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Interaction of L-*threo* and L-*erythro* isomers of 3-fluoroglutamate with glutamate decarboxylase from *Escherichia coli*

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L-*threo*-3-Fluoroglutamate and L-*erythro*-3-fluoroglutamate were tested with glutamate decarboxylase from *Escherichia coli*. Both isomers were substrates: the *threo* isomer was decarboxylated into optically active 4-amino-3-fluorobutyrate, whereas the *erythro* isomer lost the fluorine atom during the reaction, yielding succinic semialdehyde after hydrolysis of the unstable intermediate enamine. The difference between the two isomers demonstrates that the glutamic acid–pyridoxal phosphate Schiff base is present at the active site under a rigid conformation. Furthermore, although the *erythro* isomer lost the fluorine atom, yielding a reactive aminoacrylic acid in the active site, no irreversible inactivation of *E. coli* glutamate decarboxylase was observed.

Glutamate decarboxylase (L-glutamate 1-carboxy-lyase, EC 4.1.1.15) is present in animals, bacteria and plants (Boeker & Snell, 1972; Gale, 1946), but most work has been performed on the animal and bacterial enzymes. The biological function of bacterial glutamate decarboxylase is unclear (Boeker & Snell, 1972; Gale, 1946), but in animals glutamate decarboxylase is involved in regulating the concentrations of glutamate, an excitatory neurotransmitter (Roberts, 1974) and of 4-aminobutyrate, an inhibitory neurotransmitter (Wofsey *et al.*, 1971). Several mechanism-based irreversible inhibitors (Walsh, 1982) of glutamate decarboxylase have been described: serine *O*-sulphate (Likos *et al.*, 1982), allylglycine (Orlowski *et al.*, 1977), 3,4-dehydro-2-methylglutamate (Chrystal *et al.*, 1979), 2-fluoromethylglutamate (Kuo & Rando, 1981) and 4-aminohex-5-ynoate (Jung *et al.*, 1978).

These $k_{cat.}$ inhibitor studies have revealed active-site differences between the bacterial and the animal enzymes, but they have provided very little information concerning the positioning and the conformation of glutamic acid at the active site. The only information available derives from the use of analogues as competitive inhibitors of glutamate (Fonda, 1972).

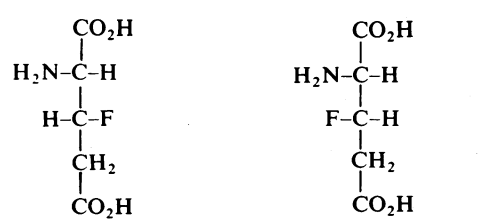
We considered that a comparison of the behaviour of the L-*threo* and L-*erythro* isomers of 3-fluoroglutamate (Fig. 1), two potential $k_{cat.}$ inhibitors of glutamate decarboxylase, could provide valuable information on the conformation of glutamic acid at the active site.

In the present paper we report on the interactions of those isomers with glutamate decarboxylase from *Escherichia coli*.

Materials and methods

Enzymes

Glutamate decarboxylase from *E. coli* (type V; 37 units/mg) and Gabase from *Pseudomonas fluorescens* (a mixture of 4-aminobutyrate transaminase and succinic semialdehyde dehydrogenase; 4 units/mg) were from Sigma Chemical Co.



(2*R*,3*R*)-3-Fluoroglutamate L-*threo* isomer (2*R*,3*S*)-3-Fluoroglutamate L-*erythro* isomer

Fig. 1. *Structures of* L-*threo and* L-*erythro isomers of 3-fluoroglutamic acid*

Chemicals

The synthesis and resolution of the L-*threo* and the L-*erythro* isomers of 3-fluoroglutamic acid are described elsewhere (Vidal-Cros *et al.*, 1985). Pyridoxal phosphate was from Sigma Chemical Co., $NADP^+$ (disodium salt) from Boehringer, ninhydrin (spectrophotometric grade) from Janssen and 2-oxoglutaric acid from Prolabo. All other chemicals were of the highest purity available.

Ninhydrin titration

Comparison of glutamic acid, L-*threo*-3-fluoroglutamic acid and L-*erythro*-3-fluoroglutamic acid with respect to ninhydrin titration (Greenstein & Winitz, 1961) yielded relative response coefficients of 1, 0.93 and 0.83.

Gabase assay

Standard assays were run in 300μl of 0.1M-potassium pyrophosphate buffer, pH8.6, containing $NADP^+$ (4mM), 2-oxoglutarate (20mM), Gabase {10 munits [stock solution containing 1 unit/ml in 75mM-phosphate buffer, pH7.2, containing 25% (v/v) glycerol]} and solution to be tested (10μl). After preincubation (25°C, 5min) of the other components the reaction was initiated by addition of 2-oxoglutarate and monitored at 340nm. Under those conditions, the reaction was completed in 40min for 4-aminobutyrate and 3.5h for 4-amino-3-fluorobutyrate.

Rate of reaction of 3-fluoroglutamic acid isomers with glutamate decarboxylase

Glutamate decarboxylase (10 units) and pyridoxal phosphate (1mM) were preincubated in 2ml of 50mM-pyridinium chloride buffer, pH4.5, for 5min at 37°C. The reaction was initiated by the addition of fluoroglutamic acid isomer (3mM). Fractions (0.2ml) were pipetted after several periods of time (0–60min) and poured on top of 2ml Dowex 1 X4 columns (200–400 mesh; acetate form). After elution with water (5ml) followed by 20% (v/v) acetic acid (5ml), the acidic fractions were freeze-dried, dissolved in water (0.5ml) and titrated with ninhydrin.

The K_m and $V_{max.}$ determinations were carried out, as indicated above, in a final volume of 0.25ml of reaction mixture containing 1 unit of glutamate decarboxylase, 0.2–2mM-glutamic acid and 0.7–10mM-3-fluoroglutamic acid isomer. The incubation times were respectively 2.5min (*threo* isomer) and 5min (glutamic acid and *erythro* isomer) in order to ensure operation under initial-rate conditions (see Fig. 2). The K_m and $V_{max.}$ parameters were derived by using a curve-fitting programme for plots of $1/v$ versus $1/[S]$. Each plot included six glutamate or 3-fluoroglutamate concentrations and yielded a correlation coefficient of 0.99.

Decarboxylation of threo-3-fluoroglutamic acid with glutamate decarboxylase: isolation and identification of (−)-4-amino-3-fluorobutyric acid

Glutamate decarboxylase (8mg, 296 units) and pyridoxal phosphate (15.9mg, 1mM) were preincubated at 37°C for 5min in 60ml of 50mM-pyridinium chloride buffer, pH4.4. L-*threo*-3-Fluoroglutamic acid (30mg, 0.18mmol) was added, and incubation was continued for 30min. The crude mixture was poured on top of a Dowex 1 X4 column (200–400 mesh; acetate form), and elution was performed with water. Ninhydrin-positive fractions were pooled and freeze-dried, yielding quantitatively (−)-4-amino-3-fluorobutyric acid. It had R_F 0.39 in butanol/pyridine/acetic acid/water (5:5:1:4, by vol.) and $[\alpha]_D^{20} = 11.5°$ (c = 1 in water). The ^1H n.m.r. spectrum (^2H_2O, tetramethylsilane as external reference: Jeol FX90Q spectrometer) for

$$H_2N\text{–}CH^4H^{4'}\text{–}CH^3F\text{–}CH^2H^{2'}\text{–}CO_2H$$

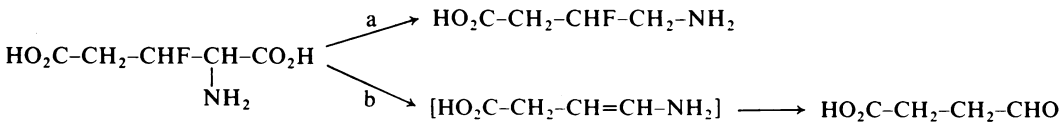
showed δ (p.p.m.) 5.19 (H^3), 3.35 (H^4), 3.16 ($H^{4'}$), 2.63 (H^2) and 2.54 ($H^{2'}$), and $J = H^3$–F, 48Hz, H^4–F, 20.6Hz, $H^{4'}$–F, 28.5Hz, H^2–F, 18.3Hz and $H^{2'}$–F, 26.7Hz; the ^{19}F n.m.r. spectrum (^2H_2O, trifluoroacetate as internal reference; Jeol FX90Q spectrometer) showed $\delta = -150$p.p.m. (multiplet = 13 peaks).

Decarboxylation of L-erythro-3-fluoroglutamic acid: search for irreversible inactivation of glutamate decarboxylase

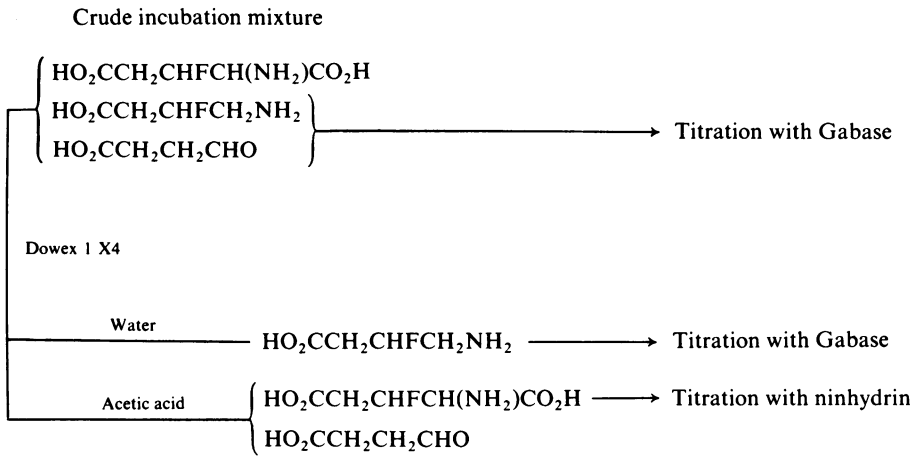
Glutamate decarboxylase (10 units) and pyridoxal phosphate (1mM) were preincubated at 37°C for 5min in 0.2ml of 50mM-pyridinium chloride buffer, pH4.5. L-*erythro*-3-Fluoroglutamic acid (10mM) was added, and 20μl fractions were sampled (over 0–40min) and diluted in 0.25ml of pyridinium chloride buffer containing pyridoxal phosphate (1mM) and glutamic acid (3mM). After 5min at 37°C, 0.2ml fractions were taken, treated as above and titrated with ninhydrin.

Results and discussion

The decarboxylation of 3-fluoroglutamate *a priori* could generate 4-amino-3-fluorobutyrate (Scheme 1, pathway a) and succinic semialdehyde via the hydrolysis of the enamine (Scheme 1, pathway b). The determination of the concentrations of fluoroglutamate, 4-amino-3-fluorobutyrate and succinic semialdehyde was achieved according to the following sequence (Scheme 2): fluoroglutamate and succinic semialdehyde were separated from 4-amino-3-fluorobutyrate by using a Dowex 1 X4 column. The aminobutyrate was eluted with water, and the mixture of fluoroglutamate and succinic semialdehyde was eluted with 20% (v/v)



Scheme 1. *Decarboxylation of 3-fluoroglutamic acid*



Scheme 2. *Titration of the decarboxylation product of 3-fluoroglutamic acid by using Gabase and ninhydrin titration*

acetic acid. Unchanged fluoroglutamate was titrated with ninhydrin (Greenstein & Winitz, 1961), whereas 4-amino-3-fluorobutyrate and succinic semialdehyde were determined with Gabase, a commercial mixture of 4-aminobutyrate transaminase and succinic semialdehyde dehydrogenase. The sum of both compounds was measured on the crude mixture before the Dowex 1 X4 step, and the amount of aminobutyrate was determined in the fraction eluted with water.

The behaviour of L-fluoroglutamates with glutamate decarboxylase is illustrated in Fig. 2. Both isomers were substrates of the enzyme. The *erythro* isomer reacted more slowly than did the *threo* isomer, and this was due to a higher K_m, since the $V_{max.}$ values were almost identical (Table 1).

Upon decarboxylation, the L-*threo* isomer yielded 4-amino-3-fluorobutyrate (Scheme 1, pathway a): the crude product gave the same response with Gabase before and after elution of the Dowex column with water, and the optically active compound was isolated and identified by ^1H and ^{19}F n.m.r. On the other hand, the L-*erythro* isomer lost the fluorine atom and yielded succinic semialdehyde (Scheme 1, pathway b): the crude product was a substrate of Gabase but was not eluted from the Dowex column with water.

Previous work on glutamate decarboxylase (Fonda, 1972) revealed that both carboxy groups are required for a good affinity, and that the glutamic acid is probably present at the active site in an extended conformation. Glutamic acid and 3-

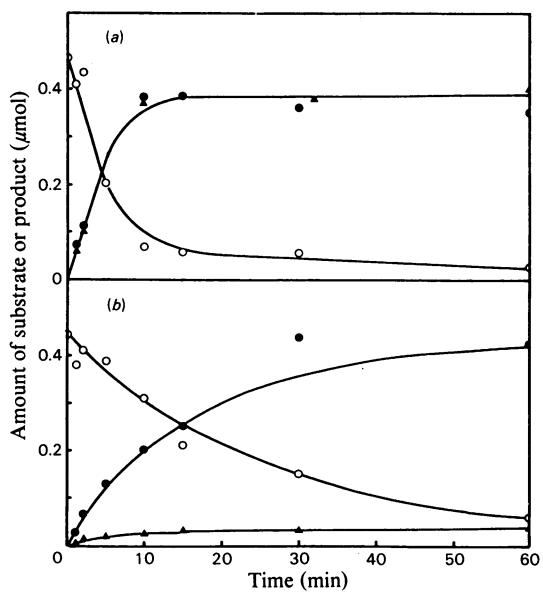


Fig. 2. *Decarboxylation of* L-*threo and* L-*erythro isomers of 3-fluoroglutamic acid by E. coli glutamate decarboxylase*
(*a*) L-*threo*-3-Fluoroglutamic acid (3mM) was incubated in the presence of glutamate decarboxylase (5 units/ml). (*b*) L-*erythro*-3-fluoroglutamic acid (3mM) was incubated in the presence of glutamate decarboxylase (6.6 units/ml). In both cases unchanged 3-fluoroglutamic acid was titrated with ninhydrin after separation of a Dowex 1 X4 column (○). Gabase measurement was carried out on a sample before (●) and after (▲) the Dowex 1 X4 step.

Table 1. *Comparison of the kinetic parameters of E. coli glutamate decarboxylase for glutamate and 3-fluoroglutamate isomers*

Amino acid	K_m (mM)	$V_{max.}$* (μmol/min per mg)
Glutamate	0.8	22.0
L-*threo*-3-Fluoroglutamate	4.0	25.3
L-*erythro*-3-Fluoroglutamate	10.0	19.9

* The commercial enzyme [37 units/mg = 30% of maximum specific activity (Strausbauch & Fischer, 1967)] had lost some activity on storage. The possible contaminating activities (glutamate–oxaloacetate transaminase and glutamate–pyruvate transaminase) could not interfere with our determinations.

fluoroglutamic acids probably adopt the same conformation at the active site. Since only one of the fluoroglutamic acid isomers eliminates the halogen atom during decarboxylation, it is probable that this conformation is rigid.

Stereoelectronic considerations imply that, according to Dunathan's (1971) hypothesis, the $C_{(\alpha)}$–CO_2^- bond to be broken is perpendicular to the planar system of the amino acid–pyridoxal Schiff base. The description of the conformation around the $C_{(\beta)}$–$C_{(\gamma)}$ bond, on the basis of the behaviour of both isomers with regard to elimination, requires the knowledge of the nature and of the stereochemistry of that reaction. The β-elimination can be concerted or E_{1CB}, i.e. with a carbanionic intermediate, but, in both cases, the $C_{(\beta)}$–F bond to be broken must be perpendicular to the planar conjugated system; if, as in most cases described so far, the elimination is anti, the conformation of glutamate will correspond to Fig. 3(*a*) (whereas, if it were syn, it would correspond to Fig. 3*b*). Although generally not invoked in enzymic reactions, an E_{1CB}-like mechanism could be taken into account with pyridoxal phosphate-dependent enzymes. In such a case, the $C_{(\beta)}$–F bond will be colinear with the $C_{(\alpha)}$ sp_2 orbital, and this will correspond to one of the orientations described in Fig. 3.

The elimination of a β-leaving group that leads to the formation of a conjugated system being a prerequisite for irreversible inactivation of pyridoxal phosphate-dependent enzymes, the L-*erythro*-3-fluoroglutamate was a candidate for the irreversible inactivation. However, when tested at 10 mM, for incubation times up to 40 min, no significant loss of activity could be detected. Thus, in contrast with 2-fluoromethylglutamate (Kuo & Rando, 1981), L-*erythro*-3-fluoroglutamate is not an irreversible mechanism-based inhibitor of glutamate decarboxylase from *E. coli*.

Two hypotheses have been proposed to account for the irreversible inhibition of pyridoxal phosphate-dependent enzymes (Likos *et al.*, 1982; Kuo & Rando, 1981; Ueno *et al.*, 1982). The difference between 2-fluoromethylglutamate and L-*erythro*-3-fluoroglutamate means either that no nucleophile is suitably located to add to the β-unsaturated Schiff base derived from 3-fluoroglutamate, or that the intermediate enamine, obtained after decarboxylation and fluorine atom elimination of the fluoro acids, can add to the lysine–pyridoxal Schiff base in the 2-fluoromethylglutamate case, but cannot when 3-fluoroglutamate is involved.

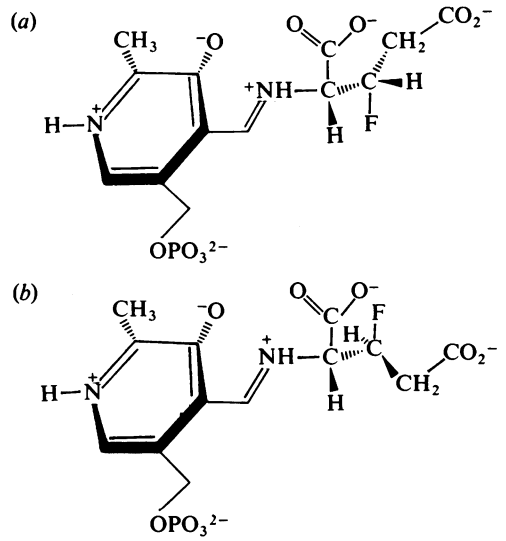


Fig. 3. *Possible conformation of 3-fluoroglutamic acid–pyridoxal phosphate Schiff base at the active site of glutamate decarboxylase*

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