The Reduction Step in Diaminopimelic Acid Biosynthesis*

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In order to facilitate the elucidation of the remaining undetermined steps in diaminopimelic acid and lysine biosynthesis, mutants blocked in the pathway between aspartic semialdehyde and α-N-succinyl-ε-ketopimelic acid were sought. A mutant (M-203) with a requirement for diaminopimelate and lysine was obtained. An examination of the enzymatic composition of the extract prepared from the mutant, coupled with its nutritional requirements, indicated that it was blocked in the desired part of the biosynthetic pathway.

It had previously been shown that crude extracts prepared from wild type Escherichia coli could synthesize diaminopimelic acid from its early precursors, aspartate and pyruvate. Similar extracts prepared from M-203 were deficient in the capacity to make diaminopimelic acid unless they were supplemented with an enzyme fraction from the wild type. This ability to restore the capacity for diaminopimelic acid synthesis to M-203 extracts was made the basis for an assay for the enzyme lacking in M-203. Although the substrate and product of the new enzyme were initially unknown, the enzyme could be extensively purified from wild type extracts with use of this assay.

When the condensation step in diaminopimelate synthesis involving aspartic semialdehyde and pyruvate was discovered (2), it was shown that M-203 could carry out this reaction as well as wild type extracts. Later it was shown that the succinyl group of α-N-succinyl-ε-ketopimelic acid arose by a transfer from succinyl-CoA to ε-tetrahydrodipicolinic acid (3). It was found that M-203 could also carry out this reaction. This left, as the most likely role for the enzyme that M-203 lacked, that of a reductase capable of converting the product of the condensing reaction to tetrahydrodipicolinic acid. This was verified by using the aspartic semialdehyde-pyruvate-condensing enzyme purified from M-203 to generate the substrate of the new enzyme. TPNH was added and remained in the reduced form until the new enzyme was also added, whereupon rapid oxidation of TPNH ensued. The enzyme was then purified with TPNH as the basis of a spectrophotometric assay. The parallelism in purification with both assays revealed that restoration and reductase activity were identical. This report deals with the discovery of the new enzyme, its partial purification from E. coli, its identification as dihydrodipicolinic acid reductase, and some of its properties. A preliminary communication of these findings has appeared (4).

MATERIALS AND METHODS

Diaminopimelic acid was determined by its reaction with acidified ninhydrin (1). Aspartic semialdehyde-pyruvate-condensing enzyme was prepared and measured as described in the preceding paper (2). Glucose 6-phosphate dehydrogenase was purchased from Worthington Biochemicals. Homoserine dehydrogenase was prepared according to Black and Wright (5). The aspartic semialdehyde was also prepared by their procedure (6). Protein was measured by the method of Warburg and Christian (7). The anion for Tris and imidazole buffers was chloride. Authentic ε-tetrahydrodipicolinic acid was made by the action of Neurospora crassa L-amino acid oxidase on mesodiaminopimelic acid.

Preparation of M-203 Crude Extract—Flasks (1 liter each) containing 500 ml of enriched medium (8) supplemented with 10 μg per ml of diaminopimelic acid were used for growing M-203. The flasks were shaken at 32° and harvested near the end of logarithmic growth in a refrigerated Sharples centrifuge. The bacterial pellet was washed twice with several volumes of 0.02 M phosphate buffer, pH 7.0. The cells were then suspended in an equal volume of 0.02 M Tris buffer, pH 8.0, and disrupted in a 10-Kc Raytheon sonic oscillator. Debris was removed by centrifugation at 10,000 × g for 20 min.

EXPERIMENTAL RESULTS

Location of Block in M-203—M-203, a new mutant of E. coli (W-ATCC 9397) with a requirement for diaminopimelic acid and lysine, was isolated with use of the penicillin technique. No further nutritional requirement was observed. At the time that M-203 was isolated it was known that aspartic semialdehyde underwent a series of uncharacterized reactions that eventually gave rise to an α-N-succinyl-ε-ketopimelic acid. This latter compound is then transformed into mesodiaminopimelic acid by a sequence of reactions involving transamination, decarboxylation, and, finally, epimerization to mesodiaminopimelic acid (9-11). This compound is then either decarboxylated to lysine (12) or incorporated into the bacterial cell wall. In order to establish whether M-203 was blocked in the central part of the diaminopimelate pathway, extracts prepared from M-203 were examined for their content of the enzymes of the terminal part of the pathway. It was already clear from the nutritional requirements of M-203 that the block had to be after aspartic semialdehyde since an earlier block would have resulted in more complex nutritional requirements (13). Table I shows that transaminase, decyclase, racemase, and decarboxylase were present in M-203 as determined by assaying for each enzyme individually. Therefore, the metabolic block in M-203 had to be at a locus between aspartic semialdehyde and \( \alpha \)-succinyl-\( \epsilon \)-amino-\( \epsilon \)-ketopimelate.
Assay of Enzyme Activity—Crude extracts of M-203 synthesized little diaminopimelic acid in comparison to those prepared from wild type. However, if these same extracts were supplemented by minimal amounts of an enzyme extract prepared from wild type, diaminopimelic acid biosynthesis could proceed readily.

This observation was made the basis of an assay for the enzyme missing in M-203 extracts. In Fig. 1 is shown the amount of diaminopimelic acid biosynthesized as increasing amounts of a wild type preparation are added to a fixed quantity of crude M-203 extract. The relationship is initially linear but eventually slopes off as some component in the M-203 extract becomes rate-limiting. It should also be noted that the curve intersects the ordinate, indicating that M-203 is a leaky mutant having only a partial block with respect to the enzyme.

Spectrophotometric Assay of Dihydrodipicolinic Acid Reductase—When it became apparent that the role of the new enzyme was that of the reductase, its substrate dihydrodipicolinic acid was unavailable. However, the substrate could be formed from aspartic semialdehyde and pyruvate with the use of the enzyme that catalyzes the condensation of these 2 molecules. This enzyme was originally obtained from M-203. The preparation used was purified until relatively free of the TPNH oxidase, homoserine dehydrogenase, and the low levels of reductase present in the mutant extracts. In a system composed of condensing enzyme, pyruvate, aspartic semialdehyde, and TPNH there was no oxidation of pyridine nucleotide until the reductase was added. Fig. 2 shows that the rate of TPNH oxidation obtained was proportional to the amount of reductase added.

Crude fractions of E. coli contain homoserine dehydrogenase and TPNH oxidase which interfere with this assay. However, the reductase is unusually resistant to heat. Therefore, in order to assay crude extracts, the interfering activity was destroyed by heating the extract to 70° for 3 min. Coagulated protein was removed by centrifugation.

After these experiments were performed, highly purified (1000-fold) condensing enzyme was obtained from the wild type (2). This preparation could also serve as the generator of substrate for the reductase. When chemically synthesized dihydrodipicolinic acid became available, it could be used in place of pyruvate, aspartic semialdehyde, and the condensing enzyme (14). The chemically synthesized material remained active if it was stored at a pH above 10.

Preparation of Dihydrodipicolinic Acid Reductase—As a source
of the reductase E. coli strain W (ATCC 9637) was used. The organism was grown in a 200-liter fermenter on minimal medium (8). At the end of logarithmic growth the cells were harvested in a Sharples centrifuge and washed twice with several volumes of 0.03 M phosphate buffer, pH 7.0.

**Step 1. Preparation of Crude Extract**—The procedure was the same as was used in the preparation of crude extract from M-203. See “Materials and Methods.”

**Step 2. Manganese Chloride Precipitation**—The supernatant fluid from Step 1 was diluted with 0.02 M Tris buffer, pH 8.0, to give 20 mg of protein per ml. To this solution, 2% of its volume of 1 M MnCl₂ was added dropwise with mechanical stirring. The stirring was continued for 30 min, after which time the precipitate which formed was removed by centrifugation at 10,000 × g for 20 min. The pH of the supernatant was adjusted to 7.0 with a buffer, until 65% saturation was attained. The stirring was continued for 30 min, after which time the precipitate was collected by centrifugation at -6°. The precipitate was dissolved in 0.01 M Tris buffer, pH 8.0; 0.001 M EDTA; and 1%, w/v, of ammonium sulfate. The volume of buffer was equal to the volume of the initial crude extract. The enzyme solution was dialyzed against 20 volumes of the same buffer for 15 hours with two changes.

**Step 3. Ammonium Sulfate Fractionation**—To the clear supernatant fluid from Step 2, solid ammonium sulfate was added until 48% saturation was reached, saturation being defined as 70 g/100 ml of water. The stirring was continued for 30 min, and the precipitate was removed by centrifugation at 10,000 × g for 20 min. Ammonium sulfate was added to the clear supernatant until 65% saturation was attained. The stirring was continued for 30 min, and the precipitate was separated by centrifugation at 10,000 × g. The precipitate was dissolved in 0.02 M Tris buffer, pH 8.0, containing 0.001 M EDTA, 0.001 M mercaptoethanol, and 1%, w/v, of ammonium sulfate. The volume of buffer was equal to the volume of the initial crude extract. The enzyme solution was dialyzed against 20 volumes of the same buffer for 15 hours with two changes.

**Step 4. Acetone Fractionation**—Acetone which had been cooled to -15° was added to the dialyzed solution at a rate of 2 ml per min with mechanical stirring. The temperature was adjusted to -6°, and addition of acetone was continued to a final concentration of 37%, v/v. Mechanical stirring was continued for 30 min. Change in volume due to addition of acetone was ignored. The precipitate was removed by centrifugation at 10,000 × g for 20 min in a refrigerated centrifuge maintained at -6°. Acetone was added to the supernatant until a concentration of 52%, v/v, was reached. Stirring was continued for 20 min, after which time the precipitate was collected by centrifugation at -6°. The precipitate was dissolved in 0.01 M Tris buffer, pH 8.0; 0.001 M EDTA; and 0.001 M mercaptoethanol. The volume of buffer used was † the volume of the initial crude extract. The enzyme solution was dialyzed for 15 hours against 20 volumes of this buffer with two changes.

**Step 5. Chromatography on DEAE-cellulose**—DEAE-cellulose was equilibrated with 10 volumes of 0.2 M Tris-chloride buffer, pH 8.0, and washed repeatedly with large volumes of 0.01 M Tris buffer, pH 8.0, until the pH of the wash buffer was the same as that of the original buffer, as measured by the glass electrode. The DEAE-cellulose was then suspended in the buffer and poured onto a glass column. A polyethylene disk was floated on the buffer solution above the resin. The dialyzed enzyme solution was applied to the top of the column. It was washed in with 1 column volume of the Tris buffer; and then gradient elution was started, with a system composed of 0.5 M KCl dissolved in 0.01 M Tris buffer, pH 8.0, in the upper reservoir and the Tris buffer in the mixing reservoir. In a typical run, 223 mg of protein were put onto a column 2.2 × 60 cm. The mixing chamber contained 1450 ml of 0.01 M Tris buffer, pH 8.0, and the upper reservoir contained 2500 ml of 0.5 M KCl dissolved in this buffer. The flow rate was 3 ml per min, and 30-ml fractions were collected. The column chromatography was done at room temperature. Glass-distilled water was used in all stages of enzyme purification. Unless otherwise stated, all steps were carried out at 0-4°. The purification procedure is summarized in Table II. The partially purified enzyme was stable for several months when stored at -15°.

**Identity of Reductase and Restoration Activities**—The last step in the purification of the reductase was DEAE-cellulose column chromatography. The enzyme purified to Step 4 was adsorbed onto the column and eluted as described. Each fraction was assayed for its ability to restore diaminopimelic acid-synthesizing activity to crude extracts of M-203 and for dihydrodipicolinic acid reductase activity. That the two activities had identical elution patterns is shown in Fig. 3.

**Identification of Product of Reductase as Δ⁻-Tetrahydrodipicolinic Acid**—When it was first suspected that the reaction catalyzed by the new enzyme was the pyridine nucleotide-linked reduction of dihydrodipicolinic acid to Δ⁻-tetrahydrodipicolinic acid a method for identifying the product was sought. Compounds such as Δ⁻-piperidines or Δ⁻-pyrrolines were known to react with o-aminobenzaldehyde to give a chromogenic product (presumably the dihydroxyquinazolinedine derivative (15). Authentic Δ⁻-tetrahydrodipicolinic acid was prepared by the oxidation of L-diaminopimelic acid with the L-amino acid oxidase of N. crassa (16). Addition of o-aminobenzaldehyde to the reaction mixture was followed by the appearance of a yellow product, indicating that this test could be used to characterize the reductase product. Pyruvate and aspartic semialdehyde were combined with the condensing enzyme, the reductase, and a TPNH-generating system in the presence of o-aminobenzaldehyde. The absorption spectrum of the product which formed was compared with that of the o-aminobenzaldehyde adduct of the authentic Δ⁻-tetrahydrodipicolinic acid, between 416 and 550 nm. Fig. 4 shows the great similarity between both adducts. When the TPNH-generating system was omitted, the yellow product did not form.

**Table II**

Purification of dihydrodipicolinic acid reductase

The fractionation procedure is described under "Experiments and Results." The method for assaying the enzyme was the coupled optical assay with aspartic semialdehyde, pyruvate, and excess generating system. See Fig. 2.

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein concentration mg/ml</th>
<th>Specific activity μmol/ min/mg</th>
<th>Total Units %</th>
<th>Yield</th>
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<tbody>
<tr>
<td>Initial extract</td>
<td>115.0</td>
<td>0.11</td>
<td>125</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂ supernatant</td>
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<td>0.33</td>
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<td>82</td>
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<td>92</td>
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<tr>
<td>Acetone fraction, 0.37-0.52</td>
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<td>2.64</td>
<td>81</td>
<td>65</td>
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<tr>
<td>DEAE-cellulose</td>
<td>0.08</td>
<td>15.4</td>
<td>68</td>
<td>55</td>
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</table>
**FIG. 2.** Dependence of TPNH oxidation upon amount of dihydrodipicolinic acid reductase. The reaction mixture contained Tris buffer, pH 7.4, 100 μmoles; TPNH, 100 μg; sodium pyruvate, 5.0 μmoles; L-aspartic semialdehyde, 2.0 μmoles; and aspartic semialdehyde-pyruvate-condensing enzyme (specific activity, 3.4 μmoles per min per mg of protein), 200 μg. After 2 min, dihydrodipicolinic acid reductase, 90 μg of protein per ml, was added. The volume of the reaction mixture was 1.0 ml. The partially purified reductase had a low TPNH oxidase activity. This activity was subtracted from the observed rate.

**FIG. 3.** Elution of dihydrodipicolinic acid reductase from DEAE-cellulose. ○—○, protein concentration as measured spectrophotometrically; —, reductase activity as assayed by the coupled optical assay with aspartic semialdehyde, pyruvate, and generating system (see Fig. 2); ○, diaminopimelate restoration activity as measured by diaminopimelic acid synthesis by M-203 extracts after supplementation with column eluates (see Fig. 1).

**FIG. 4.** The absorption spectrum of the o-aminobenzaldehyde adduct of Δ1-tetrahydrodipicolinic acid compared with the o-aminobenzaldehyde adduct of the product of dihydrodipicolinic acid reductase. ○—○, o-aminobenzaldehyde plus Δ1-tetrahydrodipicolinic acid; ——, o-aminobenzaldehyde plus the enzymatically generated compound. To form the adduct between o-aminobenzaldehyde and Δ1-tetrahydrodipicolinic acid, the reaction mixture contained imidazole buffer, pH 7.4, 100 μmoles; o-aminobenzaldehyde, 0.5 mg; and Δ1-tetrahydrodipicolinic acid, 0.99 μmole in a final volume of 1.0 ml. The reaction proceeded until there was no further increase in optical density at 440 μm (62 min). The o-aminobenzaldehyde adduct of the reductase product was formed by combining imidazole buffer, pH 7.4, 100 μmoles; L-aspartic semialdehyde, 1.0 μmole; sodium pyruvate, 10.0 μmoles; glucose 6-phosphate, 4.0 μmoles; TPNH, 0.4 μg; glucose 6-phosphate dehydrogenase, 1.5 units (17); dihydrodipicolinic acid reductase (specific activity, 1.6 μmoles per min per mg), 35.2 μg; and o-aminobenzaldehyde, 0.5 mg, in a final volume of 1.1 ml. After 85 min the optical density at 440 μm ceased to increase. The spectrum of the samples was determined after the optical density stopped increasing.

Identity of the product as Δ1-tetrahydrodipicolinic acid was shown in yet another way. Although Δ1-tetrahydrodipicolinic acid has never been isolated in a pure state, it can be reduced with sodium borohydride to piperidine dicarboxylic acid, which can be more readily characterized. It had been shown by Schweert that piperolic acid undergoes a reaction with ninhydrin in glacial acetic acid to yield a purple color (18). Piperidine dicarboxylic acid reacted similarly. The absorption maximum was at 560 μm. When the product of the reductase was reduced with sodium borohydride, the product after purification and reaction with acidic ninhydrin had an absorption spectrum identical with that of authentic Δ1-tetrahydrodipicolinic acid similarly treated (Fig. 5). When TPNH or the reductase was excluded from the reaction, no piperidine dicarboxylic acid could be detected. The reaction with acidic ninhydrin could also be used to detect piperidine dicarboxylic acid on paper chromatograms. The solvent used was methanol-pyridine-water (8:1:1), and the chromatograms were run in a descending system. The papers were dried overnight in a hood at room temperature,
sprayed with ninhydrin in glacial acetic acid (18.6 mg per ml), after reaction with acidic ninhydrin. 

authentic piperidine dicarboxylic acid was obtained with 3.2 μmoles was not added to the reaction mixture. The spectrum of au-

trum was obtained in the same manner, but glucose 6-phosphate added. The tubes were capped and heated at 100° for 8 min. 

3 N NH₄OH. Ammonia was removed under reduced pressure, and

acid was then eluted with 0.2 N HCl. The eluate was concentrated with several column volumes of water. The piperidine dicarboxylic acid was first then put on a Dowex 1-W column, which was then washed with H₂O, 0.05 ml; glacial acetic acid, 3.95 ml; and ninhydrin, 3.72 mg

ferred with a Beckman DU spectrophotometer. The second spec-

trum was obtained by combining imidazole buffer, pH 7.4, 200 μmoles; L-aspartic semialdehyde, 10 μmoles; sodium pyruvate, 60.0 μmoles; glucose 6-phosphate, 60.0 μmoles; TPNH, 30 μg; glucose 6-phosphate dehydrogenase, 30 units (17); condensing enzyme (specific activity, 3.4 μmoles per min per mg), 200 μg; and dihydrodipicolinic acid reductase (specific activity, 1.6 μmoles per min per mg), 132 μg, in a final volume of 2.0 ml.

The reaction proceeded at 26° for 25 min. Excess sodium borohy-
dride was then added, and the reaction mixture was permitted to stand overnight. Excess borohydride was destroyed by lowering the pH to 5.5 with 1 N acetic acid, and the reaction mixture was put on a Dowex 50 column in the H⁺ cycle. The column was washed with several volumes of water. The product was then eluted with 3 N NH₄OH. Ammonia was removed under reduced pressure, and the pH was adjusted to 6.0 with 2 N acetic acid. The material was then put on a Dowex 1-C⁺ column, which was then washed with several column volumes of water. The piperidine dicarboxylic acid was then eluted with 0.2 N HCl. The eluate was concentrated to 60 μl, and 10 μl were removed for paper chromatography identification of the product. The remainder was taken to dryness. 

The stoichiometry of the over-all process of forming the di-

aminopimelic acid skeleton from its early precursors, pyru-
vate and aspartate, indicated that two reduction steps would be

quired. One of these occurs when aspartyl phosphate is con-

verted to aspartic semialdehyde. The second has now been shown to occur after the precursor moieties have been joined. 

This involves removal of one of the two double bonds in dihydro-
dipicolinic acid. Other enzymatic reactions are known in which a carbon-carbon double bond is reduced by pyridine nucleotide. 

Among them are the reduction of erotic acid (19) and one of the reductive steps in fatty acid biosynthesis (20). The enzymes catalyzing these reactions have been identified as flavoproteins. At present the purification of the dihydrodipicolinic acid reductase has not proceeded to the point where any statement can be made as to whether it too is a flavoprotein.

In addition to the chemical nature of the substrate and the product, the fact that mutational loss of the reductase is ac-

panied by a requirement for diaminopimelate provides firm evidence for the participation of the reductase in diaminopimelate and lysine biosynthesis.

SUMMARY

1. A diaminopimelic acid auxotroph M-203 has been obtained. Crude extracts of M-203 cannot synthesize diaminopimelate acid unless supplemented by an enzyme from the wild type.

2. The enzyme lacking in M-203 has been shown to be dihydro-
dipicolinic acid reductase. This enzyme has been partially purified from the wild type.

3. The product of the reductase reaction has been shown to be Δ¹-tetrahydrodipicolinic acid.
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