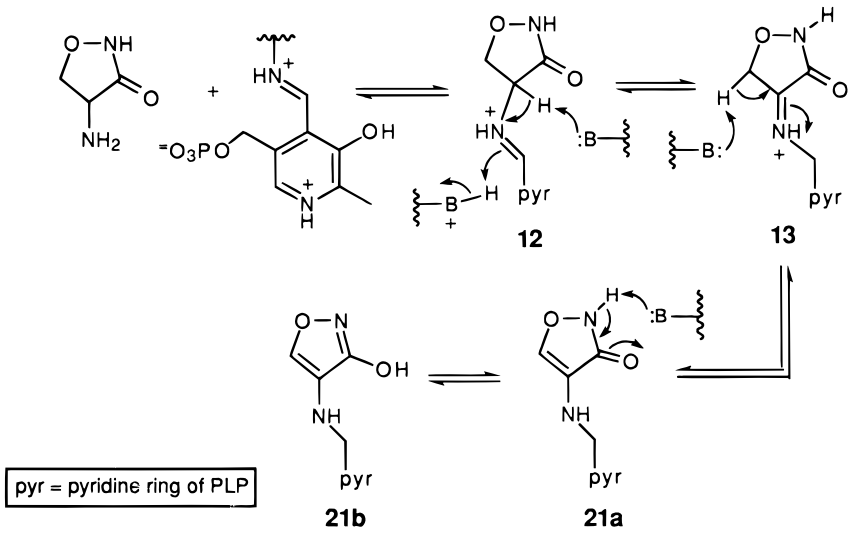

**Scheme 4.** An Enamine Inactivation Mechanism




return of the coenzyme to the PLP form. This experiment was used as evidence that even though both inactivators form oximes with the coenzyme, a portion of the cycloserine must still be covalently bonded to the enzyme, since cycloserine-inactivated enzyme did not regain much activity following elution on a Sephadex column. Finally, Karpeiskii and co-workers inactivated aspartate-glutamate aminotransferase with cycloserine at pH 9, reasoning that at this high pH cycloserine would be predominately in its amidate form, which would not allow the lactam to be opened by an active site nucleophile. Indeed, they did not see any inactivation at this pH, which was used as evidence that nucleophilic attack was involved in the inactivation mechanism.

In contrast to the above results, the UV−visible spectrum of the L-cycloserine-inactivated GABA aminotransferase did not show a shoulder at 380 nm but inactivation of GABA aminotransferase with hydroxylamine did. It is assumed that hydroxylamine produces an oxime with the coenzyme and that no covalent bond between the oxime and the enzyme forms. Inactivation of GABA aminotransferase with L-cycloserine at

**Scheme 5.** An Aromatization Inactivation Mechanism




pH 9.25 still resulted in enzyme inactivation. Therefore, the Karpeiskii mechanism is not applicable to GABA aminotransferase inactivation with L-cycloserine. However, inactivation of GABA aminotransferase with L-cycloserine resulted in formation of a UV−visible maximum at 332 nm, which is indicative of a pyridoxamine derivative.[20] We found that no GABA aminotransferase activity returned by gel filtration following L-cycloserine inactivation; denaturation of L-cycloserine-inactivated GABA aminotransferase by base, acid, or urea resulted in the complete release of radioactivity from the protein, regardless of whether the inactivator or coenzyme contained the radiolabel. Dialysis of L-cycloserine-inactivated GABA aminotransferase against α-ketoglutarate did not cause a return in enzyme activity, indicating that inactivation is not the result of conversion of the coenzyme to PMP. However, addition of PLP to the L-cycloserine-inactivated and dialyzed enzyme solution resulted in the return of 57% of the activity, suggesting the formation of a modified coenzyme that is released upon dialysis. It needs to be noted that when GABA aminotransferase was reconstituted with [$^3$H]PLP, only about 60% of the activity was recovered. Therefore, the fact that only 57% of the L-cycloserine-inactivated enzyme activity returns upon PLP incubation probably reflects the fragility of the enzyme under these conditions when the coenzyme is removed and not any permanent inactivation inflicted by the L-cycloserine. This is confirmed by the fact that microdialysis of L-cycloserine-inactivated GABA aminotransferase, when either the inactivator or coenzyme contains a radiolabel, results in complete release of radioactivity into the dialysate. On the basis of these experiments it can be concluded that the inactivator does not form a covalent bond to GABA aminotransferase but, rather, modifies the coenzyme and forms an adduct that is released by dialysis but not by rapid gel filtration.

Since a covalent bond is not formed between L-cycloserine and GABA aminotransferase, the mechanism proposed by Rando[13] also is not relevant, because it involves attack of the lactam bond by an active site nucleophile (Scheme 3). The mechanism proposed by Churchich,[12] which involves release of the Schiff base **12** (Scheme 3), is not supported because the UV−visible spectrum is indicative of a pyridoxamine derivative, not a pyridoxal derivative. Also, treatment of our released adduct with acid, even at 100 °C, did not affect its retention time by HPLC, suggesting that it is a stable species, unlike Schiff base **12** would be. Of the proposed mechanistic and adduct possibilities, only the product of the enamine mechanism (**20**, Scheme 4) and the three tautomers, **13**, **21a**, and **21b** (Scheme 5) remain.

The inactivation mechanisms presented in Schemes 3 and 4 also were shown to be invalid by HPLC and liquid scintillation analysis of denatured or microdialyzed L-cycloserine-inactivated GABA aminotransferase. When either [$^3$H]PLP GABA aminotransferase with L-cycloserine or GABA aminotransferase with L-[$^{14}$C]cycloserine was used, the released radiolabeled adduct from the two experiments comigrated by HPLC (Figure 3), indicating that the product is an adduct between cycloserine and the coenzyme. This adduct was shown to have a retention time different from both PMP (Scheme 3) and 4-[2-methyl-3-hydroxy-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid (**20**, Scheme 4) (vide infra). Therefore, the inactivation does not proceed by either the Rando mechanism (Scheme 3) or the enamine mechanism (Scheme 4).

Structures **13**, **21a**, and **21b** are pyridoxamine derivatives. Further evidence for the formation of a pyridoxamine derivative comes from UV−visible spectrophotometric studies of L-cycloserine-inactivated GABA aminotransferase. The spectrum of the inactivation product, either still in the active site or released upon denaturation at pH 12 or upon addition of 8 M urea, corresponds to that of PMP under the same conditions (Figure 4B).

It was difficult to interpret the HPLC/liquid scintillation analysis of the radiolabeled inactivation products because PMP, cycloserine, the [$^3$H]-containing inactivation products, and the [$^{14}$C]-containing inactivation products have very similar retention times, which are close to the void volume. To achieve satisfactory separation and to differentiate a primary amine-containing adduct from a substituted PMP analogue, attempted conversion of the adduct to an *o*-phthalaldehyde (OPA) derivative was carried out. The OPA derivatives of PMP and cycloserine have retention times of 16 and 28 min, respectively. Treatment of the radiolabeled inactivation product with OPA has no effect on the HPLC retention time of either the [$^3$H]- or [$^{14}$C]-containing adduct (no change to Figure 3), which still migrates at about 3 min under the conditions of the experiment. This experiment indicates that the inactivation product is not

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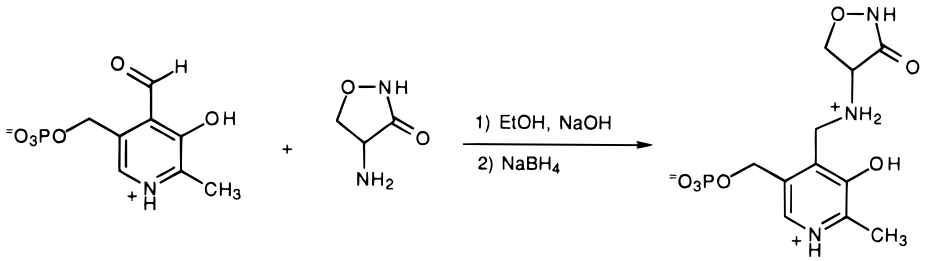
**Scheme 6.** Synthesis of the Reduced Adduct between PLP and Cycloserine




**22**

PMP or any primary amine. OPA readily derivatizes primary amines, and even a very polar amino acid like glutamate has a retention time under the same conditions as the above experiment of 13.5 min (data not shown).

To differentiate between **13** and **21a/b** (Scheme 5), derivatization of the inactivation product with phenyl isothiocyanate (PITC) was attempted. PITC generally reacts with secondary amines. Treatment of the inactivation product with PITC had no effect on its retention time, thereby giving initial evidence that the ketimine (**13**) may be the inactivation product. However, addition of $NaBH_4$ to the inactivation product, which should reduce the imine of **13** to the corresponding secondary amine-containing oxazolidinone, followed by PITC treatment also had no effect on the retention time of the product. Evidence against structure **13** is the stability of the inactivation product to both acid and base treatment. The denaturation step in the radiolabeled studies involves subjection of the sample to base at pH 12 for 1 h. This, apparently, does not affect the adduct. Also, treatment of the inactivation product with 0.5 M HCl at room temperature or with 0.1 M HCl at 100 °C has no effect on its HPLC retention time. It has been reported that ketimines formed between pyridoxal and various amino acids readily hydrolyze when subjected to aqueous conditions[18] and that the imine between cycloserine and PLP is unstable in aqueous medium.[21] However, it also has been reported that the Schiff base between pyridoxal and cycloserine can be detected by proton NMR[22] and that between PLP and cycloserine can be detected by UV−visible spectroscopy.[11]

Synthesis of isoxazole **21** (or the dephosphorylated analogue) was attempted by allowing cycloserine to react with either PLP or pyridoxal under a variety of conditions. Unfortunately, problems with isolation and solubility could not be resolved. However, the two-electron reduced product **22** (Scheme 6) was synthesized and was found to have a UV−visible spectrum comparable to that of the inactivation product, which is similar to that of a pyridoxamine derivative. Compound **22** also has an HPLC retention time similar to that of the [$^3$H]- and [$^{14}$C]-labeled inactivation products. As was the case with the inactivation product, it also does not form either an OPA or a PITC derivative, even though it is a secondary amine. However, it could be argued that steric hindrance may be a problem in forming a PITC derivative since the secondary amine is sandwiched between the two polar ring systems.

A different HPLC system was developed that cleanly separated the cycloserine−coenzyme adduct obtained by inactivation of [$^3$H]PLP-reconstituted GABA aminotransferase from PLP and PMP (Figure 6). When this experiment was repeated using gabaculine in place of cycloserine, it was found that the gabaculine−coenzyme adduct had a retention time very similar to that of the cycloserine−coenzyme adduct (data not shown). This is further support for **21** as the structure of the cycloserine−coenzyme adduct, since it resembles that of the known gabaculine−coenzyme adduct.[15]

The most conclusive evidence from these studies for an adduct of structure **21a** or **21b** comes from electrospray ionization tandem mass spectrometry of the modified coenzyme isolated by HPLC. Electrospray ionization mass spectrometry is a soft technique that is known to cause little, if any, damage to biological samples. This technique also has the advantage of being capable of analyzing small quantities of material. Electrospray ionization tandem mass spectrometry allows the selection of one ion from the first mass spectrometer to be transferred to a collision gas for fragmentation (known as collision-induced dissociation or CID), then these fragments (daughter ions) are analyzed in the second mass spectrometer. A major peak in the first mass spectrum of the cycloserine−coenzyme adduct (Figure 8A) is the molecular ion of *m*/*z* 332, corresponding to either **21a** or **21b**. This mass does not correspond to that expected for **20**. This peak was analyzed by MS/CID/MS (Figure 8B) and the molecular ion (*m*/*z* 332) and daughter ion with loss of the phosphate group (*m*/*z* 234) are the major peaks. PLP and PMP show a major daughter ion for loss of the phosphate group, indicating that this is common for PLP analogues. Possibly the strongest support for these structures is the X-ray crystal structure of D-amino acid aminotransferase inactivated by D-cycloserine, which clearly shows the cycloserine ring intact and attached to the enzyme-bound coenzyme.[23]

## Conclusion

The evidence for the structure of the product of inactivation of GABA aminotransferase by L-cycloserine strongly points to **21a** or **21b** (Scheme 5), which arise from an aromatization-type mechanism. This is only the second example of an inactivator of a PLP-dependent enzyme that functions via an aromatization mechanism; the first was the inactivation of GABA aminotransferase and other PLP-dependent enzymes by gabaculine.[15] The results described here definitively rule out the mechanisms proposed by Karpeiskii et al. (Scheme 2),[11] by Rando (Scheme 3),[13] and the enamine mechanism (Scheme 4), and do not support the simple formation of PLP oxime or a Schiff base of L-cycloserine with the PLP coenzyme of GABA aminotransferase. Given that the same conclusion is made on the basis of the X-ray crystal structure of D-amino acid aminotransferase inactivated by D-cycloserine,[23] this may be a universal mechanism for inactivation of PLP-dependent enzymes (or, at least, of aminotransferases) by cycloserine.

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## Experimental Section

**Analytical Methods.** GABA aminotransferase assays were recorded on a Perkin-Elmer Lambda 1 spectrophotometer. UV−visible scans were performed with a Beckman DU-40 spectrophotometer. HPLC analysis was done with either Beckman 110 A pumps and a Beckman 330 detector or Beckman 125P pumps and a Beckman 166 detector. The HPLC columns used were analytical or semiprep Alltech Econosil C18 10 micron columns. Radioactivity was determined by liquid scintillation counting using a Beckman LS-3133T or a Packard Tri-Carb 2100TR scintillation counter. The scintillation fluid used was Fisher ScintiSafe. NMR spectra were taken with a Gemini 300 MHz spectrometer in $D_2O$ with sodium 3-(trimethylsilyl)propionate as an internal standard; chemical shifts are reported as ppm downfield from the standard. Fast atom bombardment mass spectra were obtained on a VG 70-250SE high resolution spectrometer. Electrospray ionization mass spectra were acquired on a Micromass Quattro mass spectrometer (Fisons Instruments, Manchester, UK) with a quadrupole−hexapole−quadrupole configuration. Measurements of pH were performed on an Orion 701-A pH meter with a Ross 8301 combination electrode. Melting points were determined on a Mel-Temp capillary tube melting point apparatus and are uncorrected.

**Reagents.** All reagents were purchased from Aldrich Chemical Co. except the following: potassium pyrophosphate, Sephadex 50, α-ketoglutarate, β-mercaptoethanol, $NADP^+$, and GABA were purchased from Sigma Chemical Co.; dibasic and monobasic potassium phosphate were purchased from Mallinckrodt Chemical Co.; Centricon 30 membranes were purchased from Amicon; *o*-phthalaldehyde (OPA) and phenylisothiocyanate (PITC) derivatization solutions were purchased from Pierce.

**L-[$^{14}$C]Cycloserine.** L-[$^{14}$C]Cycloserine was synthesized according to the method of Plattner et al.,[24] except that L-[$^{14}$C]serine methyl ester was synthesized using the procedure developed by Rachele.[25] 2,2-Dimethoxypropane (15 mL, 8.13 mmol) was added to L-serine (105 mg, 1.0 mmol) and 0.5 mCi of L-[U-$^{14}$C]serine. This solution was allowed to stir for 26 h, after which time the excess 2,2-dimethoxypropane was removed by rotary evaporation. The resulting serine methyl ester (a white oil) was dissolved in a minimum amount of methanol, then 40 mL of acetone was added, and the solution was placed in the freezer overnight. The l-[$^{14}$C]serine methyl ester was separated by filtration, washed with acetone, and vacuum dried. The product was added to 6 mL of chloroform containing $PCl_5$ (400 mg, 1.92 mmol). The solution was stirred for 22 h, and the solvent was removed by rotary evaporation. The solid was cooled to 0 °C, and then water (2 mL) was added and subsequently removed by rotary evaporation. This was repeated twice more to remove excess HCl. The resulting L-[$^{14}$C]3-chloroalanine methyl ester was dissolved in 0.7 mL of water and was added dropwise to a solution containing hydroxylamine hydrochloride (80 mg, 1.15 mmol) in 280 μL of water and 10.7 M NaOH (400 pL) at −18 °C. This reaction mixture was stirred at −18 °C for 4 h and then at room temperature for 4 h. At this time, the temperature of the solution was lowered to −18 °C, and concentrated HCl was added until the pH was 4. The solution was applied to a Dowex 50 column (13 × 0.9 cm), which was washed with water until the eluant was no longer acidic. The L-[$^{14}$C]cycloserine was then eluted with 0.2 M $NH_4OH$, and the ninhydrin positive fractions were combined and rotary evaporated. The product was purified by applying to a DE 52 column (11 × 0.9 cm), which was washed with water, and the product was eluted with 1 M $NH_4OH$. Ninhydrin positive fractions were combined and the solvent was evaporated by lyophilization. This reaction pathway yielded 22.4 mg (22% yield) of L-[$^{14}$C]cycloserine. By TLC analysis, the compound was determined to be 90% radiopure. The compound had a specific radioactivity of 0.315 mCi/mmol.

**Oxime of PLP.** PLP (144 mg, 0.58 mM) and hydroxylamine hydrochloride (40 mg, 0.58 mM) were dissolved in 5 mL of water by raising the pH to 6.3 using 10 M KOH. The solution was stirred overnight at room temperature, protected from light. Then the pH of the solution was adjusted to 2 using concentrated HCl. The resulting precipitate was suction filtered and washed with cold water. After vacuum drying, the product was obtained in 30% yield (45 mg). The UV−visible spectrum had maxima at 325 and 356 nm: mp 168−170 °C (dec.); $^1$H NMR (DMSO) δ 2.45 (s, 3 H), 5.07 (d, 2 H, $J$ = 3.7 Hz), 8.07 (s, 1 H), 8.59 (s, 1 H), 10.78 (s, 1 H), 12.35 (s, 1 H). HRMS FAB$^+$: calcd for $C_8H_{12}N_2O_6P$ 263.0432, found 263.0500.

**4-[3-Hydroxy-2-methyl-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic Acid (20).** 4-[3-Hydroxy-2-methyl-5-(phosphooxymethyl)-4-pyridinyl]-2-oxo-3-butenoic acid (**20**) was synthesized according to the method of Schnackerz et al.[16] The mass spectrum and UV-visible spectrum both match those reported: mp 130−132 °C (lit.[16] 116 °C dec); $^1$H NMR ($D_2O$) δ 2.65 (s, 3 H), 5.04 (d, 2 H, $J$ = 7.7 Hz), 7.25 (d, 1 H, $J$ = 16.5 Hz), 7.72 (d, 1 H, $J$ = 16.5), 8.17 (s, 1 H).

**Attempted Synthesis of 4-{[3-Hydroxy-2-methyl-5-(phosphooxymethyl)-4-pyridinyl]methylamino}-3-hydroxyisoxazole (21).** PLP and cycloserine were heated at 100 °C at pH 7 in 0.1 M potassium phosphate buffer, according to method of Rando,[13] except substituting cycloserine for gabaculine. A mixture of five products that could not be separated was obtained. Carrying out the attempted synthesis at a variety of temperatures did not improve the result.

**Reduced Cycloserine/PLP Adduct (22).** Cycloserine (0.1 mM) and PLP (0.1 mM) were added to absolute ethanol (20 mL) containing NaOH (0.1 mM). The solution was stirred for 24 h, while protected from light, at room temperature. $NaBH_4$ (0.2 mM) was added to the cloudy, white-yellow solution, which was stirred overnight, while protected from light, at room temperature. The ethanol was removed by rotary evaporation. Water was added to the product, and the solution was evaporated again to remove the residual ethanol. The compound was further purified using a Dowex 50 column, eluting with 1 M $NH_4OH$. The product was obtained in an almost quantitative yield (33 mg). The UV−visible spectrum of the compound at pH 7.4 had absorbance maxima at 290, 325, and 365 nm: mp 98−100 °C; $^1$H NMR ($D_2O$) δ 2.30 (s, 3 H), 3.80 (t, 1 H), 3.83 (s, 2 H), 4.09 (t, 1 H), 4.35 (t, 1 H), 4.72 (d, 2 H), 7.60 (s, 1 H). LRMS FAB$^+$: calcd for $C_{11}H_{17}N_3O_7P$ 314, found 314.

**Enzymes and Assays.** GABA aminotransferase was isolated from pig brains using the procedure of Churchich and Moses.[26] Succinic semialdehyde dehydrogenase (SSDH) was obtained from GABAase (Boehringer Mannheim, West Germany), a mixture of GABA aminotransferase and SSDH, by the reported procedure.[27] GABA aminotransferase activity assays were carried out using a modification of the coupled assay developed by Scott and Jakoby.[28] 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. For each assay, excess SSDH was used. The amount of activity remaining in an enzyme solution was determined by adding an aliquot of enzyme solution to assay solution with SSDH and monitoring the change in absorbance at 340 nm, as a result of the conversion of $NADP^+$ to NADPH by SSDH. [$^3$H]PLP-reconstituted GABA aminotransferase was prepared as previously described.[29]

**Time-Dependent Inactivation of GABA Aminotransferase by L-Cycloserine.** GABA aminotransferase (15 μL; 1.72 mg/mL) was added to solutions of L-cycloserine (135 μL; final concentrations of 0, 0.25, 0.50, 0.75, and 1.0 mM), in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 6 mM α-ketoglutarate and 0.01 mM, β-mercaptoethanol at 25 °C. Experiments also were carried out in which the α-ketoglutarate and β-mercaptoethanol were omitted. At timed intervals, aliquots (10 μL) were withdrawn and added to the assay solution (580 μL) containing excess succinic semialdehyde dehydrogenase. Rates were measured spectrophotometrically at 340 nm, and the logarithm of remaining activity was plotted against time for each concentration of inhibitor. A secondary plot of 1/slope of these lines versus 1/[inactivator][10] was constructed to determine $K_I$ and $k_{inact}$ values

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for L-cycloserine. Substrate protection was demonstrated by also assaying an enzyme solution containing 0.5 mM L-cycloserine and 5 mM GABA.

**Time-Dependent Inactivation of GABA Aminotransferase by L-Cycloserine at pH 9.25.** A control of 10-fold dilute GABA aminotransferase (15 $\mu$L concentrated enzyme in 100 mM potassium pyrophosphate buffer, pH 9.25) was made. An additional 10-fold dilute enzyme solution was prepared containing 2 mM L-cycloserine. All solutions contained 0.1 $\mu$L $\beta$-mercaptoethanol and 2 mM $\alpha$-ketoglutarate. The enzyme solutions were periodically assayed for activity.

**Determination of the Equivalents of L-[$^{14}$C]Cycloserine Bound to GABA Aminotransferase After Inactivation.** GABA aminotransferase (0.25 mL) was inactivated with 50 mM L-[$^{14}$C]cycloserine (10.5 $\mu$L) and 100 mM $\alpha$-ketoglutarate (12.5 $\mu$L). Excess L-[$^{14}$C]cycloserine was removed by elution through a Penefsky column.[17] The enzyme solution was then base denatured and analyzed by HPLC and liquid scintillation counting as described in the [$^3$H]PLP-reconstituted GABA aminotransferase inactivation experiment. The total number of counts eluted was converted into mCi/mmol of enzyme and compared to the specific activity of the inactivator.

**Determination of the Amount of L-Cycloserine Covalently Bound to GABA Aminotransferase After Inactivation and Denaturation.** GABA aminotransferase and [$^3$H]PLP GABA aminotransferase were inactivated with [$^{14}$C]-labeled and unlabeled L-cycloserine, respectively. Enough trichloroacetic acid or trifluoroacetic acid was added to the enzyme solution to make it 10% in acid. The resulting enzyme precipitate was separated by centrifugation and was washed three times with a 10% solution of the respective acids. The enzyme pellet was then dissolved in 2 M NaOH and was counted for radioactivity by liquid scintillation. The determination of bound radioactivity was also performed under two different denaturing conditions. In the first method, 1 M KOH was added to the inactivated enzyme until pH 12 was reached. After being incubated for 1 h protected from light at 25 °C, the pH of the enzyme solution was lowered to 7 with 1 M HCl, and the solution was injected into the HPLC. The enzyme was eluted from the HPLC column with MeOH and was counted by liquid scintillation counting for radioactivity. In another experiment, after lowering the pH to 7, the enzyme was separated from the inactivation products by centrifugation of the solution in a Centricon 30. The enzyme was removed from the membrane by pipette and was counted. In the second denaturation method, using the same inactivation procedure, enough urea was added to the inactivated enzyme to afford an 8 M solution. After being incubated for 1 h under the same conditions as above, part of the solution was injected into the HPLC, and the rest was subjected to Centricon 30 separation.

**Inactivation of [$^3$H]PLP-Reconstituted GABA Aminotransferase by L-Cycloserine.** Two controls were prepared, each containing 200, $\mu$L of [$^3$H]PLP-reconstituted GABA aminotransferase (0.72 mg/mL, 28 000 cpm) and 0.1 $\mu$L $\beta$-mercaptoethanol. One control contained 5 mM $\alpha$-ketoglutarate, the other contained 40 mM GABA. The experimental solution contained the same amount of enzyme, $\beta$-mercaptoethanol, and $\alpha$-ketoglutarate as the controls, with the addition of 1.0 mM L-cycloserine (no GABA added). These solutions were incubated, while protected from light, at 25 °C for 9 h, at which time the experimental solution had no enzyme activity left. The pH of all three solutions was adjusted to 12 with 2 M KOH and was allowed to incubate, protected from light at 25 °C, for 1 h. The inactivation products could also be removed by microdialysis, as described below. The pH of the solutions was then lowered to 7 with 2 M HCl, and the neutral, denatured enzyme solution was injected into the HPLC, and the components were eluted with 100 mM KPi, pH 7.4 for 20 min at 0.5 mL/min. After 20 min the solvent was changed to water−0.1% TFA, and the flow rate was increased to 1 mL/min for 15 min, when a gradient to 100% methanol over 25 min at 1 mL/min was begun. Fractions of 0.5 min were collected for the first 20 min, with 2 min fractions collected thereafter. The fractions were counted by liquid scintillation counting.

**Time-Dependent Inactivation of GABA Aminotransferase by L-[$^{14}$C]Cycloserine.** Inactivation was carried out as described above using 1 mM L-[$^{14}$C]cycloserine and GABA aminotransferase with unlabeled coenzyme.

**HPLC and Liquid Scintillation Analysis of [$^3$H]- and [$^{14}$C]-Labeled OPA Derivatives of Inactivation Products.** Inactivation with either [$^3$H]PLP GABA aminotransferase or with l-[$^{14}$C]cycloserine to incorporate a radiolabel into the inactivation product was performed as described above. Penefsky columns[17] made in water were used on both inactivation solutions to remove excess inactivator and $\alpha$-ketoglutarate. Denaturation was performed the same way as in the [$^3$H]-PLP-reconstituted GABA aminotransferase inactivation, except that after the pH of the enzyme solutions was lowered to neutrality, the enzyme was removed using Centricon 30 ultrafiltration. The inactivation product solution was then lyophilized and dissolved in 10 $\mu$L of water. To this was added 10 $\mu$L of OPA reagent and was allowed to incubate for 1 min. The solution was then injected into the HPLC, eluting with 95% 100 mM sodium acetate, 4.5% methanol, 0.5% THF going to 80% methanol at 1.0 mL/min. One-min fractions were collected and analyzed by liquid scintillation counting. This experiment was repeated twice more with the following changes. After the inactivation product was lyophilized, the sample was dissolved in 25 $\mu$L of 0.5 M HCl and was allowed to incubate overnight at room temperature before OPA was added. In the other experiment, the lyophilized sample was dissolved in 40 $\mu$L of 0.1 M HCl and was heated in boiling water for 1 h, prior to the addition of OPA.

**HPLC and Liquid Scintillation Analysis of [$^{14}$C]-Labeled PITC Derivatives of Inactivation Products.** The inactivation and sample preparation incorporating the Penefsky column and Centricon 30 procedures were the same as those described above for the OPA experiment. The lyophilized sample was dissolved in 20 $\mu$L of 2:1:1 absolute ethanol:water:triethylamine and lyophilized again. The sample was then dissolved in 20 $\mu$L of 7:1:1:1 absolute ethanol:water: triethylamine:PITC, incubated at room temperature for 20 min, and lyophilized. The sample was dissolved in a mixture of 20 $\mu$L of the HPLC buffer mixture described in the OPA experiment and 10 $\mu$L of MeOH. It was then microfuged (not all the solid dissolved) and injected into the HPLC using the same conditions as those described in the OPA experiment. Fractions of 1 min also were collected and analyzed using liquid scintillation counting. In another experiment, the inactivation product was dissolved in 100 $\mu$L of absolute ethanol with an equivalent amount of NaOH and 0.5 mg of $NaBH_4$ and was vortexed for 24 h at room temperature protected from light. Then 100 $\mu$L of water was added, and the solution was vortexed an additional 8 h, at which time 1 M HCl was added until the solution was pH 6. The solution was then lyophilized, and the PITC derivatization and HPLC/ liquid scintillation analysis was carried out.

**UV−Visible Analysis of the Inactivation Product Formed from L-Cycloserine Inactivation of GABA Aminotransferase.** GABA aminotransferase (0.5 mL) was inactivated at pH 7.4 by the addition of L-cycloserine (0.19 mg, 3.72 mM) and $\alpha$-ketoglutarate (1.0 mg, 13.69 mM). The enzyme solution was assayed periodically until no enzyme activity remained (1 h). The pH of the enzyme solution was raised to 12 with 10 M KOH. After the solution was allowed to incubate for 1 h, the pH was lowered to pH 7.4 with concentrated HCl. UV−visible spectra of the enzyme solution were taken from 300−450 nm at the following times: (1) before the addition of the inactivator and $\alpha$-ketoglutarate; (2) immediately after addition of L-cycloserine and $\alpha$-ketoglutarate; (3) periodically throughout the inactivation, and (4) after denaturation of the enzyme solution at both pH 12 and 7.4. This same experiment was repeated using 8 M urea as a denaturing agent instead of raising the pH to 12. The spectra obtained were compared to standards of PLP, PMP, and the oxime of PLP.

**UV−Visible Analysis of the Inactivation Product Formed from Hydroxylamine Inactivation of GABA Aminotransferase.** GABA aminotransferase (0.5 mL) was inactivated by the addition of 25 $\mu$L of a 154 mM hydroxylamine hydrochloride solution. The enzyme solution was assayed until no activity remained. A UV−visible spectrum of the enzyme solution was taken from 300−450 nm both before addition of the inactivator and after inactivation of the enzyme.

**Dialysis of L-Cycloserine-Inactivated GABA Aminotransferase and Monitoring the Return of Activity.** Inactivated enzyme was prepared by addition of 50 mM L-cycloserine (5 $\mu$L) and 100 mM $\alpha$-ketoglutarate (5 $\mu$L) to GABA aminotransferase (50 $\mu$L). Another portion of GABA aminotransferase (50 $\mu$L) served as a control. Both

solutions were monitored for activity. When the cycloserine inactivated enzyme exhibited no activity, both enzyme solutions were dialyzed overnight against 1 L of 100 mM potassium phosphate buffer, pH 7.4 containing 50 mM α-ketoglutarate and, β-mercaptoethanol (20 μL). The enzyme solutions were assayed for activity before PLP (0.36 mM final concentration) was added. These solutions were incubated at 25 °C, protected from light, and assayed periodically for activity, until the maximum activity had returned.

**Dialysis of [$^3$H]PLP-Reconstituted GABA Aminotransferase Inactivated by L-Cycloserine and GABA Aminotransferase Inactivated by [$^{14}$C] L-Cycloserine.** [$^3$H]PLP GABA aminotransferase (0.5 mL) was inactivated with 50 mM L-cycloserine (25 μL) and 100 mM α-ketoglutarate (25 μL). Also, GABA aminotransferase (0.1 mL) was inactivated with 50 mM [$^{14}$C]L-cycloserine (5 μL) and 100 mM α-ketoglutarate (5 μL). The L-[$^{14}$C]cycloserine-inactivated enzyme solution was applied to a Penefsky column,[17] rinsing with 250 μL of 100 mM potassium phosphate buffer, pH 7.4. Then 2 × 150 μL of the [$^3$H]PLP GABA aminotransferase solution and 150 μL of the [$^{14}$C]-labeled enzyme solution were dialyzed against 1 mL of 100 mM potassium phosphate, pH 7.4 in "waterbug" dialysis systems[30] using 1.5 mL Eppendorfs caps. The enzyme solutions were removed from the waterbugs, denatured with 1 M KOH, and injected into the HPLC using the same conditions as described for the inactivation of [$^3$H]-PLP-reconstituted GABA aminotransferase by L-cycloserine experiment described above. The dialysate was injected into the HPLC using the same conditions as well. HPLC fractions were collected and counted for radioactivity using a liquid scintillation counter, as described in the inactivation of [$^3$H]PLP reconstituted GABA aminotransferase by L-cycloserine experiment.

**Modified Procedure for the Isolation of the Cycloserine-[$^3$H]-Coenzyme Adduct.** GABA aminotransferase (0.32 nmol), which had been reconstituted with [$^3$H]PLP, was protected from light and incubated at 25 °C in 100 mM potassium phosphate, pH 7.4, containing 2.0 mM L-cycloserine, 0.5 mM α-ketoglutarate, and 0.25 mM, β-mercaptoethanol. A control was run with gabaculine (2 mM) under the same conditions. After being incubated at room temperature for 8 h, the reaction mixture was assayed. Less than 1% of the enzyme activity remained. The excess of inactivators were removed by running the solutions over Sephadex G-50 using the Penefsky spin method.[17] The solutions obtained were again assayed; no enzyme activity was detected. The pH of each solution was adjusted to 12 using 1 M KOH. These were incubated at room temperature for 1 h and then were added to enough trifluoroacetic acid (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 placed into Centricon 10 microconcentrators and centrifuged. Each protein pellet was rinsed with 50 μL of 0.1% aqueous TFA three times. The protein pellets were redissolved in 200 μL of 100 mM potassium phosphate, pH 7.4 buffer. The effluents and rinses were collected and freeze-dried. Analysis of the metabolites was carried out by dissolving the resulting solid in 100 μL of water and adding 20 μL of a PLP/PMP (4 mM) standard which had also undergone the basification and acidification steps. The samples were injected onto an Alltech Alltima C18 column (4.6 × 250 mm, 5μ). The mobile phase A was water with 0.1% TFA flowing at 0.5 mL/min for 15 min. Then a 5-min gradient was run to 50% mobile phase B (mobile phase B is 80% acetonitrile). The column was eluted with 50% mobile phase B for 20 min. Under these conditions, PLP elutes at 16 min and PMP at 8 min. The HPLC eluents were analyzed for radioactivity with a Radiomatic FLO-ONE\Beta Series A-200 liquid flow scintillation counter (Figure 6).

**Preparation of the Cycloserine−Coenzyme Adduct for Electrospray Ionization Mass Spectrometry.** Care was taken in all of the experimental procedures to eliminate the introduction of glycerol, polyethylene glycol, or other detergent related polymers into the mass sample. All of the glassware used was treated sequentially with 30% v/v nitric acid in water, water, methanol, chloroform, methanol, and water. The enzyme isolated from pig brain was further purified by dialysis against 4 × 4 L of 100 mM potassium phosphate, pH 7.4 buffer for 4 h each buffer change. The dialysis tubing used, Spectra/por 2, molecular porous dialysis membrane, was soaked in 3 × 2 L of water for 4 h each change before use. The purified GABA aminotransferase (15 nmol) was inactivated with cycloserine (2 mM) in a total volume of 1.2 mL of 100 mM potassium phosphate buffer, pH 7.4, containing 1.73 mM α-ketoglutarate, and 0.25 mM, 8-mercaptoethanol at room temperature in the dark. The same procedure as described above for the [$^3$H]PLP-reconstituted enzyme was followed. The 35−37 min HPLC fractions were collected and freeze-dried.

**Electrospray Mass Spectrum of the Isolated Cycloserine−Coenzyme Adduct.** A 10-μL sample as prepared above, dissolved in methanol, was loop injected into the mass spectrometer. All data were acquired in the multichannel analysis (MCA) mode, and CsI was used to calibrate the instrument. The resolution of the instrument was adjusted to resolve isotopic peaks at ca. *m*/*z* 600, and the mass range was scanned from *m*/*z* 100 to 1200 in 4.9 s. Spectra from seven scans were computed to give one composite averaged spectrum.

For MS/CID/MS analysis, argon was used as the collision gas at a pressure of ca. $3 \times 10^{-3}$ mbar. The collision energy was 20 eV. The mass range was scanned from *m*/*z* 20 to 800, and NaI was used to calibrate the second analyzer.

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