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2-BROMOACETAMIDOPYRIDINE: A NEW CHEMICAL PROBE OF THE ACTIVE SITE OF HISTIDINE DECARBOXYLASE FROM *LACTOBACILLUS* 30a

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SUMMARY

2-Bromoacetamidopyridine was examined as a potential active site-directed alkylating agent for histidine decarboxylase from *Lactobacillus* 30a. At the optimum pH of the enzyme (pH 4.8), the reagent binds reversibly to the substrate binding site as evidenced by its ability to competitively inhibit L-histidine decarboxylation, to protect the enzyme against inactivation by the substrate analog L-histidine methyl ester and to inhibit Schiff-base binding of histamine to the pyruvoyl prosthetic group. Upon raising the pH of the reaction medium from 4.8 to 7.2, 2-bromoacetamidopyridine irreversibly inactivates the decarboxylase. Incorporation experiments with 2-bromo[1-^{14}C]acetamidopyridine demonstrate that, under inactivating conditions, one reagent molecule is covalently bound per catalytic unit of enzyme inactivated.

Studies carried out previously (1) on the interaction of histidine decarboxylase from *Lactobacillus* 30a with a variety of histidine analogs have shown that the imidazole ring is of primary importance for effective binding of ligands to the substrate combining site. A subsequent investigation (2) further indicated that only one heterocyclic nitrogen atom is apparently required for substrate recognition and turnover, since pyridine served as a potent competitive inhibitor of the enzyme and decarboxylation of β-(2-pyridyl)alanine could be demonstrated. These latter findings prompted us to explore the potential usefulness of reactive pyridine analogs as chemical probes of the topology of the histidine binding site. As a first approach to this aim, 2-bromoacetamidopyridine was synthesized and its interaction with histidine decarboxylase evaluated.

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MATERIALS AND METHODS

Enzyme: Crystalline histidine decarboxylase (sp. act. 70 μmoles CO_2 evolved min^{-1} mg^{-1}) was isolated from *Lactobacillus* 30a as described by Riley and Snell (3) and assayed manometrically at 37° in 0.2 M ammonium acetate buffer (pH 4.8) containing 8 mM L-histidine (2). Decarboxylase concentrations were determined spectrophotometrically at 280 nm using the value $E^{1\%}$ = 16.2 (3) or by the biuret procedure (5), and assuming a molecular weight of 190,000 (4).

Chemicals: 2-Bromoacetamidopyridine was synthesized by reaction of bromoacetyl bromide with 2-aminopyridine using a modification of the procedure of Chichibabin (6). 2-Bromo[1-^{14}C]acetamidopyridine (1.35 x 10^5 cpm/μmole) was prepared in a similar manner except that radioactive bromoacetyl chloride, generated from bromo[1-^{14}C]acetic acid (New England Nuclear) and thionyl chloride, was used as the acylating agent. Synthesis of N-methylpyridine iodide was carried out according to Bergman, *et al.* (7). L-Histidine hydrochloride monohydrate, histamine dihydrochloride and L-histidine methyl ester dihydrochloride were purchased from Sigma. Bromoacetylbromide, 2-aminopyridine and sodium cyanoborohydride were from Aldrich.

Radioactivity was measured with a Nuclear-Chicago Mark I liquid scintillation spectrometer. Samples were counted in 15 ml of scintillation fluid composed of 125 g of napthalene, 7.5 g of PPO and 300 mg of POPOP in 1 liter of dioxane.

RESULTS

As an initial test to determine whether 2-bromoacetamidopyridine might possess an affinity for the substrate binding site of histidine decarboxylase, the effectiveness of the reagent as an inhibitor of the decarboxylation reaction was examined at the optimum pH (pH 4.8) of the enzyme. 2-Bromoacetamidopyridine was found to inhibit the decarboxylase competitively with respect to L-histidine (Figure 1); the dissociation constant (22 mM) calculated for the enzyme-2-bromoacetamidopyridine complex compares favorably with the K_i value (11 mM) reported earlier for histamine as a competitive inhibitor (9). Under the acidic conditions employed in the enzymatic assay, no irreversible loss of catalytic activity was observed for at least one hour at the highest concentration (20 mM) of 2-BAMPyr[1] tested.

Reversible interaction of 2-bromoacetamidopyridine within the binding domain for substrate is also indicated by its protective effect against the pseudo-first order loss of decarboxylase activity observed in the presence of

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[1]The abbreviations used are: 2-BAMPyr, 2-bromoacetamidopyridine; HME, L-histidine methyl ester.

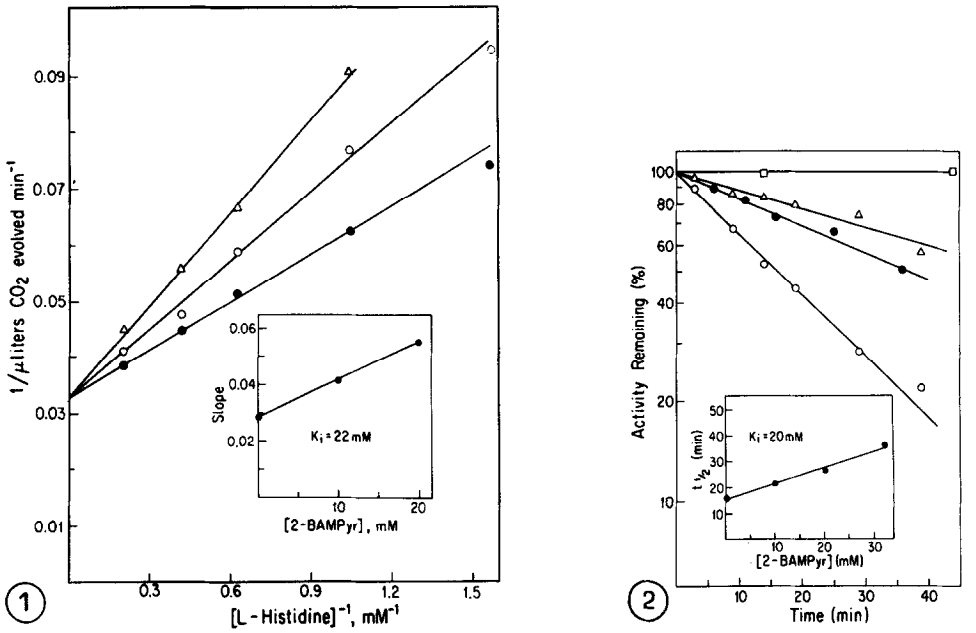


Figure 1: Competitive inhibition of histidine decarboxylase by 2-bromoacetamidopyridine. Assay solutions (3 ml) contained 0.2 M ammonium acetate buffer (pH 4.8), 3.3% methyl cellosolve, 20 μg of decarboxylase, L-histidine hydrochloride monohydrate at the concentrations indicated, and 0 mM (●), 10 mM (○), or 20 mM (Δ) 2-bromoacetamidopyridine. Initial rates of histidine decarboxylation were measured manometrically at 37° (2).

Figure 2: Protection by 32 mM 2-bromoacetamidopyridine (●) or 16 mM N-methylpyridine (Δ) against inactivation of histidine decarboxylase (1.2 mg/ml) with 0.2 mM L-histidine methyl ester (○) in 0.2 M ammonium acetate - 4% methyl cellosolve (pH 4.8) at 25°. Aliquots (50 μl) were withdrawn from the reaction mixtures at the times indicated, diluted 40-fold with 0.2 M ammonium acetate - 0.1% bovine serum albumin (pH 4.8) at 0°, and assayed for residual decarboxylase activity. The activity of a control solution with enzyme in buffer containing 4% methyl cellosolve but lacking the methyl ester is also shown (□). *Inset:* Half-life ($t_{\frac{1}{2}}$) for histidine methyl ester inactivation of the decarboxylase as a function of 2-bromoacetamidopyridine concentration.

L-histidine methyl ester (Figure 2). It has been shown previously (8) that histidine decarboxylase is inactivated by histidine methyl ester in a two-stage reaction sequence (Equation 1) whereby reversible binding of the substrate analog at the active site (K_D = 1.2 mM) precedes an irreversible binding step (k_{max} = 0.346 min^{-1}) leading to inactive enzyme (E*).

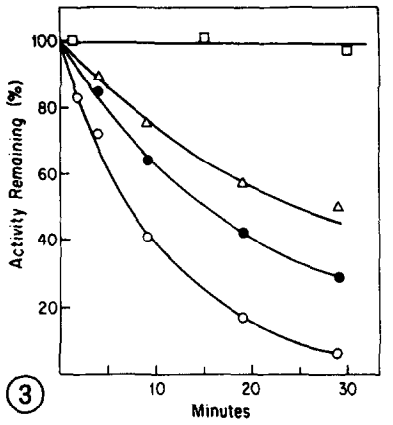
$$E + HME \rightleftharpoons E \cdots HME \xrightarrow{k_{max}} E^* \quad (1)$$

The kinetic expression derived for the situation in which the protection offered by 2-BAMPyr against HME-inactivation occurs as a consequence of competition between the reagent and methyl ester for active site binding in this reaction scheme is given by Equation 2

$$t_{\frac{1}{2}} = \frac{T}{[HME]} \cdot \frac{K_D}{K_i} \cdot [2\text{-BAMPyr}] + \left(T + \frac{T \cdot K_D}{[HME]}\right) \quad (2)$$

where K_i is the dissociation constant for 2-bromoacetamidopyridine and $T = \ln 2/k_{max}$. When the half-life ($t_{\frac{1}{2}}$) for inactivation by a fixed level of HME was plotted *vs.* increasing concentrations of 2-BAMPyr, a linear relationship was obtained (Figure 2, *inset*) as predicted from Equation 2. The K_i value for 2-BAMPyr, calculated from the slope of this line, is 20 mM. Similar protection against inactivation was provided by N-methylpyridine, another competitive inhibitor of the enzyme (K_i = 12 mM), at a concentration proportionately higher than its dissociation constant (Figure 2).

Recsei and Snell (9) have shown that histamine undergoes Schiff-base formation with the catalytically essential pyruvoyl prosthetic group of histidine decarboxylase. Thus, binding of 2-BAMPyr to the active center of the enzyme might be expected to retard the development of this azomethine complex. As shown in Figure 3 (*open circles*), formation of the histamine-pyruvate Schiff's base can be conveniently monitored by following the loss of enzyme activity in a ternary system composed of enzyme, histamine and sodium cyanoborohydride. In keeping with the known propensity for $NaBH_3CN$ to reduce imminium ions in acidic media (10, 11), the observed inactivation may be attributed to selective stabilization of the imine linkage and not to reduction of the pyruvoyl carbonyl group since $NaBH_3CN$ is without effect on enzyme activity in the absence of added histamine (Figure 3, *open squares*). Addition of 2-BAMPyr (40 mM) to the incubation mixture significantly reduced the rate of inactivation (Figure 3, *closed circles*). N-Methylpyridine at 32 mM concentration was also an effective protecting agent in this system (Figure 3, *open triangles*).



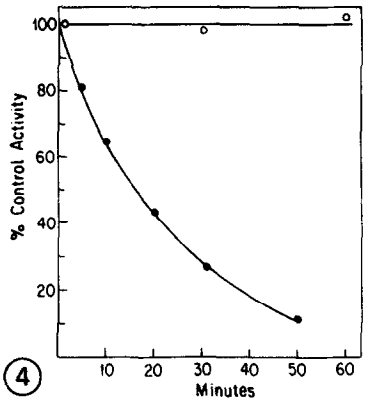


Figure 3: Inactivation of histidine decarboxylase by sodium cyanoborohydride (100 mM) *PLUS* histamine (1 mM) in the absence (0) and presence of 40 mM 2-bromoacetamidopyridine (●) or 32 mM N-methylpyridine (Δ). Other experimental conditions and procedures are described in the legend of Figure 2. (□), Control solution containing enzyme and 100 mM $NaBH_3CN$ with no histamine present.

Figure 4: Inactivation of histidine decarboxylase (0.72 mg/ml) at pH 7.2 and 25° in 0.2 M potassium phosphate - 5% methyl cellosolve containing 4 mM 2-bromoacetamidopyridine (●). Samples of the incubation mixture were periodically quenched and assayed for enzymatic activity as outlined in the legend of Figure 2. (0), no inhibitor.

Although 2-bromoacetamidopyridine failed to irreversibly inhibit histidine decarboxylase at pH 4.8, a time-dependent loss of activity was observed upon incubation of the reagent with the enzyme at pH 7.2. With 4 mM reagent, 90% of the initial activity was lost after 50 minutes under these conditions (Figure 4); complete inactivation was achieved after 90 minutes. When the enzyme was reacted with 2-bromo[1-^{14}C]acetamidopyridine and then freed of excess reagent by ammonium sulfate precipitation followed by exhaustive dialysis, substantial radioactivity remained stably associated with the enzyme. In two experiments of this type (Table I), the stoichiometry of inhibitor binding to completely inactivated enzyme was found to be 4.9 and 4.8 moles per 190,000 g of protein, in agreement with the accepted number of catalytic sites (five) in the enzyme molecule (3, 9, 12).

TABLE I

Incorporation of ^{14}C into Histidine Decarboxylase after Inactivation with 2-Bromo[1-^{14}C]acetamidopyridine[a]

Expt.	Decarboxylase Concentration	Reagent Concentration	Enzyme Activity Remaining	Counts/min/mg Protein	Moles Reagent Bound Per Mole of Enzyme[b]
	mg/ml	mM	%		
1	3.6	10	21	2758	4.9
2	8.4	12.5	<1	3354	4.8

[a] The enzyme was incubated with ^{14}C-labeled reagent (1.35 x 10^5 cpm/μmole) at 25° in 0.2 M potassium phosphate (pH 7.2) containing 5% methyl cellosolve until the degree of inactivation indicated was achieved. The reaction was then terminated by addition of the incubation mixture (~1 ml) to 8 ml of 0.2 M ammonium acetate-80% ammonium sulfate (pH 5.2). The precipitated protein was collected by centrifugation, washed twice with the ammonium sulfate solution, dissolved in 10 mM potassium phosphate - 0.5 mM EDTA (pH 6), and dialyzed against the same buffer at 4° to constant specific radioactivity.

[b] Calculated on the basis of moles of enzyme subjected to inactivation.

DISCUSSION

Several independent lines of evidence support the proposal that 2-bromoacetamidopyridine displays an affinity for the substrate binding domain of histidine decarboxylase under conditions (pH 4.8) optimal for catalysis. In the first place, the compound is an effective competitive inhibitor of decarboxylase activity. Second, the reagent protects competitively against inactivation by histidine methyl ester, a substrate analog which has been shown to function as an active site-directed irreversible inhibitor of the enzyme (8). The excellent agreement between the inhibitor dissociation constant (20 mM) for 2-BAMPyr calculated from the protection experiments with the value (22 mM) obtained from the competitive inhibition studies provides a strong indication that the observed protection is mediated by binding of the reagent to the catalytic site. Third, Schiff-base binding of histamine to the pyruvoyl prosthetic group of the enzyme, as detected by selective reduction with $NaBH_3CN$, is appreciably inhibited in the presence of the pyridine analog. Since the histamine-enzyme azomethine is considered to be an important reaction intermediate during catalysis of histidine decarboxylation (9), this result may be interpreted as being due to a mutually exclusive interaction of histamine and 2-BAMPyr at the active site.

Conditions have also been established wherein reaction of 2-BAMPyr with histidine decarboxylase produces a covalently labeled and catalytically inert enzyme. As expected for an affinity label, binding of one residue of ^{14}C-labeled reagent per enzyme active center is sufficient to produce complete inactivation. Thus, modification would appear to be highly selective and restricted to the catalytic site, with little or no non-specific labeling occurring at extraneous sites. We have, indeed, determined that 2-BAMPyr exhibits absolute specificity for a single sulfhydryl group in each of the five larger (α) subunits (12, 13) of the enzyme. These results, as well as a kinetic description of the alkylation reaction, will be presented in a subsequent paper.

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