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Time-Dependent Inhibition of γ-Aminobutyric Acid Aminotransferase, by 3-Hydroxybenzylhydrazine

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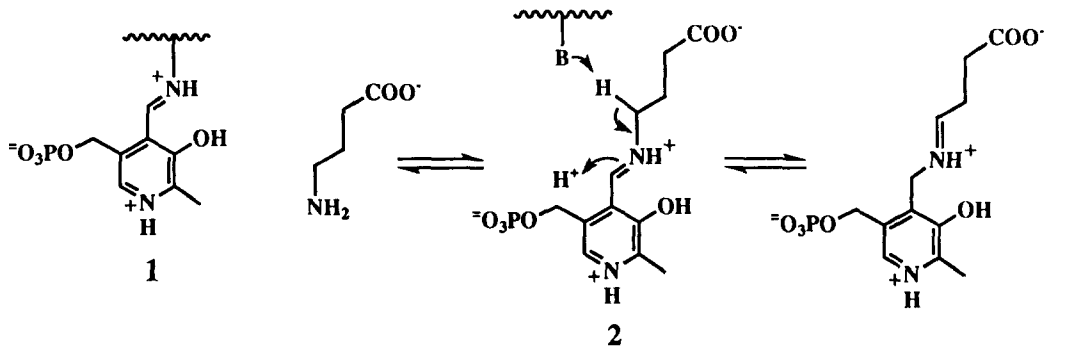
Abstract—γ-Aminobutyric acid (GABA) aminotransferase is a pyridoxal 5'-phosphate (PLP)-dependent enzyme that catalyzes the conversion of GABA into succinic semialdehyde. Hydrazine analogues have long been known to act as inactivators of PLP-dependent enzymes, including GABA aminotransferase, however, no studies of the molecular mechanism of inactivation of PLP-dependent enzymes by hydrazines have been reported. 3-Hydroxybenzylhydrazine is shown to be a potent *in vitro* time-dependent inhibitor of pig brain GABA aminotransferase. UV-visible and ^{1}H NMR studies, both with GABA aminotransferase and with PLP as a chemical model for the enzyme-catalyzed reaction, indicate that 3-hydroxybenzylhydrazine reacts both enzymatically and nonenzymatically to form the 3-hydroxybenzylhydrazone of PLP without tautomerization.

Introduction

Inactivators of γ-aminobutyric acid aminotransferase (EC 2.6.1.19; GABA-AT), particularly mechanism-based inactivators,[1] have been actively sought because of their pharmacological potential as anticonvulsant agents.[2–4] Inhibition of brain GABA-AT leads to an increase in the concentration of the inhibitory neurotransmitter GABA, which can result in an anticonvulsant effect.[5] In general, these compounds react covalently either with an active-site amino acid residue or with the pyridoxal 5'-phosphate (PLP) cofactor of GABA-AT. Hydrazine-containing compounds have long been known to be potent inhibitors of PLP-dependent enzymes;[6] 3-hydrazinopropionate has been known to be an inhibitor of GABA-AT for 25 years[7] and more recently was reported to be a mechanism-based inactivator.[8] Hydrazinosuccinate, however, was demonstrated to be a slow-binding inhibitor of aspartate aminotrans-ferase.[9] Phenelzine (2-phenylethylhydrazine) and its analogues have been shown to increase GABA levels *in vivo*,[10] suggesting that they may also be interfering with GABA metabolism.

To the best of our knowledge, mechanistic studies of the reaction of hydrazines with PLP-dependent enzymes have not been published. Since the PLP-dependent aminotransferases catalyze a tautomerization reaction (Scheme 1), it was thought that hydrazone formation (**4**, Scheme 2) of the hydrazine with lysine-bound PLP (**1**) in these enzymes may only initiate the actual inactivation mechanism. Hydrazone **4** is the nitrogen isostere of the imine formed when GABA reacts with PLP (**2**, Scheme 1). By analogy with the reaction of this GABA-PLP imine **2**, tautomerization of



Scheme 1.

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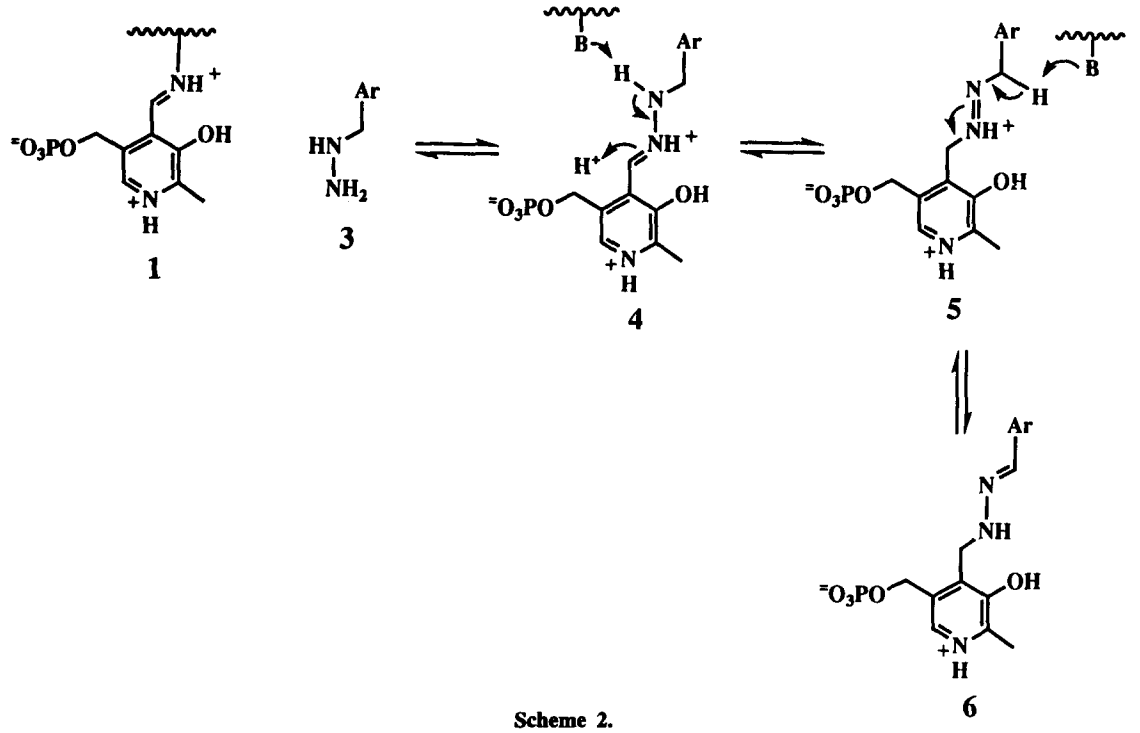


Scheme 2.

the PLP hydrazone 4 would lead to the azo adduct 5 or the isomeric hydrazone 6. According to a definition for mechanism-based inactivation,[1] a compound that inactivates an enzyme by simply reacting with its cofactor, such as in the formation of the initial hydrazone 4, is not a mechanism-based inactivator, but is classified as an affinity labeling agent, that is, a compound that does not require enzyme activation for its covalent inactivation properties. To determine whether hydrazines act as affinity labeling agents or as mechanism-based inactivators of PLP-dependent aminotransferases, the reaction of 3-hydroxybenzylhydrazine (4, Ar = 3-hydroxyphenyl) with GABA-AT was investigated. Because 3-hydrazinopropionate, a hydrazine analogue that is an even closer analogue of GABA, is unstable[7] and may have additional mechanistic pathways that may complicate the analysis, 3-hydroxybenzylhydrazine was selected for investigation. As a model for the proposed enzyme chemistry, the nonenzymatic reaction of 3-hydroxybenzylhydrazine with PLP was also studied. The results of the enzyme and model chemistry are described here.

Results

Time-dependent inhibition of GABA-AT by 3-hydroxybenzylhydrazine

3-Hydroxybenzylhydrazine was found to be a time-dependent inhibitor of GABA-AT; however, activity slowly returned upon dilution. The compound was found to be a slow, tight-binding inhibitor of GABA-AT. A detailed analysis of the kinetics of this time-dependent inhibition will be reported elsewhere (Lightcap and Silverman, unpublished results).

Change in the ultraviolet spectrum during inactivation of GABA-AT by 3-hydroxybenzylhydrazine

Ultraviolet spectra of the inactivation mixture were recorded between 250 and 500 nm as GABA-AT was titrated with 3-hydroxybenzylhydrazine (Fig. 1). The absorption peaks of bound PLP at 325 and 410 nm disappeared, while new absorption bands at 304 and 385 nm appeared, as is more clearly shown in the difference spectrum (Fig. 2). No further changes were observed at higher concentrations of 3-hydroxybenzylhydrazine. The presence of isosbestic points at 320, 348, and 417 nm is consistent with the direct interconversion of one entity into another.

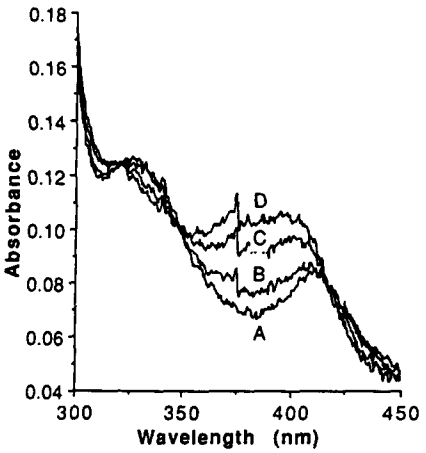


Figure 1. UV-vis spectral changes during the reaction of 3-hydroxyphenylhydrazine with GABA-AT. 3-Hydroxybenzylhydrazine (A, 0 μM; B, 1.05 μM; C, 3.5 μM; D, 6.8 μM) and GABA-AT (6.3 μM) were incubated at 26 °C in 50 mM potassium pyrophosphate buffer pH 8.5 for at least 5 min. No changes were observed for longer incubation times.

Ultraviolet spectrum of GABA-AT inactivated with 3-hydroxybenzylhydrazine then denatured compared to the product of 3-hydroxybenzylhydrazine and PLP in denaturant

3-Hydroxybenzylhydrazine was incubated with GABA-AT, excess inactivator was removed, then the inactivated enzyme was denatured with trichloroacetic acid (Fig. 3). The spectrum of the supernatant was compared to the product of the reaction of PLP with a slight excess of 3-hydroxybenzylhydrazine followed by addition of trichloroacetic acid (Fig. 3). The resultant spectra are very similar. A control for the nonenzymatic reaction in which 3-hydroxybenzylhydrazine was omitted gave an absorbance maximum at 295 nm and almost no absorbance at 355 nm. Therefore, hydrolysis of the hydrazone does not occur upon treatment with trichloroacetic acid.

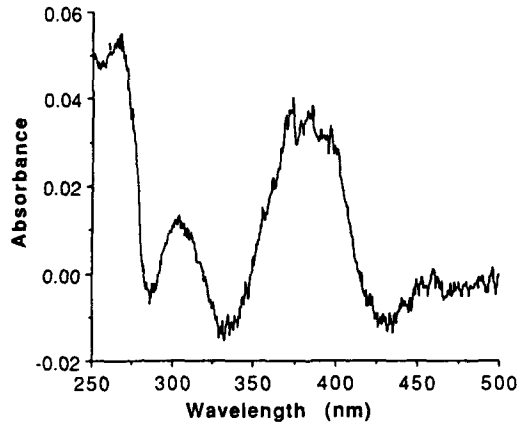


Figure 2. Difference spectrum of GABA-AT and 3-hydroxybenzylhydrazine-inhibited GABA-AT. Spectrum A from Figure 1 was substracted from spectrum D.

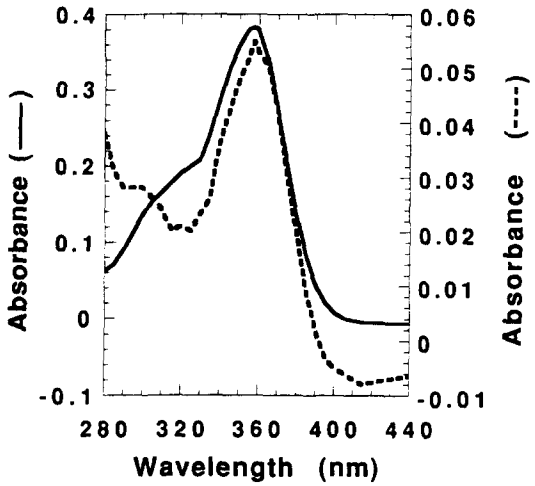


Figure 3. UV-vis spectra of the reaction product of 3-hydroxybenzylhydrazine and PLP (—)and the reaction of 3-hydroxybenzylhydrazine with GABA-AT (----). See Experimental section for details.

Change in the ultraviolet spectrum during the nonenzymatic reaction of 3-hydroxybenzylhydrazine with PLP

Upon incubation of equimolar amounts of PLP and 3-hydroxybenzylhydrazine, the absorption peak of PLP at 388 nm slowly disappeared, while new absorption bands at 298 and 325 nm appeared (Fig. 4). Both 3-hydroxybenzylhydrazine and PLP are stable to the conditions of incubation when treated independently. The presence of a single isosbestic point at 355 nm is consistent with the direct interconversion of one entity into another.

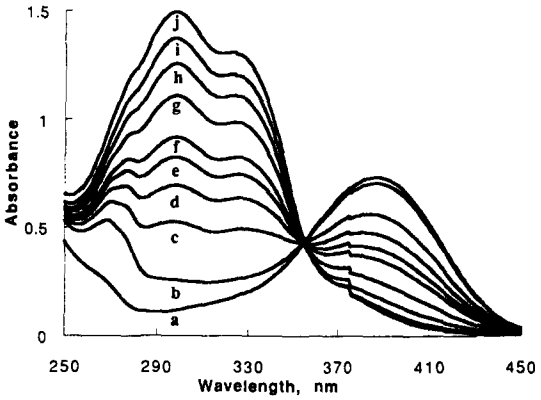


Figure 4. UV-vis spectra of the reaction of 3-hydroxybenzylhydrazine with PLP. (a) 0.14 mM PLP in 50 mM potassium pyrophosphate, pH 8.5. A slight excess of 3-hydroxybenzylhydrazine (0.15 mM) was added and the spectra were recorded at 0, 3.5, 7, 11, 15, 30, 60, 120, 240 min (b–j).

Characterization of the product of the reaction of 3-hydroxybenzylhydrazine with PLP and the inactivated enzyme adduct

Analysis of the nonenzymatic reaction mixture of 3-hydroxybenzylhydrazine with PLP by thin-layer chromatography showed that the starting materials were present only in trace amounts and a new product had appeared. The new product had maximal ultraviolet absorptions at 297 and 325 nm (Fig. 5A).

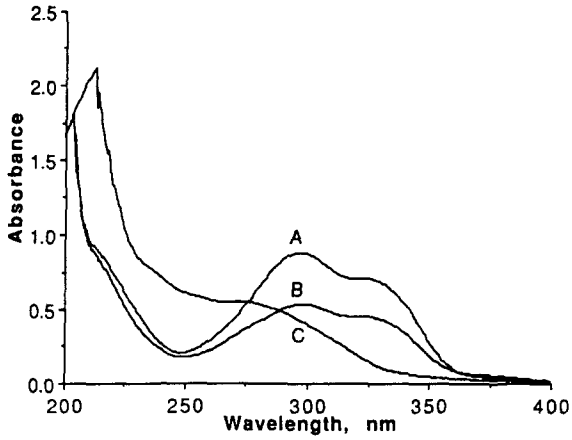


Figure 5. UV-vis spectra of the reaction product of 3-hydroxybenzylhydrazine and PLP (A), the *tert*-butylhydrazone of PLP (B), and the *tert*-butylhydrazone of 3-hydroxybenzaldehyde (C). The hydrazones were dissolved in 50 mM potassium pyrophosphate buffer pH 8.5.

Ultraviolet spectra of the tert*-butylhydrazones of PLP and of 3-hydroxybenzaldehyde*

The ultraviolet spectra of the *tert*-butylhydrazone of PLP (λ_{max} = 297 and 325 nm) and the *tert*-

butylhydrazone of 3-hydroxybenzaldehyde (λ_{max} = 275 nm) are shown in Figures 5B and 5C, respectively. The spectrum of the *tert*-butylhydrazone of PLP (Fig. 5B) is very similar to that of the product of the reaction of 3-hydroxybenzylhydrazine with PLP (Fig. 5A) and is very different from that of the *tert*-butylhydrazone of 3-hydroxybenzaldehyde (Fig. 5C).

Comparison of the ^{1}H NMR spectra of the product of the reaction of 3-hydroxybenzylhydrazine with PLP and the tert*-butylhydrazones of PLP and of 3-hydroxybenzaldehyde*

^{1}H NMR shifts for the three hydrazones are compared in Table 1. The assignments were based on coupling patterns and nuclear Overhauser effects (NOE's). The assignments of protons *g* and *i* were based on the ^{1}H NMR spectrum of the product of the reaction of 3-hydroxybenzylhydrazine and [4-^{2}H]PLP (*vide infra*). There were more NOE's than can be explained using a linear conformation of the reaction product. Therefore, the conformation of the reaction product is believed to be stacked in solution.

^{1}H NMR Spectrum of the product of the reaction of 3-hydroxybenzylhydrazine and [4-^{2}H]PLP

The ^{1}H NMR spectrum of the reaction product of 3-hydroxybenzylhydrazine and [4-^{2}H]PLP is shown in Figure 6A. This spectrum lacks a peak at 8.13 ppm, but the peak at 4.26 ppm is unaffected. The NMR sample was doped with the reaction product of 3-hydroxybenzylhydrazine and [4-^{1}H]PLP; its ^{1}H NMR spectrum is shown in Figure 6B. The peak at 8.13 ppm partially reappears, but the ratio of the 4.26 ppm integration to the other peaks in the spectrum is unchanged. This is consistent with the product of the reaction of 3-hydroxybenzylhydrazine and PLP having the structure **4** not **6** (Scheme 3).

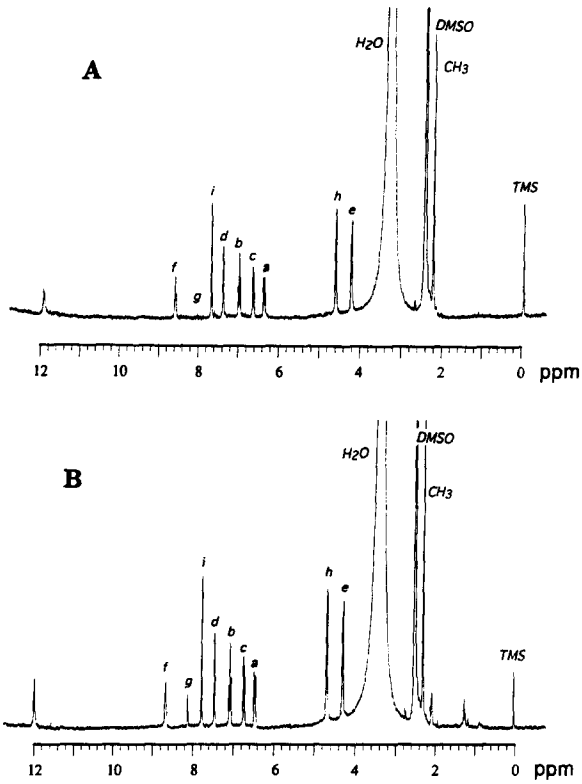


Figure 6. ^{1}H NMR spectra of the reaction product of 3-hydroxybenzylhydrazine and [4-^{2}H]PLP. (A) The reaction product of 3-hydroxybenzylhydrazine and [4-^{2}H]PLP in DMSO-d_6. (B) The reaction product of 3-hydroxybenzylhydrazine and PLP added to the sample from spectrum A.

Discussion

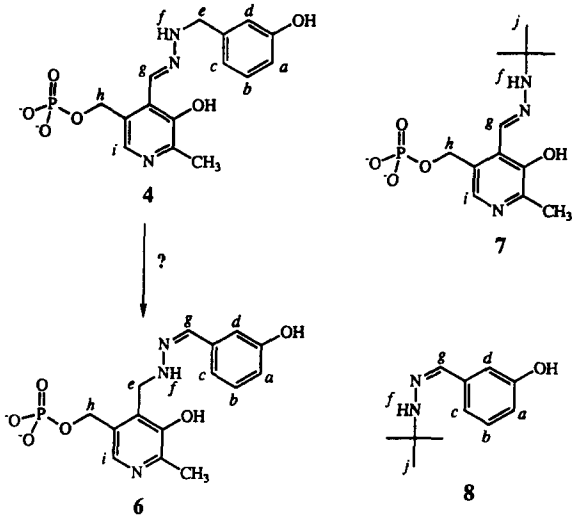
There is a clear similarity in the products of time-dependent inactivation of GABA-AT by 3-hydroxybenzylhydrazine and the corresponding nonenzymatic reaction of this compound with PLP, as evidenced

Table 1. ^{1}H NMR† Spectral characterization of the product of the reaction of 3-hydroxybenzylhydrazine with PLP and *tert*-butylhydrazones of PLP (**7**) and of 3-hydroxybenzaldehyde (**8**)

Proton	Reaction Prdt	NOE	7	8
a	6.42 ddd (8.0, 2.5,1.0 Hz)	b	-	6.62 ddd (8.0, 2.6, 1.0 Hz)
b	7.04 t (7.8 Hz)	a,c	-	7.11 t (7.8 Hz)
c	6.68 td (7.6, 1.3 Hz)	ND	-	6.86 td (7.6, 13. Hz)
d	7.50 dd (2.0 Hz)	ND	-	6.95 dd (2.0 Hz)
e	4.26 d (5.7 Hz)	c,d	-	-
f	8.60 t (5.8 Hz)	ND	8.40 s	6.74 s
g	8.13 s		8.25 s	7.57 s
h	4.67 d (4.0 Hz)	g,i	4.63 d (4.5 Hz)	-
I	7.68 s	a,b,c,e,h	7.79 s	
j	-	-	1.20 s	1.15 s
CH_3	2.26 s	ND	2.34 s	-

†All spectra were taken in d_5-DMSO with a TMS internal reference.
ND means not determined; - means not applicable

by the similarity of the ultraviolet spectra obtained after denaturation of the enzyme adduct and the nonenzymatic reaction product (Fig. 3). Therefore, characterization of the reaction product of 3-hydroxybenzylhydrazine and PLP provides evidence for the structure of the adduct formed when 3-hydroxybenzylhydrazine inactivates GABA-AT, from which mechanistic details for the time-dependent inhibition can be inferred.



Scheme 3.

To determine if inactivation was the result of simple hydrazone formation from the reaction of **3** with the active site PLP to give **4** (Scheme 2) or from the product of further tautomerization (**6**), it was necessary to differentiate these structures spectrally. To that end the *tert*-butylhydrazones of PLP (**7**, Scheme 3) and of 3-hydroxybenzaldehyde (**8**), which, by virtue of the *tert*-butyl group, are unable to tautomerize, were synthesized and their UV-vis and ^{1}H NMR spectra were compared to the spectra obtained for the compound formed from the nonenzymatic reaction of 3-hydroxybenzylhydrazine and PLP (Fig. 5; Table 1). The UV-vis spectra of this reaction product (Fig. 5A) and of **7** (Fig. 5B) are very similar, both having λ_{max} at 297 and 325 nm, whereas the spectrum of **8** is significantly different, having a λ_{max} at 275 nm. Although these data indicate that the tautomerization in Scheme 2 is not operative, this interpretation is complicated by the results of the NOE experiments described below.

The ^{1}H NMR resonances of the nonenzymatic reaction product of 3-hydroxybenzylhydrazine with PLP, of **7**, and of **8** are fully assigned in Table 1. These assignments are based on the ^{1}H–^{1}H coupling constants and NOE experiments. The assignment of protons *g* and *i* (Scheme 3) is ambiguous from these data. Attempts were made using a 600 MHz NMR spectrometer to detect possible coupling between either the benzylic protons (protons *e* of **6**, Scheme 3) or the hydrazone proton (proton *g* of **4**, Scheme 3) and the *ortho* protons of the benzene ring (protons *d* and *c* of **4** and **6**, Scheme 3); however, no coupling was found. NOE experiments were performed to see if distance measurements might provide an absolute structural determination of the reaction product. However, the large number of protons that were within NOE distance of each other indicated that the benzene ring is likely to be stacked on the pyridine ring in solution. This stacking complicates the interpretation of the UV-vis data, because stacking may result in a spectral shift.

Based on the assignment of proton *g* presented in Table 1, the ^{1}H NMR shifts also favor structure **4**. However, if the protons listed as proton *i* at 7.68 ppm for the reaction product and at 7.79 ppm for **7** are assigned to position *g* instead, then the hydrazone proton for **8** (proton *g*) could correspond to the peak at 7.57 ppm; therefore, the structure assignment is ambiguous. Without absolute assignment of protons *e* and *g*, an unambiguous structure cannot be determined. To clarify this ambiguity, the ^{1}H NMR spectrum of the reaction product of [4-^{2}H]PLP and 3-hydroxybenzylhydrazine was obtained. If the product of the reaction of 3-hydroxybenzylhydrazine with PLP has structure **4**, then the hydrazone proton would be a deuterium. If **6** is the product structure, then *one* of the methylene protons (*e*) would be a deuterium; the remaining methylene proton signal would be expected to be broadened by coupling with the deuterium. Substitution of deuterium at the 4'-position of PLP resulted in a product the ^{1}H NMR spectrum of which (Fig. 6A) had changes only in the hydrazone peak (proton *g*, 8.13 ppm) and not in the benzylic peak (proton *e*, 4.26 ppm). This is consistent with structure **4** as the reaction product. When the deuterated reaction product was incubated in H_2O, no exchange occurred over a period of 4 h. If tautomerization were reversible to either **5** or **6**, there would have been deuterium washout. Therefore, these results are consistent with the product of the reaction of 3-hydroxybenzylhydrazine with PLP being **4** without tautomerization (either reversible or irreversible).

The inhibition of GABA-AT by 3-hydroxybenzylhydrazine is time-dependent, but because it reacts with the cofactor to give a stable adduct without other enzyme-catalyzed processes, it is not mechanism-based inactivation. If an aminotransferase is incapable of catalyzing isomerization of the initially formed hydrazone, it is unlikely that other PLP-dependent enzymes would be able to catalyze hydrazone tautomerization. Therefore, it can be concluded that the mechanism of hydrazine inactivation of PLP-dependent enzymes is hydrazone formation.

Hydrazinosuccinate[9] and aminooxysuccinate[11] were found to be slow-binding inhibitors of aspartate aminotransferase; aminooxyalkylphosphonates were found to be slow-binding inhibitors of aspartate and alanine aminotransferases.[12] On the basis of a detailed kinetic analysis, it appears that 3-hydroxybenzylhydrazine also is a slow-binding inhibitor.[13]

Experimental

Chemicals and syntheses

3-Hydroxybenzylhydrazine, pyridoxal 5'-phosphate, γ-aminobutyric acid, β-mercaptoethanol, and α-ketoglutarate were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 3-Hydroxybenzaldehyde and *tert*-butylhydrazine were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. The formation of hydrazones was accomplished by mixing the hydrazine (1.6 mmol) and aldehyde (1.8 mmol) in 50 mL of 0.1 M potassium phosphate (pH 7.2) at room temperature, protected from light. After 2 h, the precipitate was collected by filtration and dried *in vacuo* overnight. The amounts of reactants and products existing at a time along the reaction coordinate for 3-hydroxybenzylhydrazine and PLP were determined from their absorbances at their respective λ_{max} values: 3 (Ar = 3-hydroxyphenyl), λ_{max} = 250 nm; PLP, λ_{max} = 390 nm; product, λ_{max} = 320 nm. The product behaved as a single spot on silica gel thin-layer plates eluted with BuOH:AcOH:H_2O (2:1:2). ^{1}H NMR (400 MHz, D_2O) δ 8.31 (*s*, 1H), 7.91 (*s*, 1H), 7.28 (*t*, 1H), 6.92 (*s*, 1H), 6.90 (*d*, 1H), 6.76 (*d*, 1H), 4.38 (*s*, 2 H), 3.58 (*s*, 1H), 2.41 (*s*, 3H). ^{1}H NMR (300 MHz, DMSO-d_6) data are presented in Table 1 for the 3-hydroxybenzylhydrazone of PLP, the *tert*-butylhydrazone of PLP, and the *tert*-butylhydrazone of 3-hydroxybenzaldehyde; HRMS (FAB$^-$): 3-hydroxybenzylhydrazone of PLP, calculated 366.0856, found 366.0847; the *tert*-butylhydrazone of PLP, calculated 316.1062, found 316.1010; *tert*-butylhydrazone of 3-hydroxybenzaldehyde, calculated 192.1263, found 192.1261. [4-^{2}H]Pyridoxal 5'-phosphate was synthesized by the method of Stock *et al.*[14] without the inclusion of $HClO_4$.

Analytical methods

Thin-layer chromatography was done on EM-HP-254-silica gel plates. GABA-AT and PLP assays were carried out on a Perkin–Elmer Lambda 1 UV-vis spectrophotometer. UV-vis spectra were recorded on a Beckman DU-40 UV-vis spectophotometer. ^{1}H NMR spectra were recorded on a Varian Gemini 300 MHz or a Varian XLA-400 MHz NMR spectrometer. Determinations of coupling constants and NOE experiments were done on a Bruker AMX-600 (600 MHz) NMR spectrometer. All spectra were taken in DMSO-d_6 with a TMS internal reference unless otherwise noted.

Enzymes and assays

GABA-AT was purified to homogeneity from pig brains by the method of Churchich and Moses.[15] The enzyme showed one band on NaDodSO$_4$-PAGE at pH 7.0 and had a specific activity of 4.2 units mg^{-1} of protein. One unit is defined as the amount of enzyme that catalyzes the transamination of 1 μmol of GABA min^{-1} at pH 8.5 and 25 °C. Enzyme activity was measured as previously described.[1] Succinic semialdehyde dehydrogenase was prepared from Gabase (Boehringer Mannheim) as described previously.[3] Concentrations of GABA-AT are reported for the active dimer.

Comparison of the UV-vis spectra for the reaction of 3-hydroxybenzylhydrazine with PLP to that with GABA-AT

3-Hydroxybenzylhydrazine (0.15 mM) and PLP (0.14 mM), dissolved in 50 mM potassium pyrophosphate buffer, pH 8.5, were allowed to react in the dark for 2 h (reaction complete). The solution was made 10% in trichloroacetic acid, and the UV-vis spectrum was run. 3-Hydroxybenzylhydrazine (219 μM) was incubated in the dark with GABA-AT (7 μM) for 45 min in 50 mM potassium pyrophosphate buffer pH 8.5 (no activity remaining). Excess inactivator was removed on a Penefsky column[16] then trichloroacetic acid to 10% was added. After 5 min the precipitated protein was removed by centrifugation, and the UV-vis spectrum was taken as above. 3-Hydroxybenzylhydrazine does not absorb appreciably in the region observed.

Acknowledgments

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