Histidine Decarboxylase of *Lactobacillus* 30a: Inactivation and Active-Site Labeling by L-Histidine Methyl Ester†

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ABSTRACT: Histidine decarboxylase from *Lactobacillus* 30a is rapidly and irreversibly inactivated upon incubation with L-histidine methyl ester. The rate of inactivation is first-order with respect to remaining active enzyme and exhibits saturation kinetics with a k_{inact} of 1.2 mM and an apparent first-order rate constant of 0.346 min^{-1} at pH 4.8 and 25 °C. On complete inactivation, 3 mol of [^{14}C]histidine (from L-[^{14}C]histidine methyl ester) and 2 mol of ^{14}C (from L-histidine [^{14}C]methyl ester) are bound in nondialyzable form per mol (190 000 g) of protein inactivated with a corresponding loss of three of the five DTNB-titratable -SH groups that are essential for activity of the native enzyme. Imidazole propionate, a competitive inhibitor of the enzyme, protects against inactivation, loss of SH groups, and incorporation of radioactivity from both the histidine and the methyl ester moieties of the labeled inhibitor, and kinetic evidence indicates that imidazole propionate and histidine methyl ester compete for binding at the active site of histidine decarboxylase in a mutually exclusive manner. Treatment of the labeled protein with either alkali or hydroxylamine results in the quantitative release of radioactivity. These data suggest that inactivation of histidine decarboxylase by L-histidine methyl ester results from two different modes of interaction between the inhibitor and the active site of histidine decarboxylase; the major interaction involves an essential SH group.

Histidine decarboxylase from *Lactobacillus* 30a catalyzes the decarboxylation of L-histidine to yield histamine and CO_2. Catalysis involves the initial formation of a Schiff base between the α-amino group of the substrate and the carbonyl moiety of a functionally essential pyruvoyl residue in the enzyme molecule. Decarboxylation of the histidine–enzyme azomethine and subsequent hydrolysis of the corresponding histamine complex yield the reaction products (Riley and Snell, 1968; Recsei and Snell, 1970). Chemical modification studies of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) also implicate a reactive cysteinyl residue as being critical for normal catalytic function (Lane and Snell, 1976).

To further define other functional residues, affinity labeling of histidine decarboxylase would be useful. The observation that L-histidine methyl ester irreversibly inactivates histidine decarboxylase from *Micrococcus* sp. n. (Mardashev et al., 1968) prompted us to examine the nature of the reaction of this substrate analogue with the homogeneous *Lactobacillus* enzyme. In this report, we show that L-histidine methyl ester also inactivates histidine decarboxylase from *Lactobacillus* 30a and that the reaction satisfies the criteria for active-site specificity. Experiments directed at identifying the principal site(s) of interaction between the decarboxylase and histidine methyl ester also are described.

Experimental Section

Materials

Crystalline histidine decarboxylase was isolated from acetone powders of *Lactobacillus* 30a cells by the procedure of Riley and Snell (1968) and was homogeneous on disc gel electrophoresis (Davis, 1964). Enzyme stock solutions were prepared by dissolving the crystals in cold 0.2 M ammonium acetate (pH 4.8) followed by overnight dialysis at 4 °C against two changes of the same buffer. Enzyme thus prepared exhibited specific activities of 70–80 μmol of CO_2 evolved min^{-1} (mg of protein)$^{-1}$ at 37 °C.

L-Histidine methyl ester dihydrochloride (Sigma), bovine serum albumin (Pentex), imidazole and histamine dihydrochloride (Calbiochem), L-histidine hydrochloride monohydrate (Nutritional Biochemicals), hydroxylamine hydrochloride (Eastman), sodium borohydride (Metal Hydrides, Inc.), and 5,5′-dithiobis(2-nitrobenzoic acid) (Aldrich) were commercial products. The latter compound was twice recrystallized from glacial acetic acid prior to use. L-[U-^{14}C]Histidine (256 mCi/mmol) and [^{14}C]methanol (3.2 mCi/mmol) were obtained from New England Nuclear; [^{14}C]methanol (12.8 mCi/mmol) was obtained from Amersham/Searle.

Methods

Enzyme Assay. Histidine decarboxylase was determined manometrically at 37 °C (Chang and Snell, 1968a) by adding a suitable aliquot of the enzyme in 0.2 M ammonium acetate–0.1% bovine serum albumin (pH 4.8) to 3 ml of 0.2 M ammonium acetate–8 mM L-histidine solution (pH 4.8), and monitoring CO_2 evolution at 1-min intervals for 10 min. In some experiments, the release of $^{14}CO_2$ from carboxyl-labeled histidine was monitored (Recsei and Snell, 1970).

Protein concentrations were determined from the absorbancy of histidine decarboxylase at 280 nm ($A_{1cm}^{1\%}$, 16.2; Riley and Snell, 1968) assuming a molecular weight of 190 000 (Chang and Snell, 1968b).

Synthesis of L-[^{14}C]Histidine Methyl Ester. L-Histidine hydrochloride monohydrate (83 mg) was suspended with 50 μCi of L-[U-^{14}C]histidine (256 mCi/mol) in 50 ml of dry, redistilled methanol and esterified by saturation of the reaction mixture with a stream of dry HCl (Greenstein and Winitz, 1961). After 4 h at room temperature under anhydrous conditions, the solution was evaporated under reduced pressure

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at 40 °C and repeatedly concentrated to dryness from methanol to remove excess HCl. To determine the specific activity of the resulting methyl ester, a sample of the dry residue was hydrolyzed at pH 12 for 90 min. A portion of the hydrolysate was then chromatographed on the short column of the amino acid analyzer (Spackman et al., 1958) which was attached to a Nuclear-Chicago flow cell. The yield of ester, as determined by amino acid analysis before and after treatment with alkali, was 98%. Two samples of the ester prepared by this general method and used in these studies had specific activities of 2.5×10^5 and 3.3×10^5 dpm/μmol, respectively.

L-Histidine [^{14}C]methyl ester was prepared by a slight modification of the above procedure. L-Histidine hydrochloride monohydrate (2.7 mg), suspended in 1 ml of dry methanol containing 0.5 mCi of [^{14}C]methanol (3.2 mCi/mmol), dissolved when dry hydrogen chloride was bubbled through the mixture. The reaction vessel was fitted with a $CaCl_2$ drying tube and incubated at 37 °C for 16 h. The methanol was then evaporated under N_2 and traces of HCl were removed by repeated lyophilization from dry methanol. The specific activity of the product was determined by dissolving the dry residue in 1 ml of methanol and counting suitable aliquots. Another aliquot was hydrolyzed and analyzed for histidine as described earlier. A nearly quantitative yield of L-histidine [^{14}C]methyl ester (specific activity 6.4×10^4 dpm/μmol) was obtained.

Sulfhydryl Determinations. Free sulfhydryl groups in histidine decarboxylase were determined by titration with DTNB[1] as described by Lane and Snell (1976). A solution (0.15 ml) of native or modified enzyme in 10 mM potassium phosphate–0.5 mM EDTA (pH 6.0) was added to 0.85 ml of 0.1 M potassium phosphate–1 mM EDTA–0.5 mM DTNB (pH 8.0) to give a final protein concentration of approximately 0.5 mg/ml. The increase in absorbancy at 412 nm was complete within 20 min at 25 °C; the SH titer was calculated using a molar extinction coefficient of 13 600 (Ellman, 1959).

Kinetics of Inactivation. Appropriate amounts of a freshly prepared solution (20 mM) of L-histidine methyl ester dihydrochloride in 0.2 M ammonium acetate (pH 4.8) were added to solutions of histidine decarboxylase (1.14 mg/ml) in the same buffer at 25 °C. At various time intervals, aliquots (50 μl) of the reaction mixture were withdrawn, diluted 40-fold with 0.2 M ammonium acetate–0.1% bovine serum albumin (pH 4.8) at 0 °C, and assayed for decarboxylase activity within 30 min. Samples (50 μl) from a control mixture lacking histidine methyl ester were removed at zero time and after the last sample was withdrawn from the incubation mixture. Apparent first-order rate constants (k_{obsd}) were calculated by least-squares analysis from the slopes of plots of ln E/E_0 vs. time where E_0 and E are activities observed at zero time and times t, respectively. The half-life for inactivation, $t_{0.5}$, was calculated from eq 1.

$$t_{0.5} = 0.693/k_{obsd} \quad (1)$$

Incorporation of Radioactivity. Histidine decarboxylase (2–6 mg/ml) was incubated in 0.2 M ammonium acetate (pH 4.8) containing L-histidine methyl ester (1 to 5 mM labeled in either the histidine or the methyl ester moiety) for 30–120 min at 25 °C. The reaction was quenched by quantitative transfer of the incubation mixture (0.5 to 1.0 ml) to centrifuge tubes containing 8 ml of 80% ammonium sulfate buffered with 0.2 M ammonium acetate (pH 5.2) at 0 °C. Precipitation occurred immediately. After 30 min at 0 °C, the precipitated protein was removed by centrifugation at 4 °C and washed twice with 5 ml of the ammonium sulfate solution at 0 °C, and the washed precipitate was dissolved in cold 10 mM potassium phosphate–0.5 mM EDTA (pH 6.0) to a final concentration of 3–4 mg/ml. Recovery of protein by this procedure was 80–90%. The derivatized protein was then dialyzed against the same buffer at 4 °C to constant specific radioactivity. The dialyzed solution was used to determine protein content, decarboxylase activity, and DTNB-titratable SH groups as described earlier. A sample of native enzyme was subjected to the same procedure as a control. Radioactivity of protein solutions (0.1–0.3 ml) was measured in a Nuclear-Chicago Unilux II liquid scintillation spectrometer using 15 ml of Bray's solution (Bray, 1960). Counting efficiency, determined by the use of [^{14}C]toluene as an internal standard, was routinely 78–80%.

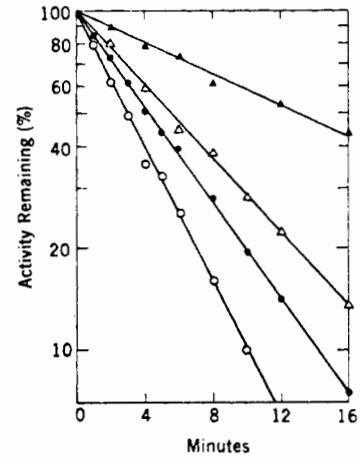

FIGURE 1: Pseudo-first-order plots of the inactivation of histidine decarboxylase at pH 4.8 and 25 °C by (▲) 0.2, (△) 0.6, (●) 1.2, and (○) 4.0 mM L-histidine methyl ester. Experimental details are outlined in Methods.

Results

Kinetics of inactivation of histidine decarboxylase by L-histidine methyl ester at pH 4.8 and 25 °C is presented in Figure 1. Loss of decarboxylase activity is first-order in active enzyme remaining with a rate constant that depends on the concentration of the histidine ester. Pseudo-first-order kinetics are obeyed to at least 90% inactivation; further incubation under these conditions results in complete inactivation. The inhibition is not reversed upon extensive (100-fold) dilution with or without overnight dialysis against 0.2 M ammonium acetate (pH 4.8) or 0.01 M potassium phosphate (pH 6.0) at 4 °C. The apparent first-order rate constant for inactivation (k_{obsd}) was found to approach a maximal value as the inactivator concentration was increased. This result is that expected if enzyme (E) and inactivator (I) form a reversible complex (EI) as an obligatory intermediate preceding the irreversible formation of inactive enzyme (E_{inact} defined in eq 2).

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E_{inact} \quad (2)$$

$$K_{inact} = [E][I]/[EI] \quad (3)$$

According to this mechanism, the dependence of k_{obsd} on inactivator concentration is represented by eq 4 (Kitz and Wilson, 1962; Fahrney and Gold, 1963; Meloche, 1967).

$$k_{obsd} = \frac{k_2}{1 + K_{inact}/[I]} \quad (4)$$

[1] The following abbreviations are used: ImPr, β(4,5)imidazole propionate; HME, L-histidine methyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid.

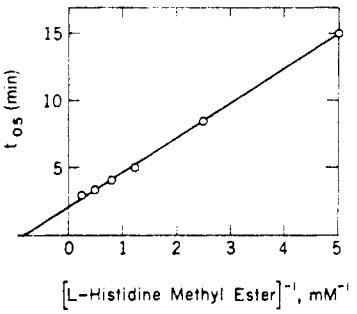

FIGURE 2: Inactivation half-life ($t_{0.5}$) of histidine decarboxylase as a function of the reciprocal of the concentration of L-histidine methyl ester. Values of $t_{0.5}$ were calculated as described in the text from plots such as those shown in Figure 1.

TABLE I: Effect of Substrate Analogues on the Inactivation Rate of Histidine Decarboxylase by L-Histidine Methyl Ester.[a]

Competitive Inhibitor Added	Inactivation Half-Life, $t_{0.5}$ (min)
None	4.2
Imidazole propionate, 0.01 M	125
Imidazole, 0.01 M	77
L-Histidinol, 0.01 M	75
Histamine, 0.1 M	39

[a] Histidine decarboxylase (1.1 mg/ml) was incubated at 25 °C in 0.2 M ammonium acetate (pH 4.8) with 1.2 mM L-histidine methyl ester. Substrate analogues were added as indicated at the beginning of the incubation period. Aliquots (50 μl) were withdrawn periodically, diluted 40-fold with 0.2 M ammonium acetate–0.1% bovine serum albumin (pH 4.8) at 0 °C, and then assayed for decarboxylase activity.

Equation 4 may be rearranged to give a linear rate expression corresponding to eq 5 (Meloche, 1967)

$$t_{0.5} = (1/[I])(TK_{inact}) + T \quad (5)$$

where $t_{0.5}$ is the time required for 50% inactivation (the half-life for inactivation), T is the minimum half-life at infinite inactivator concentration, and $K_{inact} = (k_{-1} + k_2)/k_1$ and represents the inactivator concentration giving the half-maximal rate of inactivation. Equation 5 predicts that $t_{0.5}$ should be a linear function of the reciprocal of the concentration of histidine methyl ester with the ordinate intercept at T. When the calculated half-life is plotted according to eq 5, a straight line is obtained (Figure 2) extrapolating to a minimum inactivation half-life of 2 min. The most rapid actual experimental observation, 3.0 min, was made with 4 mM L-histidine methyl ester. T was converted into k_2, the first-order rate constant for the reaction,

$$EI \xrightarrow{k_2} E_{inact} \quad (6)$$

by the use of eq 7

$$k_2 = (\ln 2)/T \quad (7)$$

to yield a value of 0.346 min^{-1} at pH 4.8 and 25 °C. From the slope of the line shown in Figure 2, a K_{inact} of 1.2 mM for L-histidine methyl ester was calculated, a value rather close to the K_m for L-histidine (0.4–0.9 mM) at this pH (Rosenthaler et al., 1965; Recsei and Snell, 1970); this result may indicate that the ester has an affinity for the histidine-binding site of histidine decarboxylase.

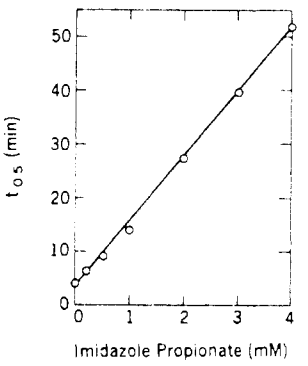

FIGURE 3: Inactivation half-life ($t_{0.5}$) of histidine decarboxylase (pH 4.8, 25 °C) as a function of imidazole propionate concentration in the presence of 1.2 mM L-histidine methyl ester.

Protection against Inactivation by Substrate Analogues. Further evidence for active-site modification of the decarboxylase by histidine methyl ester is provided by the markedly reduced rates of inactivation observed in the presence of several competitive inhibitors of the decarboxylase (Table I). For example, inclusion in the reaction mixture of 10 mM imidazole propionate increases the half-life for inactivation by 1.2 mM L-histidine methyl ester approximately 30-fold. The effectiveness of these competitive inhibitors in preventing inactivation by histidine methyl ester falls in the same order as their affinities for the active site of histidine decarboxylase, as indicated by their K_I values (Rosenthaler et al., 1965; Recsei and Snell, 1970).

If the binding of L-histidine methyl ester (I) to histidine decarboxylase is mutually exclusive with that of a competitive inhibitor (I′) in the reaction scheme of eq 2, the apparent first-order rate constant for inactivation (k_{obsd}) should vary according to the relationship

$$k_{obsd} = \frac{k_2}{1 + \frac{K_{inact}(1 + [I']/K_i)}{[I]}} \quad (8)$$

where K_i is the dissociation constant of the EI′ complex. The linear form of this expression (eq 9; Meloche, 1967)

$$t_{0.5} = T[I']\frac{K_{inact}}{K_i[I]} + \left(T + T\frac{K_{inact}}{[I]}\right) \quad (9)$$

indicates that $t_{0.5}$ should correlate in a linear manner with increasing concentrations of imidazole propionate at a constant level of histidine methyl ester. The pseudo-first-order loss of decarboxylase activity observed with 1.2 mM L-histidine methyl ester was found to be progressively reduced over an eightfold increase in imidazole propionate concentration. A plot of the calculated half-life of inactivation ($t_{0.5}$) as a function of imidazole propionate concentration (Figure 3) describes a straight line with an ordinate intercept of 4 min. This is in excellent agreement with the value of $t_{0.5}$ (4.2 min) obtained in the presence of the same concentration of L-histidine methyl ester and in the absence of the competitive inhibitor (Table I), as well as with the intercept (4.0 min) calculated from eq 9. The slope of the line is 12.0, from which a value of 0.17 mM for the dissociation constant of the enzyme–imidazole propionate complex was computed. These kinetic results, coupled with the observations (reported below) that imidazole propionate prevents incorporation of ^{14}C from [^{14}C]histidine methyl ester, thus provide convincing evidence that modification of histidine decarboxylase by the histidine ester requires an unoccupied active site.

TABLE II: Effect of Imidazole Propionate upon the Extent of Reagent Incorporation, SH Modification, and Inactivation of Histidine Decarboxylase by L-[^{14}C]Histidine Methyl Ester (HME).[a]

Additions to Enzyme	Act. Remaining (%)	Radioact. Bound (dpm/mg of Protein)	mol of ^{14}C/ mol of Enzyme	Titratable SH Groups per mol of Enzyme		SH Groups Modified per mol of Enzyme
				Control Enzyme	HME-Treated Enzyme[a]	
1. HME[b]	0	4082	3.09[f]	4.91	1.80	3.11
2. HME[b] + ImPr[c]	99	122	0.09	4.91	4.81	0.10
3. HME[b]	0	3648	2.77[f]	4.90	2.03	2.87
4. HME[d]	2	5092	2.94[f]	5.02	2.02	3.00
5. HME[d] + ImPr[c]	92	664	0.38	5.02	4.61	0.41
6. HME[b,e]	0	4430	3.36[f]	5.00	2.01	2.99

[a] Histidine decarboxylase (three different preparations) was inactivated with 1.2 mM L-[^{14}C]histidine methyl ester (HME) at various times and the resulting derivatives were analyzed as described in Methods. [b] The specific radioactivity of the L-[^{14}C]histidine methyl ester used was 2.5×10^5 dpm/μmol. [c] Imidazole propionate (50 mM) was included in the reaction mixture. [d] The specific radioactivity of L-[^{14}C]histidine methyl ester used in these experiments was 3.3×10^5 dpm/μmol. [e] The modification reaction was carried out in 0.1 M potassium phosphate (pH 8.0). [f] Calculated on the basis of moles of enzyme subjected to inactivation.

TABLE III: Release of Label from Histidine Decarboxylase Modified with L-[^{14}C]Histidine Methyl Ester.[a]

Treatment	Radioact. Bound (dpm/mg of Protein)	mol of ^{14}C/ mol of Enzyme	Radioact. Released (%)
None	4080	3.09	0
50 mM potassium[d] phosphate (pH 7.0)	4085	3.10	0
0.1 M sodium maleate (pH 6.6)[c]	3478	2.64	15
0.1 N NaOH[b]	154	0.12	96
1 M NH_2OH (pH 9)[b]	77	0.06	98
0.1 M $NaBH_4$ (pH 7)[e]	40	0.03	99

[a] Histidine decarboxylase which had been completely inactivated with L-[^{14}C]histidine methyl ester (2.5×10^5 dpm/μmol) was incubated at a concentration of 3.5 mg/ml under the conditions specified and then dialyzed for 60 h against 2000 volumes of 10 mM potassium phosphate–0.5 mM EDTA (pH 5.9) at 4 °C with three changes of dialysate. The dialyzed solutions were then used for radioactivity measurements. [b] Incubation was carried out for 12 h at 25 °C. [c] Incubation was carried out for 24 h at 25 °C. [d] Incubation was carried out for 48 h at 4 °C. [e] In this experiment, the inactivated, labeled histidine decarboxylase (1.3 mg) in 0.4 ml of 50 mM potassium phosphate (pH 7.0) was treated with four 25-μl portions of a 0.5 M aqueous solution of $NaBH_4$ over a 15-min period at 25 °C. Following incubation for an additional 60 min at 25 °C, the solution was dialyzed as described in footnote *a*.

Incorporation of L-[^{14}C]Histidine Methyl Ester and SH Group Modification. Inactivation of histidine decarboxylase by labeled L-histidine methyl ester is accompanied by incorporation of radioactivity into the protein molecule. Enzyme that has been completely inactivated at pH 4.8 contains an average of 2.93 ± 0.16 equiv of [^{14}C]histidine/mol of protein (Table II, experiments 1, 3, and 4), and this value remains constant even on prolonged (24 h) exposure of the decarboxylase to 4 mM ester. Radioactivity is not lost from the protein during extensive dialysis against phosphate buffer (pH 6) at 4 °C, or by dilution into 3 M guanidine–0.2 M ammonium acetate at pH 4.6 followed by dialysis against water, and remains associated with protein through several ammonium sulfate precipitations. The degree of ^{14}C incorporation correlates well with the observed loss of 2.99 ± 0.12 sulfhydryl groups during inactivation of the decarboxylase (Table II, experiments 1, 3, and 4). When the modification reaction is carried out in the presence of a saturating level of imidazole propionate (Table II, experiments 2 and 5), inactivation is largely prevented, negligible radioactivity is stably bound to the protein, and essentially no decrease in the SH content occurs. Inactivation of histidine decarboxylase by histidine methyl ester thus renders inaccessible to DTNB titration three of the five essential, active-site SH groups.

These five SH groups are not titrated by DTNB at pH 4.8, where these inactivation experiments were carried out, but are titrated by DTNB at alkaline pH, with complete loss of enzyme activity (Lane and Snell, 1976). Table II also shows (experiment 6) that no increase in isotope incorporation occurs when histidine decarboxylase is inactivated with L-[^{14}C]histidine methyl ester under conditions (pH 8.0, 25 °C) such that these five cysteinyl residues react with DTNB; an average of 3 equiv of ^{14}C are again introduced into the completely inactivated decarboxylase with concomitant loss of three of the five titratable SH groups.

Stability of L-[^{14}C]Histidine Methyl Ester Labeled Enzyme. Histidine decarboxylase contains ten histidine residues/190 000 daltons (Chang and Snell, 1968b; Riley and Snell, 1970). When acid hydrolysates (6 N HCl, 110 °C, 24 h) of native and histidine methyl ester inactivated decarboxylase were analyzed, three additional histidyl residues were detected in the inactivated samples. Together with the stoichiometry of [^{14}C]histidine incorporation and the reduction in SH titer of modified enzyme (Table II), these data suggested that interaction of ester and decarboxylase might occur by acylation of the thiol group of cysteine to form a histidyl thioester. The stability of the derivatized enzyme is in accord with this supposition (Table III). Thus, no radioactivity is released from the derivatized decarboxylase at neutral pH and 4 °C over a 48-h period and 85% remains associated with the protein following incubation in pH 6.6 buffer for 24 h at 25 °C. In contrast, virtually all of the label is liberated by overnight incubation in either 0.1 N NaOH or 1 M hydroxylamine (pH 9) at room temperature. Moreover, treatment of the radioactive enzyme derivative with 0.1 M sodium borohydride (pH 7, 25 °C) results in the quantitative release of isotope from the protein. The latter result was also obtained when inactivation of the enzyme with [^{14}C]histidine methyl ester and subsequent

reduction with excess borohydride were conducted at the optimum pH (pH 4.8) of the enzyme.

Recsei and Snell (1970) previously demonstrated by $NaBH_4$ trapping experiments that the functional pyruvoyl residue of histidine decarboxylase undergoes Shiff-base formation with substrate L-histidine as part of the catalytic cycle. The foregoing results argue against a similar interaction of histidine decarboxylase with histidine methyl ester for, if a Schiff base were formed, it should be reduced by borohydride and ^{14}C would remain fixed to the protein. On the other hand, if ester inactivation results in formation of a histidyl thioester, borohydride reduction of the adduct might be expected to release the histidine moiety as [^{14}C]histidinol (Hersh and Jencks, 1967; Solomon and Jencks, 1969). Despite repeated atternpts, we have been unable to demonstrate formation of this product. Thus the evidence for thioester formation, although suggestive, is not conclusive.

Incorporation of ^{14}C from L-Histidine [^{14}C]Methyl Ester. If inactivation of histidine decarboxylase by L-histidine methyl ester occurs exclusively as a result of acylation of thiol groups, inactivation with histidine methyl ester labeled in the methoxyl group should result in the formation of an enzymatically inactive but unlabeled protein derivative. The data of Table IV show that 2.13 ± 0.12 equiv of ^{14}C are stably bound per mol of inactivated enzyme (experiments 1-3) and this incorporation also is prevented by inclusion of imidazole propionate in the reaction mixture (experiment 4). Incubation of histidine decarboxylase in the presence of 10 mM [^{14}C]methanol (experiment 5) elicits no change in enzymatic activity and negligible incorporation of label. The data thus indicate that histidine methyl ester reacts with the enzyme in two different ways, both of which result in loss of activity, but which lead to different incorporations of the histidyl and methoxyl moieties of the ester. Treatment of the L-histidine [^{14}C]methyl ester labeled protein with either 0.1 N NaOH or 1 M NH_2OH (pH 9) for 12 h results in the quantitative release of radioactivity whereas only 15% of the label is liberated upon incubation of the enzyme derivative for 24 h at pH 6.6. Thus release of label was effected by the same reagents and to the same extent as when label was incorporated from L-[^{14}C]histidine methyl ester.

TABLE IV: Isotope Incorporation upon Reaction of Histidine Decarboxylase with L-Histidine [^{14}C]Methyl Ester or [^{14}C] Methanol in the Presence or Absence of Imidazole Propionate.[a]

Expt	Act. Remaining (%)	Radioact. Bound (dpm/mg of Protein)	mol of ^{14}C/ mol of Enzyme
1. HME	1	742	2.20
2. HME	0	656	1.95
3. HME	0	753	2.23
4. HME + ImPr[b]	88	150	0.46
5. [^{14}C]CH_3OH[c]	100	26	0.13

[a] Histidine decarboxylase (three different preparations) was inactivated with L-histidine [^{14}C]methyl ester (6.4×10^4 dpm/μmol) and the resulting derivatives were analyzed as described in Methods. [b] Imidazole propionate (50 mM) was included in the reaction mixture. [c] Histidine decarboxylase (5.0 mg/ml) was incubated in 0.2 M ammonium acetate (pH 4.8) with 10 mM [^{14}C]CH_3OH (3.7×10^4 dpm/μmol) for 60 min at 25 °C.

Discussion

The data presented here show that histidine decarboxylase from *Lactobacillus* 30a is rapidly and irreversibly inactivated by the substrate analogue, L-histidine methyl ester. Several lines of evidence indicate that this inactivation results from modification of the active-site region of the enzyme: (1) decarboxylase activity is completely abolished; (2) inactivation follows pseudo-first-order kinetics in the presenc of an excess of histidine ester; (3) the rate of inactivation obeys saturation kinetics implying reversible binding of the inactivator at the active site prior to an irreversible binding step; (4) competitive inhibitors of histidine decarboxylase protect against inactivation; and (5) the only competitive inhibitor studied in detail, imidazole propionate, competes with L-histidine methyl ester for the same site (or sites) on the protein. The close agreement between the extent of histidine incorporation and the loss of DTNB-titratable SH groups during inactivation could be interpreted to indicate either unusually strong noncovalent interaction of histidine methyl ester with these active-site SH groups, or covalent modification of the cysteinyl residues by the inhibitor. If noncovalent interaction occurs exclusively, incorporation of ^{14}C from the separately labeled histidine and methyl moieties of histidine methyl ester should be equivalent, and this was not observed. This unequal binding of the two portions of the inhibitor must reflect *some* covalent interaction with the enzyme protein. A nucleophilic attack of the reactive SH groups of the protein on the carboxyl ester carbon of histidine methyl ester to form the corresponding thioester containing the histidyl group is readily visualized, and the stability characteristic of the product (stability in neutral or weakly acidic solutions, lability toward alkali and hydroxylamine, and to borohydride reduction) is characteristic of acyl thiols (Jaenicke and Lynen, 1960; Hersch and Jencks, 1967). However, exclusive interaction by this route does not account for the binding of ^{14}C from the ester moiety of the inhibitor, or for the failure to detect free [^{14}C]histidinol following borohydride reduction of the appropriate labeled, inactivated enzyme. Thus more than one mode of covalent interaction or a mixed (covalent and noncovalent) mode of interaction must be invoked to explain the labeling results. The binding during inactivation of three histidine residues and two methoxyl residues at the active center of an enzyme with five catalytic sites (and five reactive thiol groups) may be purely fortuitous, or may reflect the occurrence of two mutually exclusive modes of inactivation that together involve all five of the active sites of the enzyme.

Histidine amide is almost as effective an inhibitor of the histidine decarboxylase from *Micrococcus* sp. n. as histidine methyl ester (Mardeshev and Gonchar, 1968). Recsei and Snell (unpublished) found (1) that this was also true for the enzyme from *Lactobacillus* 30a; (2) that binding of this inhibitor was not accompanied by the release of NH_3; and (3) that partial reactivation of the enzyme followed long periods of dialysis. These findings lend credence to the notion that an unusually strong noncovalent binding may suffice to explain the almost irreversible inactivation brought about by histidine amide, and may contribute importantly to inactivation by histidine methyl ester as wel.

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Purification of β-Hydroxy-β-methylglutaryl-coenzyme A Reductase from Yeast†

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ABSTRACT: β-Hydroxy-β-methylglutaryl-coenzyme A reductase of yeast has been solubilized by two different methods and then purified approximately 5000-fold. The purified enzyme shows a single precipitin band on immunodiffusion, and it moves as a single band of protein and enzyme activity on gel filtration and diethylaminoethylcellulose column chromatography. It also shows one major band on polyacrylamide gel electrophoresis. The specific activity of the pure enzyme is 18 000 to 22 000 nmol of reduced nicotinamide adenine dinucleotide phosphate oxidized per min per mg of protein. The molecular weights of the enzyme, estimated by gel filtration, and the subunits, determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, are 2.6×10^5 and 6.0×10^4, respectively.

β-Hydroxy-β-methylglutaryl-coenzyme A reductase (mevalonate:$NADP^+$ oxidoreductase (acetylating coenzyme A), EC 1.1.1.34) has generated a great deal of interest, since it is the rate-limiting enzyme of hepatic cholesterol biosynthesis. This enzyme catalyzes the reduction of D-HMG-CoA[1] by NADPH according to the following equation:

$$\text{HMG-CoA} + 2\ \text{NADPH} + 2\text{H}^+ \rightarrow \text{MVA} + 2\ \text{NADP}^+ + \text{CoASH}$$

Mammalia hepatic HMG-CoA reductase is localized in the endoplasmic reticulum, whereas in yeast this enzyme is in the mitochondria (Shimizu et al., 1973). Yeast reductase was solubilized in earlier studies (Durr and Rudney, 1960) by prolonged autolysis and then purified (Kirtley and Rudney, 1967) approximately 200-fold to a specific activity of 1400 nmol of NADPH oxidized per min per mg of protein. This partially purified enzyme was very unstable.

Rat liver microsomal reductase has also been solubilized and partially purified. The various methods used for the solubilization and partial purification of the rat liver enzyme have been reviewed by Dugan and Porter (1976). The activities reported for this enzyme are much less than the activity reported by Kirtley and Rudney (1967) for the yeast enzyme.

HMG-CoA reductase has been induced in *Pseudomonas* by growing the organism on mevalonic acid (Bensch and Rodwell, 1970). The induced bacterial enzyme differs from the naturally occurring reductases of yeast and mammalian systems in that it utilizes NADH instead of NADPH as the reducing agent. The *Pseudomonas* enzyme was purified to a specific activity of 56 000 nmol of NADH oxidized per min per mg of protein.

In the present investigation, we have used two different methods of solubilization of the yeast HMG-CoA reductase. These are autolysis of dry yeast and sonication of mitochondria obtained through disruption of cells of frozen cakes of fresh yeast. The enzyme in each of these solubilized preparations has been purified to homogeneity, or near homogeneity, by classical techniques and affinity chromatography. This is the first

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[1] Abbreviations used are: HMG-CoA, β-hydroxy-β-methylglutaryl-coenzyme A; MVA, mevalonic acid; NADH, reduced nicotinamide adenine dinucleotide; NADPH, NADH phosphate; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid.