present study, each nucleus was dissected from freeze-dried sections (100 µm) and only one dissected sample was used for each assay. In freeze-dried samples, fine structures were visualized clearly as reported by Lowry[9], and the enzyme in the sections stored in evacuated tubes (−20 °C) was stable for at least 12 months.

It is of interest that GAD activity were unevenly distributed in hypothalamus and the distribution is strikingly different from that of other neurotransmitter-related enzymes such as tyrosine hydroxylase[10], choline acetyltransferase[6,7], monoamine oxidase[11,12] and DOPA decarboxylase[13]. In addition, the present method, using microdissection technique with freeze-dried sections, may help in understanding the physiological roles of the neurotransmitter in the hypothalamic nuclei.

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Functional role of arginine residues in glutamic acid decarboxylase from brain and bacteria

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Summary. The arginine-specific reagent phenylglyoxal rapidly inactives glutamic decarboxylase from both mouse brain and *E. coli* when preincubated with the enzyme at concentrations of 3 mM to 40 mM. The rate of inactivation follows pseudo-first-order kinetics and is dependent upon the concentration of phenylglyoxal. These and other data presented support the idea that arginine residues play a key role in the mechanism of action of glutamic decarboxylase.

A number of mono- and dicarboxylic aliphatic acids are pott competitive inhibitors of brain and bacterial glutamic acid decarboxylase (GAD) (4.1.1.15; L-glutamate 1-carboxy-lyase)[1,2]. The requirement of a carboxylic moiety as an integral part of the structure of these inhibitors and the presence of 2 carboxylates in the substrate molecule (glutamate) have let us to speculate the existence of an anionic substrate or inhibitor binding site in these decarboxylases. The most likely amino acid residue involved at the anionic substrate binding site is arginine. Indeed, arginine has been shown to play a general role in the functional binding of anionic cofactors and substrates of a number of enzymes. For example, by using arginine-specific protein modifying agents such as phenylglyoxal and other dicarbonyl compounds, it has been demonstrated that arginine residues are involved in the binding of phosphate-containing cofactors or substrates of several enzymes, notably the enzymes of the glycolytic pathway (see Riordan et al.[3] and references cited therein).

We now report the existence of such functional arginine residues in GAD from mouse brain and *E. coli*. This is the first evidence of the participation of arginine residues in the catalytic action of decarboxylases.

Materials and methods. Swiss white male mice of 9 weeks of age were used as the source of the brain enzyme. The GAD was extracted and an ammonium sulphate fraction prepared as previously described[4].

The mouse enzyme was assayed after the method of Wu et al.[5]. The bacterial enzyme was bought from the Sigma

The inactivation of glutamic acid decarboxylase from mouse brain and *E. coli*

Compound added (10 mM)	Enzyme activity (percent of control) Mouse brain		*E. coli*	
	No borate	50 mM borate	No borate	50 mM borate
None	100	100	100	100
Phenylglyoxal	23	21	15	19
Glyoxal	26	23	21	23
2,4-Pentanedione	82	68	74	39
2,3-Butanedione	88	73	71	33
1,2-Cyclohexanedione	87	80	79	45

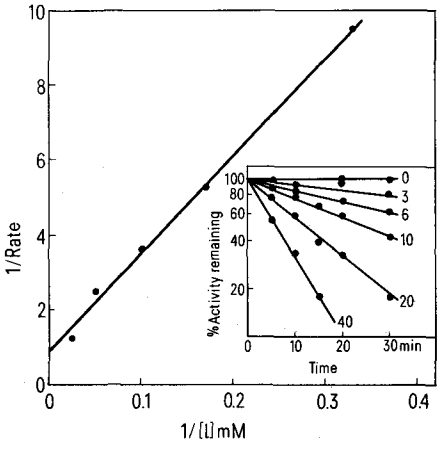

Fig. 1. Inactivation of brain glutamic acid decarboxylase against time by phenylglyoxal (I). Prior to its assay as described in the 'methods' section the enzyme was incubated at 23 °C with varying concentrations (mM) of inactivator as indicated by the numbers 0–40 (see insert). The rate of inactivation (enzyme activity in µmoles/h mg protein lost per min) was calculated and presented as a double reciprocal plot.

Chemical Company, St. Louis, Mo, as an extract from *E. coli* (5.8 units/mg). This enzyme was determined as above except that a 0.1 M pyridine HCl buffer (pH 4.6) was used in place of the phosphate buffer.

Results and discussion. Both mouse brain and bacterial glutamic acid decarboxylase are rapidly inactivated upon incubation at 25 °C with 3 mM to 40 mM of phenylglyoxal. The time courses from the inactivation of mouse brain and bacterial GAD are shown (figures 1 and 2). The incubation was carried out using 1 ml of enzyme. However, only 50 μl of this enzyme was assayed by adding to 950 μl of buffer substrate. This produced a 20fold dilution of the inactivator and effectively terminated the inactivation. The inhibition constants by phenylglyoxal of the 2 enzymes were calculated from the double reciprocal plots of the rate of inactivation against the concentrations of phenylglyoxal (figures 1 and 2). They are 24 mM and 27 mM for mouse brain and bacterial GAD respectively. From the plots of log (^{1}/t 0.5) versus log (phenylglyoxal), 2 straight lines were obtained (figure 3) with slopes equal to 0.99 for the mouse brain enzyme and 0.89 for the bacterial enzyme. These values are close to 1.0 which indicates that the inactivation is the result

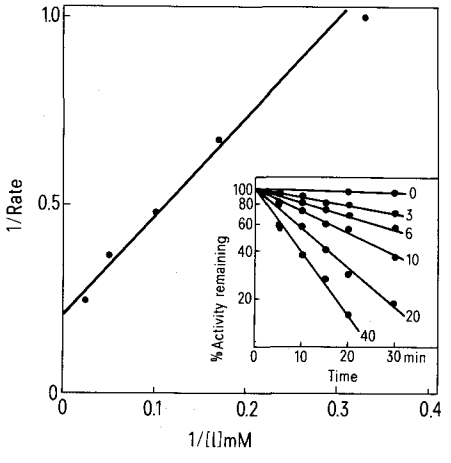

Fig. 2. Inactivation of bacterial glutamic acid decarboxylase against time by phenylglyoxal (I). Prior to its assay as described in the 'methods' section the enzyme was incubated at 23 °C with varying concentrations (mM) of inactivator as indicated by the numbers 0–40 (see insert). The rate of inactivation (enzyme activity in μmoles/h mg protein lost per min) was calculated and presented as a double reciprocal plot.

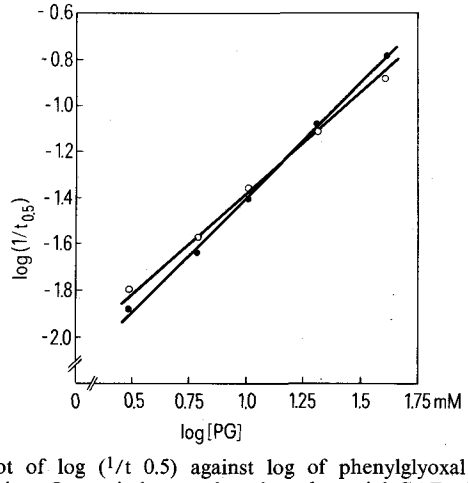

Fig. 3. Plot of log (^{1}/t 0.5) against log of phenylglyoxal (PG) concentration. Open circles are data from bacterial GAD. Closed circles are brain GAD. ^{t}0.5 = time required to reach 50% inactivation.

of reaction of one arginine residue on the enzymes with phenylglyoxal[7,8].

The effects of 5 different dicarbonyl arginine modifying reagents on the activity of brain and bacterial GAD are shown in the table. The enzymes were incubated for 1 h with 10 mM of the dicarbonyl reagents at 25 °C in the presence or absence of 50 mM sodium borate. Phenylglyoxal and glyoxal were the most potent inactivators of both enzymes. Borate does not enhance the inactivation of GAD brought about by either phenylglyoxal or glyoxal. However, it does dramatically stimulate the inactivation of the enzyme brought about by 2,4-pentanedione, 2,3-butanedione and 1,2-cyclohexanedione. These results confirm the observation of other workers who reported that the inactivation of several enzymes by the above diones is increased in the presence of borate ions. This is because the resulting dione-arginine complex is stabilized by the borate ions. Phenylglyoxal and glyoxal form complexes with greater stability and so the presence of borate ions has no effect.

D-Glutamic acid is a known competitive inhibitor of GAD from mouse brain and *E. coli*. In the presence of this inhibitor, GAD was protected against inactivation by phenylglyoxal. 30 mM D-glutamic acid afforded a 35% protection against inactivation of the bacterial decarboxylase brought about by 10 mM of phenylglyoxal and a 27% protection of mouse brain GAD.

Previous studies[1,2,4–6] have suggested the presence of cysteine, lysine and histidine residues at or near the active site of both brain and bacterial GAD. The possibility that a cationic group also is at the active site of the enzyme is suggested by the anionic nature of the substrate and various competitive inhibitors (e.g. D-glutamic acid and mercaptopropionic acid) of the enzyme[2]. Lysine can be ruled out as a residue at the cationic site, because pyridoxal 5'phosphate does not inhibit the enzyme; in fact it is required as a cofactor. Thus arginine is the most plausible amino acid constituent of the cationic binding site which could anchor the anionic substrate or inhibitors.

It is well-established that phenylglyoxal, 2,3-butanedione and other dicarbonyls are specific arginine-modifying reagents, and the borate enhancement of the inactivation is consistent with modification of arginyl residues (table).

The results presented in this paper provide evidence for the participation of arginine residues in the mechanism of action of GAD. It is already known that cysteine, lysine and histidine are involved in this mechanism[1,2]. Our data also extend the generality of the functional role of arginine in the binding of anionic substrates. Except for aspartate amino-transferase[9,10], all previous studies on the function of arginine on substrate or cofactor binding sites were performed with enzymes which utilize phosphate containing substrates or cofactors[3].

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