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L-Ornithine Decarboxylase from *Hafnia alvei* Has a Novel L-Ornithine Oxidase Activity

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A novel activity producing γ-aminobutyric acid (GABA) from L-ornithine in the presence of NAD(P)$^+$ was found in the crude extract of L-ornithine-induced *Hafnia alvei*, in addition to L-ornithine decarboxylase (ODC) activity. The reaction system for the former activity consisted of two enzymes, L-ornithine oxidase (decarboxylating, OOD) and γ-aminobutyraldehyde (GABL) dehydrogenase (GDH). OOD catalyzed the conversion of L-ornithine into GABL, CO_2, NH_3, and H_2O_2 in the presence of O_2, and GDH dehydrogenated GABL to GABA in the presence of NAD(P)$^+$. OOD, purified to homogeneity, had a high ODC activity and the activity ratio of ODC to OOD was almost constant throughout the purification (ODC/OOD=160 : 1). The molecular mass of the OOD was about 230 kDa, probably consisting of three identical subunits of a 77 kDa peptide, and OOD had an absorption maximum at 420 nm as well as at 278 nm, the specific absorption for an enzyme containing pyridoxal phosphate (PLP). The content of PLP was estimated at about 1 mol per subunit. OOD was specific to L-ornithine, and other L-amino acids and polyamines including putrescine were inert. The enzyme was activated by PLP, but not by pyridoxamine 5′-phosphate, FAD, FMN, or pyrroloquinoline quinone, and it was inactivated by hydrazine, semicarbazide, and hydroxylamine. The holoenzyme can be resolved to the apoenzyme by incubation with hydroxylamine, and reconstituted with PLP. These properties of OOD were almost the same as those of ODC separately purified to homogeneity from *H. alvei*. Zn^{2+} and Cu^{2+}, butanedione, and sodium borohydride inhibited both OOD and ODC in a similar manner. The OOD reaction required O_2 and only the ODC reaction proceeded under anaerobic conditions. The substitution of air for oxygen in the reaction vessel and the addition of catalase-H_2O_2 enhanced only the OOD reaction, resulting in an increase of the ratio of OOD/ODC to 1:30 and 1:4.1, respectively. These results suggested that OOD and ODC are identical and that the former is a side reaction of the latter in the presence of O_2.

Key words: γ-aminobutyric acid, L-ornithine decarboxylase, L-ornithine oxidation, pyridoxal 5′-phosphate, side reaction.

L-Ornithine is an important amino acid, not only as a participant in the urea cycle, but also as a precursor of polyamine biosynthesis. The enzymes involved in these conversions are L-ornithine carbamoyltransferase [EC 2.1.3.3] and L-ornithine decarboxylase (ODC, EC 4.1.1.17). In rat brain, the compound might be a source of a neurotransmitter pool of L-glutamic acid and γ-aminobutyric acid (GABA), where L-ornithine is metabolized *via* L-pyrroline-5-carboxylate by L-ornithine-oxo-acid aminotransferase [EC 2.6.1.13] (*1*). Another metabolic pathway of L-ornithine to L-proline catalyzed by L-ornithine cyclodeaminase [EC 4.3.1.12] has also been demonstrated in bacteria (*2*).

ODC from various organisms has been investigated because it is the rate-limiting enzyme in the biosynthesis of the polyamines and plays an important role in cell proliferation. In *Escherichia coli*, two types of ODC, a biosynthetic enzyme and a biodegradative enzyme, occur (*3*). The biosynthetic ODC is expressed constitutively and the activity is multiply regulated to maintain the level of internal polyamine (*4, 5*). On the other hand, the biodegradative ODC is induced in rich medium at a low pH in the presence of excess substrate (*6*), and appears to play a role in pH homeostasis by consuming protons and neutralizing the acidic by-products produced during anaerobic fermentation (*7, 8*).

Hafnia alvei (formerly named *Bacterium cadaveris*) is a Gram-negative, facultative aerobic bacterium with high lysine decarboxylase activity (*9*). During an investigation of polyamine metabolism (*10*), the organism was found to exhibit not only the activity of ODC, but also that of NAD(P)$^+$-dependent L-ornithine dehydrogenation, induced by L-ornithine. Subsequently, we found that the L-ornithine dehydrogenation was catalyzed by two separable protein fractions, L-ornithine oxidase (decarboxylating,

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Abbreviations: DNP, 2,4-dinitrophenylhydrazine; GABA, γ-aminobutyric acid; GABL, γ-aminobutyraldehyde; GDH, γ-aminobutyraldehyde dehydrogenase; OAB, *o*-aminobenzaldehyde; ODC, L-ornithine decarboxylase; OOD, L-ornithine oxidase (decarboxylating); PLP, pyridoxal 5′-phosphate.

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OOD) and γ-aminobutylaldehyde (GABL) dehydrogenase (GDH), and one of the products is GABA. Moreover, OOD had significant similarity to ODC, and could not be distinguished from the latter in terms of enzymatic properties, or during a series of enzyme purification procedures. In this report, we present evidence supporting the notion that the OOD reaction is catalyzed by ODC as a side reaction.

EXPERIMENTAL PROCEDURES

Culture of Microorganisms—Hafnia alvei IFO 3731 was cultured by shaking for 24 h at 30°C in nutrient medium (pH 7.0, 10 ml) containing 0.5% glucose, 0.5% peptone 0.1% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2O$, and 0.1% yeast extract. It was transferred to 1.0 liter of medium and shaken for a further 24 h. Induction medium (20 liters) containing 0.2% L-ornithine, 0.5% sodium L-glutamate, 0.01% glucose, 0.01% peptone, 0.01% yeast extract, 0.1% KH_2PO_4, 0.2% K_2HPO_4, and 0.01% $MgSO_4 \cdot 7H_2O$ (pH 7.0) was inoculated with this culture and incubated at 30°C for 10 h (agitation, 200 rpm; aeration, 13.4 liters/min).

Purification of OOD, ODC, and GDH—All procedures were performed below 4°C. The cells (90 g, wet weight) were suspended in 10 mM potassium phosphate buffer containing 1 mM 2-mercaptoethanol, 1 mM EDTA, and 5% (w/v) glycerol (Buffer A), and disrupted by sonication (80 W, 90 min, Tomy Seiko UR-200P). After centrifugation (7,000 rpm, 20 min), the supernatant was dialyzed against Buffer A, then applied to a DEAE-Toyopearl column (6.1 × 35 cm) equilibrated with Buffer A. The GABA-forming activity was found in the fractions eluted with Buffer A containing 0.2 M NaCl. The enzyme solution was concentrated by ultrafiltration (Amicon, PM30), and filtered through a Sephadex G-100 column (3.5 × 120 cm). The eluted enzyme was saturated with ammonium sulfate to 20% at pH 6.5. The precipitate was removed by centrifugation, then the supernatant was applied to a Butyl-Toyopearl column (2.0 × 16 cm) equilibrated with Buffer A containing 20% $(NH_4)_2SO_4$. Fractions were eluted by linearly decreasing the $(NH_4)_2SO_4$ concentration. The fraction which shows GABA-forming activity was dialyzed against Buffer A and applied to an FPLC-Mono Q column (HR5/5, Pharmacia). Two fractions involved in the GABA-forming reaction were eluted separately with an increasing NaCl concentration gradient in Buffer A (Fig. 1). The activity of the latter fractions (OOD) was traced by adding the former fractions (GDH), and *vice versa*. OOD and GDH were further purified by FPLC-HCA (Mitsui Toatsu Chemicals) column chromatography, with an increasing phosphate concentration in Buffer A without EDTA, followed by gel-filtration on FPLC-Superose 6 (HR10/30, Pharmacia).

ODC was purified from the crude extract by serial column chromatography on DEAE-Toyopearl, Butyl-Toyopearl, Sephadex G-100, Mono-Q, HCA, and Phenyl-Superose, using 10 mM KPB containing 0.1 mM PLP as the basal buffer.

GDH was additionally purified by using GABL as the reaction substrate from the crude extract of *Hafnia alvei*. The purified GDH was highly specific to GABL, whereas C_2-C_5 alkylaldehydes and their amino derivatives GABL were poor substrates. Its properties will be reported elsewhere in detail.

Enzyme Assay—GABA-forming activity in the crude preparation was measured by monitoring the increase of absorbance at 340 nm of the reaction mixture containing 20 mM L-ornithine, 0.3 mM $NADP^+$, 100 mM potassium phosphate buffer (pH 6.5), and enzyme (total 1 ml).

The OOD activity (GABL-forming activity) of the highly purified preparation was measured as follows. A reaction mixture containing 20 mM L-ornithine, 100 mM potassium phosphate buffer (pH 6.5), and enzyme (total 0.2 ml) was shaken (100 rpm) in a Wassermann tube ($\phi = 15$ mm) at 30°C for a designated period. The mixture was boiled for 3 min, then $NADP^+$ and the purified GDH were added at 30°C to final concentrations of 0.3 mM and 1.16 μg/ml, respectively, in a total volume of 1.2 ml, and the increase of the absorbance at 340 nm after 5 min was measured. One unit of OOD activity was defined as the amount which reduced 1 μmol of $NADP^+$, the same amount as that of GABL formed, per min at 30°C, pH 6.5. The specific activities are expressed as units per mg of protein.

GDH activity was assayed in a mixture (1 ml) containing 0.05 mM GABL, 0.3 mM $NADP^+$, 100 mM KPB (pH 6.5), and enzyme. The mixture was incubated at 30°C in a cuvette placed in a spectrophotometer (Hitachi, U-3000). The change in absorbance at 340 nm was monitored. One unit of GDH was defined as the amount of NADPH formed per min (molecular extinction coefficient, 6.22×10^3).

ODC activity was assayed by measuring the amount of putrescine produced by 10 min incubation of the reaction mixture containing 20 mM L-ornithine, 100 mM potassium phosphate buffer (pH 6.5), and enzyme (total 0.2 ml). One unit of ODC activity was defined as the amount which produced 1 μmol of putrescine per min at 30°C, pH 6.5. Putrescine and L-ornithine were measured by colorizing with ninhydrin after separation by paper electrophoresis (0.1 M Na-acetate buffer, pH 5.0, 500 V, 30 min).

Identification of GABL—GABL was analyzed on HPLC equipped with a μBondasphere C_8 (Waters) column after reaction with 2,4-dinitrophenylhydrazine (DNP) or *o*-aminobenzaldehyde (OAB). The reaction mixture was incubated with an equivalent volume of saturated DNP at 30°C for 30 min, then applied to the column, eluted with a gradient of 10-100% methanol and monitored at 290 nm. OAB-derivatives were prepared by incubating the reaction mixture with 4 volumes of saturated OAB at room temperature for 20 min, then eluting them on the same column by HPLC (50-100% methanol) with monitoring at 435 nm. The concentrations of OAB-derivatives of GABL were determined based on a molar extinction coefficient at 435 nm of 2.4×10^3. GABL was also analyzed directly using a gas chromatograph (Shimadzu GC 14B) equipped with an FID detector and a Thermon 3000 column (3 × 210 mm, 50°C air : H_2 = 1 : 1, 50 ml/min), and by NMR (Brucker ARX 300) after direct $CDCl_3$ extraction.

Other Analyses—GABA and ammonia concentrations were measured using an automatic amino acid analyzer (JEOL, JLC-300). H_2O_2 was assayed using horseradish peroxidase with *o*-phenylenediamine as a chromogen, and absorbance at 450 nm was measured after the reaction was stopped by adding 2 N H_2SO_4. Consumption of the dissolved oxygen in the reaction mixture was measured in the 1.2 ml cell with an oxygen electrode, under stirring with a magnet at 30°C (Freshness Meter, Oriental Electric, Tokyo). Protein in the crude extract was estimated by the method of

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Lowry *et al.* (*11*). Highly purified enzymes were estimated by measuring the absorption coefficients at 280 nm ($E_{1\%}$ of OOD; 7.63, of GDH; 8.94), obtained by the method of Scopes (*12*). SDS polyacrylamide-gel electrophoresis (SDS-PAGE) was performed according to Laemmli (*13*). The N-terminal amino acid sequence was determined by using an automatic protein sequencer (ABI, model 610). PLP of OOD was measured as pyridoxal after hydrolyzing the enzyme with 0.055 N HCl for 5 h under N_2 gas.

Materials—L-Ornithine·HCl was purchased from the Peptide Institute (Osaka). That from Sigma Chemicals and Wako Chemicals was also used as a standard. GABL (or 1-pyrroline) was prepared by hydrolyzing γ-aminobutyraldehyde diethylacetal (Wako Chemicals) at 60°C for 1 h in 2 N HCl under N_2. The solution was neutralized with 10 N NaOH and used as a substrate before or after chloroform extraction. Other reagents were all of specified grade.

RESULTS

L-Ornithine-Transforming Activity in the Crude Extract—L-Ornithine was mainly transformed to putrescine by the crude extract of *H. alvei* grown in medium containing L-ornithine, in which 1.4–4.2 units/mg of ODC activity was detected. In the presence of $NAD(P)^+$, GABA-forming activity was also evident ($5.7–14 \times 10^{-2}$ units/mg). Both ODC and GABA-forming activities were induced by L-ornithine and the activities were 10- to 12-fold higher in the crude extract grown in the medium containing 0.1% L-ornithine than that without the inducer. The activities were rather higher in the crude extract from the cells cultured at pH 7–8 than from those at acidic pH. When the culture volume in the shaking flask increased, the specific activities in the crude extract decreased (data not shown).

Purification of OOD, GDH, and ODC—The GABA-forming activity was separated by Mono Q column chromatography into two protein fractions (Fig. 1). The complete enzyme activity reconstituted from the two proteins was able to produce GABA from L-ornithine in the presence of $NADP^+$. The former fraction had GDH, and the latter fraction had OOD and ODC activities. GDH and OOD were further purified individually to homogeneity by SDS-PAGE, after a series of chromatography steps on DEAE-Toyopearl, Sephadex G-100, and Phenyl-Superose (Fig. 2). The purified GDH had a specific activity of 2.27 units/mg. The homogeneous preparation of OOD had a specific activity of 0.620 units/mg and could not be separated from ODC activity even in the final preparation (12.2 units/mg).

ODC was separately purified 348-fold from the same organism to homogeneity, in 15.4% yield (Table I). The ratio of ODC to OOD activity was almost constant throughout the purification (ODC/OOD = 160 : 1).

Properties of OOD with Regard to ODC—The molecular mass of OOD was 230 kDa according to gel-filtration on Superose 6, and 77 kDa by SDS-PAGE. The N-terminal amino acid sequence was Met-Glu-Lys-Leu-Lys-Ile-Ala-Val-Asn-Thr-Asn-Thr-Ser-Glu-Thr-Phe-Asp-Thr-Glu-Arg-Glu-Thr-Val-Pro-Ile-Asn-Asn-Thr-Asn-Phe-Thr-Asn-Thr-Asn-Phe-Thr-Asp-Val-Leu-Thr-Ala-Thr-Leu-X-A. The

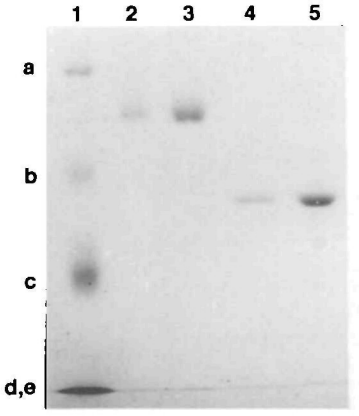

Fig. 2. **SDS-PAGE of the purified OOD and GDH.** The purified OOD (lane 2, 11 μg; lane 3, 33 μg) and GDH (lane 4, 8 μg; lane 5, 24 μg) were boiled in the sample buffer containing 0.2% 2-mercaptoethanol for 3 min, resolved by electrophoresis on a 10% acrylamide gel containing 1% SDS, and stained with Coomassie Brilliant Blue (*13*). Standard proteins (lane 1, each 10 μg) are: phosphorylase *b* (a, 94 kDa), bovine serum albumin (b, 67 kDa), ovalbumin (c, 43 kDa), carbonic anhydrase (d, 30 kDa), and soybean trypsin inhibitor (e, 20.1 kDa).

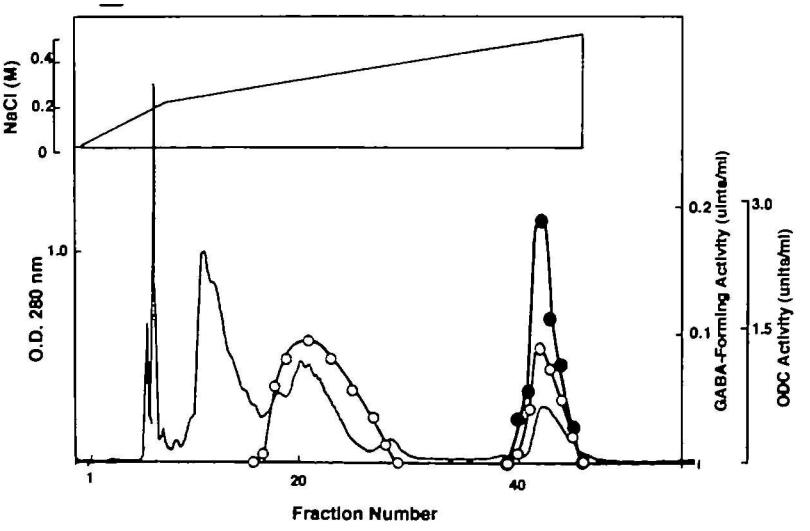

Fig. 1. **Mono Q Fast Flow column chromatography of GABA-forming enzymes.** The sample (0.73 unit, 10 mg) was applied to a Mono Q column equilibrated with Buffer A, and eluted with a linear gradient of 0–0.5 M NaCl in Buffer A. Fractions of 1 ml were collected at a flow rate of 1 ml/min. Two fractions involved in the GABA-forming reaction were separately eluted. The activity of the former fractions (No. 16–29, GDH) was traced by adding 50 μl of fraction No. 43 and that of the latter fractions (No. 39–46, OOD) was traced by adding 50 μl of fraction No. 21. Symbols: ○, GABA-forming activity; ●, ODC activity; — protein content.

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TABLE I. **Purification of L-ornithine decarboxylase.**

Step	Specific activity (U/mg)	Total protein (mg)	Total activity (units)	Yield (%)	Purification (fold)	ODC/OOD[a]
Cell-free extract	0.385	2,923	1,124	100	1	160
DEAE-Toyopearl	11.7	87	1,014	90.2	30	165
Butyl-Toyopearl	50.1	10	501	44.6	127	153
Superose 12	75.9	3.7	281	25.0	199	140
Mono Q	103	1.9	195	17.3	253	162
Superose 6	133	1.3	173	15.4	348	166

[a]Represents the activity ratio of ODC to OOD.

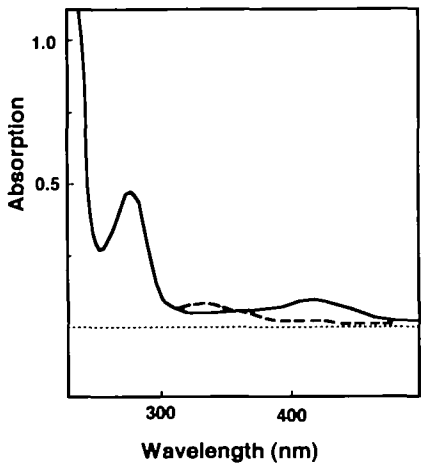

Fig. 3. **Absorption spectra of native (—) and hydrazine-treated (----) OOD.** Purified OOD in Buffer A (0.16 mg/ml) was treated with 1 mM hydrazine at 30°C for 60 min.

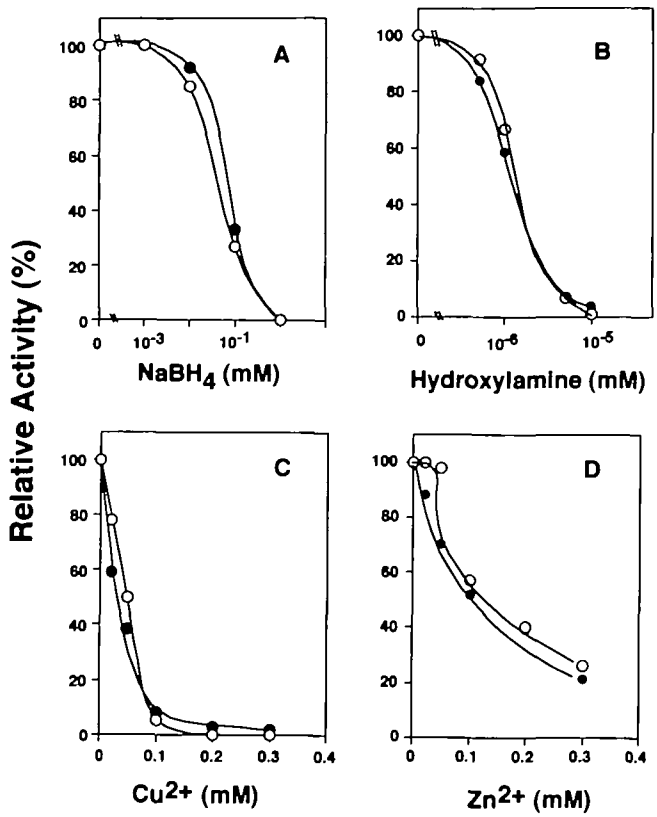

Fig. 4. **Effect of some reagents on OOD and GDH activities.** OOD (○) and ODC (●) activities were measured in the presence of various concentrations of $NaBH_4$ (A), hydroxylamine (B), $CuCl_2$ (C), and $ZnCl_2$ (D).

enzyme was slightly yellowish and had a broad absorption at 420 nm as well as that at 278 nm (Fig. 3). The molar extinction coefficients at 420 and 280 nm were 2.68×10^4 and 2.19×10^5, respectively. Hydroxylamine in the OOD solution diminished the absorption around 420 nm, and that around 340 nm appeared, while both OOD and ODC activities decreased (22 and 25% residual activity, respectively). After dialyzing the hydroxylamine-treated OOD against potassium phosphate buffer (pH 7.0) containing 0.01 mM pyridoxal 5′-phosphate, both of the activities were recovered (83 and 50% of the initial activity) with an increase of absorbance at 420 nm. Similar results were obtained when 1 mM semicarbazide and hydrazine were used instead of hydroxylamine. Phenylhydrazine (1 mM) also inhibited the OOD, as well as the ODC activity. The amount of PLP was estimated at about 1 mol per subunit ($K_m=1.75\times10^{-6}$ M for the hydroxylamine-treated enzyme). Figure 4 shows the effect of some reagents on the activities of OOD and ODC. Both activities decreased in a similar manner with increasing concentrations of borohydrate, hydroxylamine, Zn^{2+}, and Cu^{2+}. PLP (0.1 mM) activated both OOD and ODC activity (141 and 127% of that without PLP), whereas cofactors such as pyridoxamine 5′-phosphate (0.1 mM), FAD (0.1 mM), FMN (0.1 mM), and pyrroloquinoline quinone (0.01 mM) activated neither OOD nor ODC. OOD and ODC were most active at pH 6.5, and 45°C. The enzyme was specific to L-ornithine, but not to putrescine, L-lysine, L-arginine, L-glutamic acid, L-histidine, and other L-amino acids. 5-Aminovaleric acid decreased the enzyme activities to 50–60%, whereas D-ornithine, DL-norvaline, α-*N*-acetyl-L-ornithine, DL-norleucine, and all kinds of proteinous amino acids did not affect the enzyme activity.

Identification of GABL—The product of OOD reaction had the same retention time as chemically synthesized GABL on gas chromatography and HPLC after its derivatization with OAB and DNP. Derivatives of pyrroline 2-carboxylic acid and pyrroline 5-carboxylic acid gave different spectra and the R_f values on paper chromatography (data not shown). Gas chromatography (Thermon 3000) of the reaction mixture and GABL revealed sharp peaks at the same retention time (3.49 min) as that of the authentic sample. The proton NMR spectrum of the chloroform extract of the reaction mixture mostly corresponded with that of chemically synthesized GABL in chloroform: the quartet at 1.73 δ, the triplet at 2.46 δ, the multiplet

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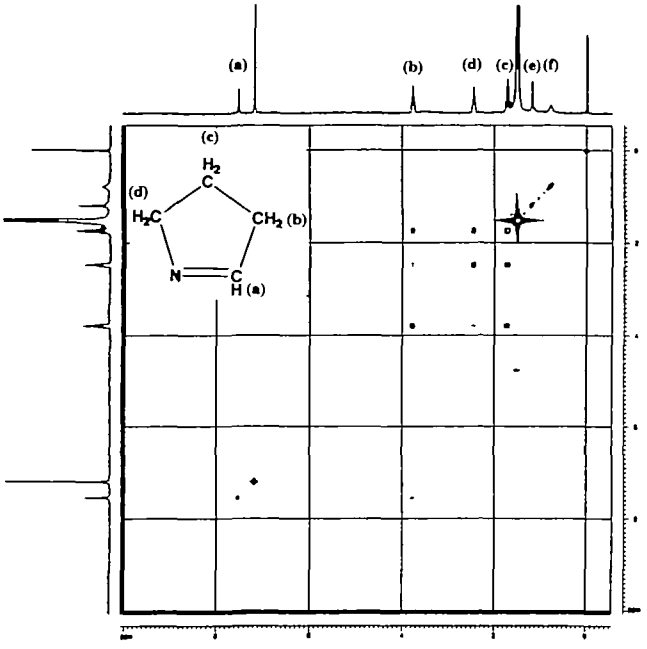

Fig. 5. **NMR spectrum of OOD reaction product.** A mixture (3 ml) containing 40 mM L-ornithine, 50 μM PLP, and 10 mM KPB (pH 7.0) was shaken for 10 h at 30°C in a Thunberg tube under oxygen. Thereafter the mixture was extracted with 0.5 ml of chloroform-*d*, then the proton NMR spectrum of the chloroform solution was measured (32 times integration). Signals (a), (b), (c), and (d) correspond to the protons indicated in the prospective structural formula. Signals (e) and (f) were from contaminants in the reaction mixture without OOD. Signals of H_2O (1.5 ppm) and chloroform (7.25 ppm) were also observed.

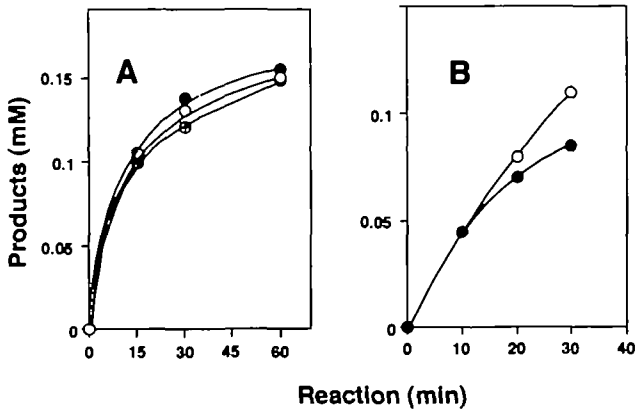

Fig. 6. **Stoichiometric relationships between the products of OOD reaction.** (A) A mixture containing 100 mM L-ornithine, 5 mM $NADP^+$, and 100 mM potassium phosphate buffer was incubated with 0.14 mg/ml OOD at 30°C for the appropriate period, then analyzed for NH_3 (○), and GABL (●). GABL was estimated as GABA using an amino acid analyzer after reaction with 0.2 mg/ml of GDH for 15 min. The amount of NADPH (⊕) formed in the GDH reaction is also presented. (B) Peroxidase (0.2 mg/ml) and *o*-phenylenediamine (10 mM) were added to the OOD reaction mixture (total 500 μl) and shaken at 30°C. The reaction was stopped with 2 N H_2SO_4, then H_2O_2 (●) was estimated from the absorbance at 450 nm. The amount of GABL formed (○) was also estimated by reacting the mixture in parallel without peroxidase and the chromogen.

TABLE II. **Effect of oxygen on OOD and ODC activities.**

Experiment number	Conditions	GABL formed (mM)	Putrescine formed (mM)	O_2 consumed (mM)	ODC/GABL[a]
1[b]	Air	0.110	17.4	0.102	158
2[c]	Air	0.16	—[g]	—	—
	N_2	0.001	—	—	—
3[d]	Air	0.09	13.7	—	152
	O_2	0.40	12.6	—	32
3[e]	Air	0.169	—	—	—
	Air, +glucose, +glucose oxidase	0.002	—	—	—
4[f]	Air	0.034	4.41	—	130
	Air, +H_2O_2	0.013	2.30	—	177
	Air, +H_2O_2, +catalase	0.518	2.13	—	4.1

[a]Represents the ratio of putrescine formed to GABL formed. [b]The reaction mixture was incubated for 1 h at 30°C in a lidded cell with gentle stirring. [c]The reaction mixtures were incubated for 24 h at 30°C with shaking under air or nitrogen. [d]The reaction mixture was incubated for 5 h at 30°C with shaking under air or O_2. [e]The reaction mixture was incubated for 1 h at 30°C with or without glucose (100 mM) and glucose oxidase (6.6 units/ml). [f]The reaction mixture was incubated for 30 min with or without H_2O_2 (200 mM) and catalase (20 units/ml). [g]Not determined.

between 3.75 and 3.80 δ, and the singlet at 7.54 δ. Their integration indicated the proton numbers of 2, 2, 2, and 1, respectively (Fig. 5). The results suggested that the product existed as 1-pyrroline in chloroform due to the dehydration and cyclization of GABL, though some contaminants were present. The results of H-H COSY supported their attribution to the carbon protons of (a)-(d).

Stoichiometry and the Oxygen Requirement of the OOD Reaction—When L-ornithine was incubated with OOD, GABL, NH_3, and H_2O_2 were formed stoichiometrically (Fig. 6), besides a large amount of putrescine and carbon dioxide. In addition, the same amount of oxygen was consumed as that of GABL formed in the lidded cell (Table II). Under a N_2 atmosphere, the OOD reaction did not proceed, while ODC activity (formation of putrescine) remained. Similar results were obtained when glucose and glucose oxidase was added to the reaction mixtures as an O_2-scavenging system. In contrast, OOD activity was 4.4-fold enhanced under an O_2 atmosphere, while putrescine formation was not changed. Catalase with H_2O_2 similarly enhanced the selective activation of OOD, resulting in a change in the activity ratio of OOD/ODC to 1 : 4.1.

DISCUSSION

In this study we found a novel oxidation reaction of L-ornithine in *H. alvei*. Various lines of evidence suggest that OOD is identical to ODC and that GABL is formed by ODC as a by-product in the presence of oxygen. The product and the stoichiometry of the OOD reaction, the identity of OOD and ODC proteins, and the hypothetical reaction mechanism are discussed below.

OOD was preliminarily purified as the enzyme that converted L-ornithine to a product that was susceptible to GDH, GABA-forming dehydrogenase. Though the findings naturally indicated that the product was GABL, this required confirmation, because PLP-dependent oxidative decarboxylation has never been reported (*14*). The instability of the product (*15*) meant that isolation was not

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feasible and so direct NMR analysis was performed. The NMR spectrum of the product coincided with that of 1-pyrroline, and this was supported by the reactivities with OAB and DNP. These indicated that GABL was dehydrated and cyclized to 1-pyrroline in chloroform. GC and HPLC analyses of the derivatives provided further evidence that one of the products of OOD reaction is GABL.

In the OOD reaction, CO_2, ammonia, H_2O_2, and GABL are formed. The consumption of O_2 and the production of GABL, H_2O_2, and NH_3 were stoichiometric. The participation of O_2 in the reaction was confirmed by replacing the atmosphere in the reaction vessel. These results suggested that OOD is a type of L-ornithine oxidase (deaminating, decarboxylating), and the reaction can be represented as follows:

$$\text{L-ornithine} + O_2 + H_2O \longrightarrow \text{GABL} + CO_2 + H_2O_2 + NH_3 \quad (1)$$

However, the amounts of GABL formed were much lower than the decrease of L-ornithine or the amount of CO_2 liberated. In addition, putrescine was formed in a similar amount to the decrease of L-ornithine. These results could be explained by the presence of ODC activity (Eq. 2) in the purified OOD: the amounts of L-ornithine lost and CO_2 produced were different from the stoichiometry of Eq. 1, because these compounds are common to both the OOD and ODC reactions.

$$\text{L-ornithine} \longrightarrow \text{putrescine} + CO_2 \quad (2)$$

The existence of a contaminant in commercial L-ornithine was examined by using L-ornithine from different lots, or recrystallized L-ornithine as substrates, and the results indicated that L-ornithine is the true substrate of the OOD reaction (data not shown). Furthermore the enzyme that was responsible for the latter half reaction of GABA formation found in the crude extract was confirmed to be identical with GDH (Eq. 3), by separate purification and characterization of them (unpublished data). Its high specificity also supported the view that the product of the OOD reaction is GABL.

$$\text{GABL} + NAD^+ \longrightarrow \text{GABA} + NADH + H^+ \quad (3)$$

The possibility of contamination of OOD by an enzyme such as a diamine oxidase (*16*) was ruled out as follows. Putrescine oxidase activity was undetectable in the final preparation and in crude extracts of *H. alvei*. The activity ratio of OOD/ODC was almost constant throughout the purification. Both activities in the final preparation coincided on gel-filtration and Mono-Q column chromatography. Both reactions were specific to L-ornithine, and all of the reagents that affected OOD activity, except for oxygen, also similarly affected ODC. They were similarly dependent upon PLP, hydrazine inactivated OOD and ODC activities with accompanying changes in their spectra, and they were simultaneously recovered by dialysis against PLP. Although there were some discrepancies, they might be due to the poor accuracy of the ODC and OOD assay systems. ODC from a separate purification had the same N-terminal amino acid sequence as OOD and had OOD activity. When culture conditions were varied, the level of OOD activity in the crude extract coincided well with that of ODC, and mutants with decreased ODC activity also had decreased OOD activity (data not shown). We found no indication from these results that the activities were separable. Thus we concluded that OOD is identical to ODC and that GABL was formed by a side reaction of ODC. This is supported by the fact that the molecular activity of the OOD reaction was very low (0.5). The cloning and expression of the ODC gene in another organism might give direct evidence that will resolve this issue.

Although there is much evidence that suggests a strong relationship between OOD and ODC protein, the reaction mechanism of the oxidative deamination *via* a decarboxylated ornithine-PLP Schiff's base intermediate cannot explain it. The deaminative oxidation of amino acids or amines is generally catalyzed by enzymes which contain flavin or TOPA-quinone as a prosthetic group (*17*). The oxidative decarboxylations of L-lysine [EC 1.13.12.2] (*18, 19*), L-tryptophan [EC 1.13.12.3] (*20*), and L-arginine [EC 1.13.12.1] (*21-23*) are catalyzed by monooxygenases with FAD as a prosthetic group, although liberation of ammonia does not occur in these cases. L-Ornithine cyclodeaminase [L-ornithine ammonia-lyase (cyclizing)] requires $NAD(P)^+$ as an inner cofactor (*2*). On the other hand, the abortive decarboxylase transamination of L-ornithine (*24*), L-glutamic acid (*25*), and L-methionine (*26*) has been reported as a side reaction of the pyridoxal enzyme, in which pyridoxamine-phosphate is formed and the enzyme is inactivated. Considering the dependence of the OOD reaction upon PLP, a possible reaction mechanism is as follows (Scheme 1). The enzyme forms an enol-imine intermediate after decarboxylation of the Schiff's base of L-ornithine. In the ODC reaction, there is an internal-external exchange of Schiff's base between the keto-imine intermediate of putrescine and peptidyl lysine. On the other hand, under aerobic conditions oxygen might attack the enol-imine intermediate and liberate 1-imido-4-aminopentane and hydroperoxy-PLP, and the former liberates ammonia nonenzymatically. The non-enzymatic oxidation of aliphatic amino acids by ionizing radiation and by metal catalysis has been investigated (*27*); the deamination from an imino compound is common to the reaction described in this report and leads to the formation of NH_4^+ and CO_2, as well as either an aldehyde or a carboxylic acid. The pyridoxal-catalyzed decarboxylation of amino acids under reflux has also been identified in the presence of oxygen and cupric ions, but no aldehyde or ammonia is generated when cupric ions are omitted. Although the reaction rate of OOD was very low compared with its "essential" activity, the enzyme and PLP retained activity and recycling, in contrast to the reported side reactions (*28*). Further experimental evidence is needed to prove the reaction mechanism proposed here.

Finally, the ODC (OOD) of *H. alvei* resembles a biodegradative ODC rather than the biosynthetic enzyme of *E. coli*. The production of the *H. alvei* ODC was stimulated by L-ornithine, although the enzyme preferentially produced aerobically at neutral pH. *H. alvei* ODC showed 46.4% similarity to the biodegradative ODC of *E. coli* (*29*), while it showed no significant homology (11.1%) to biosynthetic ODC (*30*) in the N-terminal amino acid sequences. On the other hand, Stim and Bennett reported similarity among arginine decarboxylase, lysine decarboxylase, and biosynthetic and biodegradative ODC of *E. coli*, and lysine decarboxylase of *H. alvei* in terms of their whole amino acid sequences, and suggested that the enzymes shared a com-

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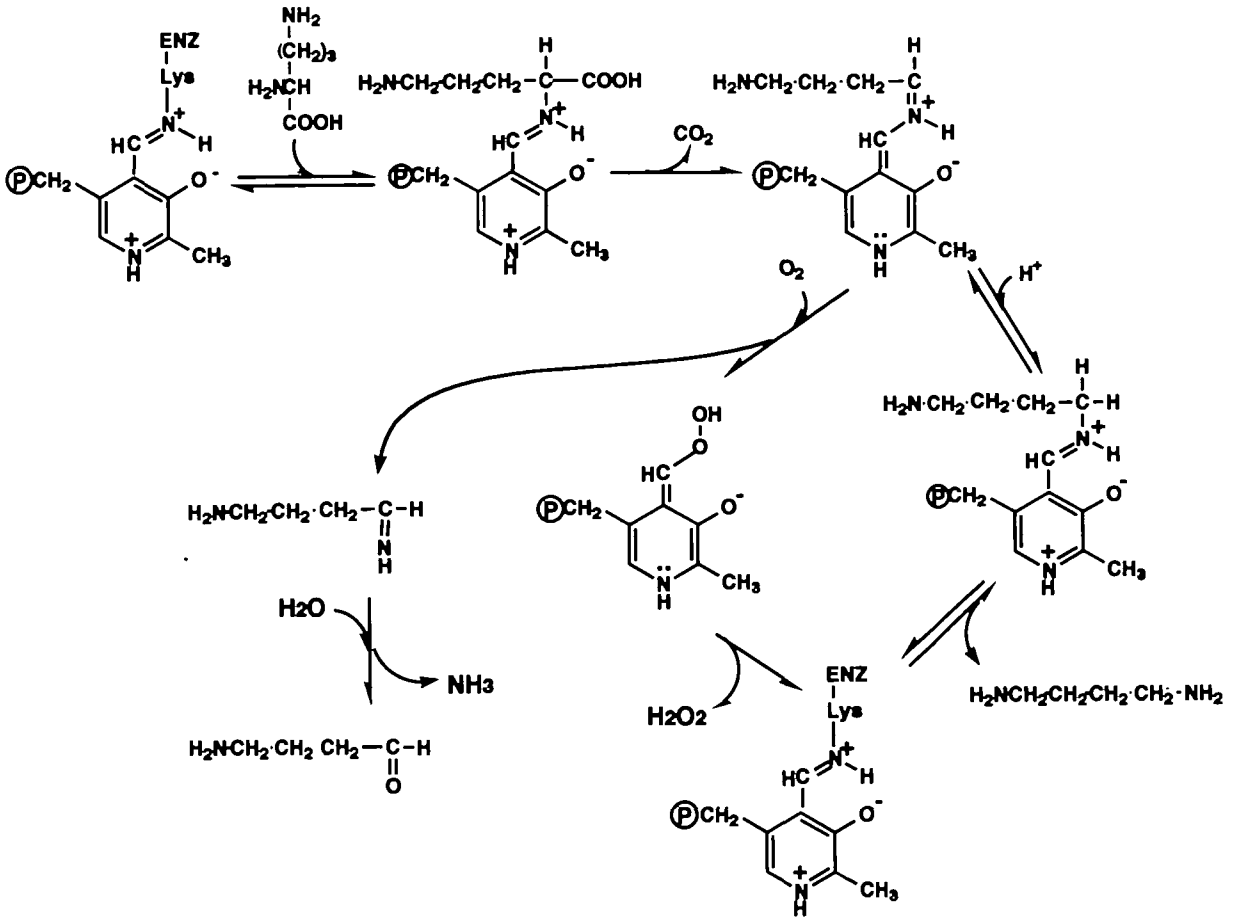

Scheme 1. **Putative mechanism of the side reaction of L-ornithine decarboxylase.**

mon ancestor (*31*). Therefore, it is of interest to know whether or not a side-oxidation reaction such as that presented here in *H. alvei* ODC generally occurs in these decarboxylase families.

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