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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

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Running title: 4-Amino-3-hydroxybenzoate 2,3-dioxygenase

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Enzymes: 4-amino-3-hydroxybenzoate 2,3-dioxygenase [EC 1.13.1.- as proposed in this paper as a new subclass of dioxygenase catalyzing the fission of the benzene ring], 2-aminophenol 1,6-dioxygenase [EC 1.13.11.x], catechol 1,2-dioxygenase [EC 1.13.11.1], catechol 2,3-dioxygenase [EC 1.13.11.2], protocatechuate 2,3-dioxygenase [EC 1.13.11.x], protocatechuate 3,4-dioxygenase [EC 1.13.11.3],

protocatechuate 4,5-dioxygenase [EC 1.13.11.8], 2,3-biphenyl 1,2-dioxygenase [EC 1.13.11.39].

SUMMARY

A bacterial strain that grew on 4-amino-3-hydroxybenzoic acid was isolated from farm soil. The isolate, strain 10d, was identified as a species of *Bordetella*. Cell extracts of *Bordetella* sp. strain 10d grown on 4-amino-3-hydroxybenzoic acid contained an enzyme that cleaved this substrate. The enzyme was purified to homogeneity with a 110-fold increase in specific activity. The purified enzyme was characterized as an *meta*-cleavage dioxygenase that catalyzed the ring fission between C2 and C3 of 4-amino-3-hydroxybenzoic acid, with the consumption of 1 mol of O_2 per mol of substrate. The enzyme was therefore designated as 4-amino-3-hydroxybenzoate 2,3-dioxygenase. The molecular mass of the native enzyme was 40 kDa based on gel filtration; the enzyme is composed of two identical 21-kDa subunits according to SDS/PAGE. The enzyme showed a high dioxygenase activity only for 4-amino-3-hydroxybenzoic acid. The K_m and V_{max} values for this substrate were 35 μM and 12 μmol $min^{-1}\cdot$ (mg protein) $^{-1}$, respectively. Of the 2-aminophenols tested, only 4-aminoresorcinol and 6-amino-*m*-cresol inhibited the enzyme. The enzyme reported here differs from previously reported extradiol dioxygenases, including 2-aminophenol 1,6-dioxygenase, in molecular mass, subunit structure, and catalytic properties.

Keywords: 4-amino-3-hydroxybenzoate-degrading bacterium; 2-aminophenol derivatives; *meta*-cleavage dioxygenase; 4-amino-3-hydroxybenzoate 2,3-dioxygenase.

INTRODUCTION

Dioxygenases catalyzing the fission of benzene rings are key enzymes in the microbial metabolic pathways of aromatic compounds. Most of these types of dioxygenases previously reported attack aromatic compounds with two adjacent hydroxyl groups, such as catechol and protocatechuic acid, and open the benzene rings through intradiol or extradiol fission [1-4] –thus their designation as intradiol or extradiol dioxygenases. Some bacterial dioxygenases are able to cleave the benzene ring of gentisic acid and hydroquinone, which have two hydroxyl groups in *para*-position [5,6]. Until a few years ago, the widely accepted theory was that two hydroxyl groups are necessary for the metabolism of aromatic compounds by bacteria. However, it has been shown that a few dioxygenases attack aromatic compounds with a single hydroxyl group, such as 2-aminophenol and salicylic acid [7–9].

Pseudomonas sp. AP-3 and *Pseudomonas pseudoalcaligenes* JS45 cleave 2-aminophenol to form 2-aminomuconic 6-semialdehyde, without the formation of catechol [10,11]. The 2-aminophenol 1,6-dioxygenase from each of these strains has been purified and characterized [8,9]. The enzymes are different from previously reported dioxygenases in substrate specificity and the deduced amino acid sequences. The enzymes catalyze

the ring fission of 2-aminophenol and its methyl- or chloro- derivatives, but not of carboxyl-group-substituted 2-aminophenols. Currently, little is known about dioxygenases that act on carboxyl-group-substituted 2-aminophenols. 3-Hydroxyanthranilic acid (2-amino-3-hydroxybenzoic acid) is metabolized via 2-amino-3-carboxymuconic 6-semialdehyde to form 2-aminomuconic 6-semialdehyde in mammalian cells and in *Pseudomonas fluorescens* strain KU-7 [12,13]. The enzyme from beef kidney that acts on 3-hydroxyanthranilic acid has been purified to homogeneity and characterized [14]. Whether the enzyme cleaves other carboxyl-group-substituted 2-aminophenols has not been elucidated. Ring fission of 2-aminophenols is a key reaction for bacterial degradation of aromatic compounds. Since 2-aminophenol 1,6-dioxygenases have played a pivotal role in understanding substrate selectivity and reaction mechanisms, it is important to characterize another type of aminophenol dioxygenase completely for comparative studies.

Here we report the isolation of a soil bacterium able to grow on 4-amino-3-hydroxybenzoic acid. The purification and characterization of a dioxygenase from this strain, catalyzing the ring fission of 4-amino-3-hydroxybenzoic acid, is described.

MATERIALS AND METHODS

Organism and growth conditions.

Strain 10d was obtained from farm soil in Hyogo Prefecture, Japan. The basal medium containing 4-amino-3-hydroxybenzoic acid used for the isolation and cultivation of strain 10d was composed of three separately prepared solutions. Solution A contained 4.5 g KH_2PO_4, 18 g $Na_2HPO_4 \cdot 12H_2O$, 1 g NaCl, 0.4 g yeast extract, and deionized water in 1 liter total volume; the final pH was adjusted to pH 6.8. Solution B contained 1 g $MgSO_4 \cdot 7H_2O$, and 1 mg each of $CaCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnCl_2$, and $FeSO_4 \cdot 7H_2O$, and deionized water in 300 mL total volume. Solution C contained 2.4 g 4-amino-3-hydroxybenzoic acid, 6.0 g $Na_2HPO_4 \cdot 12H_2O$, and deionized water in 700 mL total volume; the final pH was adjusted to pH 6.8. Solutions A and B were autoclaved, and solution C was sterilized by filtration. The three sterile solutions were mixed at room temperature. The culture was incubated at 30°C with shaking at 140 rpm. Samples were taken and 4-amino-3-hydroxybenzoic acid was quantified by the methods described below.

Morphological and phenotypic characterization.

Physiological and biochemical parameters, such as Gram reaction, flagella type, catalase activity, oxidase activity, and OF test, were determined using classical methods [15]. Alkali production of amides, organic acids, reduction of tetrazolium, and requirement for nicotinamide

were tested as described previously [16–18]. The G+C content of the DNA and isoprenoid quinones were determined using previously reported methods [19,20].

Enzyme assay.

4-Amino-3-hydroxybenzoic acid ring-fission activity was measured by monitoring the decrease in the absorbance of 4-amino-3-hydroxybenzoic acid at 294 nm. The reaction mixture contained 2.8 mL of 100 mM sodium-potassium phosphate buffer (pH 7.5) and 0.1 mL of 5 mM 4-amino-3-hydroxybenzoic acid. The reaction was started by adding 0.1 mL of enzyme solution. After incubation for 10 min at 24°C, the absorbance at 294 nm was read. One unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of 4-amino-3-hydroxybenzoic acid per min. The molar extinction coefficient of 7.53×10^3 $M^{-1}\cdot cm^{-1}$ for 4-amino-3-hydroxybenzoic acid was used. Specific activity was defined as units·$(mg\ protein)^{-1}$. Protein concentrations were measured by the method of Lowry *et al*. [21].

The initial velocity of the reaction was obtained using various concentrations (0.5–5 mM) of 4-amino-3-hydroxybenzoic acid. After incubation for 1 min, the absorbance at 294 nm was read. The method for determining the values of K_m and V_{max}, which was formulated by Hans Lineweaver-Burk, used the double reciprocal of the Michaelis-Menten equation. The K_i value was obtained using different concentrations (1.6, 3.2, and 4.8 μM) of 4-aminoresorcinol.

Enzyme purification.

All steps of the enzyme purification were carried out at 0–4°C. All centrifugations were at 20,000 × *g* and 4°C for 10 min.

Cells (30 g, wet weight) of *Bordetella* sp. strain 10d were obtained from a 4.8-L culture in basal medium containing 4-amino-3-hydroxybenzoic acid and 1% (w/v) meat extract incubated for 15 h at 30°C with shaking. The preparation of the cell extracts (step 1, Fraction 1) and the streptomycin sulfate treatment to remove nucleic acids from the cell extracts solution (step 2, fraction 2) essentially followed previously described methods [10].

Step 3. $(NH_4)_2SO_4$ fractionation. Fraction 2 was brought to 35% saturation with $(NH_4)_2SO_4$. The mixture was stirred for 30 min and centrifuged; the supernatant was collected, and the precipitate was discarded. $(NH_4)_2SO_4$ was added to the supernatant to 50% saturation. After stirring for 30 min, the precipitate was collected by centrifugation and dissolved in 20 mM Tris-HCl buffer (pH 8.0). The solution was dialyzed against buffer A [20 mM Tris-HCl buffer (pH 8.0) containing 10% (v/v) ethanol, 1 mM dithiothreitol, and 0.5 mM L-ascorbate] with two changes of buffer. The final volume of the dialyzed solution (fraction 3) was 52 mL.

Step 4. Acetone fractionation. After the protein concentration of fraction 3 was adjusted to 10 $mg \cdot mL^{-1}$ by adding buffer A, acetone was added to a final concentration of 40% (v/v). The precipitate was removed by centrifugation; acetone was then added to the supernatant to a final concentration of 60% (v/v). The precipitate was collected by centrifugation and then dissolved in buffer A. The enzyme solution was dialyzed against

buffer A. The final volume of the dialyzed solution (fraction 4) was 42 mL.

Step 5. Chromatography on DE52 cellulose. Fraction 4 was applied to a column (2.1×18 cm) of DE52 cellulose equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.4 M) of NaCl in 900 mL of buffer A. Fractions of 5 mL were collected at a flow rate 40 $mL \cdot h^{-1}$. The protein concentration and enzyme activity of the fractions were assayed. Fractions with a specific activity higher than 4.0 $units \cdot (mg\ protein)^{-1}$ were pooled to yield fraction 5 (40 mL).

Step 6. Chromatography on DEAE-Cellulofine A-800 I. Fraction 5 was applied to a column (1.6×10 cm) of DEAE-Cellulofine A-800 equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.3 M) of NaCl in 400 mL of buffer A. Fractions of 4 mL were collected at a flow rate 30 $mL \cdot h^{-1}$. Fractions with a specific activity higher than 12.0 $units \cdot (mg\ protein)^{-1}$ were pooled to yield fraction 6 (18 mL).

Step 7. Chromatography on DEAE-Cellulofine A-800 II. Fraction 6 was applied to a column (1.0×12 cm) of DEAE-Cellulofine A-800 equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.3 M) of NaCl in 200 mL of buffer A. Fractions of 3 mL were collected at a flow rate 30 $mL \cdot h^{-1}$. Fractions with a specific activity higher than 20 $units \cdot (mg\ protein)^{-1}$ were pooled to yield fraction 7 (18 mL). The enzyme preparation showed one major protein band and some indistinct bands on a SDS/PAGE.

Step 8. Chromatography on Cellulofine GCL-1000 sf. Fraction 7 was concentrated to 1.0 mL using a collodion bag (Sartorious, Goettingen,

Germany). The concentrated sample was loaded onto a column (3.2×58 cm) of Cellulofine GCL-1000 sf equilibrated with buffer A containing 0.2 M NaCl. Proteins were eluted with the same buffer. Fractions of 2 mL were collected at a flow rate 20 $mL \cdot h^{-1}$. The enzyme purity in each fraction was checked by SDS/PAGE [22]. Fractions showing a single protein band on the gel were pooled (fraction 8, 6 mL).

Identification of the reaction product (compound I) from the cleavage of 4-amino-3-hydroxybenzoic acid.

The reaction mixture contained 250 mL of 100 mM sodium-potassium phosphate buffer (pH 7.5), 2.5 mL of enzyme solution (2.5 $\mu g \cdot mL^{-1}$), and 10 mL of 5 mM 4-amino-3-hydroxybenzoic acid. After incubation at 24°C for 30 min, the reaction mixture was concentrated to 80 mL with a rotary evaporator. The pH of the concentrated solution was adjusted to pH 3.0 with 3N HCl, and the solution was extracted with ethyl acetate. The upper layer was collected and evaporated to dryness. The single reaction product reacted with methanol under acidic conditions. The esterified product (compound I) was analyzed by GC-MS and GC, as described below.

Stoichiometry of the enzyme reaction.

4-Amino-3-hydroxybenzoate-dependent oxygen uptake was measured with a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Co., USA) mounted in a water-jacketed reaction

vessel kept at 24°C. The reaction mixture (3 mL) contained sodium-potassium phosphate, 4-amino-3-hydroxybenzoic acid, and the purified enzyme (0.25 μg) as described above. The ring-fission activity with 4-amino-3-hydroxybenzoic acid as substrate was also measured. The concentrations of 4-amino-3-hydroxybenzoic acid and 2,5-pyridinedicarboxylic acid were determined by measuring the absorbance at 294 nm and 268 nm, respectively. The molar extinction coefficient of 5.77×10^3 $M^{-1} \cdot cm^{-1}$ for 2,5-pyridinedicarboxylic acid was used. All data are expressed as the mean of five determinations ± standard deviations.

Substrate specificity.

The substrate specificity of the 4-amino-3-hydroxybenzoate-fission enzyme was examined with 28 aromatic compounds, including 2-aminophenol, catechol, aniline, and benzoate compounds, using the same methods as described previously [9]. The benzene-ring cleavage of these compounds was assayed spectrophotometrically under the reaction conditions described above, using these aromatic compounds instead of 4-amino-3-hydroxybenzoic acid as substrate.

Inhibition of the 4-amino-3-hydroxybenzoate-cleaving activity by the substrate analogues (2-aminophenols, catechols, anilines, and benzoic acids described above) was examined. The enzyme (0.5 μg) was incubated with one of each of the inhibitors (0.05 mM) in 3 mL of 100 mM sodium-potassium phosphate buffer (pH 7.5) at 24°C for 1 min. The

enzyme reaction was then started by adding 0.1 mL of 5 mM 4-amino-3-hydroxybenzoic acid. After incubation for 10 min, the absorbance at 294 nm was monitored.

Unstable compounds (4-aminoresorcinol, amidol, 3-hydroxyanthralinic acid, 1,2,4-trihydroxybenzene, and pyrogallol) in aqueous solution were always freshly prepared and used immediately.

Effect of various compounds on the enzyme activity.

The effect of metal salts, and chelating and sulfhydryl agents on the enzyme activity with 4-amino-3-hydroxybenzoic acid as the substrate, was tested using the methods described previously [9]. The enzyme (0.5 μg) was incubated with 1.0 or 2.5 mM of each compound in 3 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5) at 24°C for 10 min. The enzyme reaction was started by adding 0.1 ml of 5 mM 4-amino-3-hydroxybenzoic acid. After incubation for 10 min, the absorbance at 294 nm was monitored.

Analytical methods.

UV absorption spectra of reaction products were recorded with a Beckman DU 650 spectrophotometer. The esterified compound I was 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. Iron in the enzyme was reduced to Fe^{2+} with hydroxylamine-HCl and

then measured using *o*-phenanthroline [23]. 4-Amino-3-hydroxybenzoic acid in the growing culture was determined using a diazo coupling reaction [24]. The molar extinction coefficient of 3.9×10^4 $M^{-1} \cdot cm^{-1}$ at 563 nm for the diazotized compound was used. The NH_2-terminal amino acid sequence was determined as described in detail previously [25].

Determination of molecular masses.

The molecular mass of the native enzyme was determined by gel filtration on Cellulofine GCL-1000 sf. The molecular mass of the enzyme subunit was measured using SDS/PAGE [22]. Size markers used for gel filtration were those in the calibration proteins gel chromatography kit from Boehringer Mannheim (Mannheim, Germany). The electrophoresis calibration kit LMW (Amersham Pharmacia Biotech) was used as size markers for SDS/PAGE.

Nucleotide sequence accession number.

The partial nucleotide sequence (1457 bp) of the 16S rRNA gene of *Bordetella* sp. strain 10d reported in this paper was deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB070889.

Chemicals.

4-Amino-3-hydroxybenzoic acid, 6-amino-*m*-cresol, and 2,5-pyridinedicarboxylic acid were purchased from Tokyo Kasei Kogyo

(Tokyo, Japan); meat extract (Extract Ehlrich) was from Kyokuto Seiyaku Kogyo (Osaka, Japan); 4-aminoresorcinol hydrochloride was from Aldrich (Milwaukee, Wis., USA). DE52 cellulose was from Whatman (Madison, Wis., USA), and DEAE-Cellulofine A-800 and Cellulofine GCL-1000 sf were from Seikagaku (Tokyo, Japan).

RESULTS

Identification of a 4-amino-3-hydroxybenzoate-assimilating organism.

Strain 10d grew well in the basal medium containing 4-amino-3-hydroxybenzoic acid and yeast extract and completely degraded this former compound (Fig. 1). The consumption of 4-amino-3-hydroxybenzoic acid correlated with an increase in cell density and in protein content. 2,5-Pyridinedicarboxylic acid (see Fig. 4a) in the culture broth in which strain 10d grew was not detected by HPLC. The strain could not grow on 4-amino-3-hydroxybenzoic acid without yeast extract or if the concentration of 4-amino-3-hydroxybenzoic acid exceeded 1.2 $g \cdot L^{-1}$. At high concentrations of this compound, the medium turned brown and growth ceased owing to its toxicity. Strain 10d utilized 4-amino-3-hydroxybenzoic acid as a carbon, nitrogen, and energy source, and yeast extract supplied growth factors.

Strain 10d is a rod of 0.4×1.4–2.4 μm and motile with peritrichous flagella. It is aerobic, Gram-negative, non-spore-forming, urease-negative, and catalase- and oxidase-positive. It oxidatively produced a small amount

of acid from D-glucose, D-fructose, and sucrose. Alkali was produced from L-asparagine, citrate, galactarate, and tartrate. The nucleotide sequence (1457 bp) of the 16S rRNA gene of strain 10d was 96.7% identical with that of *Bordetella avium* DSM 11334^T (accession no. AF177666), 96.7% identical with that of *Bordetella hinzii* DSM 4922^T (AF177667), and 96.0% identical with that of *Bordetella bronchiseptica* (AJ278452) [26,27]. This close phylogenic relatedness with other members of the genus *Bordetella* [27,28] was also reflected in the following characteristics of strain 10d: the DNA G+C content was 67.0 mol%; the isoprenoid quinone Q-8 was detected; tetrazolium was reduced; nicotinamide was required for growth; and potassium tellurite inhibited growth. Thus, strain 10d was identified as a species of *Bordetella*.

Purification and properties of the purified enzyme.

The 4-amino-3-hydroxybenzoate-fission enzyme from *Bordetella* sp. strain 10d was present in cell extracts. The enzyme activity was measured by monitoring the decrease in the absorbance of 4-amino-3-hydroxybenzoic acid at 294 nm. The enzyme was purified 110-fold with an overall yield of 3% (Table 1). After electrophoresis, the purified enzyme exhibited a single protein band on both native and denaturing polyacrylamide gels (Fig. 2(a) and (b)). The apparent molecular mass was determined to be 40 kDa by gel filtration and 21 kDa by SDS/PAGE. These findings indicated that the enzyme is a homodimer with 21-kDa subunits.

During the entire purification procedure, buffer A was used to stabilize the enzyme. However, the purified enzyme in buffer A lost nearly 25% of its activity after storage at 4°C for 5 days. An inactivation of the enzyme probably led to a decrease in the specific activity between purification steps 7 and 8. The enzyme showed maximal activity in 50 mM Tris-HCl buffer (pH 8.0); the activities in 100 mM sodium potassium phosphate buffer (pH 7.5) and 50 mM Tris-HCl buffer (pH 8.5) were 85% and 60% of the maximal activity, respectively. The purified enzyme was stable for 1 week in buffer A containing 10% (v/v) ethanol, 1 mM dithiothreitol, and 0.5 mM L-ascorbic acid at pH 7.0 to 9.0. The enzyme maintained 100% activity up to 30°C after 10 min incubation at pH 8.0. The enzyme activity decreased to 70% after incubation at 40°C for 10 min, and all activity was lost at 50°C.

The enzyme contained 1.9 mol Fe^{2+} per mol protein considering a molecular mass of 40 kDa. The NH_2-terminal amino acid sequence of the enzyme was determined to be MIILENFKMPNVDLEAVMRYLXEEG.

Identification of the reaction product.

The mass spectrum of the dimethyl ester of the enzyme reaction product (compound I) yielded a molecular ion at *m/z* 195 (M^+, relative intensity 1.8%), which is in agreement with the empirical formula of $C_9H_9NO_4$. Major fragment ions appeared at *m/z* 165 (M^+–OCH_2, 18), 137 (M^+–$COOCH_2$, 100), and 106 (M^+–$COOCH_2$–OCH_3, 1.1). This mass spectrum and the GC retention time (8.4 min) of the modified compound I

agreed with those of the derivatized authentic 2,5-pyridinedicarboxylic acid dimethyl ester. Compound I and authentic 2,5-pyridinedicarboxylic acid both had a peak at 268 nm in the UV absorption spectrum in buffer at pH 7.5. Compound I was thus identified as 2,5-pyridinedicarboxylic acid (see Fig. 4a).

Conversion of 4-amino-3-hydroxybenzoic acid.

Figures 3a and b show the changes in the spectrum during the enzyme reaction. When the purified enzyme was added to the reaction mixture containing 4-amino-3-hydroxybenzoic acid, the absorption peak at 388 nm increased rapidly and reached the maximum in 30 s (Fig. 3b), and then gradually decreased. The absorption peaks at 263 and 294 nm derived from 4-amino-3-hydroxybenzoic acid also decreased as the enzyme reaction proceeded (Fig. 3a) and disappeared after 10 min of incubation. The absorption peak at 268 nm was observed at this time and was judged to be due to 2,5-pyridinedicarboxylic acid (see above).

In the reaction catalyzed by the purified enzyme, 1.0±0.10 μmol of 4-amino-3-hydroxybenzoic acid and 0.90±0.08 μmol of O_2 were consumed and 1.1±0.02 μmol of 2,5-pyridinedicarboxylic acid was formed, which indicated a molar ratio of 4-amino-3-hydroxybenzoic acid:O_2:2,5-pyridinedicarboxylic acid of 1:1:1.

Substrate specificity and inhibition by substrate analogues.

The substrate specificity of the enzyme was examined with 28

aromatic compounds, including 2-aminophenol, and its methyl-, chloro-hydroxyl- or carboxyl- derivatives, catechol, and protocatechuic acid as putative substrates. The enzyme acted only on 4-amino-3-hydroxybenzoic acid. The K_m and V_{max} for 4-amino-3-hydroxybenzoic acid of the purified enzyme were 35 μM and 12 μmol·min^{-1}·(mg protein)$^{-1}$, respectively. 4-Aminoresorcinol bound to the enzyme as a competitive inhibitor with a K_i of 1.2 μM. 6-Amino-*m*-cresol (0.05 mM) decreased the enzyme activity for 4-amino-3-hydroxybenzoic acid (0.16 mM) to 85%.

Inhibition by metal salts and other compounds.

Among the metal salts tested, the enzyme was completely inhibited by 1 mM $HgCl_2$ and 1 mM $CuSO_4$; 1 mM $FeSO_4$ and 1 mM $Fe(NH_4)_2(SO_4)_2$ slightly increased the activity. Other metal salts did not affect the enzyme activity. The addition of 2.5 mM α,α'-dipyridyl, 1,2-dihydroxybenzene-3,5-disulfonate, EDTA, *o*-phenanthroline, or NaN_3 decreased the enzymatic activity to 62, 65, 58, 14, and 38%, respectively.

DISCUSSION

This is the first report of the purification of a 4-amino-3-hydroxybenzoate-fission enzyme and its characterization in terms of molecular mass, subunit structure, reaction mechanism, and catalytic properties. The new type of dioxygenase, different from the 2-aminophenol 1,6-dioxygenase reported previously [8,9], primarily and specifically attacks carboxyl-group-substituted 2-aminophenol compounds.

2-Aminophenol 1,6-dioxygenase catalyzes the production of 2-aminomuconic 6-semialdehyde from 2-aminophenol, which is then converted into picolinic acid nonenzymatically (Fig. 4b) [8–10]. 2-Aminomuconic 6-semialdehyde shows an absorption peak at 382 nm. In the experiments reported here, an absorption peak at 388 nm was observed during the enzyme reaction (Fig. 3b); we failed to isolate the compound responsible for this peak from the reaction mixture by modification with methyl chlorocarbonate and pentafluorophenylhydrazine [9]. The present and previous data together suggest that the purified enzyme catalyzes the production of 2-amino-5-carboxymuconic 6-semialdehyde from 4-amino-3-hydroxybenzoic acid with the consumption of one mol of O_2 per mol of substrate, and that 2-amino-5-carboxymuconic 6-semialdehyde is then converted to 2,5-pyridinedicarboxylic acid nonenzymatically (Fig. 4a). Therefore, we named the enzyme reported here 4-amino-3-hydroxybenzoate 2,3-dioxygenase. Strain 10d utilizes 4-amino-3-hydroxybenzoic acid as a carbon, nitrogen, and energy source. 4-Amino-3-hydroxybenzoic acid was metabolized via 2-amino-5-carboxymuconic 6-semialdehyde to 2-hydroxymuconic 6-semialdehyde by *Bordetella* sp. strain 10d (Fig. 4a, data not shown). Thus, we identified an enzyme involved in the initial steps of the metabolism of 4-amino-3-hydroxybenzoic acid.

4-Amino-3-hydroxybenzoate 2,3-dioxygenase contained 1.9 mol Fe^{2+} per mol of enzyme. Addition of Fe^{2+} increased the enzyme activity and chelating agents repressed the enzyme activity. Thus, the enzyme probably

requires Fe^{2+} for activity. Other extradiol dioxygenases, such as 2-aminophenol 1,6-dioxygenase [9] and protocatechuate 4,5-dioxygenase [4], also need Fe^{2+} for activity. Whether 4-amino-3-hydroxybenzoate 2,3-dioxygenase contains Fe^{2+} or Fe^{3+} could not be determined by EPR, because the enzyme could not be purified in large enough quantities for such studies and it gradually lost its activity after 1 week, even in buffer A containing 10% (v/v) ethanol, 1 mM dithiothreitol, and 0.5 mM L-ascorbic acid.

The 4-amino-3-hydroxybenzoate 2,3-dioxygenase reported here is similar to 2,3-dihydroxybiphenyl 1,2-dioxygenase from *Rhodococcus globerulus* strain P6 [29] and from the naphthalenesulfonate-degrading bacterium strain BN6 [30] with respect to small subunit molecular mass. However, the molecular mass of 4-amino-3-hydroxybenzoate 2,3-dioxygenase is smaller than that of well-known extradiol dioxygenases, such as catechol 2,3-dioxygenase [2], protocatechuate 2,3-dioxygenase [31], protocatechuate 4,5-dioxygenase [32], and 2-aminophenol 1,6-dioxygenase. The enzyme is a homodimer, whereas other known dioxygenases are homotetramers [2,31] or heterotetramers [9,32].

4-Amino-3-hydroxybenzoate 2,3-dioxygenase attacked 2-aminophenols with functional-group substituents at the C5 position. 2-Aminophenol 1,6-dioxygenase acts on 2-aminophenol and its methyl- and chloro- derivatives [8,9]. Other extradiol dioxygenases do not act on 4-amino-3-hydroxybenzoic acid, except for protocatechuate 2,3-dioxygenase, which has, with this substrate, 4.5% of the activity of

4-amino-3-hydroxybenzoate 2,3-dioxygenase. Protocatechuate 2,3-dioxygenase oxidizes the primary substrate protocatechuic acid and catechols with a methyl or halogen substituent at the C3 or C4 position [31]. These findings illustrate that 4-amino-3-hydroxybenzoate 2,3-dioxygenase differs from all other extradiol dioxygenases reported.

The NH_2-terminal amino acid sequence of 4-amino-3-hydroxybenzoate 2,3-dioxygenase did not show significant levels of identity to sequences of other proteins including those of extradiol dioxygenases available in the FASTA and BLAST database programs at the DNA Data Bank of Japan. The gene encoding 4-amino-3-hydroxybenzoate 2,3-dioxygenase is currently being cloned; the analysis of the entire amino acid sequence will reveal more information on the strict substrate specificity.

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Figure Legends

Fig. 1. Growth of strain 10d on 4-amino-3-hydroxybenzoic acid.

For growth experiments, strain 10d was grown in basal medium containing 4-amino-3-hydroxybenzoic acid (1.2 $g \cdot L^{-1}$) and yeast extract (0.025 $g \cdot L^{-1}$) and in basal medium containing only yeast extract (0.025 $g \cdot L^{-1}$) as a control. Each culture was incubated in a 500-mL flask at 30 °C with shaking. Disappearance of 4-amino-3-hydroxybenzoic acid (▲) was measured spectrophotometrically. Increased in cell density (● [control, ○]) was determined by measuring the optical density at 660 nm or the protein content (■ [control, □]) of the culture fluid using a modification of the method of Hartree [33].

Fig. 2. PAGE (a) and SDS/PAGE (b) of the 4-amino-3-hydroxybenzoate-fission enzyme.

PAGE: The purified enzyme (3 μg) was run on a 7.5% (w/v) polyacrylamide gel (pH 8.0) at 2 mA/tube for 2 h in a running buffer of Tris-glycine (pH 8.3) [34]. SDS/PAGE: the purified enzyme (5 μg) denatured with SDS was run on a 7.5% (w/v) polyacrylamide 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) [22]. The gels were stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 ethanol/acetic acid/H_2O (9:2:9, by vol.).

Fig. 3. Absorption spectra of the reaction products from the cleavage of

4-amino-3-hydroxybenzoate.

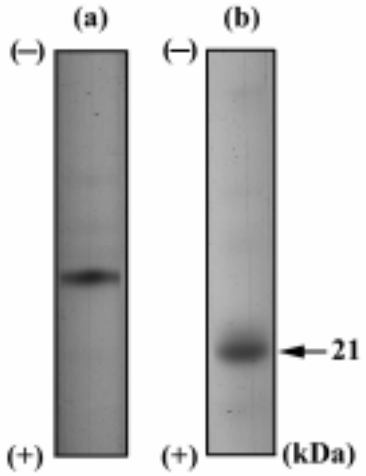
(a) Reaction conditions were as described in Materials and Methods. The reaction was started by adding 0.1 mL of the purified enzyme solution (2.5 μg mL^{-1}). After incubation at 24°C for 0 (solid line), 0.5 (dotted line), 3 (dashed line), and 10 (dash-dotted line) min, each sample was scanned with a spectrophotometer.

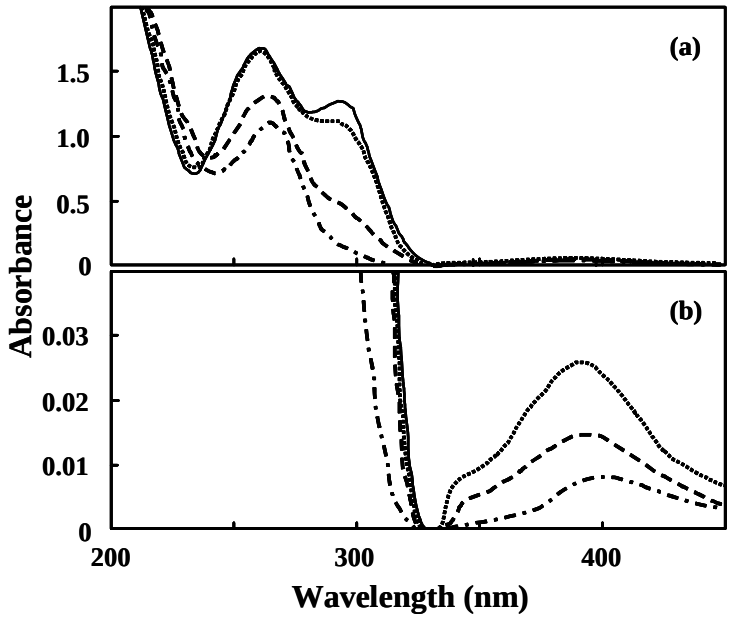
(b) The original plots shown in (a) were enlarged.

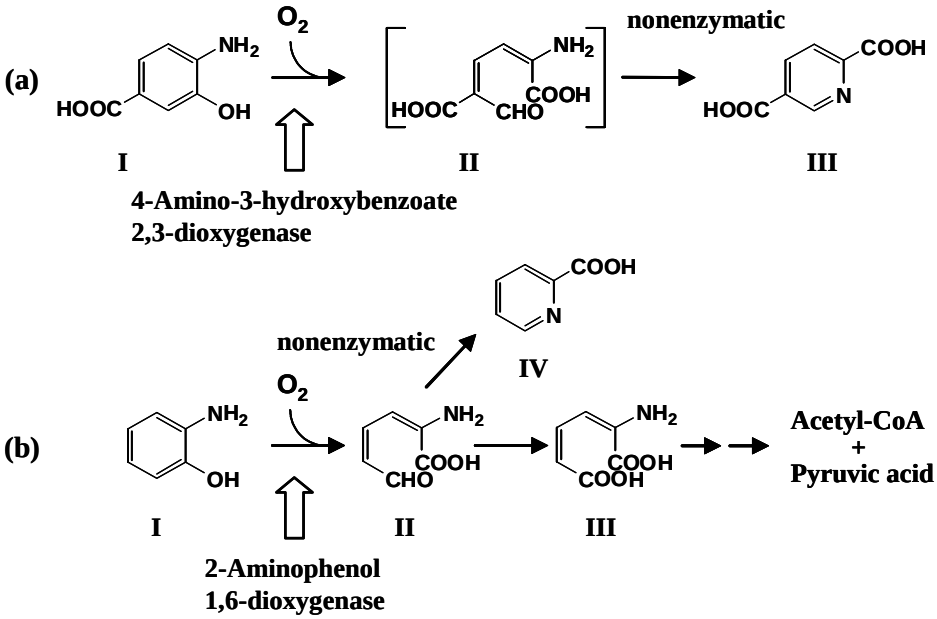
Fig. 4. (a) Proposed pathway of 4-amino-3-hydroxybenzoate cleavage in *Bordetella* sp. strain 10d and (b) comparison to the modified *meta*-cleavage pathway of 2-aminophenol in *Pseudomonas* sp. AP-3.

(a) Proposed pathway of 4-amino-3-hydroxybenzoate cleavage. I, 4-amino-3-hydroxybenzoic acid; II, 2-amino-5-carboxymuconic 6-semialdehyde; III, 2-hydroxymuconic 6-semialdehyde; IV, 2,5-pyridinedicarboxylic acid

(b) Pathway of 2-aminophenol cleavage [35]. I, 2-aminophenol; II, 2-aminomuconic 6-semialdehyde; III, 2-aminomuconic acid; IV, picolinic acid


(a)
(b)
(–)
(–)
21
(+)
(+)
(kDa)


(a)
(b)
Absorbance
1.5
1.0
0.5
0
0.03
0.02
0.01
0
200
300
400
Wavelength (nm)


(a)
O2
NH2
HOOC
OH
nonenzymatic
COOH
N
HOOC
I
II
III
4-Amino-3-hydroxybenzoate
2,3-dioxygenase
(b)
nonenzymatic
IV
O2
NH2
OH
CHO
COOH
Acetyl-CoA
+
Pyruvic acid
I
II
III
2-Aminophenol
1,6-dioxygenase