Histidine Decarboxylase from *Lactobacillus* 30a: Reconstitution from Separated Subunits†

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ABSTRACT: The α and β subunits of histidine decarboxylase from *Lactobacillus* 30a can be completely separated by first incubating the enzyme in 5 M guanidine hydrochloride at 37 °C and pH 7.4 and then chromatographing over Sephadex G-75 in the presence of 1 M guanidine hydrochloride. Neither subunit shows catalytic activity alone, but such activity was reconstituted in yields up to 40% when an equimolar mixture of the two subunits in 1 M guanidine was dialyzed against 0.2 M ammonium acetate at 4 °C. Both subunits must be present during the folding process; no active enzyme was formed when the two separated subunits were separately dialyzed and then mixed. The optimum pHs for reconstitution (pH 5.5) and for catalytic activity (pH 4.8) differ. Reconstituted enzyme was purified by chromatography on Sephadex G-200 and proved identical with native enzyme as judged by specific activity, elution volume, subunit composition, and affinity for L-histidine and several of its analogues. The same procedures for dissociation and reconstitution were applied to histidine decarboxylase obtained by incubating the inactive, homogeneous prohistidine decarboxylase from mutant 3 of *Lactobacillus* 30a at pH 7.6. Within experimental error, the reconstituted mutant enzyme and hybrid enzymes reconstituted with subunits obtained from the wild type and the mutant enzyme were identical in catalytic, kinetic, and electrophoretic behavior.

Histidine decarboxylase from *Lactobacillus* 30a appears to contain five pairs of two dissimilar subunits and can thus be represented as $\alpha_5\beta_5$ (Riley & Snell, 1970; Snell, 1977). The larger subunit (α, mol wt 28000) contains an essential pyruvoyl group in amide linkage at its N terminus and contains two –SH groups, one of which also appears essential for activity as judged by derivatization with the bulky thionitrobenzoate group (Lane & Snell, 1976). These subunits arise through cleavage and modification of a precursor subunit (π, mol wt 37000) by a process still not understood (Recsei & Snell, 1973; Snell et al., 1976). Although separation of the two types of subunits is readily effected from a derivatized enzyme (Riley & Snell, 1970), previous attempts to reconstitute an active enzyme from them have not been successful. We describe here a procedure for separation of the unmodified α and β subunits and for reconstitution of fully active histidine decarboxylase from them. The procedure will permit detailed studies of the relationship of subunit interaction and subunit structure to activity of this enzyme.

Experimental Procedure

Materials. A twice-crystallized preparation of histidine decarboxylase was obtained from *Lactobacillus* 30a as described by Chang & Snell (1968). Prohistidine decarboxylase from mutant no. 3 of this organism was purified through the third chromatography over Sephadex G-200 (Recsei & Snell, 1973) and was kept in a refrigerator in the presence of 50% saturated ammonium sulfate until use. UltraPure guanidine hydrochloride (Schwarz/Mann) was used throughout.

Enzyme activity was measured manometrically at 37 °C with a Gilson differential respirometer. Reaction mixtures contained (in 3 mL, pH 4.8) 0.2 M ammonium acetate, 0.1% bovine serum albumin, and 10 mM L-histidine hydrochloride. After 10-min equilibration, the reaction was started by adding enzyme and was monitored at 2-min intervals for 10 min.

Electrophoresis of $NaDodSO_4$-treated samples was carried out on slabs ($10 \times 16 \times 0.075$ cm^3) of 10% polyacrylamide (Laemmli, 1970) at room temperature and a constant current of 20 mA and continued for about 5 h at pH 8.8. Protein was stained with Coomassie blue according to Fairbanks et al. (1971). To check the purity of preparations and to compare the mobilities of hybrid enzymes, electrophoresis in the absence of $NaDodSO_4$ was carried out on similar slabs of acrylamide gel at a concentration of 7.5% and a pH of 6.6 (Nagai, 1967) or 9.4 (Ornstein & Davis, 1964).

Gel filtration was carried out at room temperature on Sephadex columns previously equilibrated with the developing buffer, as described in the figure legends.

Protein concentrations were determined by the method of Lowry et al. (1951) by using bovine serum albumin as the standard.

Results

Dissociation of Histidine Decarboxylase. Following 30-min treatment with 5 M guanidine hydrochloride at 37 °C, the two subunits present in homogeneous histidine decarboxylase could be readily separated by filtration over Sephadex G-75 (Figure 1). Fractions 41–55, which contain the heavy chain (chain II of Riley & Snell, 1970, designated here as the α subunit), were combined, as were fractions 65–80, which contain the light chain (chain I or β subunit). In accordance with this assignment, the α subunit, but not the β subunit, reacted with phenylhydrazine to give a phenylhydrazone (absorbance ratio, A_{328}/A_{280}, of 0.214 (cf. Riley & Snell, 1968)). To avoid precipitation of protein on storage at 4 °C, dithiothreitol and guanidine were added to each solution to give final concentrations of 1 mM and 2.0 M, respectively. Concentrations of subunits α and β were determined to be 25.2 and 31.1 μM, respectively, on the assumption that they have molecular weights of 28000 (α) and 9000 (β), as reported by Riley & Snell (1970).

Reconstitution of Active Enzyme. Neither of the isolated subunits exhibited histidine decarboxylase activity, even after dialysis against 0.2 M ammonium acetate buffer, pH 4.8. Mixtures of the two dialyzed solutions also were inactive. However, some catalytic activity was regenerated when a

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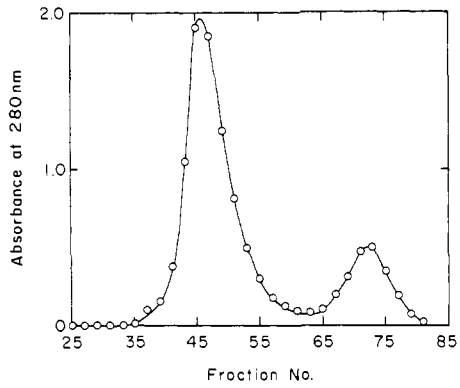

FIGURE 1: Isolation of subunits following dissociation of histidine decarboxylase by guanidine hydrochloride. A stock solution of homogeneous histidine decarboxylase (34 mg of protein) was made 75% saturated with respect to ammonium sulfate. The precipitated protein was centrifuged out and dissolved in 3 mL of a solution containing 5 M guanidine hydrochloride, 0.2 M Tris-HCl, 1.5 mM DTT, and 1 mM EDTA, pH 7.4. After 30 min at 37 °C, this reaction mixture was subjected to chromatography on a column (2.3 × 4.7 cm) of Sephadex G-75 at room temperature. The elution buffer contained 1 M guanidine hydrochloride, 0.05 M potassium phosphate, 0.5 mM DTT, and 1 mM EDTA (pH 6.6). Elution velocity was 0.4 mL/min; 1.8-mL fractions were collected.

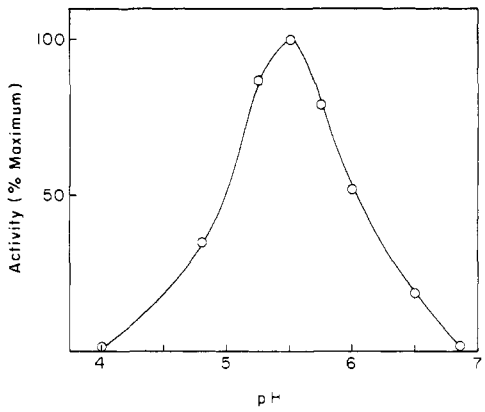

FIGURE 2: Optimal pH for reconstitution of histidine decarboxylase from its subunits. Equimolar mixtures of subunits α and β (each 1.5 μM; volume 1.0 mL) were dialyzed overnight at 4 °C against 500 mL of 0.2 M ammonium acetate buffer of various pHs containing 0.5 mM DTT and 1 mM EDTA. The maximum activity obtained was about 27 units/mg of protein.

mixture of the two subunits in 2 M guanidine was dialyzed against this buffer. The presence of both subunits during the refolding process is thus required for the reconstitution of an enzymatically active protein.

The results of Figure 2 show that maximal activity was reconstituted when dialysis was conducted at pH 5.5, a pH substantially above that optimal for catalytic activity (pH 4.8). A temperature of 4 °C was ascertained to be optimal for reconstitution; no activity was recovered when dialysis was carried out at 37 °C, and only low activity was recovered at room temperature.

To determine the optimum composition ratio for reconstitution, mixtures of the two subunits, in which the concentration of subunit α was fixed, were dialyzed overnight against 1 L of 0.2 M ammonium acetate buffer, pH 5.5, containing 0.5 mM dithiothreitol and 1 mM EDTA. Extrapolation of the linear portions of curve A indicates that the two subunits interact in a 1:1 ratio to regenerate the active enzyme. Similar results were obtained in the converse experiment in which the concentration of β was constant and that of α was varied.

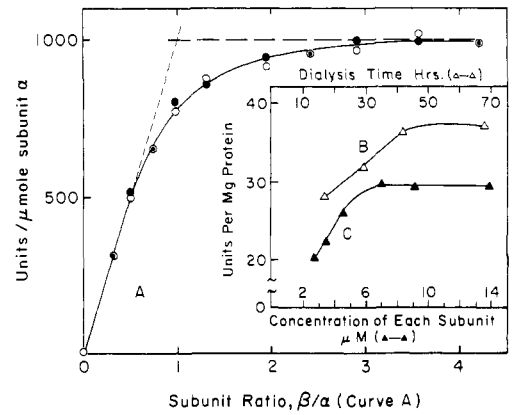

FIGURE 3: Reconstitution of histidine decarboxylase from its subunits as a function of subunit ratio (curve A), dialysis time (curve B), and concentration (curve C). For curve A, the concentration of the α subunit was 7.0 μM and that of the β subunit was varied from 2.27 μM to 29.5 μM in 2.0 mL of the mixture to give the indicated ratios. The volume of the solution to be dialyzed was adjusted to 2.0 mL in each case by addition of the elution buffer (see Figure 1). Dialysis was at 4 °C. Open and closed symbols show the results obtained with two independently dialyzed solutions. For curve B, 2.5-mL portions of a mixture of α and β subunits (each 11.3 μM) were dialyzed for the indicated times at 4 °C against 1 L of 0.2 M ammonium acetate buffer (pH 5.5) containing 0.5 mM DTT and 1 mM EDTA. All points are averages of two dialyzates, each assayed in triplicate. For curve C, 1.0 mL of the properly diluted solutions of subunits in equimolar ratio was dialyzed overnight at 4 °C against 1 L of 0.2 M ammonium acetate buffer (pH 5.5) containing 0.5 mM DTT and 1 mM EDTA. All points are averages of two dialyzates, each assayed in duplicate.

Since the enzyme activity obtained in the above experiment was considerably below that of an equivalent concentration of native enzyme, further characterization of the reconstitution reaction was required. Variation in the time of dialysis (curve B, Figure 3) at 4 °C showed that longer dialysis times, up to 40 h, increased the yield of active enzyme under these conditions. Simultaneous variation in the concentration of α and β subunits showed a constant yield of active enzyme (curve C, Figure 3) above about 6 μM.

Since none of these experiments gave the theoretically possible yield of decarboxylase activity, it was important to determine whether reconstitution was simply incomplete or whether an altered configuration of an $\alpha\beta$ complex was responsible for the low yield. For this purpose, an equimolar mixture of the α (15 mL, 22.4 μM) and β (13 mL, 26.0 μM) subunits in 2 M guanidine was dialyzed for 16 h against 2 L of 0.2 M ammonium acetate–0.5 mM dithiothreitol–1 mM EDTA buffer at pH 5.5. The dialyzed protein solution was concentrated with carboxymethylcellulose (Aquacide II A, Calbiochem) and subsequently dialyzed against 1 L of 0.2 M ammonium acetate buffer, pH 4.8. Precipitates which appeared during the dialysis were removed by centrifugation. The final volume was 6.4 mL, 4.0 mL of which was chromatographed on a Sephadex G-200 column as described in the legend to Figure 4. Three protein peaks appeared, of which only the first showed catalytic activity. The elution volumes (from left to right) of these peaks correspond to those of native enzyme, α subunit, and β subunit, respectively. Fractions 61–70 of peak 1 from this column (Figure 4) were combined and concentrated by ultrafiltration to about 3 mL. A portion of this reconstituted enzyme was subjected to $NaDodSO_4$ electrophoresis together with standards of native enzyme and of the α and β subunits. The results (Figure 5) show that reconstituted and native enzyme are indistinguishable.

The specific activity of the reconstituted enzyme was the same as that of native enzyme (64.5 and 66.2 units/mg of

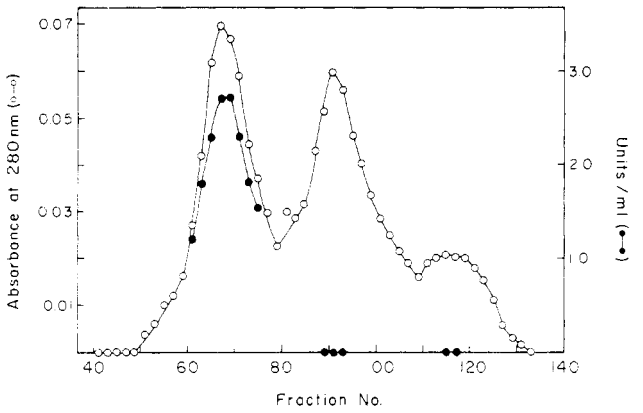

FIGURE 4: Separation of reconstituted histidine decarboxylase from residual subunits. The concentrated reconstitution mixture (4.0 mL, see text) was subjected to chromatography on a Sephadex G-200 column (2.4 × 50 cm) equilibrated with 0.2 M ammonium acetate buffer (pH 4.8). Fractions of 1.8 mL (per 5 min) were collected. Histidine decarboxylase activity was measured by using 0.3 mL of each fraction.

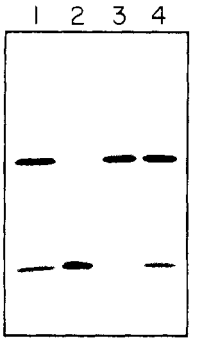

FIGURE 5: Electrophoresis on $NaDodSO_4$–polyacrylamide gel of native enzyme (1); subunit β (2); subunit α (3); and reconstituted enzyme (4). Two micrograms of each protein was applied. A constant current of 20 mA was applied at pH 8.8 and room temperature for 5 h. The concentration of acrylamide was 10%.

protein, respectively) within experimental error. Curves relating pH to activity were also the same within experimental error for reconstituted and native enzyme. A comparison of certain kinetic parameters of the reconstituted decarboxylase with those newly determined or previously reported for the native enzyme by Rosenthaler et al. (1965, values in italics) gave the following results: K_M for L-histidine, 0.7 and *0.9* mM; K_I for imidazole, 3.0 and *3.2* mM; urocanic acid, 1.6 and *2.1* mM; guanidine, 20 and 18 mM.

We conclude that there are no significant differences between the reconstituted and the native enzyme that are detectable by any of these criteria.

Dissociation and Reconstitution of Activated Prohistidine Decarboxylase. A catalytically inactive prohistidine decarboxylase, obtained from mutant 3 of *Lactobacillus* 30a (Recsei & Snell, 1973), contains a single class of subunits designated π (mol wt 37 000) and is converted by incubation above pH 7.0 to an active histidine decarboxylase in a process best represented (Recsei & Snell, 1973; Snell et al., 1976) by the equation $\pi_5 + 5H_2O \rightarrow \alpha_5\beta_5 + 5NH_3$. The procedures already described for dissociation and reconstitution of the "wild type" histidine decarboxylase, ${}^{w}\alpha_5{}^{w}\beta_5$, were applied to the activated "mutant" proenzyme, ${}^{m}\alpha_5{}^{m}\beta_5$, with similar results. Unlike the proenzyme itself, which elutes as a single band in 1 M guanidine after being subjected to the dissociation procedure in 5 M guanidine, the activated proenzyme, like the wild type enzyme, dissociated in 5 M guanidine, and the separated subunits reassociated with an optimum pH of 5.5 to yield an active enzyme. Histidine decarboxylase was reconstituted from four different mixtures of subunits: ${}^{w}\alpha{}^{w}\beta$, ${}^{w}\alpha{}^{m}\beta$, ${}^{m}\alpha{}^{w}\beta$, and ${}^{m}\alpha{}^{m}\beta$. The reconstituted enzymes were separated from residual subunits in the manner described earlier (see Figure 4) and the concentrated fractions analyzed for specific activity (low 49.9, high 55.3 units/mg of protein), mobility to the anode on acrylamide (7.5%) gel electrophoresis at pH 6.6 (low 4.2, high 4.3 cm in 6 h), and K_m for L-histidine (low 0.63, high 0.73 mM). No meaningful differences in these properties were observed among the four preparations, a result perhaps predictable from the fact that fully activated proenzyme has the same catalytic activity as wild type enzyme (Recsei & Snell, 1973).

Discussion

Although histidine decarboxylase is synthesized as a single peptide chain, its activation according to available information (Recsei & Snell, 1973; Snell et al., 1976; Snell, 1977) does not involve removal of any segment of the parent chain; only chain cleavage and conversion of a single serine residue to a pyruvate residue occur. It seems probable, therefore, that activation would result in very little loss in refolding information provided by the peptide sequence per se, although some loss in efficiency of refolding is to be expected, since two independent chains, rather than two segments of a single chain, must interact appropriately. Regeneration of enzymatic activity in 27–42% yield is obtained by the procedure described here, and the active entity appears identical with the native enzyme. Although the procedure presented is the best so far devised, further improvements in yield should be possible. Renaturation of other enzymes to active conformations is frequently favored by substrates or substrate analogues (Wetlaufer & Ristow, 1973). Although this possibility has not been tested specifically, guanidine itself may play this role, in addition to that (at higher concentrations) of denaturant, since it acts as a competitive inhibitor (K_I = 20 mM) of decarboxylase action.

A number of miscellaneous observations are of interest in connection with the dissociation–reassociation reaction described here: (1) dissociation of enzyme in 5 M guanidine at 37 °C followed by chromatography over Sephadex in 1 M guanidine at 4 °C gave incomplete separation of subunits; complete separation was obtained only when an chromatography step was carried out at room temperature; (2) reassociation of separated subunits to active enzyme was best at 4 °C; yields were very poor at room temperature and no active enzyme was formed at 37 °C; (3) refolding to active enzyme did not occur when the α and β subunits were separately dialyzed against buffer and then mixed in the buffer solution, indicating the necessity for at least a partially extended conformation of one or both subunits for appropriate refolding; (4) when β (light) subunit was dialyzed free of guanidine and then added to a 2 M guanidine solution of an α subunit, active enzyme was formed on dialysis; in the converse experiment, no activity was obtained.

These observations should permit future studies in which the effect of modifying the structure of the β chain on its ability to form active enzyme with unmodified α chain can be determined. For example, preliminary studies show that a β chain modified by incorporation of one pyridoxyl group reconstitutes an active enzyme with added α chain, although less efficiently than the unmodified β subunit.

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Catalase of *Neurospora crassa*. 1. Induction, Purification, and Physical Properties†

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ABSTRACT: We have purified to homogeneity a catalase found in extracts of *Neurospora crassa* 5297a that had been induced for nitrate reductase in a nitrate-supplemented medium. The activity is stabilized in extracts by the protease inhibitor phenylmethanesulfonyl fluoride. The enzyme has an apparent molecular weight of 3.2×10^5 and is composed of four subunits of 8×10^4 molecular weight. The catalytic constant for H_2O_2 dismutation, at pH 6.8 and 25 °C, is 4.6×10^6 M^{-1} s^{-1}, and the activation energy is 7.2 ± 0.4 kcal/mol. The protein exhibits no peroxidase activity with guiacol as the substrate. The enzyme is not reducible by sodium dithionite. The inhibitory effect of KCN, NaN_3, NaF, Na_2SO_3, KNO_2, and KNO_3 on enzyme activity at pH 7 was studied; cyanide and azide were found to be strong inhibitors of activity. Though the protein is homogeneous according to ultracentrifugation and electrophoresis, the iron content averaged 3.4 atoms of Fe/molecule, suggesting that, in common with bovine liver catalase, the isolated protein does not carry a full complement of heme groups. The electronic spectrum exhibits maxima at 280 (1.1×10^5), 400 (8.2×10^4), 590 (1.7×10^4), and 712 nm (3.8×10^3) [absorbancy per mole of iron is given in parentheses]. The inducibility of the enzyme in the presence of nitrate, its molecular weight, and its electronic spectrum all distinguish this catalase from previously reported catalases. Antibodies raised to homogeneous samples of the enzyme were used in Ouchterlony and double-antibody radioimmunoassays to indicate that a minimum of 75% of the catalase induced in the presence of nitrate is identical with the newly identified catalase. Thus, the presence of nitrate in growth media for *N. crassa* initiates a series of events including synthesis of nitrate reductase, synthesis of catalase apoprotein, and probably the synthesis of the enzymes responsible for the production of the prosthetic group.

While attempting to purify the enzyme nitrate reductase from *Neurospora crassa*, we found that the addition of phenylmethanesulfonyl fluoride, a protease inhibitor, to buffers used in purification prevents inactivation of the enzyme (Jacob, 1976). This has led to the discovery and isolation of a green protein subsequently determined to be a catalase. This catalase is induced by nitrate as is nitrate reductase. Although catalases from a number of sources have been rather thoroughly studied, the inducibility as well as the unique color of the *N. crassa* enzyme prompted us to investigate it. The present communication describes its purification to homogeneity, subunit structure, and physical and catalytic properties and indicates that the enzyme contains an unusual heme prosthetic group.

Subramanian et al. (1968) previously reported that the level of catalase in nitrate-induced mycelia increased until a maximum was reached 7 h after transfer to the induction medium. It was suggested by these authors that induction was a consequence of increased intracellular peroxide produced by the interaction of oxygen with the flavin component of nitrate reductase. This explanation was supported by their report that *Neurospora* grown in ammonia medium containing peroxide also exhibited elevated catalase levels. We report here the results of immunochemical experiments to determine whether the catalase reported in this communication is the one responsible for the increased catalatic activity observed in nitrate-induced *N. crassa*.

Materials and Methods

Materials. Spores of *N. crassa* 5297a were obtained from the Fungal Genetics Stock Collection, Humbolt College, Arcata, CA. Bovine serum albumin, carboxypeptidase, glutamate dehydrogenase, phosphorylase *a*, beef liver catalase, and phenylmethanesulfonyl fluoride were obtained from Sigma. *N. crassa* nitrate reductase was purified by the method of Jacob (1976). Purified agar and Freund's adjuvants were obtained from Difco. Carrier free $Na^{125}I$ in 0.1 N NaOH, from New England Nuclear, and anti-rabbit IgG serum, obtained from immunized goats, were generous gifts from Dr.

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