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Prohistidine Decarboxylase from *Lactobacillus 30a*. A New Type of Zymogen†

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ABSTRACT: Prohistidine decarboxylase has been purified from a histidine decarboxylaseless mutant of *Lactobacillus 30a* that was observed to synthesize large quantities of a protein that cross-reacted with antibodies to wild-type enzyme. Within experimental error the proenzyme is indistinguishable from the active wild-type enzyme in sedimentation coefficient, diffusion rate in agar gels, electrophoretic mobility in acrylamide gels, and amino acid composition. Unlike the active enzyme it contains only one type of subunit of molecular weight near 37,000 and does not contain the functionally essential pyruvoyl residues found in wild-type enzyme. The proenzyme has only slight or no decarboxylase activity, but is converted to fully active enzyme by incubation at 37° near pH 7.6 over a period of 24–48 hr. The rate-limiting step of activation is first order with respect to proenzyme and total protein, and is therefore either nonenzymatic or autocatalytic. During activation each proenzyme subunit gives rise to two smaller subunits with generation of an NH_2-terminal pyruvoyl group from an internal serine residue adjacent to the point of cleavage of the proenzyme. The activated proenzyme is indistinguishable from wild-type enzyme in molecular weight, electrophoretic properties, diffusion rate in agar and immunological properties as revealed by Ouchterlony analysis, and in subunit structure as revealed by acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by end-group analysis. The nature of the reactions that occur during activation of the proenzyme is unknown.

Histidine decarboxylase (histidine carboxy-lyase, EC 4.1.1.*x*)[1] from *Lactobacillus 30a* is a decameric enzyme composed of five subunits of mol wt 28,000–29,000 and five subunits of mol wt 9000 (Riley and Snell, 1970). Pyruvate is bound in amide linkage at the NH_2 terminus of each of the five larger subunits (Riley and Snell, 1968, 1970) and appears to function as the prosthetic group of the enzyme by undergoing Schiff base formation with the substrate L-histidine (Recsei and Snell, 1970). Riley and Snell (1970) have shown that enzyme-bound pyruvate is derived from serine and does not equilibrate with pyruvate formed as an intermediate in the fermentation of carbohydrate. The work to be described here deals further with the route of synthesis of this unusual enzyme.

Materials and Methods

Ouchterlony Analysis. Double diffusion was carried out in 20 × 60 mm petri dishes each containing 5 ml of 1% ionagar (Difco). Rabbit antiserum to wild-type histidine decarboxylase was prepared by Dr. George Chang as follows. Homogeneous wild-type histidine decarboxylase (7.2 mg), dissolved in 0.4 ml of 0.15 M sodium chloride, 1.1 ml of isotris buffer (140 mM sodium chloride, 10 mM Tris, 0.5 mM magnesium sulfate, and 0.15 mM calcium chloride) (pH 7.5), and 1.5 ml of Freund's adjuvant, was emulsified and injected into a rabbit intradermally. One month later 40 ml of blood was taken, and the rabbit was injected intravenously with 3 mg of histidine decarboxylase in 3 ml of isotris buffer. Blood (40 ml) was taken from the rabbit 1 week later, allowed to clot at 23°, and then stored at 4° for 24 hr. The serum was decanted, centrifuged at 10,000g for 10 min, and stored at −20°.

Ultracentrifugation. Sedimentation velocity and short-column sedimentation equilibrium measurements were made at 280 nm using 12-mm double-sector cells and a Beckman

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[1] Histidine decarboxylase has been listed by the Enzyme Commission as EC 4.1.1.22; however, this enzyme is defined as a pyridoxal 5′-phosphate protein. While mammalian histidine decarboxylases apparently conform to this definition, the bacterial enzyme described here does not and should be assigned a new number.

Model E analytical ultracentrifuge equipped with a photoelectric scanner and absorption optical system capable of multiplex operation. A titanium rotor was used for sedimentation velocity experiments. Fluorocarbon FC43 (0.01 ml) was added to each compartment of the double-sector cell for sedimentation equilibrium runs. Solution and solvent volumes were 0.10 ml.

Polyacrylamide Disc Gel Electrophoresis. Proteins were subjected to polyacrylamide disc gel electrophoresis following the general method of Davis (1964) except that spacer and sample gels were omitted. Gels (10 × 0.6 cm) containing 5% acrylamide (Matheson) and 0.13% *N,N'*-methylenebisacrylamide (Eastman) in 0.075 M sodium phosphate buffer (pH 7.2) were run for 2 hr at 10 mA/gel. They were then fixed in 12% trichloroacetic acid for 30 min, stained with 0.05% Coomassie Brilliant Blue (Mann) in 12% trichloroacetic acid for 1 hr, and stored in 10% trichloroacetic acid.

Sodium Dodecyl Sulfate Disc Gel Electrophoresis. The proteins used as standards in sodium dodecyl sulfate disc gel electrophoresis were all Worthington products except pepsin, which was purchased from Nutritional Biochemicals Corp. The procedures of Weber and Osborn (1969) were used with the following modifications. Protein samples (10 μg each) were incubated for 2 hr at 37° in 0.04 M ammonium acetate (pH 4.8) containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol and then dialyzed against 0.01 M sodium phosphate (pH 7.0) containing 0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol. Gel buffer was 0.1 M sodium phosphate (pH 7.0) containing 0.2% sodium dodecyl sulfate. Gels (10 × 0.6 cm) containing 10% acrylamide and 0.26% *N,N'*-methylenebisacrylamide were run for 3 hr at 8 mA/gel and stained for 3 hr in a solution of 0.12% Coomassie Brilliant Blue in 45% methanol–9% acetic acid. The gels were destained in 45% methanol–9% acetic acid overnight and then in 5% methanol–7% acetic acid for about 8 hr. They were stored in 7% acetic acid.

Amino acid analyses were performed on a Beckman 120C automatic amino acid analyzer by the method of Spackman *et al.* (1958) as modified by Hubbard (1965). Protein samples (0.41–0.44 mg) were hydrolyzed in 2 ml of 6 N hydrochloric acid in sealed, evacuated tubes at 105° for 26 hr. The hydrolysates were then dried under vacuum, dissolved in 1.5 ml of 0.2 M sodium citrate buffer (pH 2.2) and analyzed.

Amino-Terminal Residues. Protein samples (4–5 mg in 1 ml) were dialyzed against 5 M guanidine hydrochloride at 23° for 1 hr. Sodium bicarbonate (40 mg) and 0.1 ml of 1-fluoro-2,4-dinitrobenzene (Eastman) were added to the protein solutions and the mixtures were shaken in the dark at 23° for 2 hr. Then 0.25 ml of 6 N hydrochloric acid was added to each sample and the resulting suspension was extracted eight times with 3-ml portions of benzene to remove unreacted reagent. The dinitrophenyl protein was precipitated by adding 10 ml of ethanol to the solution at 0°. The precipitate was collected by centrifugation, washed with absolute ethanol followed by anhydrous peroxide-free ether, and air-dried. Dinitrophenyl protein was hydrolyzed in 6 N hydrochloric acid in sealed, evacuated tubes at 105° for 4 hr. The dinitrophenylamino acids released were examined as described by Fraenkel-Conrat *et al.* (1955).

Carboxyl-Terminal Residues. Protein samples were first dialyzed against 0.04 M ammonium acetate (pH 4.8) containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol for 2 hr at 37° and then against 0.1 M sodium phosphate (pH 7.0) containing 0.1% sodium dodecyl sulfate for 2 hr at 23°. In each case, 2.4 mg of protein and 0.10 mg of DFP-carboxypeptidase A (Worthington, treated by method two of Ambler (1967)) were incubated at 23° in a final volume of 1 ml. At various times, 0.10-ml samples were mixed with an equal volume of ice-cold 10% trichloroacetic acid, then passed through Millipore filters (0.45 μ pore size). The precipitates were washed several times with ice-cold 5% trichloroacetic acid. The filtrates were dried under vacuum, and the resulting residues were dissolved in 0.2 ml of 0.2 M sodium citrate buffer (pH 2.2) for application to the long column of the amino acid analyzer.

Pyruvic Acid Assay. A slight modification of the assay described by Weiner *et al.* (1966) was used. Protein samples (2.0 mg) were heated with 3 N hydrochloric acid for 1 hr in a boiling-water bath. The pH was then adjusted to 7.5 with concentrated sodium hydroxide. The pyruvate content of the samples was then determined spectrophotometrically following addition of NADH and lactate dehydrogenase in the usual way.

Other Methods. The manometric assay for histidine decarboxylase activity has been described by Chang and Snell (1968a). Protein concentrations of purified preparations were determined spectrophotometrically at 280 nm, using the extinction coefficient of 16.2 previously determined for histidine decarboxylase (Riley and Snell, 1968). Unless otherwise stated, activated prohistidine decarboxylase, referred to in tables and figures, was prepared by incubating the purified proenzyme (2–10 mg/ml) for 24–48 hr at 37° in 0.2 M potassium phosphate buffer (pH 7.6).

Results

Histidine Decarboxylaseless Mutants of Lactobacillus 30a Synthesize Proteins that Cross-React with Antibodies to Wild-Type Enzyme. We recently described the isolation from nitrosoguanidine-treated *Lactobacillus 30a* of seven mutants whose histidine decarboxylase activities range from less than 0.5% to 5% of the wild-type activity. Because wild-type cells decarboxylate histidine and thus raise the pH in their immediate vicinity while mutant cells do not, the two types of cells develop into colonies of different color when plated onto histidine-enriched agar supplemented with an appropriate pH indicator (Recsei and Snell, 1972). Each of these mutants synthesizes protein that cross-reacts with antibodies to homogeneous wild-type histidine decarboxylase (Figure 1). Mutants 2 and 3 make a cross-reacting protein similar in molecular weight to the wild-type enzyme; mutants 1 and 5 make a lower molecular weight cross-reacting protein; and mutants 4, 6, and 7 make both types of cross-reacting protein. The cross-reacting protein from mutant 3 has been purified and identified as a prohistidine decarboxylase on the basis of its structural resemblance to active enzyme and its conversion to active enzyme *in vitro*.

Purification of Prohistidine Decarboxylase from Mutant 3. Prohistidine decarboxylase was initially purified according to the procedures described earlier (Chang and Snell, 1968a; Riley and Snell, 1968) for the purification of wild-type enzyme. During the attempted crystallization step (in which the pH of the protein solution was adjusted from 4.8 to about 7.5 with concentrated ammonia and finely divided ammonium sulfate was added to about 70% of saturation), a 20-fold increase in histidine decarboxylase activity was observed. This activation was attributed to the increase in pH of the protein solution that is required prior to crystallization. The crystallization step was therefore omitted; instead protein was precipitated from solution at pH 4.8 by adding finely ground ammonium sulfate to 70% of saturation at 0°. The precipitated protein

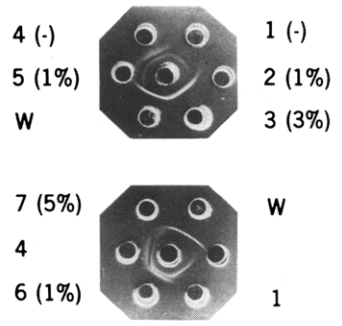

FIGURE 1: Ouchterlony analysis of cell extracts from wild-type (W) and histidine decarboxylaseless mutants 1 to 7 of *Lactobacillus 30a*. Late-log cells were harvested from 200 ml of medium containing 5 mM L-histidine, suspended in 2 ml of 0.05 M Tris buffer (pH 8.5), lysed by passage through the French press at 5000 psi, and a sample was placed in the indicated well of the agar plate. The center wells contained rabbit antiserum to purified wild-type histidine decarboxylase. The numbers in parentheses refer to the histidine decarboxylase activity of acetone-dried mutant cells, expressed as percentage of the wild-type activity. The agar was made up in 0.05 M potassium phosphate (pH 6.5). The plates were photographed after 20 hr at 23°.

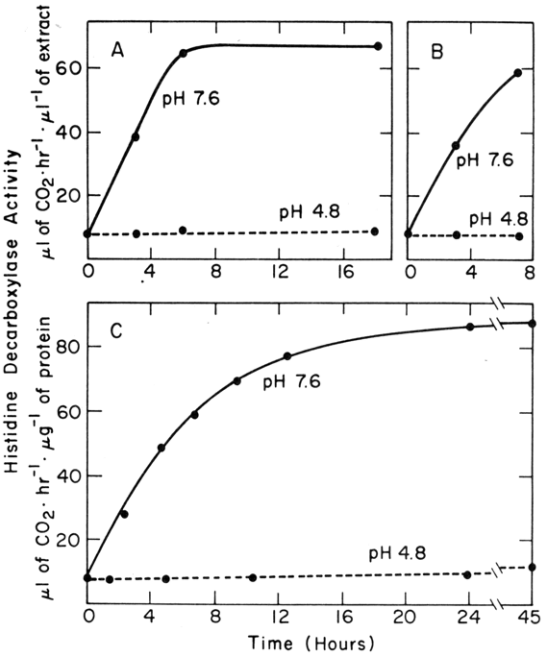

FIGURE 2: Comparative rates of activation of prohistidine decarboxylase from mutant 3 at 37°. (A) Crude extract; (B) fraction from heat step; (C) final purified preparation. Solutions at pH 4.8 were in 0.2 M ammonium acetate buffer; those at pH 7.6 were in 0.2 M potassium phosphate buffer. Activities in part B were ten times those shown at all points.

was collected by centrifugation, redissolved in water, and rerun over the Sephadex column. Active fractions were pooled and stored at 4° either in 0.2 M ammonium acetate buffer (pH 4.8) or in this buffer containing ammonium sulfate (70% of saturation).

The protocol of a typical preparation is shown in Table I. The overall yield of histidine decarboxylase activity (36%) and of protein (134 mg) are similar to that previously reported for wild-type enzyme (Chang and Snell, 1968a), indicating that negligible activation of prohistidine decarboxylase occurred during purification. The specific activity of the freshly purified proenzyme preparation (6400 μl hr^{-1} mg^{-1}) was about 6% that of the homogeneous wild-type enzyme. As shown later, this activity results from the presence of small amounts of active enzyme in the preparation, and is not an intrinsic property of the proenzyme. After four months at pH 4.8 and 4°, the specific activity had increased to 9600 μl hr^{-1} mg^{-1}.

Activation of Prohistidine Decarboxylase. Prohistidine decarboxylase in each of the fractions prepared during purification can be converted to active enzyme by incubation at 37° and pH 7.6. The rates of activation in the crude extract from cells of mutant 3 (Figure 2A), in the fraction from the heat step (Figure 2B), and in the final purified proenzyme preparation (Figure 2C) are similar; the latter preparation increases in specific activity to 90,000 μl hr^{-1} mg^{-1} within 45 hr. Homogeneous wild-type enzyme has a specific activity of 99,000 μl hr^{-1} mg^{-1} (74 IU/mg); hence, judged by this criterion, the purified proenzyme is 90% pure. Activation of the purified proenzyme is first order with respect to proenzyme (Figure 3), with a half-time of 4.5 hr. Separate experiments showed that the initial velocity of activation was also directly proportional to the protein concentration, indicating that the rate-limiting step of activation is also first order with respect to total protein. The pH–rate profile for activation is bell shaped; in 0.2 M potassium phosphate buffer at 37° the pH optimum was 7.6 and the first-order rate constant for activation, calculated as described in Figure 3, was 0.15/hr. This rate constant decreased to 0.12 hr^{-1} at pH 7.1, and to 0.06 hr^{-1} at both pH 6.8 and 8.1. The purified proenzyme activated by incubation at pH 7.6 was incubated at pH 4.8 and 37° for 16 hr. No decrease in histidine decarboxylase activity was observed, indicating that activation of the proenzyme is not reversible with respect to pH. Addition of wild-type cell extract (0.1 ml) or of pyridoxal 5′-phosphate (0.1 mM) plus ferric chloride (0.01 mM) did not increase the rate of activation of the purified proenzyme (3 mg/ml) at either pH 4.8 or 7.6.

Immunologic and Electrophoretic Properties of Prohistidine Decarboxylase. The purified proenzyme, activated proenzyme, and wild-type enzyme are indistinguishable on the basis of

TABLE I: Purification of Prohistidine Decarboxylase from Mutant 3 of *Lactobacillus 30a*.

Fraction	Vol (ml)	Total Act.[b] (μl/hr)	Overall Yield of Act.[b] (%)
1. Cell extract	300[a]	2.4×10^6	(100)
2. Heat treatment	23	1.8×10^6	75
3. First Sephadex G-200 filtration	29	1.1×10^6	46
4. Ammonium sulfate precipitation	4	1.0×10^6	42
5. Second Sephadex G-200 filtration	28	8.6×10^5	36

[a] From 14 g of acetone-dried cells. The procedure followed is that described earlier for wild-type histidine decarboxylase (see text). [b] Before activation. Fraction 5 contained 134 mg of protein of specific activity (μl hr^{-1} mg^{-1}) of 6400. Following activation by the procedure described in Figure 2 this specific activity rises to 90,000, about 90% of that of pure wild-type histidine decarboxylase.

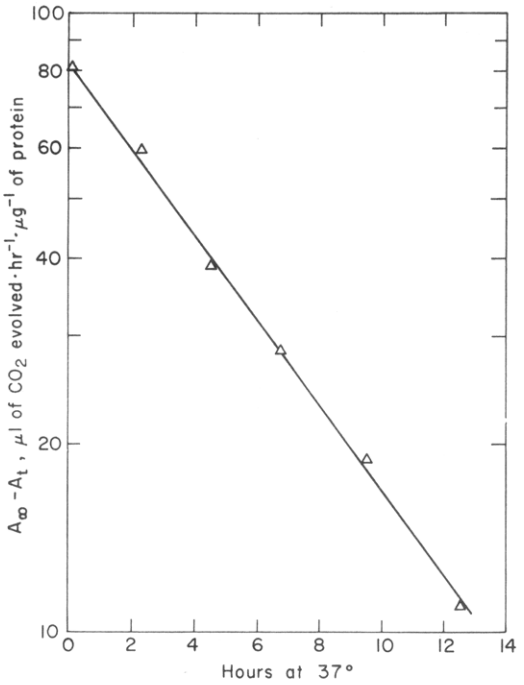

FIGURE 3: Activation of purified prohistidine decarboxylase at pH 7.6 and 37° plotted as a first-order reaction. Maximum activity of the proenzyme (A_∞) was taken as that at 45 hr; A_t is the activity at time t. The half-time of activation (4.5 hr) was read directly from the plot; the rate constant for activation (k) is equal to 0.693 divided by the half-time of activation (Jencks, 1969).

Ouchterlony analysis, which was performed at pH 4.8 to prevent proenzyme activation (Figure 4). In addition, these proteins migrate similarly when subjected to polyacrylamide disc gel electrophoresis at pH 7.2 (Figure 5). The proenzyme preparation contains a trace of contaminating protein(s) that migrates more slowly than the proenzyme; the possibility that it plays a role in the activation of the proenzyme has not been eliminated.

Sedimentation Properties of Prohistidine Decarboxylase. In sedimentation velocity experiments on freshly prepared solutions in 0.05 M ammonium acetate–0.2 M potassium chloride buffer (pH 4.8), proenzyme, activated proenzyme, and wild-type enzyme each sedimented as single, symmetrical peaks with $s_{20,w}$ values of 9.1, 9.4, and 9.2 S, respectively. High-speed sedimentation equilibrium experiments (Yphantis, 1964) were then performed on these same samples. Plots of log [C] *vs.* r^2 were linear for both the activated proenzyme and the wild-type enzyme, and corresponded to molecular weights of 192,000 and 191,000, respectively; however, similar plots for the proenzyme were concave upward indicating that the proenzyme partially dissociated during the 20-hr period of the equilibrium run. Sedimentation velocity measurements made on the same samples following these equilibrium measurements confirmed that the proenzyme had undergone partial dissociation, since it now contained two sedimenting species, one with $s_{20,w}$ of 9.1 S, the other (about 25% of the total protein) with $s_{20,w}$ of 6.7 S. The activated proenzyme and wild-type enzyme had remained monodisperse, with $s_{20,w}$ of 9.1 S in each case. The molecular weight of the proenzyme is assumed to be near 190,000, since the proenzyme and active enzyme have the same sedimentation coefficient.

Subunit Structure of Prohistidine Decarboxylase. Wild-type histidine decarboxylase is a decameric enzyme of mol wt 190,000, and is composed of five subunits of mol wt 28,000–29,000 and five subunits of mol wt 9000 (Riley and Snell, 1970). In contrast, electrophoresis of prohistidine decarboxylase in sodium dodecyl sulfate revealed only one type of subunit (Figure 6). A comparison of migration of this subunit and of several reference proteins (ovalbumin, pepsin, trypsin, chain II of wild-type histidine decarboxylase (Riley and Snell,

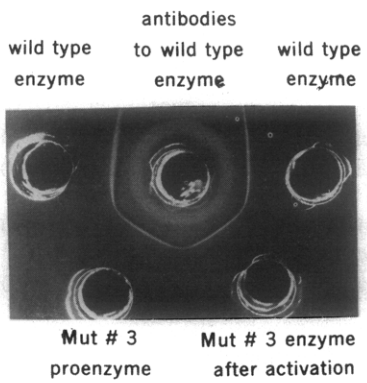

FIGURE 4: Ouchterlony analysis of prohistidine decarboxylase, activated prohistidine decarboxylase, and wild-type histidine decarboxylase at pH 4.8. Each antigen (0.5 mg/ml) was dissolved in 0.2 M ammonium acetate (pH 4.8). The center well contained antiserum to wild-type histidine decarboxylase. The agar was made up in 0.05 M ammonium acetate (pH 4.8). The plate was photographed after 15 hr at 23°.

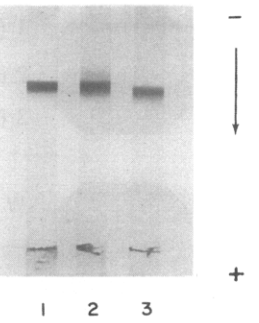

FIGURE 5: Polyacrylamide disc gel electrophoresis of (1) wild-type histidine decarboxylase, (2) prohistidine decarboxylase, and (3) activated prohistidine decarboxylase. About 25 μg of protein in 25 μl of 0.2 M sodium phosphate buffer (pH 7.2) was applied to each gel. Dye marker (Bromophenol Blue) appears at the bottom of each gel.

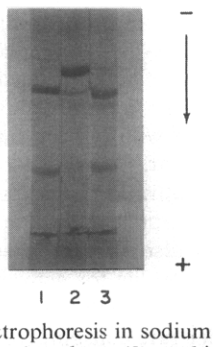

FIGURE 6: Disc gel electrophoresis in sodium dodecyl sulfate of (1) wild-type histidine decarboxylase, (2) prohistidine decarboxylase, and (3) activated prohistidine decarboxylase. About 10 μg of protein in 20 μl of dialysis buffer (see Materials and Methods) was applied per gel. Dye marker (Bromophenol Blue) appears at the bottom of each gel.

TABLE II: Amino Acid Analyses of Prohistidine Decarboxylase and Wild-Type Histidine Decarboxylase.[a]

Amino Acid	Prohistidine Decarboxylase (Residues/ 37,000 g)	Histidine Decarboxylase (Residues/ 37,000 g)
Lysine	23.0	23.6
Histidine	2.1	2.0
Arginine	11.6	10.8
Half-cystine[b]	2.0	1.8
Aspartic acid	45.8	44.9
Threonine	17.6	16.0
Serine	20.8	19.2
Glutamic acid	33.4	33.4
Proline	22.8	20.8
Glycine	26.8	28.0
Alanine	26.8	26.0
Valine	16.0	15.0
Methionine	10.0	9.7
Isoleucine	18.3	17.0
Leucine	18.8	17.7
Tyrosine	14.6	14.5
Phenylalanine	8.9	8.9
Tryptophan[c]	7.1	7.1
Σ of residue weights	37,065	36,047

[a] Protein was hydrolyzed in 6 N HCl for 26 hr at 105°. [b] Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1967). [c] Tryptophan was determined spectrophotometrically (Goodwin and Morton, 1946).

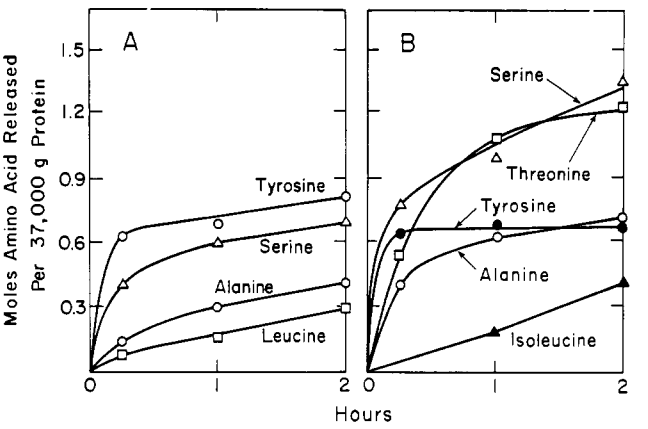

FIGURE 7: Comparative rates of release of amino acids by DFP-carboxypeptidase A from prohistidine decarboxylase (A) and activated prohistidine decarboxylase (B). Digestion and analysis were carried out as described in Materials and Methods. Leucine (not shown) was also released in part B, but at a rate slower than isoleucine.

1970), lysozyme) on electrophoresis in acrylamide gels after treatment with sodium dodecyl sulfate (see Materials and Methods) gave the usual relationship between molecular weight and distance of migration. The proenzyme subunit had a molecular weight of 37,000 while a molecular weight of 28,000 was confirmed for chain II of the wild-type enzyme. Since the native proenzyme has a molecular weight near 190,000, it is apparently a pentamer. Upon activation, each proenzyme subunit is converted into two smaller subunits that are electrophoretically identical to the two types of subunits found in wild-type enzyme (Figure 6).

Amino acid analyses of the purified proenzyme and wild-type histidine decarboxylase show that the two proteins have identical amino acid compositions (Table II) within an experimental error of ±5%. Summation of the residue weights of the nearest integer values of each of the component amino acids gave a "chemical molecular weight" of 37,065 for the proenzyme subunit, in good agreement with the value of 37,000 obtained by sodium dodecyl sulfate disc gel electrophoresis. A "chemical molecular weight" of 36,047 is obtained for the sum of the molecular weights of the two dissimilar subunits of wild-type enzyme, again in good agreement with previously reported values (Riley and Snell, 1970). In addition, the uncorrected values reported here agree well with the values reported by Chang and Snell (1968b) and by Riley and Snell (1970) for different samples of wild-type enzyme.

Amino- and Carboxyl-Terminal Residues of Prohistidine Decarboxylase. Samples of prohistidine decarboxylase and activated prohistidine decarboxylase were treated with 1-fluoro-2,4-dinitrobenzene then dialyzed and hydrolyzed with acid as described under Methods. The ether-soluble dinitrophenyl derivatives were chromatographed with appropriate standards on silica gel layers with chloroform–benzyl alcohol–acetic acid (70:30:3) as the solvent system. The water-soluble dinitrophenyl derivatives were chromatographed with 1-propanol–ammonia (7:3) as the solvent system. α-Dinitrophenylserine was obtained in roughly similar yields, as estimated from the intensity of the spot on the chromatogram, from both the proenzyme and activated proenzyme. No other α-dinitrophenylamino acids were found. These results are compatible with previous conclusions that one of the two types of subunits of wild-type histidine decarboxylase contains an NH_2-terminal serine residue; a pyruvoyl group blocked the NH_2-terminal residue of the second type of subunit (Riley and Snell, 1970).

Digestion of sodium dodecyl sulfate denatured proenzyme with DFP-carboxypeptidase A resulted in the rapid release of nearly 1 tyrosine residue/mol of proenzyme subunit; serine, alanine, and leucine were released at progressively slower rates (Figure 7). The same results were observed during digestion of the larger subunit of wild-type enzyme with carboxypeptidase, except that serine was released more slowly than alanine and leucine (Riley and Snell, 1970). Digestion of the activated proenzyme with carboxypeptidase results in the release of these same amino acids and additional serine, threonine, and alanine (Figure 7); serine is now released most rapidly indicating that one of the two subunits formed during activation (*cf.* Figure 6), like the smaller subunit of wild-type histidine decarboxylase (Riley and Snell, 1970), has serine as the carboxyl-terminal amino acid. Thus the activated proenzyme and wild-type enzyme appear to have the same carboxyl-terminal amino acid residues.

Formation of Covalently Bound Pyruvate during Conversion of Proenzyme to Active Enzyme. The pyruvoyl residues of wild-type histidine decarboxylase arise from serine by a mechanism not yet understood (Riley and Snell, 1970). They react with phenylhydrazine to form a catalytically inactive protein phenylhydrazone that is readily detected by its absorbance at 323 nm (Riley and Snell, 1968). Wild-type enzyme and the activated proenzyme were both found to bind slightly over 4 mol of phenylhydrazine/mol (190,000 g) of protein (Table III). The proenzyme, on the other hand, did not react to form

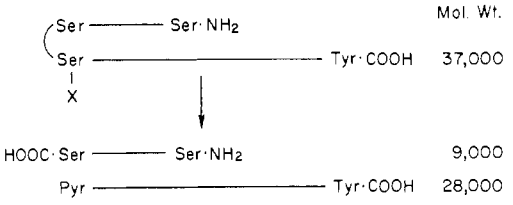

FIGURE 8: Schematic representation of the chemical nature of the prohistidine decarboxylase subunit (top) and its conversion to the two dissimilar subunits of histidine decarboxylase (bottom). The X group denoted –Ser– is converted to a pyruvate residue during the conversion.

significant amounts of a protein phenylhydrazone, indicating either that it contains no pyruvate, or that the pyruvate residue is inaccessible for reaction with phenylhydrazine. The latter alternative appears untenable, since a limited acid hydrolysis recommended for liberation of dehydroalanine residues of protein as pyruvic acid (Weiner *et al.*, 1966; Patchornik and Sokolovsky, 1964) liberated pyruvate from the activated proenzyme, but not from prohistidine decarboxylase itself (Table IV). Reduction with sodium borohydride, which converts the pyruvate residue of wild-type histidine decarboxylase to lactate and thus inactivates the enzyme (Riley and Snell, 1968) also converts dehydroalanine residues to alanine (Hanson and Havir, 1970; Hodgins, 1971; Wickner, 1969). Treatment with quantities of borohydride that completely inactivated wild-type histidine decarboxylase did not inhibit the activation of the proenzyme (Table V). It did, however, eliminate the activity of the proenzyme preparation itself, indicating that this activity results from contamination with small amounts of activated enzyme. The evidence indicates, therefore, that the proenzyme contains neither pyruvate nor dehydroalanine residues, and that activation at pH 7.6 generates a pyruvate residue in one of the two dissimilar subunits formed from each subunit present in the proenzyme.

TABLE IV: Yield of Pyruvic Acid upon Acid Hydrolysis of Prohistidine Decarboxylase and Activated Prohistidine Decarboxylase.

Sample	Mol of Pyruvic Acid/190,000 g of Protein
Prohistidine decarboxylase	0.7
Prohistidine decarboxylase plus 5 mol of pyruvate/190,000 g of proenzyme[a]	6.2
Activated prohistidine decarboxylase	5.1
Activated prohistidine decarboxylase plus 5 mol of pyruvate/190,000 g of enzyme[a]	11.5

[a] To provide an internal test of the methodology, sodium pyruvate (0.052 μmol) was added to the protein samples (2.0 mg) before acid hydrolysis.

Discussion

The relationship between the catalytically inactive mutant prohistidine decarboxylase and the active wild-type histidine decarboxylase presents several novel features. Both proteins have identical molecular weights within experimental error, as judged by rates of diffusion in agar (Figure 5), sedimentation coefficients, and amino acid analyses (Table II); they also share identical immunological determinants as judged by Ouchterlony analysis (Figure 4), and migrate identically on disc gel electrophoresis (Figure 5). The proenzyme, however, contains only one type of subunit (Figure 6), whereas wild-type enzyme and the activated proenzyme contain two types

TABLE III: Reaction of Phenylhydrazine with Prohistidine Decarboxylase, Activated Prohistidine Decarboxylase, and Wild-Type Histidine Decarboxylase.[a]

Sample	$A_{323}:A_{280}$	Mol of Phenylhydrazine Bound/190,000 g of Protein
Prohistidine decarboxylase	0.02	0.4
Activated prohistidine decarboxylase	0.21	4.4
Wild-type histidine decarboxylase	0.19	4.1

[a] In each case protein (2.8 mg) was incubated with 4.0 mM phenylhydrazine hydrochloride in 1 ml of 0.2 M ammonium acetate buffer (pH 4.8) for 30 min at 23°. Unbound phenylhydrazine was then removed by passing the reaction mixture over a column (1.7 × 26 cm) of Sephadex G-25 (coarse) equilibrated with 0.2 M ammonium acetate buffer (pH 4.8). Extinction values of the protein effluent were determined at 323 and 280 nm and used to calculate the amount of phenylhydrazine bound as phenylhydrazone (Riley and Snell, 1968).

TABLE V: Comparative Effects of $NaBH_4$ on Histidine Decarboxylase and Prohistidine Decarboxylase.[a]

Mol of $NaBH_4$ Added/190,000 g of Protein	Act., % of Control[b]	
	Histidine Decarboxylase	Prohistidine Decarboxylase
0	100	100
4	22	102
8	2	100
12	0	102

[a] To 0.44 mg of protein in 0.3 ml of 2 M potassium phosphate (pH 5.7) was added 0.01 ml of 2-octanol and varying amounts of 8 M $NaBH_4$ (Metal Hydrides, Inc.) in 0.005 N NaOH. After 20 min, wild-type enzyme solutions were diluted directly for assay of decarboxylase activity. Proenzyme solutions were freed of $NaBH_4$ and activated by dialyzing against 500 ml of 0.2 M potassium phosphate (pH 7.6) at 37° for 26 hr before assay. The pH of the protein solutions treated with $NaBH_4$ did not exceed 7.5. [b] For control samples, $NaBH_4$ was omitted. Specific activities of control samples were 95,000 and 80,000 μl hr^{-1} mg^{-1} for histidine decarboxylase and prohistidine decarboxylase (after activation), respectively.

of subunits of different size. If, as concluded earlier (Riley and Snell, 1970), the wild-type enzyme contains ten subunits (five each of two types) then the proenzyme must contain five precursor subunits. Conversion of the proenzyme subunits to active enzyme subunits occurs upon raising the pH to 7.6, and is accompanied by the changes depicted in Figure 8. The proenzyme subunit contains NH_2-terminal serine, COOH-terminal tyrosine, no pyruvate or dehydroalanine residues, and has a molecular weight approximately equal to the sum of the molecular weights of the two subunits formed from it upon activation. The larger of the latter two subunits contains the newly generated pyruvate residue blocking its NH_2 terminus and a COOH-terminal tyrosine residue; the smaller of the two subunits contains serine at both NH_2- and COOH-terminal positions. The pyruvate residue in histidine decarboxylase is known to arise from serine (Riley and Snell, 1970), but the intermediate steps in this conversion are not known. At least two steps would appear necessary: conversion of a serine residue (perhaps *via* a dehydroalanyl residue arising by elimination of HX from a free or substituted β-hydroxyl group (X in Figure 8)) to a pyruvate residue, and specific hydrolysis to yield the two peptide chains shown plus ammonia. The sequence of these steps, how they are catalyzed, and the nature of X are not known; direct analyses by the method of Bartlett (1959) show that X cannot be phosphate. The rate limiting step in the conversion under optimal conditions (37° and pH 7.6) is first order with respect to both proenzyme and total protein and therefore appears to be either nonenzymatic or catalyzed intramolecularly by the proenzyme itself; but this does not preclude the possibility that other steps are catalyzed by enzymes that copurify with the proenzyme. It should be noted that *Lactobacillus 30a* grows optimally at acidic pH, and under normal growth conditions is never exposed to pH values that permit activation of the mutant proenzyme. Despite this fact, the proenzyme accumulates only in mutant cells, and has not been observed in the parent strain of this organisms. Until the process of activation is better understood, the nature of the mutation that causes proenzyme to accumulate in mutant 3 of *Lactobacillus 30a* cannot be specified.

Studies on the biosynthesis of the pancreatic proteolytic enzymes (Neurath *et al.*, 1970; Desnuelle, 1960), pepsin (Herriot, 1962), rennin (Foltmann, 1970), collagen (Church *et al.*, 1971; Bellamy and Bornstein, 1971; Layman *et al.*, 1971), insulin (Steiner *et al.*, 1967; Steiner and Oyer, 1967; Chance *et al.*, 1968), parathyroid hormone (Kemper *et al.*, 1972), cocoonase (Berger *et al.*, 1971), streptococcal proteinase (Liu and Elliot, 1965), and the proteins involved in blood clot formation and dissolution (Magnusson, 1971) have revealed that peptides are generally released from zymogens during activation. The possibility that one or more small peptides (*e.g.*, from a segment of the peptide chain lying between the two internal serine residues of Figure 8, top) is released during activation of prohistidine decarboxylase has not been eliminated; however, any such peptide would have to be of low molecular weight, since the observed differences in molecular weight and amino acid composition between the proenzyme and active enzyme are within experimental error.

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