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Irreversible Inhibition of Glutamate Decarboxylase by α-(Fluoromethyl)glutamic Acid†

Donald Kuo and Robert R. Rando*

ABSTRACT: α-(Fluoromethyl)glutamic acid (FMG) was synthesized and shown to be an active site directed irreversible inhibitor of glutamate decarboxylase (EC 4.1.1.15) from *Escherichia coli*. The K_I for the active enantiomer is 1.4 μM, and the $k_{inh} = 5.9 \times 10^{-3}$ s^{-1}. Substrates for the enzyme, such as L-glutamate, and competitive inhibitors, such as citrate, decrease the rates of FMG-mediated inactivation of the enzyme. A profound change in the ultraviolet spectrum of the enzyme accompanies the inactivation process. When [^{3}H]-FMG is used, it can be shown that the enzyme incorporates radioactivity at the same rate as that of inactivation. There is a 1:1 stoichiometry of [^{3}H]FMG incorporated to pyridoxal phosphate binding subunits of the enzyme. From these and other studies it is concluded that FMG is a substrate for the enzyme and alkylates it as a consequence of this turnover.

Glutamate decarboxylase is a pyridoxal phosphate linked enzyme which catalyzes the decarboxylation of L-glutamate to γ-aminobutyric acid (Strausbauch & Fischer, 1967). Several active site directed irreversible inhibitors of this enzyme, including bromopyruvate, have been reported (Fonda, 1976). Reagents of this type, however, generally lack potency and selectivity. Recently, studies from different laboratories have shown that α-fluoromethyl substrate analogues can be potent mechanism-based inactivators of pyridoxal phosphate linked decarboxylases. For example, α-fluoromethyl-Dopa and α-(difluoromethyl)ornithine are potent inactivators of Dopa decarboxylase and ornithine decarboxylase, respectively (Maycock et al., 1980; Metcalf et al., 1978). Since we have been interested in the design of inactivators of glutamate decarboxylase, we have synthesized several of the fluorinated glutamate analogues as possible mechanism-based inactivators of this enzyme. In this report, we demonstrate that α-(fluoromethyl)glutamate is a potent mechanism-based inactivator of bacterial glutamate decarboxylase. The K_I for the inhibitor is 1.4 μM, and the first-order rate constant for inactivation

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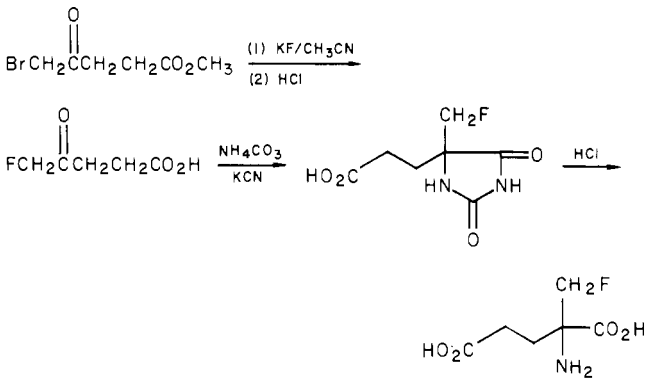
Scheme I



is 5.9×10^{-3} s^{-1}. FMG had previously been tested as an inactivator of mammalian glutamate decarboxylase and shown to possess very little activity (Kollonitsch et al., 1978).

Materials and Methods

Glutamate decarboxylase type II from *Escherichia coli* was obtained from Sigma Chemical Co. and further purified by published methods (Strausbauch & Fischer, 1967). The enzyme purified to homogeneity (specific activity 110 units/mg) was obtained from Dr. Margaret Fonda of the University of Louisville Medical School. A unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of γ-aminobutyric acid from L-glutamate under defined conditions. L-[1-^{14}C]Glutamate, specific activity 52.5 mCi/mmol, was obtained from New England Nuclear; L- and D-glutamic acids were obtained from Sigma Chemical Co. DL-Methylglutamic acid was obtained from ICN. All other chemicals and solvents were obtained from commercial sources and were of the highest purity obtainable. Elemental analyses were performed by Galbraith Laboratories Inc.

Syntheses. FMG was synthesized as in Scheme I.

5-Fluorolevulinic Acid. Methyl 5-bromolevulinate was synthesized as described in the literature (MacDonald, 1974). Methyl 5-bromolevulinate (21.9 g) and 14.5 g of potassium fluoride were refluxed in 300 mL of acetonitrile for 30 h. (The addition of 10 mol % of dicyclohexano-18-crown-6 will reduce the reflux time to 3 h.) Ether (300 mL) and 50 mL of water were added to the residue after the acetonitrile was removed. Excess sodium bicarbonate was added to the mixture, and the ether layer was separated from the aqueous layer. The aqueous layer was extracted with 200 mL of ether. The combined ether extracts were dried over magnesium sulfate, and the ether was removed by evaporation. The residue was distilled by a bulb-to-bulb distillation in vacuo (30 μm, 51–61 °C). The yield of methyl 5-fluorolevulinate was 66%. Methyl 5-fluorolevulinate (32.8 g) was stirred in 150 mL of concentrated hydrochloric acid at room temperature for 1 h. The hydrochloric acid was removed by rotary evaporation, and 100 mL of concentrated hydrochloric acid was added again. This process was repeated a third time, the residue was distilled by bulb-to-bulb distillation (30 μm, 40–90 °C) in vacuo, and 27 g of 5-fluorolevulinic acid was collected (90% yield): mp 55–56 °C; NMR (CD_3COCD_3, Me_4Si) δ 2.72 (d, 4 H) and 4.93 (s, 1 H). Anal. Calcd for $C_5H_7O_3F$: C, 44.8; H, 5.2; F, 14.2. Found: C, 44.9; H, 5.2; F, 14.0.

5-(Fluoromethyl)-5-(2-carboxyethyl)hydantoin. This synthesis was adapted from a published hydantoin synthesis (Henze & Speer, 1942).

5-Fluorolevulinic acid (13.4 g, 0.1 mol), ammonium carbonate (45.5 g, 0.4 mol), and potassium cyanide (13.0 g, 0.2 mol) were stirred with 350 mL of water at 60 °C for 3 h. The volume was reduced to about half, and then concentrated hydrochloric acid was added to bring the pH to <2. After sitting for 0.5 h, the solution was evaporated under reduced pressure, and the residue was dried overnight in vacuo. Acetone (500 mL, dried over magnesium sulfate) was added to the solid and stirred for 0.5 h. The acetone extract was filtered through the sintered-glass funnel, and the filtrate was evaporated to dryness. The solid residue was crystallized from ethanol and methylene chloride. The white crystalline material weighed 15 g (75% yield): mp 121–122 °C; NMR (CD_3-CO-CD_3, Me_4Si) δ 2.15 (m, 4 H), 4.19 (d, 1 H), 4.99 (d, 1 H), 6.02 (br, 1 H), and 7.31 (s, 1 H). Anal. Calcd for $C_7H_9FN_2O_4$: C, 41.2; H, 4.4; F, 9.3. Found: C, 41.3; H, 4.4; F, 9.2.

(RS)-2-(Fluoromethyl)glutamic Acid. The hydantoin (408 mg; 2 mmol) was refluxed with 10 mL of concentrated hydrochloric acid for 24 h. The residue was dissolved into 200 mL of water and decolorized with charcoal. The solution was evaporated to dryness and taken up into a minimum of distilled water. The solution was absorbed onto 40 g of Dowex-AG-50-H^+ (X8, 200–400 mesh) and washed with 300 mL of water. The product was eluted with 500 mL of 2 N NH_4OH, and the ammonia solution was removed on the rotary evaporator. The solid residue was dried in vacuo, and 120 mg was collected as the product (30%). The product was further purified by chromatography on Dowex-AG-1-acetate (X2, 200–300 mesh). After being washed with water, the product was eluted with 2 N acetic acid: mp 162–163 °C; NMR (D_2O) δ 2.73 (m, 4 H), 3.91 (d, 1 H), and 5.88 (d, 1 H); thin-layer chromatography behavior [silica; solvent was 1-butanol–H_2O–acetic acid (v/v) 3:1:1] FMG R_f 0.21 (compared to R_f 0.22 for methylglutamate, R_f 0.18 for L-glutamate, and R_f 0.26 for GABA), with a single ninhydrin-positive spot. Anal. Calcd for $C_6H_{10}FNO_4$: C, 36.5; H, 6.1; N, 7.1; F, 9.6. Found: C, 36.5; H, 6.0; N, 7.7; F, 9.5.

[^{3}H]-(RS)-α-(Fluoromethyl)glutamic Acid. The 3 and 5 C-H positions of 5-fluorolevulinic acid were exchanged for ^{3}H by heating a mixture containing 300 mg (2.2 mmol) of 5-fluorolevulinic acid, 0.4 mL of tritiated water (0.1 Ci/mL), and 500 mg of *p*-toluenesulfonic acid at 80 °C for 24 h in a sealed tube. In separate experiments, it was shown that 75% of the 5-protons and 100% of the 3-protons exchanged for deuterium under identical conditions in deuterium oxide. The 3- and 5-protons of 5-fluorolevulinic acid absorb at δ 2.72 and 4.63, 4.93 (NMR). The disappearance of these resonances in D_2O allowed for the determination of the amount of exchange. The excess tritiated water was removed on a vacuum line. Water (10 mL) was added to the residue, and the product was extracted into 8 × 20 mL portions of ether. After the extract was dried over magnesium sulfate, it was evaporated, and the residue was taken up in 20 mL of acetone. The acetone was filtered, dried over magnesium sulfate, and evaporated to dryness to yield 264 mg (88%) of 5-fluoro-[3,5-^{3}H]levulinic acid. This compound was converted into the 2-(fluoro[^{3}H]methyl)[3-^{3}H]glutamic acid of specific activity 1.02 Ci/mol by the procedures described above for the synthesis of nonradioactive (fluoromethyl)glutamate.

Enzyme Assays. Glutamate decarboxylase was assayed by the published method, which involved trapping and counting the $^{14}CO_2$ evolved from [1-^{14}C]glutamate (Strausbauch & Fischer, 1967). The assays were done in 0.2 M pyridine hydrochloride, pH 4.5, containing 1 mM (aminoethyl)isothiouronium bromide (AET), 1 mM pyridoxal phosphate, 5 mM L-glutamate, and 0.05 μCi of DL-[1-^{14}C]glutamate. The $^{14}CO_2$ was trapped in 5 N NaOH and counted in 10 mL of

Aquasol (New England Nuclear). Each assay contained approximately 0.3 unit of glutamate decarboxylase.

Inactivation Studies. The enzyme (~3 units) was incubated with various concentrations of α-(fluoromethyl)glutamate at 37 °C for different time periods in 50 mM pyridine hydrochloride, pH 4.5, 1 mM AET, and 1 mM pyridoxal phosphate. Aliquots of this solution were then added to the assay cocktail described above, and the remaining activity was determined. In all cases, the amount of α-(fluoromethyl)glutamate transferred to the assay cocktail was low enough that it would not interfere with the assay.

Labeling of Glutamate Decarboxylase by [^{3}H]FMG. (a) (Inhibitor) > (Enzyme). Homogeneous glutamate decarboxylase (specific activity 110 units/mg, 0.5 mL (18.9 units/mL), 1.72 nmol of active subunits) was incubated in a plastic tube with 5×10^{-2} μmol of [^{3}H]FMG (specific activity 1.01 mCi/mmol) in the assay buffer for 3 h at 25 °C. Under these conditions, the enzyme is completely inactivated. Bovine serum albumin (0.2 mL, 10 mg/mL) was added, and the reaction was quenched with 0.1 mL of 100% trichloroacetic acid (Cl_3CCOOH). The precipitate was centrifuged and the supernatant was decanted. The precipitate was suspended in 10% Cl_3CCOOH with sonication and centrifuged. This precipitate was dissolved in 0.5 mL of Protosol (New England Nuclear) in a glass vial and counted in 20 mL of Econoflor (New England Nuclear). It was found that 3542 dpm of FMG was incorporated into the above enzyme allowing for the following calculation of stoichiometry:

$$\frac{3542 \text{ dpm}/9.45 \text{ units}}{110 \text{ units/mg}} \times \frac{\mu\text{mol}}{50 \text{ mg}} = 2.06 \times 10^6 \text{ dpm}/\mu\text{mol of active subunit}$$

The specific activity of the [^{3}H]FMG = 2.03×10^6 dpm/μmol of [^{3}H]FMG. *Hence*, there are 1.01 μmol of [^{3}H]FMG/μmol of pyridoxal phosphate bearing subunits.

(b) (Enzyme) > [^{3}H]FMG. This experiment was conducted identically with that described in (a) except that 0.8 mL (2.74 nmol of active subunits) of pure enzyme was used along with 2.5 nmol. (*RS*)-[^{3}H]FMG, 1.1 nmol, was incorporated into the enzyme. Assuming the inactivation process is stereospecific, it can be calculated that on the average 80.6% of the available [^{3}H]FMG is incorporated into the enzyme.

Results

Rates of Glutamate Decarboxylase Inactivation by α-(Fluoromethyl)glutamate. FMG was synthesized as described under Materials and Methods. Bacterial glutamate decarboxylase was treated with FMG at various concentrations, and its rate of inactivation was followed. As can be seen in Figure 1, first-order rates of inactivation were observed, up to approximately 90% inactivation of the enzyme. The remainder of the enzyme was inactivated (>98%), but at a slower rate. The inactivated enzyme did not recover activity after extended dialysis against the buffer. The reason for the biphasic kinetics of inactivation is not apparent, but it is highly reproducible. It cannot be due to inactivator consumption since the same slow rate is still observed when fresh inactivator is added after 80% inactivation or when the inactivator is allowed to incubate with the buffer components for 0.5 h before the addition of the enzyme. Also shown in Figure 1 is an inverse plot for the inactivation process. Saturation kinetics are observed with a $K_I = 1.4$ μM and a $k_{inh} = 5.9 \times 10^{-3}$ s^{-1}. The rate-limiting step has not been determined yet, and it is not clear whether k_{inh} is a measure of the rate of enzymatic turnover or of the inactivation step. The measured K_I is much

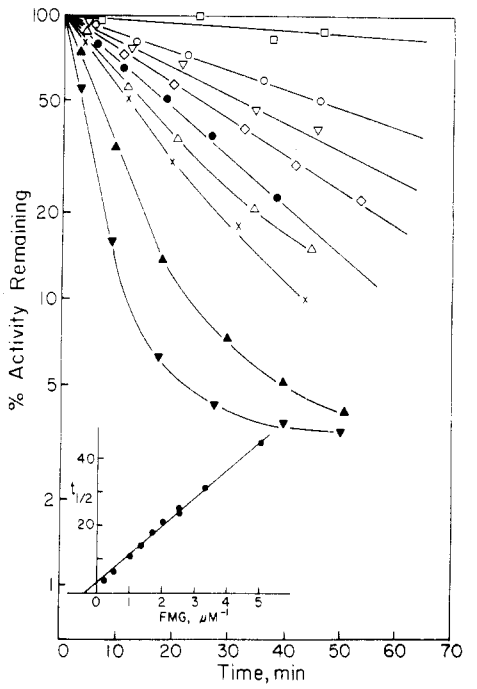


FIGURE 1: Inactivation of glutamate decarboxylase by (*RS*)-(fluoromethyl)glutamate. Pure glutamate decarboxylase (0.3 unit, specific activity 110 units/mg) was incubated with various concentrations of FMG in a total volume of 0.2 mL of inactivation medium. (□) Control (-FMG); (○) 0.2 μM FMG; (▽) 0.3 μM FMG; (◇) 0.4 μM FMG; (●) 0.6 μM FMG; (△) 0.75 μM FMG; (×) 1 μM FMG; (▲) 2 μM FMG; (▼) 4 μM FMG. At the indicated times, 25-μL aliquots were removed and transferred to the enzyme assay medium, and the remaining enzymatic activity was determined. This figure shows the pseudo first order inactivation profile. The inset shows a Lineweaver–Burk type plot ($t_{1/2}$ (min) vs. (L-FMG) (μM^{-1})) which yields the k_{inh} and K_I calculated for the *S* isomer ($K_I = 1.4$ μM, and $k_{inh} = 5.9 \times 10^{-3}$ s^{-1}). Complete inactivation of the enzyme could eventually be achieved. No reactivation of the enzyme occurred after continued dialysis against the assay buffer or after gel filtration (Sephadex G-10). The addition of 0.1 mM mercaptoethanol did not decrease the rate of enzyme inactivation.

lower than that of α-methylglutamate or of glutamate itself. The K_I for α-methylglutamate is 15 mM, and the K_M for L-glutamate is 0.88 mM (Fonda, 1972). This effect is probably due to the electronegativity of the fluorine atom vs. H, which increases the nucleophilicity of the amino group. Similar effects have been noted in the α-fluoromethyl-Dopa-mediated inactivation of Dopa decarboxylase (Maycock et al., 1980).

Effects of Competitive Inhibitors on the Inactivation Process. If FMG is an active site directed, mechanism-based inactivator of glutamate decarboxylase, substrate molecules and competitive inhibitors of the enzyme should decrease the rate off inactivation. In Figure 2, rates of enzyme inactivation are shown in the presence of D-glutamate, L-glutamate, citrate, and DL-α-methylglutamate. L-Glutamate, citrate, and α-methylglutamate protect against inactivation whereas D-glutamate does not. This is exactly what is expected since D-glutamate is not a substrate for the enzyme. In separate experiments, we observed that citrate shows perfect competitive inhibition kinetics as an inhibitor of L-glutamate decarboxylation. The measured $K_I = 0.56$ mM. In Figure 3, an inverse plot for the inactivation of glutamate decarboxylase in the presence of 1 μM FMG and various concentrations of citrate is shown. From this plot, the K_I for citrate can be calculated to be 0.52 mmol, again demonstrating the active site nature of the FMG mediated inactivation of glutamate decarboxylase.

Ultraviolet Spectral Changes Accompanying the Inactivation Process. The active site directed nature of the inactivation

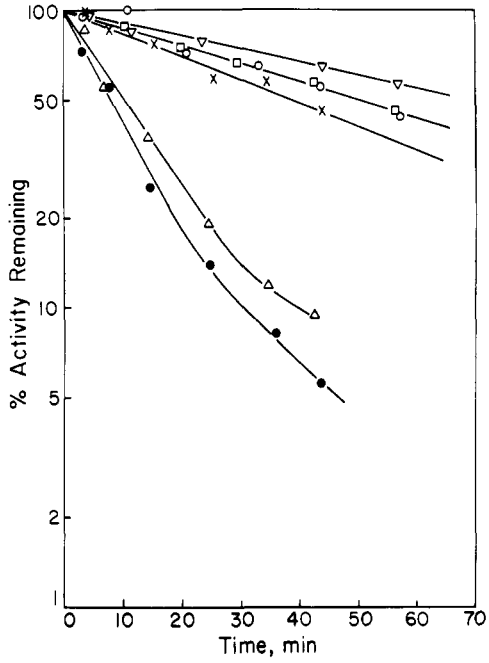


FIGURE 2: Effect of competitive inhibitors on glutamate decarboxylase inactivator. Glutamate decarboxylase (0.3 unit, specific activity 110 units/mg) was incubated as in Figure 1 and with D-glutamate, L-glutamate, DL-α-methylglutamic acid, and citrate, each independently in 0.2 mL of assay buffer for 5 min. FMG was added to give a final concentration of 1 μM; at various time points, 25-μL aliquots were removed, and the activity remaining was determined in the usual way. (▽) Control enzyme (FMG); (●) 1 μM FMG; (△) 1 μM FMG + 29 mM D-glutamate; (×) 1 μM FMG + 29 mM L-glutamate; (○) 1 μM FMG + 10 mM citrate; (□) 1 μM FMG + 50 mM DL-α-methylglutamate.

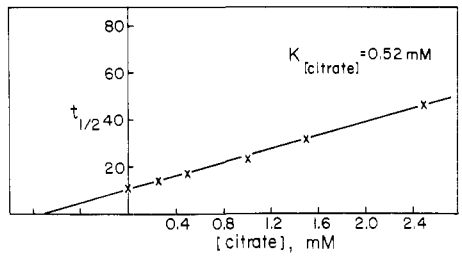


FIGURE 3: In separate experiments, it was shown that the citrate is a competitive inhibitor of L-glutamate decarboxylase. The measured K_I = 0.56 mM. In the experiment shown above, 0.3 unit of glutamate decarboxylase (specific activity 110 units/mg) was independently preincubated with the various concentrations of citrate indicated in the above plot. FMG was added to yield a final concentration of 1 μM, and the remaining enzymatic activity was determined. From the plot constructed above, a binding constant for citrate = 0.52 mM was determined.

process can be further explored by following the alteration in the ultraviolet spectrum of the enzyme as a function of its inactivation. As can be seen from Figure 4, the pyridoxal phosphate absorption at 414 nm is bleached concomitantly with inactivation, and a new band at approximately 330 nm is generated. This experiment shows that FMG must be a substrate for the enzyme prior to inactivating it. In separate experiments, it can be shown that under the conditions of the experiments the inhibitor does not react with pyridoxal phosphate and hence is not simply a pyridoxal phosphate antagonist. Enzymatic participation is required to produce the spectral change.

By studying the change in holoenzyme absorption at 414 nm as a function of inhibitor concentration, it should be possible to approximate the stoichiometry of inhibitor incorporation. This is possible because it has been determined that

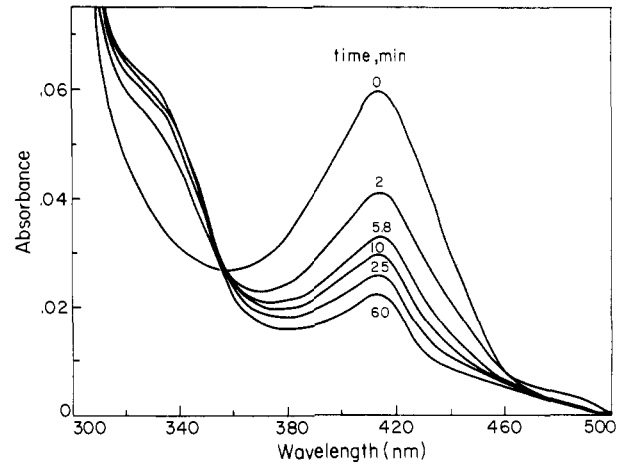


FIGURE 4: Ultraviolet spectral change of holoenzyme accompanying inactivation. To 0.8 mL of inactivation buffer containing 16 units of enzyme (specific activity 20 units/mg) was added FMG at 15 μM final concentration, all in a 1-cm cuvette. The total absorbance was set at 0.1 *A* unit on a Cary 119 spectrometer. The cuvette was scanned at the indicated times.

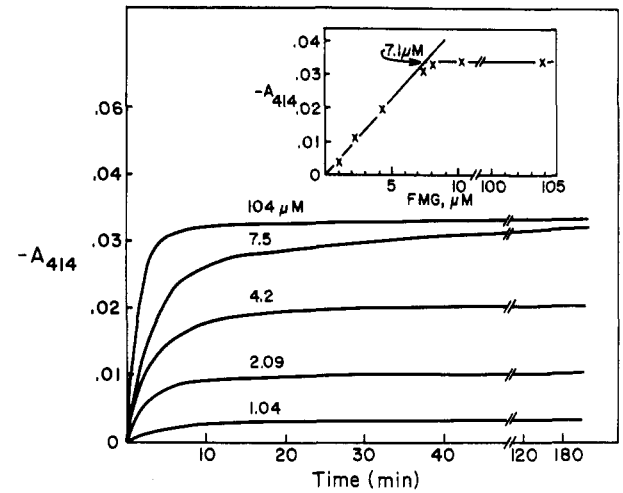


FIGURE 5: Change in holoenzyme absorption at 414 nm as a function of FMG concentration. These experiments were conducted similarly to that in Figure 4 except that enzyme of specific activity 110 units/mg was used. In the above figure, the time course for the total change in absorbance at 414 nm is plotted for various concentrations of FMG. In the inset, the total amplitude of the absorbance change at 414 nm is plotted as a function of the concentrations of FMG. Given that $\epsilon_{420}^{1cm} = 10^4$/subunit, it can be calculated that 1.04 molecules of FMG is incorporated per subunit (MacDonald, 1974).

$\epsilon_{420}^{1cm} = 10^4$/holoenzyme subunit (Fonda, 1971). This further assumes that FMG is not turned over without inactivation occurring. This point will be returned to later. In Figure 5, the time course for the change in absorbance at 414 nm is plotted for differing concentrations of FMG. In the inset in Figure 5, the absorbance change at 414 nm is plotted as a function of the amount of FMG added. Since the inactivation process is assumed to be stereospecific, 3.55 nmol of FMG is incorporated/3.5 nmol of 50 000-dalton subunits. Thus, by this method it can be calculated that 1.04 molecules of FMG are incorporated per subunit.

Titration of Glutamate Decarboxylase with [^3H]-α-(Fluoromethyl)glutamate. The studies discussed above suggest that the stoichiometry of FMG binding per subunit is 1. However, in order to demonstrate this unequivocally, a radioactive FMG analogue is required; to these ends, 2-(fluoro[^{3}H]methyl)[3-^{3}H]glutamate was prepared. This synthesis was accomplished by exchanging the hydrogen atoms at the 3 and 5 positions of 5-fluorolevulinic acid in tritium water by

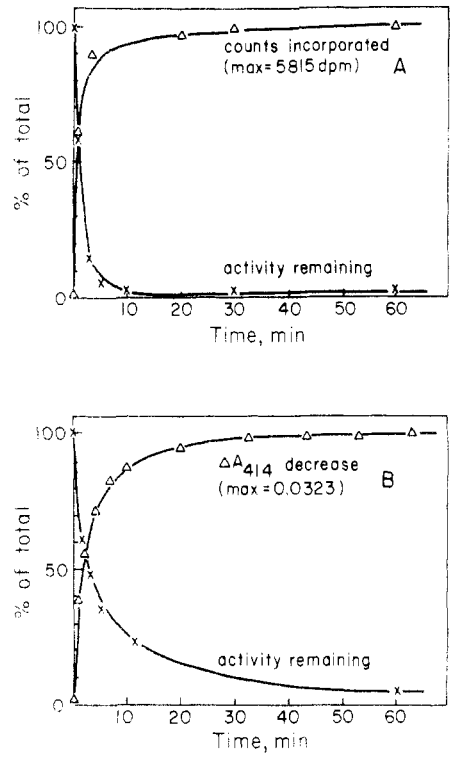


FIGURE 6: (A) Labeling of glutamate decarboxylase with [^{3}H]FMG. To 6.2 mL of the inactivation buffer containing 124 units of enzyme (specific activity 20 units/mL) was added 20 μL of 10 mM [^{3}H]FMG (specific activity 1.02 mCi/mmol) at 37 °C. At each of the indicated time points, 1-mL aliquots were removed and quenched with 0.2 mL of 100% Cl_3CCOOH. At the same time, 5-mL aliquots were removed and assayed for the remaining enzyme activity. The Cl_3CCOOH precipitate was centrifuged down and suspended in 1 mL of 10% Cl_3CCOOH. The precipitate was dissolved in 0.5 mL of Protosol and after sonication and centrifugation was counted in 20 mL of Econoflor. Under the conditions discussed above, neither apoenzyme, enzyme pretreated with 1 mM hydrazine (or 1 mM aminooxyacetic acid), nor enzyme enzyme pretreated with (*RS*)-4-aminohex-5-ynoic acid incorporated any radioactivity (Jung, 1978). The above inactivation profile was accomplished by using partially purified enzyme. In order to check the stoichiometry, the pure enzyme (specific activity 110 units/mg) was labeled with FMG, and the incorporation was precisely determined. (See Materials and Methods.) Under these conditions, the molar stoichiometry was again observed to be 1:1 (1.01 molecules of FMG incorporated/subunit). (B) Change in absorbance at 414 nm as a function of enzyme activity remaining. The experiment was conducted identically to that in Figure 4 except that the decrease in absorbance at 414 nm was followed as a function of the remaining enzymatic activity. The ordinate gives the percentage decrease in absorbance at 414 nm or the percentage decrease in enzymatic activity.

using *p*-toluenesulfonic acid as a catalyst. The tritiated 5-fluorolevulinic acid was then converted into the FMG by the standard procedures. The enzyme was inactivated with the radioactive analogue and the amount of radioactivity incorporation was plotted as a function of the rate of inactivation of the enzyme (Figure 6A). The two curves show virtually identical time courses. Similar curves can be generated by following the decrease in absorbance at 414 nm as a function of enzyme activity remaining (Figure 6B).

The stoichiometry of [^{3}H]FMG binding shows that each subunit of the enzyme contains 1.01 molecules of inactivator (see Materials and Methods). Apoenzyme does not incorporate radioactivity under the conditions of the experiments reported here. In addition, enzyme pretreated with hydrazine, aminooxyacetic acid, or 4-aminohex-5-ynoic acid does not incorporate radioactivity when treated with [^{3}H]FMG. The first two reagents are nonspecific pyridoxal phosphate antagonists whereas the last is a specific active site directed inactivator of the enzyme (Jung et al., 1978). Of further interest is the number of FMG molecules turned over per inactivation event. To study this, excess enzyme was labeled with FMG, and the amount of covalent incorporation into the protein was determined (see Materials and Methods). With the assumption that the labeling is stereospecific, approximately 80% of the theoretical radioactivity was incorporated into the protein. This suggests that the inactivation process is highly efficient, and at a minimum approximately eight enzyme molecules are labeled for every ten turnovers. These results are consistent with a specific mode of enzyme inactivation.

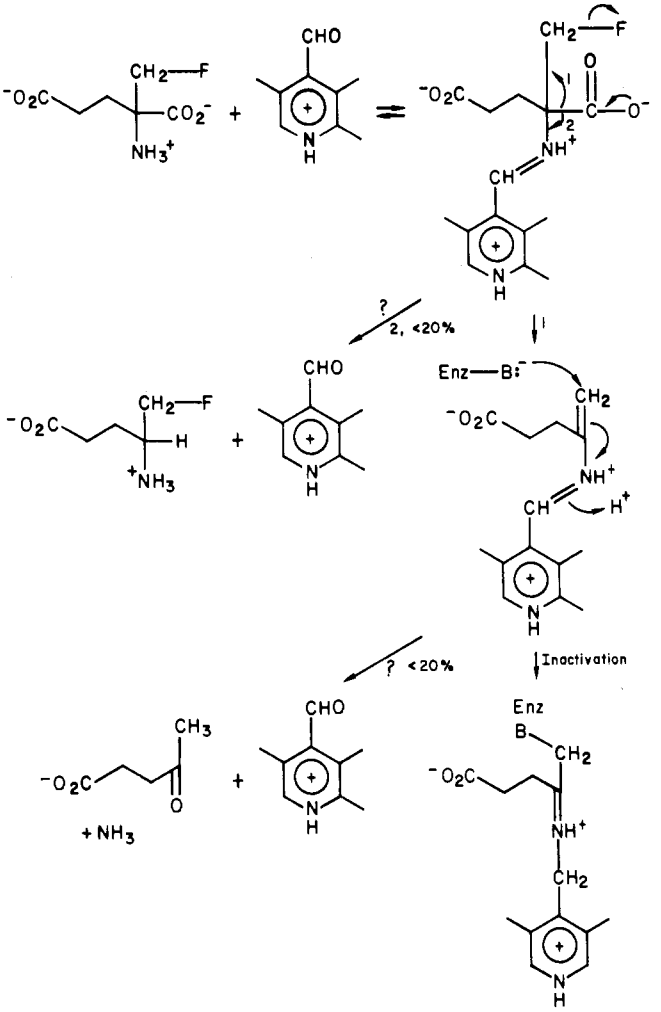
Discussion

In this article it is shown that FMG is a specific inactivator of bacterial glutamate decarboxylase. As expected of a mechanism-based, suicide, or k_{cat} inactivator, saturation kinetics for the inactivation process are observed (Rando, 1974; Abeles & Maycock, 1976). The hypothesis that direct inactivation of the enzyme occurred without the inhibitor diffusing from the active site is supported by the fact that there is no lag in the kinetics of inactivation and by the fact that trapping agents such as mercaptoethanol have no effect on the inactivation of the enzyme. The measured $K_I = 1.4\ \mu M$, and the $k_{inh} = 5.9 \times 10^{-3}\ s^{-1}$. The kinetics of inactivation, however, were not simple pseudo first order. It appears that approximately 90% of the enzyme is inactivated at a fast rate and 10% at a slower rate. Biphasic kinetics of inactivation of this type have been observed with other fluorinated-substrate–pyridoxal phosphate linked enzyme pairs. For example, Dopa decarboxylase is inactivated by *S*-α-fluoromethyl-Dopa with at least biphasic kinetics (Maycock et al., 1980). The mechanism of the observed biphasic kinetics is not clear. Glutamate decarboxylase from *Escherichia coli* is a hexamer, and it is possible that not all of the subunits are equally active (Strausbauch & Fischer, 1970). Trivial possibilities for the observed biphasic kinetics of inactivation such as enzyme purity, time-dependent enzyme reactivation, or inhibitor destruction were ruled out in this case. The measured K_I of 1.4 μM can be quite favorably compared with the K_M for L-glutamate (0.88 mM) or α-methylglutamate (15 mM) (Fonda, 1972). Doubtless the depression of the pK_A of the α-amino group by the α-fluoromethyl group leads to the increased binding affinity of FMG. As mentioned previously, this kind of enhancement has been reported for other pyridoxal phosphate linked enzymes (Maycock et al., 1980).

The notion that FMG is active site directed is supported by several lines of evidence. L- but not D-glutamate protects against FMG-mediated inactivation. Competitive inhibitors such as citrate and DL-α-methylglutamate also slow down the rate of enzymatic inactivation in the presence of FMG. Most importantly, the stoichiometry of inactivator incorporation to active subunits is approximately 1:1. In addition, the rate of enzyme inactivation in the presence of [^{3}H]FMG correlates well with the rate of radioactivity incorporation.

That enzymatic turnover of FMG precedes inactivation can be inferred from several kinds of experiments. First of all, there is a dramatic change in the ultraviolet spectrum of the holoenzyme when the enzyme is treated with inactivator. The bleaching of the 414-nm pyridoxal phosphate peak occurs concomitantly with inactivation. This change does not occur when pyridoxal phosphate itself is treated with FMG. Furthermore, pyridoxal phosphate antagonists such as hydrazine and aminooxyacetic acid protect the enzyme against inactivation and radioactivity incorporation when it is treated with [^{3}H]FMG. In addition, an apoenzyme does not incorporate

Scheme II



radioactivity when treated with [^{3}H]FMG. When excess enzyme is treated with radioactive inactivators, approximately 80% of the radioactivity expected from the *S* isomer is incorporated into the enzyme. Therefore, the inactivation process must be highly efficient in that at least four out of every five turnovers result in the labeling of an active subunit. A mechanism for the inactivation process that accounts for the data discussed above is shown in Scheme II. At least 80% of the time, enzymatic turnover of the FMG leads to enzymatic inactivation. Scheme II also shows how nonlethal turnover might occur. It is possible that direct decarboxylation could occur to afford fluoromethyl-GABA or that hydrolysis of the activated intermediate could occur to afford levulinic acid. However, no direct evidence exists for either of the two pathways, and as mentioned previously they must account for less than 20% of the original susceptible FMG.

FMG was also tested as an inactivator of the mouse and chick brain enzymes. The molecule is an exceedingly weak inactivator of these enzymes, with enzymatic half-life being several hours in the presence of millimolar concentrations of the inactivator. This is consistent with what was reported for the effect of FMG on the mammalian brain enzyme (Kollinitsch et al., 1978). The mammalian brain and the bacterial enzymes are certainly different in their structures (Strausbauch & Fischer, 1970; Wu et al., 1973). They also are markedly different in their susceptibility to mechanism-based inactivators. α-Methyl-*trans*-dehydroglutamate inactivates the mammalian brain enzyme but has little effect on the bacterial enzyme, and the opposite is true for FMG (Chrystal et al., 1979). Nevertheless, α-FMG should prove useful in understanding the structure and function of the bacterial enzymes. Studies along these lines are currently in progress.

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