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Metabolism of 4-Amino-3-hydroxybenzoic Acid by *Bordetella* sp. Strain 10d: A Different Modified *Meta*-Cleavage Pathway for 2-Aminophenols

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***Bordetella* sp. strain 10d metabolizes 4-amino-3-hydroxybenzoic acid *via* 2-hydroxymuconic 6-semialdehyde. Cell extracts from 4-amino-3-hydroxybenzoate-grown cells showed high NAD^+-dependent 2-hydroxymuconic 6-semialdehyde dehydrogenase, 4-oxalocrotonate tautomerase, 4-oxalocrotonate decarboxylase, and 2-oxopent-4-enoate hydratase activities, but no 2-hydroxymuconic 6-semialdehyde hydrolase activity. These enzymes involved in 4-amino-3-hydroxybenzoate metabolism were purified and characterized. When 2-hydroxymuconic 6-semialdehyde was used as substrate in a reaction mixture containing NAD^+ and cell extracts from 4-amino-3-hydroxybenzoate-grown cells, 4-oxalocrotonic acid, 2-oxopent-4-enoic acid, and 4-hydroxy-2-oxovaleric acid were identified as intermediates, and pyruvic acid was identified as the final product. A complete pathway for the metabolism of 4-amino-3-hydroxybenzoic acid in strain 10d is proposed. Strain 10d metabolized 2-hydroxymuconic 6-semialdehyde derived from 4-amino-3-hydroxybenzoic acid *via* a dehydrogenative route, not *via* a hydrolytic route. This proposed metabolic pathway differs considerably from the modified *meta*-cleavage pathway of 2-aminophenol and those previously reported for methyl- and chloro-derivatives.**

Key words: *Bordetella*; 4-amino-3-hydroxybenzoic acid; 4-amino-3-hydroxybenzoate 2,3-dioxygenase; 2-amino-5-carboxymuconic 6-semialdehyde deaminase; modified *meta*-cleavage pathway

2-Aminophenol[1)] and its methyl-,[2)] chloro-,[3)] and carboxy-[4)] derivatives are metabolites in the biodegradation of nitroaromatic compounds, with a few exceptions.[5–7)] The 2-aminophenols can be further transformed and mineralized *via* a modified *meta*-cleavage pathway (Fig. 1A).[8,9)] *Pseudomonas* sp. strain AP-3[9)] and *P. pseudoalcaligenes* strain JS45[8)] convert 2-aminophenol (Fig. 1A, compound I) to 4-oxalocrotonic acid (Fig. 1A, compound IV) *via* 2-aminomuconic 6-semialdehyde (Fig. 1A, compound II) and 2-aminomuconic acid (Fig. 1A, compound III). 4-Oxalocrotonic acid (Fig. 1A, compound IV) is a common intermediate in both the modified *meta*-cleavage pathway and the previously reported catechol *meta*-cleavage pathway.[10)] The two pathways differ from each other in subsequent metabolic steps. Strain AP-3 and strain JS45 metabolize the benzene ring cleavage product without 2-hydroxymuconic 6-semialdehyde hydrolase or 4-oxalocrotonate tautomerase, which are involved in the *meta*-cleavage pathway of catechol.[7,9)] Genetic studies have indicated that *P. putida* HS12[11)] and *P. fluorescens* strain KU-7[4)] also metabolize 4-oxalocrotonic acid *via* the same route that strain AP-3 and strain JS45 use to metabolize 2-aminophenol, but little is known about the complete metabolic steps and properties of enzymes involved in the steps after deamination. Only the enzymes involved in 2-aminophenol metabolism in strain AP-3 have been purified and characterized.[9)]

We have reported that 4-amino-3-hydroxybenzoic acid (Fig. 1B, compound I) is converted by 4-amino-3-hydroxybenzoate 2,3-dioxygenase to 2-amino-5-carboxymuconic 6-semialdehyde (Fig. 1B, compound II) in *Bordetella* sp. strain 10d.[12)] The ring cleavage product is deaminated to 2-hydroxymuconic 6-semialdehyde (Fig. 1B, compound IV) by 2-amino-5-carboxymuconic 6-semialdehyde deaminase and nonenzymatic decarboxylation steps.[13)] We analyzed, purified, and characterized enzymes involved in the metabolism of 2-hydroxymuconic 6-semialdehyde step by step, in order to elucidate the complete metabolic pathway for 4-amino-3-hydroxybenzoic acid in strain 10d. The proposed metabolic pathway is compared with the previously reported modified *meta*-cleavage pathway of 2-aminophenols.

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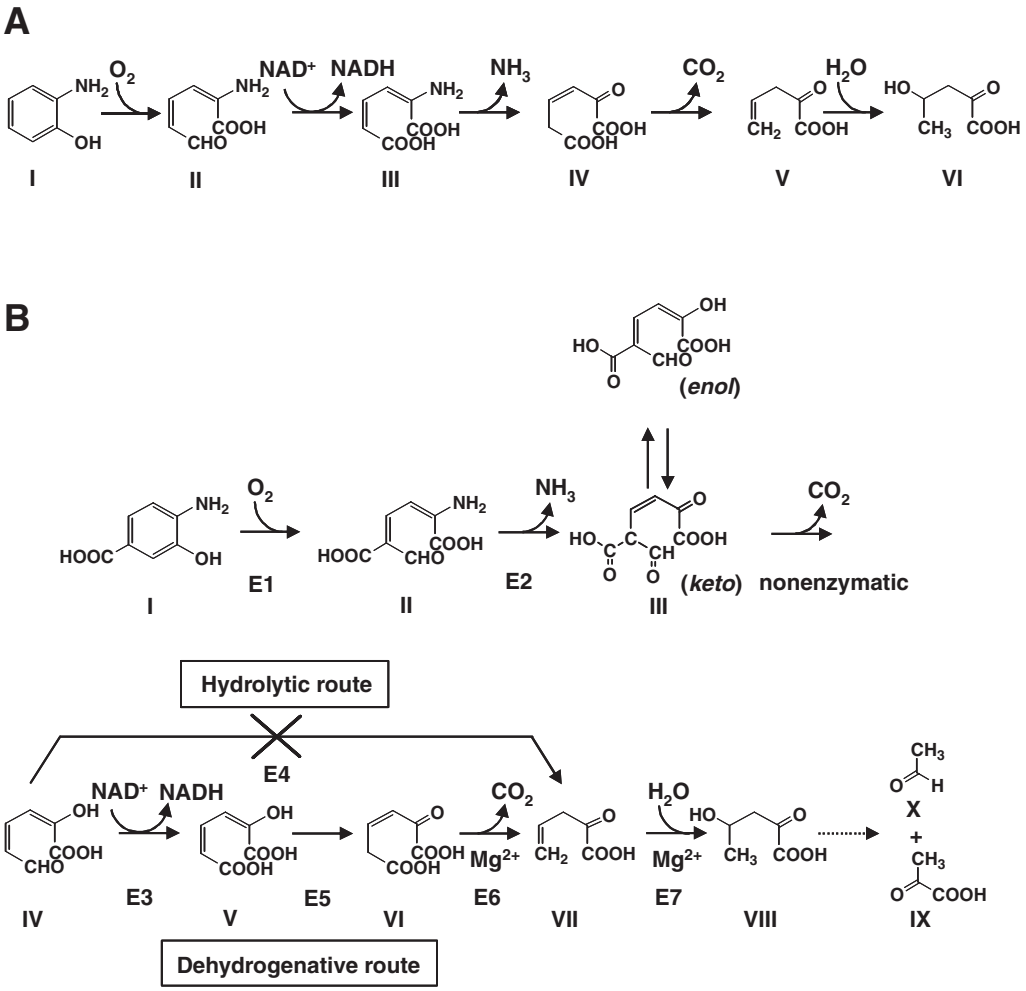

Fig. 1. Modified *Meta*-Cleavage Pathway of 2-Aminophenol (A) and Proposed Metabolic Pathway of 4-Amino-3-hydroxybenzoic Acid in *Bordetella* sp. Strain 10d (B).

A, 2-Aminophenol metabolism in *Pseudomonas* sp. strain AP-3.[9] I, 2-aminophenol; II, 2-aminomuconic 6-semialdehyde; III, 2-aminomuconic acid; IV, 4-oxalocrotonic acid; V, 2-oxopent-4-enoic acid; and VI, 4-hydroxy-2-oxovaleric acid. B, Proposed metabolic pathway of 4-amino-3-hydroxybenzoic acid in *Bordetella* sp. strain 10d.[12,13] 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-hydroxymuconic acid; VI, 4-oxalocrotonic acid (keto form); VII, 2-oxopent-4-enoic acid; VIII, 4-hydroxy-2-oxovaleric acid; IX, pyruvic acid; and X, acetaldehyde; E1, 4-amino-3-hydroxybenzoate 2,3-dioxygenase; E2, 2-amino-5-carboxymuconic 6-semialdehyde deaminase; E3, 2-hydroxymuconic 6-semialdehyde dehydrogenase; E4, 2-hydroxymuconic 6-semialdehyde hydrolase; E5, 4-oxalocrotonate tautomerase; E6, 4-oxalocrotonate decarboxylase; and E7, 2-oxopent-4-enoate hydratase.

Materials and Methods

Bacterial strain and growth conditions. Bordetella sp. strain 10d was cultured in basal medium containing 0.12% (w/v) 4-amino-3-hydroxybenzoic acid and 1% (w/v) meat extract.[12] Succinate-glucose medium was a modified basal medium containing 1% (w/v) sodium succinate, 1% (w/v) D-glucose, and 0.04% (w/v) NH_4NO_3 as the sole carbon and nitrogen sources instead of 4-amino-3-hydroxybenzoic acid.

Enzyme assays. The activities of 4-amino-3-hydroxybenzoate 2,3-dioxygenase,[12] 2-amino-5-carboxymuconic 6-semialdehyde deaminase,[13] 2-hydroxymuconic 6-semialdehyde dehydrogenase,[14] 2-hydroxymuconic 6-semialdehyde hydrolase,[14] 4-oxalocrotonate tautomerase,[14,15] 4-oxalocrotonate decarboxylase,[15,16] and 2-oxopent-4-enoate hydratase[16,17] were assayed spectrophotometrically using 4-amino-3-hydroxybenzoic acid, 2-amino-5-carboxymuconic 6-semialdehyde, 2-hydroxymuconic 6-semialdehyde, 4-oxalocrotonic acid, and 2-oxopent-4-enoic acid as substrates. Protein concentrations were determined by the Lowry method.[18]

Purification of enzymes involved in 2-hydroxymuconic 6-semialdehyde metabolism. 2-Hydroxymuconic 6-semialdehyde dehydrogenase, 4-oxalocrotonate tautomerase, 4-oxalocrotonate decarboxylase, and 2-oxopent-4-enoate hydratase were purified. All purification steps were carried out at 0–4 °C. All centrifugations were at

20,000 × *g* at 4 °C for 10 min. 4-Oxalocrotonate tautomerase, 4-oxalocrotonate decarboxylase, and 2-oxopent-4-enoate hydratase were purified using similar methods. The purification steps are described in the tables (Tables 2–4).

Typical steps of 2-hydroxymuconic 6-semialdehyde dehydrogenase purification were as follows: Cells (33 g, wet weight) of strain 10d were obtained from a 2.4-l culture in basal medium containing 0.12% 4-amino-3-hydroxybenzoic acid and 1% (w/v) meat extract incubated for 16 h at 30 °C with shaking.[12] Cell extracts (step 1, fraction 1) were prepared and treated with streptomycin sulfate to remove nucleic acids (step 2, fraction 2) following previously described methods,[12] until the mid-exponential growth phase was reached (OD_{660} of 2.1–2.3).

Step 3. $(NH_4)_2SO_4$ fractionation. Fraction 2 was brought to 30% 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 buffer A (20 mM Tris–HCl buffer, pH 8.0). The solution was dialyzed against buffer A with two changes of buffer. The final volume of the dialyzed solution (fraction 3) was 63 ml.

Step 4. Chromatography on DE52 cellulose. Fraction 3 was applied to a DE52 cellulose column (2.1 × 22 cm) equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.3 M) of NaCl in 1,100 ml of buffer A. Fractions of 5.0 ml were collected at a flow rate 42 ml·h^{-1}. The protein concentration and enzyme activity of the fractions were assayed. Fractions with a specific activity higher than 0.039 units·(mg protein)$^{-1}$ were pooled to yield fraction 4 (70.0 ml).

Step 5. Chromatography on DEAE-cellulofine. Fraction 4 was applied to a DEAE-Cellulofine A-800 column (2.1 × 25 cm) equilibrated with buffer A. Proteins were eluted with a linear gradient (0 to 0.25 M) of NaCl in 1300 ml of buffer A. Fractions of 6.4 ml were collected at a flow rate 42 ml·h^{-1}. The solution was dialyzed against buffer B (20 mM sodium-potassium phosphate buffer, pH 6.5) with two changes of buffer. Fractions with a specific activity higher than 0.06 units·(mg protein)$^{-1}$ were pooled to yield fraction 5 (45.0 ml).

Step 6. Chromatography on DEAE-toyopearl. Fraction 5 was applied to a DEAE-Toyopearl 650S column (1.6 × 15 cm) equilibrated with buffer B. Proteins were eluted with a linear gradient (0 to 0.2 M) of NaCl in 450 ml of buffer B. Fractions of 3.0 ml were collected at a flow rate 42 ml·h^{-1}. Fractions with a specific activity higher than 0.05 units·(mg protein)$^{-1}$ were pooled to yield fraction 6 (15.0 ml).

Identification of metabolic intermediates.

Metabolite from 2-hydroxymuconic 6-semialdehyde. The reaction mixture contained 14 ml of 1.42 mM 2-hydroxymuconic 6-semialdehyde, 40 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5), 2.0 ml of 1.4 mM NAD^+, and 4.0 ml of cell extract of strain 10d grown on 4-amino-3-hydroxybenzoic acid (10.6 mg·ml^{-1}). After incubation at 25 °C for 20 min, the product was extracted with ethyl ether. The extracted product (compound A, pyruvic acid) was mixed with 2,4-dinitrophenylhydrazine in methanol containing concentrated HCl. The hydrazone compound A was allowed to react with 5% (w/w) HCl in methanol at 85 °C for 1.5 h. The derivatized compound A was analyzed by GC–MS (gas chromatography–mass spectrometry) as described below.

Product of the dehydrogenation of 2-hydroxymuconic 6-semialdehyde. The reaction mixture contained 159 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5), 6.0 ml of 5 mM NAD^+, 9.0 ml of partially purified 2-hydroxymuconic 6-semialdehyde dehydrogenase solution (660 μg·ml^{-1}), and 6 ml of 0.88 mM 2-hydroxymuconic 6-semialdehyde. After incubation at 24 °C for 1 h, the reaction mixture was concentrated to 30 ml with a rotary evaporator. The pH of the concentrated solution was adjusted to pH 3.0 with 6 N HCl, and the solution was extracted with ethyl acetate. The upper layer was collected and evaporated to dryness. Compound B (2-hydroxymuconic acid) was then mixed with *N*, *O*-bis(trimethylsilyl)-trifluoroacetamide at 85 °C for 1.5 h. The trimethylsilylated compound B was analyzed by GC–MS as described below.

Products of the decarboxylation of 4-oxalocrotonate and the hydration of 2-oxopent-4-enoate. One reaction mixture contained 216 ml of 100 mM Tris–HCl buffer (pH 8.0), 24 ml of 3 mM $MgSO_4$, 10 ml of enzyme solution (310 μg·ml^{-1}), and 24 ml of 0.19 mM 4-oxalocrotonate. After incubation at 24 °C for 1 h, compound C (2-oxopent-4-enoate) was extracted with ethyl ether. The esterified compound C was analyzed by GC–MS as described below.

The other reaction mixture contained 162 ml of 0.14 mM 2-oxopent-4-enoate (pH 8.5), 18 ml of 3 mM $MgSO_4$, and 2.4 ml of enzyme solution (260 μg·ml^{-1}). After incubation at 24 °C for 30 min, compound D (4-hydroxy-2-oxovaleric acid) was extracted. The esterified and trimethylsilylated compound D was analyzed by GC–MS as described below.

Effect of various compounds on the enzyme activity. The effect of metal salts and chelating and sulfhydryl agents on the activities of the four enzymes reported here was tested using methods described previously.[19] Purified 2-hydroxymuconic 6-semialdehyde dehydrogenase, 4-oxalocrotonate decarboxylase, and 2-oxopent-4-enoate hydratase were incubated in 20 mM sodium-potassium phosphate buffer (pH 6.5) with 1.0 mM of each compound at 0 °C for 10 min. Purified 4-oxalocrotonate tautomerase was incubated in 20 mM sodium-potassium phosphate buffer (pH 7.5) with 1.0 mM of each compound at 0 °C for 10 min. After pre-incubation,

the enzyme reaction was started by adding enzyme solution to the reaction mixture.

Analytical methods. The derivatized compound A, compound B, compound C, and compound D 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. Helium gas was the carrier at a linear velocity of 2.5 cm min^{-1}. The column temperature increased from 100 to 280 °C at a rate of 10 °C min^{-1}. The molecular masses of the native enzymes reported here were determined by gel filtration on Cellulofine GCL-1000 sf. The molecular mass of the enzyme subunit was measured using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).[20] Size markers used for gel chromatography were from Boehringer Mannheim (Mannheim, Germany). The purified enzymes were electroblotted onto an Immobilon P^{SQ} membrane (Millipore, Tokyo) following the previously reported method.[21] The NH_2-terminal amino acids were sequenced using a Shimadzu PPSQ-10 protein sequencer (Shimadzu, Kyoto).

Chemicals. 4-amino-3-hydroxybenzoic acid, formaldehyde, and 4-methyl-2-oxovaleric acid were purchased from Tokyo Kasei Kogyo (Tokyo) and meat extract (Extract Ehlrich) was from Kyokuto Seiyaku Kogyo (Osaka). DE52 cellulose was from Whatman (Madison, WI), and DEAE-Cellulofine A-800 and Cellulofine GCL-1000 sf were from Seikagaku (Tokyo). DEAE-Toyopearl 650S was from Tosoh (Tokyo). Acetaldehyde, propionaldehyde, and butyraldehyde were from Nacalai Tesque (Kyoto). Benzaldehyde, 2-oxobutyric acid, 2-oxoglutaric acid, α-keto-*n*-valeric acid, *N*, *O*-bis(trimethylsilyl)-trifluoroacetamide, and 5% hydrogen chloride methanol were from Wako Pure Chemicals (Osaka). 2-Hydroxymuconic 6-semialdehyde,[22] 2-hydroxymuconic acid,[23] 4-oxalocrotonic acid,[23] and 2-oxopent-4-enoic acid[17] were synthesized by previously reported methods.

Results and Discussion

Analysis of enzymes involved in 4-amino-3-hydroxybenozate metabolism

As reported previously, the crude extract of strain 10d grown on 4-amino-3-hydroxybenzoic acid contained 4-amino-3-hydroxybenzoate 2,3-dioxygenase and 2-amino-5-carboxymuconic 6-semialdehyde deaminase.[12,13] These two enzymes oxidize and deaminate 4-amino-3-hydroxybenzoic acid, which together with nonenzymatic decarboxylation form 2-hydroxymuconic 6-semialdehyde.[13] The pathway downstream of these steps was investigated using cell extracts of strain 10d grown on 4-amino-3-hydroxybenzoic acid or on sodium succinate (Table 1). When fresh cell extracts from 4-amino-3-hydroxybenzoate-grown cells were incubated with the substrate in the presence of NAD^+, the A_{375} of 2-hydroxymuconic 6-semialdehyde decreased markedly and rapidly; no decrease in absorbance was observed in the absence of NAD^+, or in a control containing substrate only (Fig. 2). These results indicate that the cell extracts contained NAD^+-dependent dehydrogenase (Fig. 1B, E3), and not NAD^+-independent hydrolase (Fig. 1B, E4). The specific activity of NAD^+-dependent dehydrogenase was the lowest among the four enzymes reported here. 4-Oxalocrotonate tautomerase (Fig. 1B, E5), 4-oxalocrotonate decarboxylase (Fig. 1B, E6), and 2-oxopent-4-enoate hydratase (Fig. 1B, E7) activities were also found in cell extracts of strain 10d grown on

Table 1. Analysis of 4-Amino-3-hydroxybenzoate-metabolizing Enzymes from *Bordetella* sp. Strain 10d

Enzyme	Specific acitivity (U·mg^{-1})	
	Growth substrate	
	4-Amino-3-hydroxy benzoate[a]	Succinate-glucose[b]
E1: 4-Amino-3-hydroxy-benzoate 2,3-dioxygenase	0.083	0
E2: 2-Amino-5-carboxymuconic 6-semialdehyde deaminase	0.005	0
E3: 2-Hydroxymuconic 6-semialdehyde dehydrogenase	0.011	0.007
E4: 2-Hydroxymuconic 6-semialdehyde hydrolase	0	0
E5: 4-Oxalocrotonate tautomerase	0.58	0.053
E6: 4-Oxalocrotonate decarboxylase	0.081	0
E7: 2-Oxopent-4-enoate hydratase	0.103	0.006

[a] *Bordetella* sp. strain 10d was cultivated in a basal medium containing 0.12% (w/v) 4-amino-3-hydroxybenzoic acid as the sole carbon, nitrogen, and energy source until the late-exponential phase.
[b] The strain was cultivated in succinate-glucose medium until the late-exponential phase.

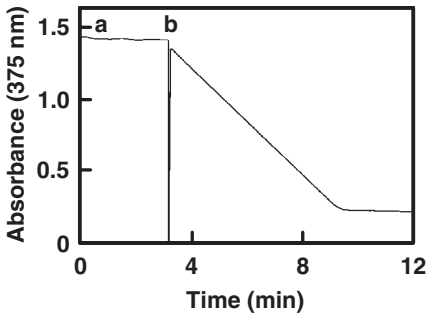

Fig. 2. Conversion of 2-Hydroxymuconic 6-Semialdehyde by Cell Extracts of *Bordetella* sp. Strain 10d in the Absence (a) and Presence (b) of NAD^+.

The reaction mixture contained 2.65 ml of 100 mM sodium-potassium phosphate buffer (pH 7.5), 0.15 ml of crude extract (11.8 mg·ml^{-1}), and 0.1 ml of 0.88 mM 2-hydroxymuconic 6-semialdehyde. a, The reaction was started by the addition of crude extract. b, After 3 min of incubation, 0.1 ml of 5 mM NAD^+ was added. The absorbance was measured with a Hitachi U-2000 spectrophotometer.

Table 2. Purification of 2-Hydroxymuconic 6-Semialdehyde Dehydrogenase

Fraction[a]	Total activity (U)	Total protein (mg)	Specific activity ($U \cdot mg^{-1}$)	Recovery (%)	Purification (fold)
1: Cell extract	16	3,900	4.1×10^{-3}	100	1.0
2: Streptomycin sulfate	14	3,800	3.7×10^{-3}	88	0.9
3: Ammonium sulfate	12	1,300	9.2×10^{-3}	75	2.2
4: DE52	2.4	52	0.046	15	11
5: DEAE-Cellulofine	0.9	12	0.075	5.6	18
6: DEAE-Toyopearl	0.1	1.4	0.071	0.6	17

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

Table 3. Purification of 4-Oxalocrotonate Tautomerase

Fraction[a]	Total activity (U)	Total protein (mg)	Specific activity ($U \cdot mg^{-1}$)	Recovery (%)	Purification (fold)
1: Cell extract	720	3,900	0.18	100	1.0
2: Streptomycin sulfate	620	3,300	0.19	87	1.1
3: Ammonium sulfate	440	420	1.0	61	5.6
4: DE52	140	26	5.4	19	30
5: DEAE-Cellulofine	78	1.4	56	11	310
6: Phenyl-Cellulofine	17	0.091	190	2	1,100

[a]20 mM Tris–HCl buffer (pH 8.0) and 20 mM sodium potassium phosphate buffer (pH 7.5) were used during steps 1–4 and 5–6 respectively.

4-amino-3-hydroxybenzoic acid. The cell extracts of strain 10d grown on sodium succinate showed 4-oxalocrotonate tautomerase and 2-oxopent-4-enoate hydratase activities, but these activities were much lower than when cells were grown on 4-amino-3-hydroxybenzoic acid. NAD^+-independent hydrolase (E4) activity was not observed either in the latter case. In strain JS45, three enzymes responsible for 2-aminophenol are inducible.[1)] In strain KU-7, enzymes of the 2-nitrobenzoate degradation pathway are inducible.[4)] In contrast, strain AP-3 constitutively expresses enzymes involved in the metabolism of 2-aminophenol.[24)]

Purification and properties of 2-hydroxymuconic 6-semialdehyde dehydrogenase

2-Hydroymuconic 6-semialdehyde dehydrogenase was enriched 17-fold with an overall yield of 0.6% (Table 2). After electrophoresis, some major protein bands appeared on both native and denaturing polyacrylamide gels (data not shown). The enzyme was inhibited (remaining activity indicated in parentheses) by the following metal salts: 1 mM $HgCl_2$ (0%), $CuSO_4$ (0%), and $ZnSO_4$ (26%). The addition of 1 mM iodoacetic acid, *p*-chloromercuribenzoic acid, and *N*-ethylmaleimide decreased the enzyme activity to 69, 41, and 18% respectively. The dehydrogenase from strain 10d was inhibited by some sulfhydryl reagents, which is reasonable since one cysteine residue in aldehyde dehydrogenases has long been thought to bind to NAD^+ and to act directly in the enzymatic mechanism of these enzymes.[25)] The enzyme showed dehydrogenase activities with the following substrates: 2-hydroymuconic 6-semialdehyde (relative activity, 100%), formaldehyde (16.5%), acetaldehyde (113%), propionaldehyde (107%), butyraldehyde (138%), and benzaldehyde (25%). A closely related dehydrogenase, 2-aminomuconic 6-semialdehyde dehydrogenase from strain AP-3, shows the highest activity with 2-aminomuconic 6-semialdehyde as substrate among various aldehyde compounds, and it does not act on benzaldehyde.[9)] The dehydrogenase from strain 10d had a broad substrate specificity and its dehydrogenase activity toward benzaldehyde was as efficient as that of the enzyme from *P. putida* strain mt-2.[16)]

Purification and properties of 4-oxalocrotonate tautomerase

4-Oxalocrotonate tautomerase was purified 1,100-fold with an overall yield of 2% (Table 3). After electrophoresis, the purified enzyme exhibited a single protein band on both native and denaturing polyacrylamide gels (Fig. 3A and B). The apparent molecular mass was determined to be 51 kDa by gel filtration and 12 kDa by SDS–PAGE. These findings indicate that the enzyme is a tetramer with 12-kDa subunits. The enzyme was inhibited (remaining activity indicated in parentheses) by the following metal salts: 1 mM $MnSO_4$ (45%), 3.3 mM $MgSO_4$ (63%), 33 mM $CaCl_2$ (72%), 1 mM $ZnSO_4$ (55%), and 40 mM KCl (46%). The addition of 1 mM iodoacetic acid, *p*-chloromercuribenzoic acid, and Tiron decreased the enzyme activity to 5, 72, and 54% respectively. The substrate analogue α-keto-*n*-valeric acid, 2-oxopent-4-enoic acid, and oxaloacetic acid decreased the tautomerase activity for 4-oxalocrotonate to 68, 39, and 36% respectively, whereas the substrate analogue 2-oxobutyric acid, 2-oxoglutaric acid, and 4-

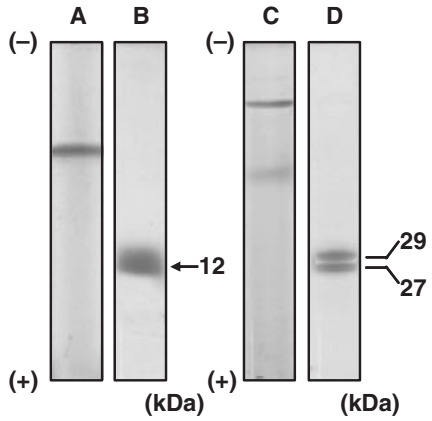

Fig. 3. PAGE (A and C) and SDS–PAGE (B and D) of the Purified Enzymes.

A, Purified 4-oxalocrotonate tautomerase (10 μg) was electrophoresed on a 12.5% (w/v) polyacrylamide gel (pH 8.0) at 2 mA/tube for 2 h in a running buffer of Tris–glycine (pH 8.3).[29] B, Purified tautomerase (10 μg) denatured with SDS was electrophoresed on a 12.5% (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).[20] C, Purified 4-oxalocrotonate decarboxylase-2-oxopent-4-enoate hydratase complex (10 μg) was electrophoresed on a 7.5% (w/v) polyacrylamide gel (pH 8.0). D, Purified decarboxylase-hydratase complex (6 μg) denatured with SDS was electrophoresed on a 7.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS.

methyl-2-oxovaleric acid did not. The tautomerase from strain 10d differed from that of strain mt-2[16] in molecular mass, subunit structure, optimal pH, and inhibition by salts (Na^+, K^+, Mg^{2+}, and Ca^{2+}). Several salts inhibited the isomerase reaction; in particular, 3.3 mM Mg^{2+} and 33 mM Ca^{2+} decreased the enzyme activity to 27.2 and 19.1% respectively.[16] The oxaloacetate keto-enol tautomerase from porcine kidney is inactivated by Na^+, Cu^{2+}, Zn^{2+}, and Mg^{2+}, and activated by Ca^{2+}, Co^{2+}, and Mn^{2+}.[25] The tautomerase from strain 10d did not require any of the 11 tested metal ions for activity. The tautomerase from strain 10d was similar to the oxaloacetate tautomerase from kidney with respect to inhibition by sulfhydryl reagents, *e.g.*, *p*-chloromercuribenzoate.[26]

Purification and properties of 4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase

Table 4 summarizes a typical enzyme purification. Both 4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase activities were found in all samples throughout the five chromatographic steps. Two enzymes in the final preparation could not be separated by gel filtration or hydrophobic chromatography. Mg^{2+} was essential for the two enzyme activities. 4-Oxalocrotonate decarboxylase was purified 360-fold with an overall yield of 3.1% (Table 4). 2-Oxopent-4-enoate hydratase was purified 420-fold with an overall yield of 3.6% (Table 4). The final enzyme preparation exhibited one major protein band with some minor bands on a polyacrylamide gel (Fig. 3C). However, on SDS–PAGE, two distinct protein bands with molecular masses of 29 and 27 kDa were observed (Fig. 3D). The molecular mass of the native enzyme complex was 470 kDa, as determined by gel filtration. 4-Oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase from strain 10d formed a complex in a molar ratio of 1:1. Such complexes have also been described for enzymes from strain AP-3 and strain mt-2.[9,16]

The NH_2-terminal amino acid sequences of the 29- and 27-kDa proteins were YLEAKVIQALAVALDIXEMNEV and MDDKKIQQYGDALYEALVERA respectively. A comparison of the sequence of the 27-kDa protein with the sequences from nucleotide sequences databases revealed a sequence identity of 80% with 2-oxopent-4-enoate hydratase (MDQQKIEQYGEELYQAFVSR) from *P. putida* (accession no. AB107791). The sequence of the 29-kDa protein did not show significant levels of identity to any other sequences available in FASTA and BLAST database programs at the DNA Data Bank of Japan. Analysis of the NH_2-terminal amino acid sequence of the 27- and 29-kDa proteins from strain 10d identified the proteins as 2-oxopent-4-enoate hydratase and 4-oxalocrotonate decarboxylase respectively. The decarboxylase and hydratase from strain AP-3 have apparent molecular masses of 28 and 29 kDa respectively, and the native enzyme complex has a molecular mass of 300 kDa, as shown by gel filtration.[9]

Table 4. Purification of 4-Oxalocrotonate Decarboxylase (4OD) and 2-Oxopent-4-enoate Hydratase (OEH)

Fraction[a]	Total activity (U)		Total protein (mg)	Specific activity ($U \cdot mg^{-1}$)		Recovery (%)		Purification (fold)	
	4OD	OEH		4OD	OEH	4OD	OEH	4OD	OEH
1: Cell extract	190	340	3,900	0.05	0.09	100	100	1	1
2: Streptomycin sulfate	130	230	3,900	0.03	0.06	68	68	0.6	0.7
3: Ammonium sulfate	120	110	860	0.14	0.13	63	32	2.8	1.4
4: DE52	51	56	120	0.43	0.47	27	16	8.6	5.2
5: DEAE-Cellulofine 1st	23	39	20	1.2	2.0	12	11	24	22
6: DEAE-Cellulofine 2nd	17	35	1.9	8.9	18	8.9	10	180	200
7: DEAE-Toyopearl 1st	10	28	0.78	13	36	5.3	8.2	260	400
8: DEAE-Toyopearl 2nd	5.9	12	0.32	18	38	3.1	3.5	360	420

[a] 20 mM Tris–HCl buffer (pH 8.0) and 20 mM sodium potassium phosphate buffer (pH 6.5) were used during steps 1–6 and 7–8 respectively.

Table 5. Mass Spectra of the Enzymatic Reaction Products

Compound	GC t_R (min)	Fragments of the derivatized product[a] [m/z, relative intensity (%)]
A	13.2	M^+ (282, 95%), M^+–OCH_3 (251, 20%), M^+–H–$COOCH_3$ (222, 58.3%), M^+–H–$COOCH_3$–CH_3 (207, 20%), M^+–H–$COOCH_3$–CH_3–C (195, 8.3%), $[C_6H_3(NO_2)_2N]^+$ (181, 100%)
B	7.9	M^+ (374, 1.3%), M^+–CH_3 (359, 11.3%), M^+–$CH_3 \times 2$ (344, 0.7%), M^+–$COOSi(CH_3)_3$ (257, 100%), $[(CH_3)_2Si{=}O{-}Si(CH_3)_3]^+$ (147, 46.2%), M^+–$COOSi(CH_3)_3$–$CHCOSi(CH_3)_3$ (143, 1.3%), $[Si(CH_3)_3]^+$ (73, 100%)
C	6.2	M^+ (128, 100%), M^+–CH_3 (113, 33.3%), M^+–CH_2CHCH_2 (87, 14.0%), M^+–$COOCH_3$ (69, 94.0%), M^+–$COCH_2CHCH_2$ (59, 100%), M^+–$COCOOCH_3$ (41, 100%)
D	6.9	M^+ (290, 15.4%), M^+–CH_3 (275, 39.8%), M^+–$COOCH_3$ (231, 3.8%), M^+–$OSi(CH_3)_3$ (201, 3.0%), $[(CH_3)_2Si{=}O{-}Si(CH_3)_3]^+$ (147, 62%), $[Si(CH_3)_3]^+$ (73, 100%), $[COOCH_3]^+$ (59, 44.3%)

[a]Methyl-esterified and 2,4-dinitrophenylhydrazone-derivatized product A, trimethylsilylated product B, methyl-esterified product C, and trimethylsilylated methyl-esterifed product D were analyzed by GC–MS.

4-Oxalocrotonate decarboxylase was inhibited (remaining activity indicated in parentheses) by the following metal salts: 1 mM $FeSO_4$ (23%), $MnSO_4$ (9%), $K_3Fe(CN)_6$ (36%), and $CoCl_2$ (47%). The addition of 1 mM iodoacetic acid, *p*-chloromercuribenzoic acid, *N*-ethylmaleimide, and Tiron decreased the enzyme activity to 64, 54, 58, and 59% respectively. 2-Oxopent-4-enoate hydratase was completely inhibited by 1 mM $HgCl_2$. The decarboxylase and hydratase from strain AP-3 are similar to each other in pH stability, optimal pH, and the effects of temperature and various metal ions on the activities, and Mg^{2+} or Mn^{2+} is essential for both enzyme activities.[9] In contrast, the decarboxylase and hydratase from strain 10d differed from each other in most of these respects. Mg^{2+} was essential for both enzyme activities, but the decarboxylase was inhibited by other metal ions, sulfhydryl reagents, and chelating reagents, while the hydratase was inhibited only by Hg^{2+}.

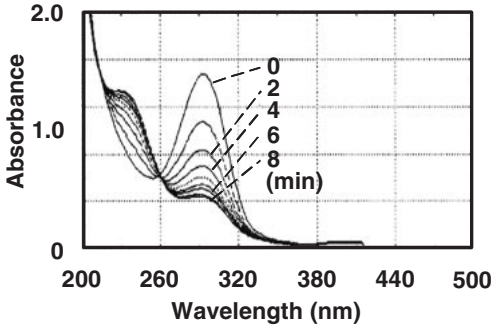

Fig. 4. Absorption Spectra of the Tautomerase Reaction Products Formed from 2-Hydroxymuconic Acid.

The reaction mixture contained 3.0 ml of 0.04 mM 2-hydroxymuconic acid in 100 mM sodium-potassium phosphate buffer (pH 7.0) and 0.02 ml of purified 4-oxalocrotonate tautomerase (5.3 mg ml^{-1}). The reaction was started by adding the enzyme solution. After incubation at 24 °C, the sample was scanned with a Beckman DU Series 650 spectrophotometer and spectra were recorded every 1 min.

Identification of the enzymatic reaction products

A reaction mixture containing 2-hydroxymuconic 6-semialdehyde, NAD^+, and cell extracts was incubated until the A_{375} of 2-hydroxymuconic 6-semialdehyde ceased to decrease (in 20 min). Mg^{2+} was excluded from the reaction, as in the previously reported method.[9] The accumulated enzyme reaction product was analyzed by GC and GC–MS after methyl-esterified and 2,4-dinitrophenylhydrazone-derivatization (Table 5). This mass spectrum and the GC retention time of the derivatized compound A agreed with those of methyl-esterified and 2,4-dinitrophenylhydrazone-derivatized pyruvic acid. 2-Hydroxymuconic acid (enol form) was gradually and spontaneously transformed to 4-oxalocrotonic acid (keto form), whose λ_{max} is at 235 nm.[16] Spectral changes were observed in the absence and the presence of purified tautomerase, which indicates that the enzymatic reaction product from 2-hydroxymuconic acid was 4-oxalocrotonic acid. 2-Hydroxymuconic acid dissolved in 100 mM sodium-potassium phosphate buffer (pH 7.0) has a λ_{max} at 295 nm.[16] The solution non-enzymatically reached equilibrium between the enol and keto forms after 15 min of incubation. Figure 4 shows the spectral changes after the addition of purified tautomerase. The absorption peaks at 295 nm derived from 2-hydroxymuconic acid decreased rapidly; a shoulder peak at 235 nm appeared concomitantly. Thus, compound VI was identified as 4-oxalocrotonic acid (keto form, $\lambda_{max} = 235$ nm) based on previously reported data.[16] The enzyme reaction product (compound B) from 2-hydroxymuconic 6-semialdehyde, the reaction product (compound C) from 4-oxalocrotonic acid, and the reaction product (compound D) from 2-oxopent-4-enoic acid were analyzed by GC and GC–MS. These mass spectra and the GC retention times of modified compound B, compound C, and compound D agreed with those of derivatized 2-hydroxymuconic acid, 2-oxopent-4-enoic acid and 4-hydroxy-2-oxovaleraic acid respectively (Table 5).

Comparison of proposed metabolic pathway for 4-amino-3-hydroxybenzoic acid and previously reported metabolic pathways for 2-aminophenols and catechols

We propose a pathway for the metabolism of 2-hydroxymuconic 6-semialdehyde in strain 10d *via* a dehydrogenative route based on our analysis of

the enzymes and the identification of intermediates (Tables 1 and 5). In the previously reported pathways for phenol and its derivatives, catechol is converted to 2-hydroxymuconic 6-semialdehyde, and then the ring-cleavage product (2-hydroxymuconic 6-semialdehyde) is generally metabolized to acetyl-CoA and pyruvate *via* a dehydrogenative route and a hydrolytic route.[16] Our results reported here indicate that strain 10d does not have a 2-hydroxymuconic 6-semialdehyde hydrolase involved in a hydrolytic route (Table 1). To our knowledge, the phenol-assimilating bacterium *Comamonas teststeroni* TA441 metabolizes catechol and the ring-cleavage product *via* a *meta*-cleavage pathway with a dehydrogenative route only.[27] Genetic and biochemical results indicate that strain TA441 does not have an NAD^+-independent 2-hydroxymuconic 6-semialdehyde hydrolase. Strain 10d and strain TA441 employ a similar pathway for the metabolism of 2-hydroxymuconic 6-semialdehyde.

He and Spain reported that the 2-aminophenol ring-cleavage pathway is not unique, but is representative of the metabolic pathways of other 2-aminophenolic compounds.[7] As they pointed out, 2-aminophenol and its methyl-, chloro-, hydroxyl-, and carboxy-derivatives are metabolized *via* a 2-aminophenol ring-cleavage pathway that we designated the modified *meta*-cleavage pathway. The metabolic pathway for 4-amino-3-hydroxybenzoic acid is similar to those for 2-aminophenol derivatives in the benzene-ring-cleavage step and the deamination step, but the 4-amino-3-hydroxybenzoic acid metabolic pathway notably differs from these metabolic pathways after the deamination step.[13] The pathway for 4-amino-3-hydroxybenozic acid in strain 10d is similar to the protocatechuic acid pathway in *Bacillus circulans* in the oxidative cleavage between positions C2 and C3 of protocatechuic acid,[12,28] but after protocatechuic acid is converted to 2-hydroxymuconic 6-semialdehyde *via* 2-hydroxy-5-carboxymuconic 6-semialdehyde, the 2-hydroxymuconic 6-semialdehyde is metabolized *via* a dehydrogenative route and a hydrolytic route. We conclude that the metabolic pathway of 4-amino-3-hydroxybenzoic acid is different from those of other 2-aminophenolic compounds.[2,3,7]

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