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Mechanism of Inactivation of γ-Aminobutyric Acid–α-Ketoglutaric Acid Aminotransferase by 4-Amino-5-halopentanoic Acids†

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ABSTRACT: (*S*)-4-Amino-5-halopentanoic acids were previously shown to irreversibly inhibit pig brain γ-aminobutyric acid–α-ketoglutaric acid aminotransferase, and a mechanism for the inactivation was proposed (Silverman, R. B., & Levy, M. A. (1980) *Biochem. Biophys. Res. Commun. 95*, 250). Evidence is presented to show that these compounds are mechanism-based inactivators, and experiments are described to elucidate their mechanism of action. The enzyme must be in the pyridoxal phosphate form for inactivation to occur, the γ proton of the inactivators is removed in a rate-determining step, and one fluoride ion is released from 4-amino-5-fluoropentanoic acid per active site labeled. The change in the optical spectrum of the enzyme during inactivation suggests that the coenzyme is converted into the pyridoxamine phosphate form. Every turnover of inactivator leads to an inactivation event, i.e., the partition ratio is zero.

It is well established that γ-aminobutyric acid (GABA)[1] is a major CNS inhibitory neurotransmitter and that the brain level of GABA is important in CNS dysfunctions such as seizures (Krnjević & Schwartz, 1967; Obata & Takeda, 1969; Baxter, 1970; Mandel & DeFeudis, 1979). The brain concentration of GABA is principally controlled by two PLP-dependent enzymes, glutamate decarboxylase, which catalyzes the biosynthesis of GABA, and GABA-T, which is responsible for its catabolism. Since GABA is not transported across the blood–brain barrier, many compounds which inhibit GABA-T have been synthesized in order to increase the brain concentration of GABA for use as potential anticonvulsant agents (Mandel & DeFeudis, 1979; Roberts et al., 1976). Numerous compounds have been shown to be irreversible inhibitors of GABA-T both in vitro and in vivo, e.g., 4-aminohex-5-ynoic acid (Jung & Metcalf, 1975), 4-aminohex-5-enoic acid (Lippert et al., 1977), gabaculine (Rando & Bangerter, 1977), isogabaculine (Metcalf & Jung, 1979), and ethanolamine *O*-sulfate (Fletcher & Fowler, 1980). Concurrent with the

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[1] Abbreviations used: GABA, γ-aminobutyric acid; GABA-T, γ-aminobutyric acid–α-ketoglutaric acid aminotransferase (EC 2.6.1.19); PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; AFPA, 4-amino-5-fluoropentanoic acid; ACPA, 4-amino-5-chloropentanoic acid; ABPA, 4-amino-5-bromopentanoic acid; α-KG, α-ketoglutarate; SSADH, succinic semialdehyde–NADP oxidoreductase (EC 1.2.1.16); NADP, nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane; Gabase, a commercial preparation from *Pseudomonas fluoroescens* of GABA-T and SSADH.

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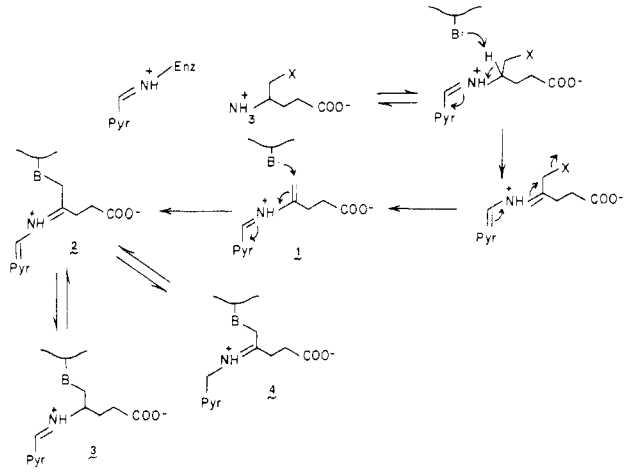
Scheme I



in vivo inactivation of GABA-T, there is an increase in the GABA brain levels and a protection against specific chemically—and audiogenically—induced seizures (Mandel & DeFeudis, 1979).

Recently we reported that the 4-amino-5-halopentanoic acids were competitive, active-site-directed, irreversible inhibitors of pig brain GABA-T (Silverman & Levy, 1980a,b). Incubation of the enzyme with (*S*)-[U-^{14}C]ACPA led to the incorporation of 1.7 mol of inactivator/mol of enzyme. Since the enzyme is a dimer containing two identical subunits, each having a PLP associated with it (Beeler & Churchich, 1978), approximately 1 mol of inactivator is incorporated per active site of enzyme. Denaturation of the labeled protein with urea did not cause release of radioactivity from the protein. Scheme I depicts the mechanism which we proposed for this irreversible inactivation (Silverman & Levy, 1980b). We report in this paper our detailed studies on the mechanism of the inactivation of GABA-T by (*S*)-4-amino-5-halopentanoic acids.

Materials and Methods

Reagents. The unlabeled inhibitors, (*S*)-4-amino-5-halopentanoic acids, were stereospecifically synthesized as previously described (Silverman & Levy, 1980a). (*S*)-[U-^{14}C]-ACPA (1.02 mCi/mmol) was prepared from L-[U-^{14}C]glutamic acid (250 μCi, Research Products International) diluted with 0.25 mmol of unlabeled L-glutamate. *N*-Acetyl-DL-[2-^{2}H]glutamic acid was prepared from *N*-acetyl-L-glutamate by the method of Upson & Hruby (1977). The DL-[2-^{2}H]glutamic acid that subsequently was obtained (Greenstein & Winitz, 1961) was used to synthesize (*RS*)-[4-^{2}H]AFPA. L-[2-^{2}H]Glutamic acid [$[\alpha]^{23}_{D}$ 31.9° (*c* 1.0, 1 N HCl)] was obtained by hydrolysis of the labeled *N*-acetylglutamate with Acylase (I) (Fang et al., 1970) and was used to make (*S*)-[4-^{2}H]ACPA. Complete (>95%) incorporation of deuterium into the α position of *N*-acetylglutamate and glutamate was determined by NMR. All other substrates and chemicals were purchased from Sigma Chemical Co.

Enzymes and Assays. Gabase was purchased from Sigma Chemical Co. and Boehringer Mannheim Biochemicals. Acylase (I) was purchased from Sigma Chemical Co. Pig brains were obtained immediately after excision from a local abattoir and kept in ice for 1 h before processing. Pig brain GABA-T was purified according to the method of John & Fowler (1976) for the rabbit brain enzyme. This enzyme preparation showed one band on NaDodSO$_4$–gel electrophoresis at pH 7.0 (Weber & Osborn, 1969) and had a specific activity of 7.5 units/mg of protein. One unit is defined as the amount of enzyme which catalyzes the transamination of 1 μmol of GABA/min at 25 °C. GABA-T activity was determined by using a modified version of a literature procedure (Scott & Jakoby, 1958). The final concentrations in the assay solution were 10 mM GABA, 5 mM α-KG, 1 mM NADP, 5 mM β-mercaptoethanol, and excess SSADH in 50 mM potassium pyrophosphate buffer, pH 8.5. Enzyme activity was determined by observing the change in absorbance at 340 nm on a Gilford 222 spectrophotometer at 25 °C after several minutes of incubation. SSADH was prepared from Gabase by inactivating the GABA-T with (*S*)-AFPA followed by exhaustive dialysis at 4 °C against 37.5 mM potassium phosphate, pH 7.2, containing 12.5% glycerol. This preparation could be stored for several months at −80 °C with no loss of SSADH activity and no gain of GABA-T activity.

Analytical Procedures. Absorption spectra were recorded on a Cary 14 spectrophotometer fitted with a 0–0.100 absorbance range slide wire. Radioactivity was measured in a Beckman LS-3100 scintillation counter using 10 mL of 3a70B scintillation fluid from Research Products International. [U-^{14}C]Toluene (4.7×10^5 dpm/mL), obtained from New England Nuclear, was used as an internal standard. Protein concentrations were estimated by the commercially available Bio-Rad method with bovine serum albumin as a standard. Fluoride ion was determined with an Orion Model 96-09 combination fluoride electrode on an Orion 701-A pH meter and was calibrated with sodium fluoride (0.1 M, Orion 94-09-06). All other analytical procedures have been previously described (Silverman & Levy, 1980a,b).

Syntheses of Labeled Inactivators. (*RS*)-[4-^{2}H]AFPA, (*S*)-[4-^{2}H]ACPA, and (*S*)-[U-^{14}C]ACPA were prepared by adapting our previously reported procedures to small-scaled syntheses.

(S)-[4-^{2}H]ACPA. A suspension of 450 mg (2.4 mmol) of L-[2-^{2}H]glutamic acid in absolute ethanol (15 mL) was treated with 1.2 mL (16.5 mmol) of thionyl chloride at 0 °C. After the resulting solution was stirred at room temperature for 30 h, the solvent was removed by atmospheric distillation under a flow of Ar and then 35 mL of dry chloroform was added. Dry NH_3 was bubbled through the solution, and the resulting NH_4Cl was removed by filtration through Celite. Following the removal of the solvent by atmospheric distillation, 40 mL of toluene was added and the solution was heated at reflux for 18 h. The solvent was removed by distillation and the residue was dried in vacuo to give 390 mg (quantitative yield) of (*S*)-5-carbethoxy-2-[5-^{2}H]pyrrolidinone. To 390 mg (2.4 mmol) of this yellow oil in 10 mL of dry THF was added a solution of lithium borohydride (95 mg, 4.36 mmol) in 3 mL of dry THF. The solution was allowed to warm to room temperature and was stirred under Ar for 24 h. The heterogeneous solution was quenched by the *slow* addition of 5 mL of 20% acetic acid at 0 °C and concentrated in vacuo. The residue was applied to 10 mL (17.5 mequiv) of Dowex 50-X8 (hydrogen form) and rinsed with H_2O. The acidic aqueous fractions were concentrated in vacuo and reevaporated from absolute ethanol (3 times) to remove the boric acid. The residue was dried in vacuo to give 276 mg (quantitative yield) of (*S*)-5-(hydroxymethyl)-2-[5-^{2}H]pyrrolidinone as a white solid. The alcohol (270 mg, 2.3 mmol) was chlorinated with triphenylphosphine (906 mg, 3.45 mmol) and carbon tetrachloride (3 mL) with chloroform (10 mL) as a cosolvent. After the mixture was stirred at ambient temperature for 20 h, the solvent was evaporated and 20 mL each of H_2O and hexane was added. The solid that formed upon vigorous agitation was removed by filtration. The layers were separated, and the aqueous phase was concentrated to an oil–solid mixture.

One-half of the crude (*S*)-5-(chloromethyl)-2-[5-^{2}H]-pyrrolidinone was hydrolyzed in 20 mL of refluxing 1 N HCl for 3.5 h. The solvent was evaporated and the residue applied to 25 mL (42.5 mequiv) of Dowex 50 (H^+). The resin was rinsed with H_2O until neutral, and then the compound was eluted with 2 N HCl. The fractions that gave a positive ninhydrin test were pooled and concentrated in vacuo at less than 30 °C. The residue was reevaporated from acetic acid and the resulting solid was recrystallized from acetic acid–ethyl acetate to give 88 mg (39%) of white, fluffy (*S*)-[4-^{2}H]ACPA, $[\alpha]^{23}_D$ 19.1° (*c* 2.5, 2 N HCl); mp 125–126.5 °C. Spectral data and physical constants of all of the intermediates and of the final product were in agreement with their structures. Complete retention of deuterium into the γ position of ACPA was determined by NMR.

(S)-[U-^{14}C]ACPA. (*S*)-[U-^{14}C]ACPA was synthesized from 36.8 mg (0.25 mmol) of L-[U-^{14}C]glutamic acid (1.02 mCi/mmol) in an overall yield of 32% (15.1 mg) by the same method used for the deuterated compound. The purity was determined to be greater than 99% by TLC (cellulose, 1-butanol–H_2O–acetic acid 12:5:3, and 1-butanol–pyridine–H_2O 1:1:1) and high-voltage electrophoresis (pH 1.9).

(RS)-[4-^{2}H]AFPA. The synthesis of (*RS*)-[4-^{2}H]AFPA, which began with DL-[2-^{2}H]glutamic acid (720 mg, 3.9 mmol), was analogous to that of (*S*)-[4-^{2}H]ACPA through its reduction to (*S*)-5-(hydroxymethyl)-2-[5-^{2}H]pyrrolidinone. The alcohol (125 mg, 1.05 mmol) was then brominated with triphenylphosphine (568 mg, 2.16 mmol) and carbon tetrabromide (716 mg, 2.16 mmol) in a total volume of 15 mL of dry acetonitrile. After the mixture was stirred at ambient temperature for 12 h, the solvent was evaporated and 20 mL each of H_2O and hexane was added. The solid which formed after vigorous agitation was removed by filtration and the aqueous phase was evaporated to a solid–oil mixture. This residue was chromatographed on a column (0.7 × 15 cm) of Florisil (5 g), with chloroform elution. Fractions 6–16 (5 mL each) contained 72 mg of product (detected by NMR). To the (*RS*)-5-(bromomethyl)-2-[5-^{2}H]pyrrolidinone in 10 mL of dry acetonitrile was added 248 mg (1.96 mmol) of silver(I) fluoride. The mixture was stirred overnight in the absence of light. The solution was filtered through Celite, concentrated to a brown oil, dissolved in chloroform (10 mL), and refiltered. The oil obtained after solvent evaporation was hydrolyzed in refluxing 1 N HCl for 4 h. Purification of (*RS*)-[4-^{2}H]AFPA was accomplished by chromatography on Dowex 50 (H^+) with H_2O and 1 N HCl as eluates. The positive ninhydrin fractions were combined and concentrated in vacuo (<30 °C) to give a yellow solid. A sample of this solid was recrystallized from acetic acid–ethyl acetate to give 7 mg of (*RS*)-[4-^{2}H]AFPA. All spectral data were consistent with the structure of all compounds. Complete retention of deuterium into the γ position of AFPA was determined by NMR.

Enzyme Inactivations. In a typical experiment, purified pig brain GABA-T (0.138 unit) was incubated at 25 °C in 0.4 mL of 50 mM buffer containing 5 mM β-mercaptoethanol and different concentrations of inhibitor and substrates. When the inhibitor concentrations exceeded 5 mM, 1 equiv of potassium hydroxide was added to maintain a constant pH. Except where noted, all incubations were done in potassium pyrophosphate buffer, pH 8.5. The incubation was stopped by diluting a 40-μL aliquot of this mixture to 1 mL in a UV semimicrocuvette with a solution containing all of the reagents necessary for the assay. K_i and k_{cat} values were determined by the method of Kitz & Wilson (1964). A least-squares linear regression program was used to determine the K_i and k_{cat} values from the secondary plots of $t_{1/2}$ vs. the reciprocal of the inhibitor concentration. In all cases, the correlation coefficient (r^2) was greater than 0.99. This procedure was used for both the nonlabeled and the γ-deuterated compounds.

Inactivated enzyme was dialyzed (3 times) at 4–6 °C vs. at least 1250 volumes each of 10–50 mM buffer at a pH range of 5.4–8.5. Samples were withdrawn and assayed for enzyme activity. The control consisted of identical dialyses of native enzyme.

Covalent Labeling of GABA-T by (S)-[U-^{14}C]ACPA. Two types of labeling experiments were performed. The first method consisted of incubating 2.76 units of enzyme at 25 °C in 1 mL of 50 mM pyrophosphate buffer, pH 8.5, containing 5 mM mercaptoethanol and 7.4 mM (7.5 μCi) (*S*)-[U-^{14}C]-ACPA. After complete inactivation, the protein was dialyzed four times, each against 700 volumes of 20 mM acetate buffer, pH 6.0, at 4–6 °C. The final dialyzate was checked for radioactivity and found to be comparable to background. Separate aliquots of the enzyme solution were analyzed for protein and radioactive content. Another aliquot was dialyzed further against 200 mL of 8 M urea at room temperature for a time sufficient to totally denature native enzyme (2 h). Protein and radioactive content were determined on the denatured enzyme.

In the second experiment, 0.86 unit of GABA-T was inactivated at 25 °C in a total volume of 250 μL containing 3.6 mM (1.84 μCi) (*S*)-[U-^{14}C]ACPA. The solution was applied to a Sephadex G-25 column (0.9 × 19 cm) and eluted with 10 mM sodium phosphate buffer, pH 7.0, at 25 °C. The protein fractions were combined, lyophilyzed to dryness, and resuspended in 0.5 mL of H_2O. Protein concentration was determined, and the sample was counted for radioactivity.

Turnover of (S)-[U-^{14}C]ACPA. The following experiment was run in duplicate. GABA-T (0.86 unit) was incubated at 25 °C in a volume of 250 μL of 50 mM pyrophosphate buffer, pH 8.5, containing 5 mM β-mercaptoethanol, 5 mM α-KG, and 9.0 mM (2.29 μCi) of (*S*)-[U-^{14}C]ACPA. At time intervals, 4-μL aliquots were removed and assayed for residual activity. A 200-μL aliquot of the completely inactivated enzyme solution was applied to a Sephadex G-25 column (0.9 × 19 cm) and eluted with 10 mM potassium phosphate buffer, pH 7.0. The fractions containing the small radioactive molecules were combined and concentrated by bulb-to-bulb distillation. The distillate was found to be void of radioactivity. The residue was applied to a column (0.5 × 5 cm) of Dowex 50-X8 (200–400 mesh) in the hydrogen form and rinsed with H_2O. The H_2O elution fractions were combined (3.7 mL) and counted for radioactivity. A control was run simultaneously in which enzyme was omitted.

Release of Fluoride Ion from AFPA Catalyzed by GABA-T. Enzyme (2.10 units) was incubated in a 440-μL solution of buffered (pH 8.5, 25 °C) (*S*)-AFPA for 120 min, at which time there was no enzyme activity remaining. A sample (200 μL) of the inactivated enzyme solution was added to 100 μL of a total ionic strength buffer (57 mL of glacial acetic acid, 58 g of NaCl, and 0.30 g of sodium citrate diluted to 500 mL with H_2O; pH 5.25) for the determination of fluoride ion. The control was identical except that enzyme was omitted. Diluted sodium fluoride solutions were used as calibration standards.

UV–Vis Absorbance Changes upon Inactivation of GABA-T by AFPA. GABA-T was dialyzed, in order to remove excess PLP, for 2 h at 4–6 °C against 2500 volumes of 20 mM sodium acetate, 10 mM HCl, and 1.25 mM β-mercaptoethanol, all of which was adjusted to pH 7.5 with Tris. The UV–vis spectrum of an aliquot (1.38 units) in a total volume

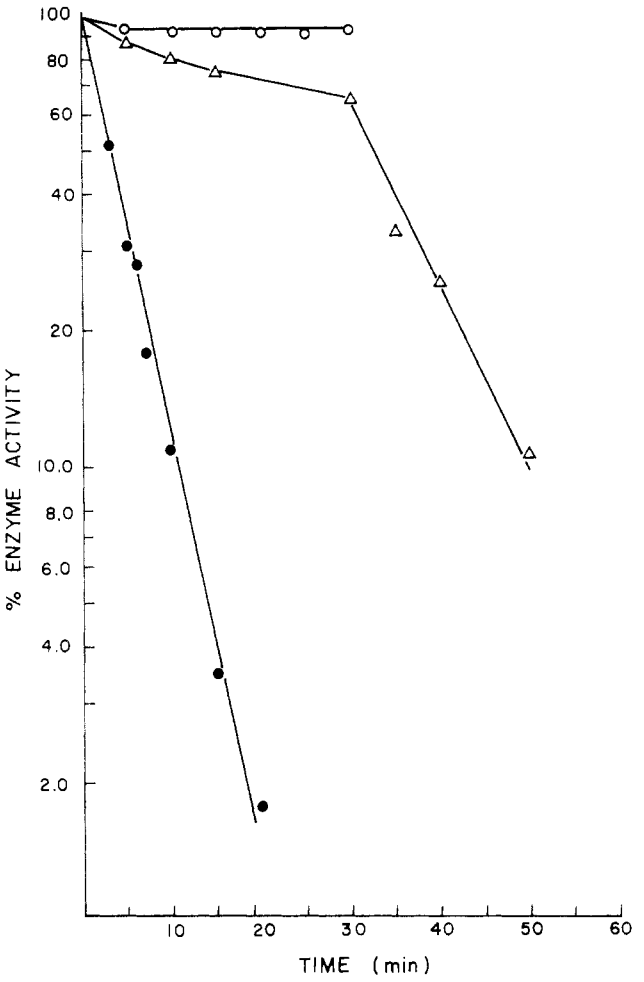


FIGURE 1: Requirement of the PLP form of GABA-T for inactivation to occur. Pig brain GABA-T (0.06 unit) was incubated with (*S*)-AFPA in 0.4 mL of 50 mM potassium pyrophosphate buffer containing 5 mM β-mercaptoethanol, pH 8.5, with (●) 1 mM GABA, 1 mM α-KG, and 0.5 mM (*S*)-AFPA, (△) 5 mM GABA and 0.5 mM (*S*)-AFPA for 30 min at which time α-KG to 1 mM was added, and (○) 0.5 mM (*S*)-AFPA after a preincubation with 1 mM GABA.

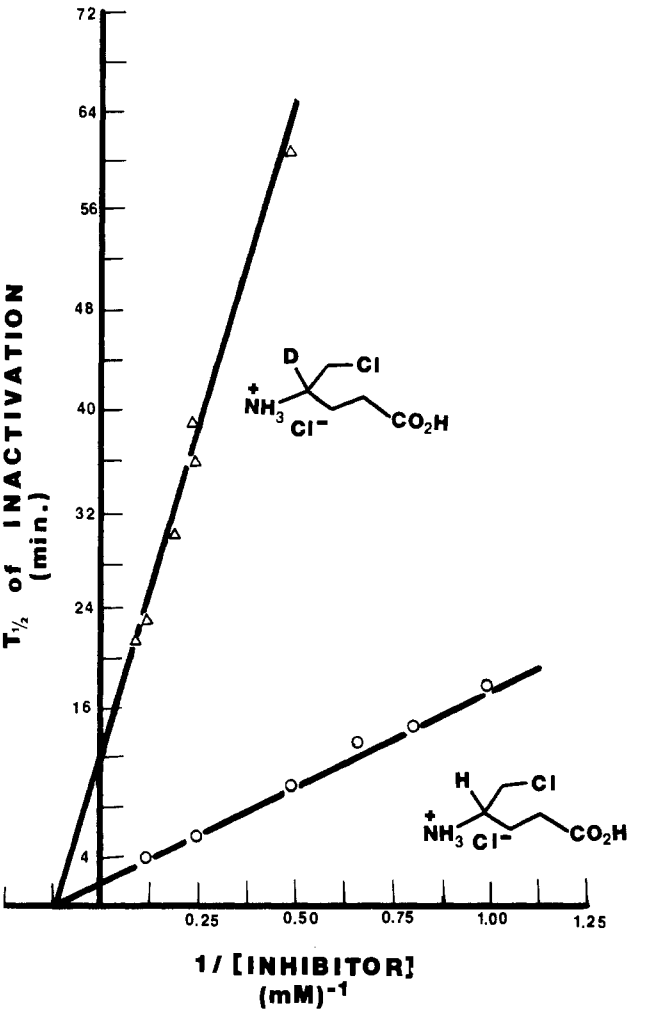


FIGURE 2: Deuterium isotope effect on the rate of inactivation of GABA-T by (*S*)-ACPA and (*S*)-[4-^{2}H]ACPA. GABA-T (0.128 unit) was incubated in 0.4 mL of 50 mM potassium pyrophosphate, pH 8.5, containing 5 mM β-mercaptoethanol with differing concentrations of (*S*)-ACPA and (*S*)-[4-^{2}H]ACPA. From a plot of the log of the remaining activity vs. the length of the incubation, values for the $t_{1/2}$ of inactivation at varying concentrations were obtained for (△), (*S*)-[4-^{2}H]ACPA and (○) (*S*)-ACPA.

of 800 μL of 6.5 mM Tris–acetate buffer, pH 8.0, was recorded. All spectra were recorded from 550 to 300 nm. To this sample was added 10 μL of 100 mM GABA, and several spectra of the conversion of PLP to PMP were recorded over a 1-h period. After 1.5 h, 10 μL of 100 mM α-KG was added to the cuvette, and the spectral changes were again monitored over a 1-h period. Spectra were recorded of dialyzed GABA-T (1.38 units) in 840 μL of a solution that was 62.5 mM in Tris–acetate buffer, pH 8.0, and contained 1.0 μmol of GABA, 1.0 μmol of α-KG, and 0.4 μmol of (*S*)-AFPA. Concurrent with the recording of the spectra at specific time intervals, aliquots were removed and assayed for enzyme activity. The inactive enzyme was dialyzed overnight against 1250 volumes of the same buffer as above, and the spectrum of this enzyme solution was recorded.

Results

Requirement of the Oxidized Form of the Coenzyme for Inactivation. As depicted in Scheme I, we have been able to show that inactivation of GABA-T by the (*S*)-4-amino-5-halopentanoic acids requires that the coenzyme be in the oxidized (PLP) form. The coenzyme was converted into the pyridoxamine phosphate (PMP) form by preincubating the active enzyme with GABA in the absence of α-KG. Treatment of this PMP enzyme with each of the 4-amino-5-halopentanoic acids gave no time-dependent inactivation whereas under the same conditions in the presence of GABA and α-KG the PLP enzyme showed complete time-dependent irreversible inactivation (Figure 1). Likewise, treatment of the PLP enzyme with GABA and one of the 4-amino-5-halopentanoic acids in the absence of α-KG resulted in time-dependent inactivation which gradually diminished. When α-KG was added, the first-order decay in activity resumed (Figure 1). These experiments support the requirement for the coenzyme to be in the PLP form in order for inactivation to occur.

Removal of the γ Proton during Inactivation. Loss of the γ proton during inactivation was monitored by comparing the rates of inactivation of GABA-T by deuterated and nondeuterated ACPA and AFPA. A kinetic deuterium isotope effect (k^H_{cat}/k^D_{cat}) for (*S*)-[4-^{2}H]ACPA of 6.7 was observed (Figure 2). The kinetic isotope effect for (*RS*)-[4-^{2}H]AFPA was 5.5 (data not shown), assuming that only the *S* isomer was involved in the inhibition. No isotope effect in the dissociation constants (K_i) was observed for either compound.

Halide Ion Release during Inactivation. To generate intermediate **1** (Scheme I), the halide ions must be eliminated from the inactivator–PLP complex. When a fluoride ion electrode was used to measure halide ion release from (*S*)-AFPA, it was found that 18.0 μmol of F^- was released after complete inactivation of 7.6 μmol of GABA-T (based on a

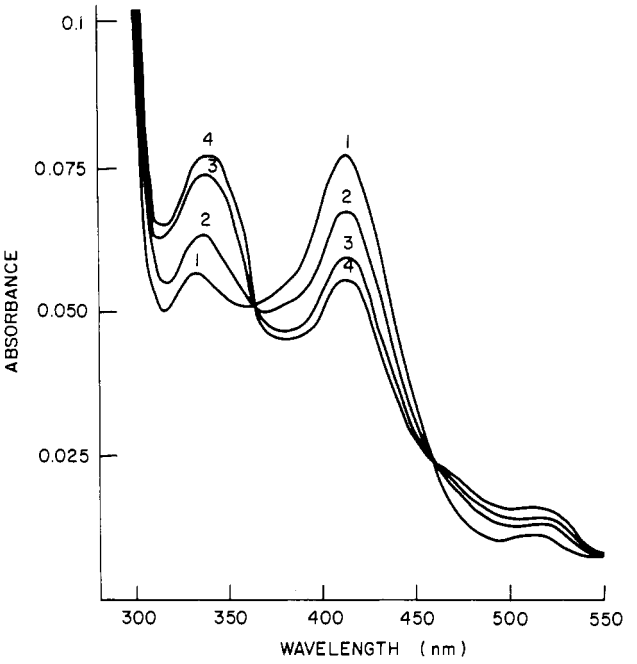


FIGURE 3: Change in the absorbance spectrum of GABA-T upon inactivation by (*S*)-AFPA. Pig brain GABA-T was incubated in the presence of 0.5 mM (*S*)-AFPA, 1.2 mM α-KG, and 1.2 mM GABA with 5 mM β-mercaptoethanol, pH 8.0. Aliquots were removed and assayed for enzyme activity concurrent with spectral recordings: (1) 100%, (2) 70%, (3) 24%, and (4) 9% enzyme activity remaining.

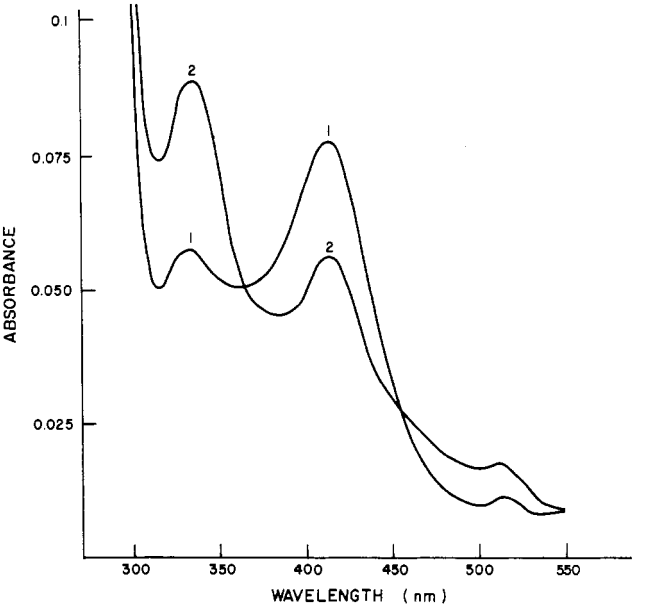


FIGURE 4: Change in the absorbance spectrum of GABA-T upon introduction of substrates. Spectra of pig brain GABA-T in the presence of (1) 1.25 mM GABA and (2) 1.25 mM GABA plus 1.25 mM α-KG were recorded from 550 to 300 nm.

molecular weight of 110000). No fluoride ions were released in the absence of enzyme. This release of 2.4 mol of F^-/mol of enzyme corresponds to approximately 1 mol of F^- released per enzyme active site (Beeler & Churchich, 1978).

Covalent Labeling of Enzyme. Several samples of GABA-T were inactivated with (*S*)-[U-^{14}C]ACPA, then either extensively dialyzed or subjected to gel filtration, and analyzed for incorporation of radioactivity. The amount of labeling varied from 1.7 to 2.5 mol/mol of active dimeric enzyme and averaged 0.98 mol of inactivator/mol of enzyme active site. No enzyme activity was found after dialysis or gel filtration. Following denaturation of the labeled enzyme with 8 M urea and dialysis, >90% of the radioactivity remained associated with the protein.

Does (S)-ACPA Generate Products during Enzyme Inactivation? Several samples of GABA-T were inactivated with (*S*)-[U-^{14}C]ACPA, and the residual small molecules were isolated by gel filtration. The radioactive amines were removed by cation-exchange chromatography. In all cases radioactivity was found in the nonamine washes; however, the same amount of nonamine radioactivity was isolated in nonenzymatic control reactions. Apparently, there is a slow nonenzymatic reaction which leads to nonamine products. The amount of ^{14}C nonamines detected in the absence of enzyme was a direct function of the incubation time at pH 8.5 (~0.08%/h). In addition, approximately 0.2% of the labeled inactivator was converted into nonamines upon storage at −10 °C for several months.

Changes in the GABA-T Optical Spectrum upon Inactivation. Attack of an active site nucleophile on **1** (Scheme I) would lead to the quinoid adduct (**2**) which could incorporate a proton at one of two positions, leading to **3**, the PLP adduct, or **4**, the PMP adduct. When inactivation of the enzyme by (*S*)-AFPA, in the presence of GABA and α-KG, was monitored by UV–vis absorbance, a time-dependent increase at 330 nm, paralleled with a decrease at 412 nm, occurred (Figure 3). This is identical with the spectral changes associated with the conversion of the native PLP enzyme to the PMP enzyme in the presence of GABA. When α-KG was added to the native PMP enzyme, a fast regeneration of the PLP enzyme was observed (Figure 4). The spectrum of the inactivated enzyme was unaltered by extensive dialysis and by treatment with α-KG.

Discussion

Recently we reported that the inactivation of GABA-T by the (*S*)-4-amino-5-halopentanoic acids was time dependent, irreversible, active site directed, and independent of added nucleophiles. In conjunction with these data, the results described in this paper show that the (*S*)-4-amino-5-halopentanoic acids are mechanism-based (suicide) inactivators of GABA-T. A mechanism-based inactivator (Abeles & Maycock, 1976; Walsh, 1977; Rando, 1977) is a relatively unreactive compound which resembles the structure of the substrate or product of the target enzyme. Through its normal catalytic mechanism, the enzyme converts these pseudosubstrates into highly reactive compounds which then form a covalent bond to an active site nucleophile. Scheme I depicts our proposed mechanism of inactivation of GABA-T by (*S*)-4-amino-5-halopentanoic acids. According to this mechanism, there is an initial Schiff base formation of the inactivators with enzyme-bound PLP. Following removal of the γ proton and delocalization of the charge throughout the pyridoxal system, the halide is eliminated, generating reactive intermediate **1**. Michael addition of an active-site nucleophile gives a ternary complex (**2**, **3**, or **4**) of the enzyme, inactivator, and coenzyme. An alternative mechanism could be transamination of the inactivator followed by S_N2 displacement of the halide by an active-site nucleophile. This mechanism, although reasonable with ACPA and ABPA, is unlikely in view of the poor reactivity of alkyl fluorides (Parker, 1963) and fluoropyruvate (Kaczorowski & Walsh, 1975) toward S_N2 reactions. Because of the instability of ABPA (Silverman & Levy, 1980b), evidence for the mechanism only could be carried out with AFPA and ACPA. The chemistry involved suggests that the mechanism of inactivation of GABA-T by ABPA should be the same as for the other two inactivators.

Consistent with the mechanism in Scheme I, we have shown that the enzyme must be in the PLP (aldimine) form, the α

proton is removed in a rate-determining step, approximately one fluoride ion is eliminated from (*S*)-AFPA per active site, and the 1:1 inactivator–enzyme adduct that forms is a ternary complex with the coenzyme in the pyridoxamine phosphate form (**4**). This mechanism is related to that proposed for the inactivation of GABA-T by 4-aminohex-5-ynoic acid (Jung & Metcalf, 1975) and by 4-aminohex-5-enoic acid (Lippert et al., 1977).

The kinetic isotope effects during inactivation of GABA-T were determined by using (*S*)-ACPA, (*S*)-AFPA, (*S*)-[4-^{2}H]ACPA, and (*RS*)-[4-^{2}H]AFPA with the assumption that the *R* isomer exhibits little or no inhibition at the concentrations used in the experiments. The bases for this assumption are that it has been reported (Mishima et al., 1976; Rando & Bangerter, 1976) that the activity for racemic gabaculine, a potent irreversible inactivator of GABA-T, was exactly half that of the *S* isomer, that only the *pro-S* proton is removed from GABA, and that only the 4-*S* isomers of γ-acetylenic GABA and γ-vinylic GABA irreversibly inactivate GABA-T (Bouclier et al., 1979). The observed difference in the deuterium isotope effects and rates of inactivation for AFPA (k^H_{cat}/k^D_{cat} = 5.5; k_{cat} = 0.50 min^{-1}) and for ACPA (k^H_{cat}/k^D_{cat} = 6.7; k_{cat} = 0.55 min^{-1}) (Silverman & Levy, 1980b) can be rationalized qualitatively on the basis of the difference in leaving group abilities of the two halides if the proton (deuterium) abstraction is a reversible process (John et al., 1979). Since the dissociation constants for the labeled and nonlabeled compounds are the same and since the enzyme–substrate binding equilibrium is a fast process relative to proton removal, an expression for the deuterium isotope effect can be derived (Northrop, 1977):

$$\frac{k^H_{cat}}{k^D_{cat}} = \frac{D_k + \frac{k_r}{k_x}}{1 + \frac{k_r}{k_x}}$$

In this expression, k_r is the rate of proton return, k_x is the rate corresponding to the halide release step, and D_k represents the relative rates of the abstraction process with the γ-protiated vs. γ-deuterated compounds. Since D_k is not expected to vary between substrates and k_r is expected to be so small relative to k_x that any difference in k_r between substrates is insignificant, both k_r and k_D can be considered to be constants. The expression for the isotope effect is reduced to a function of the rate of leaving group (halide) elimination. Thus, as the halide release rate increases (chloride > fluoride), the kinetic deuterium isotope effect also should increase, as is observed. Furthermore, a higher observed rate of inactivation would be expected from the compound that has the better leaving group (chloride). The proton return rate need not necessarily be comparable to that of the proton abstraction in order for the irreversible halide elimination rate to be reflected in the k_{cat} or the k^H_{cat}/k^D_{cat}.

The difference in the dissociation constants for (*S*)-AFPA (K_i = 0.4 mM) and (*S*)-ACPA (K_i = 13.1 mM) (Silverman & Levy, 1980b) can be attributed to steric interactions. The covalent radii of fluorine and chlorine are 0.57 and 0.99 Å, respectively (Sober, 1968). The discrepancy in the size of the halogens may account for the poorer binding of ACPA.

Following removal of the γ proton, elimination of the halide occurs, as demonstrated by the release of approximately 1 equiv of F^- from (*S*)-AFPA during enzyme inactivation. The reactive intermediate generated (**1**) could undergo active-site nucleophilic attack, potentially resulting in three different adducts, the quinoid adduct **2**, the PLP adduct **3**, or the PMP adduct **4**. In order to determine which, if any, of these adducts is generated, the change in the optical spectrum was monitored during inactivation of GABA-T by AFPA (Figure 3) in the presence of α-KG. A time-dependent increase in absorbance at 330 nm and decrease at 412 nm occurred. Two isosbestic points at about 365 nm and 460 nm were observed, suggesting the presence of only two kinetically competent species at steady state (Schnackerz et al., 1979). The observed spectral changes correspond to the conversion of the PLP–enzyme chromophore into the PMP-enzyme chromophore (Figure 4), which is consistent with the ternary complex **4** in Scheme I. The quinoid adduct **2** is unlikely since it would be expected to absorb in the 490–530-nm region of the spectrum (Jenkins, 1964; Schirch & Jenkins, 1964; Morino & Snell, 1967). The absence of a longer wavelength absorption during inactivation also suggests that **1** does not build up in any significant concentration. The active site nucleophile involved in the reaction is not yet known; the stability of the enzyme adduct is consistent with an amine, an ether, or a thioether linkage.

Two potential catalytic pathways leading from 4-amino-5-halopentanoic acids to products instead of inactivation were investigated. Transamination followed by release from the enzyme would generate 5-halolevulinic acid; elimination followed by hydrolysis would produce levulinic acid (after nonenzymatic hydrolysis of the unstable primary enamine). The results of three independent experiments[2] indicate that neither of these two potential pathways occurs. We previously reported (Silverman & Levy, 1980b) that complete inactivation of GABA-T occurs with the 4-amino-5-halopentanoic acids in the absence of α-KG; no activity returns when α-KG is added after inactivation. This suggests that there is no native enzyme in the PMP form after catalysis, implying that for every transamination process, the enzyme becomes alkylated. Release of only one fluoride ion from AFPA per active site inhibited indicates that when elimination occurs, only inactivation results. Finally when (*S*)-[U-^{14}C]ACPA is used to inactivate the enzyme, no significant amount of radioactive nonamines is detected. Both transamination and elimination would have yielded nonamines. These experiments indicate that there is a partition ratio (Walsh et al., 1978), i.e., the ratio of the number of turnovers yielding product per inactivation event, of zero. In appraising the effectiveness of a mechanism-based inhibitor, inactivation efficiency is important; inactivators exhibiting a low partition ratio are desirable when designing potential medicinal agents. If the partition ratio is large, the toxicity of metabolic byproducts may become a significant factor in the failure of the inactivators to have therapeutic value.

Lest it be thought that the design of mechanism-based inactivators simply involves conjuring up molecules which can be enzymatically transformed into reactive species, it should be noted that there are many factors involved in this type of enzyme inactivation. The enzyme must be capable of carrying out the desired transformation on the inhibitor, and once the transformation occurs, an active site nucleophile must be properly juxtaposed to react with the incipient electrophilic center. In this regard we have examined 4-amino-3-chlorobutyric acid, a known competitive inhibitor of GABA-T (Buu

[2] Added in proof: A fourth experiment further supports the conclusion that the partition ratio is zero. When GABA-T was inactivated with either 1 mM (*S*)-AFPA or 5 mM (*S*)-ACPA in the presence of 5 mM [U-^{14}C]-α-KG (0.04 μCi) followed by cation-exchange chromatography (Dowex 50, H^+ form), no radioactive glutamic acid was detected. If the inhibitors were being transaminated by the enzyme, the resulting PMP would be converted to PLP by [U-^{14}C]-α-KG with the concurrent formation of [U-^{14}C]-L-glutamic acid.

& Van Gelder, 1974), as a mechanism-based inactivator. This compound contains a β-chloro group to the amine, as does ACPA, and also hypothetically could undergo a series of reactions leading to irreversible inactivation. The reactive intermediate from the elimination of chloride would be a Michael acceptor analogous to **2** and therefore could be expected to inactivate the enzyme. We have found that this compound does not exhibit any time-dependent inactivation of GABA-T at 5 mM concentration over 60 min. Although it has been reported that this compound does not act as a substrate (Buu & Van Gelder, 1974), we have found that GABA-T does promote chloride elimination (R. B. Silverman and M. A. Levy, submitted for publication).

Preliminary pharmacological studies were carried out on AFPA. The results show that with a single 100 mg/kg body weight intraperitoneal injection in mice there is a 200% increase in brain GABA levels after 4 h, a sedative effect, and greater than a 3-fold delay in tonic convulsions induced by picrotoxinin.

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