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Histidine Decarboxylase of *Lactobacillus* 30a. III. Composition and Subunit Structure*

George W. Chang† and Esmond E. Snell

ABSTRACT: Histidine decarboxylase of *Lactobacillus* 30a has a $s^0_{20,w}$ of 9.2 S, a molecular weight of 190,000, and contains ten half-cystine residues per mole of native enzyme. In the presence of urea, ten sulfhydryl groups react with *p*-mercuribenzoate, 5,5′-dithiobis(2-nitrobenzoic acid), or iodoacetic acid, indicating that there are no disulfide bridges in the enzyme. Mercaptidation of half of these sulfhydryl groups inactivates the native enzyme. Upon complete carboxymethylation or treatment with sodium dodecyl sulfate, the decarboxylase dissociates into ten subunits of mol wt 19,000, which are not further dissociated in 5 M guanidine hydrochloride. Digestion with carboxypeptidase A removes five tyrosine residues from native histidine decarboxylase without inactivating the enzyme. Similar treatment of *S*-carboxymethylhistidine decarboxylase liberates ten tyrosine residues and on longer digestion alanine and leucine. The results are consistent with a C-terminal sequence -Leu-Ala-Tyr for each of the ten subunits. Hydrazinolysis also indicates that tyrosine is the sole C-terminal amino acid. After acid hydrolysis of dinitrophenyl enzyme, dinitrophenylserine was the only α-dinitrophenylamino acid recovered, indicating that serine is present as an N-terminal amino acid. The low recovery of this amino acid leaves open the possibility of other terminal groups. A simplified procedure for discontinuous polyacrylamide gel electrophoresis in the presence of urea was used to separate the peptides resulting from cyanogen bromide cleavage of the decarboxylase. Results of cyanogen bromide cleavage, carboxypeptidase digestion, and tryptic peptide mapping support the presence of ten subunits in this enzyme, and indicate that these are either identical or nearly so.

Preceding papers (Rosenthaler *et al.*, 1965; Chang and Snell, 1968) have described the preparation of crystalline histidine decarboxylase from *Lactobacillus* 30a, its specificity of action toward histidine and histidine analogs, and the optically specific nature of this pyridoxal phosphate independent decarboxylation reaction. This paper summarizes studies on the amino acid composition and subunit structure of this enzyme, together with a preliminary correlation of its enzymatic activity with certain of its structural features.

Materials and Methods

Materials. Unless otherwise stated, all chemicals were reagent grade and were used without further purification. Samples of guanidine hydrochloride free from

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† National Science Foundation graduate fellow.

yellowish material were chosen, and showed negligible absorbance at 300 mμ in 6 N solution. Iodoacetamide was recrystallized from carbon tetrachloride and rinsed with petroleum ether (bp 30–60°), iodoacetic acid was recrystallized from a mixture of benzene and carbon tetrachloride and washed with petroleum ether, phenol was redistilled, sodium dodecyl sulfate was recrystallized from ethanol, and urea was recrystallized twice from water at 50°. Trypsin treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone to remove chymotryptic activity (Kosta and Carpenter, 1964) was kindly supplied by Drs. S. S. Wang and F. C. Carpenter. Carboxypeptidase treated with diisopropylfluorophosphate and recrystallized twice was obtained from Worthington.

Heavy-walled ignition tubes for protein hydrolysis (18 × 150 mm) were heated before use in a mixture (1:1) of concentrated nitric and sulfuric acids for 30–60 min, then rinsed thoroughly with tap water and distilled water. Commercial dialysis tubing was freed from bisulfite and colored material by heating it to 60° for 3 hr in three changes of a 1% solution of sodium bicarbonate and then soaking it for several hours in several changes of distilled water at 60°.

Histidine decarboxylase was prepared in crystalline form by the procedure of Rosenthaler *et al.* (1965) or the modified procedure of Chang and Snell (1968). Its concentration was determined by its absorbancy at 280 mμ as described by Chang and Snell (1968).

Disc Electrophoresis in the Presence of Urea. The conventional procedure of Davis (1964) for disc electrophoresis was modified slightly as follows. (1) The running gel was made 8 M in urea; (2) the final acrylamide concentration of the running gel was reduced from 7.5 to 5% to allow the peptides to migrate faster; and (3) the protein sample was dissolved in 50 μl of buffer (0.01 M Tris, 0.076 M glycine, 10% sucrose, and 7.5 M urea (pH 8.5)) and layered directly above the running gel immediately before commencement of electrophoresis. No sample or spacer gels were used. In control runs, samples of bovine serum albumin applied by this technique gave electrophoretic patterns identical with those applied in a conventional sample gel. Electrophoresis was performed at 2 mA/tube for 10 min and then at 5 mA/tube for 24 min with a constant current power supply. The gels were then stained with Amido Black, destained by the procedure of Davis (1964), and stored in 10% acetic acid.

Amino Acid Analyses. Samples (0.5–5 mg) of native, performate-oxidized, or *S*-carboxymethylated histidine decarboxylase were hydrolyzed together with a small crystal of phenol (to reduce the destruction of tyrosine) in 1–2 ml of 6 N hydrochloric acid in sealed, evacuated tubes at 108°. After the desired time, the tubes were cooled, opened, and evaporated to dryness at 40° in a rotary evaporator. The residues were dissolved in 2–5 ml of 0.2 M sodium citrate buffer (pH 2.2) and analyzed in a Beckman 120C automatic amino acid analyzer by the method of Spackman *et al.* (1958) as modified by Hubbard (1965).

Performic acid oxidation was carried out on 2.5 mg of decarboxylase by the procedure of Moore (1963), except that hydrobromic acid was not used to destroy unreacted performic acid, and reactants were removed by lyophilization.

Reduction and carboxymethylation of histidine decarboxylase was carried out by a slight modification of the method of Crestfield *et al.* (1963). Recrystallized dialyzed decarboxylase (10–50 mg) was dissolved in 5 ml of 10 M urea, 0.1 ml of β-mercaptoethanol and a small drop of phenol red were added, and the solution was adjusted to about pH 8.5 with aqueous ammonia and gassed with nitrogen. The vial was convered tightly, and held for 4 hr at 40°. Absolute ethanol (50 ml) containing 1 ml of concentrated HCl was then added. After 0.5 hr at 0° the precipitated protein was collected by centrifugation, dissolved in 3–5 ml of 10 M urea, and a small drop of phenol red was added. The solution was again gassed with nitrogen, then iodoacetic acid (50 mg) was added, and the pH was adjusted to about 8.5 with aqueous ammonia. The vial was covered tightly and incubated in the dark at 40°. Additional ammonia was added as needed to keep the pH around 8.5. After 30 min, 0.25 ml of β-mercaptoethanol was added to react with the remaining iodoacetate and the vial was incubated in the dark at 40° for 30 min, the pH again being maintained by the occasional addition of aqueous ammonia. The product was then either precipitated with an excess of absolute ethanol (50 ml), collected by centrifugation, washed with absolute ethanol and anhydrous peroxide-free ether, and air dried, or the solution was applied to a column of Sephadex G-25 (2 × 35 cm) equilibrated with 0.1 M ammonium bicarbonate and eluted with additional bicarbonate solution. Fractions (3 ml each) were collected and those containing protein were pooled and lyophilized. Carboxymethylation of unreduced protein was carried out in this same way, except that β-mercaptoethanol was omitted from the initial incubation.

Sulfhydryl groups were determined by titration with PMB[1] (Boyer, 1954) or with DTNB; a molar absorbance of 13,600 was used for the reduction product of DTNB (Ellman, 1959).

Ultracentrifugal Studies. All ultracentrifuge runs were made in a Beckman-Spinco Model E analytical ultracentrifuge equipped with a phase plate for schlieren optics, a rotatable light source for Rayleigh interference optics, and a Wratten 77A filter. The sedimentation velocity and equilibrium techniques used were principally those of Schachman (1959) and Yphantis (1964), as described in detail elsewhere (Morino and Snell, 1967).

The partial specific volume, $\bar{v}$, of histidine decarboxylase calculated from the amino acid composition of the enzyme (Cohn and Edsall, 1943) is 0.73 ml/g, whereas a value of 0.72 ml/g was obtained from density measurements of a 3.3% solution of protein in 0.5 N KCl in a 1.5-ml pycnometer. The pycnometric value was used.

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PMB, *p*-chloromercuribenzoate; SDS, sodium dodecyl sulfate; DIP, diisopropylphosphoryl; FDNB, fluorodinitrobenzene; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

TABLE I: Amino Acid Analyses of Acid Hydrolysates of Histidine Decarboxylase.[a]

Amino Acid[a]	Residues/190,000 g: Time of Hydrolysis 23 hr	35 hr	54 hr	"Best" Values[e]	Residues/ 19,000 g
Tryptophan[b]	(30)			30	3
Lysine	111.5	110.3	102.2	111 (103)	11
Histidine	10.1	10.5	(8.9)	10.3 (9.7)	1
Ammonia	155	171	192	123	12
Arginine	63.6	63.6	59.2	62.1 (58)	6
Half-cystine[c]	(10.2)			10.2 (9.6)	1
Aspartic acid[d]	212.9	218.4	213.2	215	21
Threonine	78.9	79	73.8	86	9
Serine	99.6	96	81.9	113	11
Glutamic acid[d]	172.7	176.7	174.1	174	17
Proline	105.5	106.1	108.1	107	11
Glycine	155.1	159.3	153	156	16
Alanine	150.2	154.4	151.9	152	15
Valine	86.7	90.2	90.4	90.4	9
Methionine	55.4	52.6	55.6	54.5 (51)	5–6
Isoleucine	100	104	102.1	102	10
Leucine	100.9	102.4	99.6	101	10
Tyrosine	78.2	79.7	77.5	78.5	8
Phenylalanine	41	42.4	41	41.5	4

[a] The amino acids are listed in the order of their emergence from the short and long columns of the amino acid analyzer. [b] Tryptophan was determined spectrophotometrically. [c] Half-cystine residues were determined as *S*-carboxymethylcysteine or cysteic acid in acid hydrolysates of reduced, carboxymethylated, or performic acid oxidized protein, respectively. [d] These figures include both free and amidated residues of these amino acids. [e] Values in parentheses are based on an absorbance ($A^{1\,cm}_{1\%}$) at 280 mμ of 16.1 rather than 17.3 (see footnote 1, Chang and Snell, 1968).

Other Methods. Details of other less generally used techniques are given with the individual experiments to which they pertain.

Results

Amino Acid Analyses. Analyses of histidine decarboxylase following three periods of acid hydrolysis are shown in Table I. The 54-hr value was used for valine; other results have been averaged or extrapolated to zero time to give the "best" values. Tryptophan was determined by spectral measurments of a solution containing 0.22 mg of protein/ml in 0.1 N NaOH. A ratio of tyrosine to tryptophan of 3.05 was obtained by the method of Goodwin and Morton (1946) and 2.44 by the method of Bencze and Schmid (1957). From the tyrosine content (Table I) a tryptophan content of 29 ± 3 residues/190,000 g of native enzyme was calculated. The total half-cysteine content, determined as cysteic acid in hydrolysates of the performate-oxidized protein, agreed with the carboxymethylcysteine content of hydrolysates of the reduced carboxymethylated protein (Table II); an average of ten modified cysteine residues was recovered per mole of native histidine decarboxylase (Table II). Since the same amount of *S*-carboxymethylcysteine was recovered from the unreduced and reduced carboxymethylated enzyme, no disulfide linkages appear to be present in the enzyme.

The predominance of acidic amino acids over basic amino acids and ammonia is consistent with the isoionic point of 4.4 observed by Rosenthaler *et al.* (1965). The amino acid composition obtained accounts for 99% of the dry weight of the protein and, when the residue weights of the nearest integral values of each of the component amino acids are summed, gives a "chemical molecular weight" of 189,000, which agrees well with the values of 185,000 and 194,000 obtained by ultracentrifugal determinations described later.

Sulfhydryl Group Content. Eight SH groups of histidine decarboxylase titrate with *p*-mercuribenzoate within 15 min at room temperature and pH 4.8; two more react in the next 24 hr (Table III). In 8 M urea, the reaction was complete in less than 15 min, and about 10.5 molecules of *p*-mercuribenzoate were bound/mole of enzyme (Table III). The enzyme reacts with 5,5′-dithiobis(2-nitrobenzoic acid) with an apparent half-time of 23 min in 6.8 M urea. The reaction is complete in 6 hr, when 10.0 sulfhydryl groups/mole of enzyme had reacted. A plot of the logarithm of the unreacted material *vs.* time was linear, indicating that all of the thiol groups

TABLE II: Comparison of Half-Cystine and Cysteine Content of Histidine Decarboxylase. See Text for Details.[a]

Prepn of Histidine Decarboxylase	Cysteic Acid or Carboxymethylcysteine (mμmoles)	Glutamic Acid (mμmoles)	Cysteine[a] Residues/190,000 g of Protein
Performic acid oxidized	8.97[b]	160.2	9.8
	36.8[b]	632	10.2
Carboxymethylated	26.5[c]	428	10.8
Reduced and carboxymethylated	25.5[c]	437	10.2

[a] Glutamic acid was used as an internal standard to determine the amount of sample subjected to analysis; the presence of 175 glutamic acid residues/190,000 g of protein was assumed (Table I). [b] Determined as cysteic acid. [c] Determined as *S*-carboxymethylcysteine.

in the urea-treated enzyme react at the same rate. The sluggishness of the reaction, even in 6 M urea, indicates that SH groups are partially shielded from the reagent.

Effects of Sulfhydryl Reagents on the Activity of Histidine Decarboxylase. Inactivation of histidine decarboxylase was complete when five to six of the ten SH groups in the enzyme reacted with PMB (Figure 1A). In a similar experiment, the enzyme was inactivated completely by incubation with iodoacetamide when five to seven of its cysteine residues had been alkylated (Figure 1B). The rate of inactivation by iodoacetamide at pH 8.0 is much greater than that by iodoacetate (Figure 2). With neither reagent were any abrupt changes in rate of inactivation observed that would indicate the presence of sulfhydryl groups of different reactivities in the native enzyme.

TABLE III: Effect of Time and Urea on the Reaction of Histidine Decarboxylase with *p*-Mercuribenzoate.[a]

Time (hr)	Moles of PMB Reacted/190,000 g of Protein	
	No Urea	8 M Urea
0.25	8.13	10.6
1.0	8.58	10.5
14	9.56	
24	9.85	

[a] Samples (1 ml) contained 260 or 530 μg of protein and 2–80 μmoles of PMB in 0.2 M ammonium acetate (pH 4.8). Absorbancies were read at room temperature against the corresponding reference solution lacking added enzyme.

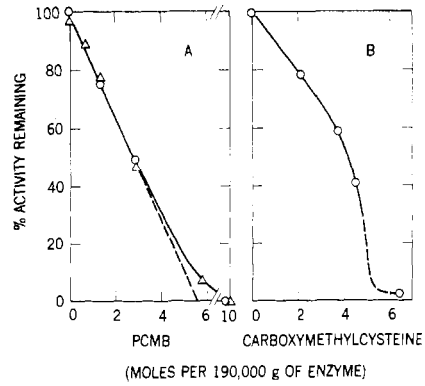

FIGURE 1: (A) Stoichiometry of inactivation of histidine decarboxylase by PMB. (○) Samples of enzyme were treated with PMB for 16 hr at room temperature before samples were withdrawn for assay. See Table III for conditions. (△) Samples (10.5 μg) of enzyme in 25–100 μl of 0.2 M ammonium acetate buffer (pH 4.8) in the side arms of the Warburg flasks were incubated with the indicated amounts of PMB for 0.5 hr at 37°, then mixed with the contents o the main compartment for assay. (B) Inactivation of histidine decarboxylase by iodoacetamide as a function of the amount of reagent bound. Decarboxylase (15.6 mg) was incubated with 0.38 mg of iodoacetamide at 37° in 1.7 ml of 0.02 M potassium phosphate buffer (pH 7.8). At appropriate intervals, samples (1–50 μl) were transferred to 0.5 ml of 0.2 M ammonium acetate buffer (pH 4.8) containing 50 μg of bovine serum albumin and 5 μl of β-mercaptoethanol in the side arms of Warburg vessels and assayed at 37°. Larger samples (200 μl) taken at these same times were mixed rapidly with 3 ml of cold acidic ethanol (2% concentrated HCl by volume). After 0.5 hr at 0°, the precipitated protein was collected by centrifugation, rinsed with 2 ml of cold, anhydrous, peroxide-free ether, dried at room temperature, hydrolyzed, and analyzed for carboxymethylcysteine on an amino acid analyzer.

Incubation of histidine decarboxylase with a large excess (2–10 mM) of cadmium chloride or sodium arsenite under the assay conditions failed to inhibit its activity. A large number of enzymes are inhibited by these compounds at these concentrations (Webb, 1966). If the free SH groups of histidine decarboxylase are essential for activity they must either not react with these inhibitors or the inhibitors must be easily displaced by the substrate, histidine.

Ultracentrifugal Behavior of Histidine Decarboxylase. A. SEDIMENTATION VELOCITY. At its optimum pH of 4.8, the sedimentation pattern of histidine decarboxylase shows a single symmetrical peak (Figure 3A); the concentration dependence of the sedimentation coefficient (Figure 3B) gives a value for $s^0_{20,w}$ of 9.2 S. The enzyme also sedimented in a single apparently symmetrical peak under several other conditions summarized in Table IV. The significant decreases in the corrected sedimentation coefficient at pH 8.5, and in the presence of urea, guanidine, or excess PMB may reflect a partial unfolding of the protein or a rapid equilibrium between fully associated enzyme and smaller subunits; the sedimentation coefficients are much higher than the value of 5.8 S expected if particles half the size of the native enzyme were formed. The sedimentation coefficient of *S*-car-

TABLE IV: Sedimentation of Histidine Decarboxylase under Various Conditions.

Enzyme Prepn	Concn (mg/ml)	pH	Solvent	$s_{20,w}$ (S)
Native	0.0 (extrapolated)	4.8	KCl + NH_4OAc[a]	9.20
Native	4.5	4.8	KCl + NH_4OAc[a]	9.12
Native	7.0	8.5	Tris–glycine buffer[b]	7.6
Native	4.4	4.8	8 M urea	7.2
Native	4.4	4.8	3 M guanidinium chloride	7.4
Native	3.0	4.8	15-fold excess of PMB[c]	8.5
S-Carboxymethyl[d]	3.4	8.5	Tris–glycine buffer[e]	1.1
Native	2.7	4.8	SDS, 0.1%[f]	2.5, 8.8
			SDS, 1.0%[f]	1.9

[a] 0.1 M potassium chloride–0.01 M ammonium acetate. [b] 0.05 M Tris–0.38 M glycine. [c] In 0.2 M ammonium acetate buffer. [d] This sample of carboxymethylhistidine decarboxylase was isolated over Sephadex columns (see text). Reagents were removed by dialysis; the sample was not precipitated or dried at any point. [e] 0.01 M Tris–0.076 M glycine. [f] 0.1 M NaCl–0.01 M β-mercaptoethanol–0.01 M ammonium acetate.

boxymethylhistidine decarboxylase on the other hand, is much lower than that of the native enzyme, indicating that extensive dissociation of the modified protein has occurred. In the presence of 0.1% sodium dodecyl sulfate (SDS), histidine decarboxylase sediments in two distinct peaks (Table IV), one sedimenting almost at the speed of the native protein and containing approximately 39% of the protein, the other sedimenting much slower and containing 61% of the protein. With larger amounts of SDS, only the slower peak is observed. By use of the analysis of Steers *et al.* (1965), an average molecular weight of protein in the slower peak of 20,000, roughly 0.1 that of the native protein, was obtained.

B. SEDIMENTATION EQUILIBRIUM EXPERIMENTS. Molecular weight determinations by low- (LaBar, 1965) and high-speed (Yphantis, 1964) equilibrium methods both gave linear plots of log C *vs.* X^2 (Figure 4) indicating the homogeneity of the native enzyme sample; values of 194,000 and 185,000 daltons, respectively, were obtained by the two procedures. Similar measurements with *S*-carboxymethylhistidine decarboxylase also gave linear plots (Figure 5A,B) that yielded an apparent molecular weight of 19,000 for the modified enzyme in buffer alone, and of 19,700 in 5 M guanidine hydrochloride (Figure 5C). These results again indicate that histidine decarboxylase is composed of subunits about 0.1 the molecular weight of the native enzyme.

Effect of Urea, Guanidine Hydrochloride, and SDS on the Activity of Histidine Decarboxylase. Histidine decarboxylase is quite resistant to dissociation by urea, guanidine hydrochloride, and SDS (Table IV). The effect of these denaturants on the activity of this enzyme was examined under conditions defined in Figure 6. Inhibition by either guanidine hydrochloride or urea is fast and freely reversible upon dilution; guanidine hydrochloride inhibits at much lower concentrations than urea. In 6 M urea, the enzyme displays 40–50% of its ac-

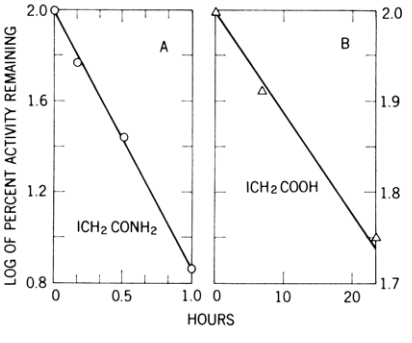

FIGURE 2: The comparative rates of inactivation of histidine decarboxylase by iodoacetamide (A) and iodoacetic acid (B). Note the differences in scales. Histidine decarboxylase (0.26 mg in 1.1 ml of 0.1 M potassium phosphate buffer, pH 8.0) was incubated in the dark at 37° with 0.5 μmole of iodoacetamide or iodoacetic acid. At the indicated times 5-μl aliquots of the reaction mixture were removed, added to 0.5 ml of 0.001 M β-mercaptoethanol in 0.2 M ammonium acetate buffer (pH 4.8), and assayed by the standard procedure.

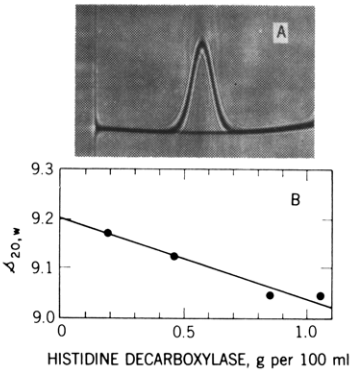

FIGURE 3: Studies on histidine decarboxylase. (A) Sedimentation velocity pattern for native histidine decarboxylase in 0.2 M potassium chloride, 0.05 ammonium acetate (pH 4.8) and 22°. The picture was taken 53 min after reaching a speed of 50,740 rpm. The schlieren diaphragm angle was 60°; the protein concentration was 0.44%. (B) Concentration dependence of the sedimentation coefficient of histidine decarboxylase. All measurements were made at 22° in 0.1 M KCl–0.01 M ammonium acetate (pH 4.8).

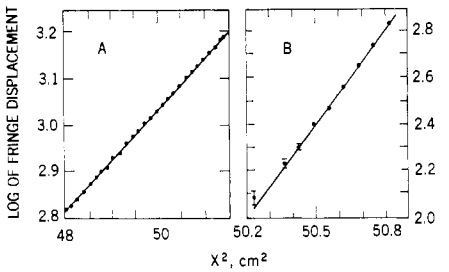

FIGURE 4: Molecular weight determination of native histidine decarboxylase. Initial protein concentration, 0.1%, in 0.25 M KCl–0.05 M ammonium acetate (pH 4.8); column height, 3 mm. X is the radial distance from the center of rotation. (A) Low-speed determination 36 hr after reaching 4609 rpm at 23°. (B) High-speed equilibrium determination 22 hr after reaching 16,200 rpm at 23°.

tivity; this falls to zero in 8 M urea, but over 70% of the activity is regained on dilution. Histidine decarboxylase is not fully dissociated by these concentrations of urea or guanidine (Table IV), perhaps explaining their relatively small effect on activity. Inactivation by sodium dodecyl sulfate, on the other hand, occurs relatively slowly and is not freely reversible, perhaps because SDS causes dissociation of the enzyme into small subunits (Table IV). The SDS-inactivated enzyme was partially reactivated when the detergent was removed by chilling, by precipitation with barium acetate, or by binding with bovine serum albumin, but complete reactivation of the enzyme was not achieved.

Chemical Evidence for the Subunit Structure of Histidine Decarboxylase. The ultracentrifugal evidence presented earlier indicates that native histidine decarboxylase contains ten subunits. We have examined this conclusion further by the techniques of peptide mapping, cyanogen bromide cleavage, and end-group determinations.

A. TRYPTIC PEPTIDE MAPS. Histidine decarboxylase

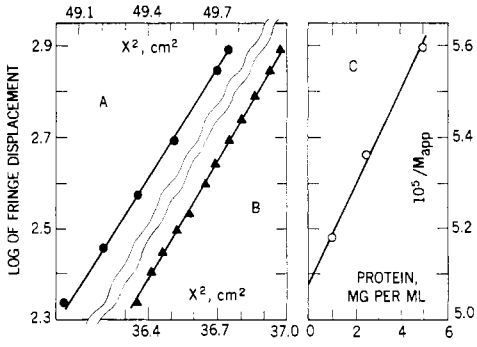

FIGURE 5: Molecular weight determination of *S*-carboxymethylhistidine decarboxylase. (A) Initial protein concentration, 0.2% in 0.05 M Tris–0.076 M glycine (pH 8.5); rotor speed, 39,460 rpm; temperature, 22°; column height, 3 mm. Equilibrium was reached within 24 hr. (B) Initial protein concentration, 0.1% in 5 M guanidine hydrochloride, 0.05 M Tris, and 0.38 M glycine (pH 8.5); rotor speed, 47,660 rpm; temperature, 22°; column height, 3 mm. Equilibrium was reached within 24 hr. (C) Concentration dependence of the apparent molecular weight of *S*-carboxymethylhistidine decarboxylase in 5 M guanidine hydrochloride. For conditions, see part B.

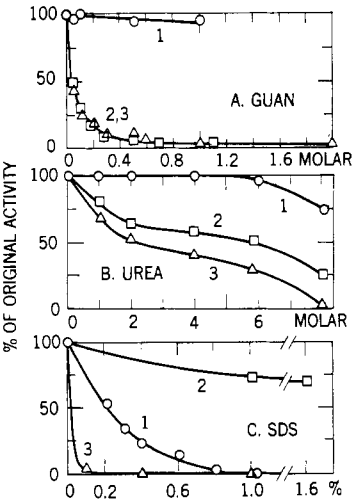

FIGURE 6: The effect of guanidine hydrochloride (GUAN, A), urea (B), and sodium dodecyl sulfate (SDS, C) on the activity of histidine decarboxylase. Activity of 20–50 μg of enzyme was determined after incubation as follows. Curve 1: incubation with denaturant for 30 min, then dilution 600-fold into the assay mixture without additional denaturant; curve 2: incubation without denaturant for 30 min, then dilution 600-fold into assay mixture containing the indicated concentration of denaturant; curve 3: denaturant present at the indicated concentration during both the 30-min prior incubation and the assay period. Solutions were prepared in 0.2 M ammonium acetate and the pH was readjusted to 4.8 when necessary. All incubations were at 37° in the side arms of the Warburg vessels used for the assays.

contains 11 lysine and 6 arginine residues/19,000 g (Table I). If the ten subunits indicated by ultracentrifugal analysis are identical, we should obtain 18 peptides after tryptic cleavage, 6 of which contain arginine. Ninhydrin-collidine spray revealed 19 major ninhydrin-positive spots, 6 or 7 of which were also Sakaguchi positive (Figure 7). A number of much fainter spots, which are not shown, were considered to be products of incomplete cleavage or cleavage at residues other than lysine and arginine. There was little difference between maps made from samples taken after 1 or 11 hr of digestion. These results are consistent with the view that histidine decarboxylase is composed of ten identical or very similar subunits of molecular weight about 19,000.

B. CYANOGEN BROMIDE CLEAVAGE. Histidine decarboxylase contains five to six methionyl residues/19,000 daltons (Table I). If the subunits are identical, a maximum of six to seven peptides should be obtained after cleavage with cyanogen bromide (Gross and Witkop, 1962). The cyanogen bromide procedure of Steers *et al.* (1965) was used to cleave 20 mg of *S*-carboxymethylhistidine decarboxylase. When subjected to disc electrophoresis in polyacrylamide gel containing urea the cleaved protein gave rise to five bands (Figure 8). When the electrophoresis was repeated with reversed polarity, no peptide band was observed, indicating the absence of any highly basic peptides large enough to be stained by Amido Black. The failure to observe the six to seven bands theoretically expected could result from analytical errors in the analyses for methionine, or from the presence of (1) one or more small peptides which failed to

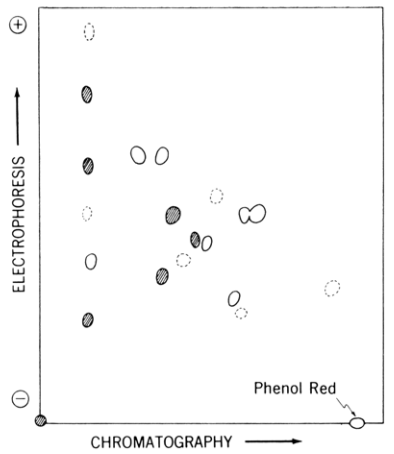

FIGURE 7: Peptide map of a tryptic digest of *S*-carboxymethylhistidine decarboxylase. To 10 mg of the *S*-carboxymethylated enzyme in 1.0 ml of water there was added a small drop of phenol red and aqueous ammonia to pH 8.5. TPCK-trypsin (25 μl of a 1% solution in 1 mM HCl) was added and the solution was held at 40° and pH 8.5 by periodic addition of 0.05 N ammonia. Samples (200 μl) were removed at variable times, adjusted to pH 4.5, and centrifuged to remove insoluble material. The soluble material was then applied to sheets of Whatman No. 3MM paper and subjected to chromatography followed by electrophoresis (Katz *et al.*, 1957). Dried chromatograms were sprayed with ninhydrin–collidine or with a modified Sakaguchi's reagent. Zones encircled with a continuous line were strongly ninhydrin positive; dotted zones were weakly ninhydrin positive; hatched zones reacted positively to Sakaguchi's reagent.

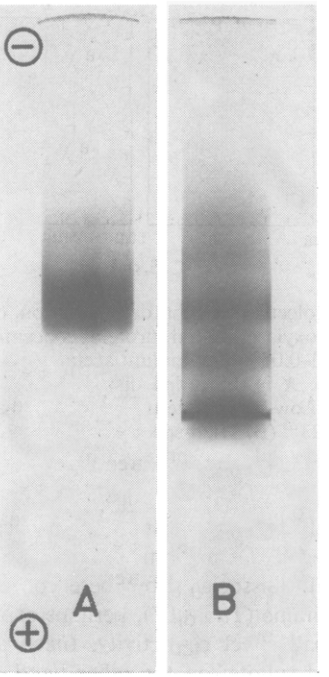

FIGURE 8: Electrophoretic patterns of (A) *S*-carboxymethylhistidine decarboxylase (100 μg) and (B) its cyanogen bromide cleavage products (300 μg), both in polyacrylamide gel containing 8 M urea at pH 9.5.

fix Amido Black, (2) more than ten identical subunits in the native protein, or (3) two peptides with identical electrophoretic mobilities among the split products. We consider 1 or 3 as most likely; in any event, the results support the conclusion that the subunits are identical.

C. NH_2 TERMINUS OF HISTIDINE DECARBOXYLASE. Samples (8 mg) of histidine decarboxylase were treated with 1-fluoro-2,4-dinitrobenzene in the presence or absence of 5 M guanidine hydrochloride. Portions were then hydrolyzed for 4 hr in 12 N HCl at 108°, and the dinitrophenylamino acids released were examined as described by Fraenkel-Conrat *et al.* (1955). No N^{α}-DNP-amino acid was recovered from the sample dinitrophenylated in the absence of guanidine hydrochloride. When the decarboxylase was dinitrophenylated in the presence of guanidine hydrochloride, DNP-serine, identified by comparison with appropriate standards on ascending chromatography, was the only N^{α}-DNP-amino acid found. The identification was confirmed by ammonolysis of the DNP-serine after isolation by paper chromatography (Lowther, 1951). Serine was the only amino acid liberated, as shown by analysis on an amino acid analyzer. The final yield (12 mμmoles, uncorrected, from 2.6 mg of DNP protein) was about 10% of theoretical on the assumption that the enzyme is composed of ten identical peptide chains. The failure to find any N-terminal amino acid other than serine supports the assumption of identical subunits; the low yield, although attributable in part to losses during the isolation procedures, leaves open the possibility of other terminal groups. The failure to obtain any DNP-amino acid from native enzyme indicates that the end group is relatively inaccessible to FDNB in the absence of a denaturant.

D. CARBOXYL TERMINUS OF HISTIDINE DECARBOXYLASE. Digestion of *S*-carboxymethylhistidine decarboxylase with DIP-carboxypeptidase A results in the rapid release of ten tyrosine residues/mole of original enzyme; alanine, leucine, and serine are released at progressively slower rates (Figure 9A). Thus tyrosine is the COOH-terminal amino acid, and the data are consistent with the view that each of the ten identical peptide chains of mol wt 19,000 ends in the sequence -Leu-Ala-Tyr. Similar digestion of native histidine decarboxylase releases only five residues of tyrosine/190,000 daltons, again followed by alanine and then leucine (Figure 9B). The catalytic activity of the native enzyme is not diminished significantly by the loss of these three residues (Figure 9B), but further digestion results in rapid loss of activity and an increasing amount of material that precipitated from solution when the digest was diluted for assay into pH 4.8 buffer.

The identity of tyrosine as the carboxyl terminal amino acid was confirmed by hydrazinolysis (Carlton and Yanofsky, 1963) which gave about 30% (uncorrected) of the expected yield of tyrosine, together with much smaller amounts of serine and glycine. The latter amino acids are common breakdown products of the aminoacyl hydrazides (Akabori *et al.*, 1956; Bradbury, 1956).

Discussion

The presence of ten cysteinyl and ten histidinyl residues/190,000 g of native histidine decarboxylase indicates that no more than ten identical subunits can be present if

the molecular weight obtained by sedimentation equilibrium is correct. The apparent homogeneity of the fully dissociated enzyme and its molecular weight (19,000) indicate that the enzyme is indeed composed of ten subunits, and the results of tryptic maps, cyanogen bromide cleavage, and NH_2- and COOH-terminal studies suggest that these subunits are either identical or very similar. Proteins containing this number of subunits are unusual (Klotz, 1967). However, on the basis of its SH content and sedimentation rate under various conditions, mitochondrial adenosine triphosphatase is thought to be composed of ten subunits (Penefsky and Warner, 1965). Arginine decarboxylase also appears to contain ten subunits and undergoes dissociation to five dimeric subunits in a reversible manner (Boeker and Snell, 1968). Reversible dissociation of histidine decarboxylase to its monomeric subunits has not been obtained; however, the undetermined changes in structure induced by high concentrations of urea or guanidine (Table IV) are largely reversible on dilution as judged from activity determinations (Figure 6).

Reaction of five of its ten SH groups with PMB inactivates histidine decarboxylase. These SH groups may be part of the active site, or lie so near it that the bulky mercuribenzoate grouping prevents binding of the substrate. In either case, the presence of five active sites on the enzyme would be indicated. Inactivation might also result simply from distortion of the quaternary structure in such a way that the configuration necessary for activity could not be assumed. The moderate reduction in the sedimentation coefficient that is observed on addition of excess PMB (Table IV) supports the idea that this reagent modifies spacial arrangements within the enzyme in some unidentified manner. The fact that only five of the ten carboxyl-terminal tyrosine residues are accessible to carboxypeptidase A in the native enzyme is consistent with a model in which two monomers associate to form a basic catalytic unit of mol wt 38,000, to yield five active sites per mole of undissociated enzyme. Alternate interpretations are also possible, however; hopefully, clarification of the basis for the inhibition of this enzyme by carbonyl reagents will provide additional information concerning the nature and number of its active sites.

Histidine decarboxylase from *Lactobacillus* 30a resembles in many respects the crystalline histidine decarboxylase obtained from a *Micrococcus* by Mardashev and his colleagues. Both enzymes are inhibited by carbonyl reagents, but neither is dependent upon pyridoxal phosphate as coenzyme (Rosenthaler *et al.*, 1965; Mardashev *et al.*, 1968). The inhibitory action of various substrate analogs on the two enzymes is similar but not identical (Rosenthaler *et al.*, 1965; Mardashev *et al.*, 1965, 1968). Major differences in molecular weights (190,000 and 110,000, respectively, for the *Lactobacillus* and micrococcal enzyme) and amino acid analyses for the two enzymes have been reported, however. Most strikingly, the histidine content of the *Lactobacillus* enzyme (Table I) is only about two-thirds that of the micrococcal enzyme, whereas the converse difference exists in their proline and alanine contents (*cf.* Table I and Mardashev *et al.*, 1968). The N-terminal amino acid

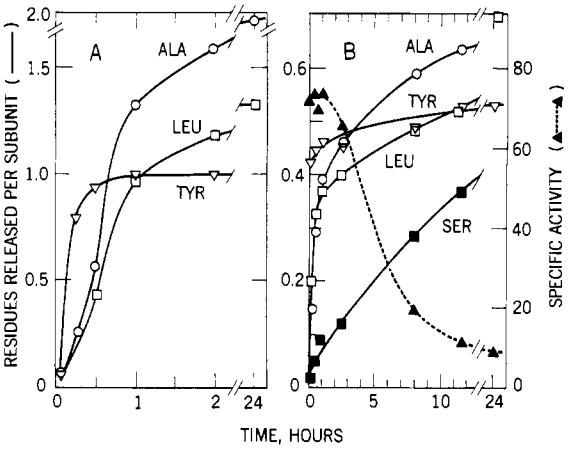

FIGURE 9: Rates of release of terminal amino acids from (A) carboxymethylated or (B) native histidine decarboxylase on digestion with carboxypeptidase A and its effect on activity of the native enzyme. Native (7 mg) or *S*-carboxymethylhistidine decarboxylase (15 mg) in 1.5 ml of 0.05 M ammonium bicarbonate buffer containing a small drop of phenol red was incubated at pH 8.3 and 40° with 0.2 mg of DIP-carboxypeptidase A. At various times, 200-μl samples were mixed with an equal volume of 10% trichloroacetic acid and centrifuged to remove the protein. The precipitated protein was rinsed with 100 μl of 5% trichloroacetic acid, centrifuged, and discarded. The rinse and the original supernatant solution were mixed and lyophilized. The residue was dissolved in 0.2 M sodium citrate buffer (pH 2.2), and applied to the long column of the amino acid analyzer. Ser, Asp, and Gly were also released in expt 9A, but at rates much lower than Tyr, Ala, and Leu. In B, additional 10–50-μl samples of the digest were added at the indicated times to 0.1 mg of bovine serum albumin in 0.5 ml of 0.2 M ammonium acetate buffer (pH 4.7) and assayed for decarboxylase activity in the usual way.

of the enzyme studied here (serine) also differs from that reported for the micrococcal enzyme (methionine); from the recovery of N-terminal methionine, the micrococcal enzyme also appears to contain several subunits (Mardashev *et al.*, 1967).

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