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Physically Associated Enzymes Produce and Metabolize 2-Hydroxy-2,4-Dienoate, a Chemically Unstable Intermediate Formed in Catechol Metabolism via *meta* Cleavage in *Pseudomonas putida*

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The *meta*-cleavage pathway of catechol is a major mechanism for degradation of aromatic compounds. In this pathway, the aromatic ring of catechol is cleaved by catechol 2,3-dioxygenase and its product, 2-hydroxymuconic semialdehyde, is further metabolized by either a hydrolytic or dehydrogenative route. In the dehydrogenative route, 2-hydroxymuconic semialdehyde is oxidized to the enol form of 4-oxalocrotonate by a dehydrogenase and then further metabolized to acetaldehyde and pyruvate by the actions of 4-oxalocrotonate isomerase, 4-oxalocrotonate decarboxylase, 2-oxopent-4-enoate hydratase, and 4-hydroxy-2-oxovalerate aldolase. In this study, the isomerase, decarboxylase, and hydratase encoded in the TOL plasmid pWW0 of *Pseudomonas putida* mt-2 were purified and characterized. The 28-kilodalton isomerase was formed by association of extremely small identical protein subunits with an apparent molecular weight of 3,500. The decarboxylase and the hydratase were 27- and 28-kilodalton polypeptides, respectively, and were copurified by high-performance-liquid chromatography with anion-exchange, hydrophobic interaction, and gel filtration columns. The structural genes for the decarboxylase (*xylI*) and the hydratase (*xylJ*) were cloned into *Escherichia coli*. The elution profile in anion-exchange chromatography of the decarboxylase and the hydratase isolated from *E. coli* XylI$^+$ XylJ$^-$ and XylI$^-$ XylJ$^+$ clones, respectively, were different from those isolated from XylI$^+$ XylJ$^+$ bacteria. This suggests that the carboxylase and the hydratase form a complex in vivo. The keto but not the enol form of 4-oxalocrotonate was a substrate for the decarboxylase. The product of decarboxylation was 2-hydroxypent-2,4-dienoate rather than its keto form, 2-oxopent-4-enoate. The hydratase acts on the former but not the latter isomer. Because 2-hydroxypent-2,4-dienoate is chemically unstable, formation of a complex between the decarboxylase and the hydratase may assure efficient transformation of this unstable intermediate in vivo.

Enzymes encoded by TOL plasmids metabolize toluene and some of its substituted derivatives via *meta* cleavage of catechol (2, 14; Fig. 1). The pathway diverges into hydrolytic and dehydrogenative routes at the ring fission product (Fig. 1, compound 2) of catechol (compound 1) and reconverges later at 2-hydroxypent-2,4-dienoate (compound 4). The hydrolytic branch converts the ring fission product (compound 2) directly to compound 4 through the action of hydroxymuconic semialdehyde hydrolase, whereas the dehydrogenative branch involves formation of 2-hydroxyhexa-2,4-diene-1,6-dionate (the enol form of 4-oxalocrotonate) or its methyl substituents (compound 3a) by NAD$^+$-dependent hydroxymuconic semialdehyde dehydrogenase, which is then converted to compound 4 by two enzymatic steps catalyzed by 4-oxalocrotonate isomerase and 4-oxalocrotonate decarboxylase (17). Each of these branched pathways metabolizes different compounds at different efficiencies. The ring fission product of 3-methylcatechol, 2-hydroxy-6-oxohepta-2,4-dienoate (compound 2; $R_1 = CH_3$ and $R_2 = H$), is metabolized exclusively by the hydrolytic branch as a result of the inability of the dehydrogenase to attack this compound, which lacks an oxidizable aldehyde group, whereas ring fission products of catechol and 4-methylcatechol, i.e., 2-hydroxymuconic semialdehyde (compound 2; R_1 and R_2 = H) and 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (compound 2; R_1 = H and $R_2 = CH_3$), respectively, are metabolized primarily via the dehydrogenative branch as a result of the high affinity of the dehydrogenase toward these compounds (10).

Some of the *meta*-cleavage pathway intermediates have been characterized with respect to their stereochemistry. The structure of compound 4, derived from 4-methylcatechol (compound 1; R_1 = H and $R_2 = CH_3$), has been determined to be 2-oxo-*cis*-hex-4-enoate (compound 4b; $R_2 = CH_3$) because this compound, but not its *trans* isomer, is a substrate of 2-oxopent-4-enoate hydratase (4). The absolute stereochemical structure of compound 5 (R_2 = H) has also been determined to be L-(*S*)-4-hydroxy-2-oxovalerate (compound 5b) (3, 4).

However, some ambiguities in the chemical structures of the intermediates still exist. Firstly, the reaction that the isomerase catalyzes has not been defined. Originally, tautomerization was proposed (17). In this model, the isomerase catalyzes the protonation of the β-carbon of 2-hydroxyhexa-2,4-diene-1,6-dioate or its derivatives (compound 3a) and the product would be 2-oxohex-4-ene-1,6-dioate or its derivatives (compound 3b). Later, Dagley and his colleagues (6, 18) proposed that isomerase could catalyze the protonation of the δ carbon of the substrate to form 2-oxohex-3-ene-

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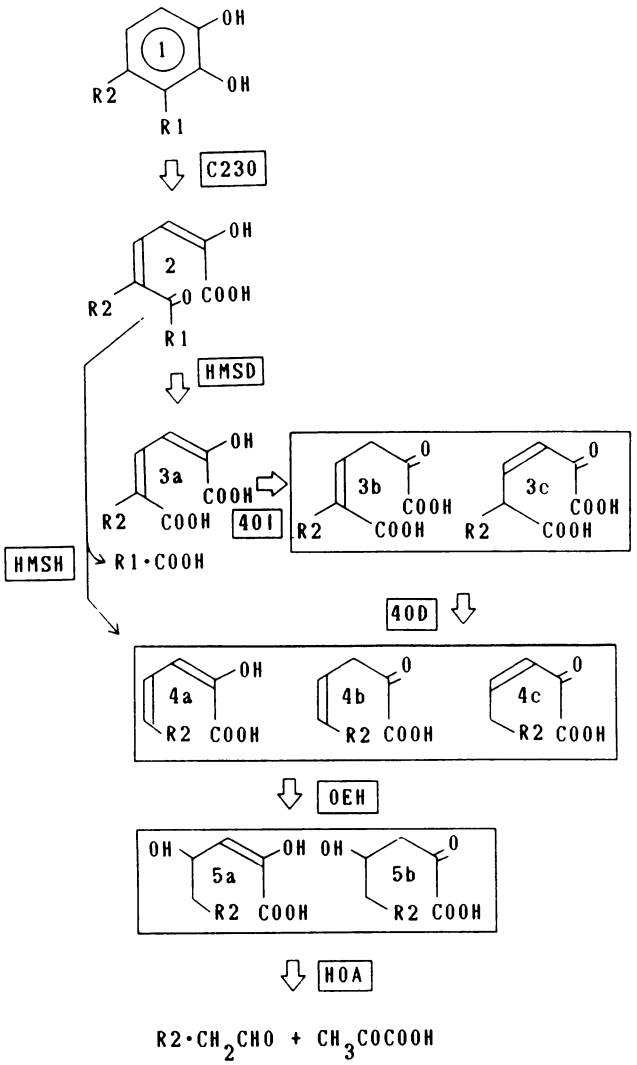

FIG. 1. The *meta*-cleavage pathway for catechol degradation. Enzyme abbreviations: C23O, catechol 2,3-dioxygenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; HMSH, 2-hydroxymuconic semialdehyde hydrolase; 4OI, 4-oxalocrotonate isomerase; 4OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase. For compounds in which R_1 and R_2 = H, 1 = catechol, 2 = 2-hydroxymuconic semialdehyde, 3a = 2-hydroxyhexa-2,4-diene-1,6-dioate (enol form of 4-oxalocrotonate), 3b = 2-oxohex-4-ene-1,6-dioate, 3c = 2-oxohex-3-ene-1,6-dioate, 4a = 2-hydroxypent-2,4-dienoate, 4b = 2-oxopent-4-enoate, 4c = 2-oxopent-3-enoate, 5a = 1,4-dihydroxypent-2-enoate, 5b = 4-hydroxy-2-oxovalerate, 6 = pyruvate, 7 = acetaldehyde, and 8 = formate. Possible chemical structures of each intermediate are presented in the boxes.

1,6-dioate (compound 3c). Secondly, compounds 4 and 5 can take either enol or keto forms (Fig. 1), but whether or not one of these forms is specifically metabolized by pathway enzymes has not been determined.

In this study, we purified 4-oxalocrotonate isomerase, 4-oxalocrotonate decarboxylase, and 2-oxopent-4-enoate hydratase to near homogeneity and examined their specificities toward different isomers.

MATERIALS AND METHODS

Plasmids, bacteria, and growth conditions. Plasmid pLV85 is an expression vector containing the lambda p_L promoter (9, 12) and confers ampicillin resistance. pGSH2915 and pGSH2829 are derivatives of pLV85 containing the catabolic genes of TOL plasmid pWW0 cloned downstream of the P_L promoter. pGSH2915 carries *xylH* and *xylI*, the structural genes for 4-oxalocrotonate decarboxylase and 4-oxalocrotonate isomerase, respectively, whereas pGSH2829 carries *xylJ*, the structural gene for 2-oxopent-4-enoate hydratase. The host strain for the plasmids was *Escherichia coli* K-12 strain K12ΔH1Δtrp [F^- *lacZ*(Am) Δ(*bio-uvrB*) Δ*trpEA2* (λ *N*am7 *N*am53 *c*I857 Δ*H1*)] (16). The strain carries a temperature-sensitive allele of the lambda repressor, *c*I857, which regulates expression from the p_L promoter. At 30°C, the *c*I857 repressor is active and expression of TOL genes from the p_L promoter is repressed, but at 42°C, the repressor is inactive and expression is derepressed. Strains containing these hybrid plasmids were cultivated in 10 liters of L broth containing 100 μg of ampicillin per ml to about 3×10^8 cells per ml at 30°C and then further cultivated at 42°C for 1 to 3 h. The cells were harvested by centrifugation. Cells of *P. putida* PaW1 harboring TOL plasmid pWW0 were grown to the stationary phase at 30°C in 10 liters of minimal M9 medium supplemented with minor salts (10), 10 mM glucose, 40 mM sodium acetate, and 5 mM *m*-toluate.

Chemicals. The enol form of 4-oxalocrotonate (compound 3a) was chemically synthesized as described previously (8). Compound 4 was synthesized enzymatically from DL-allylglycine by the method used for synthesis of 2-oxopent-4-enoate (compound 4b; 4) and further purified by reverse-phase chromatography with a Nucleosil C18 column (25 cm by 4 mm [inner diameter]; 30-nm pore size; 5-μm particle size) (Macherey-Nagel). The elution buffer was 10 mM potassium phosphate buffer (pH 2) containing $MgSO_4$ in a linear gradient from 10 to 0 mM. As described in Results, the compound obtained was 2-hydroxypent-2,4-dienoate (compound 4a). This compound was kept at −20°.

Preparation of the enol and keto forms of 4-oxalocrotonate and determination of their concentrations and extinction coefficients. Aqueous solutions of the enol form of 4-oxalocrotonate were prepared by dissolving an ethanolic solution of 4-oxalocrotonate in an appropriate buffer. The extinction coefficients of the enol form of 4-oxalocrotonate, ε(enol,λ), at wavelength λ were calculated by the equation $\varepsilon(\text{enol},\lambda) = A(0,\lambda)/C$, where $A(0,\lambda)$ is the absorbance of a fresh 4-oxalocrotonate solution at concentration C. The absorbance of the 4-oxalocrotonate solution at wavelengths longer than 260 nm decreased as the enol form was transformed to the keto form and a new λ_{max} at 235 nm appeared (Fig. 2). Assuming that the absorbance of the keto form at 295 nm or above is not significant, we calculated the concentration of the keto form of 4-oxalocrotonate, C(keto), in an equilibrium state by the equation $C(\text{keto}) = C \times [A(0,\lambda) - A(\text{eq},\lambda)]/A(0,\lambda)$, where $A(\text{eq},\lambda)$ is the absorbance in an equilibrium state at λ (>295 nm). The extinction coefficient of the keto form of 4-oxalocrotonate at 235 nm, ε(keto,235), was then calculated by the equation $\varepsilon(\text{keto},235) - \varepsilon(\text{enol},235) = [A(\text{eq},235) - A(0,235)]/C(\text{keto})$.

Enzyme assays. 4-Oxalocrotonate isomerase was assayed as described previously (8). If not specified otherwise, 4-oxalocrotonate decarboxylase was assayed by measuring the decrease in A_{235} in 100 mM Tris hydrochloride buffer (pH 7.0) containing 30 μM substrate and 3.3 mM $MgSO_4$. The standard reaction mixture for the hydratase assay contained 30 μM 2-hydroxypent-2,4-dienoate (compound 4a), 10 mM Tris hydrochloride (pH 7.0), and 3.3 mM $MgSO_4$; the enzyme activity was determined by measuring the decrease in A_{265} due to removal of the substrate. Alternatively, the

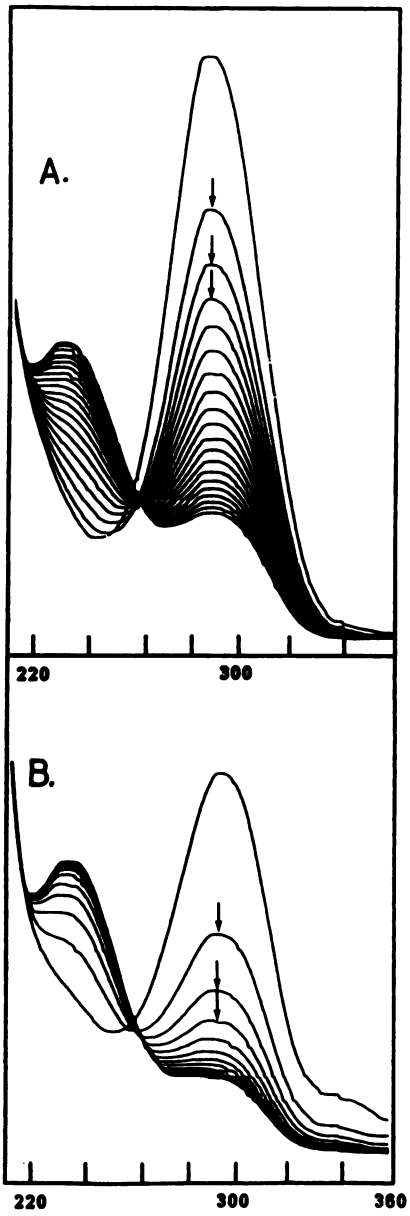

FIG. 2. Spectral change of 4-oxalocrotonate in spontaneous and isomerase-catalyzed isomerizations. (A) Spontaneous isomerization in 10 mM Tris hydrochloride buffer (pH 7.3). (B) 4-oxalocrotonate isomerase-catalyzed isomerization in the same buffer. These absorbance spectra were recorded at 2-min intervals at a scan speed of 500 nm/min.

change in the concentration of compound 4 (4a plus 4b plus 4c) in the reaction mixture was determined by measuring the A_{305} immediately after addition of 0.25 N (final concentrations) NaOH. In an alkaline solution, all of the keto forms are transformed to the enol form, which exhibits a λ_{max} at 305 nm.

Purification of enzymes. Fifteen grams (wet weight) of cells were suspended in 100 ml of ED buffer (10 mM sodium ethylenediamine hydrochloride buffer [pH 7.3] containing 5 mM β-mercaptoethanol) and disrupted by passage through a French press (FA-073; SLM Instruments Inc.) at 16,000 lb/in^2. The cell extract was centrifuged at 20,000 rpm in a Sorvall SS34 centrifuge for 30 min, and the supernatant was further centrifuged at 40,000 rpm in a Beckman Ti60 rotor for 1 h. The supernatant was then filtered through a Nalgene nitrocellulose membrane (0.45-μm pore size) and charged into a Toyo Soda Bio-Gel anion-exchange column (type TSK-DEAE-5 PW; 150 by 21.5 mm [inner diameter]; Bio Rad Laboratories) fitted on a Waters 600 liquid chromatograph (Millipore Corp.). The proteins were eluted from the column at a flow rate of 5 ml/min with a linear gradient of 0 to 0.3 M NaCl in 600 ml of ED buffer. The elute was collected in 10-ml fractions, and those containing 4-oxalocrotonate isomerase, 4-oxalocrotonate decarboxylase, or 2-oxopent-4-enoate hydratase activity were pooled. Further purification steps are described in Results. The proteins in each fraction were analyzed by electrophoresis either on a 15% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS) fitted on a conventional electrophoresis cell or on PhastGel 20% polyacrylamide gel run on a PhastSystem apparatus (Pharmacia) at 15°C in 0.112 M Tris-acetate buffer containing 0.1% SDS. The gels were silver stained. Protein concentrations were determined with a kit from Bio-Rad Laboratories.

TABLE 1. Purification of 4-oxalocrotonate isomerase

Step	Vol (ml)	Total activity (U)	Sp act (U/mg of protein)
Cell extract	170	9,800	8.1
DEAE anion-exchange chromatography	20	3,100	100
Supernatant of 70% ammonium sulfate treated with 65°C for 2 min	38	2,500	230
Phenyl-5PW hydrophobic interaction chromatography	1	2,900	3,200
Superose-12 gel filtration	1	2,200	4,400

RESULTS

Purification of 4-oxalocrotonase isomerase. A cell extract was prepared from *E. coli* K12ΔH1Δtrp(pGSH2915) grown at 42°C for 3 h and fractionated by using a DEAE-5PW anion-exchange column. 4-Oxalocrotonate isomerase activity eluted 60 min after the start of the gradient. Active fractions (20 ml) were further fractionated by ammonium sulfate precipitation. The isomerase was not precipitated, even at 70% saturation of ammonium sulfate at 4°C. The soluble fraction was incubated at 65°C for 2 min, and the precipitated proteins were discarded by centrifugation at 20,000 rpm for 20 min in a Sorvall SS34 centrifuge. Less than 20% of the isomerase was inactivated by this treatment. The supernatant was filtered through a Nalgene nitrocellulose membrane (0.45-μm pore size) and loaded onto a Toyo Soda Bio-Gel Phenyl-5PW column (75 mm by 7.5 mm [inner diameter]; Bio-Rad) preequilibrated with 10 mM ED buffer containing 2 M $(NH_4)_2SO_4$, and proteins were eluted by a linear gradient from 2 to 0 M $(NH_4)_2SO_4$ in 30 ml of ED buffer with a flow rate of 0.5 ml/min. Isomerase activity eluted 58 min after the start of the gradient. The most active fraction (1 ml) had a specific activity of 3,000 U/mg of protein (Table 1). When analyzed on an SDS-polyacrylamide gel, this fraction contained essentially one polypeptide with a very low molecular weight (Fig. 3A). By SDS-gel electrophoresis using a PhastSystem apparatus with aprotinin (molecular weight, 6,500; Bayer) and porcine insulin chain B (molecular weight, 3,400; Sigma Chemical Co.) as standards, the molecular weight of the isomerase was determined to be 3,500. This fraction was analyzed with a gel filtration column

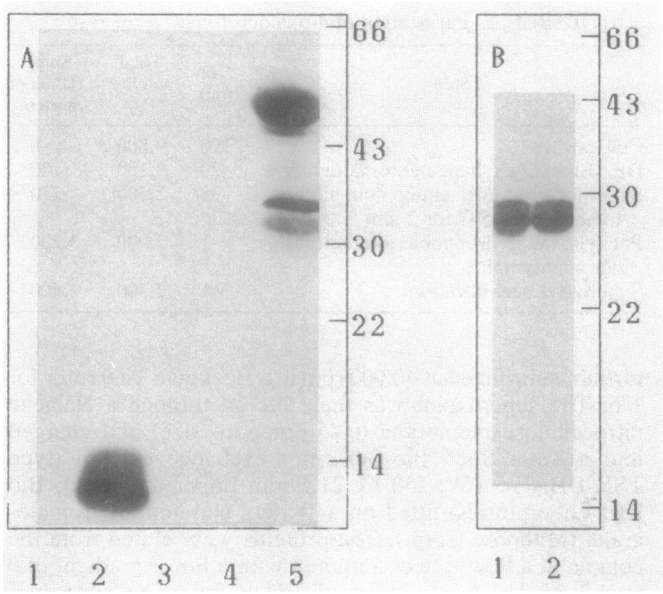

FIG. 3. (A) Purification of 4-oxalocrotonate isomerase with a Phenyl-5PW hydrophobic interaction column. A 30-μl sample of the 52- to 56-min fraction (lane 1), 10 μl of the 56- to 60-min fraction (lane 2), 30 μl of the 60- to 64-min fraction (lane 3), 30 μl of the 64- to 68-min fraction (lane 4), and 1 μl of the 68- to 72-min fraction (lane 5) eluted from a hydrophobic interaction column were separated by SDS-polyacrylamide gel electrophoresis and silver stained. The 56- to 60-min fraction contained the isomerase activity. (B) Electrophoresis of purified fractions of 4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase on an SDS-polyacrylamide gel. A fraction containing the highest hydratase activity from Superose-12 (lane 1) and a fraction containing the highest decarboxylase activity from Superose-12 (lane 2) (see Table 3) were analyzed on an SDS-polyacrylamide gel.

(Superose-12 packed in HR10/30; Pharmacia) equilibrated with 0.2 M potassium phosphate buffer (pH 7.3). Isomerase activity eluted in a peak corresponding to a size of 28 kilodaltons. The Superose-12 fraction was used as a source of isomerase in the following experiments.

The product of the isomerase. When an ethanolic solution of 4-oxalocrotonate was dissolved in 10 mM Tris hydrochloride buffer (pH 7.3), the aqueous solution initially contained the enol form of 4-oxalocrotonate, which exhibits its λ_{max} at 295 nm. However, the enol form was spontaneously transformed to the keto form, whose λ_{max} was at 235 nm. This transformation is a first-order reaction with a half-life of 6 min, and its isosbestic point was at 260 nm (Fig. 2). Using purified isomerase, we tested whether the reaction catalyzed by isomerase is different from spontaneous isomerization. If the enzymatically produced keto form of 4-oxalocrotonate has a chemical structure different from the spontaneously formed one (e.g., compound 3b versus 3c), then their UV absorbance spectra should be different (15). However, the λ_{max} of the product and the isosbestic point of the isomerase-catalyzed reaction were the same as those of the spontaneous isomerization (Fig. 2). This suggested that the same product is formed in enzymatic and nonenzymatic reactions. Recently, C. P. Whiteman and N. J. Stolowich (personal communication) identified this product as 2-oxohex-3-ene-1,6-dioate (compound 3c).

Kinetic properties and substrate specificity of 4-oxalocrotonate isomerase. In 10 mM potassium phosphate buffer (pH 7.3), the V_{max} of isomerase was 6,000 U/mg of protein and the K_m was 20 μM. The pH was optimum at 7.5 in 10 mM Tris hydrochloride buffer and at 6.5 in 10 mM 3-(*N*-morpholino)propanesulfonic acid–NaOH buffer. Several salts had an inhibitory effect on the isomerase-catalyzed reaction (Table 2). The isomerase not only stimulated isomerization of 4-oxalocrotonate but also stimulated isomerization of 2-hydroxypent-2,4-dienoate (compound 4a). The specific activity of the isomerase with this substrate in 10 mM potassium phosphate buffer (pH 7.3) was 480 μmol/min per mg of protein.

TABLE 2. Effects of salts on 4-oxalocrotonate isomerase activity

Buffer	Sp act (U/mg of protein) (%)
10 mM potassium phosphate (pH 7.3)	4,400 (100)
10 mM potassium phosphate (pH 7.3)–100 mM KCl	2,200 (50)
10 mM potassium phosphate (pH 7.3)–100 mM NaCl	1,300 (30)
100 mM potassium phosphate (pH 7.3)	840 (20)
10 mM Tris hydrochloride (pH 7.3)	1,700 (40)
10 mM Tris hydrochloride (pH 7.3)–3.3 mM $MgSO_4$	1,200 (30)
10 mM Tris hydrochloride (pH 7.3)–33 mM $CaCl_2$	100 (20)

Purification of 4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase. A crude extract from *P. putida* cells was fractionated with a DEAE-5PW anion-exchange column, and 4-oxalocrotonate decarboxylase was eluted 90 min after the start of the gradient. Fractions of 30 ml were pooled and treated with ammonium sulfate. The decarboxylase activity was precipitated between 30 and 70% saturation of ammonium sulfate at 4°C. The precipitated proteins were dissolved in 20 ml of ED buffer containing 1.2 M ammonium sulfate, and the undissolved proteins were discarded by centrifugation in a Sorvall SS34 centrifuge at 20,000 rpm for 15 min. The supernatant was filtered through a Nalgene nitrocellulose membrane (0.45-μm pore size) and loaded onto a Toyo Soda Bio-Gel Phenyl-5PW hydrophobic interaction column. Proteins were eluted from the column at a flow rate of 0.5 ml/min with a linear gradient in 30 ml of ED buffer with a descending ammonium sulfate concentration from 1.2 to 0 M but an ascending isopropanol concentration from 0 to 10% (vol/vol). The decarboxylase activity was found in a major protein peak that eluted at 31 min. A 3-ml fraction containing the highest decarboxylase activity was concentrated to approximately 400 μl with an Amicon Centricon-12 concentrator and loaded on a Superose-12 gel filtration column preequilibrated with 0.2 M potassium buffer (pH 7.3). The decarboxylase activity was found in a major protein peak corresponding to a size of 130 kilodaltons. SDS-polyacrylamide gel analysis showed that this fraction contained two polypeptides of 27 and 28 kilodaltons (Fig. 3B). As described below, it was found later that this fraction also contained 2-oxopent-4-enoate hydratase activity. The steps of the purification of the decarboxylase are summarized in Table 3.

2-Oxopent-4-enoate hydratase was purified by DEAE anion-exchange, Phenyl-5PW hydrophobic interaction, and Superose-12 gel filtration chromatography (Table 3). The elution pattern of the hydratase was very similar to that of the decarboxylase. In fact, the most purified fraction of the hydratase also exhibited the decarboxylase activity and also contained two polypeptides of 27 and 28 kilodaltons (Fig. 3B). Therefore, the two different polypeptides found in the purified fractions (Fig. 3B) are likely to be 2-oxopent-4-enoate hydratase and 4-oxalocrotonate decarboxylase. Our gene product analysis showed, in fact, that hydratase and decarboxylase are 27- and 28-kilodalton polypeptides,

TABLE 3. 4-Oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase purification

Step	Vol (ml)	4-Oxalocrotonate decarboxylase		2-Oxopent-4-enoate hydratase	
		Total activity (U)	Sp act (U/mg of protein)[a]	Total activity (U)	Sp act (U/mg of protein)[b]
Cell free extract	80	1,800	1.1	12,000	7
DEAE anion-exchange chromatography (pH 7.3)	30	740	9.1	3,000	36
Ammonium sulfate (30–70%)	20	310	16	Omitted	Omitted
Phenyl-5-PW hydrophobic interaction chromatography	1	130	33	270	72
DEAE anion-exchange chromatography (pH 8.0)	10	54	30	100	68
Superose-12 gel filtration chromatography	1	48	34[c]	63	45[d]

[a] Measured in 100 mM Tris hydrochloride buffer (pH 7.3) containing 3.3 mM $MgSO_4$.
[b] Measured in 100 mM Tris hydrochloride buffer (pH 7.3).
[c] The specific activity of 2-oxopent-4-enoate hydrolase in this fraction was 42 U/mg of protein.
[d] The specific activity of 4-oxalocrotonate decarboxylase in this fraction was 63 U/mg of protein.

respectively (Harayama et al., submitted). A fraction from the Superose-12 column chromatography containing both 4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase was used in the following experiments.

Substrate specificity of the hydratase. The λ_{max} of compound 4 prepared by the method of Colinsworth et al. (4) was at 265 nm. Its A_{265} decreased spontaneously but increased again after addition of 0.2 M (final concentration) borate. Since borate stabilizes the enol form and shifts the equilibrium between the tautomers toward the enol form (5, 13), the molecule which absorbs light at 265 nm is in the enol form (compound 4a, 2-hydroxypent-2,4-dienoate), and its keto form (compound 4b, 2-oxopent-4-enoate, or 4c, 2-oxopent-3-enoate) does not significantly absorb UV light above 220 nm.

As demonstrated by the rapid decrease in A_{265} after addition of 2-oxopent-4-enoate hydratase, 2-hydroxypent-2,4-dienoate (compound 4a) was an excellent substrate for this enzyme (4; Fig. 4A). Its keto forms (compounds 4a and 4c), however, could not be metabolized efficiently by the hydratase, as demonstrated by the existence of a population of compound 4 which was resistant to hydratase (Fig. 4A). This hydratase-resistant population increased in a solution containing more of the keto form, i.e., 25% of the population in the fresh solution (Fig. 4A) versus 50% of that in a 90-min-old solution (Fig. 4B). In the 90-min-old solution, the total concentration of compound 4 decreased to 70% of the initial concentration; 30% of compound 4 decomposed spontaneously to another, uncharacterized molecule within 90 min.

General characterization of the hydratase. Although Mn^{2+} stimulates the hydratase activity (4), it also accelerates spontaneous transformation of 2-hydroxypent-2,4-dienoate (compound 4a) to its biologically less active keto form. Therefore, Mg^{2+} was used instead of Mn^{2+} in most of the hydratase assays. The hydratase did not exhibit strong pH dependence between pHs 5.8 and 7.5. The K_m and V_{max} in 10 mM Tris hydrochloride buffer (pH 7.0) containing 3.3 mM $MgSO_4$ were determined to be 30 μM and 120 U/mg of protein, respectively.

Substrate specificity of the decarboxylase. The 4-oxalocrotonate solution in an equilibrium state contains both the enol (λ_{max}, 295 nm) and the keto (λ_{max}, 235 nm) forms. When a purified fraction containing the decarboxylase and the hydratase was added to the solution, A_{235} decreased and A_{265} initially increased (Fig. 5). A_{295}, which is due to the enol form of 4-oxalocrotonate (compound 3a), did not change initially. A short time later, both A_{265} and A_{295} started to decrease. The reactions which occurred in the experiment whose results are shown in Fig. 6 were interpreted as

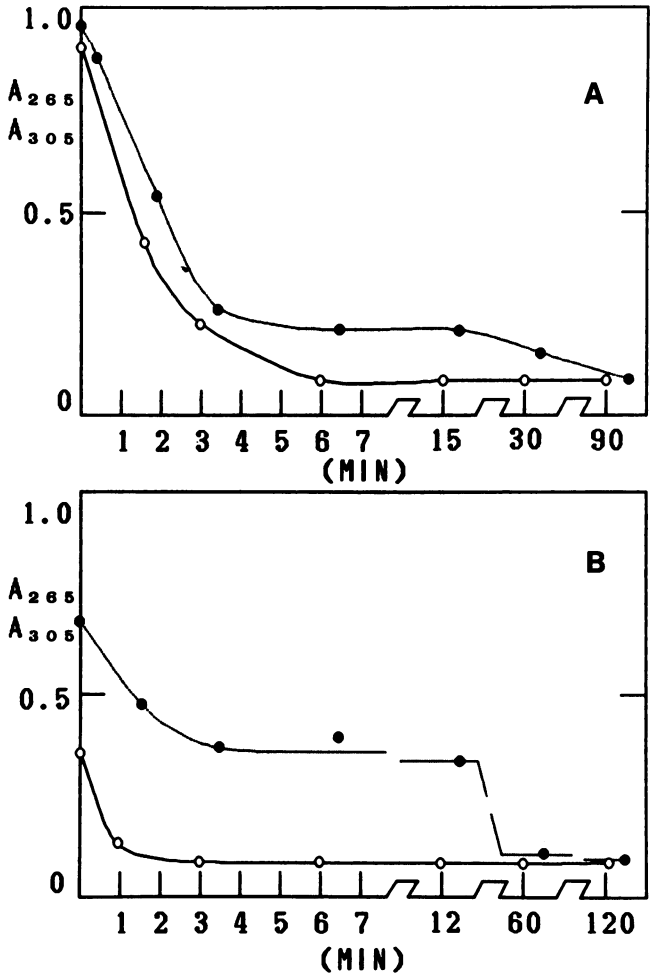

FIG. 4. Metabolism of 2-hydroxypent-2,4-dienoate by 2-oxopent-4-enoate hydratase. Purified 2-oxopent-4-enoate hydratase was added to a fresh solution of 2-hydroxypent-2,4-dienoate (compound 4a) (A) or a solution prepared 90 min before addition of the hydratase (B). The concentration of 2-hydroxypent-2,4-dienoate (compound 4a [○]) was determined by measuring changes in A_{265}, whereas the total concentration of compound 4 (4a plus 4b plus 4c [●]) was determined as follows. After a defined incubation time, a 2-ml sample of the reaction mixture was withdrawn and quickly mixed with 100 μl of 5 M NaOH in a flow cell of a spectrophotometer. The A_{305} of the mixture increased rapidly and then decreased gradually. The initial A_{305} was estimated by extrapolation of a decay curve of A_{305} to time zero.

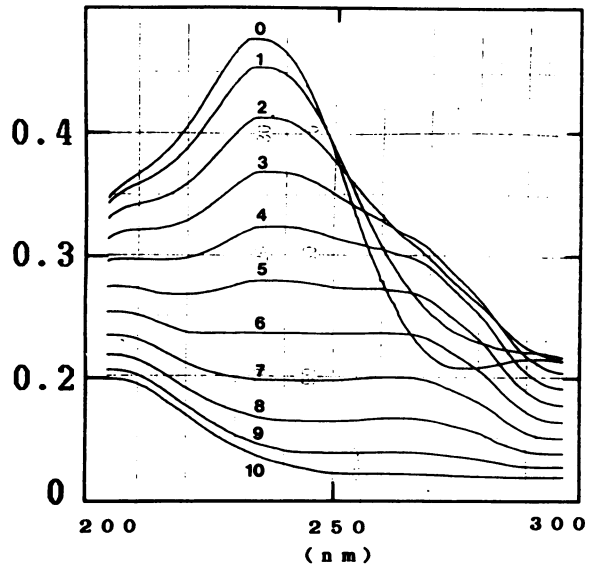

FIG. 5. Transformation of 4-oxalocrotonate by 4-oxalocrotonate isomerase, 4-oxalocrotonate decarboxylase, and 2-oxopent-4-enoate hydratase. A solution of 4-oxalocrotonate in equilibrium between its keto and enol forms was prepared in 10 mM Tris hydrochloride buffer (pH 7.0) containing 3.3 mM $MgSO_4$ (curve 0) by addition of 4 U of 4-oxalocrotonate isomerase per ml (final concentration). After completion of the isomerase reaction, hydratase and decarboxylase were added and the absorbance spectra were recorded at 1-min intervals.

follows. Initially, the keto form of 4-oxalocrotonate (compound 3c; λ_{max}, 235 nm) was metabolized to 2-hydroxypent-2,4-dienoate (compound 4a; λ_{max}, 265 nm); the enol form of 4-oxalocrotonate (compound 3a; λ_{max}, 295 nm) was not attacked by the decarboxylase, but as the concentration of the keto form of 4-oxalocrotonate (compound 3c) decreased, the enol form was transformed to the keto form (compound 3c) and therefore, A_{295} decreased. A_{265} also started to decrease as 2-hydroxypent-2,4-dienoate (compound 4a) was metabolized by the hydratase.

The concentration and the extinction coefficient of the keto form of 4-oxalocrotonate (compound 3c) at 235 nm were determined as described in Materials and Methods. Decarboxylase activity was then assayed by measuring the decrease in A_{235}. The values K_m = 15 μM and V_{max} = 51 U/mg of protein were obtained by using the substrate prepared by spontaneous isomerization of 4-oxalocrotonate, whereas the values K_m = 15 μM and V_{max} = 43 U/mg of protein, were obtained with the substrate prepared by treatment of 4-oxalocrotonate with the isomerase. The V_{max} of the decarboxylase could be underestimated in the presence of the isomerase, because the latter enzyme produces the keto form of 4-oxalocrotonate from its enol form as the decarboxylase metabolizes the keto form.

General characterization of 4-oxalocrotonate decarboxylase. 4-Oxalocrotonate decarboxylase activities at different pH values were examined in 10 mM Tris hydrochloride buffer or 10 mM 3-(*N*-morpholino)propanesulfonic acid–NaOH buffer in the presence of 3.3 mM $MgSO_4$. The extinction coefficient of the keto form of 4-oxalocrotonate at 235 nm in each buffer was obtained as described in Materials and Methods, and the relative activity of the decarboxylase at different pHs was obtained. Decarboxylase activity was optimum at pH 6.5. Mg^{2+} was essential for the activity.

Purification of 4-oxalocrotonate decarboxylase from *E. coli*(pGSH2915). *E. coli* K12ΔtrpΔH1(pGSH2915) expressed very low decarboxylase activity, i.e., 0.07 U/mg of cellular protein at 1 h after induction at 42°C and <0.01 U/mg of cellular protein at 2 h after induction. This strain does not carry *xylJ* and therefore expresses no 2-oxopent-4-enoate hydratase activity. A cell extract of this strain was fractionated by DEAE anion-exchange chromatography, and the decarboxylase activity eluted at 60 min; this was in contrast to the elution at 90 min of the decarboxylase activity isolated from *P. putida*. Further purification was not successful, because no activity was recovered after passage into a Phenyl-5PW hydrophobic interaction column. Therefore, a DEAE fraction containing decarboxylase activity was used as partially purified decarboxylase free from hydratase. When this enzyme was added to a solution containing the keto form of 4-oxalocrotonate, A_{235} decreased and A_{265} increased. A_{265}, once it reached a maximum, started to

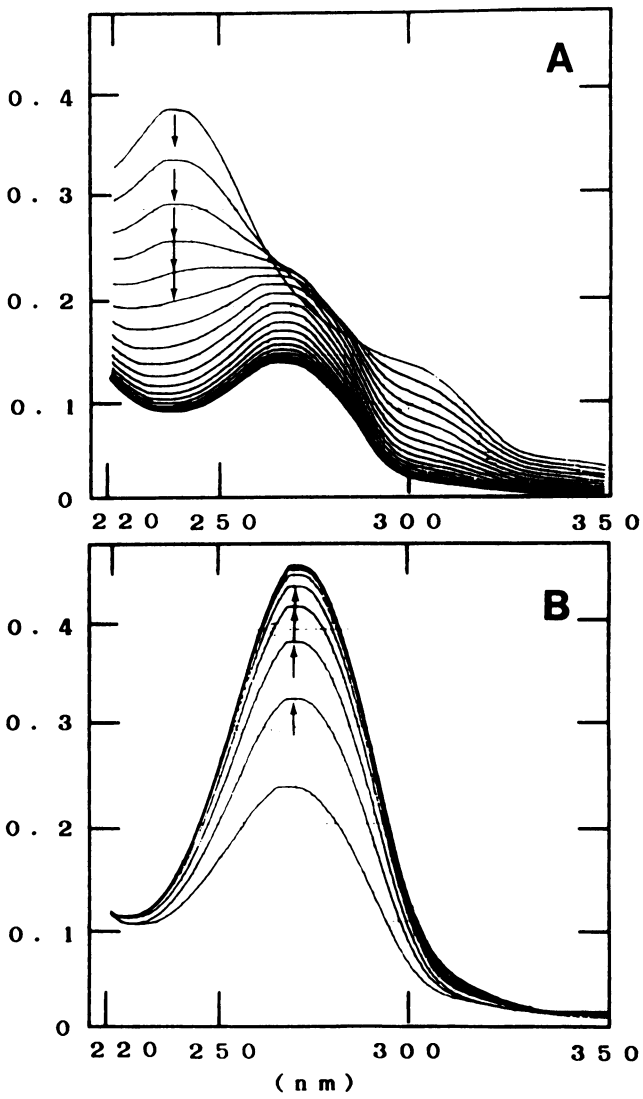

FIG. 6. Transformation of the keto form of 4-oxalocrotonate to 2-hydroxypent-2,4-dienoate by 4-oxalocrotonate decarboxylase isolated from 2-oxopent-4-enoate hydratase-negative *E. coli*. The absorbance spectra were recorded at 1-min intervals after decarboxylase addition (A). About 30 min later, 500 μl of this sample and 500 μl of 2 M sodium borate were mixed and the absorbance spectra were recorded at 5-min intervals (B).

decrease and approached a stable value (Fig. 6A). The decrease in A_{265} was due to spontaneous isomerization to the keto form, because A_{265} increased again after addition of borate (Fig. 6B).

The extinction coefficients of the keto form of 4-oxalocrotonate (compound 3c) at 235 and 265 nm in 10 mM Tris hydrochloride buffer (pH 7) containing 3.3 mM $MgSO_4$ were 7,200 and 2,400, respectively, whereas those of 2-hydroxypent-2,4-dienoate (compound 4a) were 2,900 and 13,000, respectively. If one molecule of the keto form of 4-oxalocrotonate (compound 3c) is converted to p molecules of compound 4a and $1-p$ molecules of compound 4b or 4c, the ratio of the increase in A_{265} (ΔA_{265}) to the decrease in A_{235} (ΔA_{235}) is $\Delta A_{265}/\Delta A_{235} = (13{,}000p - 2{,}400)/(7{,}200 - 2{,}900p)$, because the keto form of compound 4 (4b or 4c) exhibits no absorption above 220 nm.

Experimentally, this value was 2.0 (Fig. 6). Thus, p was calculated to be 0.9, indicating that 90% of the product of the decarboxylase was 2-hydropent-2,4-dienoate (compound 4a). Formation of this compound measured by ΔA_{265} could be underestimated because spontaneous transformation of this molecule into its keto form. Therefore, almost 100% of the product of the decarboxylase may be in the enol form (compound 4a).

Purification of 2-oxopent-4-enoate hydratase from *E. coli* (pGSH2829). *E. coli* K12ΔtrpΔH1(pGSH2829) carries the structural gene for 2-oxopent-4-enoate hydratase but that not for 4-oxalocrotonate decarboxylase. The strain expressed a hydratase activity of 1 U/mg of cellular protein after induction at 42°C for 3 h. The hydratase was fractionated by DEAE anion-exchange chromatography and eluted at 64 min after onset of the gradient. The specific activity of the 63- to 65-min fraction was 4 U/mg of protein.

DISCUSSION

In this study, three enzymes of the *meta*-cleavage pathway encoded in TOL plasmid pWW0 were purified and characterized. 4-oxalocrotonate isomerase is a multimer formed by self-association of extremely small protein subunits. The stability of this enzyme at high temperatures (4) may be due partly to the small size of its protein subunit. However, the estimation of the molecular weight of 3,500 may be erroneous if the isomerase exhibits aberrant mobility on an SDS-polyacrylamide gel. This possibility is being examined by determination of the amino acid sequence of the purified isomerase. The reaction that the isomerase catalyzes can also occur spontaneously. The isomerase, however, is essential for catabolism of benozate and *p*-toluate (1, 10). The rate of spontaneous isomerization inside the cell is probably insufficient to support cell growth. By a combination of [^{1}H]NMR and [^{13}C]NMR spectroscopy, Whiteman and Stolowich (personal communication) identified the product of the isomerase as 2-oxohex-3-ene-1,6-dioate (compound 3c).

4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase was copurified in DEAE anion-exchange, Phenyl-5PW hydrophobic interaction, and Superose-12 gel filtration chromatography. The purest fraction contained two polypeptides of 27 and 28 kilodaltons. Consistent with this result, we have identified in maxicells the products of the structural genes for the decarboxylase (*xylI*) and the hydratase (*xylJ*) as 27- and 28-kilodalton polypeptides, respectively (12). Attempts to separate these two proteins resulted in loss of decarboxylase activity. This and the following findings suggested that the two enzymes form a physical complex in vivo. (i) In DEAE anion-exchange chromatography, the decarboxylase isolated from a XylI$^+$ XylJ$^-$ clone and the hydratase isolated from a XylI$^-$ XylJ$^+$ clone were eluted differently from those isolated from *P. putida* XylI$^+$ XylJ$^+$, and (ii) the decarboxylase isolated from a XylI$^+$ XylJ$^-$ clone was extremely unstable. In contrast to the decarboxylase, the hydratase was active as a single component; *E. coli* clones carrying the structural gene for 2-oxopent-4-enoate hydratase expressed the enzyme activity at a high degree in the absence of the decarboxylase structural gene (8, 11; this paper). The hydratase from phenol-degrading *P. putida* NCIB10015 has previously been purified (4). The decarboxylase activity in the purified sample has not been examined, but SDS-polyacrylamide gel electrophoresis with β-mercaptoethanol has revealed two polypeptides in the sample (4).

The keto (compound 3c) but not the enol (compound 3a) form of 4-oxalocrotonate was the substrate for the decarboxylase. The product of the decarboxylase was 2-hydroxypent-2,4-dienoate (compound 4a), which was the substrate for the hydratase. This compound was spontaneously transformed into its keto form, which was not a substrate for the hydratase. Besides this reversible enol-keto interconversion, compound 4 was irreversibly transformed into another, uncharacterized molecule (Fig. 4B). Because of its chemical instability, effective metabolism of 2-hydroxypent-2,4-dienoate (compound 4a) requires its rapid enzymatic transformation by the hydratase. Physical associations of the decarboxylase and the hydratase may assure efficient transformation of the intermediate.

Hydrolytic cleavage of compound 2 also produces 2-hydroxypent-2,4-dienoate (compound 3a; 7). If physical association between one enzyme producing this intermediate and the other enzyme metabolizing it is required for effective metabolism of compound 3a, 2-hydroxymuconic semialdehyde hydrolase, which produces compound 3a (Fig. 1), might be expected to be associated with 2-oxopent-4-enoate hydratase, which acts on compound 3a. However, 2-hydroxymuconic semialdehyde hydrolase and 2-oxopent-4-enoate hydratase have been separated by DEAE-cellulose chromatography (our unpublished data). Therefore, the physical interaction between those two enzymes, if it exists, may be relatively weak. We could not determine in this study whether the hydratase produces 4-hydroxy-2-oxovalerate (compound 5a) or 2,4-dihydroxypent-2-enoate (compound 5b).

The results of a previous study indicated that intracellular accumulation of the enol form of 4-oxalocrotonate is toxic to host cells (10). Apparently, activities of 4-oxalocrotonate branch enzymes are adjusted so that the enol form of 4-oxalocrotonate does not accumulate during the catabolism of aromatic compounds. In induced cells of *P. putida* harboring the TOL plasmid, the activity of the dehydrogenase, the first enzyme of the 4-oxalocrotonate branch, is very low (about 0.2 U/mg of cellular protein) compared with that of the isomerase (about 6 U/mg of cellular protein). Furthermore, accumulation of 2-hydroxypent-2,4-dienoate (compound 3a) may be prevented by association of 4-oxalocrotonate decarboxylase and 2-oxopent-4-enoate hydratase.

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