Irreversible inhibition of D-3-aminoisobutyrate-pyruvate aminotransferase by gabaculine

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Gabaculine, 5-amino-1,3-cyclohexadienylcarboxylate, is an analogue of GABA and a potent irreversible inhibitor of GABA aminotransferase. However, D-3-aminoisobutyrate-pyruvate aminotransferase for which GABA was neither a substrate nor an inhibitor was also inactivated by gabaculine. The K_i for D-3-aminoisobutyrate-pyruvate aminotransferase was 8.3×10^{-6} M, and the K_{cat} fot its turnover was 0.31 min^{-1} at 25°C. β-Alanine protected the enzyme from inactivation by gabaculine, but GABA did so to much a lesser extent.

Gabaculine; D-3-Aminoisobutyrate-pyruvate aminotransferase; GABA aminotransferase; β-Alanine; γ-Aminobutyric acid

1. INTRODUCTION

Gabaculine is a naturally occurring neurotoxin isolated from *Streptomyces toyocaensis* [1]. It is an irreversible inhibitor of pyridoxal phosphate linked ω-amino acid aminotransferases such as amino butyrate aminotransferase (β-alanine-oxoglutarate aminotransferase, β-AlaAT I; EC 2.6.1.19) [2-7], β-alaninepyruvate aminotransferase (EC 2.6.1.18) [8] and ornithineoxo-acid aminotransferase [9,10]. More recent studies have shown that gabaculine inhibited L-alanine aminotransferase [11,12], L-aspartate aminotransferase [12] and D-amino acid aminotransferase [13] with a correlation with the enzymic exchange of β-protons from their normal substrate [12]. The K_i for the inactivation of ω-amino acid aminotransferase by gabaculine had a much lower value than those of α-amino acid transferase [12].

Recently, we purified β-AlaAT I [14] and R-3-aminoisobutyrate-pyruvate aminotransferase ((*R*)-3-amino-2-methylpropionate-pyruvate aminotransferase, β-AlaAT II; EC 2.6.1.40) [15] from rat liver. β-Alanine is a common amino donor to both enzymes, and 4-aminobutyrate is not broken down by β-AlaAT II. We found that gabaculine also acted as a potent irreversible inhibitor of β-AlaAT II.

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Abbreviations: GABA, 4-aminobutyrate; β-AlaAT I, GABA aminotransferase; βAlaAT II, D-3-aminoisobutyrate-pyruvate aminotransferase

2. MATERIALS AND METHODS

All reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto) unless otherwise stated. DL-Gabaculine was obtained from Calbiochem-Behring Corp. β-[2-^{14}C]Alanine was purchased from New England Nuclear.

β-AlaAT II was purified from rat liver by heat treatment, ammonium sulfate fractionation and preparative chromatography using DEAE-Sepharose CL-6B, hydroxyapatite, Sephacryl S-200 and chromatofocusing [15]. Purified β-AlaAT I from rat liver was prepared as previously reported [14]. The both enzymes were shown to be homogeneous by polyacrylamide gel electrophoresis in both the presence and absence of SDS. The specific activities of β-AlaAT I and βAlaAT II were 0.41 and 0.33 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, respectively, under standard assay conditions.

2.1. *Inactivation kinetics*

Enzyme was incubated with gabaculine in 40 mM potassium pyrophosphate, pH 8.5, at 25°C. Aliquots were removed periodically and diluted at least 1:40 with the assay mixture. The data were plotted as a semilog function of time. The slope of this plot gave the pseudo-first-order rate constant for inactivation.

2.2. *Enzyme assay*

The activities of β-AlaAT I and β-AlaAT II were determined by the amount of malonate semialdehyde produced from β-alanine using 2-oxoglutarate and pyruvate, respectively, according to the methods previously described [14,15]. The standard reaction mixture contained 50 mM triethanolamine, pH 7.3, 5 mM 2-mercaptoethanol, 0.5 mM p*f*yridoxal 5'-phosphate, 1 mM β-[2-^{14}C]alanine (spec. act. 37 GBq/mol) and 1 mM 2-oxoglutarate or 1 mM pyruvate in a final volume of 1.0 ml. The incubation was carried out in a shaking water bath for 10 min at 37°C. The reaction was terminated by the addition of 0.5 ml 2 M HCl and the tube was immediately transferred to an ice bath. After adding 0.02 ml of 1 M β-alanine and 0.2% 2,4-dinitrophenylhydrazine (in 2 M HCl), the mixture was allowed to stand at 37°C for 15 min and the dinitrophenylhydrazone formed was extracted by shaking with 5.0 ml toluene. After brief centrifugation, the radioactivity of a 2-ml aliquot of the extract was measured with a Packard Tri-Carb liquid scintillation spectrometer (460 CD type). One unit of βAlaAT I and β-AlaAT II was defined as the amount of enzyme which catalyzed the formation of 1.0 μmol malonate semialdehyde/min under the given conditions.

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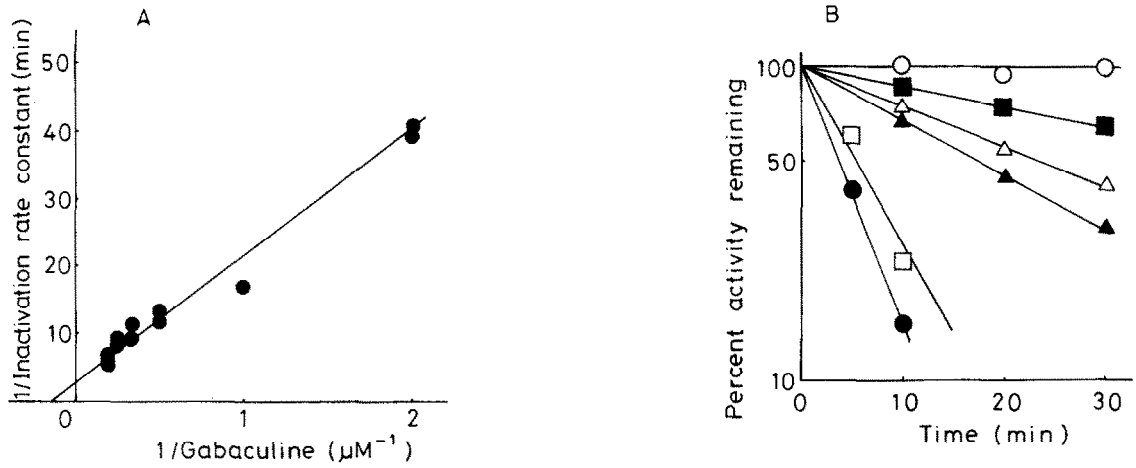

Fig. 1. Irreversible inhibition of β-AlaAT I from rat liver by gabaculine and the effects of substrates on its kinetics. (A) Concentration dependence of the inactivation of β-AlaAT I by DL-gabaculine. β-AlaAT I (44 μg = 18 mU) was incubated in 40 mM potassium pyrophosphate, pH 8.5, with the indicated concentrations of gabaculine at 25°C, and the inactivation rate constant was calculated as described in section 2. The data are presented as a double-reciprocal plot of inactivation rate constant versus concentration of gabaculine. (B) Effect of substrates on the rate of inactivation. β-AlaAT I (44 μg = 18 mU) was incubated in 40 mM potassium phosphate buffer, pH 8.5, at 25°C in a total volume of 300 μl by itself (○) or with final concentrations of the following: (●) 5 μM DL-gabaculine; (□) 5 μM DL-gabaculine and 10 mM 2-oxoglutarate; (■) 5 μM DL-gabaculine and 10 mM β-alanine; (▲) 5 μM DL-gabaculine and 5 mM GABA; (△) 5 μM DL-gabaculine and 10 mM GABA. At the indicated times, aliquots were removed and assayed for residual activity as described in section 2. The data are presented as semilog plots of the percentage of initial activity versus time.

3. RESULTS AND DISCUSSION

When gabaculine is transaminated by partially purified β-AlaAT I from *Pseudomonas fluorescens*, it is converted to a stable *m*-anthranilic acid derivative (*m*-carboxyphenylpryridoxamine phosphate) [5]. This adduct binds tightly to the active site of the enzyme and can be liberated under denaturing conditions [5]. Gabaculine acts also as a competitive inhibitor of β-AlaAT I from both brain and *Pseudomonas ovalis* [3]. Purified β-alanine-pyruvate aminotransferase from *Pseudomonas species* F-126 catalyzes the transfer of amino groups of various ω-amino acids containing GABA [16] and gabaculine inactivated the enzyme activity with a K_i value of 2.02 mM [8].

When purified βAlaAT I from rat liver was incubated with gabaculine, a rigid analogue of GABA, the enzyme was inactivated with a pseudo-first-order profile (Fig. 1A) with an apparent K_i of 7.1 μM; maximum rate of inactivation, 0.40 min^{-1}; half-life, 1.7 min (Fig. 1B). β-Alanine as well as GABA afforded substantial protection, although 2-oxoglutarate was not effective (Fig. 1A).

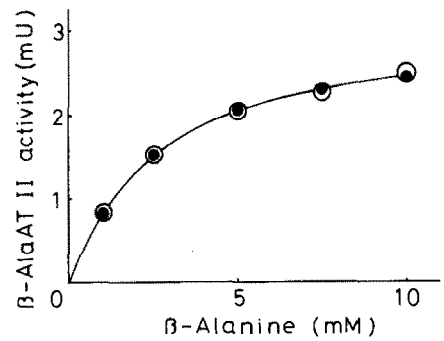

Fig. 2. β-AlaAT II activity as a function of β-alanine in the presence or absence of GABA. (○) control, (●) 5 mM GABA. Other conditions were the same as for the standard assay.

In a previous paper [15], we described that GABA is a poor substrate of β-AlaAT II. As shown in Fig. 2, the addition of 5 mM GABA did not decrease the initial reaction velocity of β-AlaAT II at various β-alanine concentrations. However, incubation of β-AlaAT II with gabaculine at various concentrations led to the inhibition of the enzyme (Fig. 3). Aliquots were removed periodically and assayed for residual enzyme activity. The control did not lose activity for up to approximately 1 h under the experimental conditions. Inactivation followed pseudo-first-order kinetics to complete loss of activity and the inhibited enzyme was not reactivated by continued dialysis against 0.1 M potassium phosphate, pH 7.5, containing 1 mM EDTA, 2 mM 2-mercaptoethanol and 40 μM pyridoxal 5′-phosphate overnight at 5°C. β-AlaAT II is saturable with gabaculine. The kinetic data calculated from Fig. 3A are as follows: apparent K_i, 8.3 μM; maximum rate of inactivation, 0.31 min^{-1}; half-life, 2.2 min. The K_i value was 240 times smaller than that of the K_i found by Burnett et al. [8] for *Pseudomonas* β-alanine-pyruvate aminotransferase. From these results, gabaculine acts as a potent inhibitor of β-AlaAT II rather like its effects on β-AlaAT I.

Substrates for β-AlaAT II from rat liver have a mark-

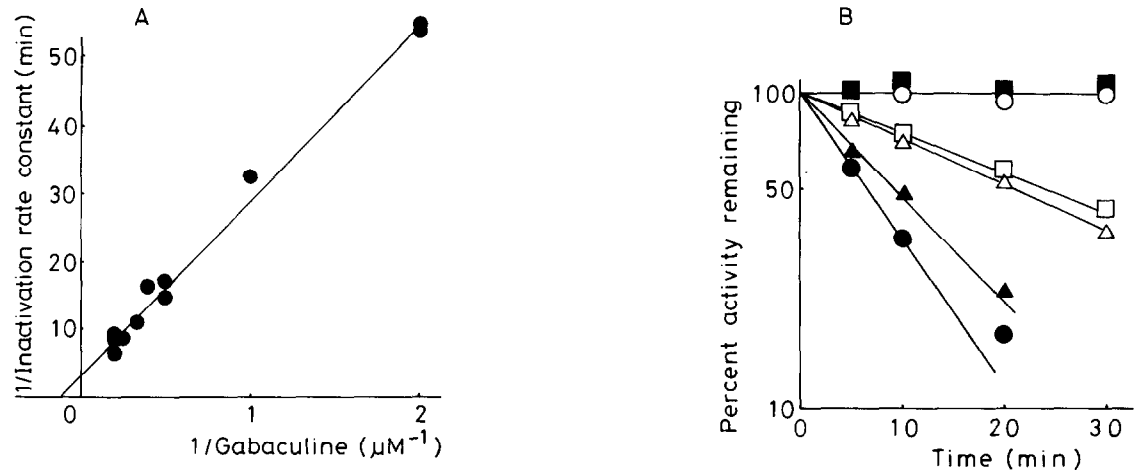

Fig. 3. Inhibition of β-AlaAT II from rat liver by gabaculine and the effects of substrates on its kinetics. (A) Concentration dependence of the inactivation of β-AlaAT II by DL-gabaculine. β-AlaAT II (5.5 μg = 1.8 mU) was incubated in 40 mM potassium pyrophosphate, pH 8.5, with the indicated concentrations of gabaculine at 25°C, and the inactivation rate constant was calculated as described in section 2. The data are presented as a double-reciprocal plot of inactivation rate constant versus concentration of gabaculine. (B) Effect of substrates on the rate of inactivation. β-AlaAT II (5.5 μg = 1.8 mU) was incubated as described in Fig. 2: (○) control; (●) 5 μM DL-gabaculine; (□) 5 μM DL-gabaculine and 10 mM pyruvate; (■) 5 mM DL-gababuline and 1 mM β-alanine; (▲) 5 μM DL-gabaculine and 5 mM GABA; (△) 5 μM DL-gabaculine and 10 mM GABA.

ed effect on the rate of inactivation (Fig. 3B). β-Alanine itself strongly retards the rate of inactivation, while GABA shows weaker effects and pyruvate has a moderate protective effect. Non-substrate 2-oxo-acids, such as 2-oxoglutarate, did not protect against inactivation by gabaculine (not shown). These experiments suggest that gabaculine preferentially interacts with the pyridoxal phosphate form of the coenzyme and does not affect the pyridoxamine phosphate form. Ornithine aminotransferase for *Bacillus sphaericus* IFO 3525 did not utilize GABA as a substrate [17] but was irreversibly inactivated by gabaculine [10]. However, the activity of ornithine aminotransferase from animals was inhibited by GABA [18–20]. On the other hand, although GABA did not act as a substrate or inhibitor or purified β-AlaAT II from rat liver, gabaculine acted as a potent irreversible inhibitor. If judged by the K_i and K_{cat} values, β-AlaAT II as well as β-AlaAT I are alternatively sensitive to gabaculine. As β-alanine and pyruvate prevent the inactivation of β-AlaAT II (Fig. 3B), the presence of substrates may inhibit binding between gabaculine and pyridoxal phosphate at the active site.

The K_m values for β-alanine with purified rat liver β-AlaAT I and β-AlaAT II were 6.3 mM [14] and 0.81 mM [15], respectively. We found the K_i values for gabaculine with rat liver β-AlaAT I and β-AlaAT II to be 7.1 μM and 8.3 μM, respectively. The K_i values for gabaculine with L-alanine transaminase and L-aspartate transaminase were 1 mM and 55 mM, respectively [13], and these values were much higher than those of the above ω-amino acid aminotransferase. Gabaculine commonly inhibits ω-amino acid aminotransferases such as β-AlaAT I, βAlaAT II, β-alanine-pyruvate aminotransferase, and ornithine aminotransferase and it is very sensitive. The binding of gabaculine to the enzymes may lead to an irreversible change in the active site. Therefore, gabaculine may be a useful tool to study the geometry and catalytic function of ω-amino acid aminotransferases.

β-Alanine formed by the degradation of uracil, is widely distributed in brain [21]. (*R*)-β-Aminoisobutyrate is an end product of the metabolic degradation of thymine. Thus, gabaculine may facilitate the study of the function of β-aminoisobutyrate as well as β-alanine including β-AlaAT II on the nervous system.

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