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THE INHIBITION OF MOUSE BRAIN GLUTAMATE DECARBOXYLASE BY SOME STRUCTURAL ANALOGUES OF L-GLUTAMIC ACID

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Abstract—A series of amino acid hydroxamates and mercapto carboxylic acids have been tested as inhibitors of glutamate decarboxylase (GAD) and GABA-aminotransferase (GABA-T). Malate, thiomalate, glutarate and L-glutamic acid γ-hydroxamate were potent competitive inhibitors of GAD with respect to glutamate, and were far less active against GABA-T. None of these compounds reacted directly with free pyridoxal phosphate. Other compounds, including cysteamine, DL-methionine hydroxamate, D-penicillamine and DL-cysteine inhibited both enzymes and formed stable complexes with free pyridoxal phosphate. Possible structural requirements for the competitive binding of an inhibitor to the active site of GAD are discussed.

There have been several studies of the inhibition of L-glutamate 1-carboxylase, EC 4.1.1.15. (GAD) by a variety of compounds [1–5] although the structural requirements for inhibition, and the mechanism of the inhibition, have not been examined in detail. Wu and Roberts [5] studied a series of dicarboxylic acids and amino acids as competitive inhibitors of GAD and, from the relative affinities of the inhibitors for the holoenzyme, were able to suggest at least two positively-charged groups at the active site separated by a distance equivalent to a 3-carbon chain, together with a thiol-sensitive sulphydryl group nearby. Most of the inhibitors in use at present are nonspecific carbonyl-trapping agents which tend to inhibit all pyridoxal-catalysed enzymes to some extent; amongst these can be included amino-oxyacetic acid [6], semicarbazide and thiosemicarbazide [7], and many aryl hydrazides [8].

The aims of the present work have been first, to discover, amongst several analogues of glutamate and mild carbonyl-trapping agents, inhibitors of GAD which are relatively specific and competitive with respect to glutamate and, secondly, to determine whether or not compounds having some affinity for other sites of glutamate binding can inhibit GAD. Possible reaction mechanisms for some of the inhibitors will also be discussed. A preliminary account of some of the present findings has already been given [9].

MATERIALS AND METHODS

Animals. The mice from which the enzyme extracts were prepared were adult LACGs of either sex and bred in the department.

Sources of chemicals. The reagents used in the assay procedures were all obtained from the Sigma Chemical Co., Kingston-upon-Thames, Surrey, England. All radiochemicals were obtained from the Radiochemical Centre, Amersham, Bucks. The compounds tested were obtained as follows: D- and L-etomidate were a gift from Janssen Pharmaceutica, Beerse, Belgium; HA-966 was kindly supplied by Prof. I. L. Bonta, University of Rotterdam, Holland; domoic and quisqualic acids were a gift from Prof. T. Takemoto, Tohoku University, Japan; succinic semialdehyde was synthesized by P.V.T.; D- and L-aspartic acid α,β-cyclic hydroxamate were synthesized by J.C.W.

Preparation of solutions. All the compounds tested were made up in sodium phosphate buffer (pH 6.4) or sodium borate buffer (pH 8.4) immediately prior to use. The free acids were converted into the sodium salts by the addition of stoichiometric amounts of sodium hydroxide to a solution in distilled water, the pH eventually adjusted to the assay pH by the addition of the appropriate buffer.

Glutamic acid decarboxylase assays. Mice were killed by cervical fracture and the brain rapidly dissected out, rinsed in ice-cold 0.9% (w/v) saline, weighed, and homogenised in 50 mM sodium phosphate buffer (pH 6.4) at 2000 rpm for 1 min at 0° in a Teflon–glass homogeniser of 0.3-mm clearance. The final tissue concentration was 20% (w/v). The preparation of a tissue extract containing GAD activity, and the assay procedure for measuring the activity using L-[1-^{14}C]glutamate as substrate were followed exactly as described previously [2].

In some experiments, the tissue extract was further purified by centrifuging the original homogenate at 60,000 *g* for 1 hr at 0° and adding solid ammonium sulphate to the supernatant to 25% saturation. After standing at 0° for 20 min the supernatant was centrifuged at 18,000 *g* for 30 min and ammonium sulphate again added to the supernatant in order to reach 70% saturation. The centrifugation was repeated and the pellet re-suspended in 5 volumes of 50 mM sodium phosphate buffer (pH 6.4) containing 0.5 mM pyridoxal phosphate and 1 mM reduced glutathione. This extract could be stored at −10° and retain enzyme activity without significant loss over a period of several weeks. After thawing, the protein which had precipitated during storage was spun down at 6,500 *g* for 10 min and the supernatant used for the assays.

GABA-aminotransferase assays. The preparation of a mouse brain tissue extract and the assay of GABA-T activity, using [U-^{14}C]GABA as substrate,

were carried out using the methods described previously [2].

Calculation of results. In both the GAD and GABA-T assays, during the initial screening of inhibitors, the compounds to be tested were present at ten times the concentration of the limiting substrates which were, in turn, present at approximately half their respective K_m values as determined under the same conditions. For the GAD assays, L-glutamate = 1 mM (K_m = 1.8 mM); for the GABA-T assays, GABA = 0.5 mM (K_m = 1 mM). The values for the apparent Michaelis constants (K_m) and the inhibitor dissociation constants (K_i) were calculated from either double reciprocal Lineweaver–Burk plots or plots of v against v/s. All lines were derived by regression line analysis from points representing the means of duplicate determinations.

Absorption spectra of pyridoxal phosphate. The absorption spectra of pyridoxal phosphate, and its alteration by carbonyl-reacting compounds, were determined on an SP 800 recording spectrophotometer. The reactions were carried out at room temperature (20°) in a 2.5 ml volume in Silica cells of 1-cm light path. Rate constants for the first-order reactions were calculated from the rate of decay of the absorption peak at 390 nm plotted on a logarithmic scale.

RESULTS

Inhibition of glutamate decarboxylase (GAD). An initial screening programme was adopted in which the test compounds, at 10 mM, were tested for their ability to inhibit GAD in the presence of suboptimal (1 mM) L-[1-^{14}C]glutamate. Under these conditions the following compounds produced no inhibition: DL-cysteic acid, DL-homoserine, 4-aminotetrolic acid, DL-serine, succinic acid, GABA, dihydroxyphenylalanine, kainic acid, quisqualic acid, domoic acid, 2-amino-4-pentenoic acid (allylglycine), L-methionine-DL-sulphoximine, 1-hydroxy-3-aminopyrrolidone-2 (HA-966) and albizzin (α-amino-β-ureidopropionic acid).

Compounds which inhibited GAD were then tested by means of dilution to determine whether or not the inhibition was reversible. If this proved, so, the inhibitor constant was then calculated except in a few cases where the very small quantity of compound available made this impossible. The results are summarized in Table 1. The most potent inhibitors tested were thiomalic acid (K_i = 0.012 mM) and mercaptopropionic acid (K_i = 0.012 mM), the latter having already been widely used as a GAD inhibitor. The D- and L-isomers of aspartic acid β-hydroxamate were equipotent inhibitors, suggesting that the inhibition was not dependent upon stereospecific binding of the inhibitor to the enzyme. L-α-Hydroxylglutaric acid was a potent irreversible inhibitor (100 per cent inhibition at 0.8 mM) whereas D-α-hydroxyglutaric acid was very much less potent (6 per cent inhibition at 10 mM) implying that, in this case, the binding of the α-hydroxy group to a site on the enzyme may well be stereospecific. The two isomers of the hypnotic drug etomidate were equipotent irreversible inhibitors of the enzyme, both compounds producing 50 per cent inhibition at 0.9 mM against 1 mM L glutamate. Glutathione, which, in its reduced form, is included in the assay to protect the sulphydryl groups on the enzyme produced significant inhibition of the enzyme at concentrations above 5 mM.

The compound HA-966 (1-hydroxy-3-aminopyrrolidone-2) and kojic acid produced a variable increase in the GAD activity in the crude brain homogenate from 25 to 100 per cent above the control value. This effect was not observed with the partially purified enzyme extract and was therefore put down to a nonspecific or detergent action on the homogenate.

Inhibition of GABA-aminotransferase (GABA-T). In order to determine the specificity of action of the GAD inhibitors listed in Table 1, they were tested at a concentration of 5 mM against GABA-T activity

Table 1. Inhibitors of glutamate decarboxylase

Compound (10 mM)	Inhibition (%)	Type of inhibition	K_i (mM)
DL-Methionine hydroxamate	74	Reversible, mixed	2.9
L-Aspartic acid α,β-cyclic hydroxamate	60	Reversible, non-competitive	7.3
L-Aspartic acid β-hydroxamate (5 mM)	80	—	
D-Aspartic acid β-hydroxamate (5 mM)	77	—	
Reduced glutathione (10 mM)	42	Reversible, non-competitive	
(5 mM)	0	—	
L-Cysteine	50	Reversible, mixed	2.5
Mercaptopropionic acid	100	Reversible, competitive	0.012
Succinic semialdehyde	30	Reversible, mixed	3.9
L-Glutamic acid γ-hydroxamate	90	Reversible, competitive	
Cysteamine	54	Reversible, mixed	
Malic acid	100	Reversible, competitive	1.6
Thiomalic acid	100	Reversible, competitive	0.012
Glutaric acid	100	Reversible, competitive	3.1
L-α-Hydroxyglutaric acid	100	Irreversible	*
D-α-Hydroxyglutaric acid	6	—	
D-Etomidate	100	Irreversible	*
L-Etomidate	100	Irreversible	*

Each compound was tested against 1 mM L-glutamate in the presence of excess pyridoxal phosphate under the assay conditions described in Methods.

* Inhibitor constant (K_i) values cannot be calculated for irreversible inhibitors.

Table 2. Inhibitors of GABA-aminotransferase

Compound (5 mM)	Inhibition (%) (mean of 4 experiments)	Type of inhibition
DL-Methionine hydroxamate	67	Reversible, mixed
L-Cysteine	92	Reversible, mixed
Mercaptopropionic acid	100	Reversible, non-competitive
L-Glutamic acid γ-hydroxamate	62	—
Cysteamine	68	Reversible, mixed
Glutaric acid	<10	—
Malic acid	14	Reversible
Thiomalic acid	14	Reversible
HA-966	16	Reversible
Allylglycine	22	Irreversible
DL-Homocysteine	41	Reversible, mixed
Kojic acid	15	Reversible

Each compound was tested against 0.5 mM GABA in the presence of excess pyridoxal phosphate (0.4 mM) and 2-ketoglutaric acid (1 mM) under the assay conditions described in Methods. Where the level of inhibition was less than 15 per cent it was not possible to determine with certainty the nature of the inhibition from double reciprocal plots.

in the presence of excess of α-ketoglutarate (1 mM) and pyridoxal phosphate (0.4 mM), but with a limiting concentration of GABA (0.5 mM). Under these conditions the following compounds produced no measurable inhibition: L-aspartic acid α,β-cyclic hydroxamate, L-α-hydroxyglutaric acid, glutaric acid, D-etomidate, and L-etomidate. The relative potencies of those compounds which did inhibit GABA-T are set out in Table 2. Mercaptopropionic acid was again the most potent inhibitor (50 per cent inhibition at 0.1 mM). HA-966 and kojic acid produced a consistent weak inhibitory action. The degree of inhibition produced by allylglycine appeared to be dependent upon the length of time the compound was in contact with the enzyme prior to the start of the assay.

Reaction of inhibitors with pyridoxal phosphate. It had earlier been observed that several compounds, when added to the enzyme assay media, caused the disappearance of the characteristic yellow colour of pyridoxal phosphate. The reaction between the enzyme inhibitors was therefore examined by following the disappearance of the 390 nm absorbance peak of pyridoxal phosphate under the same conditions as the GAD assay. The progress of the reaction between L-cysteine (4 mM) and pyridoxal phosphate (0.4 mM) is shown in Fig. 1. The loss of the absorbance peak at 390 nm can be seen to be associated with an increase in the peak at 290 nm with a single isobestic point occurring at 315 nm. Similar spectral changes were obtained with D-penicillamine, rhodanine, cysteamine, DL-homocysteine and reduced glutathione. First-order reactions were obtained with a 10-fold excess of inhibitor over pyridoxal phosphate (0.4 mM); the rate constants for the reaction are shown in Table 3. D-Penicillamine reacted at about three times the rate of the other compounds which all produced similar reaction rates. DL-Methionine hydroxamate gave a second order reaction with a rate constant of $0.012 l^3.mole.^{-1} sec.^{-1}$ derived from a plot of the reciprocal concentration of pyridoxal against

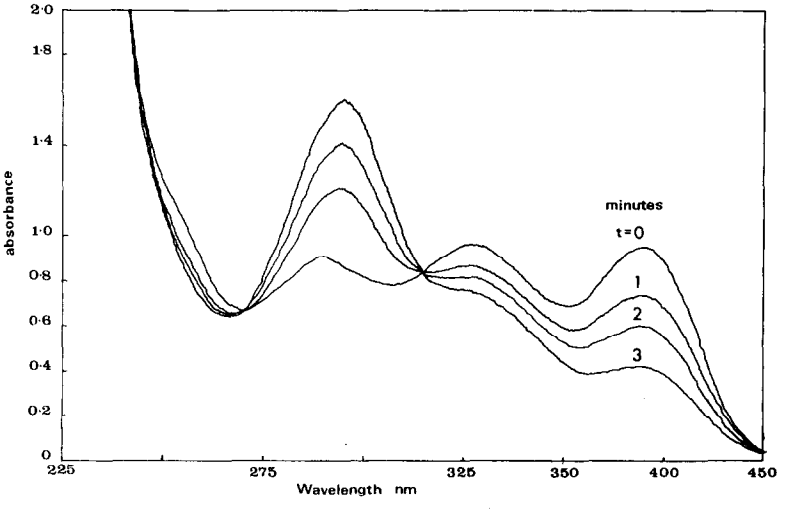


Fig. 1. Absorption spectra of the complex between pyridoxal phosphate (0.4 mM) and L-cysteine (4 mM) in 50 mM sodium phosphate (pH 6.4) buffer. Records taken at 1-min intervals following the addition of L-cysteine to the cuvette. (Other conditions see Materials and Methods).

Table 3. Rate constants for the reaction of GAD and GABA-T inhibitors with pyridoxal phosphate

Compound	Rate constant (sec^{-1})
Cysteamine	0.0016 ± 0.0001
DL-Cysteine	0.0021 ± 0.0002
DL-Homocysteine	0.0014 ± 0.0001
D-Penicillamine	0.0063 ± 0.0004
Rhodanine	0.0025 ± 0.0002
Reduced Glutathione	0.0022 ± 0.0002
DL-Methionine hydroxamate	*0.012 ± 0.001

Reactions were followed at 390 nm for 4 min and the rate constants (k) determined from the half decay time of the absorbance peak at 390 nm, obtained by plotting log E_{390} against time, where $t_{1/2} = \ln 2/k$ for a first-order reaction.

* The reaction between DL-methionine hydroxamate and pyridoxal phosphate was second order and the rate constant therefore has the dimensions $l^3 \cdot mole^{-1} \cdot sec^{-1}$.

time. Dithiothreitol reacted very rapidly with pyridoxal phosphate, producing an instantaneous fall in the absorbance at 390 nm.

The following compounds did not react with pyridoxal phosphate when present at up to twenty times the pyridoxal phosphate concentration: mercaptopropionic acid, thiomalic acid, glutaric acid, malic acid, L-glutamic acid, GABA, DL-methionine sulphoxide, L-methionine, DL-homocysteic acid and allylglycine.

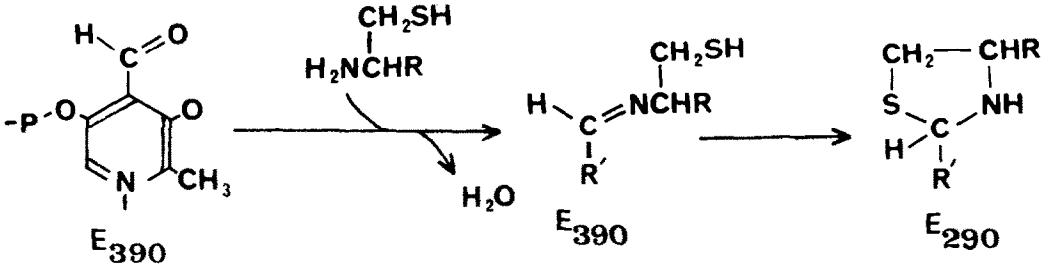
DISCUSSION

The reaction mechanisms of the pyridoxal-catalysed enzymes are relatively well-documented [10–12] and it is therefore possible to draw useful information from inhibition studies with structural analogues of the substrates. An inhibitor may react with the apoenzyme, with the enzyme–pyridoxal complex, or with free pyridoxal itself. The ideal specific inhibitor should sufficiently resemble glutamate to bind to the two positively-charged residues at the active site whilst presenting a reactive substituent at the correct position to combine with the Schiff's base formed by the carbonyl group of pyridoxal and the lysine residue of the enzyme. If the resultant ternary intermediate is stable, then the enzyme will be irreversibly inhibited. Such a mechanism has been described for the K_{cat} type of inhibitors [13, 14] including rhizobitoxine which inhibits the pyridoxal-linked β-cystathionase [15] and D-cycloserine which irreversibly inhibits alanine racemase [16].

It has already been demonstrated that L-cysteine and D-penicillamine can react with pyridoxal to form stable thiazolidine derivatives [17] and in the present work evidence has been obtained to show that cysteamine, DL-homocysteine, rhodanine, reduced glutathione and DL-methionine hydroxamate can also react to form stable products in which the carbonyl group of pyridoxal phosphate is combined with the inhibitor. The absorption peak at 390 nm in the spectrum of pyridoxal is due to the conjugated sp^2 bonding of the carbonyl carbon, and the reaction followed in Fig. 1 represents the change to sp^3 bonding occurring as the carbon–nitrogen double bond is lost, as in the formation of a thiazolidine ring for example [18].

All the compounds listed in Table 3, with the exception of rhodanine, inhibited both GAD and GABA-T by non-competitive or mixed inhibition. A similar lack of specificity was observed with the hydroxamate derivatives of DL-methionine, D- and L-aspartate and L-glutamate. The reaction of DL-methionine hydroxamate with pyridoxal phosphate was different from that observed with all the other compounds in Table 3 in that it showed second-order kinetics. The first-order reaction observed in all the other cases implied that a pyridoxal–inhibitor complex was initially formed in which the carbonyl carbon retained its sp^2 bonding followed by subsequent rearrangement to cause saturation of the carbonyl carbon which then becomes sp^3 bonded (see Scheme 1). The reaction process is essentially similar to that already proposed for the reaction of cysteine with pyridoxal [18]. This view is also supported by the evidence of the single isobestic point in Fig. 1 which implies that only two components are involved in the spectral shift. In the case of rhodanine (2-mercapto-4-hydroxythiazole) the methylene group of the thiazole ring readily condenses with free aldehydes.

The reactions of dithiothreitol (2,3-dihydroxybutane-1, 4-dithiol) and reduced glutathione with pyridoxal phosphate are interesting in that both these compounds are widely used in the assay of GAD activity in order to protect the sulphydryl groups of the enzyme from oxidation. Indeed, at concentrations above 5 mM, which is that recommended in the assay procedure of Balázs, Dahl and Harwood [19], reduced glutathione produced inhibition (see Table 1). Great care should therefore be taken in using sulphydryl compounds in GAD assay procedures, par-



Scheme 1. Reaction of pyridoxal with a mercaptoamine to form a stable complex.

ticularly when the pyridoxal phosphate concentration is not in excess*.

Reversible competitive inhibition of GAD was observed with, in addition to mercaptopropionate, malic acid, thiomalic and glutaric acid. These last three were more specific than mercaptopropionate whilst thiomalic acid was equipotent with mercaptopropionate. The 100-fold increase in potency observed between malic acid (K_i = 1.6 mM) and thiomalic acid (K_i = 0.012 mM) corresponding to the substitution of an α-hydroxyl group for a mercapto group, suggests that this substituent is important in the binding of the inhibitor to the pyridoxal–enzyme complex, despite the fact that neither inhibitor reacts with free pyridoxal phosphate. This could be explained by the increased lability of the pyridoxal carbonyl group when it is in the form of a Schiff's base in the holoenzyme. The reaction is possibly facilitated by binding of the carboxyl group of the inhibitor to a glutamate carboxylate binding site on the enzyme. This view is supported by the evidence of the potency of mercaptopropionate as an inhibitor of the decarboxylase compared to the lack of effect of mercaptoethanol (Taberner, unpublished observations). The specificity of the various carbonyl-reacting compounds used in this study may well reflect therefore the relative accessibility and lability of pyridoxal when it is bound to the appropriate apoenzyme.

The present findings indicate that a useful approach may be made towards finding a specific inhibitor of GAD by examining mercapto-substituted dicarboxylic acids of the appropriate chain length. The lack of any inhibitory action against GAD of kainic, domoic and quisqualic acids, which are glutamate agonists on spinal neurones [20], suggests that there is little in common between the structural requirements for competitors of glutamate at the receptor or enzyme active site.

* Subsequent unpublished experiments have indicated that glutathione in solution yields breakdown products which may be responsible for the inhibition.

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