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Inactivation of γ-Aminobutyric Acid Aminotransferase by (*Z*)-4-Amino-2-fluorobut-2-enoic Acid†

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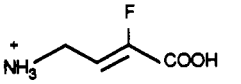
ABSTRACT: (*Z*)-4-Amino-2-fluorobut-2-enoic acid (**1**) is shown to be a mechanism-based inactivator of pig brain γ-aminobutyric acid aminotransferase. Approximately 750 inactivator molecules are consumed prior to complete enzyme inactivation. Concurrent with enzyme inactivation is the release of 708 ± 79 fluoride ions; transamination occurs 737 ± 15 times per inactivation event. Inactivation of [^{3}H]pyridoxal 5′-phosphate ([^{3}H]PLP) reconstituted GABA aminotransferase by **1** followed by denaturation releases [^{3}H]PMP with no radioactivity remaining attached to the protein. A similar experiment carried out with 4-amino-5-fluoropent-2-enoic acid [Silverman, R. B., Invergo, B. J., & Mathew, J. (1986) *J. Med. Chem. 29*, 1840–1846] as the inactivator produces no [^{3}H]PMP; rather, another radioactive species is released. These results support an inactivation mechanism for **1** that involves normal catalytic isomerization followed by active site nucleophilic attack on the activated Michael acceptor. A general hypothesis for predicting the inactivation mechanism (Michael addition vs enamine addition) of GABA aminotransferase inactivators is proposed.

Allan et al. (1979) briefly noted in an abstract that 4-amino-2-fluorobut-2-enoic acid (**1**) was a time-dependent in-



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activator of the pyridoxal 5′-phosphate dependent enzyme γ-aminobutyric acid (GABA)[1] aminotransferase; no inacti-

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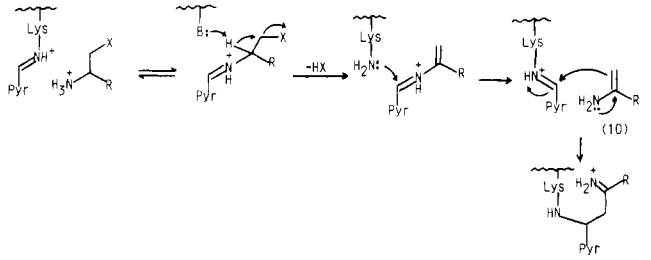
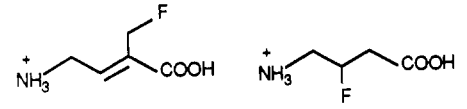
Scheme I: Enamine Mechanism for Inactivation of Pyridoxal 5′-Phosphate Dependent Enzymes[a]

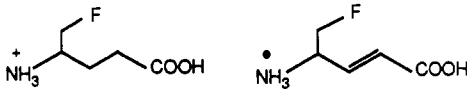


[a] Pyr represents the pyridine nucleus of PLP.

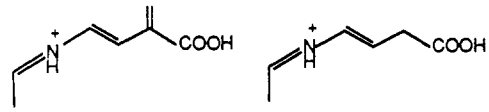
vation mechanism was suggested. This observation is intriguing because we have reported that the closely related analogues 4-amino-2-(fluoromethyl)but-2-enoic acid (**2**) (Silverman et al., 1986a) and 4-amino-3-fluorobutanoic acid (**3**) (Silverman & Levy, 1981a) are substrates, not inactivators, of GABA aminotransferase. (*S*)-4-Amino-5-fluoropentanoic acid (**4**) (Silverman & Levy, 1980, 1981b; Silverman & Invergo, 1986) and (*SE*)-4-amino-5-fluoropent-2-enoic acid (**5**) (Silverman et al., 1986b), however, are inactivators of GABA aminotransferase. Inactivation occurs *every* turnover with **4** and, on the average, once every five turnovers with **5**. The enzyme catalyzes an abnormal reaction, namely, elimination of HF from all four of these compounds (**2**–**5**). It was originally proposed that the mechanism for transformation of all of these compounds involved initial Schiff base formation with the PLP followed by enzyme-catalyzed γ-proton removal and elimination of fluoride ion to produce the PLP-bound reactive Michael acceptors **6**–**9**, respectively (Pyr represents the pyridine ring of PLP). Since only compounds **4** and **5** lead to inactivation, it was suggested (Silverman et al., 1986a; Silverman & Levy, 1981a,b) that a nucleophile in the active site was positioned near the γ-position, but not vicinal to the β- or α-positions of the enzyme-bound GABA backbone. Recently, however, we reported (Silverman & Invergo, 1986) that the mechanism for inactivation of GABA aminotransferase by **4** does not involve an elimination–Michael addition mechanism; rather, an enamine mechanism was proposed identical with that suggested by Metzler and co-workers for the inactivation of glutamate decarboxylase (Likos et al., 1982) and aspartate aminotransferase (Ueno et al., 1982) by serine *O*-sulfate and by Walsh and co-workers (Badet et al., 1984; Roise et al., 1984) for the inactivation of alanine racemase by fluoroalanine. All of these inactivation mechanisms involve the formation of an enamine intermediate (**10**) generated by transimination of the elimination product by a lysine residue as shown in Scheme I. Compound **1**, however, can be activated at the α-position by enzyme-catalyzed γ-proton removal and isomerization as in the normal catalytic mechanism; elimination of HF in this case is not possible. In this paper we show that **1** is a potent inactivator of GABA aminotransferase and provide support for an inactivation mechanism that involves isomerization–Michael addition rather than the enamine mechanism.



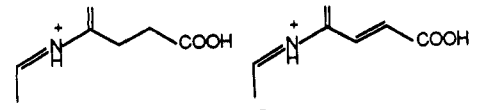
2 **3**



4 **5**



6 **7**



8 **9**

Materials and Methods

Analytical Methods. GABA aminotransferase assays were carried out on a Perkin-Elmer Lambda 1 UV/vis spectrophotometer. Radioactivity was measured in a Beckman LS-3100 scintillation counter with 10 mL of Ready-Solv MP from Beckman or 3a70B complete counting cocktail from Research Products International. [U-^{3}H]Toluene, obtained from New England Nuclear, was used as the internal standard. Protein concentrations were estimated with the use of the BCA protein assay reagent (Pierce Chemical Co.) with bovine serine albumin as standard. High-performance liquid chromatography (HPLC) was performed on a Beckman 330 HPLC with a Beckman Model 421A microprocessor controller and a Beckman 163 variable-wavelength detector at 254-nm wavelength with an Alltech Econosil 10-μm C_{18} silica gel column.

Reagents. PLP, PMP, pyridoxal, bovine serum albumin, β-mercaptoethanol, and α-ketoglutarate were purchased from Sigma Chemical Co. Trichloroacetic acid was bought from Mallinckrodt; HPLC water was from American Scientific Products.

(*Z*)-4-Amino-2-fluorobut-2-enoic acid was synthesized by the method of Bergmann and Cohen (1965) except that deprotection of the corresponding phthalimide compound was carried out by the method of Osby et al (1984); mp 190 °C dec (lit. mp 190 °C dec; ^{1}H NMR (D_2O) δ 3.9 (dd, 2 H), 6.2 (dt, J_{HF} = 33 Hz, 1 H); exact mass calcd for $C_4H_7NO_2F$ (M + 1) 120.04608, found 120.0464 (2.6 ppm). High-resolution mass spectral determinations were carried out at the Midwest Center for Mass Spectrometry, an NSF Regional Instrumentation Facility (Grant CHE-8211164). The preparation of [4-^{3}H]PLP and the reconstitution of apo-GABA aminotransferase with it were carried out as previously reported (Silverman & Invergo, 1986).

Enzymes and Assays. GABA aminotransferase was purified to homogeneity (sp act. 3.8 μmol min^{-1} mg^{-1}) from pig brain by the method of Churchich and Moses (1981) except that the order of the anion- and cation-exchange columns was reversed. Enzyme activity was measured as described previously (Silverman & Levy, 1981b). GABA aminotransferase was added to a solution containing 11 mM GABA, 5.3 mM α-ketoglutarate, 1.1 mM $NADP^+$, 5 mM β-mercaptoethanol, and excess succinic semialdehyde dehydrogenase in 50 mM potassium pyrophosphate buffer, pH 8.5. The change in absorbance at 340 nm with time as a result of the conversion of $NADP^+$ to NADPH was recorded at 25 °C.

Time-Dependent Enzyme Inactivation. In a typical experiment, purified pig brain GABA aminotransferase (6.8 μg)

[1] Abbreviations: PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; GABA, γ-aminobutyric acid.

was incubated at 25 °C in 0.5 mL of 50 mM potassium pyrophosphate buffer (pH 8.5) containing 5 mM β-mercaptoethanol, 2 mM α-ketoglutarate, and varying concentrations of (*Z*)-4-amino-5-fluorobut-2-enoic acid (40–300 μM). At predetermined time intervals the incubation was stopped by diluting a 50-μL aliquot of this solution to 0.575 mL in a UV semimicrocuvette with a solution containing all of the reagents needed for the spectrometric assay. Activity was determined by the increase in absorbance at 340 nm.

GABA Aminotransferase Catalyzed Release of Fluoride Ion from (Z)-4-Amino-2-fluorobut-2-enoic Acid. Varying concentrations of pig brain GABA aminotransferase (0.68–7.5 μg) were incubated at 25 °C with 0.5 mM (*Z*)-4-amino-2-fluorobut-2-enoic acid in a total volume of 600 μL of 50 mM potassium pyrophosphate buffer (pH 8.5) containing 5 mM β-mercaptoethanol and 2.1 mM α-ketoglutarate. A control also was run in which enzyme was excluded. After 5 h of incubation, a 550-μL aliquot of each sample was removed and added to an equal volume of total ionic strength adjusting buffer (57 mL of glacial acetic acid, 58 g of NaCl, and 0.3 g of sodium citrate diluted to 500 mL with H_2O; pH 5.25) in a plastic vial, and the fluoride ion concentration was measured with a fluoride ion specific electrode (Orion Model 96-09).

GABA Aminotransferase Catalyzed Transamination of (Z)-4-Amino-2-fluorobut-2-enoic Acid. Varying concentrations of pig brain GABA aminotransferase (0.68–7.5 μg) were incubated at 25 °C with 1.5 mM (*Z*)-4-amino-2-fluorobut-2-enoic acid in 50 mM potassium pyrophosphate buffer (pH 8.5) containing 5 mM β-mercaptoethanol and 5 mM [U-^{14}C]-α-ketoglutarate (Amersham; sp act. 0.031 mCi/mmol) in a total volume of 200 μL for 8 h. A sample containing no enzyme and one containing no inactivator served as controls. The enzyme reactions and controls were quenched with 20% (w/w) aqueous trichloroacetic acid (20 μL) and then applied to a column (0.6 × 5.5 cm) of Dowex 50-X8 (H^+ form). The column was washed with 3.5 mL of water and eluted with 2 N aqueous ammonia (5.5 mL). The last 4.5 mL of the ammonia eluate was found to contain all the [U-^{14}C]glutamate. Scintillation fluid (10 mL) (Ready-Solv MP from Beckman) was added to the glutamate-containing eluate, and radioactivity was measured in a Beckman LS-3100 scintillation counter. [U-^{14}C]Toluene (New England Nuclear) was used as an internal standard.

Titration of Pig Brain GABA Aminotransferase with (Z)-4-Amino-2-fluorobut-2-enoic Acid. GABA aminotransferase (17 μg) was incubated at 25 °C in 50 mM potassium pyrophosphate buffer (pH 8.5) containing 5 mM β-mercaptoethanol, 12.5 mM α-ketoglutarate, and amounts of (*Z*)-4-amino-2-fluorobut-2-enoic acid varying from approximately 30 to 880 times the enzyme concentration, until no further change in GABA aminotransferase activity was observed (approximately 5 h). The incubation mixtures were transferred into separate dialysis bags, and each was dialyzed against 500 mL of 50 mM potassium pyrophosphate buffer (pH 8.5) overnight at 4 °C. The percentage of enzyme activity and the protein concentration were determined for each dialyzed sample; the percentage of enzyme activity was normalized to the corresponding protein concentration.

Inactivation of [^{3}H]GABA Aminotransferase by 4-Amino-2-fluorobut-2-enoic Acid. [^{3}H]GABA aminotransferase (Silverman & Invergo, 1986) (55 μg) was incubated at 25 °C in 100 mM potassium buffer containing 14.8 mM α-ketoglutarate, 1.56 mM β-mercaptoethanol, and 11.5 mM 4-amino-2-fluorobut-2-enoic acid at pH 7.4. Two controls were run: one was identical except that the inactivator was omitted; the other was identical except that the enzyme was pretreated with GABA and α-ketoglutarate was omitted. After 5 h, enzyme activity in the control experiments was still 100%, whereas the enzyme in the inactivation experiment was completely inactive. The three samples were kept in the refrigerator overnight; then, the pH values of the solutions were raised to 12 with 0.3 M KOH. This solution was made 10% (w/w) in trichloroacetic acid by the addition of solid trichloroacetic acid. The mixture was then centrifuged in the Beckman Microfuge B (9000*g*), and the supernatant was separated from the pellet, which was washed 3 times (100 μL each) with 10% (w/w) trichloroacetic acid in water. The combined supernatants were bulb-to-bulb distilled, and the solid residue was dissolved in deionized water. To the protein pellet was added 500 μL of 2 N KOH, and then it was kept overnight. The digested protein was diluted with 10 mL of scintillation fluid and counted for radioactivity content.

HPLC Analysis of Tritiated Products Released. The residue obtained above was analyzed by HPLC with PLP and PMP as standards, which were pretreated in such a way as to mimick the conditions of the above experiment as follows: each compound (1 mg) was dissolved in water (1 mL), the pH was raised to 12 with potassium hydroxide, and then solid trichloroacetic acid was added to 10% (w/w). An aliquot of each standard was added to the aqueous solution of the residue from the above experiment, and HPLC was performed with 67 mM potassium monobasic phosphate titrated to pH 2.6 as solvent. With a flow rate of 0.5 mL/min, PMP and PLP were eluted at T_R = 12 min and T_R = 16 min, respectively. Fractions of 1 mL were collected and mixed with 10 mL of scintillation fluid, and radioactivity measurements were done.

Results and Discussion

As is apparent from the *trans*-HF coupling constant (33 Hz) in the NMR spectrum, the 4-amino-2-fluorobut-2-enoic acid (**1**) used in this study has the *Z* configuration.

Inactivation of GABA aminotransferase by **1** is time- and concentration-dependent, but not pseudo first order. The K_I and k_{inact} values, as determined by the method of Kitz and Wilson (1962), are 0.22 mM and 0.21 min^{-1}. Dialysis does not regenerate enzyme activity. Inactivation is inhibited by the presence of GABA, suggesting that an active site reaction takes place. Since β-mercaptoethanol is routinely included in all of the buffers, the inactivation does not result from return to the active site of a released reactive species generated from **1**. Rather, this is a mechanism-based enzyme inactivation (Silverman & Hoffman, 1984; Silverman, 1988).

During enzyme inactivation by **1**, fluoride ions are released. The average of six determinations with varying amounts of enzyme was 708 ± 79 fluoride ions released per enzyme molecule. Since the enzyme is dimeric with two active sites, about 350 fluoride ions are released per active site labeled. Transamination, as measured by the conversion of [U-^{14}C]-α-ketoglutarate to [U-^{14}C]glutamate, occurs 737 ± 15 times prior to the loss of each enzyme molecule. In order to determine if release of fluoride ion and transamination are separate phenomena or if they both occur with every molecule, a turnover number was determined by titration of the enzyme with **1**. As shown in Figure 1, an average of about 770 molecules of **1** is consumed per enzyme inactivation event (or 350–400 per active site). This result indicates that fluoride ion release and transamination occur concurrently, and a mechanism consistent with that is shown in Scheme II. According to this mechanism, for every active site nucleophilic attack (pathway b) there are approximately 350–400 hydro-

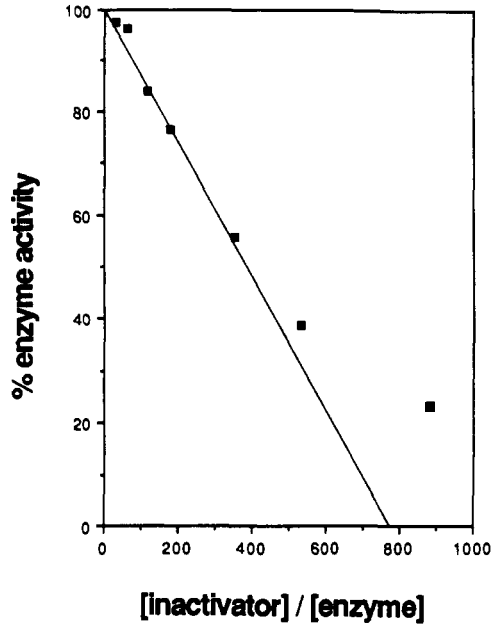
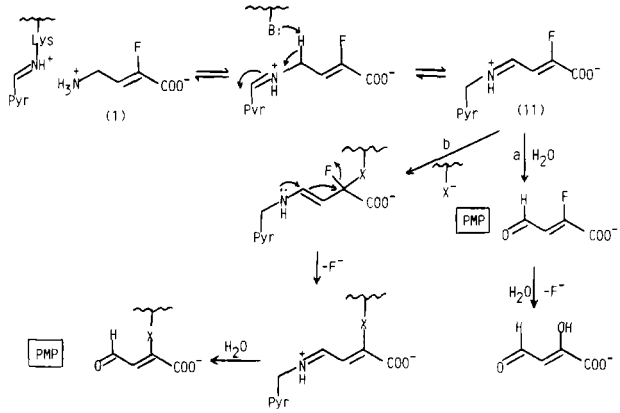


FIGURE 1: Titration of GABA aminotransferase with varying amounts of (*Z*)-4-amino-2-fluorobut-2-enoic acid. See Materials and Methods for the procedure.

Scheme II: Proposed Mechanism for Inactivation of GABA Aminotransferase by (*Z*)-4-Amino-2-fluorobut-2-enoic Acid[a]

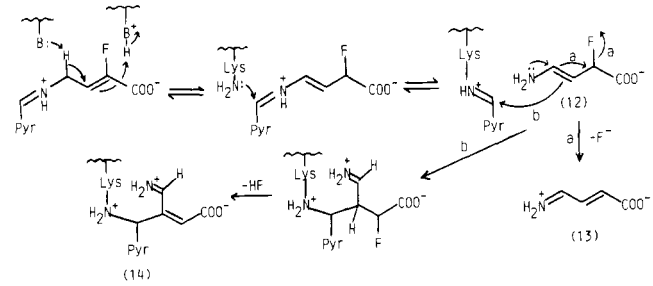


[a] Pyr represents the pyridine nucleus of PLP.

lytic reactions (pathway a). In Scheme II, Schiff base hydrolysis is shown prior to fluoride ion displacement by water; however, these two steps could be reversed. In either case, each turnover gives transamination with release of a fluoride ion, consistent with the results obtained. It is not clear why the higher concentration data points in Scheme II deviate from linearity, but it could be the result of product inhibition (Silverman, 1988).

The alternative enamine mechanism (Likos et al., 1982; Ueno et al., 1982) (Scheme III) is much less attactive for several reasons. The enzyme is configured for azallylic isomerization as shown in Scheme II; it is unclear what would be the driving force for the allylic isomerization shown in Scheme III. Furthermore, if enamine **12** were generated, it should undergo facile elimination of F^- (pathway a) to **13** rather than carbanionic attack of enzyme-bound PLP as shown in pathway b. Third, intermediate **12** is the fluoro analogue of the enamine that would be generated from **7**, which is derived from **3**. However, **3** is not an inactivator of GABA aminotransferase, and therefore, it is not clear why **12** should react any differently. In order to determine the irrelevance of the enamine mechanism shown in Scheme III, apo-GABA aminotransferase was reconstituted with [^{3}H]PLP (Silverman & Invergo, 1986)

Scheme III: Enamine Mechanism for Inactivation of GABA Aminotransferase by (*Z*)-4-Amino-2-fluorobut-2-enoic Acid[a]



[a] Pyr represents the pyridine nucleus of PLP.

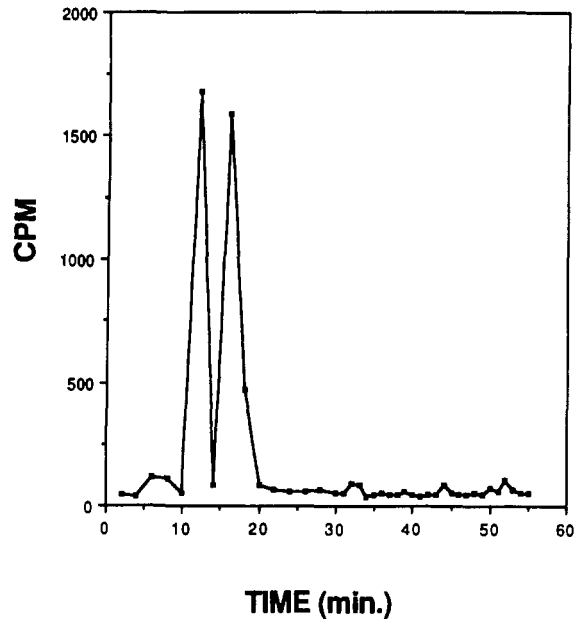


FIGURE 2: HPLC profile of the supernatant obtained by acid denaturation of [^{3}H]GABA aminotransferase inactivated by (*Z*)-4-amino-2-fluorobut-2-enoic acid. See Materials and Methods for the procedure.

and incubated with **1** until complete inactivation occurred. After the pH of the inactivated enzyme was raised to 12 and the enzyme was acid denatured, 99.9% of the radioactivity was released from the protein as a 45:55 mixture of [^{3}H]PMP and [^{3}H]PLP, monitored by HPLC (Figure 2). The [^{3}H]PLP was shown to be derived from inactive enzyme. An enzyme control, treated the same except without the addition of **1**, gave [^{3}H]PLP exclusively. Two controls were carried out to determine the genesis of the observed [^{3}H]PLP in this experiment. First, [^{3}H]PLP-reconstituted GABA aminotransferase was incubated with GABA to convert it to the [^{3}H]PMP form. Then it was treated under the same conditions as was the inactivated enzyme. The released radioactivity was identified as a 47:53 mixture of [^{3}H]PMP and [^{3}H]PLP. Since PMP under the identical conditions gives no PLP, it is concluded that only half of the ^{3}H-labeled enzyme used in the experiment was active and the observed [^{3}H]PLP was derived from the inactive enzyme. If the enamine mechanism (Scheme III) were relevant, no [^{3}H]PMP would be observed. Furthermore, inactivation by the enamine mechanism should produce an adduct (**14**) that was irreversibly attached to both the enzyme and the cofactor, and no tritium would have been released except possibly as ^3H_2O. The amount of volatile tritium obtained by bulb-to-bulb distillation, however, was the same as that obtained in the noninactivated enzyme control. All of these results, then, support the isomerization–Michael addition mechanism shown in Scheme II.

Compound **1** is the fluoro analogue of (*Z*)-4-aminobut-2-enoic acid, an excellent substrate for GABA aminotransferase (Beart & Johnston, 1973). The rationalization for the reactivity of **1** relative to the non-fluorinated analogue as described by Scheme II may be derived from the relative reactivities of β,β-dichloro- and β,β,β-trifluoroalanine toward various PLP-dependent enzymes compared with those of β-chloro- and β-fluoroalanine, respectively (Silverman & Abeles, 1976). The proposed intermediate **11** (Scheme II), containing a fluoride at the β-position of a potential Michael acceptor, is more electrophilic than the corresponding Michael acceptor without the fluorine [i.e., the intermediate generated from (*Z*)-4-aminobut-2-enoic acid]. This increased electrophilicity may be responsible for the observed inactivation reaction with **1**. Another explanation is that when the active site nucleophile attacks **11**, fluoride ion is released to form a stable adduct. When there is no fluorine, nucleophilic attack may be reversible, in which case no inactivation would result.

The presence or absence of the 2,3 double bond does not affect the inactivation mechanism as evidenced by the fact that **5** also inactivates GABA aminotransferase by the enamine mechanism (Silverman & George, 1988). Therefore, the 2,3 double bond in **1** is not responsible for the change in mechanism of **1** relative to **4**.

To date, we have investigated five different fluorine-containing GABA analogues (**1–5**); fluoride ion is released from all five during turnover. However, enzyme-catalyzed release of fluoride ion from compounds **2–5** appears to occur by initial γ-proton abstraction of these PLP-bound compounds followed by fluoride ion elimination. Compounds **2** (Silverman et al., 1986a) and **3** (Silverman & Levy, 1981a) produce intermediates (presumably **6** and **7**, respectively) that do not produce covalent bonds to the enzyme; compounds **4** (Silverman & Levy, 1981b; Silverman & Invergo, 1986) and **5** (Silverman et al., 1986b; Silverman & George, 1988) inactivate the enzyme. As shown here, the mechanism of inactivation of GABA aminotransferase by **1** differs from that by **4** (Silverman & Invergo, 1986) and **5** (Silverman & George, 1988), which proceed by the enamine inactivation mechanism (Likos et al., 1982; Ueno et al., 1982). If this small set of data can be extrapolated to a generality, it appears that azallylic isomerization (i.e., the normal catalytic process) occurs with substrates unless a leaving group is positioned in the molecule so that elimination is possible (e.g., with compounds **2–5**). When the latter pathway is followed, transimination to the active site lysine residue then occurs (i.e., the normal catalytic process) to produce the enamine. Whether or not the enamine product generated is capable of reattachment to the enzyme and produces inactivation prior to release, however, would depend upon various structural and binding constraints of the enamine and would not depend upon the geometry of the enzyme active site nucleophiles. In the cases of **2** and **3**, the enamines generated from intermediates **6** and **7**, respectively, presumably are not juxtaposed properly for carbon–carbon bond formation and, therefore, are exclusively released from the active site and hydrolyzed. The enamine from **4** must be set up in the ideal position, because it is never released (Silverman & Levy, 1981b), whereas the addition of a 2,3 double bond (i.e., compound **5**) alters the geometry sufficiently to allow four out of five enamine molecules to be released and one to become reattached to the cofactor (Silverman et al., 1986b). Since compound **1** cannot undergo HF elimination, it proceeds by the usual substrate mechanism. However, the isomerization product (**11**, Scheme II) is susceptible to nucleophilic attack, and in one out of 770 turnovers this occurs prior to the normal catalytic hydrolysis (Scheme II, pathway a).

This hypothesis, which may be relevant to PLP-dependent aminotransferases in general, predicts that ethanolamine *O*-sulfate inactivates GABA aminotransferase (Fowler & John, 1981) by the enamine mechanism, whereas 4-aminohex-5-enoic acid (Lippert et al, 1977) and 4-aminohex-5-ynoic acid (Jung & Metcalf, 1976) inactivate this enzyme by isomerization–Michael addition mechanisms. These predictions are currently under investigation.

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