Histidine Decarboxylase of *Lactobacillus* 30a. V. Origin of Enzyme-Bound Pyruvate and Separation of Nonidentical Subunits*

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ABSTRACT: When *Lactobacillus* 30a was grown in a defined medium containing L-[^{14}C]serine, the specific radioactivity of the pyruvoyl residue of the histidine decarboxylase produced was identical with that of the serine added to the medium and the serine isolated from the purified enzyme. The specific radioactivity of the lactate formed during growth was less than 0.01% that of the added serine. These results show conclusively that the covalently bound pyruvate present in this enzyme is derived from serine and does not equilibrate with pyruvate formed from glucose as an intermediate in fermentation. Following conversion into its phenylhydrazone and carboxymethylation, histidine decarboxylase can be fractionated by either ion-exchange or molecular sieve chromatography into two components which differ in molecular weight, amino acid composition, and end groups. Chain I has a molecular weight of about 9000, an N-terminal serine, a C-terminal serine, and contains no pyruvate, half-cystine, histidine, or phenylalanine. Chain II has a molecular weight of about 29,700, a C-terminal tyrosine, a pyruvate residue at the N terminus, and contains a complete assortment of amino acids. The intact enzyme (mol wt 190,000) contains five chains of each type. Separation of the subunits is also possible after reaction of the enzyme in 2 M guanidine with 5,5′-dithiobis(2-nitrobenzoic acid). The protein disulfide can then be split to yield the underivatized subunits. Neither subunit isolated in this way showed decarboxylase activity, and no reconstitution of the enzyme by mixing the two subunits has so far been achieved.

Histidine decarboxylase (histidine carboxy-lyase, EC 4.1.1.22) from *Lactobacillus* 30a contains approximately five pyruvoylphenylalanine residues located at the amino-terminal end of five of the ten polypeptide chains of this enzyme (Riley and Snell, 1968). Thus, at least two different types of subunits, differing at their amino terminus, must be present in this enzyme. The work presented here deals with two problems raised by these findings: (a) the origin of the enzyme-bound pyruvate, and (b) the separation and partial characterization of the subunits.

Experimental Methods

Preparation of [^{14}C]Histidine Decarboxylase. Cells of *Lactobacillus* 30a labeled with [^{14}C]serine were grown in the synthetic medium described by Guirard and Snell (1964), modified by replacing DL-serine by one-tenth the amount of L-[U-^{14}C]serine (0.834 mg, 0.5 mCi, per 100 ml of medium) and doubling the amount of L-histidine. This medium (10 ml) was inoculated with *Lactobacillus* 30a. After 19 hr at 39°, this culture was used to inoculate 90 ml of the same medium. After 17 hr at 39°, the radioactive cells (about 0.2 g) were collected by centrifugation and mixed with 25 g (wet weight) of unlabeled cells grown in crude medium (Chang and Snell, 1968a). The cells were then dried with acetone and the labeled histidine decarboxylase was isolated as described elsewhere (Riley and Snell, 1968).

Preparation of unlabeled histidine decarboxylase from acetone-dried cells was carried out as described previously (Riley and Snell, 1968). For comparative purposes, the crystalline enzyme was also isolated from freshly harvested cells as follows. Wet cells (40 g) were suspended in enough acetate buffer (0.2 M, pH 4.8) to bring the final volume to 100 ml. The cells were broken by sonic oscillation (Branson Sonicator, set at 80 W) while the slurry was passed repeatedly through a rosette cell cooled in an ice–salt bath. After 25 min, the suspension was diluted with buffer to 320 ml, and the insoluble debris was sedimented and discarded. The supernatant solution was then purified by the same procedure used for the extract of acetone-dried cells.

Manometric assay, reduction and carboxymethylation, and formation of the phenylhydrazone of histidine decarboxylase were conducted by procedures described previously (Riley and Snell, 1968).

Protein concentrations were determined spectrophotometrically at 280 mμ. Extinction coefficients of isolated protein fractions were calculated from the dry weights of dialyzed samples as previously described for native histidine decarboxylase ($\epsilon^{1\%}_{280\ m\mu}$ 16.1; Riley and Snell, 1968).

Sedimentation velocity and short-column sedimentation equilibrium measurements were made at 280 mμ by use of 12-mm double-sector cells and a Beckman Model E analytical ultracentrifuge equipped with a photoelectric scanner and absorption optical system capable of multiplex operation.

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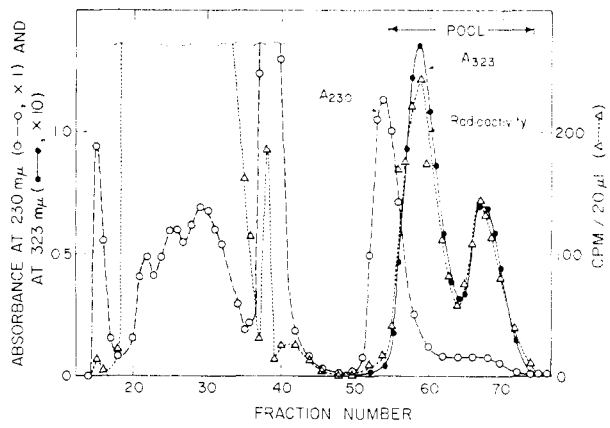

FIGURE 1: Chromatography of pronase digest of [^{14}C]histidine decarboxylase phenylhydrazone on Sephadex G-25. The concentrated digest (see text) was applied to a column (1.2 × 70 cm) of Sephadex G-25 (fine) and eluted in the cold with 0.05 M ammonium bicarbonate. The flow rate was 10 ml/hr. Each fraction contained about 2 ml.

A titanium rotor was used at rotor speeds between 60,000 and 68,000 rpm. Fluorocarbon FC43 (0.01 ml) was added to each compartment of the double-sector cells for sedimentation equilibrium experiments, and solution (and solvent) volumes of 0.10 ml were used. Both low-speed (Richards and Schachman, 1959) and high-speed (Yphantis, 1964) runs were performed, and the optical density traces were amplified several-fold by use of a 10-in. recorder (Honeywell, Lectronic 94) run at a chart speed of 60 in./min with the photomultiplier scanning at its slowest speed. For calculations of molecular weights, a value for $\bar{V}$ of 0.73 ml/g, calculated from the amino acid analyses by the method of Cohn and Edsall (1943), was used.

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 (0.5–1.2 mg) were hydrolyzed in the presence of a small crystal of phenol (to reduce the destruction of tyrosine) in 1–4 ml of 6 N HCl in sealed, evacuated tubes at 105° for periods of 18, 36, or 54 hr. The hydrolysates were then dried under vacuum, dissolved in 1–2 ml of 0.2 M sodium citrate buffer (pH 2.2), and analyzed. For isolation of serine from peptide fractions, no phenol was included and the times of hydrolysis were 16, 24, and 40 hr.

Carboxypeptidase digestions were carried out on protein samples (0.5–3.0 mg) dissolved in 0.17–0.30 ml of 0.2 M ammonium bicarbonate. DIP-carboxypeptidase A (Worthington) was treated by method 2 of Ambler (1967); the amount of enzyme used is specified together with digestion times and temperatures with the individual experiments. The enzymatic reaction was stopped by adding an equal volume of ice-cold 10% trichloroacetic acid; the mixtures were then passed through Millipore filters (0.45 μ pore size), and the precipitates were washed with ice-cold 5% trichloroacetic acid. The combined filtrates were lyophilized, redissolved in a small amount of water, and again lyophilized. The residues were then dissolved in 0.5 ml of 0.2 M sodium citrate buffer (pH 2.2) and the solutions were applied to the long column of the amino acid analyzer. Analysis of appropriate controls without substrate showed that no appreciable amounts of amino acids were released from carboxypeptidase A under the specified conditions.

Acrylamide gel electrophoresis was performed in a flat bed apparatus containing a gel slab with dimensions of 10 × 15 × 0.15 cm^3. Details of this procedure will appear elsewhere (H. Wada and E. E. Snell, in preparation). After washing, the gel was equilibrated with 7.5 M urea in 1 M acetic acid (pH 3.2), the samples (20–50 μg of protein in 15–20 μl of buffer) were pipetted into the wells, and electrophoresis was carried out at 4° at constant voltage for the desired time. The gel was stained for 30–60 min in 0.25% aniline blue-black in 7% acetic acid, destained in approximately 10% acetic acid, and placed between porous cellophane sheets, which were stretched evenly across glass plates until the gel was dry. This method provides a permanent record of the experiment which can be photographed at any time.

Concentration of protein solutions was achieved either by allowing dialysis bags filled with solutions to stand in trays of carboxymethylcellulose (Aquacide, Calbiochem) followed by dialysis against buffer, or by pressure ultrafiltration with a Diaflo apparatus. With the latter method, UM-10 membranes were normally used; for proteins of molecular weight less than 10,000 (*i.e.*, chain I; see Results), UM-2 membranes were employed.

Radioactivity measurements were made by liquid scintillation counting in Bray's (1960) solution.

Results

Origin of Pyruvate in Histidine Decarboxylase. Labeled histidine decarboxylase obtained from cells grown with L-[U-^{14}C]-serine (see Methods) was acid hydrolyzed and the hydrolysate was subjected to two-dimensional chromatography. Autoradiograms of the chromatograms thus obtained showed that serine was the only radioactive, ninhydrin-positive compound present in the protein. This correlates well with nutritional data (Guirard and Snell, 1964) which showed that *Lactobacillus* 30a was auxotrophic for each of the amino acids, including both serine and glycine.

Additional labeled enzyme (8.5 mg, 6.22 × 10^6 dpm) was converted into its phenylhydrazone and then digested with pronase. The digest was evaporated under vacuum to approximately 0.4 ml and fractionated over a column of Sephadex G-25. The elution profile (Figure 1), like that reported previously (Riley and Snell, 1968), showed that extensive digestion had occurred and that the fragments containing pyruvoyl phenylhydrazone residues (as determined by the absorbance at 323 mμ) were retarded and partially purified on the column. Analyses for radioactivity showed that, in addition to several pyruvate-free peptides, all of the pyruvate-containing fragments were heavily labeled (Figure 1). The latter fractions were pooled, as indicated in Figure 1, and digested further with chymotrypsin. This treatment yields the phenylhydrazone of *N*-pyruvoylphenylalanine which can be extracted into ethyl acetate in nearly pure form (Riley and Snell, 1968). The pyruvate content of this compound was calculated from its absorbance at 323 mμ; from the pyruvate content and the radioactive count, the specific radioactivity of the pyruvate was calculated. This value was compared with the specific activity of serine isolated from the serine-rich peptides (fractions 31–33) of histidine decarboxylase. The

TABLE 1: Comparative Specific Radioactivities of Serine and Pyruvate Residues from [^{14}C]Histidine Decarboxylase and of Lactate Isolated from the Bacterial Growth Medium.

Compound Analyzed	Sp Act. (dpm/nmole)
L-[U-^{14}C]Serine added to growth medium[a]	1310
Serine isolated from digest of purified enzyme[b]	1300
Pyruvate in CH_3—C(—C_6H_5NHN)—C(=O)—Ph fragment[c]	1320
Lactate extracted from growth medium[d]	19

[a] Histidine decarboxylase (15.5 units, 0.22 mg) from cells grown on medium containing L-[U-^{14}C]serine was diluted with 23.4 mg (1650 units) of unlabeled histidine decarboxylase (see Methods), which represents a 107-fold dilution of the radioactive serine in the enzyme. Thus, the specific activity of the L-[^{14}C]serine originally added to the medium was, in effect, decreased by a factor of 107 and is expressed accordingly. [b] Fractions 31–33 (Figure 1) were pooled and aliquots containing 2.81×10^5 dpm were dried under vacuum and subjected to acid hydrolysis. The hydrolysates were analyzed qualitatively by thin-layer chromatography on silica gel with several different solvent systems (Brenner *et al.*, 1964). Autoradiography showed that the radioactivity was present in only one spot, corresponding to serine or glycine, which are not easily resolved by this method. Quantitative analyses for serine were carried out on the amino acid analyzer; in one case, fractions were collected after passage through the colorimeter and analyzed for radioactivity. Only serine was radioactive; no counts were present in glycine. [c] Portions (0.3 ml) of the ethyl acetate solution of the pyruvoyl phenylhydrazone fragment (see text) were evaporated to dryness and the residues were dissolved in 0.8 ml of 0.05 M ammonium bicarbonate. The spectrum of these solutions was identical with that of *N*-pyruvoylphenylalanine phenylhydrazone. By use of the extinction coefficient of 15,600 (Riley and Snell, 1968) and the determined radioactivity, the specific activity was calculated. [d] A portion (45 ml) of the radioactive growth medium, with cells removed, was acidified with H_2SO_4 and extracted continuously with ether for 45 hr. The extract was evaporated, an aqueous solution of the residue was passed over a small Dowex 50 column (H^+ form), and the lactic acid present in the column effluent was converted into its *p*-bromophenacyl ester (Shriner and Fuson, 1948). The identity of this compound with the *p*-bromophenacyl derivative of lactic acid was confirmed by elemental analysis, and its specific activity was determined by counting weighed aliquots. No unlabeled lactate was added to that isolated, *i.e.*, the specific activity of the isolated lactate is <0.01% that of the added serine (see footnote *a* above).

results (Table I) show that the specific activities of the serine added to the growth medium, the serine isolated from the purified enzyme, and the pyruvate in the phenylhydrazone fragment are identical, and prove conclusively that enzyme-bound pyruvate is derived from serine.

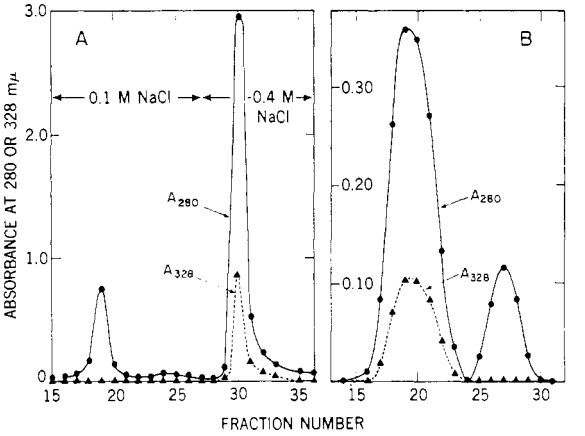

FIGURE 2: Separation of the peptide chains of histidine decarboxylase. (A) By chromatography on DEAE-cellulose. Carboxymethyl enzyme phenylhydrazone (6.5 mg in 1.7 ml) was dialyzed *vs.* 0.05 M Tris, pH 7.5, and applied to a column (1.3 × 8.0 cm) of DEAE-cellulose (Whatman DE-52), equilibrated, and washed in the cold with buffer containing 0.1 and 0.4 M NaCl. The flow rate was about 20 ml/hr; 1.8-ml fractions were collected. (B) By chromatography on Sephadex G-75. Carboxymethyl enzyme phenylhydrazone (1.1 mg in 0.3 ml of 0.05 M Tris, pH 8.0) was applied to a column (1 × 58 cm) of Sephadex G-75, equilibrated, and eluted in the cold with 0.05 M sodium phosphate, pH 7.5, at a flow rate of 2.0 ml/hr. Fractions of 1.0 ml were collected.

Pyruvate is formed as an intermediate in the fermentation of carbohydrate to lactate. That this pyruvate cannot contribute to that present in the enzyme is clear from the very low specific activity of lactate, which was isolated by ether extraction of the clarified, acidified, growth medium, and counted as its *p*-bromophenacyl ester (Table I).

Separation of the Peptide Chains in Histidine Decarboxylase. Histidine decarboxylase was first allowed to react with phenylhydrazine and then subjected to carboxymethylation (see Methods). After dialysis, the derivatized protein was applied to DEAE-cellulose and eluted with buffers of increasing salt concentration (Figure 2A). Absorbance measurements at 280 mμ show two well-separated peaks, only one of which contains pyruvate, as measured by the absorbance of the phenylhydrazone at 328 mμ. As suggested by the unequal size of the peaks, the two chains differ considerably in size, and they can also be separated by molecular sieve chromatography on Sephadex G-75 (Figure 2B). Both methods of fractionation have been used to prepare the isolated chains; with larger amounts of material, the size and capacity of the columns were increased accordingly.[1] The larger chain, eluted from Sephadex first, contains all of the pyruvate. From the total absorbance of each peak at 280 mμ and the separately determined extinction coefficients (see Methods), the ratio of the larger to the smaller chain was calculated to be 3.56:1 on a weight basis. The smaller chain, which contains no pyruvate, has been designated as chain I and the larger one as chain II. As isolated routinely, chain II is a carboxymethylated peptide phenylhydrazone. This derivative has been used for characterization except where otherwise indicated.

[1] Carboxymethyl enzyme which has not been converted into the phenylhydrazone also can be fractionated into two components by these procedures.

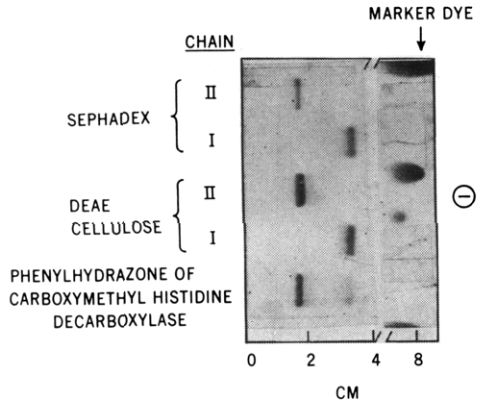

FIGURE 3: Acrylamide gel electrophoresis at pH 3.2 of the two peptide chains from histidine decarboxylase. Separate samples of the four peaks illustrated in Figure 2 were reduced in volume and dialyzed against 0.05 M sodium phosphate, pH 7.5, containing 6 M urea. The dialyzed samples were then subjected to electrophoresis for 4.5 hr at 120V. See Methods for other experimental details.

On acrylamide gel electrophoresis at pH 3.2, each chain appears pure and migrates identically whether it was obtained by DEAE-cellulose or Sephadex chromatography (Figure 3). In addition, the two chains appear in roughly the expected proportion on electrophoresis of the unfractionated carboxymethylated phenylhydrazone of histidine decarboxylase.

Absorption Spectra of Chains I and II. Chain I (Figure 4) has a complex maximum from 275 to 280 mμ but shows no absorption centering around 328 mμ where the histidine decarboxylase phenylhydrazone absorbs. The phenylhydrazone of carboxymethylated chain II (Figure 4) exhibits both maxima and also a shoulder at 290 mμ. Extinction values at 280 mμ of 12.1 and 17.2 were calculated from similar data for 1% solutions of chains I and II, respectively.

Molecular Weights and Sedimentation Coefficients of Chains I and II. Approximate values for the molecular weights of the two chains were obtained by comparing their migratory rates with those of proteins of known molecular weight on disc gel electrophoresis in the presence of sodium dodecyl sulfate by a slight modification of the method of Shapiro *et al.* (1967).

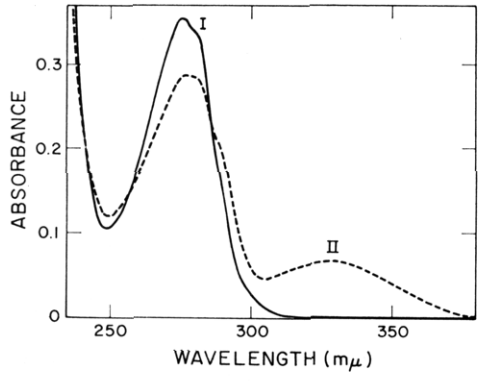

FIGURE 4: Absorption spectra of chains I and II from carboxymethylated histidine decarboxylase phenylhydrazone in 0.05 M sodium phosphate, pH 7.5. Protein concentrations were 0.27 mg/ml (chain I) and 0.17 mg/ml (chain II); measurements were made with a Cary spectrophotometer (Model 14) and 1-cm quartz cuvets.

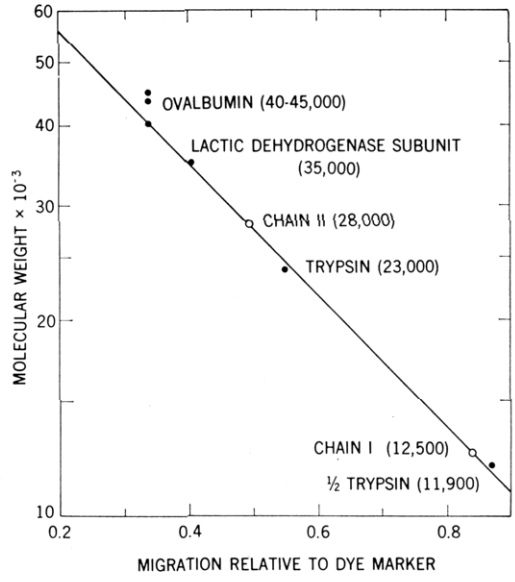

FIGURE 5: Estimation of molecular weights of chains I and II by gel electrophoresis. Samples (50 μg) were used in all cases; electrophoresis was performed at 8 V/cm for 2.5 hr in 0.075 M phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulfate.

The results (Figure 5) yield values of 12,500 for chain I and 28,000 for chain II. Since proteins with a molecular weight approaching the lower limit tested (13,900) do not migrate as far as predicted (Shapiro *et al.*, 1967), the value for chain I is probably too high.

A representative sedimentation equilibrium experiment performed at low protein concentration with the scanning

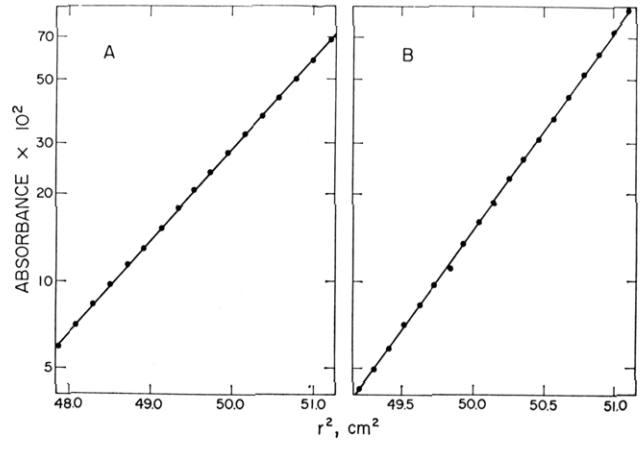

FIGURE 6: Determination of molecular weight of chains I and II by sedimentation equilibrium. (A) Low-speed determination with chain I. Initial protein concentration, 0.23 mg/ml in 0.05 M potassium phosphate–0.1 M KCl, pH 7.5; temperature, 22.4°. Equilibrium was reached within 20 hr at 36,000 rpm. After scanning, the rotor speed was increased to 68,000 rpm and the cell was scanned again after 3 hr. The latter trace was used to determine the optical base line; *i.e.*, zero concentration of protein. (B) High-speed determination with chain II. Initial protein concentration, 0.13 mg/ml in 0.05 M potassium phosphate–0.1 M KCl, pH 7.5; temperature, 23°. Equilibrium was reached within 18 hr at 30,000 rpm.

TABLE II: Amino Acid Analyses of Acid Hydrolysates of Histidine Decarboxylase and Its Subunits.

Amino Acid	Chain I (residues/9000 g)		Chain II[a] (residues/29,700 g)		Histidine Decarboxylase[b] (residues/38,800 g)	
	Best Value[c]	Nearest Integer	Best Value[c]	Nearest Integer	Best Value[c]	Nearest Integer
Pyruvate[d]	0		1.0	1		1
Lysine[e]	5.8	6	14.8	15	21.0	21
Histidine	0		2.0	2	2.1	2
Arginine	3.5	4	8.0	8	11.9	12
Half-cystine[f]	0		2.2	2	2.2	2
Aspartic acid	13.3	14	27.1	27	41.8	42
Threonine	4.5	5	12.0	12	16.8	17
Serine	4.5	5	17.5	18	22.3	22
Glutamic acid	4.7	5	30.2	30	36.0	36
Proline	2.1	2	18.2	18	20.3	20
Glycine	8.7	9	21.8	22	30.9	31
Alanine	5.6	6	24.2	24	30.3	30
Valine	5.5	6	12.2	12	18.0	18
Methionine	1.5	2	8.1	8	9.7	10
Isoleucine	5.0	5	15.2	15	20.5	21
Leucine	5.3	6	18.5	19	22.0	22
Tyrosine	4.5	5	11.0	11	15.8	16
Phenylalanine	0		9.4	10	9.4	10
Tryptophan[g]	0.9	1	6.6	7	6.2	6

[a] The sample analyzed was carboxymethyl chain II phenylhydrazone. [b] Carboxymethylated histidine decarboxylase, not further fractionated, was analyzed. Results are expressed on the assumption that a dimer composed of one chain of each type exists comprising 0.2 (mol wt 38,700) of the total molecular weight of the native enzyme. [c] Values obtained at three hydrolysis times (see Methods) were extrapolated to zero time in the case of threonine, serine, and tyrosine; 54-hr values were used for valine. Average values for the remaining amino acids are reported. Analyses of two additional independent samples of chain I for arginine gave values of 3.9 and 3.9 residues per 9000 g, indicating that all of the "best" values reported here (other than those for lysine and tryptophan) for chain I may be 10% low due to a sampling error. Application of such a correction improves agreement of best values with nearest integer values but does not change the latter values. [d] The pyruvate content was calculated from the amount of radioactivity incorporated after reaction of [^{14}C]phenylhydrazine with native enzyme (Riley and Snell, 1968). [e] Lysine was determined in separate analyses. [f] Half-cystine was determined as *S*-carboxymethylcysteine. [g] Tryptophan was determined spectrophotometrically (Goodwin and Martin, 1946).

absorption optical system of the analytical ultracentrifuge is shown in Figure 6. A series of such determinations yielded molecular weights of 9,000 ± 400 and 29,700 ± 2,000 for chains I and II, respectively; these values have been used in subsequent calculations. In addition, the molecular weight of native histidine decarboxylase (190,000), previously determined by use of interference optics at high protein concentrations (initial concentration, 1.0 mg/ml), was confirmed for the lower protein concentrations (initial concentration, 0.1 mg/ml) used with the absorption optics. Sedimentation velocity measurements gave values for $s_{20,w}$ of 0.8 S for chain I and 1.8 S for the carboxymethylated chain II phenylhydrazone.

Amino acid analyses at three different times of hydrolysis (Table II) show that the two chains differ considerably in composition. Chain I contains no histidine, half-cystine, or phenylalanine. The number of residues of any given amino acid in chain I plus chain II agrees well with the value calculated from independent analysis of the native enzyme when expressed on an equal weight basis (Table II). Since the molecular weights of chain I plus II (38,700) is very nearly one-fifth that of the native enzyme, these data are consistent with the presence of five chains of each type per molecule of native enzyme. The analysis of the unfractionated histidine decarboxylase (Table II) agrees well with the independent analysis reported by Chang and Snell (1968b) for a different sample of this enzyme. In addition, when the residue weights of the nearest integral values of each of the component amino acids are summed, chemical molecular weights of 8,970 and 27,700 (omitting any amide groups) are obtained for chains I and II, respectively. These values are in good agreement with those determined by sedimentation equilibrium measurements.

Carboxyl-Terminal Residues of Chains I and II. Digestion of chain I with carboxypeptidase A results in the rapid release of serine and threonine and a slower release of alanine and other amino acids (Figure 7A). Additional experiments (Table III) showed that serine was released at a much faster rate than threonine during the early course of the digestion. Since carboxypeptidase A is known to release serine at a slower rate than threonine from the carboxyl terminus of a peptide chain (Ambler, 1967), we conclude that serine is the

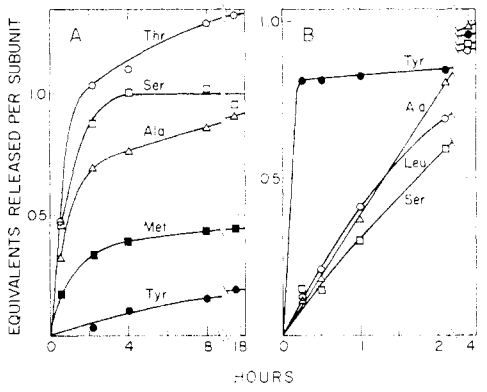

FIGURE 7: Rates of release of amino acids from chains I and II of histidine decarboxylase on digestion with DIP-carboxypeptidase A. (A) chain I: 0.92 mg of protein was dissolved as described in Methods and incubated with 0.13 mg of carboxypeptidase at 37°. (B) Chain II: 2.67 mg of protein was digested at 23° with 0.07 mg of carboxypeptidase.

carboxyl-terminal amino acid of chain I. The observation that exactly 1 equiv of serine (Figure 7A) is released supports the value of 9000 for the molecular weight of chain I.

Digestion of chain II with carboxypeptidase results in the rapid release of nearly 1 equiv of tyrosine and the much slower release of leucine, alanine, and serine (Figure 7B). Thus, tyrosine is the carboxyl-terminal amino acid of chain II. More than 1 equiv of tyrosine is released upon longer digestion; this excess, together with the small amount released from chain I, accounts for most of the tyrosine released by carboxypeptidase from the unfractionated carboxymethyl enzyme (Table III; *cf.* Chang and Snell, 1968b).

N-Terminal Amino Acids of Chains I and II. Samples of chain I (2.0 mg) and chain II (5.9 mg) were dinitrophenylated by the procedure of Fraenkel-Conrat *et al.* (1955). Residues from the ether extracts of the acid-hydrolyzed samples were sublimed at 50° for 1 hr to remove free dinitrophenol and then chromatographed on silica gel layers with water-saturated 1-butanol as the solvent system. Dinitrophenylserine was obtained from chain I and was further identified by co-chromatography with a [^{14}C]dinitrophenylserine standard; no dinitrophenylamino acid was obtained from chain II. These results are consistent with previous studies (Chang and Snell, 1968a; Riley and Snell, 1968) which showed that pyruvate occupied an N-terminal position in histidine decarboxylase, and that dinitrophenylserine was also obtained in low yield from this enzyme.

Attempts to Reversibly Dissociate Histidine Decarboxylase. Native histidine decarboxylase is remarkably resistant to urea denaturation (Chang and Snell, 1968b), and mild procedures for its dissociation into two chains have not been found. However, after reaction with 5,5′-dithiobis(2-nitrobenzoic acid) in dilute guanidine solution, the two chains could be separated by Sephadex filtration. The conditions were as follows. A solution (1.2 ml, pH 7.6) containing 6 mg of native enzyme, 0.012 mmole of EDTA, 2.4 mmoles of Gd·HCl,[2] 0.30 mmole of Tris-HCl, 0.07 mmole of ammonium acetate, and 0.0012 mmole of 5,5′-dithiobis(2-nitrobenzoic acid) was incubated at 45°. The expected absorbance (96%) at 412 mμ appeared in 15 min. The reaction mixture was then applied to a column (1.5 × 85 cm) of Sephadex G-75, equilibrated, and eluted in the cold with 0.05 M potassium phosphate buffer, pH 7.5. The elution profile was similar to that shown in Figure 2B, and the two separated fractions that were obtained, after concentration by ultrafiltration, each gave a single band on acrylamide gel electrophoresis migrating almost identically with bands I and II of Figure 3. Neither chain exhibited enzymatic activity, and no activity was generated on allowing chain II (which contains both half-cystine residues) to stand for 15–60 min at pH 7.5 with β-mercaptoethanol (1–1.7 mM); this treatment was shown by appearance of absorbance at 412 mμ to remove the 1-thio-4-nitrobenzene groups. Several attempts were made to regenerate decarboxylase activity from stoichiometric amounts of chain I and mercaptoethanol-treated chain II, both in the absence and presence of 2 M Gd·HCl; in no case was any activity observed.

[2] Gd·HCl = guanidine hydrochloride.

TABLE III: Digestion of Histidine Decarboxylase and Its Subunits with Carboxypeptidase A.[a]

Substrate	Temp (°C)	Time (hr)	Residues Released (Moles per Equivalent Weight of Protein)[b] Ser	Thr	Tyr
Chain I	23	0.25	0.11	0.01	
Chain I	37	0.25	0.16	0.09	
Chain I	37	17.7			0.19
Chain II	37	15.3			1.17
Carboxymethylhistidine decarboxylase	37	8			1.71

[a] Digestion and analysis were carried out as described in Methods. The molar ratio of carboxypeptidase to chain I (mol wt 9000) was 1:27; to chain II (mol wt 29,700), 1:18; and to carboxymethylhistidine decarboxylase (mol wt 190,000), 1:2.8. [b] Equivalent weights of chain I, chain II, and carboxymethylhistidine decarboxylase were considered to be 9,000, 29,700, and 38,700 g, respectively. The latter value corresponds to 0.2 of the molecular weight of histidine decarboxylase.

Discussion

We previously showed (Riley and Snell, 1968) that pyruvate in histidine decarboxylase was linked through an amide bond to a phenylalanyl residue of a peptide chain, and that approximately 5 equiv of such groupings were present per mole (190,000 g) of enzyme. The present results show (a) that the bound pyruvate arises entirely from serine by a process which does not permit equilibration with pyruvate arising as an intermediate in the lactic acid fermentation carried out by *Lactobacillus* 30a; (b) that this pyruvate is present in only one of two very different peptide chains that can be separated from the enzyme; and (c) that the pyruvate-containing chain has no free N-terminal group so that the pyruvoylphenylalanyl residue must be present at the N-terminus of an unbranched peptide chain.

It is unlikely that enzyme-bound pyruvate arises initially from free serine through the action of a serine dehydratase: this would require that the pyruvate formed in this way be rigorously maintained in a pool distinct from the pyruvate that arises from carbohydrate. A more likely possibility would involve dehydration of a serine residue of a macromolecule; *e.g.*, (a) of a seryl-tRNA to yield pyruvoyl-tRNA which could then be incorporated at the N terminus of a peptide chain, (b) of a preformed peptide chain to yield a pyruvoyl residue, either directly (if an N-terminal serine were involved), or by formation of a dehydroalanyl residue with subsequent chain cleavage (if an internal serine residue is involved). We hope to study this process further.

Before the presence of pyruvate in histidine decarboxylase had been established, Chang and Snell (1968b) reported that this enzyme had a molecular weight of 190,000, and was composed of ten subunits of similar size and composition with N-terminal serine (obtained in very low yield) and C-terminal tyrosine. The discovery that only five pyruvate residues were present per enzyme molecule (Riley and Snell, 1968) showed that the subunits could not be identical. The present results show that the two types of subunits present are in fact very different: chain I has a molecular weight of only about 9000; contains no pyruvate, cysteine, histidine, or phenylalanine residues; and serine is present at both the N- and C-terminal positions. Chain II has a molecular weight of about 29,700, contains a complete assortment of amino acids, has pyruvate at the N-terminal end, and tyrosine as the C terminus.

From the weight ratio of chain II to chain I (3.46:1.0, as calculated from their extinction coefficients after separation on Sephadex) and their molecular weights, 5.0 moles of chain II and 4.7 of chain I would be present per mole (190,000 g) of histidine decarboxylase. This stoichiometry agrees well with the observation that there are 5 moles of pyruvate/mole of enzyme (Riley and Snell, 1968), and also agrees with the results of amino acid analyses of the separated subunits and of the complete enzyme (Table II).[3]

In addition, the extinction coefficients of chain I ($\epsilon^{1\%}_{280\,m\mu}$ 12.1) and chain II ($\epsilon^{1\%}_{280\,m\mu}$ 17.2) give a calculated extinction of 16.1 for a 1% solution containing equimolar amounts of the two chains. An experimental value of 16.1–16.2 for native histidine decarboxylase was reported previously (Riley and Snell, 1968).

The possibility was considered that chains I and II may be artifacts arising by partial proteolysis of a single-chain enzyme during the preparation and prolonged buffer extraction of acetone-dried cells employed in the purification procedure. To test this possibility, a sample of histidine decarboxylase was purified from sonic extracts of fresh cells (see Methods). It proved identical in specific activity and molecular weight to the enzyme prepared from acetone-dried cells, and yielded both chains I and II on carboxymethylation and acrylamide gel electrophoresis.

[3] Reexamination of the earlier data (Chang and Snell, 1968b) in the light of present knowledge shows that while the molecular weight of the native enzyme was correct, the value of 19,000 for the molecular weight of the dissociated carboxymethyl enzyme obtained by sedimentation equilibrium measurements was incorrect; concavity in the plot of log C *vs.* X^2 near the top of the solution (a result expected from the presence of chain I) was overlooked. Their finding of N-terminal serine and C-terminal tyrosine agrees with present data (Table III). The number of ninhydrin-positive spots observed after "fingerprinting" tryptic digests of carboxymethylhistidine decarboxylase was lower than expected for nonidentical subunits; however, several peptides which stained only weakly with ninhydrin were discounted as products of incomplete digestion or cleavage at residues other than lysine or arginine. The number of bands observed on disc gel electrophoresis of cyanogen bromide cleaved protein was also low for nonidentical subunits; the presence of small peptides not stained by Amido Black and of insoluble peptides is thought to explain the discrepancy.

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