CATALYTIC INHIBITION OF γ-AMINOBUTYRIC ACID - α-KETOGLUTARATE TRANSAMINASE OF BACTERIAL ORIGIN BY 4-AMINOHEX-5-YNOIC ACID, A SUBSTRATE ANALOG.

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SUMMARY

γ- Aminobutyric acid-α-ketoglutarate transaminase from Pseudomonas fluorescens is irreversibly inhibited by 4-aminohex-5-ynoic acid, a new structural analog of GABA. The fact that this inhibition requires the pyridoxal form of the holoenzyme, and the formation of a Michaelis complex is in support of a catalytic mechanism. The compound is also active in vitro and in vivo on the same enzyme from mammalian brain.

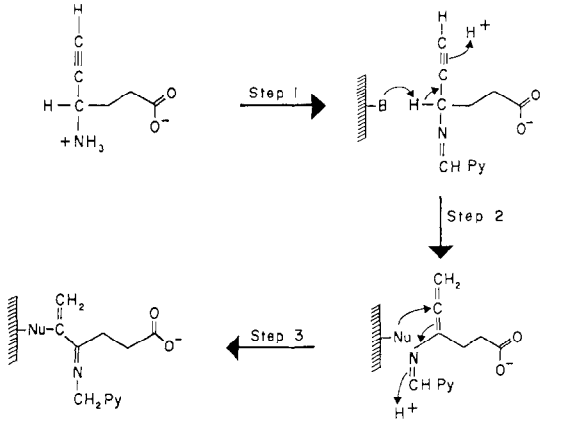
INTRODUCTION

The design of structural analogs of the substrate of an enzyme, inert as such but capable of being transformed by the target enzyme into highly reactive intermediates, has led to the discovery of specific irreversible enzyme inhibitors (1, 2, 3). Ethanolamine-O-sulfate is such a catalytic inhibitor for mammalian brain GABA-T * in vitro, and in vivo if given intraventricularly (4,5). A compound with the same type of action but active when given by a peripheral route would be of great value to neurochemists (6). Based on the accepted mechanism of action of this PLP dependent enzyme (7) and on similar examples of catalytic inhibitors (8,9,10), 4-aminohex-5-ynoic acid has been selected as a potential suicide substrate of GABA-T and synthesized (11).

The proposed mechanism for the catalytic inhibition is depicted in Scheme 1. The inhibitor will have to form a Schiff base with PLP in the active site of the enzyme (step 1) . Then the enzyme-catalysed proton abstraction by a basic group of the enzyme (B in the scheme) would lead to an allene

GABA : γ-aminobutyric acid ; GABA-T : γ-aminobutyric acid-α-ketoglutarate transaminase, E.C. 2.6.1.19 ; SSA : succinicsemialdehyde ; SSADH : succinicsemial dehyde - NADP : oxidoreductase, E.C. 1.2.1.16 ; α-KG : α-ketoglutarate ; PLP : pyridoxal phosphate ; NADP : nicotinamide adenosine triphosphate. Py in Scheme 1 represents the substituted pyridine nucleus in PLP.

SCHEME 1



rearrangement (step 2) rather than to the tautomeric Schiff base as in the normal mechanism of action. The conjugated allene could then react as a Michael acceptor with a nucleophilic residue (Nu on the scheme) in the enzyme active site (step 3).

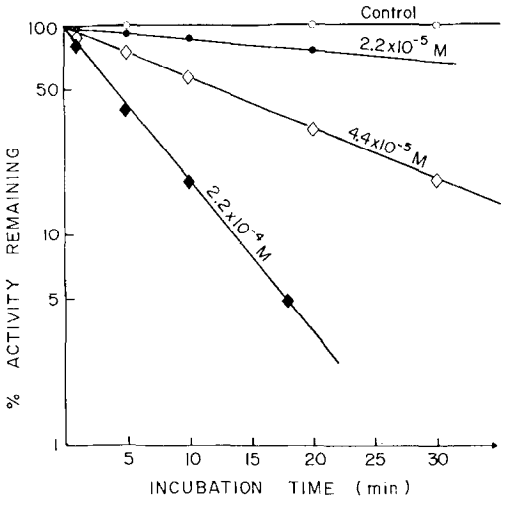
We report here the time-dependent inactivation of Pseudomonas fluorescens GABA-T, used as a model for brain GABA-T (12), by this new GABA analog.

MATERIAL AND METHODS

GABA, α-KG, and NADP were purchased from Sigma Chemical Co.,Inc., St.Louis, Mo., PLP was purchased from Merck and Co., Rahway,N.J. A commercial preparation of GABA-T (0.2 U/mg) containing SSADH and extracted from Pseudomonas fluorescens was bought from SIGMA and used without further purification. GABA-T activity was determined via the coupled SSA oxidation by measuring the increase of NADPH spectrophotometrically at 340 nm. It was found that the maximum SSADH activity is much higher than in the coupled reaction so that GABA-T is always rate limiting. SSA was prepared as a 0.1 M solution in 1N HCl according to (13). All the reactions were done at pH 8.6 in 0.1 M potassium pyrophosphate, $3X10^{-3}M$ in mercaptoethanol. One unit is the amount of enzyme which catalyzes the transamination of 1 μmol of GABA per min.

RESULTS AND DISCUSSION

On incubation with 4-aminohex-5-ynoic acid, GABA-T is inhibited in a time-dependent manner and the inhibition follows pseudo-first order kinetics (Fig. I). The activity of SSADH is not affected after complete inhibition of GABA-T as checked by adding SSA. Prolonged dialysis against several buffers at different pH's ranging from 5 to 10, containing $10^{-4}M$ PLP does not restore any GABA-T activity. This suggests that the inhibition is irreversible and that a strong covalent bond is formed between the enzyme and the inhibitor.



LEGEND FIGURE I

Time-dependent inactivation of GABA-T by 4-aminohex-5-ynoic acid.

0.04 units of the commercial enzyme solution were incubated at 25°C in 0.3 ml buffer with different concentrations of the inhibitor. The inactivation process was stopped by diluting an aliquot of this incubation mixture 100 times into a U.V. cuvette containing the same buffer (6 mM GABA, 5 mM α-KG, 1.5 mM NADP). The linear increase of U.V. absorption at 340 nm was recorded for 6 min. at 26°C and taken as measure of remaining enzyme activity.

The process for enzyme inactivation may be represented by the following scheme :

$$E + I \underset{k_2}{\overset{k_1}{\rightleftarrows}} [E - I] \xrightarrow{k_{cat}} [E - I'] \xrightarrow{k_{inh}} E_i$$

where E represents the active enzyme ; I the catalytic inhibitor ; E-I , E-I' the ternary enzyme - PLP - inhibitor complexes resulting respectively from step 1 and step 2 ; Ei the covalently modified enzyme after step 3 ; k_{cat} and k_{inh} the rate constants of step 2 and step 3 respectively.
Assuming that I >> E, $[E - I]$ is constant and $k_{inh} >> k_{cat}$, the following equation can be derived (14) :

$$\frac{dE}{E} = \frac{k_{cat}}{1 + \frac{K_I}{I}} \, dt$$

or after integration :

$$\ln \frac{E}{E_o} = \frac{-k_{cat}\, t}{1 + \frac{K_I}{I}}$$

where E_o represents the total amount of enzyme
E the active enzyme at time t
$K_I = k_1 / k_2$

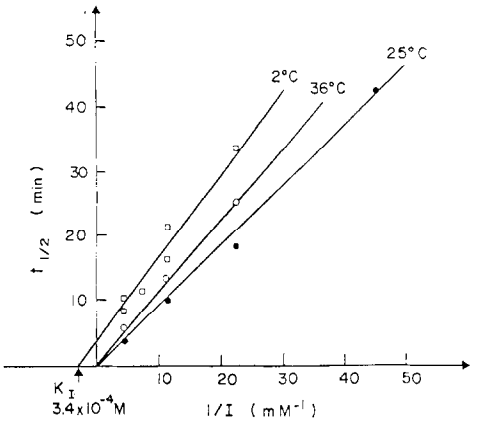
$$\text{or } t_{1/2} = \frac{0.69}{k_{cat}} + \frac{0.69}{k_{cat}} \frac{K_I}{I}$$

As shown in Fig. II, at 25°C and 36°C $t_{1/2}$ is proportional to 1/I. This means that E-I ~ O and that $k_{cat} > k_1$ at these temperatures, in other words the formation of the binary complex is rate limiting. However if the incubation is carried out at 2°C, the catalytic mechanism is slowed down and the kinetic consta were found to be $K_I = 3.4 \times 10^{-4}$M, $t_{1/2}$ for I ~ ∞ = 3.3 min. and $k_{cat} = 3.45 \times 10^{-3}$ sec.$^{-1}$. The K_I of the inhibitor is lower than the K_M of GABA (10^{-3}M).

To ascertain whether the inactivation is catalytic and active site directed, the effect of adding the enzyme substrates was studied (Fig. III). If GABA is added to the incubation mixture, even at a concentration lower than the K_M, the rate of inactivation is greatly decreased. However, if both GABA and α-KG are present in the incubation, the protection against inactivation is lost. At a low inhibitor concentration the rate of inhibition is even higher than in the absence of the substrates. Again, it seems that the formation of the binary complex is rate limiting. GABA-T like many transaminases follows a bi-bi ping pong mechanism (7,15). The protection against enzyme inhibition by GABA is explained by the tran formation of the holoenzyme from the pyridoxal into the pyridoxamine form :

$$E - PLP + GABA \rightleftharpoons E - Pyridoxamine + SSA$$

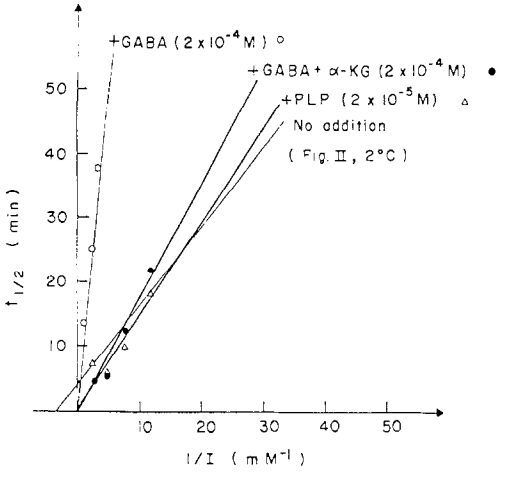
The pyridoxamine form is then unable to bind and transform the inhibitor. The addition of α-KG displaces the equilibrium to the left, regenerating the PLP holoenzyme which can then bind the inhibitor. The addition of PLP has no marked effect on the inactivation process.



LEGEND FIG. II

Temperature-dependent mechanism of the inhibition.

The experimental conditions were as in Fig. I except for the temperature of the inactivation mixture. $t_{1/2}$ was determined graphically from the semi-logarithmic plots of enzyme activity versus time.



LEGEND FIG. III

Effect of the substrates on the rate of inhibition.

The incubations were done at 2°C in the same buffer containing one or both substrates of the enzyme at the given concentrations. $t_{1/2}$ was determined as described in Fig. II.

Preliminary investigations have shown that 4-aminohex-5-ynoic acid is also a catalytic inhibitor of pig brain GABA-T in vitro and that the administration of the compound to rats or mice by a peripheral route results in the reduction of brain GABA-T activity and the elevation of brain GABA levels, both effects being long-lasting and dose-dependent and will be reported in full at a later date.

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