Articles

Slow-Binding Inhibition of γ-Aminobutyric Acid Aminotransferase by Hydrazine Analogues[†,‡]

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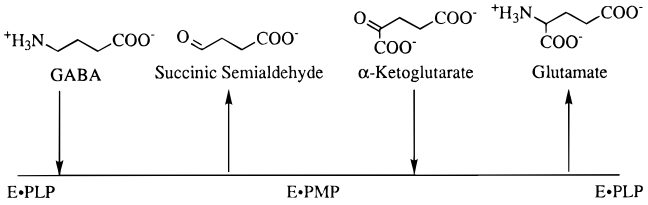
(3-Hydroxybenzyl)hydrazine and methylhydrazine have been found to be potent slow-binding inhibitors of the pyridoxal 5-phosphate (PLP)-dependent enzyme γ-aminobutyric acid aminotransferase (GABA-AT). Both compounds follow mechanism A (Morrison, J. F.; Walsh, C. T. *Adv. Enzymol.* **1988**, *61*, 201−301) which does not involve formation of a rapidly reversible enzyme−inhibitor complex before the formation of the final tight complex. The rate constant for formation of the enzyme−inhibitor complex determined from the slow-binding kinetics was 2.08×10^3 and 1.98×10^4 M^{-1} min^{-1} for methylhydrazine and (3-hydroxybenzyl)hydrazine, respectively. The rate constant for dissociation of the enzyme−inhibitor complex determined for the slow-binding kinetics was 4.6×10^{-3} and 5×10^{-3} min^{-1}, respectively. The inhibition constants calculated from the slow-binding inhibition kinetics are 2.2 μM for methylhydrazine and 0.3 μM for (3-hydroxybenzyl)hydrazine. Reactivation of the inhibited enzyme was not first order, perhaps due to a side reaction of the hydrazine, but was consistent with the results obtained from the slow-binding kinetics. Inhibition constants were calculated from the level of enzyme activity at equilibrium inhibition. These constants are 2.8 and 0.46 μM for methylhydrazine and (3-hydroxybenzyl)hydrazine, respectively, in good agreement with those calculated from the slow-binding inhibition kinetics. 3-Hydrazinopropionate also behaved as a slow-binding inhibitor. However, the dependence of its kinetics on the concentration of inhibitor could not be described by the slow-binding or slow, tight-binding inhibition models. These kinetics could not be described by the tight-binding character of the inhibition because the addition of the competitive inhibitor propionic acid at 100 times its K_i did not affect the shape of the curve for inhibitor concentration dependence. The slow-binding inhibition appeared to require 2−4 molecules of 3-hydrazinopropionate/enzyme. The reactivation of enzyme inhibited by 3-hydrazinopropionate was first order with a rate constant of 6.9×10^{-3} min^{-1}. Its equilibrium inhibition constant was calculated to be <20 nM. However, the inhibition constant calculated was dependent on the concentration of inhibitor because of the unusual character discussed above and may be much lower. Only 1 PLP/enzyme dimer reacted with methylhydrazine or (3-hydroxybenzyl)hydrazine, as indicated by Scatchard plots, or with 3-hydrazinopropionate, as shown by a spectrophotometric titration. Slow-binding inhibition does not appear to be the result of a significant enzyme conformational change because there is no change in the tryptophan fluorescence of GABA-AT upon binding either methylhydrazine or 3-hydrazinopropionate. Implications for the design of hydrazine inhibitors of GABA-AT are discussed.

γ-Aminobutyrate aminotransferase (EC 2.6.1.19; GABA-AT), an important target site for the development of anticonvulsants,[1] is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that carries out the degradation of the inhibitory neurotransmitter GABA to succinic semialdehyde while stoichiometrically producing the stimulatory neurotransmitter glutamate from α-ketoglutarate. GABA-AT follows a ping-pong mechanism (Scheme 1) in which GABA reductively transaminates the lysyl aldimine of PLP to pyridoxamine (PMP). The α-ketoglutarate then reacts with the PMP to give glutamate and the lysine-bound PLP.

Scheme 1



The inhibition of GABA-AT has been the target of a great deal of research because of the importance of maintaining GABA levels in the prevention of convulsions and for other psychopharmacological effects.[1−4]

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[‡] Abbreviations: $NADP^+$, nicotinamide adenine dinucleotide phosphate; GABA, γ-aminobutyric acid; $NaDodSO_4$−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GABA-AT, GABA aminotransferase; HPA, 3-hydrazinopropionate.

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Chart 1

GABA

3-Hydroxybenzylhydrazine

3-Hydrazinopropionate

Methylhydrazine

The enzymatic mechanism of PLP-dependent enzymes is well understood and has provided a wealth of mechanism-based inactivators.[5]

Some of the earliest potent inhibitors of GABA-AT to be evaluated were hydrazines and hydroxylamines[6–8] which generally had low micromolar or nanomolar inhibition constants. A number of these compounds were demonstrated to raise GABA levels or alter GABA-AT activity in the brain.[9–12]

Several hydrazine analogues have been successfully used as pharmaceutical agents. Important examples include hydralazine, an antihypertensive; phenelzine and isocarboxazid, antidepressives; procarbazine, an antineoplastic; and isoniazid, the most important treatment of tuberculosis.[13] However, hydrazines and hydroxylamines are general carbonyl-trapping agents that inhibit a number of PLP- and pyruvate-requiring enzymes and are, therefore, generally cytotoxic. Isoniazid is administered with pyridoxine to prevent nervous system disorders, including convulsions.[13] In this fashion, isoniazid is relatively nontoxic,[14] demonstrating that, with sufficient specificity, other hydrazine analogues should be good pharmaceutical agents. Since the mechanism of inhibition of GABA-AT by hydrazine analogues is not well characterized, careful study of their mechanism should provide insights into how to develop more specific hydrazine-based inhibitors.

Over the past decade, a greater understanding of the kinetics of slow-binding inhibitors has developed.[15] More interest in the kinetics of hydrazine and hydroxylamine analogue time-dependent inhibition has resulted in the characterization of the inhibition of aspartate aminotransferase,[16–18] alanine aminotransferase,[18] and the quinoprotein methylamine dehydrogenase.[19] These authors found the hydrazine and hydroxylamine analogues to be slow-binding inhibitors. 3-Hydrazinopropionate was reported to be a mechanism-based inactivator of GABA-AT from the nematode *Nippostrongylus basiliensis*[20] with a $K_i = 174$ nM and a $k_{inact} = 2.7$ min^{-1}; however, their studies did not exclude the possibility that 3-hydrazinopropionate was acting as a slow, tight-binding inhibitor. According to a definition for mechanism-based inactivation,[5] a compound that inactivates an enzyme simply by reacting with its cofactor is not a mechanism-based inactivator but an affinity-labeling agent. We recently demonstrated that mechanism-based inactivation cannot account for the time-dependent inhibition of GABA-AT from pig brain by (3-hydroxybenzyl)hydrazine.[21]

The kinetics of three time-dependent inhibitors of GABA-AT, methylhydrazine, (3-hydroxybenzyl)hydrazine, and 3-hydrazinopropionate, which vary significantly in their structural similarity to the substrate GABA (Chart 1) are described here. Furthermore, since mechanism-based inactivation had been previously ruled out for (3-hydroxybenzyl)hydrazine,[21] reversal kinetics for all three time-dependent inhibitors were

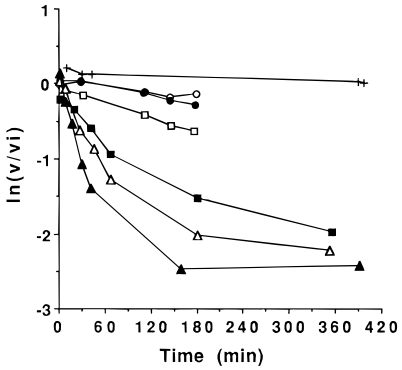


Figure 1. Time-dependent inhibition kinetics of methylhydrazine with GABA-AT. Methylhydrazine was incubated with 0.82 μM GABA-AT in 50 mM potassium pyrophosphate, pH 8.5, 25 °C. Concentration of methylhydrazine: 0 (+), 0.5 (○), 1.3 (●), 2.5 (□), 5.0 (■), 10 (△), and 20 (▲) μM.

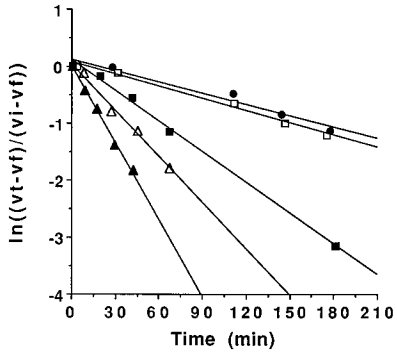


Figure 2. Time-dependent inhibition kinetics of methylhydrazine with GABA-AT plotted as slow-binding inhibition. The raw data are the same as in Figure 1. The dissociation constant was assumed to be 1.3 μM as calculated from the final velocities for 5.0, 10, and 20 μM methylhydrazine.

Scheme 2

$$E + I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E{\bullet}I$$

Scheme 3

$$E + I \underset{K_i}{\rightleftharpoons} E{\bullet}I \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{\bullet}I^*$$

evaluated to see if they were consistent with slow-binding inhibition. Tryptophan fluorescence of the enzyme was used to observe conformational changes of GABA-AT upon binding of the inhibitors. Finally, a comparison of the results is made to see whether any predictors of specificity of inhibition can be established. Such predictors might allow for the synthesis of hydrazine and hydroxylamine analogues that are selective for GABA-AT.

Results

Slow-Binding Inhibition of GABA-AT by Methylhydrazine and (3-Hydroxybenzyl)hydrazine. The kinetics of slow-binding inhibition by methylhydrazine are shown in Figure 1. As the inhibition of GABA-AT approaches equilibrium, the first-order plot exhibits curvature. A linear plot would be expected for irreversible inhibition, including most cases of mechanism-based inactivation.[22] The same data can be plotted as $\ln((v_t - v_f)/(v_i - v_f))$ vs time, where v_t is the remaining activity at time t, v_f is the activity at equilibrium inhibition, and v_i is the activity in the absence of inhibitor. This plot,

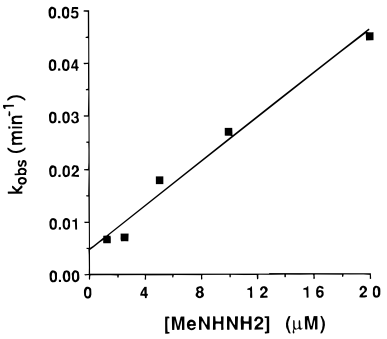


Figure 3. Replot of the slow-binding kinetics versus methylhydrazine concentration. k_{obs} is the slope from the linear fit in Figure 2. Data are fitted to the equation $k_{obs} = (4.6 \times 10^{-3}\ min^{-1}) + (2.08 \times 10^{3}\ M^{-1}\ min^{-1})[I]$.

Table 1. Kinetic Constants for Slow-Binding Inhibition of GABA-AT by Hydrazines

$RNHNH_2$	mech	k_3 ($\times 10^3\ M^{-1}\ min^{-1}$)	k_4 ($\times 10^{-3}\ min^{-1}$)	K_i (μM)	r^2
R = Me	A	2.08 ± 0.16	4.6 ± 1.7	2.2 ± 0.8	0.980
R = 3-HOBn	A	19.8 ± 1.8	5 ± 4	0.3 ± 0.2	0.920

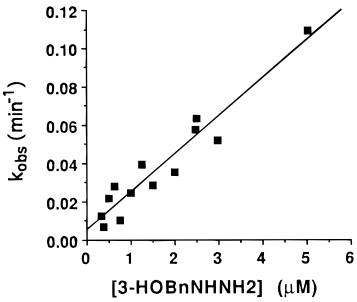


Figure 4. Replot of the slow-binding kinetics for (3-hydroxybenzyl)hydrazine versus inhibitor concentration. (3-Hydroxybenzyl)hydrazine (0.31−5.0 μM) was incubated with 0.10 μM GABA-AT in 50 mM potassium pyrophosphate, pH 8.5, 25 °C. Data are fitted to the equation $k_{obs} = (5 \times 10^{-3}\ min^{-1}) + (19.8 \times 10^{3}\ M^{-1}\ min^{-1})[I]$.

shown in Figure 2, results in the linearization of the data. The rate constants determined from Figure 2 (k_{obs}) are plotted vs inhibitor concentration in Figure 3. These data were fitted to two different slow-binding mechanisms described by Morrison and Walsh,[15] the mechanism in Scheme 2 ($r^2 = 0.980$, Table 1) and the mechanism in Scheme 3 ($r^2 = 0.981$, data not shown). Note that in the case of slow-binding inhibition, the *y*-intercept describes the constant k_4 (eq 3; see the Experimental Section) or k_6 (eq 5). Therefore, it is expected to be nonzero. In the case of enzyme inactivation, the plot is expected to be hyperbolic and pass through the origin.[23]

Enzyme that had not undergone preincubation with methylhydrazine was introduced into an assay mixture containing variable amounts of methylhydrazine. The reaction rate was followed for <5 min. The rapidly reversible inhibition constant was determined from these data to be 1.8 mM; however, this constant should be considered a minimum because some slow-binding inhibition may have occurred within 5 min. Since this inhibition constant is at least 820 times that in Table 1 and at least 90 times the highest concentration of methylhydrazine studied, the contribution of a rapidly reversible complex to the overall kinetics would be exceedingly small. Therefore, taking into consideration

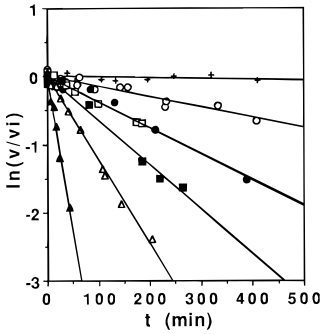


Figure 5. Time-dependent inhibition kinetics of 3-hydrazinopropionate with GABA-AT. 3-Hydrazinopropionate was incubated with 102 nM GABA-AT in 50 mM potassium pyrophosphate, pH 8.5, 25 °C. Concentration of 3-hydrazinopropionate (HPA): 0 (+), 75 (○), 113 (●), 151 (□), 226 (■), 302 (△), and 453 (▲) nM.

the lack of saturation in Figure 3 and the lack of rapidly reversible inhibition described above, the mechanism in Scheme 2 is favored for methylhydrazine.

Using the same analysis described above, the rate constants for slow-binding inhibition by (3-hydroxybenzyl)hydrazine are plotted vs inhibitor concentration in Figure 4. These data were fitted to the mechanism in Scheme 2 ($r^2 = 0.920$, Table 1) and the mechanism in Scheme 3 ($r^2 = 0.920$, data not shown). The fit to the mechanism in Scheme 3 resulted in the constants K_i and k_5 having exceedingly high values and high standard deviations, indicating that the mechanism in Scheme 2 provides the better fit. Therefore, both methylhydrazine and (3-hydroxybenzyl)hydrazine are slow-binding inhibitors of GABA-AT that do not require rapidly reversible complexes with the enzyme.

Attempts were made to evaluate the slow-binding inhibition of GABA-AT by 3-hydrazinopropionate. The kinetics were linear when plotted as $\ln(v/v_i)$ vs time (Figure 5), indicating that $v_f \approx 0$. The replot of the slopes demonstrated that neither the mechanism in Scheme 2 nor the mechanism in Scheme 3 fit the data (Figure 6). The data also were not fit by the slow, tight-binding model (eq 7). Upon the addition of the competitive inhibitor propionic acid (106 mM), a nonzero equilibrium velocity became apparent (Figure 7). The shape of the curve was not altered by the inclusion of the competitive inhibitor at a concentration 100 times its inhibition constant (Figure 7). Assuming that the hydrazine cannot bind to the GABA-AT−propionic acid complex, the inclusion of the competitive inhibitor reduces the amount of free enzyme available for binding by the hydrazine by 100-fold. The concentration of free enzyme in the experiment described in Figure 7 is ca. 2 nM, sufficiently below the lowest concentration of 3-hydrazinopropionate (280 nM) to be outside the range of tight-binding inhibition. At the least, it is expected that the curve should become more linear. This did not occur (Figure 6B). Therefore, neither attempt to fit the data to tight, slow-binding inhibition was successful. The kinetic profiles do suggest that more than one inhibitor is required to provide optimal inhibition. With this in mind, the data were fitted to eqs 8 and 9 (Figure 6A,B, respectively). The data with no propionic acid were best fitted by eq 8: $k = 0.70 \pm 0.03\ M^{-3.5}\ min^{-1}$, $b = (2.3 \pm 0.4) \times 10^{-3}\ min^{-1}$, and $n = 3.52 \pm 0.07$. The data with 106 mM propionic were best fitted by eq 9: $k = (4 \pm 2) \times 10^{-3}\ M^{-2.3}\ min^{-1}$ and $n = 2.3 \pm 0.4$. The value *n*

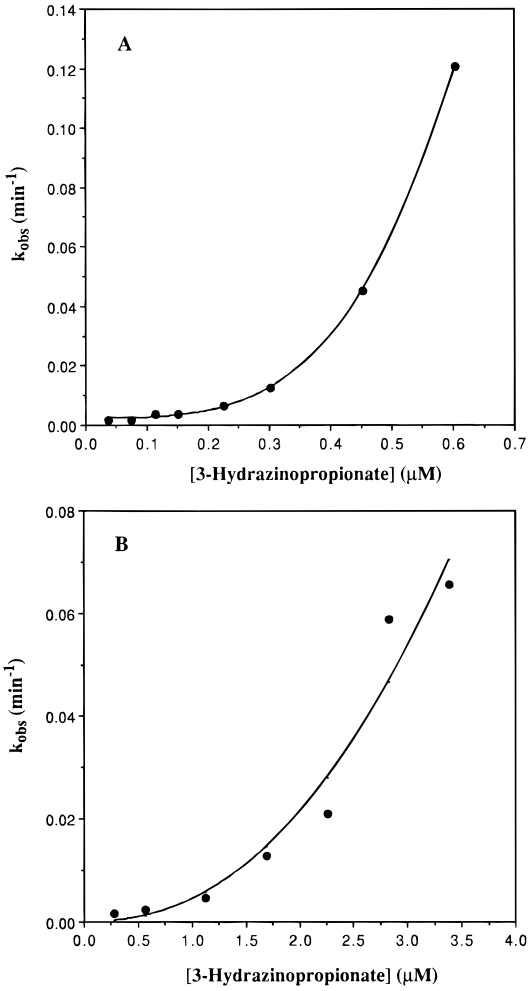


Figure 6. (A) Replot of the slow-binding kinetics for 3-hydrazinopropionate versus inhibitor concentration. 3-Hydrazinopropionate (38−604 nM) was incubated with 102 nM GABA-AT in 50 mM potassium pyrophosphate, pH 8.5, 25 °C. Data are fitted to the equation $k_{obs} = (0.70 \pm 0.03\ M^{-3.5}\ min^{-1})[I]^{3.52\pm0.07} + (2.3 \pm 0.4) \times 10^{-3}\ min^{-1}$. (B) 3-Hydrazinopropionate (0.28−2.8 μM) incubated with 198 nM GABA-AT and 106 mM propionic acid (K_i = 1.01 mM) in 50 mM potassium pyrophosphate, pH 8.5, 25 °C. Data are fitted to the equation $k_{obs} = ((4 \pm 2) \times 10^{-3}\ M^{-2.3})[I]^{2.3\pm0.4}$.

indicates the number of molecules of 3-hydrazinopropionate required to interact with the enzyme to get optimal inhibition. The inhibition constant cannot be calculated from this fit since k is not equivalent to k_3.

Competitive Inhibition of GABA-AT by Propionic Acid. The inhibition constant for propionic acid vs GABA was determined. Analysis of the data by nonlinear regression indicated that competitive inhibition provided the best fit, evaluated according to Mannervik.[24] This conclusion was in agreement with the Lineweaver−Burk analysis. The following values were determined by nonlinear regression: $V_{max} = 3.45 \pm 0.08$ μmol of GABA/min/mg of protein, $K_m = 1.35 \pm 0.09$ mM GABA, and $K_{is} = 1.01 \pm 0.07$ mM propionic acid. Surprisingly, the inhibition constant for propionic acid is similar to the Michaelis constant for GABA, even though propionic acid is incapable of reacting with PLP.

Kinetics of Reactivation of GABA-AT Inhibited by Methylhydrazine, (3-Hydroxybenzyl)hydrazine, and 3-Hydrazinopropionate. GABA-AT inhibited by any of the three hydrazines was reactivated upon dilution (Table 2). The data from the reactivations for

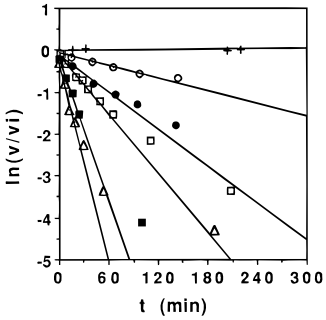


Figure 7. Time-dependent inhibition kinetics of 3-hydrazinopropionate with GABA-AT in the presence of propionic acid. 3-Hydrazinopropionate was incubated with 198 nM GABA-AT and 106 mM propionic acid (K_i = 1.01 mM) in 50 mM potassium pyrophosphate, pH 8.5, 25 °C. Concentration of 3-hydrazinopropionate (HPA): 0 (+), 1.1 (○), 1.7 (●), 2.3 (□), 2.8 (■), and 3.4 (△) μM. Linear fits were done on the first four data points of each curve.

Table 2. Reactivation Kinetic Constant and Dissociation Constant for GABA-AT with Hydrazines

$RNHNH_2$	k_{react} ($\times 10^{-3}$ min^{-1})	K_d (μM)
R = Me	~4.0	2.8 ± 0.4
R = 3-HOBn	~20.0	0.46 ± 0.19
R = $HOOCCH_2CH_2$	6.9	<0.02

methylhydrazine and (3-hydroxybenzyl)hydrazine did not fit well to a first-order model, and the determined rate constants were influenced by inhibitor concentration. The observation of these reactivations may have been complicated by another reaction, perhaps involving the reaction of the hydrazine with succinic semialdehyde (the product formed from GABA). However, the reactivation constant (k_{react}) for methylhydrazine is the same as k_4, and that for (3-hydroxybenzyl)hydrazine is only a factor of 4 different from k_4 determined by slow-binding inhibition methods (Table 1). The reactivation of 3-hydrazinopropionate was first order, possibly because the reaction between 3-hydrazinopropionate and succinic semialdehyde was too slow to affect this determination at the low concentrations of the hydrazine used.

Determination of the Dissociation Constant Using Final Velocities of Slow-Binding Inhibition. The final velocities were determined for GABA-AT incubated with the hydrazines until apparent equilibrium was attained. These final velocities were used to determine the equilibrium level of enzyme−inhibitor complex using eq 1. A Scatchard plot of methylhydrazine inhibition indicates that 1.2 methylhydrazines bind per enzyme with $K_i = 4.2$ μM ($r^2 = 0.863$). A Scatchard plot for (3-hydroxybenzyl)hydrazine indicates that 1.0 (3-hydroxybenzyl)hydrazine binds per enzyme with K_i = 0.60 μM ($r^2 = 0.972$). The data were fitted to eq 2, and the calculated dissociation constants are shown in Table 2. Both of these fits are in good agreement with the inhibition constants determined from slow-binding inhibition.

3-Hydrazinopropionate (75−604 nM) was incubated with 102 nM GABA-AT for 114−408 min. The remaining enzyme activity indicated that the K_i for 3-hydrazinopropionate was <20 nM. When 3-hydrazinopropionate (2.9−3.4 μM) was incubated with 198 nM GABA-AT in the presence of the competitive inhibitor propionic acid (106 mM) for 100−188 min, a $K_{i,app} = 47$ nM was

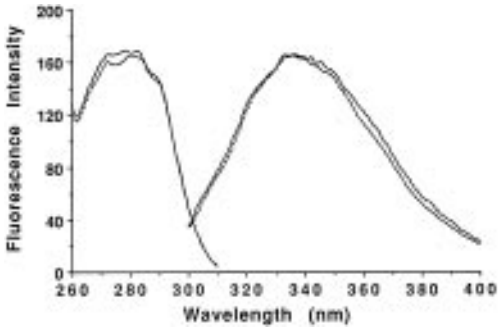


Figure 8. Fluorescence spectra of GABA-AT and GABA-AT inhibited by 3-hydrazinopropionate. 3-Hydrazinopropionate (19 μM) was incubated with 5.6 μM GABA-AT for 1 h in potassium pyrophosphate, pH 8.5, at room temperature. The remaining enzyme activity was 0.5%. The spectra of native GABA-AT (PLP-imine form) are indicated by the solid lines, whereas the spectra of 3-hydrazinopropionate-inhibited GABA-AT are indicated by the dashed lines. The excitation spectra (260−310 nm) were observed by emission at 340 nm. The emission spectra (300−400 nm) were excited at 278 nm.

determined. If 3-hydrazinopropionate were simply a slow-binding or slow, tight-binding inhibitor and propionic acid were competitively inhibiting the slow-binding inhibition, this would indicate K_i = 0.45 nM. However, the inclusion of propionic acid did not result in the linearization of the replot (Figure 6B). Therefore, we can only conclude that 3-hydrazinopropionate binds very tightly to GABA-AT.

Fluorescence of GABA-AT and GABA-AT Inhibited by 3-Hydrazinopropionate or Methylhydrazine. The tryptophan fluorescence spectra of the aldimine form of GABA-AT and 3-hydrazinopropionate-inhibited GABA-AT are compared in Figure 8. No change in the fluorescence intensities of the eight tryptophans per active enzyme is observed. No fluorescence was seen upon excitation at 340 nm. Also, no change in the fluorescence intensity is observed upon inhibition of GABA-AT by methylhydrazine (data not shown).

Titration of PLP on GABA-AT with 3-Hydrazinopropionate. GABA-AT (6.3 μM) was titrated with 3-hydrazinopropionate (2.3−16 μM) by observing its UV−vis spectrum from 300−430 nm as shown in Figure 9. The UV−vis absorbance spectra indicate that the aldimine form of GABA-AT has λ_{max} at 330 and 410 nm, whereas 3-hydrazinopropionate-inhibited GABA-AT has λ_{max} at 304 and 385 nm but lacks peaks at 330 and 410 nm (Figure 9). No further changes in the spectrum are observed above 5.8 μM 3-hydrazinopropionate (Figure 10). The difference spectrum obtained (Figure 11) is similar to that obtained with (3-hydroxybenzyl)hydrazine[21] with absorbance maxima at 304 and 385 nm (the spike at 375 nm is due to instrument error).

Discussion

Determination of the kinetics of slow-binding inhibition of substituted hydrazines by incubation of GABA-AT with the inhibitor in the presence of substrate[25] was attempted. When the reaction was followed by the change in absorbance with time, the initial profile was the one expected, namely, exponential loss of enzyme activity with time. However, as the reaction progressed, enzyme reactivation began to occur. This is consistent with consumption of the hydrazine, e.g., by reaction with

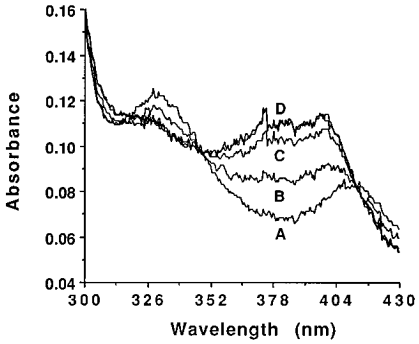


Figure 9. Changes in the UV−vis spectrum of GABA-AT upon binding of 3-hydrazinopropionate. All spectra were done in 50 mM potassium pyrophosphate, pH 8.5, 26 °C. Spectra have been corrected for dilution: (A) 6.3 μM GABA-AT, (B) 6.0 μM GABA-AT incubated with 2.0 μM 3-hydrazinopropionate, (C) 5.6 μM GABA-AT incubated with 4.1 μM 3-hydrazinopropionate, (D) 5.4 μM GABA-AT incubated with 5.8 μM 3-hydrazinopropionate superimposed on 5.1 μM GABA-AT incubated with 7.3 μM 3-hydrazinopropionate. Higher concentrations of 3-hydrazinopropionate (8.8 μM with 4.9 μM GABA-AT, 10.1 μM with 4.7 μM GABA-AT, and 11.2 μM with 4.5 μM GABA-AT) were superimposible on spectrum D.

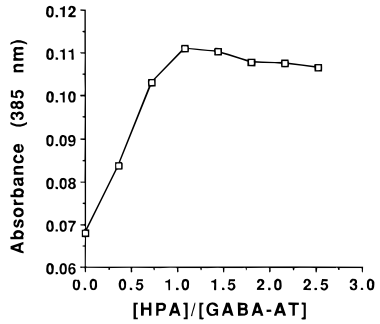


Figure 10. Titration of GABA-AT by 3-hydrazinopropionate. Data are from Figure 9.

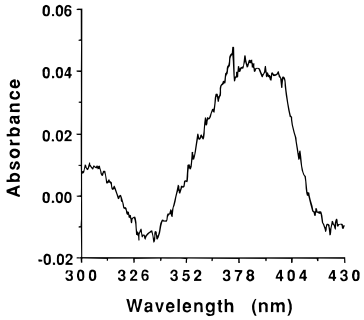


Figure 11. Difference spectrum for GABA-AT inhibited by 3-hydrazinopropionate and GABA-AT. GABA-AT (6.3 μM) was in 50 mM potassium pyrophosphate, pH 8.5, 26 °C. The sample was brought to 7.3 μM 3-hydrazinopropionate and 5.1 μM GABA-AT. The spectra were corrected for dilution before subtraction.

the intermediate succinic semialdehyde; therefore, the Morrison approach could not be used to characterize these kinetics.

A different approach described by Morrison and Walsh[15] was employed. In this approach, enzyme is incubated with the slow-binding inhibitor in the presence of buffer. Aliquots are withdrawn at appropriate times, and the amount of remaining enzyme activity is determined. The data obtained are then fitted to the equations described in the Experimental Section. This approach is similar to that used for mechanism-based inactivation.[22] It is limited by the fact that higher

concentrations of enzyme must be used in the incubation mixture rather than direct observation of the onset of slow-binding inhibition.

If the final complex is tight enough, as with 3-hydrazinopropionate, it may be difficult to distinguish kinetically between slow-binding inhibition and mechanism-based inactivation. However, if the reactivation kinetics of the enzyme (k_{react}) are consistent with those determined for slow-binding inhibition (k_4), then slow-binding inhibition is indicated. If the final complex is less tight, as seen for 3-hydrazinopropionate in the presence of propionic acid and for methylhydrazine and (3-hydroxybenzyl)hydrazine, slow-binding inhibition will approach an equilibrium enzyme activity. Mechanism-based inactivation will result in complete loss of enzyme activity or eventual complete metabolism of the inactivator. If the equilibrium attained after time-dependent inhibition is consistent with the equilibrium constant calculated from the slow-binding kinetics, then slow-binding inhibition is indicated. Finally, mechanism-based inactivation is expected to give a hyperbolic plot of k_{obs} vs I that passes through the origin.[23] A positive y-intercept would be indicative of slow-binding inhibition.

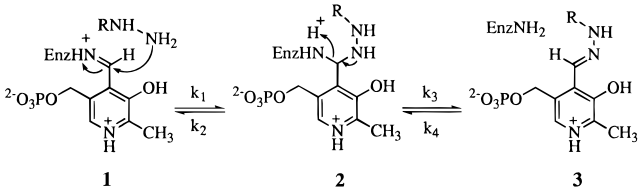
The kinetic constants determined by the incubation method are given in Table 1. Both sets of data were fitted to the mechanisms in Schemes 2 and 3.[15] In neither case does a rapidly reversible enzyme−inhibitor complex form; the mechanism in Scheme 2 fully accounts for the slow-binding inhibition. The difference in the inhibition constants of methylhydrazine and (3-hydroxybenzyl)hydrazine appears to be entirely due to differences in k_3.

The kinetics of reactivation provide another method for measuring k_4. The kinetics for methylhydrazine and (3-hydroxybenzyl)hydrazine reactivation were complicated by an apparent side reaction, possibly the reaction of the hydrazine with succinic semialdehyde, the product from GABA. However, the rate constants for reactivation (Table 2) are not too different from those determined by the incubation method (Table 1). In addition, the rate constants for reactivation are within experimental error of each other whether the inhibitor is methylhydrazine, (3-hydroxybenzyl)hydrazine, or 3-hydrazinopropionate. The tightness of the inhibition appears to be controlled by k_3.

The equilibrium inhibition constants also can be determined by fitting to eq 1 the enzyme activity remaining after extensive incubation with the hydrazine analogue (Table 2). The inhibition constants for both methylhydrazine and (3-hydroxybenzyl)hydrazine agree well with those shown in Table 1. This equation assumes that one hydrazine analogue interacts with one active GABA-AT (a dimer). The Scatchard plot results are consistent with this conclusion. Only 1 PLP/enzyme was reactive to titration of the enzyme by 3-hydrazinopropionate (Figure 10). Churchich and Moses[26] found only one molecule of PLP/dimer for homogeneous GABA-AT as isolated, but they could bind a second molecule of PLP with a $K_d = 3\ \mu M$. The k_{cat} is not increased upon binding the second molecule of PLP; however, both sites appear to be equally catalytically active.[27]

The fact that the method of determination does not affect the value of either k_4 or K_i indicates that the slow-binding model is a good one for methylhydrazine and

Scheme 4



(3-hydroxybenzyl)hydrazine. This conclusion is corroborated by mechanistic studies on (3-hydroxybenzyl)-hydrazine inhibition of GABA-AT[21] which show that no further chemistry occurs after initial hydrazone formation.

The data for 3-hydrazinopropionate suggest that it is a slow, tight-binding inhibitor that requires two to four molecules of 3-hydrazinopropionate interacting with the enzyme in order to get optimal inhibition. Since we know that only one PLP interacts with 3-hydrazinopropionate (Figure 10), this implies that the other ~2 inhibition sites do not contain PLP. Multiple sites would help explain why 100 times the K_i of a competitive inhibitor would shift the curve 6-fold instead of 101-fold ($1 + I/K_i$) as expected by a competitive inhibitor of GABA-AT. The reduction of the number of 3-hydrazinopropionate molecules binding for inhibition of GABA-AT from 3.5 to 2.3 suggests that propionic acid may be preventing binding of 3-hydrazinopropionate at one of these sites. It is indicated that the binding of propionic acid does not prevent inhibition by 3-hydrazinopropionate. Although other theories might also fit these data, this explanation appears to be the most reasonable.

These kinetics are in contrast to the results for the 3-hydrazinopropionate inhibition of GABA-AT from the nematode *N. brasiliensis*[20] which appear to support mechanism-based inactivation. However, the kinetics seen in this study with 3-hydrazinopropionate are not consistent with mechanism-based inactivation.

Slow-binding inhibition is often attributed to a slow conformational change of the enzyme upon binding of the inhibitor.[15] Conformational changes of GABA-AT have been successfully observed by tryptophan fluorescence.[27−29] For methylhydrazine and 3-hydrazinopropionate, the fluorescence spectra from the tryptophans of GABA-AT (λ_{ex} = 278 nm, λ_{em} = 340 nm) indicated no changes in the fluorescence intensities upon binding either methylhydrazine or 3-hydrazinopropionate (Figure 8). Although conformational changes that might affect small regions of the protein cannot be ruled out, any conformational change that might occur could not affect any of the eight tryptophan residues.

Since a slow conformational change of the enzyme seems unlikely from these results, the time-dependent kinetics must result from slow hydrazone formation or conformational changes of the PLP hydrazone, such as a bond rotation that does not significantly affect the enzyme structure. There are two steps to hydrazone formation as shown in Scheme 4. If the formation of the hydrazone **3** were slow, breakdown of intermediate **2** to imine **1** also must be slow or the slow-binding inhibition would follow the mechanism in Scheme 3. In other words, intermediate **2** must be kinetically insignificant (i.e., k_2 and k_3 in Scheme 4 cannot be rate limiting) for the slow-binding inhibition kinetics to follow the mechanism in Scheme 2. If the conformational change were the slow step, then hydrazone **3**

would also have to be kinetically insignificant. Considering the chemical stability of hydrazones, this latter condition seems unlikely.

Slow-binding inhibition of a number of PLP-dependent enzymes by hydrazines and *O*-alkylhydroxylamines has been reported.[16−18] Yamada et al.[16] noted that *l*-hydrazinosuccinate and aminooxyacetate both dissociated from aspartate aminotransferase with similar rate constants ($1.7-2.0 \times 10^{-3}$ min^{-1}), about the same as those reported in this paper, even though their overall inhibition constants vary by nearly 500-fold ($K_i = 0.2$ and 95 nM, respectively). Furthermore, the majority of dissociation rate constants of aminooxyphosphonates from aspartate and alanine aminotransferases reported by Lacoste et al.[18] falls between 1.0×10^{-3} and 1.3×10^{-2} min^{-1}. The dissociations of *l*-aminooxysuccinate[17] and 2-aminooxyethanephosphonate[18] from aspartate aminotransferase appear to deviate from this generalization with $k_{off} = 9.0 \times 10^{-2}$ and 8.4×10^{-2} min^{-1}, respectively. Even so, the overall range of reported dissociation rate constants for hydrazine and hydroxylamine analogues from the enzyme−inhibitor complexes varies by a factor of 90, even though the inhibition constants vary by 11 000-fold. Also, there is no correlation between those that dissociate more slowly and those that have tighter overall inhibition constants.

The UV−vis spectral changes observed upon inhibition of aspartate aminotransferase by *d*- and *l*-hydrazinosuccinate,[30] *l*-aminooxysuccinate,[17] and aminooxymethanephosphonate[18] are remarkably similar to the ones reported here. The PLP maximum at 362 nm in aspartate aminotransferase is shifted to 378 nm,[30] to a broad peak at ∼380 nm,[17] and to 380 nm.[18] The inhibition of GABA-AT by (3-hydroxybenzyl)hydrazine results in the loss of absorbance peaks at 410 and 325 nm with new absorption bands appearing at 385 and 304 nm.[21] 3-Hydrazinopropionate inhibition of GABA-AT results in very similar changes (Figures 9 and 11).

However, the (3-hydroxybenzyl)hydrazone of PLP and the *tert*-butylhydrazone of PLP have absorption maxima at 325 and 297 nm.[21] Acid denaturation of GABA-AT inhibited by (3-hydroxybenzyl)hydrazine results in a spectrum nearly identical to that of the (3-hydroxybenzyl)hydrazone of PLP.[21] These results suggest that the aldimine may be held in conjugation with the pyridine ring on the enzyme but is less conjugated off the enzyme. Rotation of the aldimine relative to the pyridine ring might be the slow step general to hydrazine and hydroxylamine analogue kinetics. Regardless, the physical environment surrounding the hydrazone on the enzyme must be significantly different from that in solution.

Interestingly, the UV−vis spectrum of aspartate aminotransferase inhibited by *l*-hydrazinosuccinate continues to change over 30 min.[30] The first complex formed has a $\lambda_{max} = 374$ nm, but the final complex has $\lambda_{max} = 345$ and 390 nm. *d*-Hydrazinosuccinate does not proceed beyond formation of a complex absorbing at 374 nm. *l*-Hydrazinosuccinate binds 15-fold more tightly than does *d*-hydrazinosuccinate. It is not known whether this spectral change involves an enzyme conformational change as well.

This similarity in the kinetics and spectra of hydrazine and hydroxylamine inhibition of a number of aminotransferases strongly indicates a common mechanism. One possibility is that formation of intermediate **2** or elimination of the amine moiety on the enzyme to form the hydrazone **3** is the slow step of association (i.e., k_1 and k_4 in Scheme 4 can be rate limiting). Likewise, the reversal of either of these steps could be the slow step of dissociation. A conformational change of the hydrazone that does not significantly affect the tryptophan conformations might instead be the slow step.

The strength of binding of all of the reported hydrazine and hydroxylamine analogues, as well as the ones reported here, appears to be dictated by the rate of association.[16−18] Structurally, all of the tightest inhibitors are isosteres of the substrate where the amine has been converted to a hydrazine or hydroxylamine with or without deletion of one carbon. This may indicate that correct collision geometry is critical to successful formation of the tight complex and that hydrazine and hydroxylamine analogues that are close structural mimics of the bound conformation would be expected to be especially tight inhibitors.

Since these inhibitors do not appear to form reversible complexes with the enzyme, the slow step of inhibition could be the ability to form the correct collision complex. The correct collision complex could then proceed rapidly to the hydrazone. The slow step of the reverse reaction could be aminolysis of the hydrazone, a step that could have similar rates for all hydrazones and oximes. The rate constant k_4 must be slower than k_3I in order for inhibition to occur.[15] These reactions would then be expected to follow the mechanism in Scheme 2. This last hypothesis, regarding the formation of the correct collision complex, most easily explains all of the results.

Experimental Section

Reagents. $NADP^+$, α-ketoglutaric acid, β-mercaptoethanol, and GABA were obtained from Sigma Chemical Co., St. Louis, MO. Methylhydrazine, (3-hydroxybenzyl)hydrazine dihydrochloride, and propionic acid were bought from Aldrich Chemical Co., Milwaukee, WI. 3-Pyrazolidinone hydrochloride was procured from Pfaltz & Bauer, Waterbury, CT. GABAse was obtained from Boehringer Mannheim, Indianapolis, IN; succinic semialdehyde dehydrogenase was isolated using the method of either Jeffery et al.[31] or Hopkins et al.[32] GABA-AT was purified to homogeneity from frozen porcine brain by the method of Churchich and Moses.[26] Porcine brain was obtained from FDL Foods, Inc., Dubuque, IA, and preparation was started within 24 h of freezing. The enzyme appeared as one band by $NaDodSO_4$−PAGE and had a specific activity of 3.8−4.6 U/mg of protein. One unit is defined as the amount of enzyme that catalyzes the transamination of 1 μmol of GABA/min at pH 8.5, 25 °C. The concentration of GABA-AT referred to throughout this text is the concentration of the dimer, the active form of the enzyme.

Analytical Methods. Enzyme kinetics were performed on a Perkin-Elmer Lambda 1 UV−vis spectrophotometer with a Lauda MT-20 circulating bath. Enzyme incubations were done in an Eppendorf Thermostat 5320 instrument. UV−vis spectra were recorded on a Beckman DU-40 UV−vis spectrophotometer. Data were fitted to appropriate equations using nonlinear regression on Poly Software International PSI-Plot with Marquardt−Levenberg minimization run on a Packard Bell 486DX computer or on Jandel Scientific SigmaPlot with Marquardt−Levenberg minimization run on a Macintosh Quadra 650 workstation. Fluorescence spectra were recorded on a Perkin-Elmer LS50B fluorimeter.

Hydrolysis of 3-Pyrazolidinone to 3-Hydrazinopropionate. 3-Pyrazolidinone hydrochloride (1.63 mmol) was dissolved in 10 mL of 0.4 M KOH and refluxed for 1 h under N_2. TLC on cellulose plates (2:2:3 H_2O:EtOH:*n*-BuOH) visualized by ninhydrin (0.3%, w/v, with 3%, v/v, HOAc in *n*-BuOH)

indicated complete conversion of 3-pyrazolidinone ($R_f = 0.64$) to 3-hydrazinopropionate ($R_f = 0.27$). ^{1}H NMR (300 MHz, D_2O with sodium (trimethylsilyl)propane sulfonate as the internal reference): δ 2.63 (t, $J = 7$ Hz, 2 H), 3.36 (t, $J = 7$ Hz, 2 H). The hydrolyzed sample was diluted into 50 mM potassium pyrophosphate buffer, pH 8.5, and used for kinetics within 3 days of hydrolysis, generally within 24 h.

Determination of GABA-AT Activity. GABA-AT was assayed in 50 mM potassium pyrophosphate, pH 8.5, containing 4.8 mM α-ketoglutarate, 9.1 mM GABA, 1.0 mM $NADP^+$, 3.6 mM β-mercaptoethanol, and 0.1 U of succinic semialdehyde dehydrogenase, at 26 °C, by observing the change in A_{340}.

Slow-Binding Inhibition Kinetics of GABA-AT by Hydrazines. Methylhydrazine (0.50−20 μM), (3-hydroxybenzyl)hydrazine (0.31−5.0 μM), or 3-hydrazinopropionate (38−604 nM) was incubated with GABA-AT (820, 101, or 102 nM, respectively) in 50 mM potassium pyrophosphate buffer, pH 8.5, 25 °C. 3-Hydrazinopropionate (0.28−2.83 μM) with 106 mM propionic acid was incubated with GABA-AT (198 nM). The stability of GABA-AT in 50 mM potassium pyrophosphate buffer, pH 8.5, 25 °C, was confirmed concurrently with the slow-binding inhibition experiments. The pH of the incubation mixture was determined after equilibrium inhibition was achieved.

Aliquots (10−40 μL) were removed from the incubation mixture at various time points and added to the assay mixture (1 mL). Velocities were determined within the first 5 min of reaction. Incubations containing higher concentrations of the hydrazine were allowed to incubate until their remaining activity no longer changed. This velocity (v_f) was used to calculate the equilibrium level of enzyme−inhibitor complex (EI) using the following equations:[33]

$$\mathrm{EI} = E_t\left(1 - \frac{v_f}{v_i}\right) \tag{1}$$

$$\mathrm{EI} = \frac{E_t + I_t + K_d - \sqrt{(E_t + I_t + K_d)^2 - 4E_tI_t}}{2} \tag{2}$$

where v_i is the velocity in the absence of inhibitor, E_t and I_t are the total enzyme concentration and total inhibitor concentration, respectively, in the incubation mixture, and K_d is the dissociation constant for the enzyme−inhibitor complex. This equation assumes a 1:1 enzyme−inhibitor complex and accounts for depletion of enzyme or inhibitor in the case of tight-binding inhibition. The final velocity for each inhibitor concentration was then calculated with the use of eqs 1 and 2. The time-dependent kinetic data then were plotted as $\ln((v_t - v_f)/(v_i - v_f))$ vs time, where v_t is the velocity of the enzymatic reaction at time t. The slopes (k_{obs}) from the linear fits to this plot were fitted to the slow association mechanism (Scheme 2) and the isomerization mechanism (Scheme 3) using the following equations (mechanisms A and B, respectively[15]).

slow association mechanism: $k_{obs} = k_4 + k_3I$ (3)

$$K_i = \frac{k_4}{k_3} \tag{4}$$

Isomerization mechanism: $k_{obs} = k_6 + \frac{k_5I}{K_i + I}$ (5)

$$K_i^* = \frac{K_ik_6}{k_5 + k_6} \tag{6}$$

where I is the concentration of inhibitor in the incubation mixture and K_i^* is the dissociation constant for the tight enzyme−inhibitor complex. Mechanism A implies that the association of inhibitor with enzyme is slow, whereas mechanism B implies that the association of inhibitor with enzyme is rapid but the complex is slowly altered to form a tighter complex.

Data were fitted to slow, tight-binding inhibition for the mechanism in Scheme 2 by the following equation:[23]

$$k_{obs} = k_3^*\sqrt{(E_t + I_t + K_d)^2 - 4E_tI_t} \tag{7}$$

Eq 7 accounts for the loss of inhibitor as the slow-binding inhibition proceeds, since kinetics are done where $E_t \approx I_t$. An equation for slow, tight-binding inhibition for the mechanism in Scheme 3 has not been derived.[23]

The data were fitted to the following equations assuming formation of enzyme complexes with more than one inhibitor molecule bound:

$$k_{obs} = b + kI^n \tag{8}$$

$$k_{obs} = kI^n \tag{9}$$

The constant k is a complex rate constant of undefined order; n, the number of inhibitor molecules interacting with the enzyme to provide optimal inhibition, defines the order of k.

Inhibition of GABA-AT by Propionic Acid. The assay mixture was identical with that for determination of GABA-AT activity except that GABA levels were varied from 0.26 to 5.1 mM and propionic acid concentrations were varied from 0.73 to 13.2 mM. The velocities were fitted by nonlinear regression to the equations for competitive (eq 10), uncompetitive, and noncompetitive inhibition:[34]

$$v = \frac{V_{max}A}{K_m\left(1 + \frac{I}{K_{is}}\right) + A} \tag{10}$$

where v is the initial velocity, V_{max} is the maximum velocity, A is concentration of GABA, K_m is the Michaelis constant for GABA, I is the concentration of propionic acid, and K_{is} is the inhibition constant for propionic acid calculated from the slope replot. Propionic acid had been previously demonstrated to be a competitive inhibitor of GABA with GABA-AT.[35]

Reactivation of GABA-AT Inhibited by Methylhydrazine, (3-Hydroxybenzyl)hydrazine, and 3-Hydrazinopropionate. GABA-AT (820 nM) was incubated with 10 μM methylhydrazine or 1.2−9.2 μM (3-hydroxybenzyl)hydrazine; GABA-AT (102 nM) was incubated with 0.60 μM 3-hydrazinopropionate. An aliquot (10−20 μL) was removed from the incubation mixture and diluted into the assay mixture (1 mL). The reaction kinetics were followed for at least 2 h. The data were digitized and adjusted using eq 11:

$$A_{340} = A_{340obs} - (vt + b) \tag{11}$$

where the maximum velocity (v) was estimated from the slope of the asymptote to the final data and is the rate at equilibrium inhibition of the diluted sample, t is the time at which A_{340obs} was taken, and b is a correction factor to make sure all values are positive after adjustment. The data were then fitted to the first-order eq 12:

$$\ln(A_{340}) = -k_{react}t + c \tag{12}$$

where k_{react} is the rate constant for reactivation of GABA-AT inhibited by a hydrazine analogue. This provides a fit to a first-order approach to a maximum velocity.

Determination of Final Velocities of Slow-Binding Inhibition. Methylhydrazine (0.95−19 μM) was incubated with 820 nM GABA-AT for 96−286 min, and (3-hydroxybenzyl)hydrazine (0.091−8.90 μM) was incubated with 820 or 102 nM GABA-AT for at least 2 h. The relative remaining enzyme activities were used to determine the concentration of the enzyme−inhibitor complex using eqs 1 and 2.

Fluorescence of GABA-AT. All samples for fluorescence spectra contained 50 mM potassium pyrophosphate buffer, pH 8.5, and were incubated at room temperature. GABA-AT (4.6 μM) was incubated with 20 μM methylhydrazine for 2 h; the equilibrium inhibition is expected to be 88% based on eq 2. A

standard sample containing 4.6 μM GABA-AT was incubated for 1.5 h. GABA-AT (5.6 μM) was incubated with 19 μM 3-hydrazinopropionate for 1 h. A standard sample containing 5.6 μM GABA-AT was incubated for 1 h. After 1 h, an aliquot drawn from the 3-hydrazinopropionate sample had 0.5% the activity of an aliquot from the standard sample. The fluorescence spectra used an excitation wavelength of 278 nm and an emission wavelength of 340 nm, as optimized by the fluorimeter software.

Titration of the PLP of GABA-AT by 3-Hydrazinopropionate. The absorbance spectrum of GABA-AT (6.3 μM) was obtained in potassium pyrophosphate buffer, pH 8.5, 26 °C. To this sample was added 2.1 μM 3-hydrazinopropionate, resulting in a GABA-AT concentration of 6.0 μM, and the incubation was continued for 10 min. The absorbance spectrum was reobtained. Incubation for 22 and 30 min gave superimposible spectra. This process was repeated with 3-hydrazinopropionate at 4.1, 5.8, 7.3, 8.8, 10.1, and 11.2 μM. The addition of 3-hydrazinopropionate resulted in the dilution of GABA-AT to 5.6, 5.4, 5.1, 4.9, 4.7, and 4.5 μM, respectively.

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