Reaction of *Lactobacillus* Histidine Decarboxylase with L-Histidine Methyl Ester†

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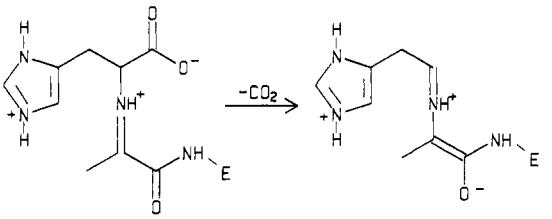
ABSTRACT: L-Histidine methyl ester inactivates histidine decarboxylase in a time-dependent manner. The possibility was considered that an irreversible reaction between enzyme and inhibitor occurs [Recsei, P. A., & Snell, E. E. (1970) *Biochemistry 9*, 1492–1497]. We have confirmed time-dependent inactivation by histidine methyl ester and have investigated the structure of the enzyme–inhibitor complex. Upon exposure to either 8 M guanidinium chloride or 6% trichloroacetic acid, unchanged histidine methyl ester is recovered. Formation of the complex involves Schiff base formation, most likely with the active site pyruvyl residue [Huynh, Q. K., & Snell, E. E. (1986) *J. Biol. Chem. 261*, 4389–4394], but does not involve additional irreversible covalent interaction between inhibitor and enzyme. Complex formation is a two-step process involving rapidly reversible formation of a loose complex and essentially irreversible formation of a tight complex. For the formation of the tight complex, K_i = 80 nM and $k_{off} = 2.5 \times 10^{-4}$ min^{-1}. Time-dependent inhibition was also observed with L-histidine ethyl ester, L-histidinamide, and DL-3-amino-4-(4-imidazolyl)-2-butanone. No inactivation was observed with glycine methyl ester or histamine. We propose that in the catalytic reaction the carboxyl group of the substrate is in a hydrophobic region. The unfavorable interaction between the carboxylate group and the hydrophobic region facilitates decarboxylation [Crosby, J., Stone, R., & Liehard, G. E. (1970) *J. Am. Chem. Soc. 92*, 2891–2900]. With histidine methyl ester this unfavorable interaction is no longer present; hence, there is tight binding. The effect of substrate ester analogues on the activity of other decarboxylases was examined to determine whether this approach to inhibition has general applicability. Enzymes investigated were (with substrate analogues in parentheses) acetoacetate decarboxylase from *Clostridium acetobutylicum* (methyl acetoacetate), thiamin pyrophosphate dependent pyruvate decarboxylase from brewers' yeast (methyl pyruvate), pyridoxal phosphate dependent tyrosine/phenylalanine decarboxylase from *Streptococcus faecalis* (L-tyrosine methyl ester, L-phenylalanine methyl ester, DL-2-amino-4-phenyl-2-butanone), and arginine and ornithine decarboxylases from *Escherichia coli* (L-arginine methyl ester, D-arginine methyl ester). No time-dependent inhibition was observed in those cases.

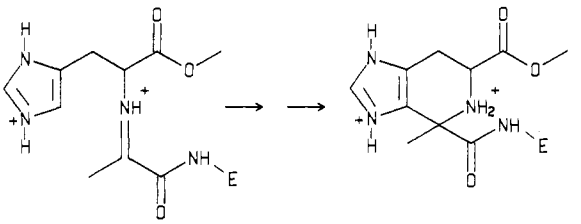
Histidine decarboxylase of *Lactobacillus* 30a employs a covalently bound pyruvyl residue as a prosthetic group (Recsei & Snell, 1979). The pyruvoyl group forms an imine with the substrate to stabilize a transient carbanion formed during decarboxylation. The pyruvoyl group plays a role similar to that of pyridoxal phosphate in other enzymes that catalyze decarboxylation of amino acids (Scheme I).

The *Lactobacillus* enzyme and a similar decarboxylase from *Micrococcus* sp. n. are curiously subject to practically irreversible inactivation by L-histidine methyl ester (Mardashev et al., 1968; Lane et al., 1976). The mechanism of inactivation is not known. Histidine methyl ester binds very tightly to the enzyme. Bound ester is not released nor is activity regained upon prolonged dialysis at 4 °C. Furthermore, inhibitor is not dissociated upon exposure to 3 M guanidinium chloride (Lane et al., 1976). These properties suggest, but do not require, that inhibition involves formation of a stable covalent bond between enzyme and inhibitor. The possibility was suggested, but not extensively pursued, that histidine methyl ester may acylate a nucleophile at one of the active sites of the hexameric enzyme (Lane et al., 1976). Another possibility, which occurred to us, is that the enzyme-bound inhibitor may undergo the Pictet–Spengler reaction proposed in Scheme II (Neuberger, 1944). This investigation was carried out to determine the basis for the tight binding of enzyme and inhibitor.

Scheme I: Mechanism of Decarboxylation



Scheme II: Pictet–Spengler Reaction



Experimental Procedures

Enzymes. Lactobacillus 30a was obtained from the American Type Culture Collection (33222). The cells were cultured, and the decarboxylase was isolated as described by Chang and Snell (1968) except that some material absorbing at 260 nm was removed by chromatography on [2-(diethylamino)ethyl]cellulose equilibrated with 50 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate at pH 7.0 and 4 °C. Upon elution with 100 mL of the same buffer containing 0–400 mM NaCl in linear gradient, the enzyme was found

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in earlier fractions than the nucleic acid. After dialysis against 50 mM ammonium acetate, pH 4.8, the decarboxylase was concentrated by ultrafiltration and used without crystallization. The protein migrated as the expected two polypeptides during sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Enzyme activity was assayed by following the hydrogen ion loss that accompanies the decarboxylation of histidine in lightly buffered solutions at slightly acidic pH (Vienozinskiene et al., 1985). Activity was followed continuously through increasing absorbance at 412 nm with 4-nitrophenol as a chromogenic pH indicator. The assay solution contained 10 mM L-histidine hydrochloride, 4.0 mM 4-nitrophenol, and 50 mM KCl adjusted to pH 4.8 at 25 °C with KOH. One micromole of KOH per milliliter increased the optical density at 412 nm of this solution from 0.46 to 1.12 cm^{-1} as the pH was raised to 5.2. Enzymatic rates of absorbance change appeared linear for at least 1.0 cm^{-1} but were typically followed for changes of less than 0.5 cm^{-1}.

Acetoacetate decarboxylase, a gift from K. Nazaretian, was isolated from *Clostridium acetobutylicum* (Zerner et al., 1966). Other decarboxylases were purchased from Sigma and tested without further purification.

Reagents. L-[3-^{3}H]Histidine (diluted to 1.4 Ci/mol) was obtained from New England Nuclear and purified by thin-layer chromatography (cellulose, 3:1:1 2-propanol/NH_4OH/water). DL-[2-^{3}H]Histidine (2.4 Ci/mol) was prepared from tritiated water and *N*-acetyl-L-histidine via the dicyclohexylcarbodiimide-generated oxazoline (Battersby et al., 1980). Pilot reactions with D_2O confirmed that, as reported, only α-hydrogen exchange occurred. Amino ester hydrochlorides were prepared by Fischer esterification and crystallized from alcohol–ether. Stock solutions of radioactive methyl esters were prepared in anhydrous methanol and stored at −20 °C. DL-3-Amino-4-(4-imidazolyl)-2-butanone dihydrochloride and DL-3-amino-4-phenyl-2-butanone hydrochloride were synthesized via the Dakin–West reaction of L-histidine or L-phenylalanine with acetic anhydride (Smissman & Weiss, 1971). L-Histidine was cyclized with formaldehyde, and histamine was cyclized with benzaldehyde in base-catalyzed Pictet–Spengler reactions (Neuberger, 1944; Stocker et al., 1966). Synthesized products were obtained as crystalline solids and exhibited the expected ^{1}H nuclear magnetic resonance spectra. Radiochemical purities appeared >98% on thin-layer chromatograms.

Optical experiments were performed with a Perkin-Elmer 559 recording spectrophotometer. Radioactivity was measured in a Beckman LS-100C liquid scintillation counter using Amersham ACS scintillant.

Results

Inactivation by Histidine Methyl Ester and Related Compounds. Incubation of histidine decarboxylase with L-histidine methyl ester results in time-dependent loss of enzymatic activity (Lane et al., 1976). The inactivation is a pseudo-first-order process exhibiting saturation kinetics. In 50 mM ammonium acetate buffer at pH 4.8 and 25 °C, a maximal inactivation rate constant of 0.63 min^{-1} is approached, the half-maximal rate occurring at 0.2 mM ester. Other compounds that inactivate in a time-dependent manner are L-histidine ethyl ester (1.2 min^{-1}, 0.3 mM), L-histidinamide (1.6 min^{-1}, 0.6 mM), and DL-3-amino-4-(4-imidazolyl)-2-butanone (2.1 min^{-1}, 0.6 mM), an analogue of histidine in which a keto, rather than an ester, moiety replaces the carboxylate group. The amino ketone has previously been noted to inhibit micrococcal non-pyridoxal histidine decarboxylase and the mammalian pyridoxal phosphate dependent decarboxylase (Mardashev et al., 1969; Smissman & Weis, 1971), but time dependence or irreversibility have not been reported in those cases. The facile inactivation of the *Lactobacillus* decarboxylase by the amino ketone suggests that the amino ester does not inactivate the enzyme in a process involving an acylation reaction by the methoxyformyl group.

No inactivation was observed when the following compounds (10 mM) were incubated with the enzyme for 30 min at pH 4.8 and 25 °C before dilution into assay solutions containing saturating levels of L-histidine: histamine, D-histidine methyl ester, DL-α-methylhistidine methyl ester, glycine methyl ester (±10 mM imidazole), L-phenylalanine methyl ester, DL-2-amino-4-phenyl-2-butanone, and DL-*m*-tyrosine methyl ester.

Stoichiometry of Inhibition. Enzyme (3.0 mg) completely inactivated with L-[3-^{3}H]histidine methyl ester (1.4 Ci/mol) in 50 mM ammonium acetate at pH 4.8 and 25 °C was filtered through a 1.0 × 20 cm column of Bio-Gel P-6 to remove excess inhibitor. From the amount of radioactivity associated with the protein peak, it was determined that 0.5 mol of inhibitor was associated with 39000 g of protein, in good agreement with previous results (Lane et al., 1976). Thus 1 mol of inhibitor binds per two subunits. The experiment was repeated with DL-[2-^{3}H]histidine methyl ester (2.4 Ci/mol). The same result was obtained as with L-[3-^{3}H]histidine methyl ester; i.e., 1 mol of inhibitor binds per 2 mol of subunit. This experiment not only confirms the stoichiometry of labeling but also establishes that reaction of inhibitor and enzyme does not involve labilization of the α-hydrogen of the inhibitor. Further evidence aginst the possible formation, and accumulation, of a delocalized carbanion was the failure of the inactivated enzyme to exhibit enhanced near-ultraviolet absorbance (not shown).

Reversibility of Inhibition. Less than 2% of control activity is found after the *Lactobacillus* decarboxylase is inactivated by either L-histidine methyl ester or DL-3-amino-4-(4-imidazolyl)-2-butanone and then dialyzed for 24 h against 50 mM ammonium acetate buffer at pH 4.8 and 4 °C. The rate of dissociation of the enzyme–inhibitor complex was further investigated. After complete inactivation of the enzyme by L-[3-^{3}H]histidine methyl ester (1.4 Ci/mol) and separation of unbound radioactivity by gel filtration, the radiolabeled enzyme (26 μN) was incubated with 5.0 mM unlabeled L-histidine methyl ester in 50 mM ammonium acetate buffer at pH 4.8 and 25 °C. Aliquots containing 0.5 mg of protein were periodically subjected to gel filtration through a 1.0 × 20 cm column of Bio-Gel P-6 in order to determine the extent of dissociation of the radiolabel from the protein. In a first-order process, 36% of the radioactivity dissociated in 24 h at 25 °C. The rate constant for dissociation (k_{off}) is, therefore, 2.5×10^{-4} min^{-1}.

Structure of Enzyme-Bound Inhibitor. Snell and co-workers found that treatment with 3 M guanidinium chloride does not dissociate the bound inhibitor from the enzyme inactivated by histidine methyl ester (Recsei & Snell, 1970). Similarly, we found no dissociation of bound inhibitor when the enzyme was inactivated with L-[3-^{3}H]histidine methyl ester, freed from unbound material by gel filtration, dialyzed for 7.0 h against 6 M urea at pH 4.8 and 4 °C, and then subjected to high-pressure gel filtration through a 0.75 × 30 cm Altex Spherogel-TSK column equilibrated with 6 M urea at pH 4.8 and 25 °C. However, rather than indicating an irreversible covalent modification of the protein by the inhibitor, these observations are attributable to high resistance of the enzyme–inhibitor complex to denaturation by chaotropic agents. The radiolabeled inhibitor is readily dissociated from the enzyme upon treatment with a higher concentration of guanidinium

chloride. After inactivation with tritiated ester and isolation by gel filtration, the enzyme solution was treated with solid guanidinium chloride to achieve a concentration of 8 M at pH 4.8 and 25 °C. After 1.0 h, all of the radioactivity had dissociated, as determined by chromatography on a Bio-Gel P-6 column equilibrated with 8 M guanidinium chloride and 50 mM ammonium acetate at pH 4.8 and 25 °C.

Since identification of the released radioactive material was not possible in the presence of high concentrations of guanidinium chloride, we have also used trichloroacetic acid to dissociate the enzyme–inhibitor complex. After complete inactivation by excess L-[3-^{3}H]histidine methyl ester (1.4 Ci/mol) and isolation by gel filtration, the radiolabeled protein (0.85 mg in 0.8 mL) was chilled to 0 °C and mixed with 0.8 mL of cold 12% trichloroacetic acid. After 15 min, the denatured protein was removed by centrifugation, and the supernatant solution was treated 3 times with 2.0 mL of water-saturated diethyl ether at 0 °C to extract the trichloroacetic acid. The protein precipitation and ether extraction caused loss of 5.0% of the radioactivity initially present. Therefore, essentially all of the bound inhibitor was released by the acid. The radioactivity released from the the protein was examined by cation-exchange chromatography (Pharmacia FPLC aparatus, 0.5 × 5.0 cm Mono S column, 0–500 mM NaCl gradient, pH 3.5), cellulose thin-layer chromatography (4:1:1 1-butanol/acetic acid/water), and electrophoresis (Whatman 3MM paper, 3000 V, pH 6.5). In all cases >90% of the radioactivity comigrated with carrier histidine methyl ester (ninhydrin detected). Thus, any covalent modification of histidine methyl ester during its inactivation of the decarboxylase must be readily reversible under mild conditions after enzyme denaturation.

Release of unchanged histidine methyl ester by trichloroacetic acid strongly mitigates against the Pictet–Spengler reaction proposed in Scheme II. We have furthermore confirmed that the condensation product of L-histidine with formaldehyde and that of histamine with benzaldehyde are quantitatively recovered intact after such treatment.

Although irreversible covalent modification of the enzyme by the amino ester does not occur, reversible covalent reactions such as Schiff base formation may participate in the inactivation process. Previously, trapping with borohydride established that the substrate histidine ordinarily forms the imine linkage shown in Scheme I (Recsei & Snell, 1970). Similarly, inactivation of *Lactobacillus* decarboxylase by L-histidine methyl ester is accelerated by cyanoborohydride under conditions in which cyanoborohydride alone does not inactivate the enzyme (Huynh & Snell, 1986). This observation demonstrates that imine linkage of the amino ester to the enzyme is kinetically competent to occur during the process of inactivation but does not demonstrate that the inactivated decarboxylase bears the imine functionality. We have corroborated the presence of the imine linkage by treating the enzyme with cyanoborohydride after, rather than concurrently with, inactivation by the ester. Thus, histidine decarboxylase was completely inactivated by excess L-[3-^{3}H]histidine methyl ester (1.4 Ci/mol) and isolated by gel filtration through a 1.0 × 20 cm column of Bio-Gel P-6 equilibrated with 200 mM ammonium acetate at pH 4.8 and 25 °C. The radiolabeled enzyme was divided into two 0.6-mL portions, each containing 0.42 mg of protein. One sample was treated for 1.0 h with 20 mM $NaCNBH_3$, and then both samples were chilled on ice and treated with 1 volume of 10% trichloroacetic acid. After 1.0 h, the samples were centrifuged, and the supernatants (1.0 mL) were checked for unbound radioactivity. The protein pellets were washed with 1.0 mL of cold 10% trichloroacetic acid and then checked for the irreversibly bound radioactivity. In the absence of $NaCNBH_3$, >99% of the radioactivity was freed from the protein by the trichloroacetic acid. In contrast, 72% of the radioactivity precipitated with the protein (3510 cpm) rather than remaining in solution (1400 cpm) in case of the $NaCNBH_3$-treated sample.

Other Decarboxylases. Several decarboxylases were incubated for 30 min at 25 °C with the indicated substrate analogues. Activity was then assayed after 100-fold dilution. In no case was inactivation observed. The enzymes were (with substrate analogues in parentheses) cofactor-independent acetoacetate decarboxylase from *C. acetobutylicum* (methyl acetoacetate, pH 7.0), thiamin pyrophosphate dependent pyruvate decarboxylase from brewers' yeast (methyl pyruvate, pH 6.0), pyridoxal phosphate dependent tyrosine/phenylalanine decarboxylase from *Streptococcus faecalis* (L-tyrosine methyl ester, L-phenylalanine methyl ester, DL-2-amino-4-phenyl-2-butanone, pH 4.8), and arginine and ornithine decarboxylases from *Escherichia coli* (L-arginine methyl ester, D-arginine methyl ester, pH 4.8). Only with acetoacetate decarboxylase was the substrate analogue tested as reversible inhibitor. In 100 mM potassium phosphate buffer at pH 7 and 25 °C, methyl acetoacetate exhibited a K_i value of 0.14 mM while the substrate exhibited a K_m value of 8.0 mM.

Discussion

The data presented establish that the inactivation of histidine decarboxylase by histidine methyl ester and related compounds involves no irreversible covalent modification of the inhibitors other than the previously established imine linkage to the enzyme. The inhibitors bind tightly and have slow dissociation rates. Thus for L-histidine methyl ester K_i = 80 nM and k_{off} = 2.5 × 10^{-4} min^{-1} at pH 4.8 and 25 °C.

The question then arises as to why these compounds bind so tightly. It was pointed out by Lienhard (1970) that decarboxylation of the anionic form of a carboxylic acid bearing an electron sink in the β-position can be greatly accelerated by placing the molecule in a hydrophobic environment. Decarboxylation is accelerated because the carboxylate group is destablilized in the hydrophobic environment. Decarboxylation leads to loss of negative charge and is, therefore, energetically favorable. It was proposed that this type of destabilization mechanism is a component of thiamin adduct decarboxylation. A very effective inhibitor of pyruvate decarboxylase was designed on the basis of this principle (Gutowski & Lienhard, 1976). We propose that a similar mechanism is operative with histidine decarboxylation. Placing the carboxyl group into the hydrophobic region facilitates decarboxylation, but it also requires energy to introduce the charged group into a hydrophobic region. This energy is derived from substrate–enzyme interaction. With substrate analogues that do not have a negative charge, the unfavorable interaction with the hydrophobic region is no longer involved. The energy normally required to introduce the negative charge into the hydrophobic region now manifests itself as binding energy, hence the very low K_i.

Such a destabilization mechanism should be operative with other decarboxylation reactions. However, in no other case examined was inactivation by substrate analogues (methyl esters) observed. Possibly steric constraints could be responsible; i.e., the ester cannot be accommodated in the hydrophobic region. It is also possible that the negative charge is required for initial binding and that it is kinetically not possible to reach the state in which the interaction with the hydrophobic region becomes important without formation of the initial

complex. A similar explanation has been invoked to account for the failure of lactyl thiamin pyrophosphate to bind to wheat germ apopyruvate decarboxylase (Kluger & Smyth, 1981).

Neither imidazole (K_i = 0.003 M) (Rosenthaler et al., 1965) nor glycine methyl ester (K_i > 0.01 M) are good inhibitors of the decarboxylase although they are components of histidine methyl ester ($K_i = 8 \times 10^{-8}$ M). Effective inhibition is the result of incorporating these components into a single molecule. The binding advantage obtained from forming a single molecule is a factor of at least 3×10^3 (Jencks, 1975).

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Registry No. $AcCH_2CO_2Me$, 105-45-3; $AcCH_2CO_2H$, 541-50-4; histidine decarboxylase, 9024-61-7; L-histidine methyl ester, 1499-46-3; L-histidine ethyl ester, 7555-06-8; L-histidinamide, 7621-14-9; DL-3-amino-4-(4-imidazolyl)-2-butanone, 108212-43-7; acetoacetate decarboxylase, 9025-03-0.

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Construction and Evaluation of the Kinetic Scheme Associated with Dihydrofolate Reductase from *Escherichia coli*†

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ABSTRACT: A kinetic scheme is presented for *Escherichia coli* dihydrofolate reductase that predicts steady-state kinetic parameters and full time course kinetics under a variety of substrate concentrations and pHs. This scheme was derived from measuring association and dissociation rate constants and pre-steady-state transients by using stopped-flow fluorescence and absorbance spectroscopy. The binding kinetics suggest that during steady-state turnover product dissociation follows a specific, preferred pathway in which tetrahydrofolate (H_4F) dissociation occurs after NADPH replaces $NADP^+$ in the ternary complex. This step, H_4F dissociation from the E·NADPH·H_4F ternary complex, is proposed to be the rate-limiting step for steady-state turnover at low pH because $k_{off} = V_M$. The rate constant for hydride transfer from NADPH to dihydrofolate (H_2F), measured by pre-steady-state transients, has a deuterium isotope effect of 3 and is rapid, k_{hyd} = 950 s^{-1}, essentially irreversible, K_{eq} = 1700, and pH dependent, pK_a = 6.5, reflecting ionization of a single group in the active site. This scheme accounts for the apparent pK_a = 8.4 observed in the steady state as due to a change in the rate-determining step from product release at low pH to hydride transfer above pH 8.4. This kinetic scheme is a necessary background to analyze the effects of single amino acid substitutions on individual rate constants.

Dihydrofolate reductase (DHFR)[1] (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase) catalyzes the reduction of 7,8-dihydrofolate (H_2F) by NADPH to form 5,6,7,8-tetrahydrofolate (H_4F). Tetrahydrofolate is a coenzyme utilized in a number of one-carbon-transfer reactions and is essential for the biosynthesis of purines, thymidylate, and several amino acids. Hence, DHFR is a target for both anticancer and antibacterial drugs. DHFR has also been the subject of in-

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[1] Abbreviations: DHFR, dihydrofolate reductase; H_2F, dihydrofolate; H_4F, tetrahydrofolate; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; $NADP^+$, nicotinamide adenine dinucleotide phosphate; TNADPH, thionicotinamide adenine dinucleotide phosphate, reduced; APADPH, acetylpyridine adenine dinucleotide phosphate, reduced; MTX, methotrexate; TMP, trimethoprim.

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