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4-Amino-hex-5-enoic Acid, a Selective Catalytic Inhibitor of 4-Aminobutyric-Acid Aminotransferase in Mammalian Brain

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Incubation of rat brain 4-aminobutyrate aminotransferase with 4-amino-hex-5-enoic acid, a substrate analog of 4-aminobutyric acid, results in a time-dependent irreversible loss of enzymatic activity. In the presence of 0.1 mM inhibitor the half-life of the inactivation process is approximately 6 min. Low concentrations of L-glutamic acid or 4-aminobutyric acid protect against this inactivation, while 2-oxoglutarate prevents this protection, suggesting that only the pyridoxal form of the enzyme is susceptible to inhibition by 4-amino-hex-5-enoic acid.

The irreversible inhibition of mammalian 4-aminobutyrate aminotransferase by 4-amino-hex-5-enoic acid is selective. There is no inhibition of this enzyme from *Pseudomonas fluorescens* with the inhibitor at mM concentrations. Even at 10 mM there is no irreversible inhibition of mammalian glutamate decarboxylase or of aspartate aminotransferase, while alanine aminotransferase is inhibited over 500 times more slowly than rat brain 4-aminobutyrate transaminase.

4-Aminobutyric acid is believed to be an important inhibitory neurotransmitter in mammalian brain [1]. The major pathway for its degradation is *via* transamination with 2-oxoglutarate. Fowler and John [2] have described the specific irreversible inhibition of brain aminobutyrate transaminase by ethanolamine-*O*-sulphate, a substrate analog of 4-aminobutyric acid, which was effective *in vivo* when given intracisternally. Recently, 4-amino-hex-5-enoic acid (4-acetylene derivative of aminobutyric acid) was shown to be a potent catalytic inhibitor of aminobutyrate transaminase from *Pseudomonas fluorescens* [3,4]. In addition, it is effective against mammalian brain aminobutyrate transaminase *in vitro* [5] and *in vivo* [5,6] when administered peripherally (p.o., i.p., i.v.).

The concept of catalytic inhibition of enzymes as an approach toward the rational design of specific, irreversible, active-site-directed inhibitors of enzymes has attracted much attention, and has recently been reviewed by Rando [7–9]. Based on the accepted mechanism of action of aminobutyrate transaminase and on the fact that 2-amino-but-3-enoic acid inhibits aspartate aminotransferase [10], the hitherto unknown 4-amino-hex-5-enoic acid (4-vinyl derivative of aminobutyric acid) was conceived as a potential suicide substrate of aminobutyrate transaminase, synthesised and tested. The results presented in this communication confirm that 4-amino-hex-5-enoic acid is a specific irreversible inhibitor of this enzyme.

Abbreviations. Pyridoxal-*P*, pyridoxal phosphate.

Enzymes. Aminobutyrate transaminase or 4-aminobutyric acid : 2-oxoglutaric acid aminotransferase (EC 2.6.1.19); aspartate transaminase or L-aspartic acid : 2-oxoglutaric acid aminotransferase (EC 2.6.1.1); alanine transaminase or L-alanine : 2-oxoglutaric acid aminotransferase (EC 2.6.1.2); succinate-semialdehyde dehydrogenase [$NAD(P)^+$] or succinate-semialdehyde : $NAD(P)^+$ oxidoreductase (EC 1.2.1.16); malate dehydrogenase or L-malate : NAD^+ oxidoreductase (EC 1.1.1.37); lactate dehydrogenase, L-lactate : NAD^+ oxidoreductase (EC 1.1.1.27); glutamate decarboxylase or L-glutamic acid 1-carboxy-lyase (EC 4.1.1.15).

MATERIALS AND METHODS

4-Amino-hex-5-enoic acid was synthesised *via* Li/NH_3/$(NH_4)_2SO_4$ reduction [11] of racemic 4-amino-hex-5-ynoic acid [4] and was recrystallised from acetone/water, m.p. 209 °C. Alternatively, the methyl ester acetamide of 4-amino-hex-5-ynoic acid was semi-hydrogenated using Lindlar's catalyst [12] and 4-amino-hex-5-enoic acid obtained by acid hydrolysis of the reduction product.

Spectral (infrared and proton-magnetic-resonance spectrometry) and elemental analyses were consistent with the expected structure. No 4-amino-hex-5-ynoic acid remained as verified by thin-layer chromatography in two solvent systems (butanol/acetic acid/water, 60/25/15) · (ethanol/ammonia/water, 70/5/25) under conditions where approximately 1% of the contaminant could be detected. All the biochemical

experiments were performed with this racemic mixture of 4-amino-hex-5-enoic acid.

A preparation of 4-aminobutyric acid-transaminase (0.2 U/mg protein) and succinate-semialdehyde dehydrogenase extracted from *Pseudomonas fluorescens*, 4-aminobutyric acid, 2-oxoglutarate, NAD, L(+)-glutamic acid, dithiothreitol, lactate dehydrogenase (0.4 U/μg protein) and malate dehydrogenase (10 U/μg protein) were purchased from Sigma Chemical Co., glutamic acid from E. Merck (Darmstadt).

Enzyme Preparations

Rat brain aminobutyrate transaminase was partially purified by following the method of Bloch-Tardy *et al.* [13] through the second $(NH_4)_2SO_4$ precipitation. The resulting preparation had a specific activity of 13.5 $\mu mol \times h^{-1} \times mg\ protein^{-1}$. Succinate-semialdehyde dehydrogenase was partially purified by following the method of Pitts *et al.* [14] through the second $(NH_4)_2SO_4$ precipitation.

Protein was determined by the fluorescamine method of Böhlen *et al.* [15] using bovine serum albumin as a standard.

Rat-brain glutamic acid decarboxylase was partially purified by the following procedure carried out at 5 °C: brains were homogenized in a Waring Blender in 4 volumes of chilled buffer (100 mM potassium phosphate, pH 7.0, containing 0.2 mM; pyridoxal-*P*; 1 mM dithiothreitol; 1 mM Na_4EDTA; 0.13% Triton X-100). The homogenate was centrifuged at $20000 \times g$ for 30 min and the supernatant glutamate decarboxylase activity precipitated with $(NH_4)_2SO_4$ between 30 – 60% saturation, redissolved in a minimum volume of a solution similar to the homogenization buffer except that it contained no Triton-X, and then dialyzed against this buffer. The resulting preparation had a specific activity of 1.2 $\mu mol \times mg\ protein^{-1} \times h^{-1}$ at 37 °C.

The source of both aspartate aminotransferase and alanine aminotransferase was the low-speed supernatant from a 9% (w/v) rat-brain homogenate. The preparation was dialyzed against a phosphate buffer, 10 mM (containing 1 mM Na_4EDTA; 0.1 mM dithiothreitol; 0.1 mM 2-oxoglutarate; 0.1 mM pyridoxal-*P*) adjusted to pH 6.8 with acetic acid followed by a buffer similar to the above except that it contained no 2-oxoglutarate.

Determination of Irreversible Enzyme Inhibition by 4-Amino-hex-5-enoic Acid

Rat-Brain Aminobutyrate Transaminase. 50 μl aminobutyrate transaminase; 78 μl 300 mM pyrophosphate buffer, pH 8.6, and 12 μl 100 mM mercaptoethanol were incubated at 25 °C with or without 20 μl of different effectors such as 2-oxoglutarate, L-glutamate, pyridoxal-*P etc.* and sufficient H_2O so that the total volume of the incubates was 200 μl after the addition of 4-amino-hex-5-enoic acid. At the indicated times 20-μl aliquots of the incubate were withdrawn, diluted to 2.00 ml in a spectrophotometric cuvette and immediately assayed for residual aminobutyrate transaminase activity using a modification of the method of Scott and Jacoby [16]. The cuvette contained the following medium: 100 mM pyrophosphate buffer, pH 8.6; 6 mM aminobutyrate; 5 mM 2-oxoglutarate; 3 mM mercaptoethanol; 1 mM NAD; and sufficient succinate-semialdehyde dehydrogenase so that aminobutyrate transaminase was always rate-limiting. The rate of increase of NADH absorption at 340 nm was linear for at least 1 h and was routinely recorded for 10 – 30 min using a Beckman Acta III or Cary 118 spectrophotometer equipped with an automatic sample changer. Temperature of both the preincubate and spectrophotometric assay was maintained at 25.0 °C using a Lauda circulating water bath. In the absence of inhibitor the enzyme was stable for at least 1 h.

Aspartate Aminotransferase and Alanine Aminotransferase. Both aspartate aminotransferase and alanine aminotransferase were tested at pH 7.4 and pH 8.6 for inhibition by employing a method analogous to the one described above for aminobutyrate transaminase. Aliquots of an incubate of buffered enzyme and inhibitor were removed at the indicated times. Residual enzymatic activity [17] was then assayed spectrophotometrically by following the disappearance of NADH using malate dehydrogenase or lactate dehydrogenase respectively as the auxiliary enzyme.

Rat Brain Glutamate Decarboxylase. 20 μl 100 mM mercaptoethanol; 12.5 μl 500 mM phosphate buffer, pH 6.5; 14.5 μl H_2O; 133 μl of the glutamate decarboxylase preparation; 20 μl of 4-amino-hex-5-enoic acid (1 – 100 mM) were incubated together. At the indicated times 25-μl aliquots were removed and assayed for residual glutamate decarboxylase activity by a modification of the method of Roberts and Simonsen [18]. Each assay (2 ml) contained 0.5 μCi DL-[1-^{14}C]glutamic acid (New England Nuclear NEC-548). To decrease the blank and increase the observed radioactivity, no additional pyridoxal-*P* was added and L-glutamate was added to a final concentration of 0.5 mM.

RESULTS AND DISCUSSION

Incubation of 4-amino-hex-5-enoic acid (0.1 mM) with rat brain aminobutyrate transaminase results in a rapid, irreversible and complete loss of enzymatic activity. The inactivation is progressive with time and follows pseudo-first-order kinetics. Enzymatic half-lives range from 11 min to 1 min with concentrations

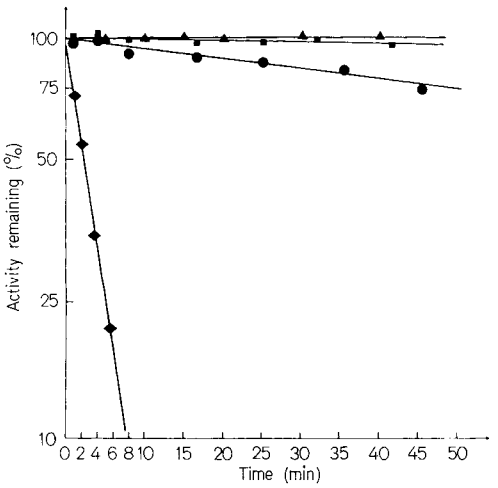

Fig. 1. *The effect of 4-amino-hex-5-enoic acid on rat brain glutamate decarboxylase, aspartate transaminase, alanine transaminase and aminobutyrate transaminase.* Glutamate decarboxylase (▲), aspartate transaminase (■) and alanine transaminase (●) were preincubated with 10 mM 4-amino-hex-5-enoic acid, while aminobutyrate transaminase (◆) was preincubated with 0.5 mM of the inhibitor. Aliquots of the preincubates were taken at the indicated times, and the residual enzymatic activity determined as described in the Methods section

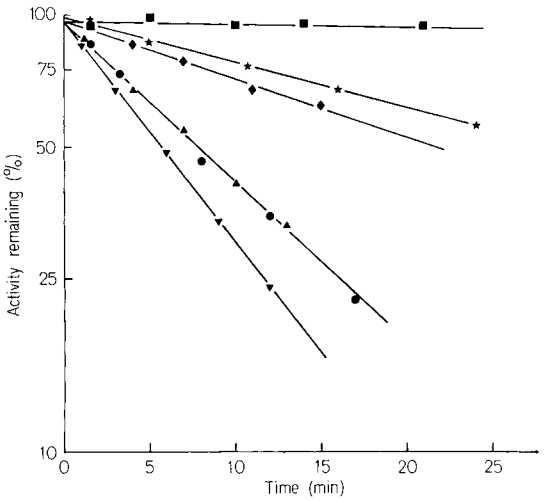

Fig. 2. *Protection and deprotection against the inactivation of rat brain aminobutyrate transaminase by 4-amino-hex-5-enoic acid; the effect of* D-*glutamate,* L-*glutamate and 2-oxoglutarate.* The enzyme was incubated with 0.1 mM 4-amino-hex-5-enoic acid alone (●) and with 0.1 mM 4-amino-hex-5-enoic acid in the presence of the following ligands; (▼) 1 mM 2-oxoglutarate, (■) 1 and (★) 0.01 mM L-glutamate, (◆) 1 mM D-glutamate, (▲) 1 mM L-glutamate plus 1 mM 2-oxoglutarate

of inhibitor between 0.05 and 1 mM[1]. Only 5–10% of control activity is restored upon exhaustive dialysis for 4 days at pH values ranging from 5–9 with buffers containing pyridoxal-*P* (0.1 mM) and 2-oxoglutarate (0.1 mM), suggesting that the observed inhibition is due to covalent bond formation between the inhibitor and enzyme.

The irreversible inhibition of mammalian brain aminobutyrate transaminase by 4-amino-hex-5-enoic acid appears to be selective in that no inhibition of aminobutyrate transaminase from *Pseudomonas fluorescens* was observed at mM concentrations. In addition, even at 10 mM there is no irreversible inhibition of rat brain glutamate decarboxylase or aspartate transaminase but at this concentration at pH 8.6 there is inactivation ($t_{1/2}$ = 120 min) of rat brain alanine transaminase that is about 1000-times slower than that of rat brain aminobutyrate transaminase (Fig. 1).

To help determine if the inhibition of aminobutyrate transaminase by 4-amino-hex-5-enoic acid is catalytic and active-site directed, the effect of various ligands on the rate of inactivation of this enzyme was studied. Both L-glutamate (Fig. 2) and aminobutyrate (Fig. 3) effectively decreased the rate of inhibition of

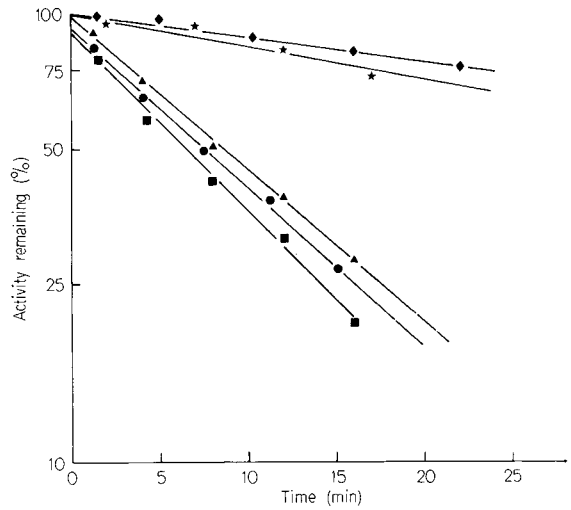

Fig. 3. *Protection and deprotection against the inactivation of aminobutyrate transaminase by 4-amino-hex-5-enoic acid; the effect of aminobutyrate, 2-oxoglutarate, oxaloacetate and pyridoxal phosphate.* The enzyme was incubated with 0.1 mM 4-amino-hex-5-enoic acid alone (■), and with 0.1 mM 4-amino-hex-5-enoic acid in the presence of the following ligands; (◆) 0.1 mM aminobutyrate, (★) 0.1 mM aminobutyrate plus 0.01 mM oxaloacetate, (▲) 0.1 mM aminobutyrate plus 0.01 mM 2-oxoglutarate, (●) 0.1 mM pyridoxal phosphate

aminobutyrate transaminase by 4-amino-hex-5-enoic acid. D-Glutamate was about 100-fold less effective than the natural enantiomer. Since optical rotation measurements indicated that the D-isomer was contaminated with about 1% of the natural isomer, the

[1] The K_i for the inactivation process was determined by the method of Kitz and Wilson [19] and was found to be approximately 10 mM. In addition, the inactivation of rat brain aminobutyrate transaminase by 4-amino-[4-^{2}H]hex-5-enoic acid exhibits a primary kinetic isotope effect. Details will be given in a separate communication.

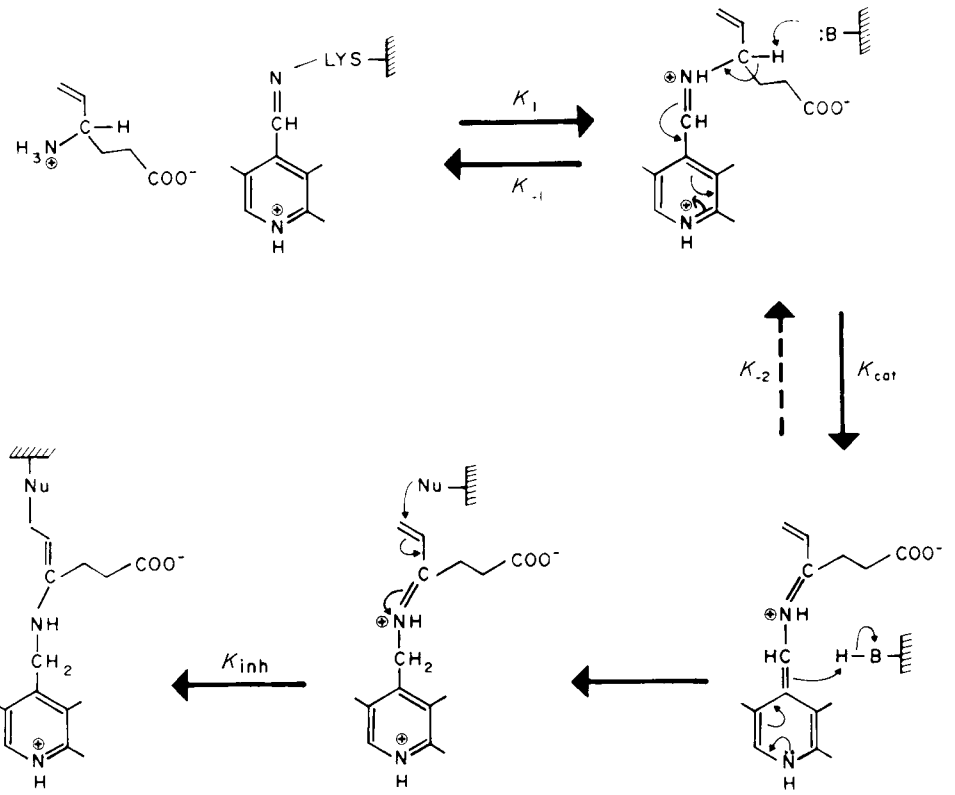

Fig. 4. *Proposed mechanism of inhibition of aminobutyrate transaminase by 4-amino-hex-5-enoic acid.* B = base in enzyme active site; Nu = nucleophilic residue in enzyme active site

observed protection by the D-isomer was probably due to the contaminating L-glutamate. The addition of 2-oxoglutarate (0.01 mM) to enzyme previously protected by either L-glutamate or aminobutyrate restored the original rate of inactivation of aminobutyrate transaminase by 4-amino-hex-5-enoic acid. Oxaloacetate (0.01 mM) was ineffective.

Aminobutyrate transaminase is a pyridoxal-*P* enzyme which follows a bi-bi ping-pong mechanism [20] and in the pyridoxamine form can readily transaminate only with succinate semialdehyde and 2-oxoglutarate. The above results strongly suggest that only the pyridoxal form of the enzyme is capable of reacting with 4-amino-hex-5-enoic acid.

Pyridoxal-*P* addition (0.1 mM) is without effect on the rate of inhibition of aminobutyrate transaminase by 4-amino-hex-5-enoic acid (Fig. 3). In addition, as determined by nuclear magnetic resonance spectrometry there is no detectable exchange of the proton at C-4 of 4-amino-hex-5-enoic acid with ^2H_2O in the presence of an equimolar concentration of pyridoxal-*P* even after an incubation of two days at pH 10. Therefore, the inhibition is due neither to the formation of a stable complex between inhibitor and pyridoxal-*P* nor to transamination of inhibitor with pyridoxal-*P* in solution. The fact that the inhibition of aminobutyrate transaminase by 4-amino-[4-^{2}H]-hex-5-enoic acid displays a primary kinetic isotope effect necessitates that, in analogy to normal transamination with aminobutyrate or glutamate, the enzyme must abstract a proton from carbon-4 of the inhibitor before inhibition can occur.

The incubation of aminobutyrate transaminase with 4-amino-hex-5-enoic acid was routinely carried out in the presence of 6 mM dithiothreitol or mercaptoethanol. This fact rules out the possibility that the observed inhibition is due to enzyme-induced release of 4-keto-hex-5-enoic acid into the medium, analogous to the acrolein-induced inhibition of yeast alcohol dehydrogenase by allyl alcohol [21].

The results presented strongly suggest that 4-amino-hex-5-enoic acid irreversibly inhibits mammalian brain aminobutyrate transaminase because it is accepted as a substrate. In analogy to the mechanism of inhibition of aspartate transaminase by vinylglycine [10], the following mechanism for the inhibition of aminobutyrate transaminase by 4-amino-hex-5-enoic acid is proposed (Fig. 4): 4-amino-hex-5-enoic acid forms a Schiff base with the active site pyridoxal-*P*; the enzyme then abstracts the labilized proton at C-4 from this aldimine Schiff base. The charge stabilization by the pyridine ring then induces the aldimine to ketimine tautomerism as in the normal transamination mechanism. This reactive (C-5, C-6) unsaturated ketimine undergoes a Michael addition with a nucleophilic residue of the active site before it can desorb

from the enzyme surface. The fact that 2-oxoglutarate increases the rate of inactivation of aminobutyrate transaminase by 4-amino-hex-5-enoic acid may indicate that either 2-oxoglutarate can interact with the pyridoxal form of the enzyme or that 10–20% of the reactive ketimine Schiff bases complete the transamination reaction without inhibiting the enzyme. An increased rate of inactivation in the presence of 2-oxoglutarate would also be expected if 10–20% of the enzyme were in the pyridoxamine form at the start of the incubation. However, this is extremely unlikely to have occurred, because before the start of the experiment the enzyme was dialyzed extensively against a phosphate buffer containing 2-oxoglutarate and then against buffer alone. Work is now in progress to further document the proposed mechanism of inhibition of mammalian brain aminobutyrate transaminase by 4-amino-hex-5-enoic acid.

4-Amino-hex-5-enoic acid is also active *in vivo*. When administered by a peripheral route to rats or mice it results in a dose-dependent inhibition of brain aminobutyrate transaminase leading to an elevation of brain aminobutyrate levels (*e.g.* 400 mg/kg triples the whole brain content of aminobutyrate [5]. In summary, 4-amino-hex-5-enoic acid is a rationally designed specific irreversible inhibitor of aminobutyrate transaminase effective *in vitro* and *in vivo* which should prove useful in elucidating the role of aminobutyrate in the central nervous system.

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