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Kinetic Studies on the Inhibition of GABA-T by γ-Vinyl GABA and Taurine

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γ-Aminobutyric acid transaminase (GABA-T, EC 2.6.1.19) is a pyridoxal phosphate (PLP) dependent enzyme that catalyzes the degradation of γ-aminobutyric acid. The kinetics of this reaction are studied *in vitro*, both in the absence, and in the presence of two inhibitors: γ-vinyl GABA (4-aminohex-5-enoic acid), and a natural product, taurine (ethylamine-2-sulfonic acid). A kinetic model that describes the transamination process is proposed. GABA-T from *Pseudomonas fluorescens* is inhibited by γ-vinyl GABA and taurine at concentrations of 51.0 and 78.5 mM. Both inhibitors show competitive inhibition behavior when GABA is the substrate and the inhibition constant (K_i) values for γ-vinyl GABA and taurine were found to be 26 ± 3 mM **and** 68 ± 7 mM **respectively. The transamination process of α-ketoglutarate was not affected by the presence of γ-vinyl GABA, whereas, taurine was a noncompetitive inhibitor of GABA-T when α-ketoglutarate was the substrate. The inhibition dissociation constant (K_{ii}) for this system was found to be** 96 ± 10 mM. **The Michaelis-Menten constant (K_m) in the absence of inhibition, was found to be** 0.79 ± 0.11 mM, **and** 0.47 ± 0.10 mM **for GABA and α-ketoglutarate respectively.**

Keywords: γ-Aminobutyric acid transaminase (GABA-T); γ-Vinyl GABA (4-aminohex-5-enoic acid); Taurine (ethylamine-2-sulfonic acid); Kinetics; Inhibitors

Abbreviations: GABA, γ-Aminobutyric acid; GABA-T, γ-Aminobutyric acid transaminase; SSDH, succinic semialdehyde dehydrogenase; GAD, glutamic acid decarboxylase; PLP, pyridoxal phosphate

INTRODUCTION

γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the mammalian central nervous system. It is the most prevalent and abundant neurotransmitter in the brain with as many as one-third of all neurons in the brain using GABA as an inhibitory neurotransmitter.[1]

The adequate control of GABA levels in the brain is attributed to the regulation of the levels of the anabolic enzyme, glutamic acid decarboxylase (GAD; EC 4.1.1.15), and the catabolic enzyme, γ-aminobutyric acid transaminase (GABA-T; EC 2.6.1.19).[2] This can be represented by the following equations:

$$\text{L–Glutamic Acid} \xrightarrow{\text{GAD}} \text{GABA} + CO_2 \qquad (1)$$

$$\text{GABA} + \alpha - \text{Ketoglutarate} \xrightarrow{\text{GABA–T}} \text{Succinic Semialdehyde} + \text{Glutamate} \qquad (2)$$

It is well known that reaction (2) undergoes Schiff base formation at the active site of GABA-T that uses pyridoxal-5-phosphate (PLP) as a co-enzyme.[3]

In disorders such as epilepsy, Huntington's chorea, and schizophernia, GABA levels are detected to be lower than normal. Here, inhibition of GABA-T becomes necessary to increase the levels of GABA and to bring them back to normal.[4]

Inhibition of GABA-T can be accomplished by a number of different inhibitors that vary in their inhibitory effect towards GABA-T. Examples of structural analogue inhibitors include amino oxoacetic acid (AOAA),[5] sodium-n-dipropylacetate (n-DPA),[3] and γ-vinyl GABA. In fact, over the last three decades, there have been several studies suggesting γ-vinyl GABA, known as vigabatrin, as a potent inhibitor of GABA-T *in vivo* and *in vitro*.[6,7,4,8]

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Taurine, on the other hand, is a natural product found in the central nervous system, skeletal muscles, and is very concentrated in the heart and brain.[9] Taurine is a sulfur containing amino acid which is available in the diet but can be synthesized in the body from cysteine. It is also obtained as a cleavage product of taurcholic acid (the sodium salt of this acid occurs in the bile). Nevertheless, the functional role of taurine in the body remains unknown. There are reports that describe taurine as an inhibitory neurotransmitter in the brain.[10,11] Other workers prefer to consider taurine as modulator rather than a neurotransmitter.[12,13] There have been reports on the benefits of taurine supplementation for epileptics, membrane stabilization for the muscles and nerves, and in the prevention of heart failure. In this work, we study the kinetics of inhibition of GABA-T using two inhibitors, γ-vinyl GABA and taurine, in an attempt to comparatively assess their inhibitory effect towards this enzymatic system.

METHODOLOGY

Materials and Methods

Enzymes, Substrates, and Co-Factors. GABase from *Pseudomonas fluorescens* was purchased from Sigma-Aldrich Chemical Co (Germany). The cell free preparation contains both γ-aminobutyric acid transaminase (GABA-T) and succinic semialdehyde dehydrogenase SSDH (EC 1.2.1.24) with 44% protein (biuret) and total activity of 0.995 units/mg protein. One unit of GABase converts one μmole of γ-aminobutyric acid to succinic semialdehyde and then to succinate per minute with a stoichiometric reduction of one μmole of $NADP^+$ at pH 8.6 and 25°C. The chemicals GABA, sodium salt of α-ketoglutarate, $NADP^+$, potassium pyrophosphate, potassium phosphate, and 2-mercaptoethanol were reagent grade from Sigma-Aldrich Chemical Co. γ-Vinyl GABA (Vigabatrin) was purchased from Marion Merrell Dow Co, and mega taurine was purchased from Twin Laboratories Inc. (Newyork, U.S.A).

Initial Velocity Measurements

GABA-T activity was measured by a coupled spectrophotometric assay using a Diode Array spectrophotometer HP 8453. In this procedure GABA is converted into succinic semialdehyde by GABA-T and then to succinate by succinic semialdehyde dehydrogenase (SSDH) with a stoichiometric reduction of $NADP^+$ to NADPH according to the following equation.

$$\text{Succinic Semialdehyde} + \text{NADP}^+ \xrightarrow{\text{SSDH}} \text{Succinate} + \text{NADPH} \quad (3)$$

The redox reaction was followed spectrophotometrically by recording the rate of increase in absorption at 340 nm at a temperature of 25°C. Reaction mixtures were made up to 3.00-ml containing potassium pyrophosphate (0.1 M) pH 8.6, GABA (0.2–7.0 mM), α-ketoglutarate (0.09–5.0 mM), $NADP^+$ (1.66 mM), and inhibitor (51.0 and 78.5 mM) for the kinetic studies in presence of inhibitor. The reaction was initiated by adding 0.02 ml of the standardized enzyme solution. Initial velocities were based upon the early linear portions of the reaction curve. The kinetic studies were performed by following the time course for GABA-T catalyzed transamination of GABA at different concentrations of α-ketoglutarate (4.0, 6.8 and 10.0 mM) at a pH of 8.6 and a fixed temperature of 25°C. Initial velocities were obtained on the basis of the initial linear portion of the time courses for the reactions.

Data Analysis

Kinetic parameters were obtained by nonlinear regression analysis using the programs EZ-Fit™ curve fitter[14] and Enzfitter [Biosoft]. The substrate saturation curves of a Ping-Pong mechanism were fitted to equation (4). Data conforming to competitive and non-competitive inhibition were fitted to equations (5) and (6) respectively.

$$\nu = \frac{V_{\max}[A][B]}{(K_A[B] + K_B[A] + [A][B])} \quad (4)$$

$$\nu = \frac{V_{\max}[A]}{(K_A(1 + [I]/K_i) + [A])} \quad (5)$$

$$\nu = \frac{V_{\max}[B]}{(K_B(1 + [I]/K_i) + [B](1 + [I]/K_{ii}))} \quad (6)$$

where ν is the initial velocity, V_{max} is the maximum velocity. A and B are the substrates GABA, and α-ketoglutarate respectively. K_A and K_B are K_m values for the substrates A and B respectively; K_i and K_{ii} are the inhibition dissociation constants for the inhibitor (I). EZ-fit uses two nonlinear methods, Gauss–Newton method, and the simplex method of Nelder and Mead.[15] In all cases the best model selected is always the simplest one that passes all statistical tests; the one that has the lowest χ^2 and best correlation coefficient.

RESULTS AND DISCUSSION

γ-Aminobutyric acid transaminase (GABA-T) is the enzymatic system that catalyzes the reaction:

$$\text{GABA} + \alpha - \text{Ketoglutarate} \xrightarrow{\text{GABA-T}} \text{Succinic Semialdehyde} + \text{Glutamate} \quad (7)$$

The enzyme (GABA-T) is tightly bound to the co-enzyme pyridoxal phosphate. This holo-enzyme

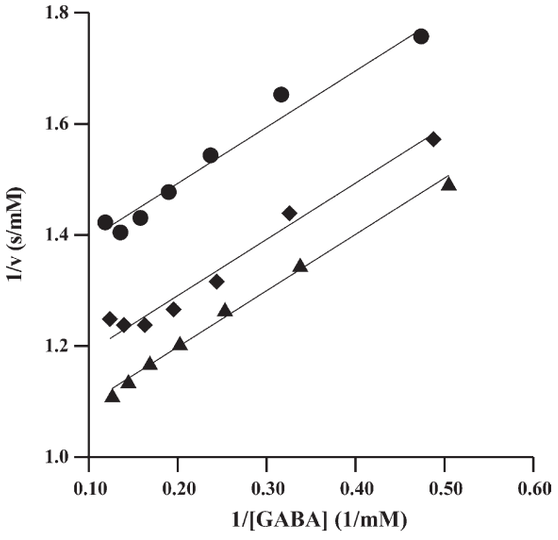


FIGURE 1 Initial velocity patterns of GABA transamination at varying concentration of α-ketoglutarate concentration: (▲) 4.0 mM, (■) 6.8 mM, (●) 10.0 mM. pH 8.6, temperature 25°C. Points are experimental and solid lines are from a fit using Equation (4).

transaminates with each substrate in turn, thus involving two forms of the holo-enzyme; the aldo-form (E—CHO), and amino form ($E—CH_2NH_2$).[3] Therefore, the above reaction can be conveniently divided into two half-reactions:

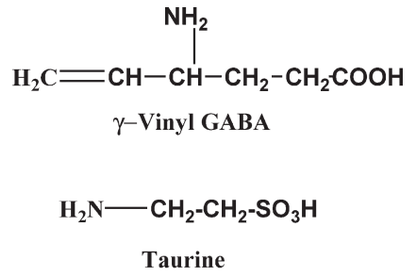
$$\text{GABA} + \text{E—CHO} \rightleftharpoons \text{Succinic Semialdehyde} + \text{E—CH}_2\text{NH}_2 \quad (8)$$

$$\text{E—CH}_2\text{NH}_2 + \alpha - \text{Ketoglutarate} \rightleftharpoons \text{Glutamate} + \text{E—CHO} \quad (9)$$

Figure 1 shows a double reciprocal plot in the direction of GABA transamination. A group of parallel lines is obtained conforming with a Ping-pong mechanism. The K_m values for each substrate were determined using Equation (4) and the kinetic parameters obtained are summarized in Table I. The Michaelis-Menten constants obtained are comparable to K_m values reported for GABA-T from *Pseudomonas fluorescens*.[3,16]

TABLE I Kinetic parameters for GABA transamination by GABA-T in the absence and presence of the inhibitors γ-vinyl-GABA and taurine

Parameter	GABA	α-Ketoglutarate
V_{max}	0.91 ± 0.03 mM	
K_m	0.79 ± 0.11 mM	0.47 ± 0.10 mM
K_i (γ-vinyl-GABA)	26 ± 3 mM	-
K_i (Taurine)	68 ± 7 mM	
K_{ii} (γ-vinyl-GABA)	-	-
K_{ii} Taurine)		96 ± 10 mM



SCHEME 1 Structures of γ-vinyl GABA and taurine.

Inhibition Studies

Two structural analogues of the substrate were tested for their ability to inhibit GABA-T: γ-vinyl GABA and taurine (Scheme 1). γ-Vinyl GABA is a drug used by patients suffering from epilepsy and schizophrenia whereas the latter is a natural product that has proved useful to those patients. Few studies have been performed on the kinetics and the mechanism of action of taurine.

The steady state assays in the presence of γ-vinyl GABA demonstrated competitive inhibition with respect to GABA as shown in Figure 2. The inhibition dissociation constant, K_i, for this inhibitor was found by fitting the data to Equation (5) where a value of 26 ± 3 mM was obtained. Competitive inhibition behaviour indicates the affinity of the inhibitor for the substrate-binding site. The different degree of

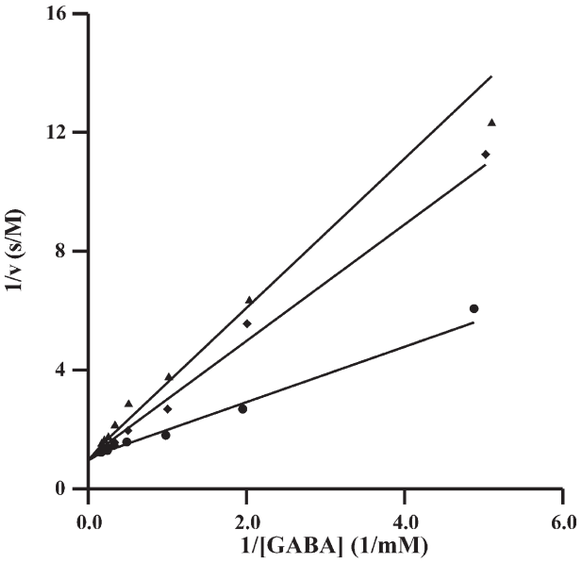


FIGURE 2 Inhibition by γ-vinyl GABA with respect to GABA. Assays were carried out at pH 8.6, [α − ketoglutarate] = 6.8 mM and temperature 25°C: γ-vinyl-GABA = (•) 0 mM, (■) 51.0 mM, (▲) 79.0 mM. Points are experimental and solid lines are from a fit using Equation (5).

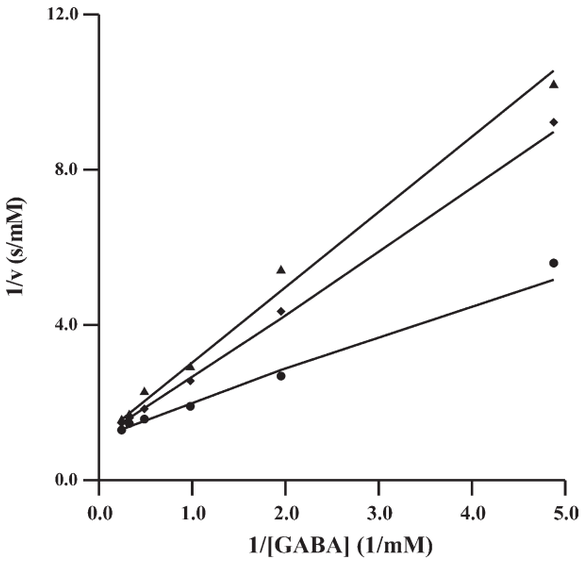


FIGURE 3 Inhibition by taurine with respect to GABA. Assays were carried out at pH 8.6 and temperature 25°C: taurine = (●) 0 mM, (■) 51.0 mM, (▲) 79.0 mM. Points are experimental and solid lines are from a fit using Equation (5).

inhibition can be explained by the similarities between the substrate and its analogues.

Interestingly, when α-ketoglutarate is varied at fixed concentrations of GABA, γ-vinyl GABA had no effect on the initial rates. This result indicates clearly that γ-vinyl GABA has no inhibitory effect on the amino-form of the enzyme. This agrees with published information such as the studies on incubation of rat brain GABA-T with γ-vinyl GABA that was found to decrease the enzymatic activity.[17] Other studies showed that inhibition *in vivo* follows pseudo first order kinetics and was described as being in accord with a ping-pong bi bi mechanism.

The reciprocal plots of varied GABA concentration (α-ketoglutarate concentration is fixed at 6.8 mM) in the presence of different taurine concentrations demonstrated a similar effect on GABA-T to that of γ-vinyl GABA (Figure 3). The patterns in Figure 3 provide clear evidence of a competitive inhibition. However in this case taurine is a weaker inhibitor with respect to GABA with a K_i value of 68 ± 7 mM compared to a K_i of 26 ± 3 mM for γ-vinyl GABA. Competitive inhibition indicates that taurine and γ-vinyl GABA can bind to the same form of the enzyme, which is the aldehyde form. By comparison γ-vinyl GABA and taurine are similar to GABA with respect to the amino part of the molecule, and therefore expected to bind in a similar fashion to the same enzyme site. However, it is clear that the presence of the $-SO_3^-$ group in taurine in place of the $-CH_2-COOH$ group resulted in the poorer binding of taurine. This could be due to the fact that the structural differences result in more stable intermediates or the enzyme achieves a high degree of specificity that favours certain configurations of the substrates. The mechanism by which γ-vinyl GABA and taurine inhibit GABA-T could be similar to the well-known mechanism for the inhibition of GABA-T by γ-ethynyl-GABA.[18] In this mechanism an aldimine intermediate is formed between the inhibitor and the pyridoxal-5′-phosphate that might be stabilized internally by hydrogen bonding. Loss of an α-hydrogen produces a resonance-stabilized species by electron pair delocalization. This species is expected to be more stable for γ-vinyl-GABA than for taurine. This is then followed by the tautomerization of the imine to form another intermediate which upon attack of an enzyme active-site nucleophile would give a covalently inactive enzyme. Another possible mechanism involves formation of an imine between the coenzyme and the inhibitor followed by a step in which the enzyme catalyzes the isomerization of the intermediate into a reactive conjugate olefin.[19] Nucleophilic addition by an active site nucleophile would result in the formation of a covalently modified enzyme.

Taurine was also found to inhibit GABA-T in its amino form when GABA concentration was fixed and α-ketoglutarate concentration was varied. Figure 4 represents a double reciprocal plot of the initial velocity *vs*. α-ketoglutarate concentration and shows that taurine demonstrates a non-competitive pattern of inhibition with respect to the amino form of the enzyme. The value of the inhibition dissociation constant K_{ii} for taurine is 96 ± 10 mM and is obtained by fitting the initial velocity data to Equation (6). In this type of inhibition a decrease in the amount of the active enzyme would probably

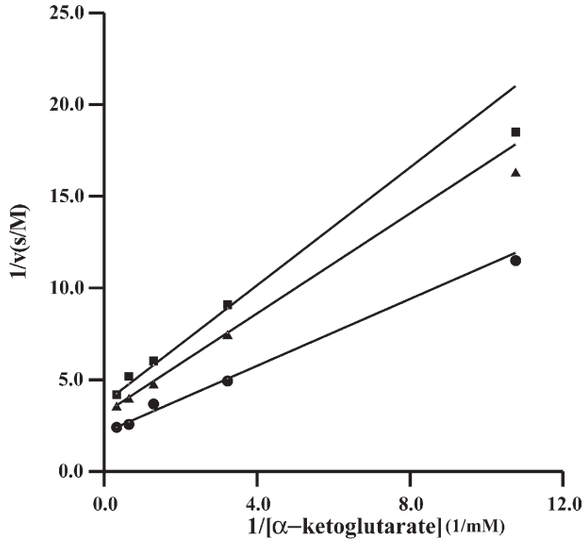


FIGURE 4 Inhibition by taurine with respect to α-ketoglutarate. Assays were carried out at pH 8.6 and temperature 25°C: taurine = (●) 0 mM, (▲) 51.0 mM, (■) 79.0 mM. Points are experimental and solid lines are from a fit using Equation (6).

result from the formation of an enzyme-substrate-inhibitor complex. This reaction decreases the proportion of the central complexes that proceed to products, and as a result non-competitive inhibition is observed.

In this study the slight upward curvature in the double reciprocal plots of the initial velocity of GABA (Figure 1) is a clear indication of substrate inhibition. Furthermore when the data in Figure 1 were subjected to a fit involving substrate inhibition, slight improvement in the results was obtained but without a significant change in the values of the Michaelis-Menten constants. Similar findings were reported in the literature, as substrate inhibition is very common in ping-pong mechanisms.[3,13] Usually substrate inhibitions may result from the interaction of the substrate with improper forms of the enzyme that would form dead-end complexes. In general dead-end complexes may result from two substrates competing for a single enzyme-binding site that exists in two forms, or one substrate binding at two sites in a two-site enzyme.

It is worth mentioning here that the enzymatic mechanism and the inhibition patterns of GABA-T from *Pseudomonas fluorescens* and from mammalian sources obey a ping-pong bi bi reaction mechanism. It is therefore imperative to indicate that GABA-T from a bacterial source is similar to a certain extent to that from the mammalian source. However, reported elsewhere, K_m values for GABA and α-ketoglutarate were significantly lower for the mammalian brain GABA-T compared to the bacterial enzyme. This may be due to the existence of possible specific isozymes of GABA-T.[3,20]

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