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IRREVERSIBLE INACTIVATION OF PIG BRAIN γ-AMINOBUTYRIC ACID-α-KETOGLUTARATE TRANSAMINASE BY 4-AMINO-5-HALOPENTANOIC ACIDS

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SUMMARY

γ-Aminobutyric acid-α-ketoglutarate transaminase from pig brain is irreversibly inactivated by 4-amino-5-halopentanoic acids. Protection from inactivation by the natural substrates, the pH dependence of inactivation and the incorporation of 1.7 moles of radioactive inhibitor per mole of enzyme from (S)-[U-^{14}C]-4-amino-5-chloropentanoic acid suggest a covalent adduct at the active site of the enzyme. A mechanism-based inactivation is proposed.

INTRODUCTION

It is well established that GABA is an inhibitory neurotransmitter (1). Its concentration in the brain is regulated by two enzymes, GAD, which converts glutamate to GABA, and GABA-T, which catalyzes the degradation of GABA to succinic semialdehyde. Both of these enzymes require PLP as a coenzyme for activity (2). When brain levels of GABA diminish, convulsions may result (1). Since GABA does not cross the blood-brain barrier under normal circumstances (3), a chemotherapeutic approach to the treatment of convulsive diseases, e.g., epilepsy and Huntington's chorea, has been to block the degradation of GABA by inhibiting GABA-T (1). The widely used epilepsy drug, sodium valproate, has been shown to inhibit GABA-T reversibly (4). Several compounds, 4-aminohex-5-ynoic acid (6), 4-amino-

Abbreviations: GABA: γ-aminobutyric acid; GABA-T: γ-aminobutyric acid-α-ketoglutarate transaminase, E.C. 2.6.1.19; GAD: glutamate decarboxylase, E.C. 4.1.1.14; PLP: pyridoxal phosphate; α-KG: α-ketoglutaric acid; SSADH: succinic semialdehyde-NADP: oxidoreductase, E.C. 1.2.1.16; NADP: nicotinamide adenosine triphosphate; Pyr in Scheme 1 represents the substituted pyridine nucleus in PLP.

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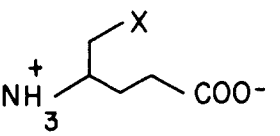
hex-5-enoic acid (7), gabaculine (8), isogabaculine (9), and ethanolamine-O-sulfate (10), have been proven to be mechanism-based irreversible inactivators (5) of GABA-T. Based on the accepted mechanism of action of GABA-T (11), we have synthesized (12) a series of 5-substituted-4-aminopentanoic acids (1) as potential anticonvulsant agents and report here the results of our inhibition studies with purified pig brain GABA-T.



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1a, X = F
1b, X = Cl
1c, X = Br
1d, X = OH

MATERIALS AND METHODS

Pig brain GABA-T was purified by the method of John and Fowler for the rabbit brain enzyme (13). The enzyme showed one band on SDS gel electrophoresis and had a specific activity of 7.5 units per mg protein. One unit is defined as the amount of enzyme which catalyzes the transamination of 1 μmole of GABA per min at 25° C. GABA-T activity was determined using a modification of the method of Scott and Jakoby (11). 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 at 25° C after several minutes of incubation. SSADH was prepared from GABASE (Sigma Chemical Co. or Boehringer Mannheim) by inactivating the GABA-T with (S)-4-amino-5-fluoropentanoic acid 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. (S)-[U-^{14}C]-4-Amino-5-chloropentanoic acid (specific activity 1.02 mCi/mmol) was prepared in an overall 32% yield from [U-^{14}C]-L-glutamic acid (Research Products International) diluted with 0.25 mmol of unlabeled L-glutamic acid by carrying out the synthesis of 1b as previously reported (12). The purity was determined to be greater than 99% by TLC (cellulose, n-butanol-H_2O-acetic acid, 12:5:3 and n-butanol-pyridine-water, 1:1:1) and by high voltage electrophoresis (pH 1.9). Radioactivity was determined on a Beckman LS-3100 scintillation counter using 3a 70B scintillation fluid (Research Products International) with [U-^{14}C]toluene (New England Nuclear) as an internal standard. All other materials and methods have been reported previously (12) or are commercially available.

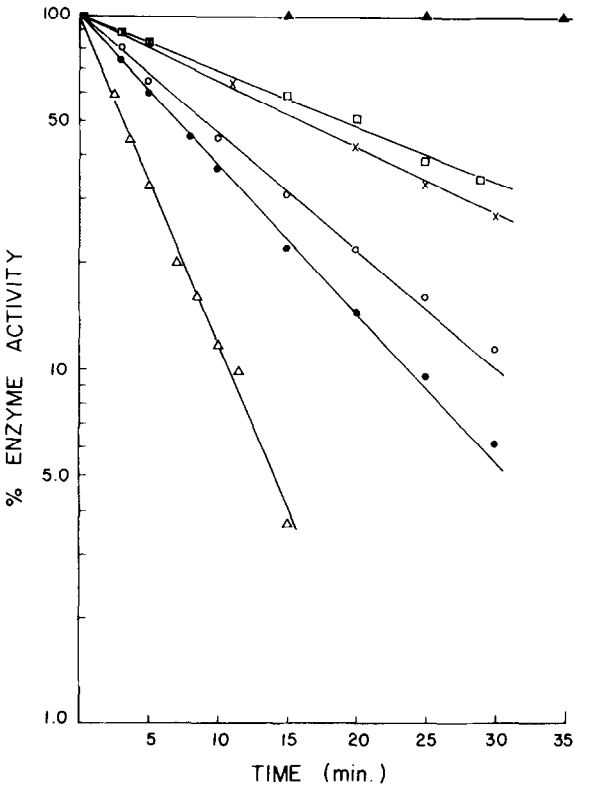


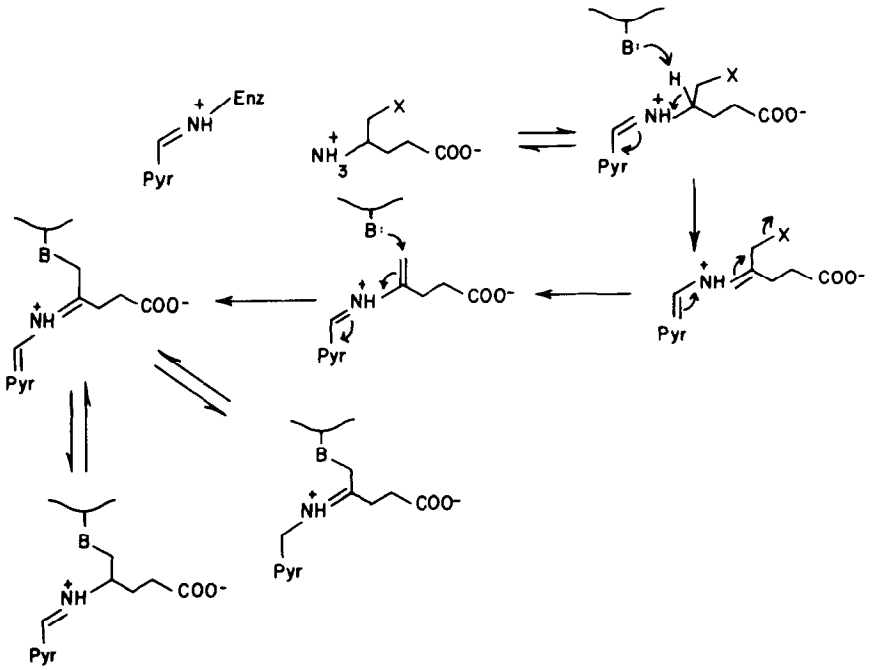
Figure 1. Inactivation of GABA-T by (S)-4-Amino-5-fluoropentanoic acid.

Pig brain GABA-T (0.138 units) was incubated at 25° C in 0.4 ml of 50 mM buffer containing 5 mM mercaptoethanol and different concentrations of inhibitor and substrates. The incubation was stopped by diluting a 40 µl aliquot of this mixture to 1 ml in a U.V. cuvette with buffer containing all of the necessary reagents for the assay. The inhibitor concentrations and the substrates depicted are: ▲ , control; ✕, 0.100 mM inhibitor, pH 7.5; △ , 0.200 mM inhibitor, pH 8.5; ● , 0.100 mM inhibitor, pH 8.5; 0.100 mM inhibitor, pH 8.5, ○ , plus 1.0 mM α-KG and □ , plus 1.0 mM α-KG and 1.0 mM GABA.

RESULTS AND DISCUSSION

The 4-amino-5-halopentanoic acids (1a, b, c) were found to be irreversible inactivators of pig brain GABA-T. Incubation of GABA-T with 1a or 1b led to a pseudo first-order time-dependent loss of enzyme activity (Figure 1). The K_I and k_{cat} (6) for 1a are 395 µM and 0.50 min^{-1} (pH 8.5, 25° C) and for 1b are 13.1 mM and 0.55 min^{-1}, respectively. It is noteworthy that the K_I for 1a is 12 times lower than the K_M (4.8 mM)

for GABA (14). Incubation of GABA-T with 1c showed a time-dependent loss of enzyme activity which was not pseudo first-order; the rate of inactivation gradually diminished with time. In a separate experiment we found that preincubation of 1c in pH 8.5 buffer in the absence of enzyme for 20 minutes produced a solution whose components did not inactivate the enzyme. High voltage electrophoresis of this solution showed that it may contain 4-amino-5-hydroxypentanoic acid (1d), a compound which we have found to be a reversible inhibitor of GABA-T. Presumably, the rate of inactivation of GABA-T by 1c under the conditions of the experiment is slower than the rate of its non-enzymatic hydrolysis. The rate of inactivation of GABA-T by 1a and 1b is slower at pH 7.5 than at pH 8.5 (Figure 1), the pH optimum for the enzyme (14). Since the rate of inactivation corresponds with the activity of the enzyme, the mechanism of inactivation may be enzyme-dependent. The enzyme was protected from inactivation by its second substrate, α-KG, and by GABA with α-KB (Figure 1). The degree of protection was dependent on substrate concentration, implying that the inactivation is active site-directed. The rate of inactivation was unaffected by β-mercaptoethanol, suggesting that a reactive species does not escape from the active site and then return to effect inactivation. Exhaustive dialysis of the inactivated enzyme at pH 5.4 to pH 8.5 did not regenerate any enzyme activity. Treatment of GABA-T with (S)-[U-^{14}C]-4-amino-5-chloropentanoic acid followed by exhaustive dialysis led to incorporation of 1.7 moles of inactivator per mole of enzyme. This is consistent with the fact that the enzyme is a dimer containing two moles of PLP per mole of enzyme (14). Denaturation of the labeled enzyme in 8 M urea resulted in little or no release of radioactivity from the enzyme, thus confirming covalent attachment of the inactivator to the protein.



Scheme 1. Proposed Mechanism of Inactivation of GABA-T by 4-Amino-5-halopentanoic Acids.

These results show that 4-amino-5-halopentanoic acids are irreversible inactivators of GABA-T. We believe that these compounds are mechanism-based (suicide) inactivators of this enzyme as described in Scheme 1, and are currently investigating this proposed mechanism of inactivation.

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