The Pyruvate-Aspartic Semialdehyde Condensing Enzyme of Escherichia coli*

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SUMMARY

The pyruvate-aspartic semialdehyde condensing enzyme, which occurs at the branch point in the aspartic acid family of amino acids leading to diaminopimelic acid and lysine biosynthesis, has been purified 5000-fold from crude extracts of Escherichia coli W. The protein has been shown to be homogeneous by polyacrylamide gel electrophoresis and of amino acids leading to diaminopimelic acid and lysine (2). In agreement with this conclusion is the observation that the enzyme, in Escherichia coli, can be inhibited by lysine thus providing feedback regulation for this branch of the aspartic acid family of amino acids (3).

Previous studies from this laboratory have shown the presence in bacterial extracts of an enzyme that catalyzes the condensation of aspartic semialdehyde with pyruvate (1). A variety of evidence supports the conclusion that the enzyme participates in the biosynthetic pathway leading to diaminopimelic acid and lysine (2). In agreement with this conclusion is the observation that the enzyme, in Escherichia coli, can be inhibited by lysine, thus providing feedback regulation for this branch of the aspartic acid family of amino acids (3).

The product of the enzymatic condensation was found to be labile, severely limiting efforts for its thorough characterization. Nevertheless, several experiments have led to the tentative assignment of 2,5-dihydrodipicolinic acid as a product of the condensation. This molecule would be expected to be in equilibrium with 2,3-dihydrodipicolinic acid and 4-hydroxy-Δ1-tetrahydrodipicolinic acid (Fig. 1). It is not clear which of these 3 molecules is the immediate product of the enzymatic reaction. Moreover, these experiments were performed with partially purified preparations of the enzyme. Consequently, it was not certain whether more than one enzyme was responsible for producing the heterocyclic molecule. Before undertaking detailed studies on the mechanism of the enzymatic reaction and the nature of its product, it was deemed important to obtain a pure preparation of the enzyme.

This paper presents the purification of the condensing enzyme to the point of homogeneity, a study of some of its molecular parameters, and some preliminary observations on its mechanism in an effort to gain some insight into this complex reaction system.

EXPERIMENTAL PROCEDURE

Materials
14C-Pyruvate and tritiated water were purchased from New England Nuclear; sodium borohydride and α-aminobenzaldehyde were from K and K Laboratories. Lysine was obtained from Nutritional Biochemicals; α-ketobutyrate was from Aldrich. Dowex resins were from Bio-Rad. All inorganic chemicals used were of reagent grade with the exception of ammonium sulfate, which was an enzyme grade product of Mann.

Horse heart cytochrome c (type II) and yeast alcohol dehydrogenase (crystalline) were purchased from Sigma; bovine liver catalase (crystalline suspension) was from Worthington. Dextran blue was purchased from Pharmacia.

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Coomassie brilliant blue, L amino acid oxidase from Neurospora crassa, and ε-N-carbobenzoxy-L-lysine were gifts from Dr. Bruce Alberts, Dr. Paul Shapshak, and Mr. Louis Lebetkin, respectively. ε-N-(1-Carboxyethyl)-L-lysine and α-N-(1-carboxyethyl)-L-lysine were the generous gifts of Dr. Leslie Hellerman.

Sources of other chemicals are cited in the text.

Methods

Chemical Syntheses—The synthesis of ε-N-(1-carboxyethyl)-L-lysine was performed by the method of Rosso and Adams (4). Poly-L-lysine (Pilot Chemicals, Inc.), 210 mg, was dissolved in 2 ml of water to which were added 2.5 mmoles of 2-bromopropionic acid (Eastman). This mixture was adjusted to pH 8.0 with 10 N KOH. After maintaining the solution for 12 hours at 50°, another 2.5 mmoles of 2-bromopropionic acid were added and the pH was again raised to 8.0 with 10 N KOH. The reaction was judged complete, after an additional 2 hours, by the absence of blue color in spot testing with ninhydrin spray. The mixture was extremely viscous at this point.

The reaction mixture was diluted 5-fold with water and dialyzed overnight against 200 volumes of distilled water. The dialyzed material was taken to dryness under reduced pressure, and the residue was dissolved in 1.0 ml of 6 N HCl and heated for 6 hours at 120°. The hydrolysate was taken to dryness several times under reduced pressure, after redissolving in water. The final residue was resuspended in 1.0 ml of water and applied to a Dowex 50 (H+ form) column (0.5 x 33 cm), which was developed with an exponential acid gradient (1 N HCl in the reservoir and 1 liter of water in the mixing chamber).

Two peaks of ninhydrin-positive material were eluted from the column (Fig. 2). The material eluting between 125 and 140 ml (Peak II) was identified as ε-N-(1-carboxyethyl)-L-lysine by the following criteria. (a) The ratio of color yields of the peak fractions in the ninhydrin blue assay of Moore and Stein (5) (color yield: 1.07, compared to leucine) and the lysine-specific assay of Shimura and Vogel (6) (color yield: 0.85, compared to lysine) corresponded to that for the authentic compound. (b) Peak fractions had the same RF as authentic compound in the following solvent systems: 0.31, ethanol-water (77:33) v/v; 0.10, n-butyl alcohol-acetic acid-water (4:1:5) v/v; 0.51, n-propyl alcohol-ammonia-water (6:3:1) v/v. (c) When treated with L-amino acid oxidase from Neurospora crassa, 1 eq of oxygen was taken up per eq of amino group. A control experiment with lysopine1 revealed no uptake of oxygen. Peak II fractions were pooled, concentrated by evaporation under reduced pressure, and crystallized several times from 95% ethanol by addition of approximately 0.5 volume of pyridine. No attempt was made to resolve diastereomers. A 40% yield of ε-N-(1-carboxyethyl)-L-lysine was obtained.

The compound eluting between 110 and 120 ml (Peak I) was tentatively assigned as ε-N-bis(1-carboxyethyl)-L-lysine based upon the following. (a) Although the color yield of peak fractions in the quantitative ninhydrin assay was the same as that of Peak II, the color yield of these fractions in the lysine assay was low (approximately 0.1, compared to lysine). This differential sensitivity to the lysine assay of a secondary (i.e. monoderivatized) ε-amino nitrogen as compared to a tertiary (i.e. doubly derivatized) ε-amino nitrogen might be expected, since the color yield given by proline, which contains an imino nitrogen in its structure, is nearly identical with that of lysine (6). (b) Peak I fractions migrated toward the anode under the same conditions of paper electrophoresis in which Peak II fractions migrated toward the cathode. This difference in electrophoretic behavior can be understood on the basis of an added acidic group in the structure of the Peak I compound. (c) The uptake of 1 eq of oxygen per eq of amino group in the presence of L-amino acid oxidase indicated the presence of a free ε-amino function. These fractions were treated in the same manner as those of Peak II with respect to concentration and crystallization. A 45% yield was obtained.

Lysopine was synthesized by the method of Biemann et al. (7) with 920 mg of ε-N-carbobenzoxy-L-lysine. The final reaction mixture was purified on a Dowex 50 (H+ form) column (1 x 20 cm) and eluted with 2 N NH4OH. The eluate was taken to dryness under reduced pressure, the residue was taken up in a minimum amount of water, and the compound was precipitated with addition of 1 to 2 volumes of absolute ethanol. Recrystallization was performed three times from the ethanol-water system; final crystals were dried under vacuum for 40 hours. RF values were identical with those for the authentic compound: 0.31, ethanol-water (77:33), v/v; 0.10, 2,4,6-collidine-water (1:1:2), v/v; 0.09, n-butyl alcohol-glacial acetic acid-water (4:1:5), v/v. There was no uptake of oxygen in the presence of L-amino acid oxidase from N. crassa as compared to a control experiment with

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1 Lysopine, α-N-(1-carboxyethyl)-L-lysine.
L-lysine, indicating that lysopine is not a substrate for the oxidase. No attempt was made to resolve disteromers. The synthesis resulted in 22% yield.

D,L-Aspartic β-semialdehyde was prepared by ozonolysis of D,L-allylglycine according to the procedure of Black and Wright (8).

Preparation of Trinitrated Pyruvate—Trinitrated pyruvate was prepared by allowing 61.2 mg of pyruvic acid (Calbiochem) to incubate in 1.0 ml of tritiated water (specific activity 100 mCi per ml) for 5 months at room temperature. The incubation mixture was then distilled under vacuum to remove nonvolatile impurities, and 87.1 mg of K₂HPO₄ were added to the distillate. This mixture was repeatedly taken up in 1.0 ml of water, which was subsequently removed under vacuum, until only 1.7% of the total radioactivity occurred in the final distillate. At this point 69.6 mg of K₂HPO₄ were added, bringing the pH to 6.4. Redistillation revealed less than 1.0% of the total radioactivity present in the water. The concentration of pyruvate was assayed with lactic acid dehydrogenase (9). Specific activity of the final preparation was 4.0 × 10⁴ cpm per μmol.

Gel Filtration—For the determination of molecular weight by gel filtration, a column of Sephadex G-200 (Pharmacia), 2.5 × 36 cm, equipped with flow adapters for ascending chromatography, was equilibrated and developed at 4° with 50 mM phosphate buffer, pH 7.5, after being calibrated with the following standards: horse heart cytochrome c, mol wt 12,400 (10); bovine liver catalase, mol wt 250,000 (11); yeast alcohol dehydrogenase, mol wt 150,000 (12). Dextran blue was used to determine the void volume. The effluent volume corresponding to maximum concentration of a protein (i.e., elution volume) was estimated for the nearest 0.1 ml from an elution diagram by extrapolating both sides of the protein concentration peak to an apex.

Density Gradient Centrifugation—Linear gradients (4.5 ml total volume) of sucrose concentration were prepared in cellulose nitrate tubes from 6 and 20% (w/v) sucrose solutions in 50 mM potassium phosphate buffer, pH 7.5. Gradients were stored at 4° for 36 hours prior to use. Linearity of the gradient was monitored by addition of 9,000 cpm of tritiated water to the sucrose solution of higher concentration. Following centrifugation and collection of fractions, radioactivity of 10⁻¹ aliquots was determined, and total radioactivity per fraction was found to increase linearly with fraction number. Proteins, dissolved in buffer, were layered onto the gradient to a total sample volume of 0.1 ml. Centrifugation was performed for 12.7 hours at 37,500 rpm (average revolutions per min) at 4° in a Beckman model L-2 ultracentrifuge, with an SW 56-L swinging bucket rotor. Fractions of approximately 0.1 ml were collected after piercing the bottom of the tube with a hypodermic needle.

Paper Chromatography and Electrophoresis—Descending paper chromatography was carried out at room temperature. Whatman No. 1 paper was routinely used; solvent fronts were allowed to run 25 to 35 cm from the origin. Solvent systems used were: A, ethanol-water (77:33); B, pyridine-glacial acetic acid-water (30:35:15); C, n-propyl alcohol-ammonium-water (6:3:1); D, 2,4 lutidine-2,4,6 collidine-water (1:1:2); E, n-butyl alcohol-glacial acetic-acid-water (4:1:0). All systems were prepared on a volume per volume basis.

Paper electrophoresis was performed at 25° in a model LT-36 electrophoresis tank with an HV-5000 power supply (Savant Instruments, Inc.); electrophoresis coolant model EC-123 was used in all experiments. Compounds were spotted on Whatman No. 1 paper and run at a potential of 2000 volts for 1 hour, except where noted otherwise. Pyridine-glacial acetic acid-water (1:10:89), pH 3.5, was used as buffer in each case.

All chromatograms were dried in a hood for several hours and then sprayed with 0.5% ninhydrin in 65% acetone or 0.1% brom thymol blue, when detecting amino acids or carboxylic acids, respectively.

Radioactivity was detected by cutting the chromatogram into pieces, 1.0 × 1.0 cm, along the direction of the solvent (or in both directions from the origin in the case of electrophoresis) and inserting them into scintillation vials containing 10 ml of scintillation fluid.

Polyacrylamide Gel Electrophoresis—Gel electrophoresis was performed in a model EC470 vertical gel electrophoresis cell (E-C Apparatus Corporation) on 3-mm thick slabs of 5% Cyanogum 41 at 4°. Gel buffers used were: (a) 0.18 % Tris-Na₂EDTA-boric acid, pH 9.2, 6.5; (b) 50 mM potassium phosphate, pH 6.5, 7.0, 7.5. The electrode and gel buffers were at the same pH in each experiment. Samples were made 10% in sucrose, bromphenol blue was added as a marker, and the sample was introduced beneath the buffer into slots (10 × 10 × 3 mm) in the gel.

Migration was allowed to proceed for 2 hours at 300 volts, 120 ma, at 4° with EC-478 power supply (E-C Apparatus Corporation). The gel was stained by placing it in 50 ml of 0.2% Coomassie brilliant blue or 0.2% Amido black 10B in methanol-glacial acetic acid-water (5:5:1 v/v) for 1 hour. Destaining was performed by rinsing the gel of excess dye with tap water and putting it in a 4-liter plastic container containing 1.5% acetic acid. The gel was washed for 24 hours by shaking at the lowest setting on a reciprocating shaker; wash solution was changed twice during this period.

Enzymatic activity on unstained gels was located by cutting the slab into strips 0.5 inch wide. Each strip corresponded to a channel in which the protein had migrated. The strip of gel was then cut into pieces 0.3 cm long along the direction of migration from the origin. Each slice was placed into a tube containing the components of the γ-aminovaleraldehyde assay in 1.0-ml volume. Following a 30-min incubation period, the supernatant solution was removed, transferred to a cuvette, and read at 10-min intervals against a blank at 540 μµ.

Radioactivity was located in gel slices after extraction overnight into 1.0 ml of water. The supernatant was removed from each tube and taken to dryness under reduced pressure, and the residue was suspended in 10 ml of scintillation fluid.

Radioactivity Determination—Radioactivity was measured by counting in a Packard Tri-Carb scintillation spectrometer, model 3050. In all cases Bray's solution (13) was used as the scintillation fluid. Radioactive disintegrations were counted for a time sufficient to give a probable error of less than 3%.

Protein Determination—Protein was routinely estimated by the procedure of Warburg and Christian (14). With purified preparations of the enzyme the method of Lowry et al. (15) was used.

Optical Measurements—Spectrophotometric measurements were made with a Beckman DU spectrophotometer or a Zeiss PMQ II instrument with cuvettes having a 1.0-cm light path. Buffer Anions and Cations—Cation for phosphate buffer, K⁺; anion for Tris and imidazole buffer, Cl⁻.

Enzyme Assay Systems—The condensing enzyme may be assayed by any one of the following methods (1):
of enzymatic activity is defined according to the 270 nm assay activity is defined differently in each system: 1, coupled, micro-
 brief lag.

is expressed in units per mg of protein, although a unit of enzyme
ence of imidazole buffer, pH 7.4, is followed with time after a
increase in absorption at 540 nm following a 30-min lag period.

Assay is by the 270 nm method.

-- Azw,; O-O, enzyme activity.

1. Enzymatic reduction of dihydrodipicolinic acid by TPNH to tetrahydrodipicolinic acid in the presence of dihydrodipicolinic acid reductase is followed spectrophotometrically at 340 nm.

2. The rate of formation of a complex between o-aminobenzaldehyde and dihydrodipicolinic acid at pH 7.4 is followed by the increase in absorption at 540 nm following a 30-min lag period.

3. The increase in absorption, at 270 nm, of a reaction mixture containing the condensing enzyme and its substrates in the presence of imidazole buffer, pH 7.4, is followed with time after a brief lag.

Standard reaction mixtures for the above systems were pre-
pared as described previously (1). In each case specific activity is expressed in units per mg of protein, although a unit of enzyme activity is defined differently in each system: 1, coupled, micro-

molecules per min; 2, o-aminobenzaldehyde ΔA 440 per min; 3, 270 nm, ΔA 370 per min.

Except where stated otherwise, in all tables and figures 1 unit

of enzymatic activity is defined according to the 270 nm assay system, i.e. the amount of condensing enzyme which catalyzes an increase in optical density, measured at 270 nm, of 0.001 per min after reaching maximal velocity (about 5 min).

Yeast alcohol dehydrogenase was assayed by the method of

Racker (16). Catalase was assayed by the method of Chance and Meabhy (17).

RESULTS

Purification of Enzyme

The pyruvate-aspartic semialdehyde condensing enzyme was isolated from extracts prepared from E. coli (strain W, ATCC 9637) that had been grown in minimal medium. The first three

stages of the purification procedures are those previously de-

scribed (1). The present procedure, accordingly, begins with a

modification of the zinc ethanol precipitation.

Step 4: Zinc-Ethanol Precipitation—A 20-ml aliquot of the

depth yellow solution from Step 3 is made 0.002 M with respect to

ZnCl₂ (an 0.05 M solution of the salt is consistently used).

The solution is stirred vigorously until it reaches 1° and the pH

is adjusted, if necessary, to within 0.05 unit of pH 7.5. Ethanol-

ZnCl₂ solution (absolute ethanol made 0.002 M in ZnCl₂ with a

0.05 M solution of ZnCl₂) is chilled to 1° and added beneath the

surface of the protein solution within 4 min to give a final alcohol

concentration of 35% (v/v). Centrifugation in a refrigerated

Sorvall SS-1 rotor must then be accomplished within 5 min at

20,000 × g. The precipitate is thoroughly drained of superna-

tant and dissolved in a minimal volume of 0.05 M Tris buffer, pH 8.0-0.005 M EDTA. This precipitation is extremely sensitive to (a) pH variation and (b) time of alcohol addition. An attempt to use larger aliquots resulted in significantly decreased yields of enzyme.

Step 5: Second Acid Treatment—The solution from Step 4 is quickly brought to pH 4.25 with 4.0 M acetic acid and clarified by centrifugation at 20,000 × g for 7 min. The precipitate is discarded and the supernatant is brought to pH 7.0 with 3.0 M Tris base.

Step 6: Hydroxylapatite Chromatography—The supernatant solution is dialyzed against 50 volumes of 0.005 M phosphate buffer, pH 7.0, for 1.25 hours. Hydroxylapatite is prepared by the method of Tiselius (18) and stored in 0.001 M phosphate buffer, pH 7.0. A column, 2.0 × 150 cm, is prepared and equilibrated with 0.005 M phosphate buffer, pH 7.0-0.25 M KCl-0.001 M mercaptoethanol; 10 mg of protein per ml of bed volume are placed on the column and eluted with an exponential salt gradient formed by placing 175 ml of equilibration buffer in the mixing chamber and 550 ml of 0.30 M phosphate buffer, pH 7.2-0.25 M KCl-0.001 M mercaptoethanol in the reservoir. At a

flow rate of 0.40 ml per min, the enzyme elutes within 13 hours after initiation of the gradient. A profile of the hydroxylapatite chromatography of the protein solution is presented in Fig. 3. Active fractions are pooled and dialyzed against 50 volumes of

0.005 M phosphate buffer, pH 7.0-0.005 M KCl-0.001 M mercapto-
ethanol-10⁻² M EDTA for 2 hours. The dialyzed solution is al-

lowed to settle slowly into a 3-ml bed volume of hydroxylapatite previously equilibrated with 0.005 M phosphate buffer, pH 7.0. The flow rate should be such that 24 ml of dialyzed solution pass through the column within 12 hours. Protein is then eluted with 1.0 M phosphate buffer, pH 7.6.

Step 7: Second Ammonium Sulfate Fractionation—To the concentrated hydroxylapatite fraction is added 0.40 g of solid ammonium sulfate (enzyme grade) per ml of solution. The solution is stirred vigorously for 15 min, clarified by centrifugation at 20,000 × g for 10 min, and drained of supernatant. The precipitate is dissolved in 1.0 ml of 0.005 M phosphate buffer, pH 7.5-0.20 M KCl. To this solution is added 0.30 g of solid ammonium sulfate.
The condensing enzyme can be assayed in three different systems. The coupled assay remains an effective method for determining a single rate-limiting activity throughout the purification, and it has been found that the ratio of the specific activity by this method and by two other assay systems (which do not depend on the addition of other proteins) remains the same to the point of apparent homogeneity. This is reasonable evidence that the reaction in question is catalyzed by one protein. Based upon the specific activity in crude extracts and turnover number for the enzyme, it has been calculated that there are approximately 200 molecules of the enzyme per cell, which represents the constitutive level of this protein. Under growth conditions in which other enzymes in the lysine pathway are derepressed, such as dihydrodipicolinic acid reductase and meso-diaminopimelic acid decarboxylase (19), there is no change in the concentration of the condensing enzyme.

**Amino Acid Analysis**

Solutions of enzyme were dialyzed overnight against distilled water. The samples were lyophilized and taken up in 1.0 ml of 6 N HCl and hydrolyzed in evacuated, sealed ampoules for 17 hours at 120°. Amino acid analyses were carried out with a Beckman model 120 B amino acid analyzer (20). Table II lists the average number of residues per 134,000 g of pure condensing enzyme from two independent determinations on different enzyme preparations. Since hydrolysis was performed for one time period only, it was not possible to estimate the amount of destruction that occurred during hydrolysis. No estimate was made of the tryptophan content or amide ammonia.

**Criteria for Homogeneity**

Polycrylamide gel electrophoresis, at pH 8.5, of 200 µg of protein from one of several individual preparations having the highest specific activity (100 µmoles per min per mg) reveals a single stainable band (Fig. 4). Control experiments with varied amounts of protein indicate that 10 µg afford the smallest amount of protein that yields an easily visible band. Consequently, the condensing enzyme at the final stage of purification contains no single impurity in excess of 5% of the total protein. In a parallel experiment, enzyme was run in an adjacent channel, which was then sectioned and assayed for enzymatic activity. Enzymatic activity corresponded to the stained region, as judged by migration relative to a marker dye. In several experiments the major zone (by protein stain) extended from 0.36 to 0.43 (Rf relative to...
TABLE III
Methionine content of protein hydrolysates at various stages of purification

Aliquots from each stage were dialyzed against 3 liters of distilled water for 72 hours. Aliquots of dialysate containing at least 1.0 mg of protein were hydrolyzed at 110° for 17 hours in 6 N HCl; each hydrolysate was analyzed with a Beckman model 120B amino acid analyzer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Methionine contenta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>1.52</td>
</tr>
<tr>
<td>First acid precipitation</td>
<td>1.11</td>
</tr>
<tr>
<td>Zinc ethanol precipitation</td>
<td>1.18</td>
</tr>
<tr>
<td>Hydroxylapatite column</td>
<td>0.27</td>
</tr>
<tr>
<td>Final ammonium sulfate precipitation</td>
<td>≤0.000b</td>
</tr>
</tbody>
</table>

a Uncorrected for methionine destruction.
b Hydrolysate contained only 500 µg of protein.

Fig. 6. Determination of the molecular weight of the condensing enzyme by gel filtration on Sephadex G-200. Each point represents the position of activity for each marker used (X, experimental run; O, calibration run). The arrow indicates the position of the peak of activity for the condensing enzyme. Development of column is described under "Methods." Cytochrome c was located by absorption at 412 mp while catalase and yeast alcohol dehydrogenase were located by enzymatic assay. Marker dye under conditions noted in Fig. 4). Enzymatic activity was located between RF 0.34 and 0.45. Only one band of protein is visible in the pH range 6.0 to 9.2 (Fig. 5). Further, at each pH there is a correspondence between the position of the band and enzymatic activity.

When it was observed from amino acid analyses that the enzyme appeared to lack methionine, it became apparent that this could be used as an added criterion for determining homogeneity. Accordingly, the methionine content of the protein in various stages of the purification was determined (Table III). The barely detectable levels of methionine in the amino acid analysis of the final fractions, even if corrected for losses of up to 50%, indicate that, if the purified protein is not less than 95% homogeneous, as suggested by gel electrophoresis, then the added restriction of being methionineless must also be imposed upon any contaminants.

Molecular Weight Studies

The molecular weight of the condensing enzyme was estimated from its elution volume on Sephadex G-200 according to the procedure of Andrews (21). A plot of the logarithm of the molecular weight for the standard proteins against emergence volume gave a straight line (Fig. 6). Elution volumes for the reference compounds differed by less than 0.2 ml in the calibration and experimental runs. From an interpolation of the elution position of the condensing enzyme, the molecular weight can be estimated as 126,000 ± 6,000. This is to be contrasted with a molecular weight of 112,000, which has been assigned to the condensing enzyme occurring in E. coli K12 HfrH (22).

Siegel and Monty (23) have demonstrated that the behavior of a series of proteins during chromatography on a column of Sephacryl is correlated with the molecular (Stokes) radius of proteins rather than with molecular weight. A correlation of molecular weight with elution position is virtually indistinguishable from a correlation with molecular radius only if proteins used as calibrating standards have closely similar frictional ratios and partial specific volumes.

The Stokes radius, a, of the condensing enzyme was obtained from gel filtration data according to the method of Ackers (24) with yeast alcohol dehydrogenase (a = 46 A) serving as the reference molecule. This method yields a = 43 A as the Stokes radius of the condensing enzyme. This value agreed within 1% with that calculated with catalase (a = 52 A) as the reference molecule.

The sedimentation coefficient of the condensing enzyme was obtained by the method of sucrose density gradient centrifugation.
Inactivation of condensing enzyme with sodium borohydride

The reaction mixture contained condensing enzyme, 6 units (specific activity: 20 ΔA260 units per min per mg), and phosphate buffer, pH 7.5, 100 μmoles. Following a 5-min incubation period with the compound indicated, 5 μmoles of NaBH₄ in 0.01 ml were added, bringing the total volume to 1.0 ml. The reaction was carried out at 0-4°. The total volume of the reaction mixture was put onto a Sephadex G-25 column (0.9 × 19.5 cm), previously equilibrated with 0.05 M phosphate buffer, pH 7.5, and developed with the same buffer. Fractions (2.6 ml) were collected and assayed for enzymatic activity. All aliquots were withdrawn 2 hours after addition of borohydride, and examined for enzyme activity.

<table>
<thead>
<tr>
<th>Addition to reaction mixture</th>
<th>Amount</th>
<th>Enzymatic activity recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>NaBH₄ + sodium pyruvate</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>NaBH₄ + 2-aminoaspartic semialdehyde</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>NaBH₄ + α-ketobutyrate</td>
<td>10</td>
<td>97</td>
</tr>
<tr>
<td>NaBH₄ + L-lysine</td>
<td>1</td>
<td>98</td>
</tr>
</tbody>
</table>

TABLE V
Dependence of inactivation of condensing enzyme on sodium borohydride in presence of 5 × 10⁻³ M pyruvate

Each reaction mixture contained condensing enzyme, 0.45 units (specific activity, 200 ΔA260 units per min per mg); sodium pyruvate, 2.5 μmoles; phosphate buffer, pH 7.5, 100 μmoles. An appropriate aliquot of a fresh solution of NaBH₄ was added to bring the total volume to 0.6 ml. After 2 hours at 0-4°, aliquots were assayed directly for enzymatic activity. There was no change in activity when assays were repeated at 4 hours after borohydride addition.

<table>
<thead>
<tr>
<th>Concentration of NaBH₄ in reaction medium</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>%</td>
</tr>
<tr>
<td>0.30</td>
<td>35</td>
</tr>
<tr>
<td>0.50</td>
<td>50</td>
</tr>
<tr>
<td>1.0</td>
<td>80</td>
</tr>
<tr>
<td>1.5</td>
<td>93</td>
</tr>
<tr>
<td>2.0</td>
<td>100</td>
</tr>
</tbody>
</table>

according to the procedure of Martin and Ames (25). A sedimentation pattern, showing the movement relative to catalase and alcohol dehydrogenase after 12 hours of centrifugation at 35, is presented in Fig. 7. Assuming a partial specific volume (ϕ) of 0.725 cm³ per g, the sedimentation coefficient of the condensing enzyme, defined as s₂₀,₅₀°, is 7.1.

A crude estimation of molecular weight can be obtained from the sedimentation constant alone (25) with the following expression:

$$\frac{s_1}{s_2} = \left(\frac{M_1}{M_2}\right)^{2/3}$$

The molecular weight (M) of the condensing enzyme, evaluated on the basis of its sedimentation constant as compared to that of alcohol dehydrogenase (s₂₀,₅₀° = 7.3), is 139,000.

A more accurate determination of the molecular weight was obtained from a combination of the Stokes radius with the sedimentation coefficient. With the value of these molecular parameters obtained above, together with an assumed ϕ of 0.725 cm³ per g, the molecular weight and frictional ratio of the condensing enzyme were calculated from the following equations (23):

$$M = \frac{6\pi N\eta a}{(1 - B\rho)}$$

Where η = viscosity of medium, ρ = density of medium, N = Avagadro’s number; other symbols as noted above. Values of DeDuve, Berthet, and Beaufay (26) for the density and viscosity of sucrose solutions as functions of concentration and temperature were used to determine η and ρ from the position of the condensing enzyme in the sucrose density gradient experiments. Solution of these equations yields: mol wt 134,000; f/φ = 1.26.

A large error in the assumed value of ϕ may lead to quite a significant error in the calculated values for sedimentation coefficient, molecular weight, and frictional ratio. However, since most proteins have partial specific volumes between 0.700 and 0.750 cm³ per g, the assumption of a partial specific volume of 0.725 cm³ per g will result in less than 3% error in the estimation of s₂₀,₅₀° for most proteins (27). However, this uncertainty can be eliminated by calculation of ϕ according to Cohn and Edsall (28). With the data obtained from an amino acid analysis of purified condensing enzyme, ϕ has been calculated as 0.724 cc per g, which is well within the assumed value used in the above determination of molecular weight. Therefore, a value of 134,000 daltons has been assigned as the molecular weight of the pyruvate aspartic semialdehyde condensing enzyme of E. coli W.

Enzyme Inactivation

Studies with various aldolases have shown that imine formation between a keto substrate and an ε-amino group of a lysine residue on the enzyme is an obligatory step in enzyme-catalyzed aldol condensation reactions (29-32). In view of these precedents, experiments were carried out to determine whether either of the substrates of the condensing enzyme could form an enzyme-substrate imine bond. A well established criterion for Schiff base formation is the inactivation of the enzyme in the presence of substrate by sodium borohydride (31-33).

The effect of sodium borohydride treatment on the condensing enzyme in the presence and absence of its substrates is shown in Table IV. It may be seen that only in the presence of pyruvate...
FIG. 8. Column chromatography of condensing enzyme partially inactivated by NaBH₄ in the presence of labeled sodium pyruvate. The reaction mixture contained 100 µmoles of phosphate buffer, pH 7.5, 56 µg of condensing enzyme (specific activity, 110 µmoles per min per mg), and 4.44 µmoles of sodium pyruvate (1.25 × 10⁶ cpm per µmole) in 1.0 ml volume. Within 5 min after addition of the ¹⁴C-pyruvate, 0.02 ml of 1 × 10⁻² M sodium borohydride was added. After 5 min incubation at 0°, the mixture was admitted to a Sephadex G-25 column (0.9 × 27 cm), previously equilibrated with 0.05 M phosphate buffer, pH 7.5, and eluted with the same buffer. Fractions of 0.8 ml were collected and assayed for enzymatic activity; radioactivity was determined as described under "Methods." Enzymatic activity: O — O (control); ▲ ▲ (after borohydride treatment). Radioactivity: X — X (control); — (after borohydride treatment).

FIG. 9. Polyacrylamide gel electrophoresis of condensing enzyme that was 50% inactivated by NaBH₄ in the presence of ¹⁴C-pyruvate. Reaction conditions were the same as for Fig. 8, except that only 1.11 µmoles of ¹⁴C-pyruvate were added. After separation of excess radioactivity by gel filtration, peak fractions were pooled, lyophilized, and taken up in 0.02 ml of 0.05 M phosphate buffer, pH 7.5. Gel electrophoresis and relevant assays on sections thereof were performed as described under "Methods." Native enzyme: O — O (enzymatic activity); radioactivity: ▲ ▲; A₄₅₀, X — X.

### Table VII

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total radioactivity bound</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no NaBH₄)</td>
<td>7,500</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>7,300</td>
<td>6,800</td>
</tr>
<tr>
<td>2</td>
<td>14,700</td>
<td>14,000</td>
</tr>
<tr>
<td>3</td>
<td>23,900</td>
<td>20,600</td>
</tr>
<tr>
<td>4</td>
<td>52,400</td>
<td>21,000</td>
</tr>
</tbody>
</table>

Each reaction mixture, maintained at 4°, contained condensing enzyme (56 µg; specific activity, 110 µmoles per min per mg); phosphate buffer, pH 7.5, 100 µmoles. Sodium L-¹⁴C-pyruvate (1.25 × 10⁶ cpm per µmole) was added in varying amounts, and the total volume was brought to 1.0 ml. Within 5 min after addition of the pyruvate, 0.02 ml of 10⁻² M freshly prepared NaBH₄ was added (except for control). Each reaction mixture was then treated in the manner described in Fig. 8.

### Table VIII

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chromatographic mobility (Rᶠ)</th>
<th>Electrophoretic mobility with L-α-amino acid oxidase treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Lysine</td>
<td>0.22 0.38 +12.0 +1.4</td>
<td></td>
</tr>
<tr>
<td>e-N-(1'-carboxyethyl)-L-lysine</td>
<td>0.55 0.28 +2.5 −8.5*</td>
<td></td>
</tr>
<tr>
<td>Protein hydrolysate</td>
<td>0.34 0.29 +2.4 −8.5*</td>
<td></td>
</tr>
<tr>
<td>α-N-(1'-carboxyethyl)-L-lysine</td>
<td>0.33 0.31 +2.5 +2.4</td>
<td></td>
</tr>
</tbody>
</table>

* Toward anode; all others toward cathode.

The dependence of the extent of inactivation on sodium borohydride concentration was studied by treating the condensing enzyme with varying amounts of sodium borohydride in the presence of a fixed concentration of pyruvate. From results shown in Table V, it is clear that the rate of reduction of the enzyme-substrate complex by sodium borohydride must be very much greater than the rate of reduction of the carbonyl group of pyruvate by the same reductant. Even when the molar concentration of pyruvate is approximately 600-fold in excess over the available reducing equivalents of sodium borohydride, 100% inactivation is still observed. It is the preferential, rapid rate
of reduction of the pyruvate-enzyme complex that necessitates the complete removal of all traces of borohydride before meaningful assays of enzymatic activity can be made. The technique of gel filtration was initially required to do this; later excess aspartic semialdehyde was added to reaction mixtures before taking aliquots to ensure the removal of residual borohydride reduction potential.

Protein inactivation by sodium borohydride in the presence of pyruvate appears to be pH-dependent (Table VI). Reduction experiments were routinely carried out at pH 7.5.

Binding of Pyruvate to Enzyme

To determine whether the sodium borohydride-pyruvate-dependent inactivation of the enzyme leads to the formation of a covalent bond between enzyme and substrate, the borohydride treatment was performed in the presence of $^{14}C$-1-pyruvate. Following an inactivation experiment, the reaction mixture was placed on a Sephadex G-25 column. Fig. 8 illustrates the elution profile obtained from this column. A control experiment was first carried out under the same conditions, except that no borohydride was added. The reaction mixture was chromatographed on the same Sephadex column. Ninety-eight per cent of the enzymatic activity was recovered, with no detectable radioactivity associated with the protein peak. In another experiment, 50% inactivated enzyme, separated from excess radioactivity by gel filtration as described above, was subjected to polyacrylamide gel electrophoresis (Fig. 9). Of the radioactivity applied to the gel, 98% was recovered and migrated with the same mobility, relative to a marker dye, as the native enzyme. Peaks for enzymatic activity and radioactivity were superimposed. A marker of native enzyme was run in an adjacent gel channel. Whether the activity of the partially inactivated enzyme preparation represents a mixture of molecules some of which have completely escaped any site derivatization or species in which only some sites have been derivatized cannot be decided until the sequence of site inactivation has been studied.

These experiments indicate that, in the sodium borohydride reduction of the enzyme in the presence of pyruvate, the substrate is covalently bound to the protein.

Titrator of Binding Sites

Substrate amounts of enzyme were titrated with increasing concentrations of radioactively labeled pyruvate in the presence of borohydride. Radioactivity bound to protein correlates quantitatively with units of enzyme activity lost (Table VII). The absence of binding of additional radioactivity in the presence of 2-fold molar excess of pyruvate indicates the absence of nonspecific pyruvate-binding sites or contaminants. It is evident that these experiments suggest the presence of four binding sites for pyruvate in the enzyme.
sites per mole of enzyme, based upon the molecular weight value of 134,000 daltons derived from previous studies. Calculation of the number of binding sites was the same when an equivalent amount of protein from another independent preparation of enzyme having the same specific activity was used.

Characterization of Covalent Enzyme-Substrate Complex

A sample of radioactivity-labeled, fully inactivated protein (192 μg, 23,600 cpm) was lyophilized, dissolved in 6 N HCl, and hydrolyzed in a sealed, evacuated ampoule at 115° for 12 hours. The hydrolysate was taken to dryness under reduced pressure to remove excess HCl and examined by descending paper chromatography and paper electrophoresis. Table VIII indicates that radioactivity from the hydrolysate has the same electrophoretic mobility and nearly the same chromatographic Rf in the solvent systems used. Consequently, an aliquot of the protein hydrolysate was treated with L-amino acid oxidase and subjected to the same electrophoretic conditions as control experiments in which the two isomers were similarly treated. Radioactivity of the treated hydrolysate then migrated toward the anode with the same mobility as the ε isomer control. These experiments establish that the derivative formed by reduction with sodium borohydride in the presence of pyruvate is indeed ε-N-(1-carboxyethyl)-L-lysine. No attempt was made to determine whether the reduction at the former carboxyl carbon atom of pyruvate is stereospecific and leads to the production of one diastereomer.

Tritiated Pyruvate Exchange Studies

The above experiments give ample evidence that pyruvate can form a Schiff base intermediate with the condensing enzyme. In several enzyme-catalyzed reactions, notably aldol condensations (31, 32) and decarboxylation (33), stabilization of intermediate carbanions is made possible by Schiff base formation between enzyme and substrate. It became of interest, therefore, to determine whether the pyruvate aspartic semialdehyde condensing enzyme catalyzed a proton exchange between the methyl hydrogens of pyruvate and the medium. Fig. 10 shows that, when tested at several different concentrations, the enzyme does indeed catalyze a first order exchange between tritiated pyruvate and the medium, and that the rate of exchange is directly proportional to enzyme concentration.

The pH profile of the exchange reaction does not mimic that for the enzymatic reaction. In fact, the pH optimum is shifted from pH 8.4 (for the over-all enzymatic reaction) to near neutral pH values. Since anion formation may not be the rate-limiting step in the net reaction, it is not necessary that the pH optimum of exchange and over-all reaction overlap. Indeed, proton exchange is not a necessary concomitant of Schiff base formation: transaldolase, a Class I aldolase, does not exhibit proton exchange between its C-3 carbanion adduct (dihydroxyacetone) and water (34). In this connection it may be noted that the ratio of the first order reaction constants of enzyme rate (k2) to exchange at pH 8.4 is 8 (Fig. 10). This disparity in rates could be due to a direct kinetic isotope effect. Alternatively the exchange may not be a true measure of carbonium formation because of the ability of the enamine to compete successfully for the proton bound to a basic group on the enzyme with those processes that result in exchange of that proton with the medium. Unfortunately, whenever proton exchange with the medium does occur, there is no general way of distinguishing between direct loss of a proton or transfer to the enzyme.

DISCUSSION

By surveying the fructose diphosphate aldolases in representative phylogenetic groups, Rutter (35) has been able to describe two major classes. (a) Class I are those enzymes that form a Schiff base between a substrate bearing a carbonyl group and an ε-amino group of a reactive lysine residue of the enzyme. The molecular weight of these aldolases from plant sources is 120,000 to 140,000; from animal sources 150,000 to 160,000 (36); estimates of molecular weight after dissociation under a variety of conditions indicate the presence of three to four subunits. (b) Class II includes those enzymes that are reversibly inhibited by metal chelators. A divalent ion, in most cases zinc, has been found to be an integral and essential component. Compared to Class I, these metalloaldolases are small, having a molecular weight of approximately 70,000 daltons (36). Enzymes of this type are widely distributed among the protozoa and green algae and in virtually all higher plants and animals. The metallo-aldolases are more sensitive to inhibition by sulfhydryl-binding reagents than are the Class I enzymes.

One of the chief differences between Class I and Class II aldolases is the differential sensitivity of these enzymes to treatment with sodium borohydride in the presence of substrate. The sodium borohydride treatment has been used repeatedly as a method of stabilizing azomethine (i.e., Schiff's base) linkages by reduction to stable secondary amines (29-33).

The loss of enzymatic activity that accompanies incubation of the pyruvate-aspartic semialdehyde condensing enzyme in the presence of pyruvate and sodium borohydride suggested the presence of a Schiff's base intermediate in the enzymatic reaction. The specificity of the inactivation is shown by its selective dependence upon pyruvate and by the correlation between the extent of inactivation and the extent of labeling of the protein. It can be concluded that the inactivation results from the irreversible binding of pyruvate at the active site of the enzyme.

The nature of the complex between the condensing enzyme and pyruvate has been established by the chromatographic and electrophoretic identity of the radioactive derivative of the hydrolyzed protein with ε-(1-carboxyethyl)-L-lysine. The absence of the α isomer (lysopine) from the protein hydrolysate indicates that no α-amino group of an NH₂-terminal lysine residue is involved in imine formation. Therefore, it can be concluded that the condensing enzyme-pyruvate complex, prior to reduction and hydrolysis, exists as the Schiff base formed between the carbonyl of pyruvate and the ε-amino group of a lysine residue on the enzyme. In studies aimed at investigating the sulfhydryl content, it was found that the enzyme is unaffected by 10⁻⁴ M p-chloromercuribenzoate.

The formation of a Schiff base bond between the carbonyl group of pyruvate and an ε-amino group of a lysine residue, the direct involvement of 4 lysine residues, presumably at the same active site as the condensation, which suggests a four-subunit enzyme, an insensitivity to sulfhydryl inhibitors, and a molecular weight in the range 120,000 to 140,000—all properties of nonmetal-containing aldolases—show that the pyruvate-aspartic semialdehyde condensing enzyme can be classified as a Class I aldolase (according to Rutter) with respect to carbon-carbon bond formation in the net reaction that it catalyzes.
freeing the product from the active site. This reaction sequence would be analogous to that suggested by Nandi and Shemin (37) for δ-aminolevulinic acid dehydratase, in which 2 molecules of δ-aminolevulinic acid condense to form porphobilinogen. Alternatively, the displacement of the ε-amino nitrogen of the enzyme by the ε-amino nitrogen of the semialdehyde could occur first to produce a conjugate of the two substrates (Part II of Fig. 12), followed by the generation of a carbanion; condensation would now become the method of ring closure. If transimination occurs initially, then the resulting ketimine could not be in a covalent linkage to the enzyme, although it is in a favorable conformation for aldol condensation to ensue.

An attempt was made to decide between these two routes by allowing a system of condensing enzyme, 14C-pyruvate, and aspartic semialdehyde to come to equilibrium and adding an excess of borohydride. It was postulated that, if condensation preceded transimination, a new lysine derivative containing the elements of pyruvate and aspartic semialdehyde would be isolated after protein hydrolysis. In all cases in which the incubation system contained sufficient radioactivity to derivatize all binding sites, only the ε-N-(1-carboxyethyl)-L-lysine derivative could be detected. These experiments do not rule out the "condensation before transimination" hypothesis, because the particular enzyme intermediate could exist in very low steady state concentration, requiring a higher specific radioactivity of pyruvate or, alternatively, a higher concentration of protein in order to be detected.

Dehydration must occur after the carbon-carbon bond has formed, and it may precede or follow ring closure. The preparation and testing of 4-hydroxy-Δ1-tetrahydrodipicolinic acid as an intermediate should help to clarify the position of this step. It may be that the production of this hydroxylated ring compound is the true function of the enzyme, dihydrodipicolinic acid resulting as the product of spontaneous dehydration of the ring.

Acknowledgment—We are grateful to Miss Barbara Bannman for expert assistance in performing amino acid analyses.

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The Pyruvate-Aspartic Semialdehyde Condensing Enzyme of *Escherichia coli*

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