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 (xylJ) and the hydratase (xylI) 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 reconverts 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-dione (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; R2 = CH3 and R3 = 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; R1 and R2 = H) and 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate (compound 2; R2 = H and R3 = CH3), 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; R1 = H and R2 = CH3), has been determined to be 2-oxo-cis-hex-4-enoate (compound 4b; R2 = CH3) 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 (R2 = 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, tautomeration 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 8 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 hydroxylase; 4O1, 4-oxalocrotonate isomerase; 4OD, 4-oxalocrotonate decarboxylase; OEH, 2-oxopent-4-enoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase. For compounds in which R1 and R2 = H, 1 = catechol, 2 = 2-hydroxymuconic semialdehyde, 3a = 2-hydroxyhexa-2,4-diene-1,6-dioate (enol form of 4-oxalocrotonate), 3b = 2-oxo-hex-4-ene-1,6-dioate, 3c = 2-oxo-hex-3-ene-1,6-dioate, 4a = 2-hydropent-2,4-dieneoate, 4b = 2-oxopent-4-enoate, 4c = 2-oxopent-3-enoate, 5a = 4,1-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 pL 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 pL promoter. pGSH2915 carries xyIH and xyIJ, the structural genes for 4-oxalocrotonate decarboxylase and 4-oxalocrotonate isomerase, respectively, whereas pGSH2829 carries xyIL, the structural gene for 2-oxopent-4-enoate hydratase. The host strain for the plasmids was Escherichia coli K-12 strain K12ΔH1Δtrp [F′ lacZΔM15 Δ(bio-uvrB) ΔtrpEA2 (λ Nam7 Nam53 cI857 ΔH1)]. The strain carries a temperature-sensitive allele of the lambda repressor, cI857, which regulates expression from the pL promoter. At 30°C, the cI857 repressor is active and expression of TOL genes from the pL 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 LB broth containing 100 μg of ampicillin per ml to about 3 × 10^9 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 D-glutamate 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 MgSO4, in a linear gradient from 10 to 0 mM. As described in Results, the compound obtained was 2-hydropent-2,4-dieneoate (compound 4a). This compound was kept at −20°C.

**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 ε=enol,λ = A(0,λ)/C(keto), where A(0,λ) 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(keto) = C × [A(0,λ) − A(eq,λ)]/A(0,λ), where A(eq,λ) 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 ε(keto,235) = [A(eq,235) − A(0,235)]/C(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 MgSO4. The standard reaction mixture for the hydratase assay contained 30 μM 2-hydropent-2,4-dieneoate (compound 4a), 10 mM Tris hydrochloride (pH 7.0), and 3.3 mM MgSO4; the enzyme activity was determined by measuring the decrease in A_{265} due to removal of the substrate. Alternatively, the
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 EDT 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) or on a Pharmacia 20% polyacrylamide gel run on a Pharmacia apparatus at 15°C in 0.11 M Tris-acetate buffer containing 0.1% SDS. The gels were silver stained. Protein concentrations were determined with a kit from Bio-Rad Laboratories.

### Results

**Purification of 4-oxalocrotonase isomerase.** A cell extract was prepared from E. coli K12ΔH1aTrp(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 (NH₄)₂SO₄, and proteins were eluted by a linear gradient from 2 to 0 M (NH₄)₂SO₄ 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 antiprotein (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.

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

**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 300 nm/min.
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 λmax at 295 nm. However, the enol form was spontaneously transformed to the keto form, whose λmax 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 λmax 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. Stolwisch (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 Vmax of isomerase was 6,000 μM of protein and the Km 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

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Sp act (U/mg of protein) (%)</th>
</tr>
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<tbody>
<tr>
<td>10 mM potassium phosphate (pH 7.3)</td>
<td>4,400 (100)</td>
</tr>
<tr>
<td>10 mM potassium phosphate (pH 7.3)–100 mM KCl</td>
<td>2,200 (50)</td>
</tr>
<tr>
<td>10 mM potassium phosphate (pH 7.3)–100 mM NaCl</td>
<td>1,300 (30)</td>
</tr>
<tr>
<td>100 mM potassium phosphate (pH 7.3)</td>
<td>840 (20)</td>
</tr>
<tr>
<td>10 mM Tris hydrochloride (pH 7.3)</td>
<td>1,700 (40)</td>
</tr>
<tr>
<td>10 mM Tris hydrochloride (pH 7.3)–3 mM MgSO4</td>
<td>1,200 (30)</td>
</tr>
<tr>
<td>10 mM Tris hydrochloride (pH 7.3)–35 mM CaCl2</td>
<td>100 (20)</td>
</tr>
</tbody>
</table>

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 Toyoda 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,
respective (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 \( \lambda_{\text{max}} \) of compound 4 prepared by the method of Colinsonworth 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 \( V_{\text{max}} \) in 10 mM Tris hydrochloride buffer (pH 7.0) containing 3.3 mM MgSO\(_4\) were determined to be 30 \( \mu \text{M} \) and 120 U/mg of protein, respectively.

Substrate specificity of the decarboxylase. The 4-oxalocrotonate solution in an equilibrium state contains both the enol \( \lambda_{\text{max}} \) (295 nm) and the keto \( \lambda_{\text{max}} \) (235 nm) forms. When a purified fraction containing the decarboxylase and the hydratase was added to the solution, \( A_{295} \) 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 \( A_{295} \) and \( A_{265} \) started to decrease. The reactions which occurred in the experiment whose results are shown in Fig. 6 were interpreted as...
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 MgSO4 (curve 0)
by addition of 4 U of 4-oxalocrotonate isomerase per ml (final
concentration). After completion of the isomerase reaction, hy-
dratase and decarboxylase were added and the absorbance spectra
were recorded at 1-min intervals.

follows. Initially, the keto form of 4-oxalocrotonate (com-
 pound 3c; \( \lambda_{\text{max}} = 235 \) nm) was metabolized to 2-hydroxypent-
2,4-dienoate (compound 4a; \( \lambda_{\text{max}} = 265 \) nm); the enol form of
4-oxalocrotonate (compound 3a; \( \lambda_{\text{max}} = 295 \) 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. Decar-
boxylase activity was then assayed by measuring the de-
crease in \( A_{235} \). The values \( K_m = 15 \) \( \mu \)M and \( V_{\text{max}} = 51 \) U/mg
of protein were obtained by using the substrate prepared by
spontaneous isomerization of 4-oxalocrotonate, whereas the
values \( K_m = 15 \) \( \mu \)M and \( V_{\text{max}} = 43 \) U/mg of protein, were
obtained with the substrate prepared by treatment of 4-
oxalocrotonate with the isomerase. The \( V_{\text{max}} \) of the deca-
boxylase 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 decarbox-
ylase metabolizes the keto form.

**General characterization of 4-oxalocrotonate decarboxyl-
ase.** 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 MgSO4. 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. Mg2+ 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 xyU and therefore expresses no 2-oxopent-4-enoate
hydratase activity. A cell extract of this strain was fraction-
ated 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-SPW 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
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 3.5 mM MgSO$_4$, were 7,200 and 2,400, respectively, whereas those of 2-hydroxy-pent-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 $A_{265}$ ($\Delta A_{265}$) to the decrease in $A_{235}$ ($\Delta A_{235}$) is $\frac{\Delta A_{265}/\Delta A_{235}}{1} = (13,000 - 2,400)/(7,200 - 2,900) = 2.0$, because the keto form of compound 4 (4b or 4c) exhibits no absorption above 200 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 $\Delta A_{265}$ 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 K12ATrDH1(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 benzoate 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 $^1$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 were copurified in DEAE anion-exchange, Phenyl-SPW 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 (xyL) and the hydratase (xyD) 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 suggest that the two enzymes form a physical complex in vivo. (i) In DEAE anion-exchange chromatography, the decarboxylase isolated from a Xyl$^+$ Xyl$^-$ clone and the hydratase isolated from a Xyl$^-$ Xyl$^+$ clone were eluted differently from those isolated from P. putida Xyl$^+$ Xyl$^+$, and (ii) the decarboxylase isolated from a Xyl$^+$ Xyl$^+$ 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.

Hydrolitic 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-oxoalate (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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