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A Novel Coupled Enzyme Assay Reveals an Enzyme Responsible for the Deamination of a Chemically Unstable Intermediate in the Metabolic Pathway of 4-Amino-3-Hydroxybenzoic Acid in *Bordetella* sp. Strain 10d

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Running Title: 2-Amino-5-carboxymuconic 6-semialdehyde deaminase

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Enzymes: 2-amino-5-carboxymuconic 6-semialdehyde deaminase [EC 3.5.99.- as proposed in this paper as a new subclass of deaminases], 4-amino-3-hydroxybenzoate 2,3-dioxygenase [EC 1.13.1.-],

2-aminophenol 1,6-dioxygenase [EC 1.13.11.x], 2-aminomuconic 6-semialdehyde dehydrogenase [EC 1.2.1.32], 2-aminomuconate deaminase [EC 3.5.99.5], catechol 2,3-dioxygenase [EC 1.13.11.2], protocatechuate 2,3-dioxygenase [EC 1.13.11.x], 2,3-dihydroxybenzoate 3,4-dioxygenase [EC 1.13.11.14].

Keywords: 4-Amino-3-hydroxybenzoic acid; *Bordetella* sp. strain 10d; 2-amino-5-carboxymuconic 6-semialdehyde; 2-hydroxymuconic 6-semialdehyde; 2-amino-5-carboxymuconic 6-semialdehyde deaminase.

ABSTRACT

2-Amino-5-carboxymuconic 6-semialdehyde is an unstable intermediate in the *meta*-cleavage pathway of 4-amino-3-hydroxybenzoic acid in *Bordetella* sp. strain 10d. In vitro, this compound is nonenzymatically converted to 2,5-pyridinedicarboxylic acid. Crude extracts of strain 10d grown on 4-amino-3-hydroxybenzoic acid converted 2-amino-5-carboxymuconic 6-semialdehyde formed from 4-amino-3-hydroxybenzoic acid by the first enzyme in the pathway, 4-amino-3-hydroxybenzoate 2,3-dioxygenase, to a yellow compound (λ_{max} 375 nm). The enzyme in the crude extract carrying out the next step was purified to homogeneity. The yellow compound formed from 4-amino-3-hydroxybenzoic acid by this purified enzyme and purified 4-amino-3-hydroxybenzoate 2,3-dioxygenase in a coupled assay was identified as 2-hydroxymuconic 6-semialdehyde by GC-MS analysis. A mechanism for the formation of 2-hydroxymuconic 6-semialdehyde via enzymatic deamination and nonenzymatic decarboxylation is proposed based on results of spectrophotometric analyses. The purified enzyme, designated 2-amino-5-carboxymuconic 6-semialdehyde deaminase, is a new type of deaminase that differs from the 2-aminomuconate deaminases reported previously in that it primarily and specifically attacks 2-amino-5-carboxymuconic 6-semialdehyde.

The deamination step in the proposed pathway differs from that in the pathways for 2-aminophenol and its derivatives.

INTRODUCTION

2-Aminophenol and its derivatives are intermediates in the biodegradation of nitrobenzenes (1–4). 2-Aminophenols serve not only as a carbon source, but also as a nitrogen source for growth of the assimilating bacteria. Deaminases, which catalyze the release of ammonia, are a key enzyme in the metabolic pathways of 2-aminophenol and its derivatives. However, little is known about the metabolic steps that lead to the release of ammonia and the properties of the deaminase.

Pseudomonas sp. strain AP-3 and *Pseudomonas pseudoalcaligenes* strain JS45 convert 2-aminophenol to 4-oxalocrotonic acid via 2-aminomuconic 6-semialdehyde and 2-aminomuconic acid in the modified *meta*-cleavage pathway (Fig. 1(B)). The 2-aminomuconate deaminase from strain AP-3 and that from strain JS45 have been purified and characterized in detail (5, 6). The nucleotide sequence of the gene encoding the deaminase from strain AP-3 is not similar to any nucleotide sequences present in the databases, other than the recently reported nucleotide sequences of the gene encoding 2-aminomuconate deaminase from *Pseudomonas putida* HS12 and from *Pseudomonas fluorescens* strain KU-7 (6–8). Although

other deaminases have been detected in crude extracts of nitrobenzene-assimilating bacteria, the progress in the purification and characterization of the enzymes is slow (2, 4), probably because the substrate for the enzyme assay, 2-aminomuconic 6-semialdehyde, which is formed by ring cleavage of 2-aminophenol, is unstable and is converted nonenzymatically to picolinic acid in vitro (9).

We have previously isolated *Bordetella* sp. strain 10d, which grows on 4-amino-3-hydroxybenzoic acid, and purified and characterized the 4-amino-3-hydroxybenzoate 2,3-dioxygenase involved in the initial step of the metabolism of this substrate (10). The enzyme catalyzes the ring fission of 4-amino-3-hydroxybenzoic acid to form 2-amino-5-carboxymuconic 6-semialdehyde (Fig. 1(A)). The cloning and nucleotide sequence of the gene encoding the dioxygenase (AhdA) have also been reported (11). However, the subsequent metabolism, including the deamination step, have not been elucidated since 2-amino-5-carboxymuconic 6-semialdehyde is immediately converted nonenzymatically to 2,5-pyridinedicarboxylic acid in vitro.

Here we report the purification and characterization of an enzyme from strain 10d that uses 2-amino-5-carboxymuconic 6-semialdehyde as a substrate. Insights into the metabolic fate of 4-amino-3-hydroxybenzoic acid in strain 10d are revealed.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Bordetella sp. strain 10d was isolated previously (10). Strain 10d was cultured in medium containing 0.12% (w/v) 4-amino-3-hydroxybenzoic acid and 1% (w/v) meat extract (10).

Enzyme assay

2-Amino-5-carboxymuconic 6-semialdehyde was formed from 4-amino-3-hydroxybenzoic acid in a coupled assay by purified 4-amino-3-hydroxybenzoate 2,3-dioxygenase provided in excess. The enzyme activity in the crude extract and in the reaction mixture that used 2-amino-5-carboxymuconic 6-semialdehyde as substrate was measured by monitoring the increase in the absorbance of the reaction product at 375 nm. The reaction mixture contained 2.9 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5), 0.1 ml of 5 mM 4-amino-3-hydroxybenzoic acid, and 0.05 ml of crude extract. The reaction was started by adding 0.1 ml of 4-amino-3-hydroxybenzoate 2,3-dioxygenase (0.8 U/ml). After incubation for 10 min at 24°C, the absorbance at 375 nm was read. One unit of enzyme activity was defined as the amount of enzyme that converted 1 µmol of 2-hydroxymuconic 6-semialdehyde per min. The molar extinction coefficient of

4.4×10^4 for 2-hydroxymuconic 6-semialdehyde was used (12). Specific activity was defined as units (mg protein)$^{-1}$. Protein concentrations were measured by the method of Lowry *et al.* (13).

The substrate specificity of the purified enzyme was examined with 2-aminomuconic 6-semialdehyde and 2-aminomuconic acid using the same methods as described previously (5, 14, 15).

Enzyme purification

All steps of the purification of the enzyme that used 2-amino-5-carboxymuconic 6-semialdehyde as substrate were carried out using modifications of methods described previously (10). Cells [14.8 g (wet wt.)] of strain 10d were suspended in 20 mM Tris-HCl buffer (pH 8.0). Cell extract (fraction 1, 150 ml) was prepared and treated with streptomycin sulfate (fraction 2, 149 ml) as described previously (9). Fraction 2 was fractionated with ammonium sulfate (38–60% saturation). After centrifugation (20,000×*g* for 10 min), the pelleted precipitate was dissolved in 20 mM Tri-HCl buffer (pH 8.0). The solution was dialyzed against buffer A [20 mM Tris-HCl buffer (pH 8.0) containing 1 mM dithiothreitol and 0.5 mM L-ascorbate] (fraction 3, 46 ml). Fraction 3 was applied to a DE52 cellulose column (2.1×19 cm), and proteins were eluted with a linear

gradient (0 to 0.4 M NaCl) at a flow rate of 40 ml h^{-1}. The active fractions were pooled (fraction 4, 30 ml). Fraction 4 was applied to a DEAE-Cellulofine A-800 column (1.7×22 cm), and proteins were eluted with a linear gradient (0 to 0.35 M) of NaCl at a flow rate of 30 ml h^{-1}. The active fractions were pooled (fraction 5, 20 ml). Fraction 5 was applied to a Phenyl-Cellulofine column (1.6×13.7 cm), and proteins were eluted with a linear gradient (0.5 to 0 M) of ammonium sulfate at a flow rate of 30 ml h^{-1}. The active fractions were pooled (fraction 6, 24.5 ml). The enzyme purity was checked by SDS-PAGE (16).

Production and isolation of enzymatic reaction products in a coupled enzyme assay

The reaction mixture contained 107 ml of 50 mM sodium-potassium phosphate buffer (pH 7.5), 9 ml of 5 mM 4-amino-3-hydroxybenzoic acid, 5.1 ml of 4-amino-3-hydroxybenzoate 2,3-dioxygenase solution (8.8 µg ml^{-1}), and 6 ml of purified enzyme solution (1.0 µg ml^{-1}). After incubation at 24°C for 2.7 h with shaking at 100 rpm, the concentrations of 4-amino-3-hydroxybenzoic acid, 2,5-pyridinedicarboxylic acid, ammonia, and 2-hydroxymuconic 6-semialdehyde in the reaction mixture were determined as described below. The reaction mixture was concentrated to 10 ml with a rotary evaporator. The pH of the concentrated solution

was adjusted to pH 3.0 with 5 M metaphosphoric acid, and the solution was extracted with ethyl acetate. The upper layer was collected and concentrated to 10 ml. The extracted products were mixed with an equimolar concentration of pentafluorophenylhydrazine at 24°C for 30 min. The reaction mixture was then evaporated to dryness. The hydrazone derivative was then mixed with *N*, *O*-bis(trimethylsilyl)-trifluoroacetamide at 85°C for 1.5 h. The derivatized products were analyzed by GC-MS as described below.

Analytical tests

UV-visible absorption spectra of reaction products and the purified enzyme were recorded with a Beckman DU 650 spectrophotometer. Fluorescence spectra of the purified enzyme and a cofactor released from the enzyme were recorded using a Hitachi F-2500 fluorescence spectrophotometer. **The trimethyl-sililated or hydrazone-derivatized enzyme reaction products were analyzed with a Hitachi M-2500 mass spectrometer at an ionization potential of 70 eV, coupled to a Hitachi G-3000 gas chromatograph.** A TC-1 fused silica capillary column (0.25 mm ×30 m; GL Science, Tokyo) was used. A Hitachi L-6200 HPLC system equipped with an Inertsil ODS-2 column (4.6×150 mm, 5 μm; GL Science) was used for measuring

4-amino-3-hydroxybenzoic acid and 2,5-pyridinedicarboxylic acid. The flow rate through the column at room temperature was 0.4 mL/min. Samples were eluted with a solvent of 0.05 M phosphoric acid/methanol (65:35, v/v) with monitoring at 278 nm. The cofactor from the purified enzyme was detected by fluorescence (F-1050) at an excitation and emission wavelength of 450 and 530 nm, respectively. Ammonia release was determined by measuring the decrease in A_{340} concomitant with NADPH oxidation in the presence of glutamate dehydrogenase (18). The N-terminal amino acid sequence was determined with a Shimadzu PPSQ-10 protein sequencer using the method reported previously (10). The molecular mass of the native enzyme was determined by gel filtration on Cellulofine GCL-1000 sf using the method reported previously (10). The molecular mass of the enzyme subunit was determined by SDS-polyacrylamide gel electrophoresis (16) using the LMW calibration kit (Amersham Pharmacia Biotech) as size markers.

Chemicals

4-Amino-3-hydroxybenzoic acid and 2,5-pyridinedicarboxylic acid were purchased from Tokyo Kasei Kogyo (Tokyo, Japan); 2-aminophenol, catechol, metaphosphoric acid, dithiothreitol, L-ascorbate, *N,O*-bis(trimethylsilyl)-trifluoroacetoamide, NADPH, and glutamate dehydrogenase were from Wako Pure

Chemicals (Osaka, Japan); meat extract (Extract Ehlrich) was from Kyokuto Seiyaku Kogyo (Osaka, Japan); and pentafluorophenylhydrazine was from Pfaltz & Bauer. (Waterbury, Conn., USA). DE52 cellulose was from Whatman (Madison, Wis., USA), and **DEAE-Cellulofine A-800, Phenyl-Cellulofine, and Cellulofine GCL-1000 sf were from Seikagaku (Tokyo, Japan).** 2-Aminophenol 1,6-dioxygenase, 2-aminomuconic 6-semialdehyde dehydrogenase, and 4-amino-3-hydroxybenzoate 2,3-dioxygenase were prepared as described previously (6, 10, 19). 2-Aminomuconic 6-semialdehyde was prepared enzymatically from 2-aminophenol using purified 2-aminophenol 1,6-dioxygenase (6). 2-Aminomuconic acid was synthesized by the methods of He and Spain (5). 2-Hydroxymuconic 6-semialdehyde was prepared by incubating catechol with resting cells of a mutant, strain Y-2, of the aniline-assimilating *Pseudomonas* sp. strain AW-2 (20).

RESULTS

Spectral changes during metabolism of 4-amino-3-hydroxybenzoic acid by crude extracts of strain 10d

Strain 10d grows well in 4-amino-3-hydroxybenzoate medium and completely degrades this substrate (10). In the culture broth, 2,5-pyridinedicarboxylic acid, which is nonenzymatically

converted via 2-amino-5-carboxymuconic 6-semialdehyde, cannot be detected by HPLC (10). Cells of strain 10d grown on 4-amino-3-hydroxybenzoic acid were washed and suspended in 50 mM sodium-potassium phosphate buffer (pH 6.8) containing 4-amino-3-hydroxybenzoic acid. The substrate was also degraded without accumulation of 2,5-pyridinedicarboxylic acid in the reaction mixture. To reveal the subsequent metabolism in vivo, including the deamination step the concentrated crude extracts of strain 10d grown on 4-amino-3-hydroxybenzoic acid were prepared by ammonia sulfate fractionation (35–75% saturation). Figure 2(A) shows the changes in the spectrum during the reaction in a coupled enzyme assay of 4-amino-3-hydroxybenzoic acid and the prepared crude extracts. The absorption peaks at 263 and 294 nm characteristic of 4-amino-3-hydroxybenzoic acid decreased as the enzyme reaction proceeded and were almost completely absent after 10 min of incubation. The maximum absorption peak shifted to 268 nm and the absorption peak at 375 nm derived from an intermediate increased during this incubation time. The peak at 268 nm was assigned to 2,5-pyridinedicarboxylic acid based on the wavelength (10).

Purification and properties of the purified enzyme

The activity of the enzyme present in the crude extract of strain 10d that used 2-amino-5-carboxymuconic 6-semialdehyde as

substrate was measured by monitoring the increase in the absorbance at 375 nm [Fig. 2(A)], but was not present in cell extracts of succinate/glucose-grown cells; therefore, the synthesis of the enzyme was inducible. Table I shows a summary of a typical enzyme purification. The enzyme was purified 103-fold with an overall yield of 2%. The specific activity of the purified enzyme was 0.27 units (mg protein)$^{-1}$. After electrophoresis, the purified enzyme exhibited a single protein band on both native and denaturing polyacrylamide gels [Figs. 3(A) and (B)]. The apparent molecular mass was determined to be 34 kDa by gel filtration and 15 kDa by SDS-PAGE [Fig. 3(B)]. Therefore, the enzyme is a homodimer with 15-kDa subunits. The N-terminal amino acid sequence of the enzyme was determined to be PKILVHSDAAPTTGFTNXHTP.

The purified enzyme was stable between pH 5.5 and 7.5 in 50 mM sodium-potassium phosphate buffer containing 1 mM dithiothreitol and 0.5 mM L-ascorbate. The enzyme maintained 80% activity up to 70°C after 10 min incubation at pH 7.5. The enzyme activity decreased to 70% after incubation at 75°C for 10 min, and all activity was lost at 80°C.

The two compounds tested, 2-aminomuconic 6-semialdehyde and 2-aminomuconic acid, were shown not be substrates of the purified enzyme. The enzyme was inhibited (remaining activity indicated in parentheses) by the following

metal salts: 1 mM $FeSO_4$ (0%), 1 mM $FeCl_3$ (29%), 1 mM $MnSO_4$ (0%), 1 mM $CoCl_2$ (0%), 1 mM $NiSO_4$ (0%), and 1 mM $ZnSO_4$ (7%), $K_3Fe(CN)_6$ and $MgSO_4$ did not affect the enzyme activity. The addition of 1 mM iodoacetic acid, *p*-chloromercuribenzoic acid, 5,5'-dithiobis-(2-nitrobenzoic acid) and 2,2'-bipyridyl decreased the enzyme activity to 95, 91, 86, and 95%, respectively.

Spectroscopic characterization of the purified enzyme

The concentrated enzyme solution (fraction 6) was yellow in color. The enzyme solution showed one main absorption peak at 266 nm and a broad absorption band in the visible region (Fig. 4). The excitation spectrum of the heat-treated enzyme with emission at 530 nm showed a maximum at 367 nm and a shoulder around 449 nm [Fig. 4 (A)]. A peak at 514 nm was observed in the emission spectrum [Fig. 4 (B)]. Authentic FAD in 50 mM sodium potassium phosphate buffer (pH 7.0) showed maxima at 372 and 449 nm in the excitation spectrum with emission at 530 nm. A peak at 527 nm was observed in the emission spectrum. These results suggested that the enzyme contains a flavin derivative. The flavin cofactor of the purified enzyme was subsequently characterized using HPLC; a major peak with a retention time of 5.9 min was observed. In contrast, authentic FAD and FMN showed a peak at 16.3 and 18.0 min,

respectively.

Reaction products from 2-amino-5-carboxymuconic 6-semialdehyde

Figures 2(B) and (C) show the changes in the absorption spectrum during the coupled enzyme reaction of purified 4-amino-3-hydroxybenzoate 2,3-dioxygenase and the enzyme purified here with 4-amino-3-hydroxybenzoic acid as substrate. First the absorption around 350 nm increased, and then the absorption peak at 375 nm appeared.

4-Amino-3-hydroxybenzoic acid (0.42 mM) was degraded completely, 2,5-pyridinedicarboxylic acid (0.41 mM) and 2-hydroxymuconic 6-semialdehyde (0.028 mM) accumulated, and ammonia (0.017 mM) was released during the enzyme reaction. Most of the 2-amino-5-carboxymuconic 6-semialdehyde formed by the action of 4-amino-3-hydroxybenzoate 2,3-dioxygenase was nonenzymatically converted to 2,5-pyridinedicarboxylic acid (10), and the remainder was converted (via two steps, one enzymatic and one nonenzymatic, see below) to 2-hydroxymuconic 6-semialdehyde and an almost equimolar concentration of ammonia. The proposed pathway is shown in Fig. 1(A). Attempts to clarify the stoichiometory by adding a small amount of the purified dioxygenase to the reaction mixture with a large excess of the purified enzyme reported here to avoid the formation of

2,5-pyridinedicarboxylic acid from 2-amino-5-carboxymuconic 6-semialdehyde failed. The enzymatic reaction did not proceed well because the dioxygenase is more unstable than the purified enzyme reported here (10).

The enzyme reaction products were analyzed by GC and GC-MS. Major ion peaks at 11.0 min [Fig. 1(A), compound V] and 13.2 min [Fig. 1(A), compound IV] were observed. The mass spectra (Table 2) and the GC retention times (R_t) of compound IV and compound V agreed with those of trimethylsilylated pentafluorophenylhydrazone 2-hydroxymuconic 6-semialdehyde (R_t=11.0 min) and trimethylsilylated 2,5-pyridinedicarboxylic acid (R_t=13.2 min), respectively.

DISCUSSION

Although 2-amino-5-carboxymuconic 6-semialdehyde is very labile, an enzyme able to use this compound as a substrate was found in crude extracts of *Bordetella* sp. strain 10d. The enzyme was purified to homogeneity and characterized using a new coupled enzyme assay with 4-amino-3-hydroxybenzoate 2,3-dioxygenase. A pathway for the metabolism of 2-amino-5-carboxymuconic 6-semialdehyde in strain 10d was proposed [Fig. 1(A)] based on results of absorption spectra in a coupled enzyme assay, the enzyme reaction product identified by

GC-MS analysis, and the determination of released ammonia. The coupled enzyme assay revealed the mechanism of the deamination reaction and the subsequent metabolism, including the deamination step.

The product formed from 4-amino-3-hydroxybenzoic acid by the action of purified 4-amino-3-hydroxybenzoate 2,3-dioxygenase and the purified enzyme reported here was identified as 2-hydroxymuconic 6-semialdehyde [Fig. 1(A), compound IV]. The accumulation of 2-hydroxymuconic 6-semialdehyde points to two possible deamination and decarboxylation steps. The first possibility is that 2-amino-5-carboxymuconic 6-semialdehyde [Fig. 1(A), compound II] is converted to 2-hydroxymuconic 6-semialdehyde via 2-aminomuconic 6-semialdehyde [Fig. 1(A), compound VI]. In vitro, 2-aminomuconic 6-semialdehyde [Fig. 1(B), compound II (λ_{max} 382 nm)] is immediately converted to picolinic acid [Fig. 1(B), compound V, λ_{max} 264 nm] (9). The absorption peak at 382 nm increases rapidly and reaches the maximum in 30 s, and then gradually decreases in 10 min (9). It cannot reasonably be assumed that 2-hydroxymuconic 6-semialdehyde accumulated via these steps based on the changes in the absorption spectrum [Fig. 2 (B) and (C)]. In addition, picolinic acid was not detected in the reaction mixture after the coupled enzyme assay. The other possibility is that 2-amino-5-carboxymuconic

6-semialdehyde is converted to 2-hydroxymuconic 6-semialdehyde via 2-hydroxy-5-carboxymuconic 6-semialdehyde [Fig. 1(A), compound III]. During a coupled assay with two purified enzymes, a reaction product with an absorption around 350 nm transiently accumulated [Fig. 2 (B) and (C)]. We failed to isolate and identify such a compound; however, we propose that the compound is 2-hydroxy-5-carboxymuconic 6-semialdehyde and that this compound is converted to 2-hydroxymuconic 6-semialdehyde by spontaneous decarboxylation, based on electronic theory and previously reported spectrophotometric data (21–23). 3-Ketoacids readily undergo decarboxylation under mild conditions, and loss of CO_2 can occur readily only from the free carboxylic acid (23). Decarboxylation has a concerted mechanism with an aromatic transition state.

2-Hydroxy-5-carboxymuconic 6-semialdehyde has an aldehyde group and a C-5 carboxyl group, which is a 3-ketoacid. As shown in Fig. 1(A), compound III in the keto form possibly releases CO_2. Crawford *et al.* and Nozaki *et al.* have reported that protocatechuate 2,3-dioxygenase and catechol 2,3-dioxygenase catalyze the ring fission of protocatechuic acid (2,3-dihydroxybenzoic acid) to form 2-hydroxy-5-carboxymuconic 6-semialdehyde (λ_{max} 350 nm) (21, 22). The absorption peak at 350 nm derived from 2-hydroxy-5-carboxymuconic 6-semialdehyde is observed and later an absorption peak at 375

nm derived from 2-hydroxymuconic 6-semialdehyde appears (22). 2,3-Dihydroxybenzoate 3,4-dioxygenase from *Pseudomonas fluorescens* 23D-1 catalyzes the ring fission of 2,3-dihydroxybenzoic acid to form 2-hydroxymuconic 6-semialdehyde and CO_2 (24). Therefore, strain 10d converts 2-amino-5-carboxymuconic 6-semialdehyde to 2-hydroxymuconic 6-semialdehyde in the deamination and nonenzymatic decarboxylation steps [Fig. 1(A)]. We named the enzyme reported here 2-amino-5-carboxymuconic 6-semialdehyde deaminase.

2-Amino-5-carboxymuconic 6-semialdehyde deaminase from strain 10d differs from previously reported 2-aminomuconate deaminases in substrate specificity, thermo-stability, subunit structure, and N-terminal amino acid sequence (5, 6). The native enzyme of *Pseudomonas* sp. strain AP-3 has a molecular mass of 67 kDa and consists of four identical subunits, while the enzyme from *P. pseudoalcaligenes* strain JS45 has a molecular mass of 100 kDa and consists of six identical subunits. The enzymes from strain AP-3 and strain JS45 maintain 80% activity up to 50°C. The enzyme from strain JS45 is colorless and does not have an absorbance peak at 300 nm (5). A cofactor is not required for the enzyme activity. In contrast, the deaminase from strain 10d contained an FAD-like cofactor, similar to D-amino acid oxidases (25–27), as indicated

by the absorption peak of the purified enzyme at 266 nm. The typical protein absorption peak of 280 nm shifts to ~265 nm if the protein contains a flavin-type cofactor (28). We failed to identify the cofactor of the deaminase from strain 10d because the enzyme could not be purified in large enough quantities.

We previously reported the identification of the enzyme involved in the initial step of the metabolism of 4-amino-3-hydroxybenzoic acid in *Bordetella* sp. 10d (10). This first step, catalyzed by 4-amino-3-hydroxybenzoate 2,3-dioxygenase [Fig. 1(A)], is similar to the first step in the modified *meta*-cleavage pathway for 2-aminophenol in *Pseudomonas* sp. strain AP-3 catalyzed by 2-aminophenol 1,6-dioxygenase (10) [Fig. 1(B)]. However, 4-amino-3-hydroxybenzoate 2,3-dioxygenase differs from 2-aminophenol 1,6-dioxygenase in subunit structure and substrate specificity (4, 10). The deamination steps in these pathways differ from each other (Figs. 1(A) and 1(B)). Recently, Muraki e*t al.* reported that the carboxyl-group-substituted 2-aminophenol, 3-hydroxyanthralinic acid (2-amino-3-hydroxybenzoic acid), is metabolized to form 4-oxalocrotonate via 2-amino-3-carboxymuconic 6-semialdehyde and 2-aminomuconate through an enzymatic decarboxylation step (2-amino-3-hydroxymuconic 6-semialdehyde decarboxylase) and a deamination step (2-aminomuconic 6-semialdehyde

deaminase) in *P. fluorescens* strain KU-7 (7). The decarboxylation mechanism in the metabolic pathways for 3-hydroxyanthralinic acid differs from that in the pathway for 4-amino-3-hydroxybenzoic acid.

The N-terminal amino acid sequence of the purified enzyme did not show significant levels of identity to sequences of 2-aminomuconate deaminases (6, 8, 27) or to any other sequences available in FASTA and BLAST database programs at the DNA Data Bank of Japan. **Recently, we reported the cloning and sequencing of the gene encoding 4-amino-3-hydroxybenzoate 2,3-dioxygenase from strain 10d (11). Unfortunately, the cloned 4.2-kb fragment does not contain the gene encoding the deaminase reported here. In the cloned 5.2-kb fragment from *P. pseudoalcaligenes* JS45, there are no genes involved in the 2-aminophenol-metabolic pathway, except for *amnBA,* which encodes 2-aminophenol 1,6-dioxygenase, and *amnC,* which encodes 2-aminomuconic 6-semialdehyde dehydrogenase (29).** Analysis of the entire amino acid sequence of 2-amino-5-carboxymuconate 6-semialdehyde deaminase will reveal more information on the narrow substrate specificity and the cofactor.

REFERENCES

1. Hasegawa, Y., Muraki, T., Tokuyama, T., Iwaki, H., Tatsuno, M., and Lau, P. C. (2000) A novel degradative pathway of 2-nitorobenzoate via 3-hydroxyanthranilate in *Pseudomonas fluorescens* strain KU-7. *FEMS. Microbiol. Lett.* **190**, 185-190
2. Katsivela, E., Wray, V., Pieper, D. H., and Wittich, R-F. (1999) Initial reactions in the biodegradation of 1-chloro-4-nitrobenzene by a newly isolated bacterium, strain LW1. *Appl. Environ. Microbiol.* **65**, 1405-1412
3. Nishino, S. F., and Spain, J. C. (1993) Degradation of nitrobenzene by a *Pseudomonas pseudoalcaligenes*. *Appl. Environ. Microbiol.* **59**, 2520-2525
4. Spiess, T., Desiere, F., Fischer, P., Spain, J. C., Knackmuss, H. J., and Lenke, H. (1998) A new 4-nitrotoluene degradation pathway in a *Mycobacterium* strain. *Appl Environ Microbiol.* **64**, 446-52
5. He, Z., and Spain, J. C. (1998) A novel 2-aminomuconate deaminase in the nitrobenzene degradation pathway of *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.* **180**, 2502-2506
6. Takenaka, S., Murakami, S., Kim, Y-J., and Aoki, K. (2000) Complete nucleotide sequence and functional analysis of the

genes for 2-aminophenol metabolism from *Pseudomonas* sp. AP-3. *Arch. Microbiol.* **174**, 265-272

7. Muraki, T., Taki, M., Hasegawa, Y., Iwaki, H., and Lau, P. C. (2003) Prokaryotic homologs of the eukaryotic 3-hydroxyanthranilate 3,4-dioxygenase and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase in the 2-nitrobenzoate degradation pathway of *Pseudomonas fluorescens* strain KU-7. *Appl. Environ. Microbiol.* **69**, 1564-1572
8. Park, H-S., and Kim, H-S. (2001) Genetic and structural organization of the aminophenol catabolic operon and its implication for evolutionary process. *J. Bacteriol.* **183**, 5074-5081
9. Aoki, K., Takenaka, S., Murakami, S., and Shinke, R. (1997) Partial purification and characterization of a bacterial dioxygenase that catalyzes the ring fission of 2-aminophenol. *Microbiol. Res.* **152**, 33-38
10. Takenaka, S., Asami, T., Orii, C., Murakami, S., and Aoki, K. (2002) A novel *meta*-cleavage dioxygenase that cleaves a carboxyl-group-substituted 2-aminophenol. Purification and characterization of 4-amino-3-hydroxybenzoate 2,3-dioxygenase from *Bordetella* sp. strain 10d. *Eur. J. Biochem.* **269**, 5871-5877
11. Murakami, S., Sawami, Y., Takenaka, S., Aoki, K. (2004)

Cloning of a gene encoding 4-amino-3-hydroxybenzoate 2,3-dioxygenase from *Bordetella* sp. 10d. Biochem. Biophys. Res. Commun. **314,** 489-94

12. Nozaki, M. (1970) Metapyrocatechase (*Pseudomonas*). *Methods. Enzymol.* **17A**, 522-525.

13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Foline phenol reagent. *J. Biol. Chem.* **193**, 265-275

14. He, Z. , Davis, J. K., and Spain, J. C. (1998) Purification, characterization, and sequence analysis of 2-aminomuconic dehydrogenase from *Pseudomonas pseudoalcaligenes* JS45. *J. Bacteriol.* **180**, 4591-4595

15. Nishizuka, Y., Ichiyama, A., and Hayaishi, O. (1970) Metabolism of the benzene ring of tryptophan (mammals). *Methods. Enzymol.* **17A**, 463-491

16. Weber, K., and Osborn, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406-4412

17. Klatt, P., Schmidt, K., Werner, E. R., and Mayer, B. (1996) Determination of nitric oxide synthase cofactors: heme, FAD, FMN and tetrahydrobiopterin. *Methods Enzymol.* **268**, 358-365

18. Koike, K., Hakamada, Y., Yoshimatsu, T., Kobayashi, T., and Ito, S. (1996) NADP-specific glutamate dehydrogenase from

alkalophilic *Bacillus* sp. KSM-635 purification and enzymatic properties. *Biosci. Biotech. Biochem.* **60**, 1764-1767

19. Takenaka. S., Murakami, S., Shinke, R., Hatakeyama, K., Yukawa, H., and Aoki, K. (1997) Novel genes encoding 2-aminophenol 1,6-dioxygenase from *Pseudomonas* species AP-3 growing on 2-aminophenol and catalytic properties of the purified enzyme. *J. Biol. Chem.* **272**, 14727-14732
20. Aoki, K., Kodama, N., Murakami, S., and Shinke, R. (1997) A high level of accumulation of 2-hydroxymuconic 6-semialdehyde from aniline by the transpositional mutant Y-2 of *Pseudomonas* species AW-2. *Microbiol. Res.* **152**, 129-135
21. Crawford, R. L., Bromley, J. W., and Perkins-Olson, P. E. (1979) Catabolism of protocatechuate by *Bacillus macerans*. *Appl. Environ. Microbiol.* **37**, 614-618.
22. Nozaki, M., Kotani, S., Ono, K., and Senoh, S. (1970) Metapyrocatechase. 3. substrate specificity and mode of ring fission. *Biochim. Biophys. Acta.* **220**, 213-223
23. Vollhardt, K. P. C. & Schore, N. E. (1998) In Organic chemistry: structure and function (Vollhardt, K. P. C. & Schore, N. E., eds.), pp. 1045-1046, W. H. Freeman, NY.
24. Ribbons, D. W., and Seinior, P. J. (1970) 2,3-Dihydroxybenzoate 3,4-oxygenase from *Pseudomonas*

fluorescens—Oxidation of a substrate analog. *Arch. Biochem. Biophys.* **138**, 557-565.

25. Job, V., Marcone, G. L., Pilone, M., S., and Pollegioni, L. (2002) Glycine oxidase from *Bacillus subtilis*. Characterization of a new flavoprotein. *J. Biol. Chem.* **277**, 6985-6993

26. Nishiya Y., and Imanaka, T. (1998) Purification and characterization of a novel glycine oxidase from *Bacillus subtilis*. *FEBS Lett.* **438**, 263-266

27. Pollegioni, L., Ceciliani, F., Curti, B., Ronchi, S., and Pilone, M. S. (1995) Studies on the structural and functional aspects of *Rhodotorula gracilis* D-amino acid oxidase by limited trypsinolysis. *Biochem. J.* **310**, 577-583

28. Cook, S. A., and Shiemke, A. K. (2002) Evidence that a type-2 NADH: quinone oxidoreductase mediates electron transfer to particulate methane monooxygenase in *Methylococcus capsulatus*. *Arch. Biochem. Biophys.* **398**, 32-40

29. Davis, J. K., He, Z., Somerville, C. C., Spain, J. C. (1999) Genetic and biochemical comparison of 2-aminophenol 1,6-dioxygenase of *Pseudomonas pseudoalcaligenes* JS45 to *meta*-cleavage dioxygenases: divergent evolution of 2-aminophenol *meta*-cleavage pathway. *Arch. Microbiol.* **172,** 330-9

30. Davis, B. J. (1964) Disc electrophoresis. II. Method and

application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**,404-427

Table 1. Purification of the 2-amino-5-carboxymuconic 6-semialdehyde deaminase from *Bordetella* sp. strain 10d

Fraction[a]	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)
1：Cell extract	4.2	1,600	2.6×10^{-3}	100
2: Streptomycin sulfate	4.1	1,100	3.7×10^{-3}	98
3: Ammonium sulfate	2.8	290	9.7×10^{-3}	67
4: DE52	0.5	16	0.031	12
5: DEAE-Cellulofine A-800	0.25	5.0	0.050	6
6: Phenyl-Cellulofine	0.08	0.3	0.27	2

[a] Fractions 1–6 refer to the fractions obtained at the end of steps 1–6 of the purification procedure. See the text for details.

Table 2. Mass spectra of the enzyme reaction products from 4-amino-3-hydroxybenzoic acid.

Compound	Fragments of the derivatization product [*m/z* (assignment, relative intensity)]
II: 2-Hydroxymuconic 6-semialdehyde[a]	466 (M^+, 18.7%), 451 (M^+-CH_3, 100%), 436 (M^+-CH_3×2, 0.53%), 421 (M^+-CH_3×3, 0.53%), 377 (M^+-$OSi(CH_3)_3$, 0.64%), 363 (M^+-$Si(CH_3)_3$-CH_3×2, 4.8%), 299 (M^+-C_6F_5, 65.1%), 195 ($[C_6F_5N_2]^+$, 8.7%), 147 ($[(CH_3)_2$=O-$OSi(CH_3)_3]^+$, 24.3%), 73 ($[Si(CH_3)_3]^+$, 98.4%)
IV: 2,5-Pyridine-dicarboxylic acid[b]	311 (M^+, 30.6%), 296 (M^+-CH_3, 100%), 266 (M^+-CH_3×3, 39.3%), 238 (M^+-$Si(CH_3)_3$, 11.7%), 222 (M^+-$Si(CH_3)_3$-O, 62.7%), 194 (M^+-$COOSi(CH_3)_3$, 39.3%), 147 ($[(CH_3)_2$=O-$OSi(CH_3)_3]^+$, 100%), 73 ($[Si(CH_3)_3]^+$, 100%), 77 (M^+-$COOSi(CH_3)_3$-$COOSi(CH_3)_3$, 90.9%)

[a]Pentafluorophenylhydrazine and trimethylsilylated product
[b]Trimethylsilylated product

Figure Legends

Fig. 1. (A) Proposed pathway of 4-amino-3-hydroxybenzoate metabolism in *Bordetella* sp. strain 10d compared to (B) the modified *meta*-cleavage pathway of 2-aminophenol in *Pseudomonas* sp. strain AP-3.

(A) Proposed pathway of 4-amino-3-hydroxybenzoic acid in *Bordetella* sp. strain 10d (10). I, 4-amino-3-hydroxybenzoic acid; II, 2-amino-5-carboxymuconic 6-semialdehyde; III, 2-hydroxy-5-carboxymuconic 6-semialdehyde; IV, 2-hydroxymuconic 6-semialdehyde; V, 2,5-pyridinedicarboxylic acid; and VI, 2-aminomuconic 6-semialdehyde. (B) Pathway of 2-aminophenol metabolism in *Pseudomonas* sp. strain AP-3 (6). I, 2-aminophenol; II, 2-aminomuconic 6-semialdehyde; III, 2-aminomuconic acid; IV, 4-oxalocrotonic acid; and V, picolinic acid.

Fig. 2. Absorption spectra of the reaction products formed from 4-amino-3-hydroxybenzoic acid in (A) an assay with crude extract and (B, C) a coupled assay with two purified enzymes.

(A) The reaction mixture consisted of 2.9 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5), 0.1 ml of 5 mM 4-amino-3-hydroxybenzoic acid, and 0.05 ml of the crude extract (35–75% ammonia sulfate saturation) (61 mg ml^{-1}). The reaction

was started by adding the enzyme solution. After incubation at 24°C, the sample was scanned with a spectrophotometer and spectra were recorded every 2 min.

(B) The reaction mixture consisted of 2.9 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5), 0.1 ml of 5 mM 4-amino-3-hydroxybenzoic acid, 0.1 ml of purified 4-amino-3-hydroxybenzoate 2,3-dioxygenase solution (6 μg ml^{-1}) and 0.1 ml of purified 2-amino-5-carboxymuconic 6-semialdehyde deaminase (71 μg ml^{-1}). The reaction was started by adding the enzyme solution. After incubation at 24°C, the sample was scanned with a spectrophotometer and spectra were recorded every 2 min. (C) Enlargement of the original plots shown in (B).

Fig. 3. (A) PAGE and (B) SDS-PAGE of the 2-amino-5-carboxymuconic 6-semialdehyde deaminase. (A) The purified enzyme (10 μg) was electrophoresed on a 12.5% (w/v) polyacrylamide disc gel (pH 8.0) at 2 mA/tube for 2 h in a running buffer of Tris-glycine (pH 8.3) (30). (B) The purified enzyme (10 μg) denatured with SDS was electrophoresed on a 12.5% (w/v) polyacrylamide disc gel containing 0.1% (w/v) SDS at 6 mA/tube for 3.5 h in a running buffer of 0.1% (w/v) SDS-0.1 M sodium phosphate (pH 7.2) (16). Standards were run separately. The gels were stained with 0.25% (w/v) Coomassie

Brilliant Blue R-250 in a solvent of ethanol/acetic acid/H_2O (9:2:9, by vol.).

Fig. 4. UV-visible and fluorescence spectra of the purified enzyme. The main figure shows the UV-visible absorption spectrum of the purified enzyme (1.1 mg) recorded using 50 mM sodium-potassium phosphate buffer (pH 7.0) as reference. The insets show (A) the fluorescence excitation spectrum (detected at 530 nm) and (B) the emission spectrum (excited at 450 nm) of the supernatant of the heat-treated enzyme (1.2 mg protein ml^{-1}). The cofactor derived from the purified enzyme was released by heat treatment as described previously (17).

Fig. 1. Orii et al.

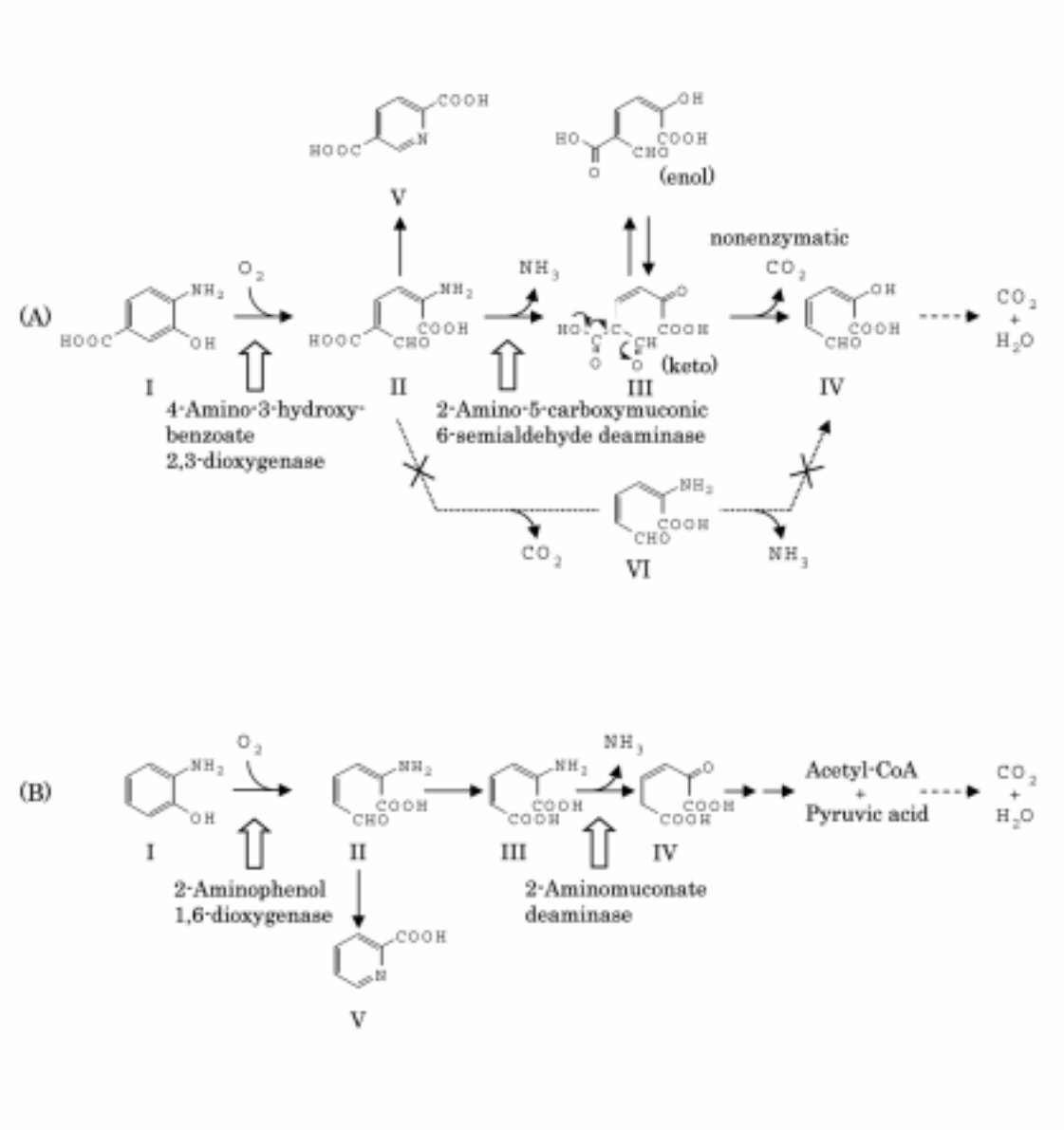

Fig. 2. Orii et al.

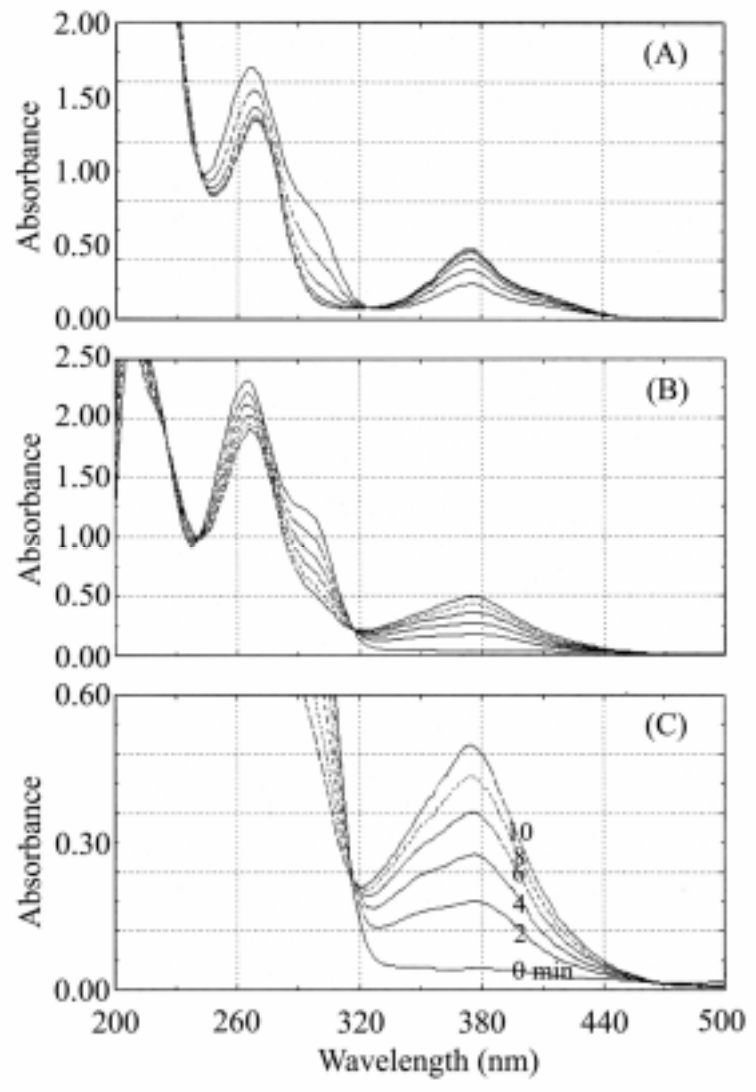

Fig. 3. Orii et al.

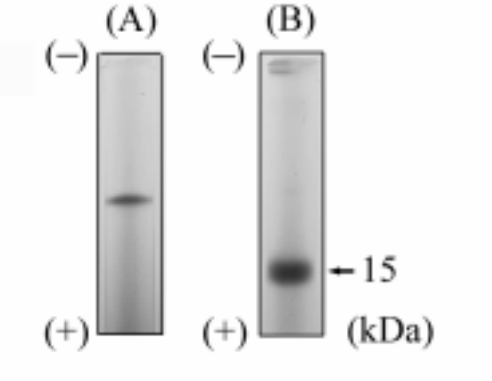

Fig. 4. Orii et al.