Mechanism of Inactivation of γ-Aminobutyric Acid Aminotransferase by 3-Hydrazinopropionic Acid

Richard B. Silverman* and Gregory T. Olson

Department of Chemistry and Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, Illinois 60208-3113

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3-Hydrazinopropionic acid (**1**) was designed as a structural mimic of the inhibitory neurotransmitter γ-aminobutyric acid (GABA).[1] It was shown to be a very potent in vitro and in vivo inhibitor of the enzyme GABA aminotransferase.[1] Subcutaneous injection into mice produced a time-dependent rise in the brain concentration of GABA[2] and a sedative effect.[1] Time-dependent inactivation of nematode[3] and pig brain[4] GABA aminotransferase has been demonstrated. The mechanism of inactivation of GABA aminotransferase by 3-hydrazinopropionic acid has not been reported, although it has been suggested that it does not function by formation of a stable hydrazone with the pyridoxal 5′-phosphate (PLP) cofactor, because addition of high concentrations of PLP does not interfere with inhibition by 3-hydrazinopropionate.[1]

The accepted mechanism for oxidation of GABA to succinic semialdehyde is shown in Scheme 1. To rationalize time-dependent inactivation of GABA aminotransferase leading to a stable adduct that is not a hydrazone, we contemplated the inactivation mechanism shown in Scheme 2. The driving force for this decarboxylative fragmentation mechanism would be the formation of three stable gases (highly favorable entropy) with concomitant elimination of 4′-deoxypyridoxine 5′-phosphate (**4**). Because the pK_a of *N*-methyl-4-methylpyridinium ion in DMSO is about 11,[5] it is reasonable that decarboxylation should be efficient. In fact, O'Leary and coworkers[6] have studied the nonenzymatic decarboxylation of 4-pyridylacetic acid (**5**; Scheme 3) as a model for PLP-dependent decarboxylases and found that decarboxylation occurred at 25 °C in water–dioxane mixtures. More recently, Ashley et al.[7] raised a catalytic antibody that catalyzes the decarboxylation of 4-pyridylacetic acid and derivatives. Despite the strong rationalization for a decarboxylative mechanism, evidence is presented that shows that decarboxylation does not occur and the mechanism of inactivation of GABA aminotransferase by 3-hydrazinopropionic acid is simple hydrazone formation (**2**, Scheme 2).

Results and Discussion

apo-GABA aminotransferase was reconstituted with [4′-^{3}H]PLP, and the tritiated enzyme was inactivated with 3-hydrazinopropionate. Following trifluoroacetic acid denaturation, the supernatant was analyzed by HPLC for cofactor and analogues.[9] No [^{3}H]-4′-deoxypyridoxine 5′-phosphate (**4**) was detected (in Figure 1, (—) is representative of four different runs). The major radioactive peak corresponded to the hydrazone of 3-hydrazinopropionate and PLP[10] as demonstrated in a control experiment in which [^{3}H]PLP was allowed to react with 3-hydrazinopropionate in the absence of enzyme (Figure 1 (- - -)). If the inactivated enzyme is first run through a Penefsky[8] column to remove the excess 3-hydrazinopropionate prior to denaturation, the same radioactive peak of the hydrazone of 3-hydrazinopropionate with PLP was observed (data not shown). PLP is very tightly bound to GABA aminotransferase (you do not have to add PLP to assay mixtures and gel filtration or dialysis does not remove the PLP under ordinary conditions). This indicates that the hydrazone did not arise from the nonenzymatic reaction of excess 3-hydrazinopropionate with the [^{3}H]PLP which would be released upon denaturation; instead, it suggests that the hydrazone is formed at the active site, and this is the cause for the time-dependent inactivation of the enzyme.

Experimental Section

Reagents. 3-Hydrazinopropionate was prepared by alkaline hydrolysis of 3-pyrazolidinone hydrochloride (Pfaltz & Bauer, Waterbury, CT)[1] and stored in solution in the freezer for up to a week without decomposition. Potassium pyrophosphate, α-ketoglutarate, β-mercaptoethanol, $NADP^+$, 4′-deoxypyridoxine 5′-phosphate, and GABA were purchased from Sigma Chemical Co.

Enzyme and Assay. Pig brain GABA-AT (specific activity 3.75 units/mg), Gabase, and succinic semialdehyde dehydrogenase were obtained and assayed as previously described.[11]

Inactivation of [^{3}H]PLP-Reconstituted GABA-AT by 3-Hydrazinopropionate. [^{3}H]PLP and [^{3}H]PLP-reconstituted GABA-AT were prepared as described previously.[12] [^{3}H]PLP-reconstituted GABA-AT (200 μL of 0.72 mg/mL solution; 28 000 cpm) in 100 mM potassium phosphate buffer, pH 7.4, containing 5 mM α-ketoglutarate, 0.1 μL of β-mercaptoethanol, and 0.3 mM 3-hydrazinopropionate, was incubated, while protected from light, at 25 °C for 9 h, at which time no enzyme activity remained. The pH was adjusted to 12 with 2 M KOH and was allowed to incubate for 1 h. Then trifluoroacetic acid was added to afford a 10% solution. After being incubated for 5 min, the solutions were centrifuged for 5 min in a Beckman Microfuge B. The supernatant was removed, and the pellet was washed with 50 μL of 10% trifluoroacetic acid. This was centrifuged again for 5 min, and the supernatant was collected. This process was repeated twice more. The pellet was dissolved in 0.5 mL of 2 M KOH. The combined supernatants were lyophilized. PLP, PMP, and 4′-deoxypyridoxine 5′-phosphate were subjected to the same conditions as described above for the enzyme reaction and were lyophilized. The lyophilized supernatants were dissolved in 125 μL of water, 25 μL of each standard was then added to the reconstituted supernatant from the enzyme reaction, and

* Address correspondence to this author at Department of Chemistry, Northwestern University, Evanston, Illinois 60208-3113. Phone: (708) 491-5653. FAX: (708) 491-7713. e-mail: Agman@chem.nwu.edu.

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(9) The sum of all of the radioactivity obtained by HPLC corresponded to 99% of the radioactivity initially in the enzyme.
(10) We have isolated the product obtained from a nonenzymatic reaction of PLP with 3-hydrazinopropionate, purified it by HPLC, and analyzed it by NMR and HRMS to be the proposed hydrazone (see the Experimental Section). This product comigrates with the major radioactive product from the enzyme reaction.
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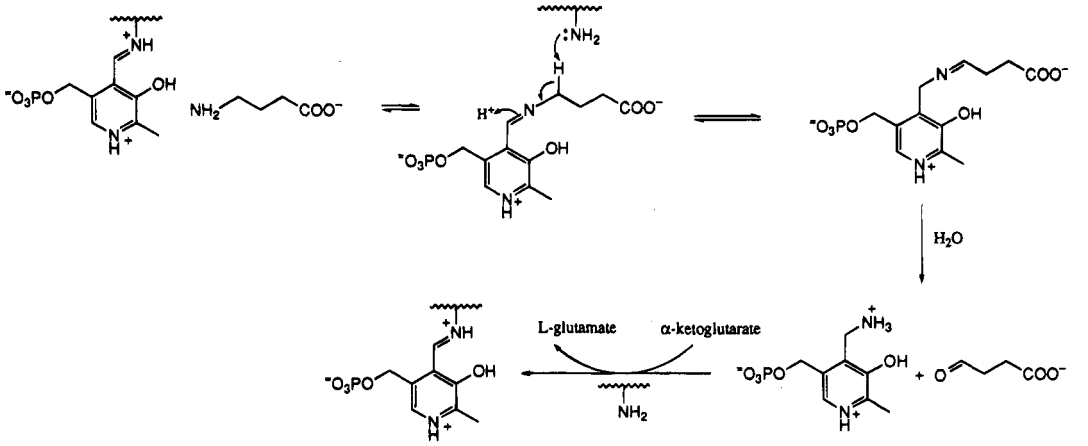
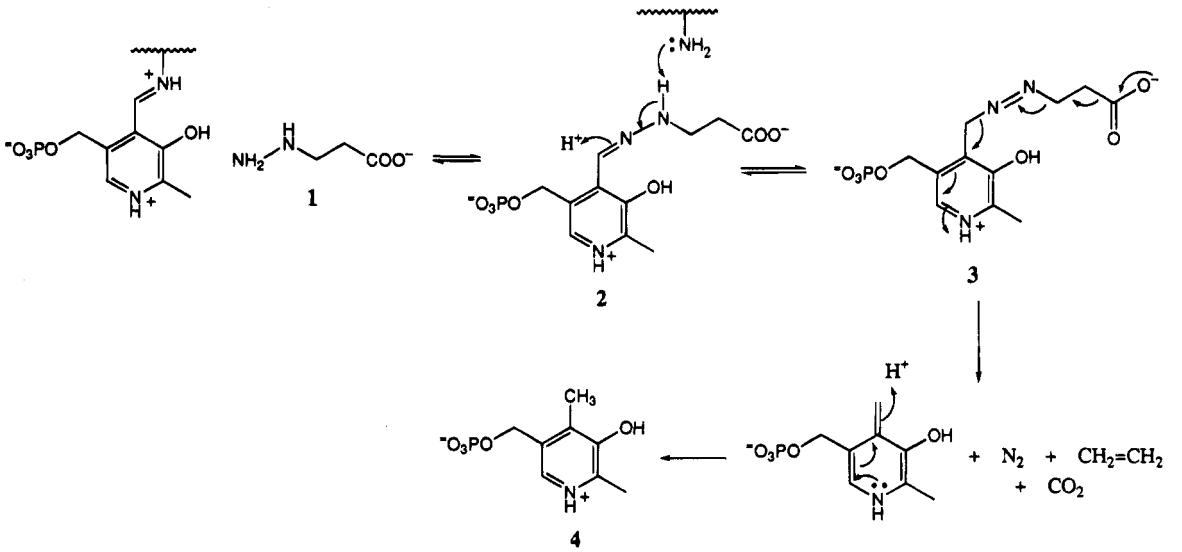
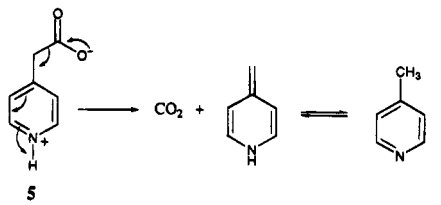
Scheme 1



Scheme 2



Scheme 3



the solution was injected into the HPLC (Beckman 330). The compounds were eluted with water–0.1% TFA at 0.5 mL/min for 15 min, then the flow rate was increased to 1 mL/min over 5 min, and finally the solvent was switched to 100% methanol over 60 min. During this time, 1 min fractions were collected for the first 30 min and 2 min fractions were collected for the last 30 min. Scintillation cocktail (10 mL) was added to the fractions, as well as to the dissolved pellets, and they were counted for radioactivity. This same experiment was repeated, except that the enzyme solution was run through a Penefsky column[8] (Sephadex G-50) after inactivation. The column was washed with 150 μL of the potassium phosphate buffer, and the protein fraction was treated as above, starting with the adjustment of the pH to 12 with 2 M KOH.

The experiment described above was repeated, substituting [^{3}H]PLP for the [^{3}H]PLP-reconstituted enzyme. All of the conditions were the same as above. HPLC was used to identify the radioactive peaks observed for the enzyme experiment.

Synthesis of the Hydrazone of Hydrazinopropionate and PLP. PLP (28.7 mg, 0.116 mmol) was added to hydrazinopropionate (36.2 mg, 0.348 mmol) in a water solution (3 mL) at pH 5. The pH of the reaction was adjusted to 7.5, and the solution was allowed to stir overnight. The desired product was isolated from the reaction solution by semipreparative HPLC

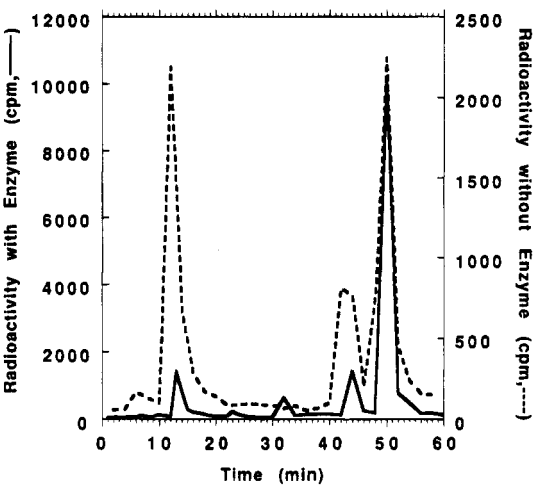


Figure 1. HPLC of tritiated products following inactivation of [^{3}H]PLP-reconstituted GABA-AT by 3-hydrazinopropionate. The solid line is the radioactivity after denaturation of [^{3}H]-PLP-reconstituted GABA-AT inactivated with 3-hydrazinopropionate. The dashed line is the nonenzymatic control of [^{3}H]PLP with 3-hydrazinopropionate. 4′-Deoxypyridoxine 5′-phosphate (**4**) elutes at 22 min, PLP elutes at 13 min, and the hydrazone of 3-hydrazinopropionate with PLP elutes at 50 min under the conditions described in the Experimental Section.

(Alltech Econosil C_{18}, 10μ), eluting with a gradient of water–0.1% TFA to 100% methanol over 100 min at 1.0 mL/min: ^{1}H NMR (D_2O) δ 2.59 (s, 3 H), 2.59 (t, 2 H), 3.63 (t, 2 H), 5.02 (s, 1 H), 5.04 (s, 1 H), 7.88 (s, 1 H), 8.03 (s, 1 H); HRMS (FAB$^+$) calcd for $C_{11}H_{17}N_3O_7P$ 334.0804, found 334.0769.

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