Properties of meso-α,e-Diaminopimelate D-Dehydrogenase from Bacillus sphaericus*

Haruo Misono‡ and Kenji Soda§

From Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611, Japan

meso-α,e-Diaminopimelate D-dehydrogenase, which has been purified to homogeneity from the extract of Bacillus sphaericus IFO 3525, has a molecular weight of about 80,000 and consists of two subunits identical in molecular weight (approximately 40,000). The enzyme has a high substrate specificity. In addition to meso-α,e-diaminopimelate, lanthionine is deaminated by the enzyme to a far lesser extent. NADP+ is the exclusive cofactor. The pH optima were at about 10.6 for the deamination of meso-α,e-diaminopimelate and at 7.6 for its amination. L and D isomers of α,ε-diaminopimelate are aminated by the enzyme to a far lesser extent. NADP+ is the exclusive cofactor. The pH optima were at about 10.6 for its amination. L and D isomers of α,ε-diaminopimelate and meso-α,ε-diaminopimelate competitively inhibit the oxidation of meso-α,ε-diaminopimelate. Initial velocity and product inhibition studies show that the reductive amination proceeds through a sequential ordered ternary-binary mechanism. NADPH binds first to the enzyme followed by L-α-amino-ε-ketopimelate and ammonia, and the products are released in the order of meso-α,ε-diaminopimelate and NADP⁺. The Michaelis constants are as follows: meso-α,ε-diaminopimelate (2.5 mM), NADP⁺ (85 mM), NADPH (0.2 mM), L-α-amino-ε-ketopimelate (0.24 mM), and ammonia (12.5 mM). The pro-S hydrogen at C-4 of the dihydronicotinamide ring of NADPH is transferred to the substrate; the enzyme is B-stereospecific. Fluorometric study on the binding of NADPH to the enzyme revealed that the enzyme contains two coenzyme binding sites per molecule.

meso-α,ε-Diaminopimelate, a key intermediate in bacterial lysine biosynthesis and a constituent of the cell wall of certain bacteria, is metabolized through a few pathways (1). Recently, we found the occurrence of a novel dehydrogenase acting on meso-α,ε-diaminopimelate in Bacillus sphaericus, Corynebacterium glutamicum, Brevibacterium sp., and several other bacteria (2, 3). The enzyme catalyzes the reversible oxidative removal of an amino group with D configuration in the substrate to yield L-α-amino-ε-ketopimelate in the presence of NADP⁺ (3). Thus, we have designated the enzyme meso-α,ε-diaminopimelate D-dehydrogenase, and have purified it from B. sphaericus IFO 3525 to investigate some of the fundamental properties (4). meso-α,ε-Diaminopimelate does not occur in the cell wall of B. sphaericus (5). Although the physiological function of the enzyme has not been elucidated, the enzyme probably plays a role in lysine biosynthesis, because it is found predominantly in the bacterial strains that are known as good lysine producers (6).

The enzymological and physicochemical characteristics of the purified enzyme are described here with emphasis on the kinetic mechanism of the enzyme reaction and stereospecificity of hydrogen transfer by the enzyme.

EXPERIMENTAL PROCEDURES

RESULTS

Molecular Weight and Subunit Structure—The molecular weight of the enzyme was determined to be approximately 80,000 by gel filtration method as reported in a previous paper (4). A molecular weight of 78,400 ± 4,000 was also obtained by sedimentation equilibrium method assuming a partial specific volume of 0.74. The subunit structure was examined by sodium lauryl sulfate-polyacrylamide gel electrophoresis. The sodium lauryl sulfate-treated enzyme was subjected to electrophoresis in the presence of 0.1% sodium lauryl sulfate and migrated as a single protein (Fig. 1). The molecular weight of the subunit was estimated to be about 41,000 from a semilogarithmic plot of molecular weight against mobility. These results show that the enzyme is composed of two subunits identical in molecular weight.

Stability of Enzyme—The enzyme can be stored at −20°C in 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and 10% glycerol for periods of over 1 year without loss of the activity. However, the enzyme activity was rapidly lost in the absence of glycerol at −20°C (about 60% loss for 3 days). The enzyme is also stable at 4°C in the presence of 0.01% 2-mercaptoethanol and 20% glycerol for about 1 month, but the enzyme activity decreased gradually in the absence of glycerol.

The enzyme was stable up to 45°C, when heated for 10 min in 0.1 M potassium phosphate buffer (pH 7.2). When incubated at 55°C for 10 min, the enzyme was most stable at about pH 7.0.

Isoelectric Point—The isoelectric point of the enzyme was determined to be 4.3 by isoelectric focusing on polyacrylamide gels.

Effect of pH on the Enzyme Activity—The enzyme shows the maximum reactivity at pH 10.5 for the oxidative deamination of meso-α,ε-diaminopimelate (Fig. 2A). The pH optimi

* Portions of this paper (including “Experimental Procedures,” Figs. 1 to 9, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document 80M-745, cite author(s), and include a check or money order for $2.70 per set of photocopies. Full sized photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
mum for the reductive amination of L-α-amino-β-ketopimelate is about 7.5 (Fig. 2B). The rate of the amination reaction declines markedly above pH 9.0, and is about 11 times higher than that of the oxidative deamination at pH 7.5.

**Equilibrium Constant**—The apparent equilibrium constant was determined to be 4.46 × 10⁻¹⁰ at pH 7.29 and 25°C.

**Substrate Specificity**—The enzyme acts almost specifically on meso-α,ε-diaminopimelate, although lanthionine (a mixture of L, L, and meso) also is deaminated only slightly (0.58%). None of the following amino acids are substrates: L and D isomers of α,ε-diaminopimelate, DL-α-amino-β-ketopimelate, DL-α,β-diamino-L-ketopimelate, L-djenkolate, L-cystine, L-lysine, S-(β-aminomethyl)-L-homocysteine, L-ornithine, L-arginine, L-α,β-diaminobutyrate, L-histidine, L-phenylalanine, L-tyrosine, L-glutamate, L-aspartate, L-leucine, L-valine, L-methionine, L-serine, L-alanine, L-α-amino L-butyrate, D-lysine, D-glutamate, D-leucine, D-alanine, D-phenylalanine, ε-aminocaproate, 7-aminoheptanoate, and 8-amino-octanoate.

L-α-Amino-β-ketopimelate is the exclusive substrate for the amination reaction, although α-keto analog of lanthionine has not been examined because of its unavailability. None of α-ketoglutarate, pyruvate, oxalacetate, glyoxylate, α-keto butyrate, α-ketovalerate, α-keto L-crotonate, α-ketoisocaproate, α-ketoisovalerate, and phenylpyruvate are substrates.

**Coenzyme Specificity**—The enzyme requires NADP⁺ as a coenzyme for the oxidative deamination of meso-α,ε-diaminopimelate. NAD⁺ and NAD⁺ analogs (3- pyridine aldehyde-NAD⁺, 3-acetylpyridyl-NAD⁺, 3-acetylpyridyldeaminono-NAD⁺ and deaminono-NAD⁺) are inactive and have no effect on the NADP⁺-dependent deamination of meso-α,ε-diaminopimelate. NADPP⁺ (1 mM), an analog of NADP⁺ (7), inhibits slightly (10%) the reaction.

**Inhibitors**—L and D isomers of α,ε-diaminopimelate and meso-α,β-diamino L-decapeptide behave as competitive inhibitors against meso-α,ε-diaminopimelate. The inhibition constants for L- and D-α,ε-diaminopimelate and meso-α,ε-diamino L-decapeptide are 3.12, 4.0, and 4.16 mM, respectively. DL-α-Aminocaproate, DL-α,β-diamino L-ketopimelate, and ε-aminocaproate, and 8-amino-ocanooacetae also inhibit the deamination reaction, although slightly, L- and D-Lysine, L- and D-glutamate, pimelate, L-djenkolate, L-α-amino L-ketopimelate, L-ε-cystine, and S-(β-aminomethyl)-L-homocysteine are not inhibitory.

Glyoxylate (5 mM) inhibited the reductive amination of L-α-amino-β-ketopimelate about 30%, but none of the following keto acids showed appreciable influence: α-ketoglutarate, pyruvate, oxalacetate, α-keto butyrate, α-ketovalerate, α-keto L-crotonate, α-ketoisocaproate, and phenylpyruvate.

The enzyme was inhibited completely by p-chloromercuribenzoate (1 μM), and HgCl₂ (10 μM), typical inhibitors of —SH enzymes. Some other metal ions (1 mM), such as Mg²⁺, Mn²⁺, Zn²⁺, Cu²⁺, Ni²⁺, and Mo⁶⁺ were not inhibitory. EDTA, a,dipryridyl, NaF, NaN₃, Na₂SO₄, and pyridoxal 5'-phosphate had no effect on the oxidative deamination of meso-α,ε-diaminopimelate. None of the following purine bases, nucleosides, and nucleotides affected the activity: adenine, adenosine, AMP, ADP, ATP, guanine, GMP, GDP, and GTP.

**Kinetic Mechanism**—A series of steady state kinetic analyses was conducted to investigate the reaction mechanism.

Initial velocity studies for the oxidative deamination were first performed with NADP⁺ as a variable substrate in the presence of several fixed concentrations of meso-α,ε-diaminopimelate. Plots of reciprocals of initial velocities against reciprocals of NADP⁺ concentrations gave a family of straight lines which intersect in the left quadrant (Fig. 3). The apparent Michaelis constant for one substrate is dependent on the concentration of the other. According to Cleveland (16), the data shown in Fig. 3 indicate that the reaction proceeds via the formation of ternary complex of the enzyme with NADP⁺ and meso-α,ε-diaminopimelate. The Michaelis constants for NADP⁺ and meso-α,ε-diaminopimelate were calculated to be 0.83 μM and 2.5 mM, respectively, from the secondary plots of intercepts versus reciprocal concentrations of the other substrate (16).

A kinetic analysis in the reductive amination was performed to investigate several possible reaction mechanisms (16-19). Fig. 4A shows double reciprocal plots of velocities against L-α-amino-β-ketopimelate concentration at several concentrations of ammonia and a high and constant concentration of NADPH. The double reciprocal plots gave straight lines intersecting on the abscissa. At a high level of ammonia, the double reciprocal plots of velocities against L-α-amino-β-ketopimelate concentration at several fixed concentrations of NADPH also gave straight intersecting lines (Fig. 4B). However, with L-α-amino-β-ketopimelate at a saturating concentration, the double reciprocal plots of velocities against NADPH concentrations at several different concentrations of ammonia were quite different from those shown in Fig. 4A and B and gave parallel lines (Fig. 4C). These Eadie-Hofstee kinetic patterns rule out the possibility of random addition of substrates and represent a sequential ordered mechanism in which L-α-amino-β-ketopimelate binds to the enzyme between NADPH and ammonia (16, 19). The Kₘ values for NADPH, L-α-amino-β-ketopimelate, and ammonia were determined to be 0.20, 0.24, and 12.5 mM, respectively.

The product inhibition studies in the oxidative deamination were performed to determine the order of substrate addition and product release according to the method of Cleveland (16-19). With NADPH as an inhibitor, the double reciprocal plots of velocities against NADP⁺ concentrations at a high and constant concentration of meso-α,ε-diaminopimelate showed competitive inhibition (Fig. 5B). NADPH showed noncompetitive inhibition with respect to meso-α,ε-diaminopimelate in the presence of a high and constant NAD⁺ concentration (Fig. 5A). These results suggested that NADP⁺ and NADPH can bind to the free form of the enzyme. Thus, NADP⁺ binds first to the enzyme and NADPH is the last product which is released from the enzyme. The other product inhibition patterns for the oxidative deamination observed with L-α-amino-β-ketopimelate and ammonia as the inhibitors (Fig. 5, D to F) were identical with the predicted patterns for the sequential ordered binary-ternary kinetic mechanism except the inhibition by L-α-amino-β-ketopimelate with respect to meso-α,ε-diaminopimelate (Fig. 5C). The noncompetitive inhibition by L-α-amino-β-ketopimelate with meso-α,ε-diaminopimelate as a variable substrate (Fig. 5C) rule out a mechanism of Theorell-Chance type (24), and is different from the predicted pattern for the sequential ordered binary-ternary kinetic mechanism (Table I, Part A). However, if L-α-amino-β-ketopimelate binds also to the free enzyme as a dead-end inhibitor, the noncompetitive inhibition pattern will be obtained as described by Cleveland (25). This is the case also for microbial glutamate dehydrogenase (26). These results show that the sequence of addition of the substrates in the oxidative deamination is NADP⁺ and meso-α,ε-diaminopimelate, and that of release of products is ammonia, L-α-amino-β-ketopimelate, and NADPH.

In order to confirm this order of substrate addition, the product inhibition studies were carried out on the reductive amination. As shown in Table I, Part B, the product inhibition pattern observed with NADP⁺ and meso-α,ε-diaminopimelate as the inhibitors are identical with the predicted pattern for the sequential ordered ternary-binary kinetic mechanism. These results obtained from initial velocity and product
inhibition studies show that the sequence of addition of the substrates in the reductive amination is NADPH, L-α-aminoadipate, and ammonia, and that of release of products is meso-α,ε-diaminopimelate and NADP⁺.

**Stereospecificity of Hydrogen Transfer between Coenzyme and Substrate**—When L-α-amino-ε-ketopimelate was reduced by NADPH with specific activity [4B-3H]NADPH, the significant amount of radioactivity of [4B-3H]NADPH was transferred to meso-α,ε-diaminopimelate (Table II). However, a marked decrease in the specific radioactivity was noted upon transfer of the tritium from the pyridine nucleotide to meso-α,ε-diaminopimelate. It is probably due to the isotope effect, because the enzyme reactions did not proceed completely and the ratio of NADPH to the specific radioactivity of NADPH used to that of meso-α,ε-diaminopimelate formed decreased with an increase in the incubation time (Table II, Experiments 1 and 2). The isotope effect factor of 4 has been reported for the tritium transfer from [4B-3H]NADPH to steroids catalyzed by 3β-oxosteroid 5β-reductase (27-29), and also for the tritium transfer of the tritium from Dl-isocitrate to NAD⁺ by isocitrate dehydrogenase (30). This magnitude of the isotope effect is comparable with that observed in the present experiment. The results suggest that the transfer of hydrogen from the pyridine nucleotide cofactor is the rate-limiting step of the overall dehydrogenase reaction. Thus, meso-α,ε-diaminopimelate d-dehydrogenase catalyzes the transfer of the pro-S hydrogen at C-4 of the dihydronicotinamide ring of NADPH to the substrate: the enzyme is B-stereospecific.

**Difference Spectrum**—The addition of NADPH to the enzyme induced a spectral change. The difference spectrum of the enzyme/NADPH mixture versus the enzyme plus NADPH shows a positive peak at 360 nm (Fig. 6). This change shows that the binding of NADPH to the enzyme and the occurrence of a red shift. This red shift is characteristic of B-stereospecific dehydrogenases according to the observations of Fisher et al. (31) and Biellman et al. (32). This, meso-α,ε-diaminopimelate d-dehydrogenase-catalyzed transfer of hydrogen is B-stereospecific, and is in accordance with the result of the hydrogen transfer study described above.

**Fluorescence Spectra of the Enzyme-NADPH Binary Complex and Determination of the Number of NADPH Binding Sites**—The fluorescence of protein is associated with the ultraviolet absorption, with a major contribution of the tryptophan residue (33). When excited at 280 nm, the enzyme has a single emission band around 340 nm (Fig. 7A). The fluorescence of the enzyme is quenched by forming a complex with NADPH. The fluorescence spectra of NADPH, the enzyme, and their complex are shown in Fig. 7B. The fluorescence of NADPH showed the emission maximum at 470 nm, when excited at 340 nm. The fluorescence intensity of NADPH was enhanced significantly by addition of the enzyme. This fluorescence enhancement was observed similarly for the several other nicotinamide nucleotide-dependent dehydrogenases (21, 22, 24, 25). The fluorescence spectra of the enzyme, NADPH and the enzyme plus NADPH were also taken by excitation at 285 nm. By addition of NADPH to the enzyme, the NADPH fluorescence at 470 nm is increased markedly with a concomitant decrease in the protein fluorescence at 340 nm (Fig. 8). An increase in the fluorescence at 470 nm is greater than that obtained by excitation at 340 nm. This suggests that the energy transfer from tryptophan residue of the enzyme protein.

The enzyme was treated with various concentrations of NADPH by measuring fluorescence at 445 nm (Fig. 9A). The maximum difference in NADPH fluorescence was estimated to be 50 from a double-reciprocal plot according to Price and Radda (36). The data analysis according to Klotz (23) leads to a straight line (Fig. 9B) extrapolating to a value of two binding sites per mol of enzyme. Dissociation constant (Kd) obtained from reciprocal slope of the straight line was 12.3 μM.

**DISCUSSION**

The enzyme from B. sphaericus is unique as a pyridine nucleotide-dependent dehydrogenase in catalyzing oxidative deamination of an amino group with D configuration. It is highly specific for meso-α,ε-diaminopimelate. Since meso-α,δ-diamino adipate was not a substrate, the length of carbon chain of diaminomonocarboxylic acids is critical in the susceptibility to the enzyme. Lanthionine is a very poor substrate and its meso form probably is a substrate by analogy with α,ε-diaminopimelate. Thus, the substitution of γ-CH₂ of meso-α,ε-diaminopimelate with a sulfur atom leads to a marked decrease in the reactivity. Both amino and carboxyl groups of meso-α,ε-diaminopimelate participate in binding of the molecule to the enzyme, because L- and D-α,ε-diaminopimelate, DL- and meso-α,δ-diamino adipate, L-α-aminoadipate, and ε-aminocaproate inhibited the reaction of meso-α,ε-diaminopimelate.

The enzyme is inhibited strongly by p-chloromercuribenzoate and HgCl₂: the —SH group of the enzyme plays an important role in catalysis as shown for alanine dehydrogenase (37, 38) and leucine dehydrogenase (39). It is not affected by pyridoxal 5'-phosphate, and in this respect is not analogous to leucine dehydrogenase (13) and glutamate dehydrogenase (40), which contain a catalytically essential lysine residue, but is more similar to alanine dehydrogenase (37).

Amino acid dehydrogenases studied so far catalyzes the reaction in a sequential ordered mechanism (13, 26, 37, 41-46), except bovine liver glutamate dehydrogenase which shows a random mechanism (47, 48). meso-α,ε-Diaminopimelate d-dehydrogenase is different from NAD⁺-specific glutamate dehydrogenase (42) and alanine dehydrogenase (NAD⁺) (37) in the sequence of addition of the substrates, and is similar to NAD⁺-specific glutamate dehydrogenase (26, 41-46) and leucine dehydrogenase (NAD⁺) (13) in the kinetic mechanism. These suggest that the sequence of substrate addition is characteristic of the enzymes, but does not depend on the cofactor.

Nicotinamide nucleotide-dependent dehydrogenases show A or B stereospecificity for hydrogen removal from the C-4 of the nicotinamide moiety of the reduced coenzyme (49, 50). We have shown here that the pro-S hydrogen at C-4 of the dihydronicotinamide ring of NADPH is transferred to the substrate: the enzyme is B-stereospecific. This supports the generalization of You et al. (51) on the enzyme stereospecificity for hydrogen removal from nicotinamide nucleotides.

Titration of meso-α,ε-diaminopimelate d-dehydrogenase with NADPH shows the presence of two coenzyme binding sites per mol of enzyme. The enzyme consists of two subunits identical in molecular weight; one subunit involves one catalytic site. A similar dimeric structure has been reported for D-erythro-3,5-diaminohexanoate dehydrogenase (52) and 2,4-diaminopimelate dehydrogenase (53) of Clostridium sticklandii, although leucine dehydrogenase (13), alanine dehydrogenase (37), and glutamate dehydrogenases from various sources (40) are hexamer or tetramer. Each subunit of leucine dehydrogenase also has one coenzyme binding site (38). The involvement of tryptophan residue in the coenzyme binding site has been reported for bovine and Neurospora glutamate dehydrogenases and the energy transfer between the reduced coenzyme and tryptophan residue has been observed (40).

The similar energy transfer occurred in meso-α,ε-diaminopimelate d-dehydrogenase. This suggests that tryptophan res-
ide probably presents in or near the active site of the enzyme, and its function in the catalytic action is currently under investigation. Recently, we developed the spectrophotometric procedure for specific microdetermination of meso-α,ε-diaminopimelate with the enzyme (54).

Acknowledgments—We thank Dr. T. Yamamoto and Dr. S. Nagasaki for their helpful advice, and Dr. M. Kuwahara for the generous gift of an NADP⁺ analog.

REFERENCES
**SUPPLEMENTAL MATERIAL**

**TO**

**PROPERTIES OF meso-a,-e-DIAMINOPIMELATE D-DEHYDROGENASE FROM BACILLUS SPHAERICUS**

Haruo Misono and Kenji Soda

**EXPERIMENTAL PROCEDURES**

Materials — NADH, NADP⁺ and NADPH were obtained from Sigma, Na⁺H₄PO₄ and Na⁺₂HPO₄ from Kanto, Tokyo; NAD⁺ analogs from Sigma Chemical; 7-L-glutamic acid from Nitta, Tokyo; 7-L-glutamic dehydrogenase from Rocheing-Manheim; DL-arginine deiminase from Sigma Chemical; DL-arginine from Nakarai, Kyoto; and a-amino acid from Nakarai, Tokyo. NAD⁺, NADP⁺ and NADPH were synthesized according to the methods given in the literature. Glycine-NaCl and glycine-KCl-hydroxylamine hydroxide buffers were made up by adding 5 M NaOH and hydroxylamine, respectively, to a mixture containing 0.9 M glycine and 0.5 M KCl to adjust the pH. Other chemicals were analytical grade reagents.

**Decomposition** — The enzyme was purified to homogeneity from a partially purified thrice previously described preparation. The activity was measured by increasing the absorbance at 250 nm of the reaction mixture containing 20 μM of each NAD⁺, NADP⁺, and NADPH and 0.8 μM of the enzyme. The absorbance coefficient was obtained from the absorbance at 280 nm of the enzyme dilution. The reaction was started by addition of enzyme (or substrate) and followed by measuring the change in absorbance at 250 nm of the mixture. Kinetic measurements were conducted at 25°C with a Hitachi spectrophotometer using 1.0-cm light path. The data for titration of the enzyme were obtained by absorbance and dry weight determinations. The absorbance coefficient was used throughout, obtained by absorbance and dry weight determinations.

**Ultracentrifugal Analysis** — The molecular weight of the enzyme was determined by the ultracentrifugal sedimentation equilibrium method, which was carried out according to the method of Richetti and Sydansk (15). The enzyme was dialyzed against 0.25 M sodium phosphate buffer and then isolated by using 11.4 ml sodium lauryl sulfate solution containing 0.1% mercuric phosphate at 37°C for 7 h. Standard proteins: bovine serum albumin (Mr = 67,000), soybean trypsin inhibitor (Mr = 20,000), ovalbumin (Mr = 44,000), bovine heart lactate dehydrogenase (Mr = 140,000), Lysophosphatidylethanolamine (Mr = 20,000), myoglobin (Mr = 17,000), myoglobin (Mr = 11,900), and cytochrome C (Mr = 12,400) were treated in the same way.

**NAD⁺ and NADP⁺ and NADPH**

**Enzyme Preparation** — The enzyme was prepared from meso-a,-e-Diaminopimelate D-dehydrogenase. Twenty micromoles of the enzyme was subjected. The direction of migration is from the cathode (top) to the anode (bottom).

**Fig. 1. Sodium lauryl sulfate-disc gel electrophoresis of meso-a,-e-Diaminopimelate D-dehydrogenase.** Ten micromoles of the enzyme was subjected. The direction of migration is from the cathode (top) to the anode (bottom).

**Fig. 2. Effect of pH on the oxidative denamination and the reduction of the enzyme.** The enzyme activity was measured in the following buffers (pH 2-7) and concentration of each substrate: A, Mesotel buffer; B, potassium phosphate buffer; C, glycine-NaCl buffer; D, glycine-KCl buffer; E, glycine-NaCl-KCl buffer; F, glycine-KCl-NaCl buffer; G, glycine-KCl-NaCl-HCl buffer; H, glycine-NaCl-HCl buffer; I, glycine-KCl-HCl buffer; J, glycine-KCl-NaCl-HCl buffer. The experimental data were obtained from the secondary plots of intercross various concentrations of the substrate (I-K).

Fig. 6. Difference spectrum of the enzyme-NADPH complex with the enzyme plus NADPH at 25°C. Enzyme and NADPH were used at concentrations of 1.9 μM and 0.07 μM per ml, respectively, in 10 mM potassium phosphate buffer (pH 7.4). ——: reference and ——: the difference spectrum.

Fig. 7. Fluorescence change of enzyme (A) or NADPH (B) by formation of the NADPH-enzyme complex. Excitation wavelength was 290 nm (A) and 340 nm (B). The fluorescence was measured at 25°C and pH 7.5 (0.1 M potassium phosphate buffer), fluorescence of the enzyme (11.19 μM); II, Fluorescence of the enzyme (11.19 μM) in the presence of NADPH (34.1 μM) and III, Fluorescence of NADPH (34.5 μM).

Fig. 8. Fluorescence spectra of enzyme, NADPH and enzyme-NADPH complex. The fluorescence spectra were taken at pH 7.5 (0.1 M potassium phosphate buffer) and 25°C. Excitation wavelength was 290 nm. I, enzyme (11.19 μM); II, enzyme (11.19 μM) plus NADPH (37.2 μM) and III, NADPH (37.2 μM).
**meso-\(\alpha,\varepsilon\)-Diaminopimelate D-Dehydrogenase**

![Graph](image)

**Figure 9. Titration Curve of the enzyme with NADPH.**

**Table I**

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Predicted</th>
<th>Observed</th>
<th>Predicted</th>
<th>Observed</th>
<th>Predicted</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH (\alpha)</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>NC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>DAP</td>
<td>NC</td>
<td>NC</td>
<td>UC</td>
<td>UC</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>L-(\alpha)-AFP</td>
<td>UC</td>
<td>UC</td>
<td>UC</td>
<td>UC</td>
<td>UC</td>
<td>UC</td>
</tr>
<tr>
<td>DAP</td>
<td>NADPH</td>
<td>UC</td>
<td>UC</td>
<td>UC</td>
<td>UC</td>
<td>UC</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
<th>Exp. 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH used</td>
<td>1.54 x 10^4</td>
<td>6.54 x 10^4</td>
<td>2.24 x 10^4</td>
<td>6.59 x 10^4</td>
</tr>
<tr>
<td>DAP formed</td>
<td>1.49 x 10^4</td>
<td>2.06 x 10^4</td>
<td>0.59 x 10^4</td>
<td>1.00 x 10^4</td>
</tr>
</tbody>
</table>

**NADPH/DAP**

| Ratio (NADPH/DAP) | 3.70 | 3.69 | 3.80 | 3.55 |

**Notes:**

- Abbreviations used: L-\(\alpha\)-AFP, L-\(\alpha\)-amino-\(\varepsilon\)-ketopimelate; DAP, meso-\(\alpha,\varepsilon\)-diaminopimelate; J, competitive inhibition; UC, uncompetitive inhibition and NC, noncompetitive inhibition.
- Predicted product inhibition patterns were obtained from the steady state algebraic equation for the sequential ordered binary-ternary or ternary-binary mechanism proposed by Cleland (16).