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Inactivation of γ-Aminobutyric Acid Aminotransferase by L-3-Chloroalanine Hydroxamate

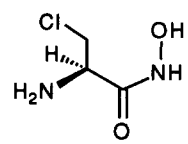
Richard B. Silverman* and Gregory T. Olson

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL 60208-3113, U.S.A.

Abstract—The mechanism of inactivation of γ-aminobutyric acid aminotransferase (GABA-AT) by L-3-chloroalanine hydroxamate (1) was investigated. Inactivation of [^{3}H]PLP-reconstituted GABA-AT with **1** followed by denaturation gave no PMP or enamine adduct to the PLP; however, a new unknown metabolite was observed which was identical to the metabolite formed upon inactivation of GABA-AT by L-cycloserine. Time-dependent inactivation occurs, but the kinetics are second order; the rate of inactivation increases with time. After inactivation occurs the addition of fresh enzyme results in a faster rate of inactivation than prior to the initial inactivation. This indicates that the actual inactivator is generated from L-3-chloroalanine hydroxamate, and is not L-3-chloroalanine hydroxamate itself. Added gabaculine-inactivated enzyme to fresh enzyme does not increase the rate of inactivation, suggesting that the conversion of L-3-chloroalanine hydroxamate to the active form is not catalyzed by peripheral amino acid residues. L-3-Chloroalanine hydroxamate was shown to undergo buffer-catalyzed cyclization to L-cycloserine, which is the actual inactivator of GABA-AT.

Introduction

The pyridoxal 5'-phosphate (PLP) dependent enzyme, γ-aminobutyric acid aminotransferase (GABA-AT; EC 2.6.1.19) is an important regulatory enzyme in the brain, catalyzing the transformation of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) to succinic semialdehyde.[1] When GABA levels in the brain fall too low, seizures can result.[2] Since GABA is too polar to pass through the blood–brain barrier, it is not useful as an anticonvulsant agent. Another strategy to increase the GABA levels in the brain employs more lipophilic compounds that can cross the blood–brain barrier and selectively inactivate GABA-AT.[3] These compounds then can be used for the treatment of such diseases as epilepsy and Huntington's disease.[4] One compound that was reported to be a time-dependent inactivator of GABA-AT *in vitro* and *in vivo* and to delay the onset of drug-induced seizures is L-3-chloroalanine hydroxamate (**1**),[5] the synthetic precursor of L-cycloserine (**2**),[6] another known time-dependent inactivator of brain GABA-AT.[7] There are several inactivation mechanisms that can be imagined for **1**, summarized in Schemes 1 and 2. Scheme 1 depicts three possibilities. Pathway a involves Schiff base formation with the PLP followed by normal isomerization to an activated α-chloro imine (**3**) then active site nucleophilic attack, leading to a covalent adduct (**4**). Pathway b is the same except that instead of S_N2 attack, there is elimination–Michael addition; however, the same adduct results. In pathway c the active site nucleophile is a lysine residue, which undergoes transimination to the enamine (**5**), which attacks the lysine-bound PLP, reminiscent of the inactivation of aspartate aminotransferase[8] and glutamate decarboxylase[9] by serine *O*-sulfate, thereby producing the PLP adduct **6**. Denaturation of the adduct generated by pathways a and b would produce PMP, but denaturation of the adduct by pathway c would produce the same PLP adduct (**7**) observed by Metzler and coworkers[8,9] from serine *O*-sulfate inactivation of aspartate aminotransferase and glutamate decarboxylase. Scheme 2 depicts an enzyme-catalyzed cyclization of the initial PLP-bound **1** to give PLP-bound cycloserine. The mechanism of inactivation of GABA-AT by cycloserine is unknown. In this paper we describe our studies on the inactivation of GABA-AT by **1** which show that **1** is a cycloserine prodrug that is activated non-enzymatically.



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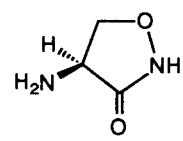

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Results

Inactivation of [^{3}H]PLP-reconstituted GABA-AT by L-3-chloroalanine hydroxamate (1)

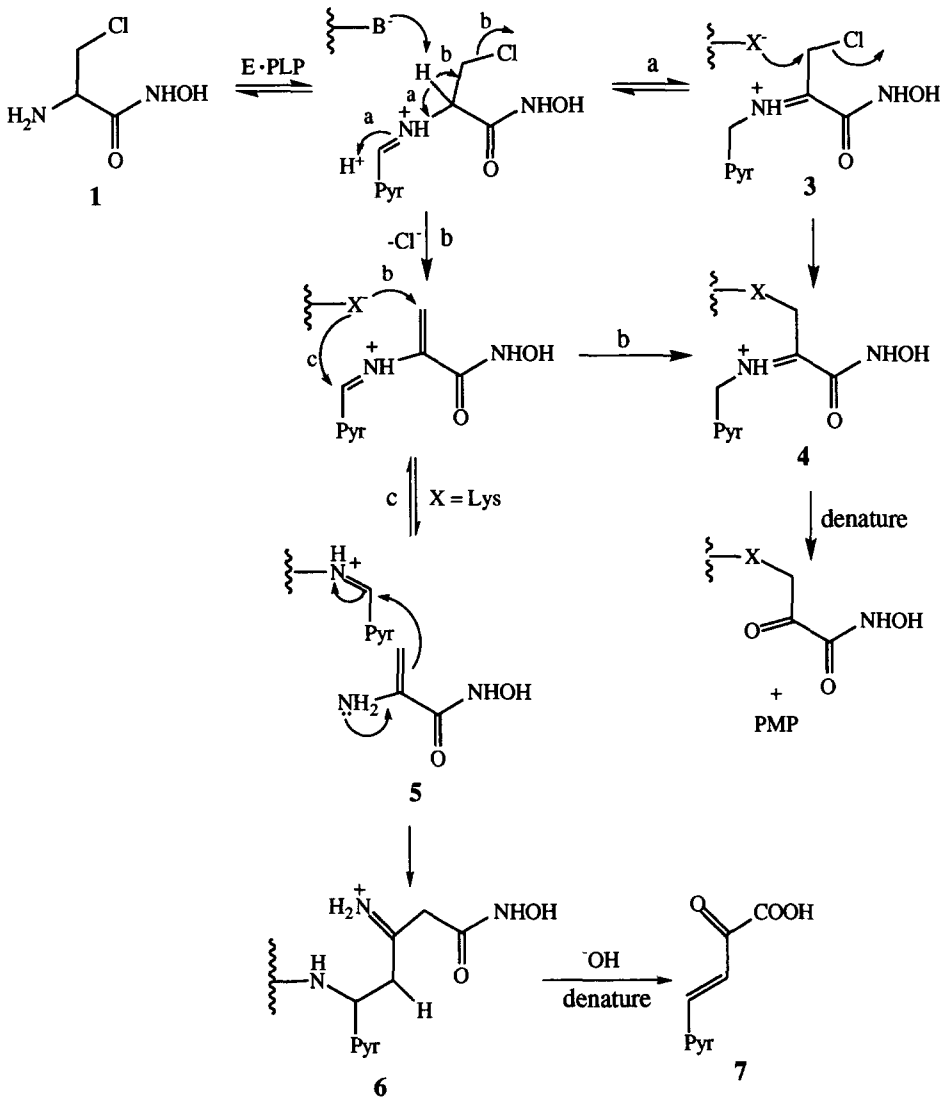
GABA-AT, which was reconstituted with [^{3}*H*]PLP, was allowed to incubate with L-3-chloroalanine hydroxamate (**1**) until complete inactivation occurred. The pH of the inactivated enzyme was raised to 11, then the enzyme was acid denatured and the supernatant was analyzed by HPLC. No compound **7**[8,9] (Scheme 1) or PMP was observed; only a new unknown metabolite was formed, which was identical to that observed when L-cycloserine was the inactivator.

Inactivation of GABA-AT by L-3-chloroalanine hydroxamate (1) and L-cycloserine (2)

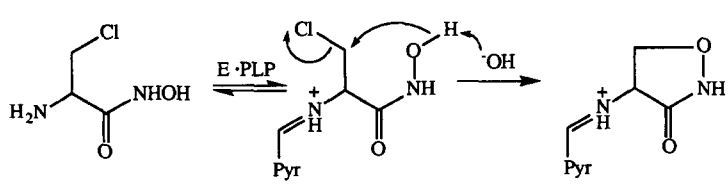
L-Cycloserine was a pseudo-first-order time-dependent inactivator of purified pig brain GABA-AT (Fig. 1A); the K_I and k_{inact} values determined from the Kitz and Wilson[10] replot (Fig. 1B) are 360 μM and 0.005 min^{-1}, respectively. L-3-Chloroalanine hydroxamate also was a time-dependent inactivator, but it did not exhibit pseudo-first-order kinetics and appeared to be concentration independent, even at a concentration as low as 2.5 μM (Fig. 2; see below for an explanation of the concentration independence). The rate of inactivation increased with time and the data could be fitted to a second-order polynomial equation. When a fresh aliquot of enzyme was added to the inactivated solution, the rate of inactivation increased (Fig. 2).

Effect of gabaculine-inactivated GABA-AT on the rate of inactivation of GABA-AT by L-3-chloroalanine hydroxamate

In order to determine if a peripheral group on the enzyme was responsible for conversion of L-3-chloroalanine hydroxamate into the active form, GABA-AT was inactivated with a known active-site inactivator, gabaculine.[11] The excess gabaculine was removed by dialysis, and then this inactivated enzyme was added to



Scheme 1.



Scheme 2.

fresh GABA-AT. Inactivation of GABA-AT by L-3-chloroalanine hydroxamate in the presence of gabaculine-inactivated GABA-AT was monitored relative to that of just GABA-AT (Fig. 3). There was essentially no difference in the rates of inactivation in the presence or absence of gabaculine-inactivated GABA-AT either when measured immediately after addition of L-3-chloroalanine hydroxamate or 2 h after L-3-chloroalanine hydroxamate was added (Fig. 3).

Reaction of L-3-chloroalanine hydroxamate with buffer

The reaction of L-3-chloroalanine hydroxamate with buffer was followed by NMR spectroscopy (Fig. 4), which indicated that the product formed was cycloserine. The conversion of L-3-chloroalanine hydroxamate to cycloserine results in the decrease in the L-3-chloroalanine hydroxamate peaks between 3.65 and 3.85 ppm and the build-up of cycloserine peaks between 4.0 and 4.5 ppm. The cycloserine hydrogens beta to the amino group (3.8–4.2 ppm) shift downfield by a little more than 0.1 ppm over the 54 h period. This shift is independent of pH changes between pH 8.1 and 8.5 and concentration changes from 17.8 to 97.5 mM. The spectra in Figure 4 also show the formation of some unknown degradation product(s) (1.8–2.2 ppm). Perhaps this product(s) is somehow coordinating to the cycloserine, causing the shift in the spectra.

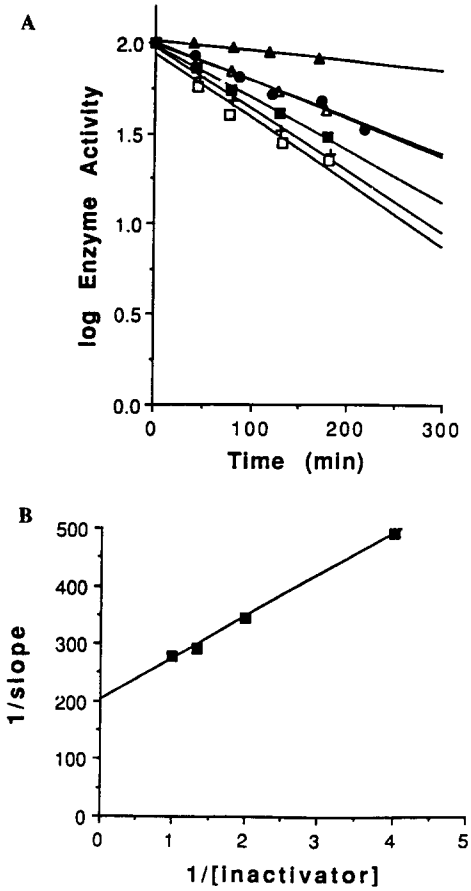


Figure 1. Time-dependent inactivation of GABA-AT by L-cycloserine. (A) Log of remaining enzyme activity vs time for L-cycloserine: 0.0 (▲), 0.25 (Δ), 0.50 (■), 0.75 (+), 1.0 (❑), 0.5 mM L-cycloserine and 5 mM GABA (●); (B) Absolute value of the slopes from the lines in A vs the inverse of the inactivator concentration.

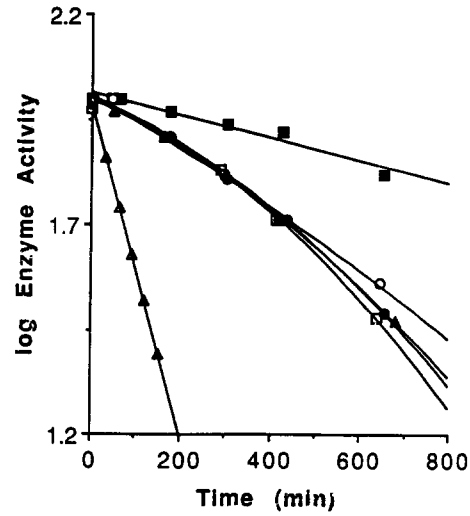


Figure 2. Time-dependent inactivation of GABA-AT by L-3-chloroalanine hydroxamate. Log of remaining enzyme activity vs time for L-3-chloroalanine hydroxamate: 0.00 (■), 0.25 (❑), 0.50 (●), 0.75 (○), and 1.0 (▲) mM. Addition of fresh enzyme to the inactivated solution to give a 0.75 mM solution (Δ). The 0.25–1.0 mM points are fitted to second order polynomial equations (lines). See the Experimental Section for details.

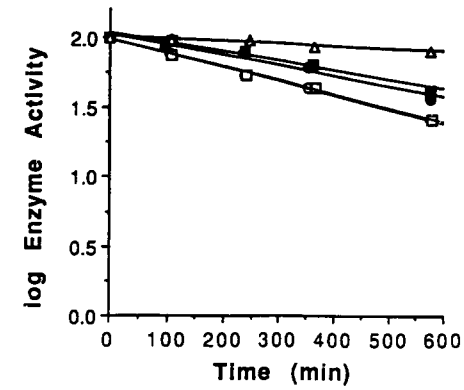


Figure 3. Effect of added gabaculine-inactivated GABA-AT (1.26 μM final concentration) on the rate of inactivation of GABA-AT (1.26 μM final concentration) by L-3-chloroalanine hydroxamate. Using 1.0 mM solutions of 3-chloroalanine hydroxamate, the rate of inactivation was studied with (■) and without (●) added gabaculine-inactivated enzyme. This experiment was repeated using hydroxamate solutions which were allowed to react in 50 mM KPPi, pH 8.5 buffer for 2 h prior to addition of fresh GABA-AT, again with (❑) and without (○) added gabaculine-inactivated enzyme. A control (Δ) contained gabaculine-inactivated enzyme but no L-3-chloroalanine hydroxamate.

Deuterium isotope effect upon cyclization of L-3-chloroalanine hydroxamate

If the mechanism of cyclization involves removal of the hydroxamate hydroxyl hydrogen in the rate-determining step, there should be a solvent deuterium isotope effect on this reaction. It was not possible to get accurate kinetic data by NMR spectroscopy; however, there is a 40-fold difference in the extinction coefficient at 230 nm for L-3-chloroalanine hydroxamate and cycloserine and, therefore, the conversion can be monitored by following the change in the absorption spectrum with time (Fig. 5). Conversion of L-3-chloroalanine hydroxamate into cycloserine

exhibits a small primary solvent deuterium isotope effect; at pH 7 the isotope effect is 2.0 and at pH 8.5 it is 1.2. In the lower pH solution the O–H bond cleavage would be expected to be more rate determining than at the higher pH because of the lower base strength at the lower pH. At pH 6.0 and 6.5 no cyclization occurs after 1 h of incubation.

Correlation of the buffer-catalyzed reaction and inactivation of GABA-AT

To show that the buffer-catalyzed conversion of L-3-chloroalanine hydroxamate into L-cycloserine is responsible for the inactivation of GABA-AT by L-3-chloroalanine hydroxamate, the hydroxamate was incubated in 50 mM potassium phosphate buffer, pH 8.5 for 20 h (time sufficient for complete conversion of L-3-chloroalanine hydroxamate to cycloserine), and the rate of GABA-AT inactivation by that solution was monitored relative to a solution of authentic (commercial) L-cycloserine incubated under identical conditions. There was no significant difference in the rates of inactivation by the two solutions; the $K_{I\ and}\ k_{inact}$ values obtained were 400 μM and 0.004 min^{-1}, respectively.

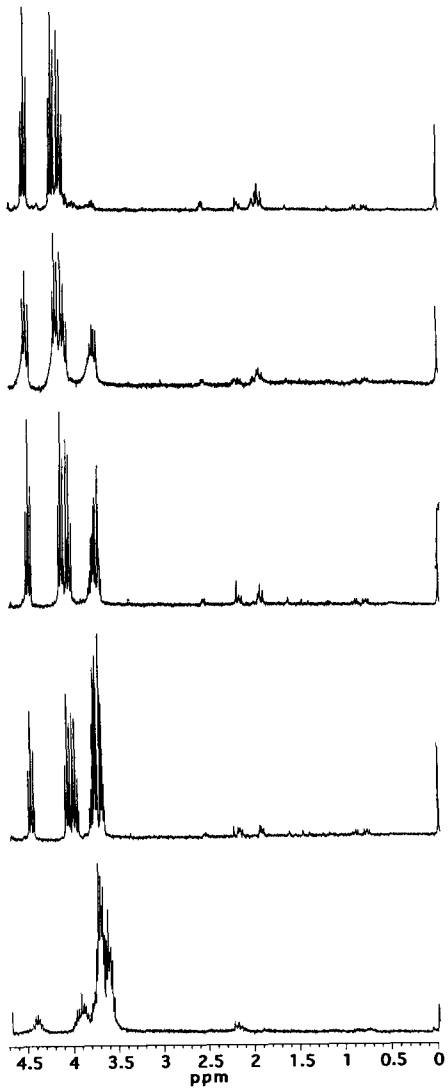


Figure 4. NMR spectra of the reaction of L-3-chloroalanine hydroxamate in deuterated buffer (50 mM potassium pyrophosphate, pH 8.5). Reaction times from the bottom spectrum to the top spectrum are 20 min, 2.5, 7, 14, and 54 h.

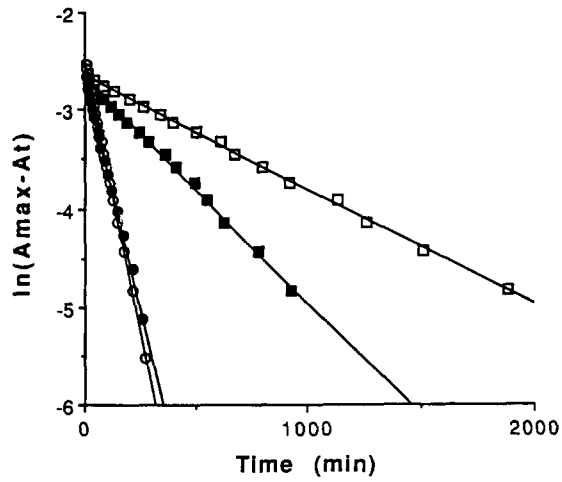


Figure 5. Rate of conversion of L-3-chloroalanine hydroxamate to cycloserine. $\ln[A_{230}\ (max) - A_{230}\ (t)]$ for the cyclization of 3-chloroalanine hydroxamate to cycloserine at pH 7.0 in protonated (❑) and deuterated (■) buffer and at pH 8.5 in protonated (○) and deuterated (●) buffer.

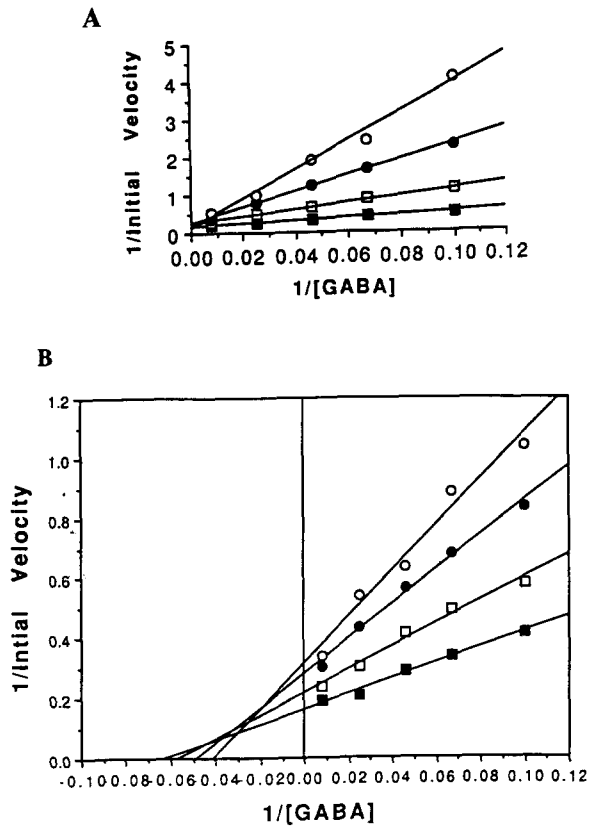


Figure 6. (A) Lineweaver–Burk analysis[12] of initial velocity measurements using varying concentrations of L-cycloserine [0.0 (■), 1.0 (❑), 2.0 (●), and 3.0 (○) mM] and GABA (10–120 mM). (B), Lineweaver–Burk analysis as in A except using the same concentrations of L-3-chloroalanine hydroxamate.

Reversible inhibition of GABA-AT by L-cycloserine and L-3-chloroalanine hydroxamate

To show that cycloserine acts at the active site of GABA-AT and L-3-chloroalanine hydroxamate does not, Lineweaver–Burk analyses[12] of these two compounds were carried out. The rate of inactivation of GABA-AT is slow enough that initial rate data is possible. Figure 6 shows that cycloserine (A) is a competitive inhibitor of GABA-AT (K_i = 35 μM) but L-3-chloroalanine hydroxamate (B) is a mixed inhibitor. In order to prevent the L-3-chloroalanine hydroxamate from cyclizing to cycloserine prior to its addition to the enzyme in this experiment, the L-3-chloroalanine hydroxamate solution was made up in water and added last to the enzyme solution; no cyclization occurs in unbuffered water (pH about 4) for over a period of at least a week. L-Cycloserine also was shown to be competitive by the fact that its inactivation of GABA-AT is inhibited by GABA (Fig. 1A).

Concentration independence of the inactivation of GABA-AT by L-3-chloroalanine hydroxamate

When α-ketoglutarate was omitted from the incubation buffer, the kinetic constants for the inactivation of GABA-AT by L-cycloserine (Fig. 7) were K_I = 310 μM and k_{inact} = 0.325 min^{-1} (in the presence of α-ketoglutarate they were K_I = 360 μM and k_{inact} = 0.005 min^{-1}). Under these conditions inactivation of GABA-AT by L-3-chloroalanine hydroxamate is time- and concentration-dependent, but still not pseudo-first-order (Fig. 8).

Discussion

There are several potential mechanisms for the inactivation of GABA-AT by L-3-chloroalanine hydroxamate which are shown in Schemes 1 and 2. The key experiment that eliminated all of the pathways in Scheme 1 was that inactivation followed by denaturation

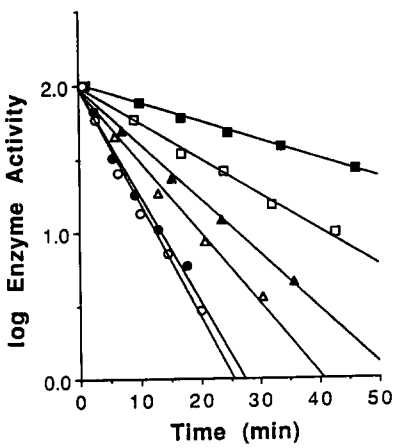


Figure 7. Time-dependent inactivation of GABA-AT by L-cycloserine in the absence of α-ketoglutarate. (A) Log of remaining enzyme activity vs time for L-cycloserine: 0.0125 (■), 0.025 (❑), 0.04 (▲), 0.055 (Δ), 0.085 (●), and 0.1 (○) mM.

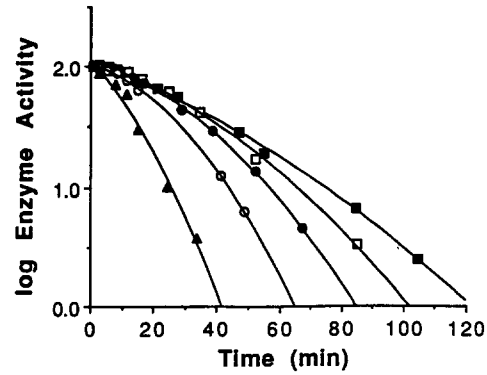


Figure 8. Time-dependent inactivation of GABA-AT by L-3-chloroalanine hydroxamate in the absence of α-ketoglutarate. Log of remaining enzyme activity vs time for L-3-chloroalanine hydroxamate: 0.06 (■), 0.08 (❑), 0.1 (●), 0.15 (○), and 0.3 (▲) mM. The data are fitted to second order polynomial equations (lines). See the Experimental Section for details.

does not produce any PMP (pathways a and b) or **7** (pathway c). However, a new metabolite is generated which is identical to the unknown metabolite produced under these conditions when L-cycloserine is the inactivator. Three other mechanisms can be considered, an enzyme-catalyzed conversion of PLP-bound L-3-chloroalanine hydroxamate to PLP-bound L-cycloserine (Scheme 2), an enzyme-catalyzed conversion of unbound L-3-chloroalanine hydroxamate to L-cycloserine prior to inactivation, or a nonenzymatic (buffer-catalyzed) conversion of L-3-chloroalanine hydroxamate to L-cycloserine prior to reaction with the enzyme-bound PLP. L-3-Chloroalanine hydroxamate was a time-dependent inactivator, but it did not exhibit pseudo-first-order kinetics (Fig. 2). Instead, the rate of inactivation increased with time and the data could be fitted to a second-order polynomial equation. If the inactivation were the result of an enzyme-catalyzed conversion of PLP-bound L-3-chloroalanine hydroxamate to PLP-bound L-cycloserine followed directly by inactivation, then pseudo-first-order loss of enzyme activity should have been observed. This anomalous kinetic behavior can be explained in several different ways: L-3-chloroalanine hydroxamate could be a slow-binding inhibitor,[13] it could be converted into the actual inactivator by some group on the enzyme, or it could be converted into the inactivator by the buffer. When fresh aliquots of enzyme were added to the inactivated solutions, the rates of inactivation increased (Fig. 2). This result is consistent with the conversion of L-3-chloroalanine hydroxamate into the actual inactivating species over time; after the initial inactivation period, a higher concentration of the actual inactivator would be in solution, so fresh enzyme added would be inactivated at a faster rate than the initial aliquot of enzyme. If L-3-chloroalanine hydroxamate were a slow-binding inhibitor, the rate of inactivation of a fresh aliquot of enzyme would be the same as that for the initial aliquot of enzyme.

To determine if an enzyme-catalyzed conversion of non-enzyme-bound L-3-chloroalanine hydroxamate to L-cycloserine was occurring, a comparison of the rate of inactivation of GABA-AT by L-3-chloroalanine

hydroxamate was made with the rate of inactivation of the same concentration of L-3-chloroalanine hydroxamate in the presence of gabaculine-inactivated GABA-AT. Gabaculine is known to attach to the active-site PLP;[11] therefore, only peripheral groups would be available for catalysis. No difference was observed in the two reactions (Fig. 3). Therefore, a peripheral group on GABA-AT is not responsible for the conversion of L-3-chloroalanine hydroxamate to the activated form.

The remaining possibility considered is that L-3-chloroalanine hydroxamate is converted to L-cycloserine by the buffer. The nonenzymatic conversion of L-3-chloroalanine hydroxamate into L-cycloserine was followed by NMR spectroscopy (Fig. 4) and UV–vis spectroscopy (Fig. 5), and it was found to be a facile process. Furthermore, when L-3-chloroalanine hydroxamate was allowed to incubate in buffer long enough to be converted to cycloserine, then this solution was used to inactivate GABA-AT, the same rate of inactivation was observed as that for an authentic sample of cycloserine. This supports a buffer-catalyzed conversion of L-3-chloroalanine hydroxamate into cycloserine as the mechanism of activation of L-3-chloroalanine hydroxamate and suggests that L-3-chloroalanine hydroxamate itself is not the inactivator of GABA-AT. The observation that L-3-chloroalanine hydroxamate is not a competitive inhibitor of GABA-AT (Fig. 6) also is consistent with the notion that it is not directly responsible for enzyme inactivation.

The rate of inactivation of GABA-AT by L-cycloserine in the presence of α-ketoglutarate was much slower than in the absence of α-ketoglutarate (Fig. 7). Also, inactivation of GABA-AT by L-3-chloroalanine hydroxamate in the presence of α-ketoglutarate was found to be concentration independent, but in the absence of α-ketoglutarate, it was concentration dependent (Fig. 8). The function of α-ketoglutarate is to convert the PMP that is formed during transamination back to PLP. In its absence, the enzyme becomes inactivated because it remains in the PMP form. Therefore, in the absence of α-ketoglutarate the more rapid rate of inactivation by L-cycloserine reflects its rate of transamination (which results in inactive enzyme), not the rate of mechanism-based inactivation. The rate of inactivation of GABA-AT by L-3-chloroalanine hydroxamate becomes concentration independent in the presence of α-ketoglutarate because the rate of conversion of L-3-chloroalanine hydroxamate to L-cycloserine is fast relative to the rate of inactivation of GABA-AT by L-cycloserine (the rate-determining step), and saturation is reached at all concentrations of L-3-chloroalanine hydroxamate used.

In conclusion, L-3-chloroalanine hydroxamate is a time-dependent inactivator of GABA aminotransferase, but the mechanism of inactivation involves buffer-catalyzed conversion of L-3-chloroalanine hydroxamate to cycloserine prior to inactivation. The mechanism of inactivation of GABA-AT by L-cycloserine is currently under investigation.

Experimental

General procedures

Proton NMR spectra were recorded on a Varian Gemini 300 MHz instrument. Mass spectra were obtained on a VG 70-250SE high resolution spectrometer. Melting points were done on a Mel-Temp capillary tube melting point apparatus and are uncorrected. pH measurements were made with an Orion 601A pH meter and a 8115SC electrode. Enzyme activity was measured on a Perkin-Elmer Lambda 1 spectrophotometer with a constant temperature cuvette holder as described under Enymes and Assays.

Reagents

All reagents were purchased from Aldrich Chemical Co. with the exceptions of potassium pyrophosphate, α-ketoglutarate, β-mercaptoethanol, $NADP^+$, and GABA, which were purchased from Sigma Chemical Co. Dibasic and monobasic potassium phosphate were purchased from Mallinckrodt Chemical Co.

N-tert-*Butylcarbonyl-L-3-chloroalanine*. The method of Bodanszky and Bodanszky[14] was modified as follows. L-3-Chloroalanine (1 g, 6.25 mmol) was dissolved in 1 M NaOH (12.5 mL, 12.5 mmol) and dioxane (12.5 mL). This solution was cooled in an ice bath and di-*tert*-butyl dicarbonate (1.6 mL, 6.88 mmol) was added and stirred for 0.5 h at room temperature. The solution was concentrated to 8 mL, covered with 20 mL EtOAc, cooled in an ice bath, and acidified with 1 M $KHSO_4$ to pH 2–3. The aqueous layer was extracted twice with 10 mL of EtOAc. The EtOAc layers were pooled and washed twice with 20 mL H_2O. This solution was dried over Na_2SO_4 and the solvent was removed by rotary evaporation. The product was recrystallized from EtOAc–hexane to give 1.25 g (89%) of *N-tert*-butylcarbonyl-L-3-chloroalanine as a white solid; mp 124–126 °C; ^1H NMR ($CDCl_3$): δ 1.48 (*s*, 9H), 3.88 (*d*, 1H, J = 11 Hz), 4.04 (*d*, 1H, J = 11 Hz), 4.79 (*d*, 1H, J = 8 Hz), 5.47 (*d*, 1H, J = 8 Hz), 9.36 (*s*, 1H). Anal. calcd for $C_8H_{14}NO_4Cl$: C, 42.96; H, 6.31; N, 6.26. Found: C, 42.75; H, 6.26; N, 6.05.

L-3-Chloroalanine hydroxamate hydrochloride (1). The method of Bodanszky and Bodanszky[14] was modified as follows. *N-tert*-Butylcarbonyl-L-3-chloroalanine (0.67 g, 3 mM) was dissolved in THF (10 mL), cooled to –18 °C, and neutralized with Et_3N (0.418 mL). Isobutyl chlorocarbonate (0.428 mL, 3.3 mM) was added, and after 1 min, hydroxylamine hydrochloride (0.312 g, 4.5 mM), dissolved in a minimum amount of DMF and neutralized with Et_3N (0.570 mL), was added; residual hydroxylamine was washed into the reaction flask with THF (6 mL). The solution was allowed to warm to room temperature and was stirred for 0.5 h, after which time the Et_3NH^+ salt was removed by filtration. The filtrate was rotary evaporated to an oil, which was dissolved in EtOAc (5 mL) and washed twice with H_2O (10 mL each). The solvent was removed by rotary evaporation to give an oil, which was dissolved in EtOAc containing 3 M HCl, causing cleavage of the

protecting group. The resulting white, needle-like solid (0.19 g, 36%) that formed overnight was filtered and washed with EtOAc; mp 194 °C (dec.) (lit.[15] mp 191 °C (dec.)); ^{1}H NMR (D_2O): δ 4.35 (*dd*, 1H, *J* = 5.7 and 4.4 Hz), 3.99 (*dd*, 2H, *J* = 5.7, 4.4 Hz). HRMS: calcd for $C_3H_8O_2N_2Cl$ 139.0274, found 139.0246.

Cyclization of L-3-chloroalanine hydroxamate to L-cycloserine

L-3-Chloroalanine hydroxamate (5.0 mg, 57.5 mM) was added to 500 μL (57.5 mM) of 50 mM potassium pyrophosphate buffer in D_2O at pH 8.5 containing 3-(trimethylsilyl)propionic acid as a NMR standard. After the addition was complete the pH was brought back to pH 8.5 with NaOD. The NMR spectra were taken at the time intervals noted in Figure 4.

Deuterium isotope effect on cyclization of L-3-chloroalanine hydroxamate to cycloserin

For the experiment at pH 8.5, 0.05 mM L-3-chloroalanine hydroxamate solutions were made up in 200 mM potassium pyrophosphate buffer in H_2O and D_2O. The absorbance at 230 nm was observed until it no longer increased. The natural logarithm of the maximum absorbance minus the absorbance at various time intervals vs time was plotted. The slope of the line from the protonated buffer was divided by the slope of the line generated in deuterated buffer for the calculation of the isotope effect. This experiment was repeated at pH 7.0 using 200 mM H_3PO_4 (or D_3PO_4) and NaOH or NaOD to adjust the pH.

Enzymes and assays

Pig brain GABA-AT (sp. act. 3.75 units mg^{-1}), Gabase, and succinic semialdehyde dehydrogenase were obtained and assayed as previously described.[16]

Inactivation of [^{3}H]PLP-reconstituted GABA-AT by L-3-chloroalanine hydroxamate (1)

This experiment was carried out as reported previously for γ-vinylGABA.[17]

Time-dependent inactivation of GABA-AT by L-cycloserine and L-3-chloroalanine hydroxamate

Purified GABA-AT (15 μL; 1.72 mg mL^{-1}) was added to solutions of either L-cycloserine or L-3-chloroalanine hydroxamate (135 μL; final concentration ranges are shown in Figs 1 and 2), in 50 mM potassium pyrophosphate buffer, pH 8.5 containing 6 mM α-ketoglutarate and 0.01 mM β-mercaptoethanol at 25 °C (the hydroxamate was in a H_2O stock solution and was added to the buffer solution immediately before use). Experiments also were carried out in which the α-ketoglutarate and β-mercaptoethanol were omitted. At timed intervals aliquots (10 μL) were withdrawn and added to the assay solution (580 μL) containing excess succinic semialdehyde dehydrogenase. Rates were measured spectrophotometrically at 340 nm and the logarithm of remaining activity was plotted against time for each concentration of inhibitor. A secondary plot of 1/slope of these lines vs 1/[inactivator][10] was constructed to determine K_I and k_{inact} values for cycloserine. To show substrate protection against L-cycloserine inactivation, solutions containing 0.5 mM cycloserine and 5 mM GABA were assayed for inactivation.

Effect of gabaculine-inactivated GABA-AT on the rate of inactivation of GABA-AT by L-3-chloroalanine hydroxamate

GABA-AT (1.26 μM) was incubated with gabaculine (57 mM) in 50 mM potassium pyrophosphate, pH 8.5 buffer. When no activity remained, the excess inactivator was removed by the method of Penefsky[18] then an equal amount of this inactivated enzyme solution or buffer was added to a fresh aliquot of GABA-AT (1.26 μM). The enzyme solution with and without the gabaculine-inactivated GABA-AT were incubated with 3-chloroalanine hydroxamate (final conc. 1.0 mM) and aliquots were assayed periodically.

Time-dependent inactivation of GABA-AT by L-cycloserine obtained from the buffer catalyzed cyclization of 3-chloroalanine hydroxamate

A 7.81 mM solution of 3-chloroalanine hydroxamate in 50 mM potassium pyrophosphate, pH 8.5 buffer was allowed to incubate at room temperature for 20 h. GABA-AT (1.26 μM) was incubated with this solution (final concentration 1 mM) and with commercially available L-cycloserine (final concentration 1 mM) and assayed as described above.

Inhibition of GABA-AT by L-cycloserine and L-3-chloroalanine hydroxamate

GABA-AT (1.72 mg mL^{-1}) was incubated at 25 °C with 5 mM α-ketoglutarate, 1 mM $NADP^+$, 5 mM β-mercaptoethanol, excess succinic semialdehyde dehydrogenase, and varying concentrations of GABA (10–120 mM) in 400 mM potassium phosphate buffer, pH 7.4. L-Cycloserine or L-3-chloroalanine hydroxamate were prepared in a dionized H_2O solution and were added to the cuvette last after addition of enough NaOH to neutralize the inhibitor. The final concentration of the inhibitor ranged from 0.0 to 3.0 mM. Initial rates were measured spectrophotometrically, and the kinetic parameters were derived from Lineweaver–Burk plots[12] with five different GABA concentrations for every concentration of L-cycloserine or L-3-chloroalanine hydroxamate.

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