Diaminopropionate Ammonia-lyase from *Salmonella typhimurium*

PURIFICATION AND CHARACTERIZATION OF THE CRYSTALLINE ENZYME, AND SEQUENCE DETERMINATION OF THE PYRIDOXAL 5'-PHOSPHATE BINDING PEPTIDE

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We have found a wide occurrence of $\alpha,\beta$-diaminopropionate ammonia-lyase in bacteria and actinomycetes. Considerable amounts of this enzyme were found in *Salmonella typhimurium*. The enzyme was purified and crystallized from *S. typhimurium* (IFO 12529). The relative molecular mass of the native enzyme, estimated by the ultracentrifugal equilibrium method, is 89,000 Da, and the enzyme consists of two subunits identical in molecular mass. The enzyme exhibits absorption maxima at 278 and 413 nm and contains 2 mol of pyridoxal 5'-phosphate (pyridoxal-P)/mol of enzyme. The enzyme catalyzes the $\alpha,\beta$-elimination reaction of both L- and D-$\alpha,\beta$-diaminopropionate, the most suitable substrates, to form pyruvate and ammonia. The L- and D-isomers of serine were also degraded, though slowly. After the internal Schiff base with pyridoxal-P had been reduced with sodium borohydride, followed by trypsin or lysyl endopeptidase digestion of the enzyme, we determined the sequence of about 20 amino acid residues around the lysine residue which binds pyridoxal-P. No homology was found in either the amino acid sequence of the pyridoxal-P binding peptide or the amino-terminal amino acid sequence between the enzyme and other pyridoxal-P-dependent enzymes.

L-$\alpha,\beta$-Diaminopropionate (DAP)$^1$ is a constituent of the polypeptide antibiotics, viomycin (1) and edeine (2, 3), and also occurs in the free state in the seeds of some plants belonging to the *Mimosaceae* (4). D-DAP is present in the free amino acid pool of the digestive fluid of *Bombby x mori* (5). Studies on the biosynthesis of L-DAP indicated that serine is the precursor in a *Streptomyces* sp. (6), but not on *Lathrus sativus* (7). Seneviratne and Powden (8) studied the metabolism of L-DAP in *Acacia* and *Phaeisulus* seedlings and found that it predominantly undergoes acetylation, although a small proportion undergoes transamination. Rajagopal Rao et al. (9) studied the metabolism of DAP in a pseudomonad and demonstrated that it is metabolized to pyruvate through an ammonia-lyase reaction. The DAP ammonia-lyase found in the pseudomonad was purified and characterized (10). However, since then no studies on the enzyme have been carried out. Little information is available on the enzyme, and many of its physicochemical and catalytic properties remain unclear. It is noteworthy that the enzyme is the first reported pyridoxal-P-dependent enzyme that catalyzes elimination of the amino group at the $\beta$-position.

In the present study, we purified and crystallized the DAP ammonia-lyase from *Salmonella typhimurium*. We characterized the crystalline enzyme in detail and determined the amino acid sequence of the pyridoxal-P binding peptide of the enzyme.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**DISCUSSION**

The study described here dealt with the enzymological and physicochemical characterization of the *Salmonella* DAP ammonia-lyase. The enzyme was purified and crystallized from *S. typhimurium*, as DAP ammonia-lyase occurs most abundantly in this species. Previously, DAP ammonia-lyase was shown to be present in a pseudomonad for the first time and purified by Vijayalakshmi et al. (10). However, the homogeneity of the enzyme purified from the pseudomonad seems to be doubtful in view of its catalytic and structural properties. Our crystalline enzyme catalyzes the $\alpha,\beta$-elimination of L-DAP to pyruvate at the rate of 46.9 pmol/min/mg of protein at 30°C. However, the highest specific activity of the Pseudomonas enzyme was only 4.0 pmol/min/mg of protein at 37°C (10). Although the molecular mass (89,000) and subunit structure (two subunits) of the *Salmonella* DAP ammonia-lyase resembled those of the Pseudomonas enzyme (10), the pyridoxal-P contents and absorption spectra are different (*Salmonella* enzyme, 2 mol of pyridoxal-P/mol of the enzyme, $\lambda_{max} = 413$ nm; Pseudomonas enzyme, 1 mol of pyridoxal-P/mol of the enzyme, $\lambda_{max} = 388$ nm). The Pseudomonas enzyme was highly specific for L-DAP, d-DAP being about 10% as effective as L-DAP as a substrate, although the optical purity of the d-compound was unclear (10). None of the structurally related compounds tested were affected by the enzyme (10). As to the *Salmonella* enzyme, the D-isomer of DAP was about 66.3% as effective as L-DAP as a substrate. D-Serine acted as a more preferred substrate than the L-isomer, though their activities were much lower than those of L- and D-DAP. Other physicochemical and catalytic properties of the Pseudomonas enzyme remain unknown.

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$^1$ The abbreviation used is: DAP, $\alpha,\beta$-diaminopropionate.
The Salmonella enzyme is very unique in that it acts on not only L-isomers but also D-isomers of DAP and serine. In addition, both L- and D-alanine competitively inhibited the enzyme. Thus, the Salmonella DAP ammonia-lyase has almost no optical specificity for substrates, although its substrate specificity, itself, is very high. It is known that tyrosine phenol-lyase also catalyzes the \( \alpha \)-elimination reaction of both L- and D-serine (47), which is likely to be related to the enzyme ability to catalyze the slow racemization of alanine (48). By contrast, the racemization of alanine and methionine was not catalyzed by DAP ammonia-lyase, even though a large amount of the enzyme was added and the incubation was carried out for a long time. The D- and L-isomers of 3-chloroalanine which are known as suicide substrates for some pyridoxal-P-dependent enzymes were also attacked by the enzyme at very low rates. The removal of the \( \alpha \)-proton of 3-chloroalanine results in the simultaneous elimination of C1-carbon position. The amino acid sequence of the DAP ammonia-lyase in-
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**EXPERIMENTAL PROCEDURE**

Potassium phosphate buffer, 0.2 M, was prepared by dissolving 78 g of potassium dihydrogen phosphate and 70 g of potassium chloride in 1 L of water. The buffer was adjusted to pH 7.0 with concentrated hydrochloric acid. Protein solution was prepared by dissolving the lyophilized enzyme in potassium phosphate buffer at pH 7.0, containing 0.2 M potassium phosphate. The concentration of the protein solution was determined by the biuret method on a spectrophotometer.

**RESULTS**

The enzyme was purified by the method of Winter and Hahn. The enzyme solution was dialyzed against 0.1 M potassium phosphate buffer, pH 7.0, and then placed on a column of Sephadex G-100 (500 ml) equilibrated with the same buffer. Fractions of 1 ml were collected. The fractions containing the enzyme were combined and then dialyzed against water. The enzyme solution was then concentrated to 20 ml by reduced to 20 ml by dialysis against 10 mM potassium phosphate buffer at pH 7.0. The enzyme solution was then dissolved in a minimum volume of the same buffer.

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Diaminopropionate-lyase from S. typhimurium

A. Diaminopropionate-lyase

The enzyme was purified by double ammonium sulfate precipitation of a 75-mg ammonium sulfate extract obtained by fractionation of the enzyme's ammonium sulfate fraction (500-ml volume) on DEAE-cellulose. The protein peak was found in the 60-80% ammonium sulfate fraction. The purified enzyme was stable for at least 1 year when stored at -20°C.

B. Molecular Weight and Configuration

The molecular weight of the purified enzyme was determined by gel filtration on a Sephadex G-150 column. The molecular weight was estimated to be approximately 150,000.

C. Enzyme Activity

The enzyme activity was determined using the chromogenic substrate L-lysine. The enzyme activity was measured by monitoring the decrease in absorbance at 280 nm due to the formation of L-lysine.

D. Purification of Diaminopropionate-lyase

The enzyme was purified from S. typhimurium by ammonium sulfate fractionation and chromatography on DEAE-cellulose. The purified enzyme had a specific activity of 350 U/mg protein and a yield of 15%.

E. Substrate Specificity

The enzyme was found to have high specificity for L-lysine, with low activity towards other substrates such as D-lysine and L-arginine.

F. Inhibitors

The enzyme was found to be inhibited by high concentrations of sodium chloride and by the addition of EDTA.

G. Enzyme Mechanism

The mechanism of action of the enzyme was investigated using various substrates and inhibitors. The enzyme was found to have a double substrate mechanism, with the initial step involving the formation of a covalent intermediate.

H. Enzyme Kinetics

The enzyme kinetics were studied using a variety of L-lysine concentrations. The enzyme exhibited Michaelis-Menten kinetics with a Michaelis constant of 1 mM.

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DAP-lyase was inhibited by various amines such as methylene, ethylene, propylene, benzylmethylene and chloroforminhibitions at 10 mM: 91, 81.9, 72.7, 66.2 and 63.1%, respectively, at pH 8.2. Propylene was found to be competitive with L-DAP from the Lineweaver-Burk plot (r = 0.94). D- and L-lysine competitively inhibited the N-elimination reaction catalyzed by the enzyme. KI values for D-lysine were calculated to be 0.02 and 0.003 mM, respectively, from the intercept and slope of the double reciprocal plot. Glutathiosemialdheyde was also a competitive inhibitor of the enzyme (K = 0.5 mM).

Further Characterization of DAP-lyase

It is known that some pyridoxal P-dependent enzymes, e.g., ornithine aminotransferase (3), serine transhydroxymethylase (3) and amino acid transaminase (6), catalyse the N-elimination of 3-chlorolysine to pyruvate and ammonia, and the chloropirodyl P-lyase from Pseudomonas diminuta is completely N-eliminated (21). The chloropirodyl P-lyase from Salmonella typhimurium was also purified and characterized as described under "Experimental Procedures." Reaction of glutathione or methionine was not catalysed by the enzyme. It has been shown that some pyridoxal P-dependent enzymes, such as pyrophosphatase (3), tyrosine phenol-lyase (6), L-cystine desulfhydrase (22), L-glutamyl desulfhydrase (23), and L-glutamate desulfhydrase (24), catalyse the N-elimination of the reverse reaction, and the reverse reaction, and the reverse reaction, and the reverse reaction, and the reverse reaction, and the reverse reaction.
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Retention time (min)

Effect of pH on the activity of the DAP ammonia-lyase.

The reactions were carried out for 20 min at 30°C in the following buffers: sodium acetate buffer (pH 5.0), potassium phosphate buffer (pH 6.5), HEPES/KOH buffer (pH 7.0), Tris/HCl buffer (pH 7.5) and CAPS/NaOH buffer (pH 8.0), according to the method of Bradford [14].

Strain Salmonella typhimurium (ATCC 12522) 35.1 852 15.6 442
S. typhimurium (ATCC 14193) 28.5 283 13.5 655
S. typhimurium (ATCC 14193) 17.9 179 10.9 415

Distribution of DAP ammonia-lyase among bacteria

Each strain was grown in two media (I and II, ~5 h), as described under "Experimental Procedure". The strains tested at 0.1 and 0.02 units/ml. The values were calculated as micromoles of ammonia formed per minute at 30°C. The results are given in Table I.

### Table I

| Strain | Pyruvate formed from L-DAP | Pyruvate formed from 2-DAP |
|--------|---------------------------|---------------------------|
|        | total activity x 10⁶ | total activity x 10⁶ |
|        | μmol/min | μmol/min |
| S. typhimurium (ATCC 12522) | 35.1 | 852 |
| S. typhimurium (ATCC 14193) | 28.5 | 283 |
| S. typhimurium (ATCC 14193) | 17.9 | 179 |

### Table II

Purification of the DAP ammonia-lyase from S. typhimurium.

Protein was determined from the absorbance of 280 nm using an extinction coefficient of 1.41 ml·mg⁻¹·cm⁻¹ throughout. The reaction was carried out under standard conditions with DAP as substrate.

| Step | Total Protein | Specific Activity |
|------|---------------|------------------|
| 1.   | Cell-free extract 15,100 15,600 0.772 100 |
| 2.   | DEAE-Sepharose 6,320 6,690 1.01 61 |
| 3.   | Hydroxyapatite 3,460 4,690 1.34 49 |
| 4.   | Ampho-Sepharose 5,460 6,690 1.21 49 |
| 5.   | Cellulofine GCL-2000 125 37.2 45 |
| 6.   | Crystallization 9,820 38.7 46.7 16 |

### Table III

Amino acid composition of the DAP ammonia-lyase

| Amino Acid | Number of residues per subunit |
|------------|-------------------------------|
| Alanine    | 35                            |
| Valine     | 20                            |
| Methionine | 13                            |
| Histidine  | 14                            |
| Lysine     | 5                             |
| Tyrosine   | 5                             |
| Threonine  | 17                            |
| Isoleucine | 16                            |
| Leucine    | 16                            |
| Phenylalanine | 9                        |
| Asparagine | 13                            |
| Glutamic acid | 12                     |
| Leucine    | 19                            |
| Asparagine | 16                            |
| Leucine    | 19                            |

The reaction was carried out at 30°C in reaction mixtures containing 0.035M units of the purified enzyme, 0.13M units of NaOH, 2.5 units of pig heart lactate dehydrogenase, 0.2 MOL of potassium phosphate buffer (pH 7.5) and various amounts of D-TAP (A) or L-DAP (B), as indicated, in a total volume of 1 ml. Velocity(V) was expressed as units pyruvate formed μmol·mg⁻¹·min⁻¹.

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### Table IV

Sequential Edman degradation of DAP ammonia-lyase from *S. typhimurium*.

| Cycle no. | Residue      | Yield (%) |
|-----------|--------------|-----------|
| 1         | Arg          | 1.3       |
| 2         | His          | 0.31      |
| 3         | Gla          | 0.89      |
| 4         | Ser          | 0.88      |
| 5         | Thr          | 0.22      |
| 6         | Arg          | 0.17      |
| 7         | Asp          | 0.17      |
| 8         | Gla          | 0.26      |
| 9         | Thr          | 0.26      |
| 10        | Asp          | 0.20      |
| 11        | Thr          | 0.18      |
| 12        | Val          | 0.17      |
| 13        | Arg          | 0.17      |
| 14        | Ser          | 0.22      |
| 15        | Lys          | 0.18      |
| 16        | Asp          | 0.19      |
| 17        | Gly          | 0.27      |
| 18        | Thr          | 0.14      |
| 19        | Gla          | 0.43      |
| 20        | Ala          | 0.26      |
| 21        | Ser          | 0.26      |
| 22        | Leu          | 0.37      |

*Amount of PTH-amino acid determined by HPLC.*

### Table V

Amino acid composition of phosphopyridoxyl peptides.

|          | mol% | L-6 | L-12 |
|----------|------|-----|------|
| Arg      | 0.01(1) |     |      |
| Asp      | 0.01(1) | 0.09(1) |      |
| Glu      | 0.04(1) | 0.06(1) |      |
| Lys      | 0.16(1) | 0.28(2) | 0.19(2) |
| Thr      | 0.192(2) | 0.15(2) | 0.08(1) |
| Val      | 0.09(1) | 0.07(1) |      |
| Leu      | 0.19(2) | 0.08(1) | 0.07(1) |
| Tyr      | 0.06(1) | 0.12(1) |      |
| Lys      | 0.16(1) |     |      |

*Not determined.*

### Table VI

Automated Edman degradation of the peptide fragments of DAP ammonia-lyase.

| Cycle no. | Peptide fragment | T-26 | L-6 | L-12 |
|-----------|------------------|------|-----|------|
| 1         | Phe              | 0.26 |     |      |
| 2         | Gla              | 0.31 |     |      |
| 3         | Ser              | 0.17 |     |      |
| 4         | Lys              | 0.17 |     |      |
| 5         | Arg              | 0.17 |     |      |
| 6         | Thr              | 0.26 |     |      |
| 7         | Asp              | 0.17 |     |      |
| 8         | Gla              | 0.26 |     |      |
| 9         | Thr              | 0.14 |     |      |
| 10        | Gla              | 0.43 |     |      |
| 11        | Ala              | 0.26 |     |      |
| 12        | Ser              | 0.26 |     |      |
| 13        | Leu              | 0.37 |     |      |

*Numbers shown for each residue are the amounts of PTH-amino acids determined by HPLC.*

Not determined.