INACTIVATION OF GLUTAMATE DECARBOXYLASE BY BROMOPYRUVATE

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SUMMARY

Bromopyruvate was shown to inhibit E. coli glutamate decarboxylase competitively with respect to L-glutamate. High concentrations of bromopyruvate caused a time-dependent inactivation of glutamate decarboxylase. However, the apoenzyme was rapidly and irreversibly inactivated by bromopyruvate with an inactivation constant of 490 1 $mole^{-1}$ min^{-1} at pH 5.7. Studies with labeled bromopyruvate indicated that approximately 1.7 moles of inhibitor were bound per subunit of apoenzyme.

INTRODUCTION

A variety of aliphatic dicarboxylic acids have been shown to inhibit bacterial L-glutamate decarboxylase competitively with respect to glutamate (1-5). The dicarboxylic acids, glutaric, adipic, and pimelic acids, and the monocarboxylic acid, *n*-valeric acid, were good competitive inhibitors (4,5). In addition, monocarboxylic acids, especially acetic acid, are potent inhibitors of the recombination of the apodecarboxylase with its coenzyme, pyridoxal phosphate (6,7).

Since halo acids have been found to be fairly specific alkylating agents of some enzymes, and carboxylic acids inhibit the binding of both substrate and coenzyme to glutamate decarboxylase, we have examined various halo carboxylic acids for their ability to inhibit either holo or apo glutamate decarboxylase.

MATERIALS AND METHODS

Pyridoxal phosphate, dithiothreitol, and α-methyl-DL-glutamic and bromopyruvic acids were purchased from Sigma Chemical Co. 2-[^{14}C]-Bromopyruvate was prepared according to a modification of the procedure of Meloche (8).

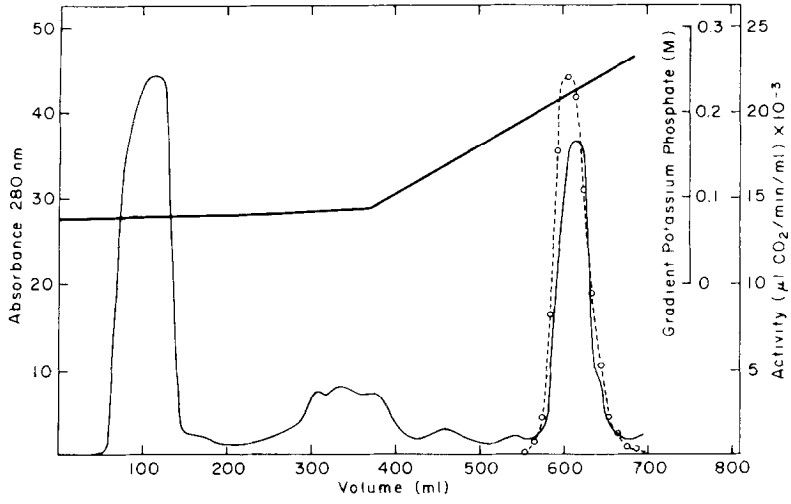

Fig. 1. Chromatography of glutamate decarboxylase on DEAE-Sephadex A-50. Protein was eluted with potassium phosphate (pH 6.0) in 10-ml fractions at a flow rate of 25 ml/hr. Absorbance at 280 nm, — ; glutamate decarboxylase activity, - - - - ; and potassium phosphate concentration, ▬ .

Radioactivity was determined using an aqueous toluene scintillation fluid (628 ml toluene, 330 ml Triton X-100, and 42 ml Liquiflor [New England Nuclear Corp.]) and a Nuclear Chicago Mark II liquid scintillation counter.

Glutamic decarboxylase was isolated from Escherichia coli (ATCC 11246) as previously described (9, 10) except that the enzyme was purified on a column of DEAE-Sephadex rather than DEAE-cellulose as the last step in purification. In this step, the enzyme which had been dialyzed against 0.1 M potassium phosphate (pH 6.0) was applied to a 2.5 x 45 cm column of DEAE Sephadex A-50 equilibrated with the same buffer. The column was developed at a flow rate of approximately 25 ml/hr with 0.1 M potassium phosphate (pH 6.0) until all the opalescent material was eluted. A linear gradient between 400 ml of 0.1 M potassium phosphate (pH 6.0) and 400 ml of 0.4 M potassium phosphate (pH 6.0) was used to elute the enzyme. A typical elution profile is shown in Fig. 1. An approximately 10-fold purification is obtained with this step. Enzyme with specific activity of 2,900 to 3,300 µl CO_2/min/mg protein at 37° has been obtained with this procedure. The enzyme was stored at 4° at

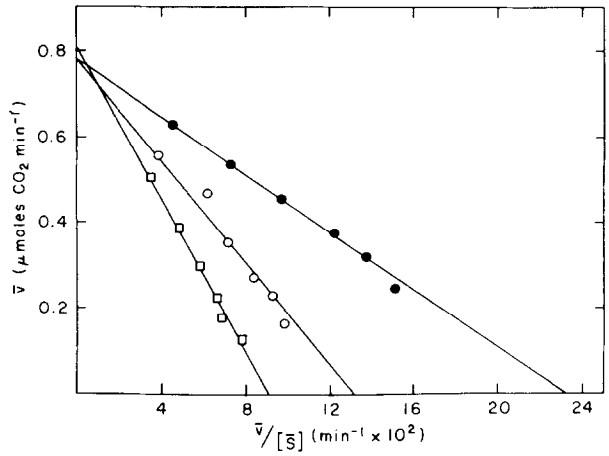

Fig. 2. Eadie plot showing competitive inhibition of glutamate decarboxylase by bromopyruvate. Glutamic acid concentration was varied from 0.67 to 5.33 mM. Reaction mixtures contained 0.1 M pyridine·HCl (pH 4.6), 0.2 M total chloride, 0.067 mM pyridoxal phosphate, 10 μg of glutamate decarboxylase, glutamic acid, and bromopyruvate as indicated, in a total volume of 3 ml. No inhibitor (●), 20 mM bromopyruvate (○), and 40 mM bromopyruvate (□).

a concentration of approximately 5 mg/ml in 0.1 M pyridine·HCl (pH 4.6), containing 0.2 mM pyridoxal phosphate and 0.1 mM dithiothreitol.

Glutamate apodecarboxylase was prepared by modifications of the procedures of Huntley and Metzler (11) and O'Leary and Malik (6). Holoenzyme was precipitated with 70% saturation ammonium sulfate, centrifuged at 10,000 rpm, and dissolved in a minimal volume of 0.1 M pyridine·HCl buffer (pH 4.9) containing 0.3 mM dithiothreitol. The solution was applied to a 0.9 x 15 cm column of Sephadex G-25-80 equilibrated with the same buffer and was eluted with the same buffer. The volume of the holoenzyme was adjusted so that the concentration was approximately 10 mg/ml. Solid DL-α-methylglutamic acid was added to the holoenzyme to a concentration of 0.1 M, and the pH of the solution was adjusted to pH 4.5 with 1 M phosphate buffer (pH 7.0). The solution was stirred gently at room temperature for 1 to 2 hr and was then dialyzed for 15 to 24 hr against two changes of 500 ml each of buffer containing 0.3 mM dithiothreitol. The apoenzyme retained less than 2% of its original activity, but was completely active upon addition of pyridoxal phosphate. Molar concentrations of apodecarboxylase were estimated from the

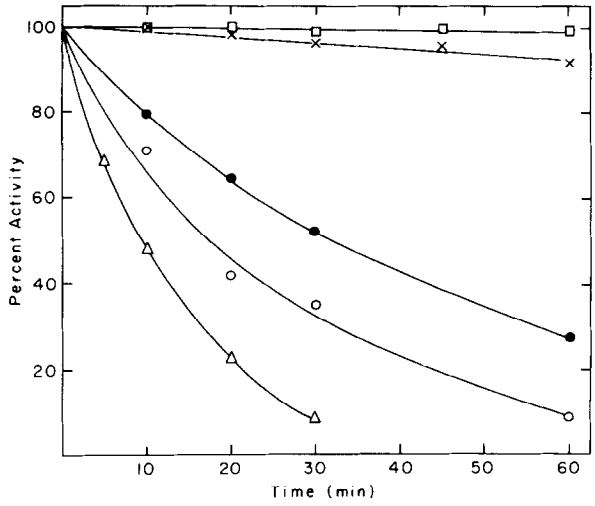

Fig. 3. Time-dependent inactivation of glutamate apodecarboxylase by bromopyruvate at 23°. The reaction mixtures contained 50 mM piperazine·PO_4 (pH 4.6), 0.02 mM dithiothreitol, 0.024 mM apoenzyme, and inhibitor as indicated. No inhibitor (□), 3.3 mM bromopyruvate (●), 10 mM bromopyruvate (o), 16.7 mM bromopyruvate (△). (x), 0.025 mM holoenzyme and 33 mM bromopyruvate.

absorbance at 280 nm using an apparent extinction coefficient of 86.1×10^3 (per subunit) (10, 12).

A Gilson differential respirometer was used for enzyme assays and all kinetic studies as described previously (5).

RESULTS AND DISCUSSION

Iodoacetate, 2- and 3-bromopropionate, 2- and 3-bromobutyrate, bromopyruvate, and bromosuccinate were all found to inhibit glutamate decarboxylase competitively with respect to glutamate. In Fig. 2 is an Eadie plot showing the competitive inhibition of the enzyme by bromopyruvate. The inhibitor dissociation constant (K_i) for bromopyruvate is 25 mM.

To determine whether these compounds inhibited glutamate decarboxylase in a time-dependent reaction, the inhibitor at a concentration approximately twice its K_i value was incubated at room temperature in the dark with 0.05 mM glutamate decarboxylase and 0.1 mM pyridoxal phosphate in 0.1 M pyridine·HCl (pH 4.6). After various times, aliquots were removed and assayed for activity. No inhibition was observed after 2 hours incubation with iodoacetate,

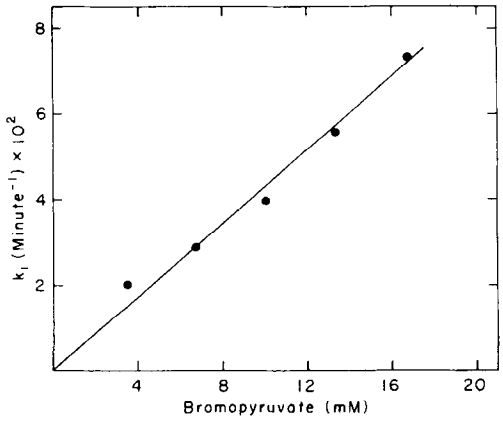

Fig. 4. The effect of bromopyruvate concentration on the first order rate constants of glutamate apodecarboxylase inactivation at pH 4.6 and 23°. The reactions were prepared as in Fig. 3.

2-bromopropionate, 3-bromopropionate, 2-bromobutyrate, 3-bromobutyrate, or bromosuccinate. After 2 hrs incubation, 50 mM bromopyruvate inhibited glutamate decarboxylase approximately 15%.

Inhibition of glutamate apodecarboxylase was investigated by incubating 0.05 mM apoenzyme with the inhibitor at a concentration approximately twice its K_i value at room temperature in the dark. After various times aliquots were removed, added to 2 mM pyridoxal phosphate, and assayed for activity. All of the halo carboxylic acids inhibited the recombination of pyridoxal phosphate and the apodecarboxylase reversibly, except for bromopyruvate which inactivated the apoenzyme rapidly and irreversibly. In Fig. 3 is shown the inhibition of pyridoxal phosphate binding to the apoenzyme by three different concentrations of bromopyruvate as a function of time. Pyridoxal phosphate protects the enzyme against this inactivation as the holoenzyme is inhibited only 8% by 33 mM bromopyruvate after 60 min of incubation. A plot of log activity against time gives a straight line for each inhibitor concentration indicating a pseudo first order reaction. The rate constants obtained from the slopes of these lines plotted against the concentration of bromopyruvate gives a straight line with a slope of 4.38 1 mole^{-1} min^{-1} (Fig. 4). The inactivation of the apodecarboxylase at pH 5.7 is much more rapid and has an inactivation constant of approximately 490 1 mole^{-1} min^{-1}.

The stoichiometry of bromopyruvate binding to the apodecarboxylase was determined using 2-[^{14}C]-bromopyruvate (specific activity of 5.42 x 10^4 dpm per mole). Glutamate apodecarboxylase incubated in duplicate for 1 hr with 0.594 mM 2-[^{14}C]-bromopyruvate had 1.70 to 1.75 moles of bromopyruvate stably incorporated per mole of enzyme subunit (Table I). The modified enzyme, after incubating with 2 mM pyridoxal phosphate, had approximately 3% as much activity as the control. In a second experiment, glutamate decarboxylase was pretreated with nonradioactive bromopyruvate in the presence of pyridoxal phosphate so that any nonspecific alkylation could occur. This pretreated enzyme was converted to apoenzyme which was then incubated with 2-[^{14}C]-bromopyruvate in duplicate experiments. The incorporation was the same as with apoenzyme obtained from untreated holoenzyme indicating that no nonspecific binding of bromopyruvate is occurring under these conditions.

Analog and inhibition studies with glutamate apodecarboxylase indicate that there are ionic interactions between the phosphate group of the coenzyme and the protein (6, 10). Anions, especially sulfate, phosphate, and carboxylic acids, inhibit the formation of the Schiff base between pyridoxal phosphate and the protein (6, 7). The concentration of bromopyruvate necessary to inactivate the apoenzyme is similar to the concentration of propionate (8 mM) or *n*-butyrate (2 mM) that cause a 50% decrease in the rate of reconstitution of apodecarboxylase with pyridoxal phosphate (7).

The protection afforded the enzyme by pyridoxal phosphate may indicate bromopyruvate is interacting at or near the coenzyme binding site of the enzyme. It is also possible that conformational changes of the protein are responsible for this protection. Phosphate also protects the enzyme against inactivation by bromopyruvate. Inactivation of the apodecarboxylase by bromopyruvate is approximately 20 times faster in 50 mM piperazine·Cl (pH 4.6) than in piperazine·PO_4 (pH 4.6).

The lack of inactivation of the enzyme by iodoacetate or 2-bromopropionate may indicate that the functional group of the enzyme with which bromo-

Table I. Incorporation of 2-[^{14}C]-Bromopyruvate into Glutamate Apodecarboxylase.

Sample	2-[^{14}C]-Bromopyruvate bound per subunit	Activity
	moles	m moles CO_2/min/ mole enz.
Control	0	1.678
^{14}C-Bromopyruvate + apoenzyme	1.73	0.056
^{14}C-Bromopyruvate + apoenzyme prepared from holoenzyme pretreated with cold bromopyruvate*	1.69	0.077

The glutamate apodecarboxylase labeled with 2-[^{14}C]-bromopyruvate was prepared by incubating, at room temperature for 60 min in the dark, 0.059 mM apoenzyme in 50 mM piperazine·PO_4 (pH 5.7), 0.05 mM dithiothreitol, and 0.594 mM 2-[^{14}C]-bromopyruvate. The reaction mixture was dialyzed against several changes of water for 12 hrs. The control was incubated under the same conditions in the absence of 2-[^{14}C]-bromopyruvate and dialyzed against water.

*Glutamate decarboxylase was first treated with 0.584 mM bromopyruvate for 60 min, the protein was precipitated with ammonium sulfate, and was desalted on a 0.9 x 15 cm column of Sephadex G-25-80. Apoenzyme was prepared from this fully active pretreated holoenzyme in the usual manner.

pyruvate reacts covalently is at least 3 to 4 A° from the carboxylate interaction site.

Bromopyruvate has been shown to bind specifically at the active sites of several enzymes. It alkylates a histidyl residue of ribonuclease (13), alkylates a cysteinyl residue of aspartate aminotransferase (14), and esterifies a carboxyl group or alkylates a cysteinyl residue of 2-keto-3-deoxy-6 phosphogluconic aldolase (15).

Currently, experiments are in progress to elucidate further the kinetics of inactivation of glutamate apodecarboxylase and to identify the amino acid residue(s) which react with bromopyruvate.

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