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ESSENTIAL ARGININE RESIDUES AT THE PYRIDOXAL PHOSPHATE BINDING SITE OF BRAIN γ-AMINOBUTYRATE AMINOTRANSFERASE

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SUMMARY

Mouse brain γ-aminobutyrate aminotransferase was inactivated by phenylglyoxal in a time and concentration dependent manner. Pyridoxal phosphate but not γ-aminobutyric acid or α-ketoglutarate afforded some protection against the drug-induced inactivation. [^{14}C]Phenylglyoxal was incorporated into the enzyme at a similar rate to that of the inactivation. Subsequent hydrolysis of the enzyme demonstrated the presence of a [^{14}]phenylglyoxal-arginine complex. The phenylglyoxal is probably modifying an arginine residue at or near the pyridoxal phosphate binding site of γ-aminobutyrate aminotransferase.

INTRODUCTION

γ-Aminobutyric acid is the major inhibitory neurotransmitter in mammalian brain [1]. Its action at the synapse is terminated by its swift removal by an active uptake process [2]. γ-Aminobutyric acid is degraded by the action of γ-aminobutyrate aminotransferase (EC 2.6.1.19) which is located in the mitochondria.

There is some suggestion that increased brain γ-aminobutyric acid concentrations lead to protection against convulsions [3]. This can be engendered by the inhibition of γ-aminobutyrate aminotransferase and could be of potential use in the treatment of epilepsy. A detailed knowledge of the active site of this enzyme should make it easier to design potential inhibitors.

The substrates of γ-aminobutyrate aminotransferase, γ-aminobutyric acid and α-ketoglutarate, and its cofactor pyridoxal phosphate, each possess at least one anionic group. Often such groups form an ionic attraction with arginine residues at the active

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site of an enzyme [4]. An important inhibitor frequently used to detect essential arginine residues is phenylglyoxal. This compound reacts fairly specifically with arginine to form a stable complex possessing two phenylglyoxal moieties for each guanidino group [5]. Phenylglyoxal has already been employed to show the presence of essential arginine residues at the glutamate binding site [6] and the pyridoxal phosphate binding site [7] of glutamic decarboxylase. This enzyme is responsible for the biosynthesis of γ-aminobutyric acid. The present study was initiated to determine whether or not arginine residues are present at the active site of γ-aminobutyrate aminotransferase.

MATERIALS AND METHODS

[^{14}C]Phenylglyoxal (28 mcuries/mmol) was obtained from Research Products International, Elk Grove Village, IL. [^{3}H]γ-Aminobutyric acid (66 curies/mmol) was supplied by Amersham, Arlington Heights, IL. Phenylglyoxal and γ-aminobutyric acid were purchased from Aldrich Chemical Company, Milwaukee, WI. Pyridoxal phosphate, pyridoxal, pyridoxine, pyridoxamine and pyridoxamine phosphate were obtained from Sigma Chemical Company, St. Louis, MO. Pyridoxine phosphate was supplied by ICN Pharmaceuticals Inc., Cleveland, OH.

Male Swiss White mice were killed by cervical dislocation and the brains homogenised in 9 vol of ice-cold 0.32 M sucrose. The enzyme was extracted and partially purified by ammonium sulphate fractionation and Sephadex G200 column chromatography as described by Schousboe *et al.* [8]. The enzyme was dissolved in 50 mM Tris-HCl (pH 8.5).

The inactivation of the enzyme was studied by incubating for various lengths of time and with different concentrations of phenylglyoxal at 23°C. The reaction was stopped with the transfer of 1 µl of enzyme to 40 µl of buffer-substrate. The activity of the enzyme was measured by the method of Sterri and Fonnum [9]. Protein was determined by the Lowry method [10]. To investigate the incorporation of [^{14}C]phenylglyoxal into the enzyme, 10 µl of γ-aminobutyrate aminotransferase was incubated with 2 mM of radioactive inhibitor at 23°C for up to 60 min. The reaction was terminated by adding 1 ml of distilled water and pouring over nitrocellulose filter paper under vacuum [11]. The filter paper was washed three times with 5 ml of water and then dissolved in 1 ml of methyl cellosolve. The radioactivity was counted after the addition of 10 ml of Aquasol (NEN, Boston).

In one experiment 10 µl of enzyme was incubated with [^{14}C] phenylglyoxal for 60 min at 23°C. Dilute hydrochloric acid (pH 2.2) was added (0.1 ml) and the solution was immersed in

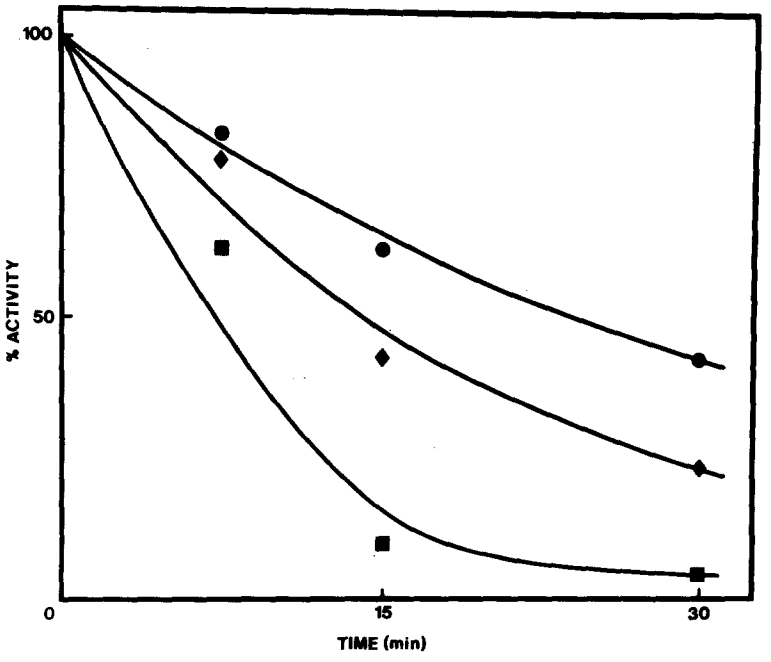

Fig. 1. Time course of inactivation of γ-aminobutyrate aminotransferase by various concentrations of phenylglyoxal (PG). Closed circles = 2.5 mM PG; diamonds = 10 mM PG; squares = 20 mM PG. Each point is the mean of 3 determinations. 100% activity represents 0.81 mol. min^{-1} mg $protein^{-1}$

boiling water for 90 min. Ten microlitres were applied to Whatman no. 4 filter paper and the chromatogram developed in n-butanol, acetic acid, water, concentrated HCl (80:80:20:1.5).

RESULTS

γ-Aminobutyrate aminotransferase was inactivated by phenylglyoxal in a manner resembling first-order kinetics (Fig. 1). The maximum rate of inactivation was calculated as 0.22 min^{-1}.

If the enzyme was incubated with 20 mM phenylglyoxal in the presence of 20 mM substrate, cofactor, or cofactor analogues, then protection against inactivation could be demonstrated (Table 1). Neither γ-aminobutyric acid nor α-ketoglutarate provided any protection. However, pyridoxal phosphate and to a lesser extent pyridoxine phosphate and pyridoxamine phosphate, gave rise to a substantial protection against phenylglyoxal-induced inactivation.

TABLE 1. Influence of various cofactor analogues on the inactivation of γ-aminobutyrate aminotransferase by phenylglyoxal

Compound	Percent Protection Against Inactivation
Pyridoxal phosphate	84.1 ± 9.2
Pyridoxal	1.1 ± 0.6
Pyridoxine phosphate	28.0 ± 3.1
Pyridoxine	2.2 ± 2.3
Pyridoxamine phosphate	46.3 ± 4.9
Pyridoxamine	0.0 ± 1.2
γ-Aminobutyric acid	3.0 ± 0.9
α-Ketoglutarate	0.9 ± 2.5

Enzyme was incubated for 30 min in the presence of 20 mM phenylglyoxal and 20 mM of the compound being tested.

Pyridoxine, pyridoxamine and pyridoxal gave no protection.

The rate of incorporation of radioactivity into the enzyme when incubated with 2 mM [^{14}C]phenylglyoxal gave a good correlation with the rate of inactivation (Fig. 2). Chromatography of the hydrolysed enzyme after incubation with radioactive phenylglyoxal yielded a spot with an Rf value of 0.28. This corresponds to an Rf value of 0.29 for the [^{14}C]phenylglyoxal-arginine complex.

DISCUSSION

The use of phenylglyoxal and other arginine-specific reagents has demonstrated that the presence of arginine residues as anionic binding sites at the active site of an enzyme is not an uncommon occurrence [4]. It has now been shown that γ-aminobutyrate aminotransferase possesses such residues. Other enzymes involved in neurotransmitter metabolism that possess essential arginine residues are glutamic decarboxylase [6,7], catechol-O-methyltransferase [12], and adenylate cyclase [13].

The arginine residue at the γ-aminobutyrate aminotransferase active site appears to be necessary for the binding of pyridoxal

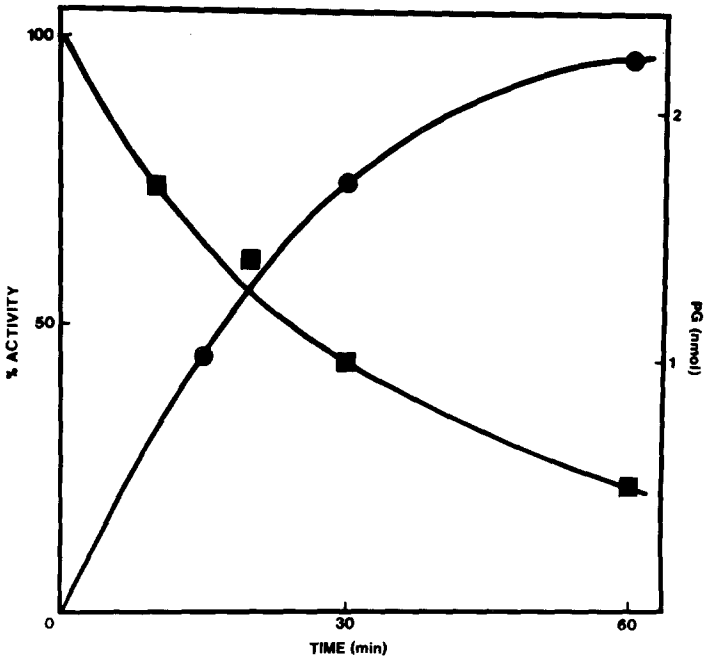

Fig. 2. Correlation between inactivation of γ-aminobutyrate aminotransferase and incorporation of [^{14}C]phenylglyoxal as a function of time of incubation. 10 μg of enzyme protein was incubated with 2 mM [^{14}C] phenylglyoxal (PG). Squares represent enzyme activity. Closed circles represent incorporation of radioactivity. Each point is the mean of 3 determinations.

phosphate rather than for either of the substrates γ-aminobutyric acid or α-ketoglutarate. Furthermore, it is probable that it is the phosphate moiety of pyridoxal phosphate that binds to the arginine residue. This conclusion is based on the observation that pyridoxal phosphate, pyridoxamine phosphate and pyridoxine phosphate all offer some protection against inactivation of the enzyme whereas the unphosphorylated compounds had no such effect.

Several other pyridoxal phosphate-dependent enzymes possess essential arginine residues. Aspartate aminotransferase, cystathionase, and tryptophanase all have an arginine that is thought to play a part in substrate binding [14,15,16]. In addition to γ-aminobutyrate aminotransferase, D-serine dehydratase requires an

arginine residue for pyridoxal phosphate binding [17]. Finally, the activity of glutamic decarboxylase is dependent upon the presence of arginine for both substrate and cofactor binding [6,7].

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