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Occurrence of L-Lysine ε-Dehydrogenase in *Agrobacterium tumefaciens*

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A novel amino acid dehydrogenase catalyzing the oxidative deamination of the ε-amino group of L-lysine was found in the crude extract of *Agrobacterium tumefaciens* ICR 1660. The enzyme required NAD^+ and was specific for L-lysine. The enzyme was optimally active at about pH 9.7.

L-Lysine is metabolized via saccharopine in mammals (8, 9). The microbial degradation of lysine, however, is diverse and is initiated by the following reactions: decarboxylation in *Hafnia alveri* (= *Bacterium cadaveris*) (17) and *Escherichia coli* (13); ε-transamination in *Flavobacterium lutescens* (= *Flavobacterium fuscum* and *Achromobacter liquidum* [14]) (15, 16); α-oxidase reaction of *Trichoderma viride* (10); monooxygenase reaction in *Pseudomonas fluorescens* (18, 19) and *Pseudomonas putida* (3); mutase reaction in clostridia (4, 5); and ε-*N*-acetylation in *Hansenula saturnis* (12) and *Rhizoctonia leguminicola* (7). During a search for lysine metabolism of *Agrobacterium tumefaciens*, which is known to cause a crown gall, a neoplastic disease of dicotyledonous plants (1), we found lysine dehydrogenase activity in the crude extract. Although the occurrence of a lysine dehydrogenase in human liver homogenates was reported (2), the reaction product and enzymological properties of the enzyme have not been investigated, and the enzyme was speculated to act on the α-amino group of L-lysine.

We present here evidence for the occurrence of a novel bacterial amino acid dehydrogenase that catalyzes the oxidative deamination of the ε-amino group of L-lysine.

The dehydrogenase activity was determined at 30°C by measuring the rate of increase in the absorbance at 340 nm with a Hitachi 124 spectrophotometer. The standard reaction mixture consisted of 20 μmol of L-lysine, 1 μmol of NAD^+, 200 μmol of glycine-KCl-KOH buffer (pH 10.0), and enzyme in a final volume of 1.0 ml. Protein was determined by the procedure of Lowry et al. (11) with bovine serum albumin as a standard. *Agrobacterium tumefaciens* ICR 1660 was grown in a medium containing 0.5% L-lysine, 0.5% peptone, 0.2% NaCl, 0.2% K_2HPO_4, 0.2% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.01% yeast extract. The pH of the medium was adjusted to 7.2 with 4 N NaOH. The bacteria were grown aerobically in 2-liter flasks containing 500 ml of the medium on a reciprocating shaker at 30°C for 20 h. The cells were harvested by centrifugation and washed twice with 0.85% NaCl. The washed cells were disrupted by grinding in a mortar with levigated aluminum oxide and extracted with 0.1 M potassium phosphate buffer (pH 7.2) containing 0.02% 2-mercaptoethanol. The supernatant solution obtained by centrifugation was dialyzed against 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and used as the crude extract. The crude extract was brought to 40% saturation with solid ammonium sulfate, and the precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant solution to achieve 60% saturation. The precipitate obtained by centrifugation was dissolved in 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and placed on a DEAE-cellulose column equilibrated with 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol. After the column was washed thoroughly with the buffer supplemented with 0.05 M KCl, the enzyme was eluted with the buffer supplemented with 0.1 M KCl. The active fractions were pooled and concentrated by the addition of ammonium sulfate (70% saturation). The precipitate was dissolved in 0.01 M potassium phosphate buffer (pH 7.4) containing 0.01% 2-mercaptoethanol and dialyzed against the same buffer. The enzyme was purified about 30-fold with 61% yield by these procedures. All operations were carried out at 5°C. This enzyme preparation, unless otherwise specified, was used in this experiment.

When L-lysine was incubated with the crude extract of *A. tumefaciens* in the presence of

NAD^+, the reduction of NAD^+ was observed. The reaction occurred only in the presence of L-lysine and enzyme and proceeded linearly as a function of the amount of enzyme and incubation time. L-lysine was oxidized with the liberation of an equimolar amount of ammonia (Fig. 1). The enzymatic ammonia formation occurred only in the presence of both L-lysine and NAD^+. No activity of L-amino acid oxidase (6) or L-lysine α-oxidase (10) was found in the enzyme preparation.

The enzyme was specific for L-lysine among various amino acids and amines tested, and $NADP^+$ could not replace NAD^+. The enzyme has an optimum reactivity at about pH 9.7.

One mole of ammonia was released per mole of L-lysine, suggesting that one of two amino groups of lysine is removed. The reaction mixture (2.0 ml) containing 20 μmol of L-lysine, 20 μmol of NAD^+, 200 μmol of Na_2CO_3 buffer (pH 9.5), and 0.492 mg of enzyme was incubated at 30°C for 1 h. After the addition of 0.2 ml of 50% trichloroacetic acid and centrifugation, samples of the supernatant solution were examined by high-voltage paper electrophoresis in 1 N formic acid at 1,000 V for 2 h. In addition to lysine, an unknown compound was observed only in the complete system. This compound reacted with *o*-aminobenzaldehyde and ninhydrin to develop an orange and a yellow color, respectively, but did not react with 2,4-dinitrophenylhydrazine. These suggest that a cyclized form of the keto analog of lysine, i.e., Δ^1-piperideine-2-carboxylate or Δ^1-piperideine-6-carboxylate is produced (16). The reaction product showed the R_f value (0.43) closely similar to those of Δ^1-piperideine-2-carboxylate and Δ^1-piperideine-6-carboxylate, which were prepared by oxidation of L-lysine

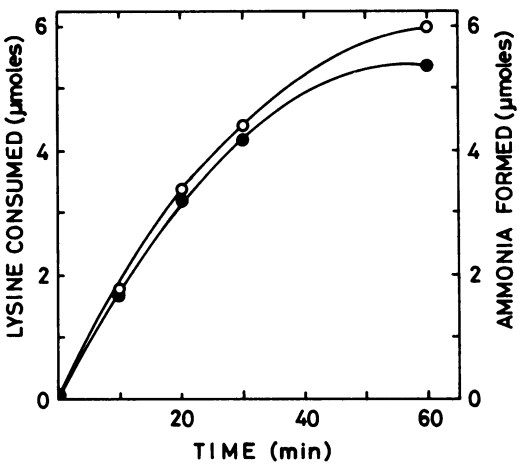

FIG. 1. Enzymatic formation of ammonia from L-lysine. The reaction mixture was composed of 10 μmol of L-lysine, 10 μmol of NAD^+, 200 μmol of Na_2CO_3 buffer (pH 9.5), and 0.492 mg of enzyme in a final volume of 2.0 ml. After incubation at 30°C for the indicated times, the reaction was terminated by the addition of 0.2 ml of 50% trichloroacetic acid, and the mixture was centrifuged. Lysine and ammonia were determined with a Hitachi 034 automatic amino acid analyzer. Symbols: ○, lysine consumed; ●, ammonia formed.

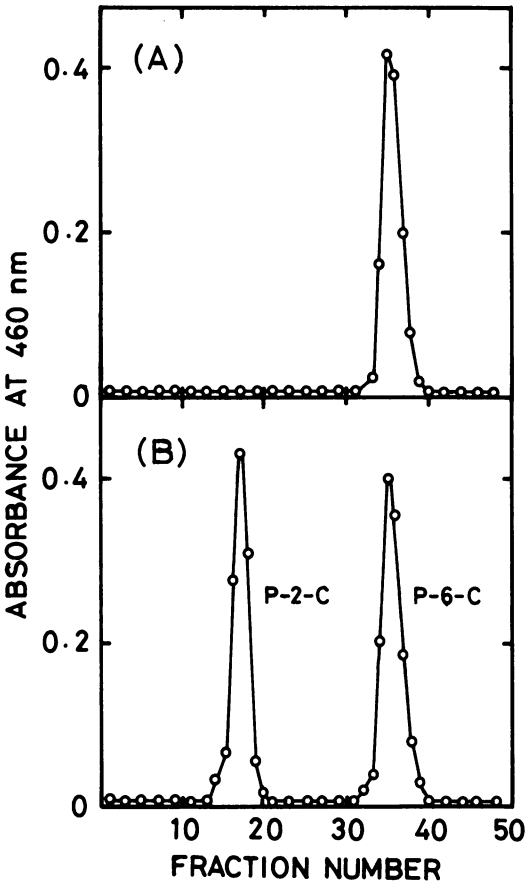

FIG. 2. Ion-exchange chromatography of the reaction product formed from L-lysine. The reaction mixture (2.0 ml) containing 20 μmol of L-lysine, 20 μmol of NAD^+, 200 μmol of Na_2CO_3 buffer (pH 9.5), and 0.492 mg of enzyme was incubated at 30°C for 1 h. After the addition of 0.2 ml of 50% trichloroacetic acid and centrifugation, the supernatant solution (1.0 ml) was applied to a column (0.9 by 25 cm) of a Hitachi 034 automatic amino acid analyzer, equilibrated with 0.2 M citrate buffer (pH 3.25). The column was eluted with the same buffer at a flow rate of 1.0 ml per min at 55°C. The eluate was collected in 1.0-ml portions and mixed with 0.2 ml of 0.05 M *o*-aminobenzaldehyde in 0.2 M potassium phosphate buffer (pH 8.0). Absorbance was measured at 460 nm after incubation at 37°C for 2 h. (A) Reaction product; (B) authentic Δ^1-piperideine-2-carboxylate (P-2-C) and Δ^1-piperideine-6-carboxylate (P-6-C).

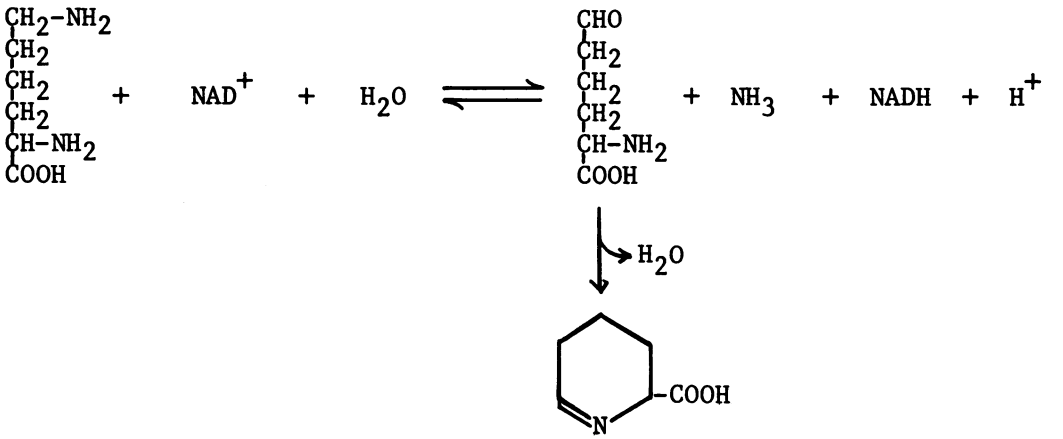

FIG. 3. L-Lysine ε-dehydrogenase reaction.

with L-lysine α-oxidase of *T. viride* (10) and by transamination of L-lysine with L-lysine-α-ketoglutarate ε-aminotransferase of *F. lutescens* (15), respectively, on paper chromatography with the solvent of *n*-butanol–acetic acid–water (4:1:1, vol/vol/vol) (16). However, the product was separated from Δ^1-piperideine-2-carboxylate and behaved identically with Δ^1-piperideine-6-carboxylate, when examined by high-voltage paper electrophoresis. The reaction mixture was analyzed also by cation-exchange chromatography (Fig. 2). The product from lysine was identified as authentic Δ^1-piperideine-6-carboxylate. In addition, both of the orange complexes of the reaction product and the authentic Δ^1-piperideine-6-carboxylate with *o*-aminobenzaldehyde showed the same absorption spectrum with a maximum at 460 nm, whereas the *o*-aminobenzaldehyde adduct of Δ^1-piperideine-2-carboxylate exhibited an absorption maximum at 445 nm.

The results obtained show that the ε-amino group of L-lysine is oxidatively deaminated by the enzyme to yield α-aminoadipate δ-semialdehyde, which is spontaneously converted into the dehydrated form, Δ^1-piperideine-6-carboxylate (Fig. 3). Thus, we designated this novel enzyme L-lysine ε-dehydrogenase (EC 1.4.1.). This is the first amino acid dehydrogenase shown to work specifically on the terminal amino group of amino acids.

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