Mechanisms of Inactivation of γ-Aminobutyric Acid Aminotransferase by the Antiepilepsy Drug γ-Vinyl GABA (Vigabatrin)

Shrenik M. Nanavati and Richard B. Silverman*

Contribution from the Department of Chemistry, the Department of Biochemistry, Molecular Biology, and Cell Biology, and the Institute for Neuroscience, Northwestern University, Evanston, Illinois 60208-3113. *Received March 28, 1991*

Abstract: The molecular mechanism of inactivation of pig brain γ-aminobutyric acid (GABA) aminotransferase by the anticonvulsant drug vigabatrin (**1**, γ-vinyl GABA) has been investigated. Inactivation of GABA aminotransferase that had been reconstituted with [^{3}H]pyridoxal 5′-phosphate (PLP) produced two tritiated metabolites upon denaturation, pyridoxamine 5′-phosphate and a cofactor adduct in the ratio of about 2:1. Inactivation with [6-^{14}C]-γ-vinyl GABA led to the incorporation of 1.02 equiv of radioactivity under nondenaturing conditions and 0.60–0.76 equiv, depending upon the pH, under denaturating conditions. This indicates the formation of two different adducts, one that is stable to denaturation and one that is not. The identity of the structure of the stable adduct was determined with the use of chemical model studies on hypothesized adducts and the application of this chemistry to the [^{14}C]-labeled enzyme. Base treatment and sodium borohydride reduction followed by sodium periodate oxidation indicated that the structure of the stable adduct was 6-X-4-oxohexanoic acid (**10**, Scheme I). The adduct that was released upon denaturation (**18**, Scheme IV) was identified as the product of an enamine rearrangement onto the PLP. Therefore, two distinct mechanistic pathways are implicated during inactivation. Azaallylic isomerization of the Schiff base of γ-vinyl GABA with PLP (that is, the normal catalytic mechanism) leads to a reactive intermediate (**6**, Scheme I), which is attacked by an active-site nucleophile to give adduct **10** after Schiff base hydrolysis. Alternatively, hydrolysis of **6** to **6a** followed by active-site nucleophilic attack (prior to release of **6a** from the active site) also could give **10**. This adduct is stable to denaturation and accounts for 70–75% of the total active site labeling. The other pathway, allylic isomerization (Scheme II), leads to reactive intermediate **12**; however, transimination by the active-site lysine residue (Scheme IV), which, apparently, is faster than Michael addition to **12**, leads to enamine formation. The enamine reacts with the lysine-bound PLP to give adduct **17**. This adduct, which accounts for 25–30% of the total inactivation adducts, is unstable to denaturation and is released as **18**. Since no product of hydrolysis of the enamine could be detected, it appears that when the enamine is generated, it does not escape the active site. Although 1.8 transamination events appear to be occurring for every inactivation event, the product of transamination could not be detected.

When epilepsy is defined broadly as any disease characterized by recurring convulsive seizures, then almost 1% of the entire world population can be classified as having this disease.[1] Convulsions arise from such diverse reasons as heredity, head trauma, brain tumors, heat stroke, acute intoxication, and labor.[2,3] The biochemical mechanisms for epilepsy are unknown, but when there is an imbalance in L-glutamic acid, an excitatory neurotransmitter, and γ-aminobutyric acid (GABA), an inhibitory neurotransmitter, convulsions can occur. Increasing the brain concentrations of GABA can have an anticonvulsant effect.[4] However, since GABA does not cross the blood-brain barrier, it cannot be used as an anticonvulsant drug.[5] Consequently, an important approach that has been taken to increase the brain GABA levels is to use a compound that does cross the blood-brain barrier and then inactivates GABA aminotransferase, the enzyme that catalyzes the degradation of GABA.[6] This rational approach to the design of anticonvulsant agents led to the discovery by Lippert et al.[7] of 4-amino-5-hexenoic acid (**1**, Scheme I; γ-vinyl GABA, vigabatrin), a mechanism-based inactivator[8] of GABA aminotransferase, which exhibited potent anticonvulsant activity and low toxicity.[9] It has been shown to be an effective treatment for epilepsies that are resistant to other anticonvulsant drugs[10] and is on the European drug market.

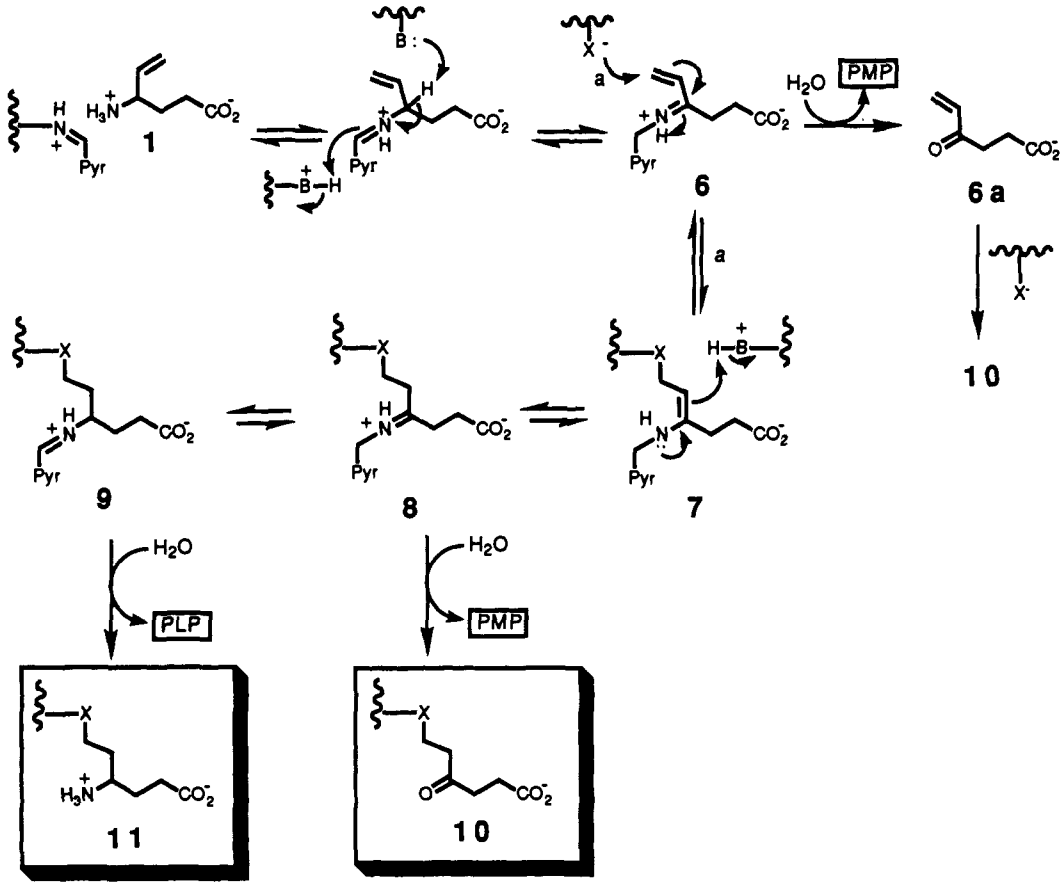
γ-Vinyl GABA was shown[7] to be a time-dependent inactivator of GABA aminotransferase. Inactivation is protected by substrate, indicating that it is active-site directed; an added external nucleophile does not block inactivation, suggesting that inactivation does not result from release of a reactive species that returns to inactivate the enzyme. Lippert et al.[7] also found that 4-amino-[4-^{2}H]-5-hexenoic acid displays a primary kinetic isotope effect on inactivation, suggesting that the rate-determining step in inactivation is enzyme-catalyzed removal of the γ-proton. On the basis of these results and reasonable physical organic chemical logic, an azaallylic isomerization mechanism for inactivation of GABA aminotransferase by **1** was proposed[7] (Scheme I, up to **7**). In addition to this inactivation mechanism, John et al.[11] suggested an allylic isomerization mechanism (Scheme II, up to **13**). Both of these mechanisms are standard hypotheses for vinyl-substituted mechanism-based inactivators,[8] but definitive mechanistic studies have not been carried out to elucidate the mechanism. In the preceding paper in this issue we reported the mechanism of inactivation of GABA aminotransferase by 4-amino-5-hexynoic acid (γ-ethynyl GABA)[12] and showed that a propargylic isomerization is the primary inactivation mechanism, although two different adducts are produced as a result. Here we report a detailed mechanistic study of the inactivation of GABA aminotransferase by γ-vinyl GABA and show that both azaallylic and allylic pathways are important, but the adducts produced are

* Author to whom correspondence should be addressed at the Department of Chemistry.

(1) Rogawski, M. A.; Porter, R. J. *Pharmacol. Rev.* **1990**, *42*, 223–286.
(2) Wyke, B. *Principles of Neurology*; Elsevier: London, 1969.
(3) Biemond, A. *Brain Diseases*; Elsevier: London, 1970.
(4) (a) Gale, K. *Epilepsia* **1989**, *30* (Suppl. 3), S1; (b) Tower, D. B. In *GABA in Nervous System Function*, Roberts, E., Chase, T. N., Tower, D. B., Eds., Raven Press: New York, 1975.
(5) Meldrum, B. S.; Horton, R. W. In *Epilepsy*, Harris, P., Mawdsley, C., Eds., Churchill Livingston: Edinburgh, 1974.
(6) Nanavati, S. M.; Silverman, R. B. *J. Med. Chem.* **1989**, *32*, 2413–2421.
(7) Lippert, B.; Metcalf, B. W.; Jung, M. J.; Casara, P. *Eur. J. Biochem.* **1977**, *74*, 441–445.
(8) Silverman, R. B. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*, Vol. I and II; CRC Press: Boca Raton, FL, 1988.
(9) Loscher, W. *Neuropharmacol.* **1982**, *21*, 803–810.
(10) (a) Tassinari, C. A.; Michelucci, R.; Ambrosetto, G.; Salvi, F. *Arch. Neurol.* **1987**, *44*, 907–910; (b) Browne, T. R.; Mattson, R. J.; Penry, J. K.; Smith, D. B.; Treiman, D. M.; Wilder, B. J.; Ben-Menachem, E.; Miketta, R. M.; Sherry, K. M.; Szabo, G. K. *Br. J. Clin. Pharmacol.* **1989**, *27*, 95S–100S. (c) Sivenius, J. M. R.; Ylinen, A.; Murros, K.; Matilainen, R.; Riekkinen, P. *Epilepsia* **1987**, *28*, 688–692.
(11) John, R. A.; Jones, E. D.; Fowler, L. J. *Biochem. J.* **1979**, *177*, 721–728.
(12) Burke, J. B.; Silverman, R. B., preceding paper.

0002-7863/91/1513-9341$02.50/0

Scheme I. Azaallylic Isomerization–Michael Addition Mechanism Proposed for the Inactivation of GABA Aminotransferase by γ-Vinyl GABA



not those predicted in these earlier studies.[7,11]

Results

Compounds Released after Denaturation of γ-Vinyl GABA-Inactivated [^{3}H]GABA Aminotransferase. Almost all of the radioactivity (>98%) associated with the enzyme was released upon treatment of γ-vinyl GABA-inactivated [^{3}H]GABA aminotransferase with trichloroacetic acid. After control radioactivity was subtracted, 61% of the radioactivity was found to comigrate with pyridoxamine phosphate (PMP) and 39% to comigrate with the released enamine adduct produced by inactivation of GABA aminotransferase with 4-amino-5-fluoropentanoic acid (Figure 1).[13]

Equivalents of [6-^{14}C]-γ-Vinyl GABA Bound to GABA Aminotransferase. Following dialysis of GABA aminotransferase that was inactivated with [6-^{14}C]-γ-vinyl GABA, 1.02 equiv of radioactivity was covalently bound per enzyme molecule. Upon denaturation with 6 M urea at pH 12, 7, and 1, however, only 0.76, 0.72, and 0.65 equiv of radioactivity, respectively, remained bound. Trichloroacetic acid precipitation resulted in the attachment of only 0.60 equiv of radioactivity.

Acid and Base Stabilities of 4-Oxo-6-piperidinohexanoic Acid and 4-Oxo-5-piperidinohexanoic Acid. The best medium for selective degradation of these two model compounds was found to be 7 M urea pH 13.3 at 76 °C. Under these conditions 4-oxo-6-piperidinohexanoic acid is completely degraded after 12 h, while 4-oxo-5-piperidinohexanoic acid is only slightly degraded over that period of time.

Equivalents of [6-^{14}C]-γ-Vinyl GABA Bound to GABA Aminotransferase after Dialysis under Basic Denaturing Conditions. About two-thirds of the radioactivity bound to the enzyme after dialysis against 6 M urea pH 7 (25 °C) is released upon dialysis against 7 M urea pH 13.3 at 76 °C (0.76 equiv vs 0.27 equiv).

Sodium Periodate Oxidation of Sodium Borohydride Reduced 5-Amino-4-oxopentanoic Acid and 6-Amino-4-oxohexanoic Acid. In the presence of sodium periodate, sodium borohydride reduced 5-amino-4-oxopentanoic acid was oxidized to succinic semialdehyde as determined by ^{1}H NMR spectroscopy. There was no change over the same period of time in the control (without sodium periodate). No oxidation of sodium borohydride reduced 6-amino-4-oxohexanoic acid was seen in the presence or absence of sodium periodate under the conditions described.

Acetaldehyde Stability. Acetaldehyde was stable to sodium periodate for over 30 h as determined by ^{1}H NMR spectroscopy and HPLC under the conditions used to oxidize the enzyme adduct with sodium periodate.

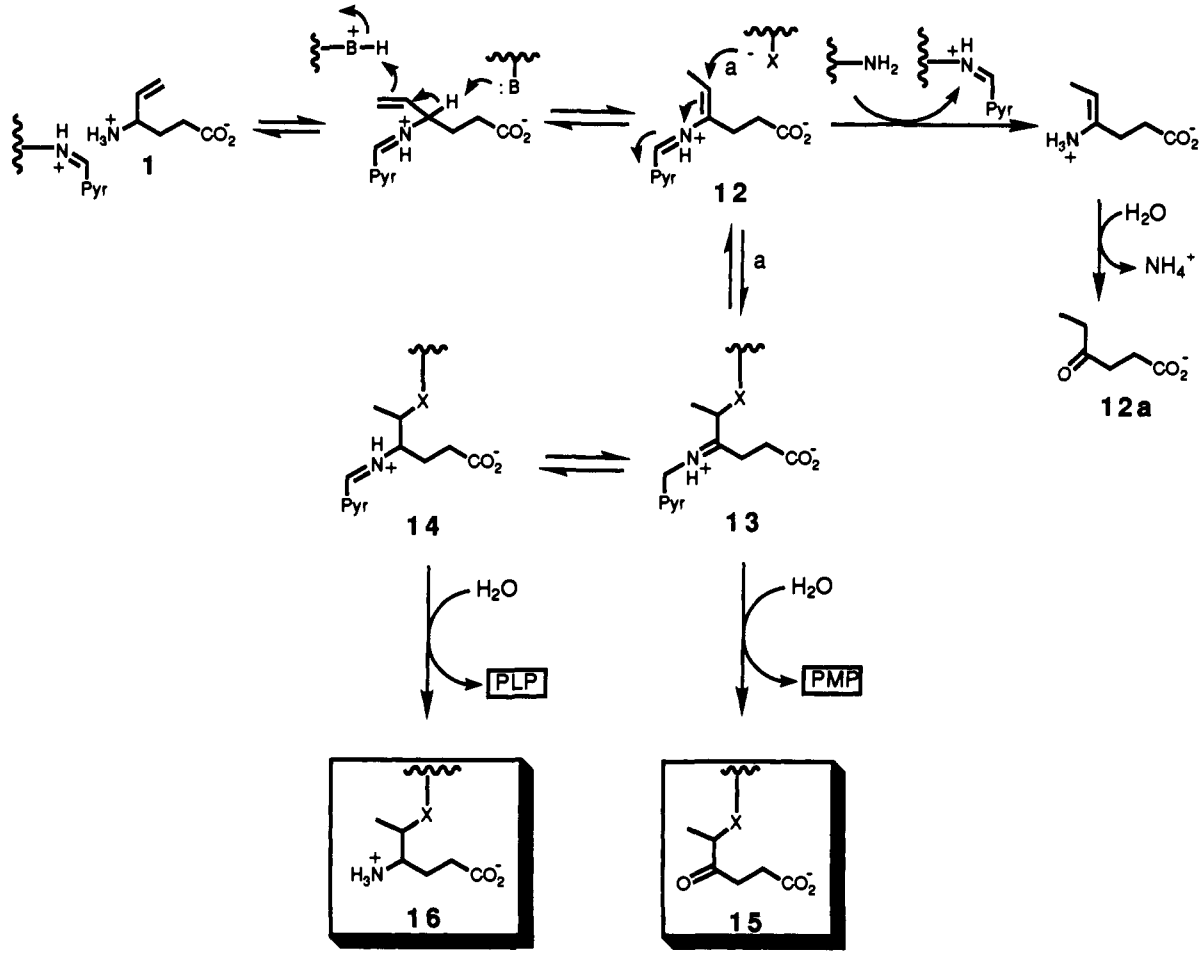
Sodium Periodate Oxidation of [6-^{14}C]-γ-Vinyl GABA-Inactivated, Sodium Borohydride Reduced GABA Aminotransferase. No radiolabeled acetaldehyde or 2,4-dinitrophenylhydrazone of acetaldehyde was found after the sodium periodate oxidation of GABA aminotransferase that was inactivated by [6-^{14}C]-γ-vinyl GABA and then treated with sodium borohydride. There also was no significant reduction of radioactivity bound to the enzyme after treatment with sodium periodate. Again this indicates that the adduct is stable to these conditions.

***Identification of the Adduct* Released after Denaturation of [6-^{14}C]-γ-Vinyl GABA-Inactivated GABA Aminotransferase under Harsh and Neutral Conditions.** The [^{14}C]-containing compound that was released under both extreme pH and neutral pH denaturing conditions comigrated by HPLC with both the 39% of radioactivity produced under the harsh conditions following γ-vinyl GABA inactivation of [^{3}H]GABA aminotransferase (see **Compounds Released After Denaturation of γ-Vinyl GABA-Inactivated [^{3}H]GABA Aminotransferase**) and with the radioactivity released following inactivation of GABA aminotransferase by 4-amino-5-fluoropentanoic acid.[13]

Transamination of γ-Vinyl GABA by GABA Aminotransferase. Incubation of GABA aminotransferase with γ-vinyl GABA in the presence of [5-^{14}C]-α-ketoglutarate led to the formation of 1.8 equiv of [^{14}C]glutamate per inactivation event. This equals the average number of conversions of PLP to PMP per inactivation event.

(13) Silverman, R. B.; Invergo, B. J. *Biochemistry* **1986**, *25*, 6817–6820.

Scheme II. Allylic-Michael Addition Mechanism Proposed for the Inactivation of GABA Aminotransferase by γ-Vinyl GABA



Equivalents of Nonamines Generated upon Inactivation of GABA Aminotransferase by [6-^{14}C]-γ-Vinyl GABA. Following inactivation of GABA aminotransferase by [6-^{14}C]-γ-vinyl GABA in the presence of α-ketoglutarate, 0.9 equiv of [^{14}C]-labeled nonamines per inactivation event were detected. When **6a** and **12a** were added as standards, only 0.15 equiv of **12a** and 0.1 equiv of **6a** were detected per inactivation event.

Discussion

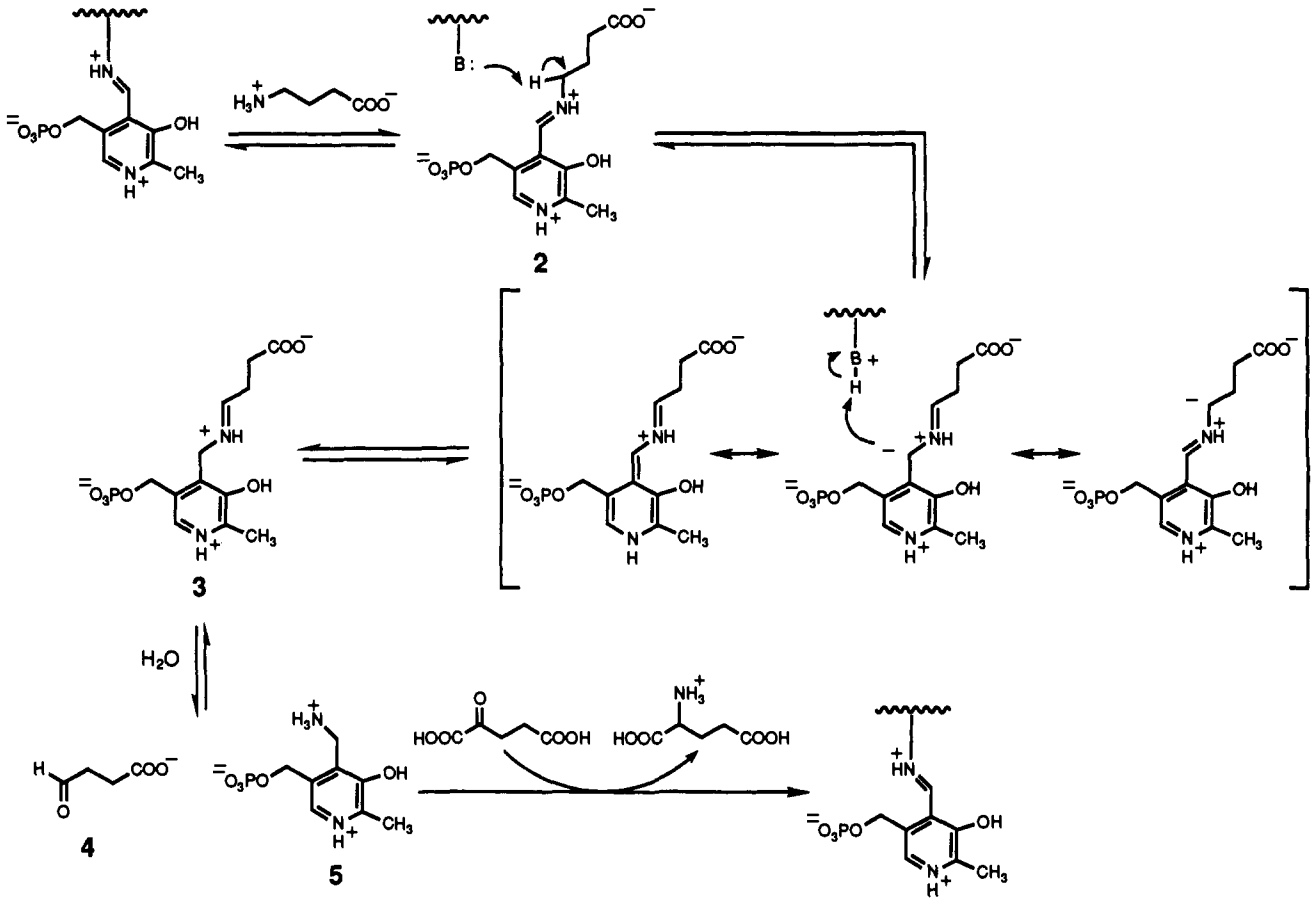
The well-accepted mechanism for pyridoxal 5′-phosphate (PLP)-dependent aminotransferases is shown in Scheme III for GABA aminotransferase. The first half reaction involves Schiff base formation between GABA and the lysine-bound PLP (**2**) followed by active-site base removal of the γ-proton to form a resonance-stabilized carbanion, which is reprotonated on the coenzyme to give the aldimine **3**. Hydrolysis of this adduct gives the product succinic semialdehyde (**4**) and pyridoxamine 5′-phosphate (**5**, PMP). The PMP is converted back to the lysine-bound PLP with concomitant conversion of α-ketoglutarate to L-glutamate in the second half reaction whose mechanism is the same as the first half reaction except that the electrons are shuttled in the opposite direction.

On the basis of this mechanism, several mechanisms (Schemes I, II, and IV) can be envisaged for the inactivation of GABA aminotransferase by γ-vinyl GABA (**1**). One mechanism (Scheme I) would involve azaallylic isomerization as described for the normal substrate. As in the case of GABA, Schiff base formation of **1** with the lysine-bound PLP would be followed by γ-proton removal and azaallylic isomerization to give aldimine **6**. Unlike the aldimine formed from GABA, however, **6** is a reactive Michael acceptor, and an active-site nucleophile could react with it to produce the covalent adduct **7**, which could be in equilibrium with two other adducts, **8** and **9**. Hydrolysis of **8** would release PMP and produce adduct **10**, whereas hydrolysis of **9** would release PLP and yield adduct **11**. Adduct **10** also could be derived from hydrolysis of **6** to **6a**, which, prior to release from the active site, could be attacked by an active-site nucleophile. An alternative inactivation mechanism involves allylic isomerization (Scheme II). In this case, subsequent to Schiff base formation, γ-proton removal leads to an allylic isomerization which produces **12**. This, too, is a Michael acceptor and could undergo nucleophilic attack by an active-site residue to give the covalent adduct **13**, which may be in equilibrium with **14**. Hydrolysis of **13** leads to release of PMP and formation of adduct **15**, whereas hydrolysis of **14** releases PLP and produces adduct **16**. A third possible inactivation mechanism for **1** (Scheme IV) starts at the allylic isomerized intermediate **12**. Metzler and co-workers[14,15] showed that for inactivators of PLP enzymes that lead to the formation of enamine Schiff base adducts with the PLP (such as **12**), a possible mechanism of inactivation is derived from active-site lysine-mediated transimination to give the enamine and lysine-bound PLP (see Scheme IV). If the enamine is properly juxtaposed, it can undergo nucleophilic addition to the lysine-bound PLP, as shown in this case, to give **17**. Consequently, there are five possible adducts that could be derived from the three different mechanisms depicted.

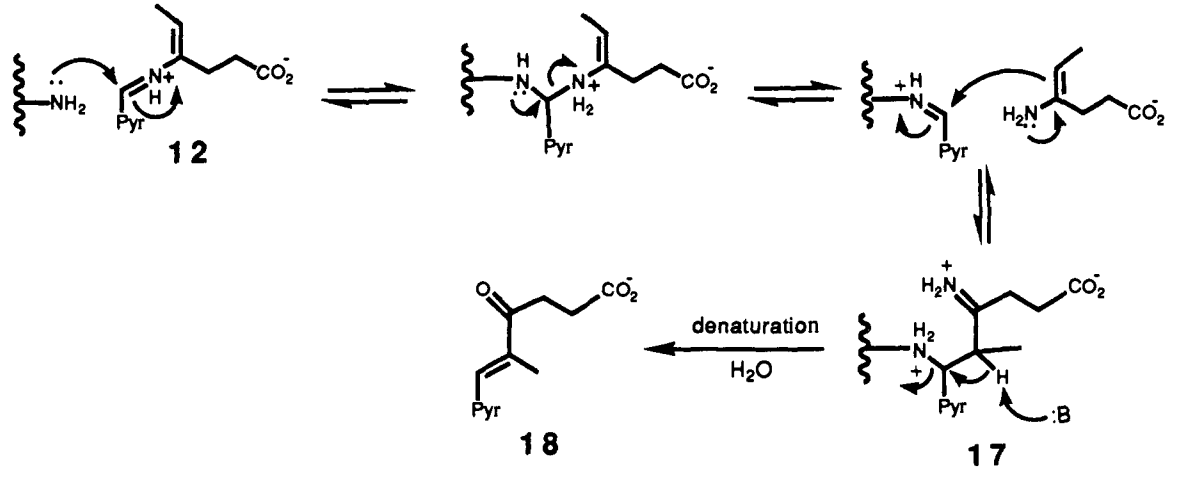
As is apparent from inspection of Schemes I, II, and IV, the coenzyme could end up as PLP, PMP, or the enamine adduct depending upon which mechanism and which adduct is formed. Therefore, it may be possible to eliminate one or more of the pathways by determination of the fate of the coenzyme after inactivation. ApoGABA aminotransferase was reconstituted with [7-^{3}H]PLP (tritium in the aldehyde hydrogen), and then the

(14) Likos, J. J.; Ueno, H.; Feldhaus, R. W.; Metzler, D. E. *Biochemistry* **1982**, *21*, 4377–4386.
(15) Ueno, H.; Likos, J. J.; Metzler, D. E. *Biochemistry* **1982**, *21*, 4387–4393.

Scheme III. Catalytic Mechanism for GABA Aminotransferase



Scheme IV. Enamine Mechanism of Inactivation of GABA Aminotransferase by γ-Vinyl GABA



tritiated GABA aminotransferase was inactivated with **1**. Three different peaks of tritium were observed by HPLC corresponding to PLP, PMP, and an unknown product (identified below as the enamine adduct metabolite). However, a control experiment in which the tritiated GABA aminotransferase was incubated under the same conditions (in the absence of α-ketoglutarate so PMP that is formed is not converted back to PLP) with GABA, which should convert all of the PLP to PMP, showed that a 75:25 mixture of PMP/PLP resulted. This may be because no succinic semialdehyde dehydrogenase was added to oxidize the succinic semialdehyde that was formed, and it is known[16] that succinic semialdehyde inhibits GABA aminotransferase. If the percentage of PLP in the control reaction is subtracted from the inactivation experiment, then it becomes apparent that no PLP is produced during inactivation, only PMP and the unknown product (Figure 1). Since no PLP is formed, then there are two adducts that can be ruled out, **11** and **16**. Therefore, only **10**, **15**, and an enamine product remain as possible adducts. Adducts **10** and **15** are the same two adducts that were possibilites for the stable adduct produced upon inactivation of GABA aminotransferase by γ-ethynyl GABA.[12] In order to differentiate these possibilities, GABA aminotransferase was inactivated with [6-^{14}C]-γ-vinyl GABA. This led to the incorporation, after dialysis, of 1.02 equiv of radioactivity per enzyme molecule, but one-quarter to one-third of the bound radioactivity was released upon denaturation at pH 12, 7, or 1. This suggests that there are two different adducts, one that is stable to denaturation and one that is unstable to denaturation in the ratio of about 3:1, respectively. Experiments were carried out to identify both of these adducts.

(16) Alston, T. A.; Porter, D. J. T.; Wheeler, D. M. S.; Bright, H. J. *Biochem. Pharmacol.* **1982**, *31*, 4081–4084.

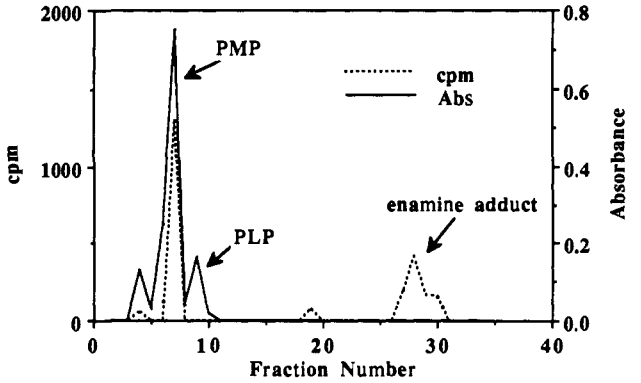
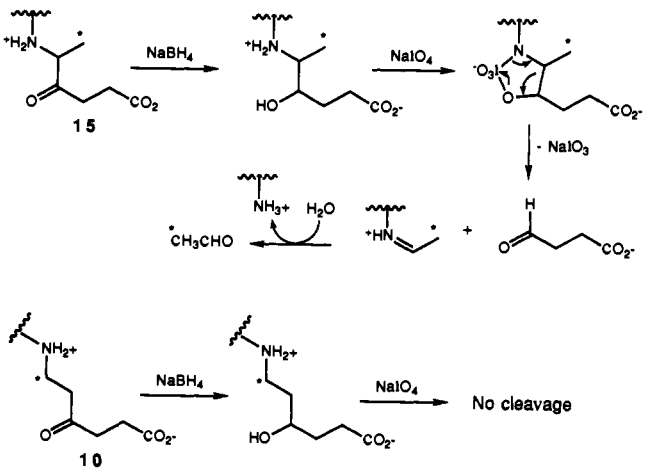


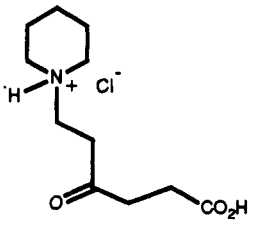
Figure 1. HPLC of Cofactor and Cofactor Adduct After Denaturation of γ-Vinyl GABA-Inactivated [^{3}H]GABA Aminotransferase. [^{3}H]GABA aminotransferase (0.08 units) was incubated with 3.6 mM γ-vinyl GABA, 1.08 mM α-ketoglutarate in 100 mM potassium phosphate buffer (pH 7.1) at 25 °C. Three controls also were run: one was identical except that inactivator was omitted; the second was identical except that 0.28 mM 4-amino-5-fluoropentanoic acid was used instead of γ-vinyl GABA; the third was identical except that GABA was used instead of γ-vinyl GABA and α-ketoglutarate was omitted. After the enzyme in the samples containing γ-vinyl GABA and 4-amino-5-fluoropentanoic acid had lost its activity as compared to the control with no inactivator (about 4 h), the pH of every incubation solution was raised to between 12.1 and 12.4 with 2N KOH. After a 2 h incubation period enough solid trichloroacetic acid was added to give a 10% (w/v) solution. The mixture was centrifuged in a Beckman Microfuge B (9000 × g) to spin down the resultant precipitated enzyme. The supernatant was separated from the pellet which was then washed with 3 × 100 μL of a 10% solution of trichloroacetic acid. The supernatant of each incubation solution was combined with the respective washings. PMP (0.3–0.6 mM) and PLP (1–1.7 mM) were added as standards to the samples to be analyzed by HPLC. HPLC analyses were carried out on a C18 reversed-phase analytical column at 254 nm using a dual solvent system: solvent A was 67 mM potassium phosphate buffer titrated to pH 2.6 with phosphoric acid; solvent B was methanol. The elution program used was solvent A at 0.5 mL/min for 30 min and then an increase in flow rate to 1 mL/min over 5 min. After a total of 35 min a solvent gradient to 15% solvent B was run over 15 min. Fractions were collected every 2 min over a period of 80 min and then counted for radioactivity content. With this program PMP and PLP eluted at T_R = 13 min and 17 min, respectively; the enamine adduct (**21**, R = H) eluted at 56 min. The protein pellet from above was taken up in 400 μL of 2N KOH and counted for radioactivity content. The [^{3}H]PLP and [^{3}H]PMP generated in the control was subtracted from the total tritium prior to plotting Figure 1.

Scheme V. Sodium Borohydride Reduction and Sodium Periodate Oxidation of Adducts **10** and **15**

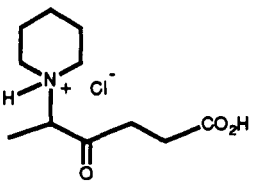


It was suspected that the active-site amino acid to which the inactivator was attached may be a lysine residue, since it has been reported[17] that inactivation of human liver GABA aminotransferase by γ-vinyl GABA results in labeling of the active-site lysine to which the PLP is bound. If the active site of human liver enzyme is homologous with that of the pig brain enzyme, which was used in the studies reported here, then attachment to a lysine residue is reasonable. γ-Ethynyl GABA appears to be attached to an active-site lysine residue of the pig brain enzyme.[12]

The initial approach taken was to determine if there is a difference in the acid or base stability of adducts **10** and **15**. Consequently, on the assumption that the active site nucleophile to which the inactivator is attached is a lysine residue, 4-oxo-6-piperidinohexanoic acid (**19**) and 4-oxo-5-piperidinohexanoic acid (**20**) were synthesized as model compounds for adducts **10** and



19



20

15, respectively, and the acid and base stabilities of these compounds were tested. Both model compounds were stable to a variety of acid and base conditions; however, there was a difference when they were incubated with 7 M urea, pH 13 at 76 °C. In 12 h the β-substituted ketone (**19**) degraded significantly, but the α-substituted model (**20**) was stable. However, the expected β-elimination degradation product from **19**, namely, 4-oxo-5-hexenoic acid, was not observed, presumably because it decomposed under these conditions. When GABA aminotransferase, which was labeled with [6-^{14}C]-γ-vinyl GABA, was incubated under these latter conditions, only 0.27 equiv of radioactivity, instead of 0.76 equiv, remained bound. This could be rationalized that the adduct is somewhat unstable under these conditions (thus favoring adduct **10**), but it is not conclusive support for or against either adduct.

Another approach taken to identify the stable adduct was periodate oxidation of the sodium borohydride reduced adduct. Sodium periodate is known to oxidatively cleave compounds that contain oxygens or nitrogens on adjacent carbons,[18] such as α-amino ketones[19] and α-amino alcohols.[20] In this case periodate oxidation of the borohydride reduced enzyme adduct should produce acetaldehyde and succinic semialdehyde (Scheme V). Since the radioactive label in the γ-vinyl GABA is only at C-6, only the acetaldehyde would be detectable. A great amount of research has been done on the periodate cleavage of α-amino alcohols,[20] with the conclusion that both primary and secondary amino alcohols are cleaved, but only when the nitrogen and oxygen are on adjacent carbons.[21] Therefore, reduction of **15** would give an α-amino alcohol which should be susceptible to periodate cleavage, but reduction of **10** would give a β-amino alcohol which should be immune to periodate cleavage (Scheme V). Production of acetaldehyde under these conditions would indicate that **15** was the adduct; no acetaldehyde would signal the presence of **10** as the adduct. This same approach was taken to identify the stable adduct formed by inactivation of GABA aminotransferase by γ-ethynyl GABA.[12] In that case **15** was shown to be the adduct; however, since the radioactivity was at the 2-position (next to the carboxylate group) in that inactivator, the label showed up in the succinic semialdehyde that was released. Since acetaldehyde was shown to be stable to the conditions of this experiment, the *absence* of radioactive acetaldehyde would be strong evidence *against* adduct **15**. The model compounds used to test the chemistry for this experiment were 5-amino-4-oxopentanoic acid (i.e., δ-ami-

(17) DeBaise, D.; Bolton, J. B.; Barra, D.; Bossa, F.; John, R. A. *Biochimie* **1989**, *71*, 491–495.

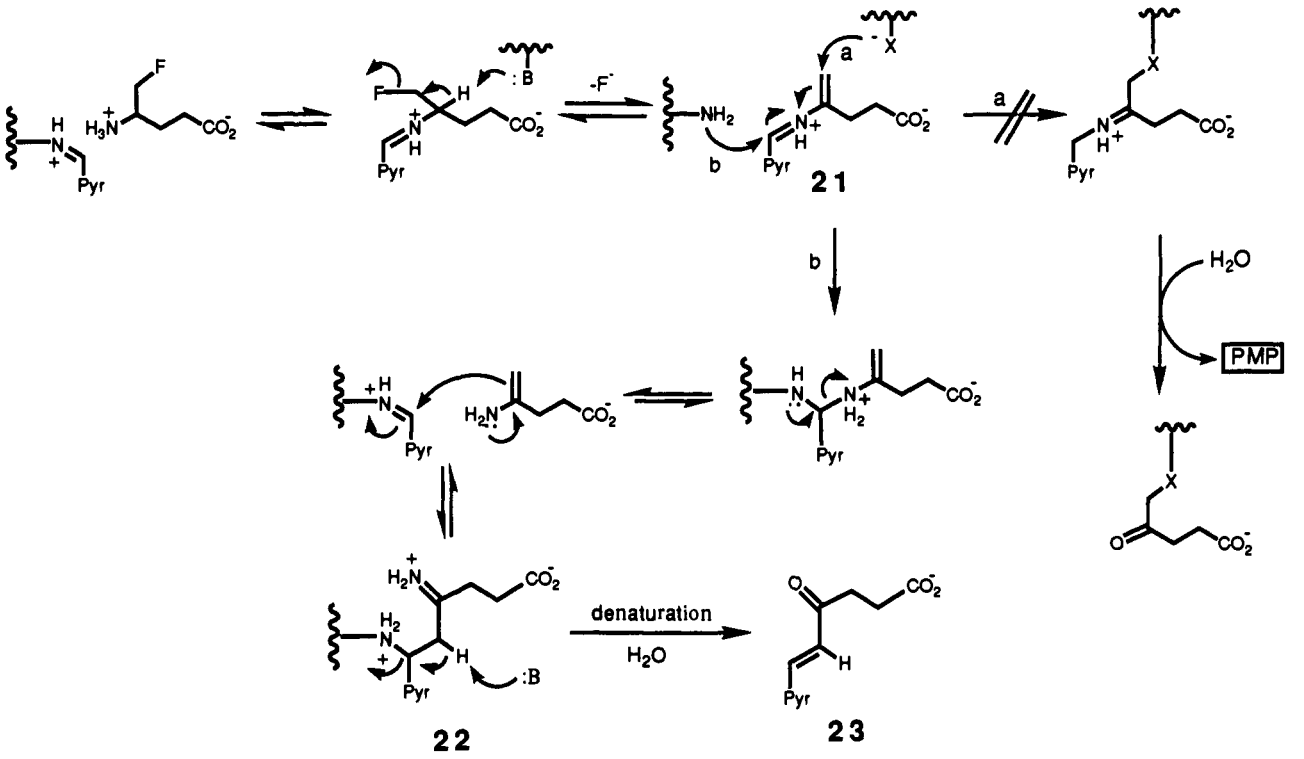
(18) March, J. *Advanced Organic Chemistry*, 3rd ed.; John Wiley & Sons: New York, 1985; p 1063.

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

(20) Maros, L.; Molnar-Perl, I.; Schissel, E.; Szerdahelyi, V. *J. Chem. Soc. Perkin Trans. II* **1980**, 39–45.

(21) Jackson, E. L. *Org. React.* **1944**, *2*, 341–375.

Scheme VI. Mechanism of Inactivation of GABA Aminotransferase by 4-Amino-5-fluoropentanoic Acid[13]



nolevulinic acid), which is a model for **15**, but with no methyl group, and 6-amino-4-oxohexanoic acid (the exact model for **10**). Sodium borohydride reduction followed by sodium periodate oxidation of these compounds confirmed that 5-amino-4-oxopentanoic acid produced succinic semialdehyde and, in this case, formaldehyde (this model does not have the methyl group, so formaldehyde is formed), but reduced 6-amino-4-oxohexanoic acid was not degraded. When these conditions were carried out on the enzyme inactivated with [6-^{14}C]-γ-vinyl GABA, *no* acetaldehyde was generated. Furthermore, essentially none of the radioactivity bound to the enzyme prior to periodate treatment was released, consistent with no degradation occurring. These results suggest that adduct **10**, derived from the azaallylic isomerization–Michael addition mechanism (Scheme I), is the stable adduct that accounts for 70–75% of the bound radioactivity after inactivation by γ-vinyl GABA and argues against the allylic isomerization–Michael addition mechanism shown in Scheme II.

This conclusion is consistent with our previous results on the mechanism of inactivation of GABA aminotransferase by 4-amino-5-fluoropentanoic acid[13] (Scheme VI). This inactivation mechanism proceeds via intermediate **21** which is almost identical to **12** in Scheme II except that it does not have the methyl substituent. In the study with 4-amino-5-fluoropentanoic acid it was found that intermediate **21** does *not* undergo a Michael addition with an active-site residue (pathway a), rather, it exclusively proceeds by the enamine mechanism (pathway b) related to that described earlier by Metzler and co-workers.[14,15] Since the only difference between **12** and **21** is the β-methyl group, and Michael addition reactions are hindered by substitution at the β-position of a Michael acceptor,[22] it is highly unlikely that intermediate **12** would undergo a Michael addition if **21** does not.

The results with 4-amino-5-fluoropentanoic acid indicate that although the Michael addition to **12** appears to be unimportant, an enamine mechanism (Scheme IV) may be quite relevant and may give a clue to the identification of the unstable adduct. The enamine mechanism with 4-amino-5-fluoropentanoic acid was characterized by determination of the structure of the adduct released from the enzyme upon denaturation. This adduct, which was characterized by spectral and chromatographic comparison to an authentic sample synthesized by an independent route, was shown to have structure **23** (Scheme VI). By analogy, then, adduct **17** (Scheme IV) should undergo elimination to **18**. This compound could not be synthesized by a variety of approaches; however, it is highly likely that the retention time on reversed-phase HPLC for **23** would be almost identical with that for **18** and that the former could be used as a HPLC standard for the latter. Compound **23** was generated by inactivation of GABA aminotransferase with 4-amino-5-fluoropentanoic acid and was shown to comigrate by HPLC with the "unknown" tritium-containing peak observed when GABA aminotransferase reconstituted with [^{3}H]PLP was inactivated with γ-vinyl GABA and denatured (Figure 1, enamine adduct). Furthermore, **23** also comigrated with the [^{14}C]-labeled unstable adduct released following inactivation of GABA aminotransferase with [6-^{14}C]-γ-vinyl GABA and neutral denaturation. These results indicate that the unstable adduct contains the cofactor (from the tritium experiment) and the inactivator (from the ^{14}C experiment), and its structure after denaturation is very similar to that of **23**. It is quite reasonable to suggest, then, that the unstable adduct is **17**. The unstable adduct is derived from allylic isomerization of the γ-vinyl GABA-PLP Schiff base to give **12** (Scheme II) followed by transfer of the PLP to the active-site lysine residue (Scheme IV), which produces an enamine that adds to the lysine-bound PLP thereby producing **17**. This accounts for the remainder of the 1 equiv of radioactivity bound to GABA aminotransferase.

In addition to covalent adduct formation, intermediates **6** (Scheme I) and **12** (Scheme II) may be released and produce metabolites **6a** and **12a**, respectively. The ratio of products generated per inactivation event is known as the partition ratio. In order to completely characterize the chemistry of interaction of γ-vinyl GABA with GABA aminotransferase, the metabolism of this compound by GABA aminotransferase was studied. In the presence of [^{14}C]-α-ketoglutarate 1.8 equiv of L-[^{14}C]glutamate are produced; therefore, one to two transaminations appear to occur during inactivation. This suggests that following azaallylic isomerization to **6** (Scheme I), on average, one to two times per inactivation event, the normal catalytic pathway (see Scheme III) becomes important and presumably leads to **6a** and PMP. Another potential metabolic pathway is shown in Scheme II in which

(22) Bergmann, E. D.; Ginsburg, D.; Pappo, R. *Org. React.* **1959**, *10*, 179–555.

intermediate **12** releases the enamine which then is hydrolyzed to **12a**. This pathway leaves the coenzyme as PLP, so it is not along the transamination pathway. When 4-amino-5-fluoropentanoic acid was used as an inactivator,[13] which produces a very similar intermediate during metabolic processing (compare **21** in Scheme VI with **12** in Scheme II), no hydrolysis to the corresponding ketone occurred, so it is not clear that **12** would be predicted to lead to **12a**. Both the conversion of **6** to **6a** and **12** to **12a** produce nonamine metabolites. Consequently, the total radioactive nonamines were isolated after inactivation by [6-^{14}C]-γ-vinyl GABA; however, only 0.9 equiv of nonamine metabolites was isolated. It is not clear why there is not a correlation between the amount of transamination products formed and the nonamine metabolites. Furthermore, both **6a** and **12a** were synthesized and were used as HPLC standards; only small amounts (0.1–0.15 equiv) of either of these compounds comigrated with the radioactive nonamine metabolite. The poor stability of **6a** under the conditions of the experiment may account for the inability to observe this metabolite; however, no conclusion can be drawn about the transamination metabolism on the basis of these results. Since **12a** was stable to the conditions of the experiment and none was observed, it suggests that, as in the case of 4-amino-5-fluoropentanoic acid, no enamine hydrolysis apparently occurs, and, whenever the enamine is generated, it inactivates the enzyme as shown in Scheme IV.

Conclusions

γ-Vinyl GABA inactivates GABA aminotransferase by two different mechanisms, each leading to different covalent adducts. Approximately 2.5 out of every 3 molecules of γ-vinyl GABA that is processed by GABA aminotransferase, subsequent to Schiff base formation with the active site PLP, undergoes azaallylic isomerization (that is, the normal catalytic mechanism), leading to a reactive intermediate (**6**, Scheme I). This intermediate is hydrolyzed (again, by the normal catalytic mechanism) approximately 1.8 times out of 2.5 molecules presumably to give the transamination product (**6a**). The other 0.7 out of 2.5 molecules undergoes nucleophilic attack to give adduct **10**, which is stable to denaturation and accounts for 70–75% of the total active-site labeling. Adduct **10** also could be derived from active-site nucleophilic attack on **6a** prior to its release from the active site. Approximately 0.3 out of the 3 molecules of γ-vinyl GABA that are processed by GABA aminotransferase are metabolized by allylic isomerization (Scheme II), leading to reactive intermediate **12**; however, transimination by the active-site lysine residue (Scheme IV), apparently, is faster than Michael addition to **12**. This leads to the formation of an enamine, which attacks the lysine-bound PLP to give adduct **17**. This adduct, which accounts for 25–30% of the total bound adduct, is unstable to denaturation. None of the enamine escapes from the active site and gets hydrolyzed.

It is interesting to compare this inactivator to γ-ethynyl GABA,[12] which also forms two adducts to GABA aminotransferase, one that is stable to denaturation and one that is not. In the case of γ-ethynyl GABA both adducts are derived from propargylic isomerization to a conjugated allene. Attack by a lysine residue on the allene gives an adduct that, upon denaturation, forms the stable and unstable adducts. No inactivation appears to be derived from the normal catalytic pathway of azaallylic isomerization, although metabolite isolation suggests that this isomerization does occur. The reactive intermediate formed by azaallylic isomerization is released without inactivation. The difference in these two inactivators, then, is the apparent ease of propargylic rearrangement of PLP-bound γ-ethynyl GABA and the high reactivity of the conjugated allene that is thereby generated. The propensity for the propargylic rearrangement and conjugated allene-induced inactivation is evident in the observation that both (*R*)-(−)- and (*S*)-(+)-γ-ethynyl GABA irreversibly inactivate both rat brain and bacterial GABA aminotransferase.[23] This was rationalized that GABA aminotransferase has two active-site bases that could potentially remove the *pro-R* or *pro-S* γ-proton. With normal substrates only the base that removes the *pro-S* proton is functional, but because of the greater acidity of the γ-proton adjacent to the acetylene group, both protons can be removed with γ-ethynyl GABA.

Experimental Section

Analytical Methods. All synthetic reactions were carried out under an atmosphere of nitrogen. Flash column chromatography was carried out with Merck silica gel 60 (230–400 mesh). ^{1}H NMR spectra were recorded on either a Varian XLA-400 or a Varian EM 390 A spectrometer at 400 and 90 MHz, respectively. Chemical shifts are reported as δ values in parts per million downfield relative to tetramethylsilane (δ 0.00) used as an internal standard in $CDCl_3$. For samples run in D_2O, the HOD resonance was arbitrarily set at δ 4.874. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Optical spectra and GABA aminotransferase assays were recorded on either a Perkin-Elmer Lambda 1 or Beckman DU-40 UV–vis spectrophotometer equipped with a constant temperature cuvette holder. GABA aminotransferase activity was measured as described under **Enzymes and Assays**. An Orion Research Model 601 pH meter with a general combination electrode was used for pH measurements. Radioactivity content was measured in a Beckman LS-3133 scintillation counter after the addition of 10 mL of 3a70B scintillation fluid (Research Products International) to the sample. [U-^{14}C]Toluene (4.0×10^5 dpm/mL), obtained from New England Nuclear, was used as an internal standard. HPLC was performed with Alltech analytical (4.6 mm × 25 cm) C18 reversed-phase columns on a Beckman system consisting of a controller (Model 421A), variable wavelength detector (Model 163), and two solvent delivery pumps (Model 110B).

Reagents. All HPLC solvents (B&J, Merck, Mallinckrodt) were filtered and degassed before use. The in-house distilled water was further purified by passage through a deionizer (Continental Water Conditioning Corp.) before use. Diethyl ether was distilled under nitrogen from sodium metal with sodium benzophenone ketyl used as an indicator. Ethyl acetate and hexane (predried over sulfuric acid) were distilled from anhydrous potassium carbonate. The syntheses of 4-oxo-6-piperidinohexanoic acid hydrochloride (**19**),[24] 4-oxo-5-hexenoic acid,[25] 6-amino-4-oxohexanoic acid,[12] and [7-^{3}H]pyridoxal 5′-phosphate[13] were reported. (±)-γ-Vinyl GABA and [6-^{14}C]-γ-vinyl GABA (specific activity 2.71 mCi/mmol) were generous gifts of the Marion Merrell Dow Research Institute. Ultrapure urea was purchased from Schwarz/Mann Biotech. Dowex 50W-X8 (H^+ form) ion exchange resin, 2,4-dinitrophenylhydrazine, potassium pyrophosphate, α-ketoglutarate, $NADP^+$, β-mercaptoethanol, pyridoxal phosphate, pyridoxamine phosphate, and GABA were purchased from Sigma. GABASE was obtained from Boehringer Mannheim Biochemicals. Potassium monobasic phosphate, potassium dibasic phosphate, sodium monobasic phosphate, and sodium dibasic phosphate were products of Mallinckrodt. All other reagents were purchased from the Aldrich Chemical Co. The 2,4-dinitrophenylhydrazine reagent was made as described by Shriner et al.[26]

Ethyl 4-Oxo-5-piperidinohexanoate. A solution of ethyl 5-bromo-4-oxohexanoate[27] (2.5 g, 10.6 mmol) in ether (15 mL) was added dropwise to a solution of piperidine (1.8 g, 21.1 mmol) in ether (25 mL) in a 100-mL, three-necked flask equipped with a mechanical stirrer and a condenser. A white solid started precipitating out immediately. After completion of the addition, the mixture was filtered, and the filtrate was concentrated in vacuo to give a yellow paste. The desired compound (2.34 g, 92%) was obtained by means of silica gel chromatography (3:1 hexane/ethyl acetate): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.17 (d, 3 H), 1.25 (t, 3 H), 1.43 (m, 2 H), 1.59 (m, 4 H), 2.44 (m, 4 H), 2.57 (m, 2 H), 2.96 (m, 2 H), 3.18 (q, 1 H), 4.13 (q, 2 H); high-resolution mass spectrum calcd for $C_{13}H_{23}NO_3$ 241.1678, found 241.1652 (11 ppm deviation). Anal. Calcd for $C_{13}H_{23}NO_3$: C, 64.73; H, 9.54; N, 5.81. Found: C, 64.13; H, 9.44; N, 5.63.

4-Oxo-5-piperidinohexanoic Acid Hydrochloride (20). The ethyl ester from above (0.52 g, 2.2 mmol) was refluxed in 15 mL of 6 N HCl for a period of 5 h. The solvent was removed in vacuo to give a brown oil. The residue was dissolved in water and applied to a Dowex 50W-X8

(23) Danzin, C.; Claverie, N.; Jung, M. J. *Biochem. Pharmacol.* **1984**, *33*, 1741–1746.

(24) Choudhari, N.; Mukharji, P. C. *J. Ind. Chem. Soc.* **1952**, *29*, 336–340.

(25) Condon, M. E. et al *J. Med. Chem.* **1982**, *25*, 250–258.

(26) Shriner, R. L.; Fuson, R. C.; Curtin, D. Y.; Morrill, T. C. *The Systematic Identification of Organic Compounds*; John Wiley & Sons: New York, 6th ed.; p 162.

(27) (a) Wehrli, P. A.; Chu, V. *Org. Syn.* **1978**, *58*, 79–82. (b) Lesiak, T.; Marzec, K. *J. Prakt. Chem.* **1980**, *322*, 479–484.

column (H^+ form, 1 cm × 8 cm) and washed with 20 mL of water. The amino acid was eluted with 2 N HCl, and the ninhydrin positive fractions were combined and concentrated in vacuo to give a yellow oil (0.31 g, 58%): ^1H NMR (400 MHz, D_2O) δ 1.46 (m, 1 H), 1.61 (d, 3 H), 1.81 (m, 3 H), 1.91 (m, 2 H), 2.72 (m, 2 H), 2.82–3.18 (m, 4 H), 3.32 (d, 1 H), 3.46 (d, 1 H), 4.35 (q, 1 H); fast atom bombardment high-resolution mass spectrum calcd for $C_{11}H_{20}NO_3$ 214.1443, found 214.1442 (−0.5 ppm deviation).

Enzymes and Assays. Pig brain GABA aminotransferase was isolated as described previously.[28] Succinic semialdehyde dehydrogenase was obtained from commercially available GABASE as previously described.[12] Protein assays were carried out with Pierce Coomassie protein assay reagent using bovine serum albumin for standard curves.

Formation of ApoGABA Aminotransferase. GABA aminotransferase (20.63 units) was incubated in the dark at 25 °C with 60 mM GABA, 0.25 mM β-mercaptoethanol in 100 mM potassium phosphate buffer (pH 7.4) for 35 min. The solution was titrated to pH 6.0 with 1 M potassium phosphate (dibasic) containing 0.25 mM β-mercaptoethanol and then refrigerated for 1 h. The solution was dialyzed in the dark successively against 2 L of 1 M potassium phosphate (dibasic) containing 0.25 mM β-mercaptoethanol for 1 h and 2 × 2 L of 100 mM potassium phosphate buffer (pH 7.4) containing 0.25 mM β-mercaptoethanol. The dialyzed solution had no GABA aminotransferase activity. The above procedure also was carried out on a control sample consisting of GABA aminotransferase (8.25 units) but without any substrate GABA.

Reconstitution of ApoGABA Aminotransferase with [7-^{3}H]PLP. ApoGABA aminotransferase was incubated with [7-^{3}H]PLP in the dark. Activity assays were carried out at time intervals, and once enzyme activity had stopped increasing (about 4 h) the excess [7-^{3}H]PLP was removed by dialysis against 2 × 4 L of 100 mM potassium phosphate buffer (pH 7.4) containing 0.25 mM β-mercaptoethanol. The specific activity of [^{3}H]GABA aminotransferase was determined to be 15.9 mCi/mmol.

Equivalents of [6-^{14}C]-γ-Vinyl GABA Bound to GABA Aminotransferase. GABA aminotransferase (6.64 units) was incubated with 0.87 mM [6-^{14}C]-γ-vinyl GABA, 0.53 mM α-ketoglutarate in 100 mM potassium phosphate buffer (pH 7.3) at 25 °C. A control (without inactivator) also was run. After the enzyme in the sample containing inactivator had lost its activity as compared to the control, the sample was dialyzed against 400 mL of 100 mM potassium phosphate buffer (pH 7.3) until the dialysate contained no more radioactivity (about five changes). Aliquots were removed to analyze for radioactivity (100 μL) and protein content (150 μL). The rest was split up into 6 portions of 240 μL. To 2 portions was added 1 mL of 6 M urea pH 1, to 2 portions was added 1 mL of 6 M urea pH 7, and to the rest was added 1 mL of 6 M urea pH 12. They were placed in dialysis bags and dialyzed against 4 × 900 mL of 6 M urea at their respective pH's. Aliquots were removed from each to analyze for radioactivity content (900 μL) and protein content (200 μL). Equivalents of inactivator bound to the enzyme were taken as the ratio of the number of μmol of inactivator bound to enzyme (determined by the radioactivity content of the dialyzed solution) to the number of μmol of enzyme present (determined by protein content of the dialyzed solution).

Acid and Base Stabilities of 4-Oxo-6-piperidinohexanoic Acid (19) and 4-Oxo-5-piperidinohexanoic Acid (20). 4-Oxo-6-piperidinohexanoic acid hydrochloride (20 mg) was incubated at temperatures of 25, 60, and 80 °C in 833 μL of the following solvents: 100 mM potassium phosphate pH 7.3, 100 mM potassium phosphate pH 11.4, 100 mM potassium phosphate pH 1.4, 7 M urea pH 13.1, 7 M urea pH 1.2, 10% (w/v) TCA solution. Additional incubations of 4-oxo-5-piperidinohexanoic acid hydrochloride (20 mg) and 4-oxo-6-piperidinohexanoic acid hydrochloride (20 mg) in 630 μL of 7 M urea pH 13.3 and 0.5 N NaOH solution (pH 13) at 25, 38, 58, and 76 °C were set up. Aliquots of every incubation solution were taken at time intervals and analyzed for degradation of 4-oxo-5-piperidinohexanoic acid and 4-oxo-6-piperidinohexanoic acid by injecting aliquots onto a C18 reversed-phase analytical column (λ = 254 nm) using water as the eluant at a flow rate of 1 mL/min. Under these conditions the T_R of 4-oxo-5-piperidinohexanoic acid and 4-oxo-6-piperidinohexanoic acid are 8.9 and 9.3 min, respectively.

Equivalents of [6-^{14}C]-γ-Vinyl GABA Bound to GABA Aminotransferase after Dialyzing under Degradation Conditions. GABA aminotransferase (4.6 units) was incubated with 1.04 mM [6-^{14}C]-γ-vinyl GABA and 1.67 mM α-ketoglutarate in 100 mM potassium phosphate buffer (pH 7.3) at 25 °C. A control (without inactivator) also was run. After the enzyme in the sample containing inactivator had lost its activity as compared to the control, the sample was dialyzed sequentially against 7 × 400 mL of 100 mM potassium phosphate buffer pH 7.3 (for at least 3 h each), 4 × 400 mL 6 M urea pH 7 (for at least 3 h each), and 4 × 400 mL 7 M urea pH 13.1 at 76 °C (for at least 4 h each). Aliquots were removed from the dialysis bag after each dialysis, and equivalents bound to the enzyme were determined as described under **Equivalents of [6-^{14}C]-γ-Vinyl GABA Bound to GABA Aminotransferase.**

Sodium Periodate Oxidation of Sodium Borohydride Reduced 5-Amino-4-oxopentanoic Acid and 6-Amino-4-oxohexanoic Acid. 5-Amino-4-oxopentanoic acid (27.8 mg) and 6-amino-4-oxohexanoic acid (40 mg) were incubated with 190 mM sodium borohydride, 6 M urea, 150 mM NaOD in D_2O (1 mL) and 6 M urea, 100 mM sodium phosphate pH 7.0 in D_2O (1 mL) for a period of 12 h. The solution was made acidic with DCl/D_2O and allowed to sit for 20 min. The solution was next neutralized with NaOD/D_2O. To 500 μL of the resulting solution was added sodium periodate (6 mg) to give a 50 mM solution. Another 500 μL of the solution served as control (without sodium periodate). ^1H NMR spectra of the incubation as well as the control were taken at various time intervals over a period of 35 h.

Acetaldehyde Stability. Acetaldehyde (200 μL) was dissolved in 1 mL of a buffer solution consisting of 6 M urea and 100 mM sodium phosphate pH 7.0 in D_2O, and then sodium periodate (12 mg) was added. The sample was analyzed by ^1H NMR and HPLC (C_{18} reversed-phase analytical column; eluant = water containing 0.1% trifluoroacetic acid; λ = 275 nm; flow rate = 1 mL/min; T_R for acetaldehyde = 5.4 min) at various time intervals over a period of over 30 h.

Sodium Periodate Oxidation of [6-^{14}C]-γ-Vinyl GABA-Inactivated, Sodium Borohydride Reduced GABA Aminotransferase. GABA aminotransferase (2.67 units) was incubated with 0.96 mM [6-^{14}C]-γ-vinyl GABA and 2.1 mM α-ketoglutarate in 100 mM potassium phosphate buffer (pH 7.3) at 25 °C. A control (without inactivator) also was run. After the enzyme in the sample containing inactivator had lost its activity as compared to the control, the sample was dialyzed against 6 M urea, 100 mM sodium phosphate buffer pH 7.4 (1 × 900 mL, 1 × 1500 mL) for 4 h each. Sodium borohydride (190 mM) in a 150 mM aqueous sodium hydroxide solution was added to the dialyzed enzyme in 4 portions (100 μL each) at 2 h intervals. The sample was incubated at 25 °C for 12 h after which time it was neutralized by the addition of glacial acetic acid. The reduced enzyme was then dialyzed sequentially against 1 L of 6 M urea, 50 mM citric acid pH 3.3 and 1 L of 6 M urea, 100 mM sodium phosphate buffer pH 7.4 for 4 h each. An aliquot was removed from the dialyzed enzyme to analyze for equivalents of inactivator bound to the enzyme. The rest of the dialyzed enzyme was split into two parts. Sodium periodate (4 mg) was added to one portion to make a 50 mM solution of periodate. The other portion served as a control. Both of them were incubated at 25 °C for 28 h. The periodate-containing sample was split up into three parts: the first for direct HPLC analysis as described below; the second for dialysis against 6 M urea, 100 mM sodium phosphate buffer pH 7.3 (2 × 1 L) and subsequent equivalents bound analysis; the third for 2,4-dinitrophenylhydrazone formation and HPLC analysis as described below. The control (without periodate) was split up into two parts: one for direct HPLC analysis and the other for 2,4-dinitrophenylhydrazone formation and HPLC analysis. Direct HPLC analysis was carried out on a C18 reversed-phase analytical column (λ = 275 nm) after the addition of acetaldehyde as a standard using water containing 0.1% trifluoroacetic acid as the eluant at a flow rate of 1 mL/min. Under these conditions T_R of acetaldehyde = 5.4 min. Acetaldehyde and 2,4-dinitrophenylhydrazine reagent were added to the solutions that were to be analyzed for 2,4-dinitrophenylhydrazone formation and incubated at 25 °C for 30 min. The samples were extracted with chloroform (2 × 1 mL), and the organic solvent was evaporated under a stream of nitrogen gas. The resulting residue was dissolved in acetonitrile (200 μL) and injected onto a C18 reversed-phase analytical column (λ = 254 nm) using 50% methanol/water as the eluant at a flow rate of 2 mL/min. Under these conditions the T_R of the 2,4-dinitrophenylhdrazone of acetaldehyde = 14.8 min.

Adduct Released after Denaturation of [6-^{14}C]-γ-Vinyl GABA Inactivated GABA Aminotransferase. GABA aminotransferase (1.92 units) was incubated with 1.2 mM [6-^{14}C]-γ-vinyl GABA, 2.75 mM α-ketoglutarate in 100 mM potassium phosphate buffer (pH 7.3) at 25 °C. A control (without inactivator) also was run. After the enzyme in the sample containing inactivator had lost its activity as compared to the control, the sample was dialyzed against 400 mL of 100 mM potassium phosphate buffer (pH 7.3) until the dialysate contained no more radioactivity (about five changes). The denaturation and metabolite identification was carried out as described in Figure 1.

Identification of the Adduct Released after Denaturation of [6-^{14}C]-γ-Vinyl GABA-Inactivated GABA Aminotransferase under Neutral Conditions. GABA aminotransferase (9.95 units) was incubated with 1.04 mM [6-^{14}C]-γ-vinyl GABA, 1.6 mM α-ketoglutarate in 100 mM potassium phosphate buffer (pH 7.3) at 25 °C. A control (without inactivator) also was run. After the enzyme in the sample containing inactivator had lost its activity as compared to the control, the sample was dialyzed against

(28) Silverman, R. B.; Levy, M. A. *Biochemistry* **1981**, *20*, 1197–1203.

400 mL of 100 mM potassium phosphate buffer (pH 7.3) until the dialysate contained no more radioactivity (about five changes). The sample was next microdialyzed against 6 M urea (9 mL, pH 7.0) for a period of 14 h. The dialysate was lyophilyzed, and the residue was taken up in water (4 mL). Enough solid trichloroacetic acid was added to give a 10% (w/v) solution. The sample was then analyzed as described in Figure 1.

Transamination of γ-Vinyl GABA by GABA Aminotransferase. Varying concentrations of GABA aminotransferase (0.07–0.13 units) were incubated with 15 mM γ-vinyl GABA in 50 mM potassium pyrophosphate buffer (pH 8.5) at 25 °C containing 2.6 mM [5-^{14}C]-α-ketoglutarate (Amersham; specific activity 0.803 mCi/mmol). A sample containing no enzyme and one containing no inactivator served as controls. After the enzyme in the samples containing inactivator had lost its activity as compared to the control without inactivator, all the samples were quenched with 20% (w/v) aqueous solution of trichloroacetic acid (30 μL each) and then applied to a column (0.6 cm × 5.5 cm) of Dowex 50W-X8 (H^+ form). The column was washed with 5 mL of water and 6 mL of 2 N aqueous ammonia. The last 5 mL of the ammonia eluate was found to contain all of the [^{14}C]glutamate. The glutamate containing eluate was measured for radioactivity content.

Equivalents of Nonamines Generated upon Inactivation of GABA Aminotransferase by [6-^{14}C]-γ-Vinyl GABA. Varying concentrations of GABA aminotransferase (0.2–0.46 units) were incubated with 1.05 mM [6-^{14}C]-γ-vinyl GABA, 3.97 mM α-ketoglutarate in 100 mM potassium phosphate buffer (pH 7.3) at 25 °C. A sample containing no enzyme and one containing no inactivator served as controls. After the enzyme in the samples containing inactivator had lost their activity as compared to the control without inactivator, solid trichloroacetic acid was added to one of the samples to make it a 10% (w/v) solution. After a period of 2 h, the samples and controls were applied separately to a column (0.6 cm × 5.5 cm) of Dowex 50W-X8 (H^+ form). The columns were washed with water and eight 5-mL fractions were collected and measured for radioactivity content.

Equivalents of 4-Oxohexanoic Acid (12a) and 4-Oxo-5-hexenoic Acid (6a) Generated during Inactivation of GABA Aminotransferase by [6-^{14}C]-γ-Vinyl GABA. GABA aminotransferase (1.28 units) was incubated with 1.04 mM [6-^{14}C]-γ-vinyl GABA, 2.7 mM α-ketoglutarate in 100 mM potassium phosphate buffer (pH 7.3) at 25 °C. A sample containing no enzyme and one containing no inactivator served as controls. After the enzyme in the samples containing inactivator had lost its activity as compared to the control without inactivator, **6a** and **12a** were added as standards, and the samples were injected separately onto a C18 reversed-phase analytical column (λ = 214 nm) using 100 mM sodium phosphate (pH 6) as eluant at a flow rate of 0.9 mL/min. Under these conditions the T_R of **12a** and **6a** were 14 and 11 min, respectively. Fractions were collected and analyzed for radioactivity content.

Acknowledgment. The authors are grateful to the Marion Merrell Dow Research Institute, Marion Merrell Dow, Inc. for a generous gift of γ-vinyl GABA and [6-^{14}C]-γ-vinyl GABA and to the National Institutes of Health (NS 15703) for financial support of this research.

Carbon Assignments and Heteronuclear Coupling Constants for an RNA Oligonucleotide from Natural Abundance ^{13}C–^{1}H Correlated Experiments[1]

Gabriele Varani and Ignacio Tinoco, Jr.*

Contribution from the Department of Chemistry and Laboratory of Chemical Biodynamics, University of California, Berkeley, California 94720. *Received April 8, 1991*

Abstract: Complete assignments of the proton-linked carbons for the oligoribonucleotide 5′(GGACUUCGGUCC) have been obtained from 2D ^{13}C–^{1}H correlated experiments at natural abundance. High-quality spectra have been collected at RNA concentrations commonly used in proton experiments (2 mM). Heteronuclear one-bond scalar couplings have been measured from high-resolution spectra. The results demonstrate the editing power of the ^{13}C–^{1}H correlation in the very crowded sugar region of the proton NMR spectrum of RNA. Thus, 2D ^{13}C–^{1}H correlated experiments can be one of the main tools used for RNA proton spectral assignments. Both the carbon chemical shifts and the ^{13}C–^{1}H couplings are shown to be sensitive to the conformation of the sugar and the phosphodiester backbone, previously characterized for this oligoribonucleotide using proton and phosphorus NMR data. The potential of ^{13}C NMR in the structural analysis of RNA is evident from the data reported here.

Structural studies of RNA oligonucleotides by NMR spectroscopy are severely limited by the spectral overlap of the crowded sugar proton region. Spectral assignments are difficult, and the crucial information on the phosphodiester backbone conformation contained in scalar couplings and NOEs involving sugar protons may be impossible to obtain.[2] Complete proton and phosphorus spectral assignments have been obtained for an RNA 12-mer (~4000 Da) from a combination of homo- and heteronuclear (^{31}P–^{1}H) correlated experiments and NOE spectroscopy.[3,4] At larger molecular weight, the overlap of the sugar protons makes it increasingly difficult to use correlated techniques for assignments and structural analysis. With the exception of the H1′, all other sugar protons resonate between 4 and 5 ppm; thus, assignments and the extraction of constraints for structural analysis are very difficult tasks. So far, one has had to rely mostly on NOESY-based information above 5000–6000 Da;[5] this information necessarily relies on conformational assumptions. Carbon NMR can help overcome these problems by editing the proton chemical shifts in 3D experiments on uniformly or selectively[6,7] labeled com-

(1) Abbreviations used: NMR, nuclear magnetic resonance spectroscopy; ppm, parts per million; 1D, 1-dimensional; 2D, two-dimensional; 3D, three-dimensional; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; TPPI, time proportional phase incrementation; TSP, (trimethylsilyl)propionic acid; RNA, ribonucleic acid.

(2) (a) Altona, C. *Recl. Trav. Chim. Pays-Bas* **1982**, *101*, 413–433. (b) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

(3) Cheong, C.; Varani, G.; Tinoco, I., Jr. *Nature* **1990**, *346*, 680–682.

(4) Varani, G.; Cheong, C.; Tinoco, I., Jr. *Biochemistry* **1991**, *30*, 3280–3289.

(5) (a) Varani, G.; Wimberly, B.; Tinoco, I., Jr. *Biochemistry* **1989**, *28*, 7760–7772. (b) Puglisi, J. D.; Wyatt, J. R.; Tinoco, I., Jr. *Biochemistry* **1990**, *29*, 4215–4226. (c) Puglisi, J. D.; Wyatt, J. R.; Tinoco, I., Jr. *J. Mol. Biol.* **1990**, *214*, 437–453.

(6) (a) Kline, P. C.; Serianni, A. S. *J. Am. Chem. Soc.* **1990**, *112*, 7373–7381. Manoharan, M.; Gerlt, J. A.; Wilde, J. A.; Withka, J. M.; Bolton, P. H. *J. Am. Chem. Soc.* **1987**, *109*, 7217–7219.

(7) (a) Williamson, J. R.; Boxer, S. G. *Biochemistry* **1989**, *28*, 2819–2831. (b) *Ibid.* 2831–2836.

0002-7863/91/1513-9349$02.50/0