Influence of Side-Chain Substituents on the Position of Cleavage of the Benzene Ring by 

*Pseudomonas fluorescens*¹

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Received for publication 14 October 1968

*Pseudomonas fluorescens* was grown on mineral salts media with phenol, *p*-hydroxybenzoic acid, *p*-hydroxy-phenylacetic acid, or *p*-hydroxy-*trans*-cinnamic acid as sole carbon and energy source. Each compound was first hydroxylated, ortho to the hydroxyl group on the benzene ring, to give catechol, protocatechuic acid (3,4-dihydroxy-benzoic acid), homoprotocatechuic acid (3,4-dihydroxy-phenylacetic acid), and caffeic acid (3,4-dihydroxy-*trans*-cinnamic acid), respectively, as the ultimate aromatic products before cleavage of the benzene nucleus. Protocatechuic acid and caffeic acid were shown to be cleaved by ortho fission, via a 3,4-oxygenase mechanism, to give *β*-substituted *cis*, *cis*-muconic acids as the initial aliphatic products. However, catechol and homoprotocatechuic acid were cleaved by meta fission, by 2,3- and 4,5-oxygenases, respectively, to give *α*-hydroxy-muconic semialdehyde and *α*-hydroxy-*γ*-carboxymethyl muconic semialdehyde as initial aliphatic intermediates. Caffeic acid: 3,4-oxygenase, a new oxygenase, consumes 1 mole of *O₂* per mole of substrate and has an optimal *pH* of 7.0. The mechanism of cleavage of enzymes derepressed for substituted catechols by *P. fluorescens* apparently changes from ortho to meta with the increasing nephelauxetic (electron donor) effect of the side-chain substituent.

Aromatic compounds are oxidatively dissimilated by two distinct mechanisms: (i) cleavage of a catechol between adjacent carbon atoms bearing hydroxyl groups (11, 13) and (ii) cleavage of the ring between a carbon atom bearing a hydroxyl group and the adjacent nonhydroxylated carbon atom (3, 4, 8, 13). It is generally accepted that a particular species of *Pseudomonas* employs either ortho or meta ring fission for a given aromatic substrate and that both types of oxygenase are not derepressed simultaneously by one inducer (15). Ortho or meta cleavage of protocatechuic acid has been used as one of the parameters to classify pseudomonads (15).

In this communication, we report that, in the case of *P. fluorescens* [this organism was classified as *P. fluorescens* according to Stanier, Palleroni, and Doudoroff (15)], the electron-donating or electron-withdrawing capacity of the side-chain substituent appears to determine whether ortho- or meta-cleaving enzymes are derepressed; the whole molecule seems to be unimportant. In this organism, both types of ring fission mechanism are not derepressed simultaneously as is found with some microorganisms (6, 9). Details of the properties of the ring fission products from catechol, protocatechuic acid, and homoprotocatechuic acid have been published elsewhere (3, 5, 12). However, caffeic acid: 3,4-oxygenase has not been reported, and details of the properties of this enzyme and those of the ring fission product are presented.

**MATERIALS AND METHODS**

Culture methods. *P. fluorescens* was grown on mineral salts media containing, per liter: *KH₂PO₄*, 2 g; (NH₄)₂SO₄, 1 g; MgSO₄, 10 mg; and FeSO₄, 5 mg. The desired carbon source was added in the following amounts, per liter: phenol, 0.3 g; *p*-hydroxybenzoic acid, 1.0 g; *p*-hydroxy-phenylacetic acid, 0.7 g; and *p*-hydroxy-*trans*-cinnamic acid, 0.7 g. Cultures were grown with forced aeration at 30 °C in 16-liter carboys, by stepwise increase in the volume of the culture from 50 ml to 1 to 16 liters. Preparation of cell-free extracts. Cells were harvested in the late exponential phase in a Sharples centrifuge and were washed in 0.05 M tris(hydroxymethane)-

¹ This work was taken in part from a thesis submitted by M. M. Seldman for a B.S. degree in chemistry at the University of Illinois.
fluorescens were found conditions consisted assay chloroform-soluble was anhydrous acetate.

j8-carboxy 0.05 M buffer KOH of 0.001 Oxygraph (model H2SO4 treated to hydrogen sulfate. The residue dryness.

The residue was subjected to centrifugation at 23,000 X g for 20 min at 0 C. Cell debris was removed by centrifugation at 23,000 X g for 20 min at 0 C. Protein was determined by the method of Lowry et al. (10).

**Heat treatment.** Meta-cleaving enzymes from *P. fluorescens* were found to be heat stable, but enzymes for the further degradation of the semialdehyde product were totally inactivated by treatment at 70 C for 5 min. The protein precipitated by heat treatment was removed by centrifugation, and the supernatant fluids were shown to contain active catechol 2,3-oxygenase (EC 1.13.1.2; phenol-grown cells) or homoprotocatechuic acid: 4,5-oxygenase (p-hydroxy-phenylacetic acid-grown cells).

**Separation of ortho-cleaving enzymes.** Protocatechue: 3,4-oxygenase (from p-hydroxy-benzoate-grown cells) was shown to accumulate p-carboxy cis,cis-muconic acid when crude extracts were diluted to a level at which the lactonizing enzyme, which converts p-carboxy cis,cis-muconic acid to p-carboxy-muconolactone, would not function but at which the protocatechuic 3,4-oxygenase was still quite active.

Crude extracts from cells grown with p-hydroxy-trans-cinnamic acid were fractionated with a saturated solution of (NH4)2SO4 (pH 7.0). The fraction which precipitated at between 50 and 70% saturation was shown to contain caffeic acid: 3,4-oxygenase. Dialysis of this fraction for 18 hr against distilled water resulted in a preparation containing caffeic acid: 3,4-oxygenase but lacking the enzymes necessary for the subsequent degradation of the ring fission product.

**Enzymatic assays.** Oxygen uptake was determined with a Gilson differential respirometer. Warburg flasks contained (in a total volume of 3.0 ml) 1.8 ml of 0.05 M Tris buffer (pH 7.0), 0.5 ml of enzyme (15 to 20 mg of protein), and 0.5 ml of 0.01 M substrate. Carbon dioxide was removed with 0.2 ml of 20% KOH on Whatman 324 filter paper in the center well. Specificity of enzymes was checked on a Gilson Oxygraph (model K) oxygen electrode. Caffeic acid: 3,4-oxygenase could be conveniently assayed by following the change in absorbancy at 320 nm with a Zeiss PMQ II spectrophotometer (Fig. 1). Typical assay conditions consisted of 0.8 ml of 0.05 M Tris buffer (pH 7.0), 0.1 ml of fractionated enzyme preparation containing 1 to 2 mg of protein, and 0.1 ml of 0.001 M caffeic acid in a cuvette having a 1-cm light path.

**Isolation of catechols from culture filtrates.** An 18-liter amount of culture filtrate of *P. fluorescens* grown with p-hydroxy-trans-cinnamic acid, in the absence of Fe3+*, was acidified with 20 ml of concentrated H2SO4 and extracted with 12 liters of ethyl acetate. The ethyl acetate was dried by filtration through anhydrous Na2SO4 before evaporation to dryness. The residue was taken up in a small volume of chloroform and filtered. The material that was not chloroform-soluble was dissolved in diethyl ether and subjected to silica gel column chromatography. A silica gel (Merck; 0.05 to 0.2 mm) column (3 by 60 cm) was prepared with diethyl ether as the solvent.

**RESULTS**

**Characterization of catechols.** Catechol, protocatechuic acid, and homoprotocatechuic acid have been established as intermediates in the oxidative degradation of phenol, p-hydroxy-benzoic acid, and p-hydroxy-phenylacetic acid (or phenylacetic acid) in *P. fluorescens* (3, 5). Extracts of *P. fluorescens* grown with p-hydroxy-trans-cinnamic acid oxidized both caffeic acid and proto-
catechelic acid with the consumption of 1 mole of O$_2$ per mole of substrate (Fig. 2). Since side- 
chain attack on p-hydroxy-trans-cinnamic acid may occur to give protocatechelic acid as the 
ultimate aromatic product before ring cleavage, it was necessary to determine which of these two 
substituted catechols was the natural inter-

mediate in this metabolic pathway. When cul-
tures were grown with p-hydroxy-trans-cinnamic acid medium, in which Fe$^{++}$ was omitted, caffeic 
acid was isolated in significant yield, but proto-
catechelic acid could not be detected even on thin-layer chromatographic analysis. Caffeic acid 
could be detected in lower yields by thin-layer chromatography from culture filtrates in which iron 
was included. This unsaturated acid is readily distinguishable from protocatechelic acid, since 
the former compound fluoresces under ultraviolet light. Caffeic acid was characterized by a melting point of 195°C (no depression on admixture with authentic caffeic acid) and by infrared spectral analysis in a Nujol mull (Fig. 3). These data indicate that caffeic acid is most likely the natural intermediate in the degrada-
tion of p-hydroxy-trans-cinnamic acid.

Characterization of ring fission products. Heat-
treated extracts of cells of P. fluorescens grown 
either with phenol or p-hydroxy-phenylacetic acid, were reacted with catechol and homopro-
tocatechelic acid, respectively. After the con-
sumption of 1 mole of O$_2$ per mole of substrate, reactions were stopped; then the protein was precipitated with 0.5 ml of 5 N HCl and removed by centrifugation. Samples (0.1 ml) of superna-
tant liquid were taken and subjected to spectral analysis in acid (2 N HCl) and alkali (5 N NaOH) 
in a Cary model 14 recording spectrophotome-
ter. The spectral properties of these ring fission products were found to be identical to those 
reported for $\alpha$-hydroxy-muconic semialdehyde and $\alpha$-hydroxy-$\gamma$-carboxymethyl muconic semi-
aldehyde (3, 5; Table 1).

![Fig. 2. Oxidation of protocatechelic acid and caffeic acid by cell-free extracts of P. fluorescens grown with p-hydroxy-trans-cinnamic acid.](image)

![Fig. 3. Infrared spectra of authentic caffeic acid (upper spectrum) and the catechol isolated from culture filtrates of cells grown with p-hydroxy-trans-cinnamic acid.](image)
Table 1. Ring fission mechanism of oxygenases of _P. fluorescens_ grown with different aromatic substrates

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Catechol utilized</th>
<th>Absorption maxima of ring fission products</th>
<th>Ring fission mechanism</th>
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<tbody>
<tr>
<td>Phenol</td>
<td>Catechol</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>p</em>-Hydroxy-benzoic acid</td>
<td>Protocatechuate</td>
<td></td>
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<tr>
<td><em>p</em>-Hydroxy-phenylactic acid</td>
<td>Homoprotocatechuate</td>
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<tr>
<td><em>p</em>-Hydroxy-trans-cinnamic acid</td>
<td>Caffeic acid</td>
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<table>
<thead>
<tr>
<th>Acid</th>
<th>Alkali</th>
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<tr>
<td>317</td>
<td>375</td>
<td>Meta</td>
</tr>
<tr>
<td>230</td>
<td>257</td>
<td>Ortho</td>
</tr>
<tr>
<td>320</td>
<td>380</td>
<td>Meta</td>
</tr>
<tr>
<td>242, 272</td>
<td>235, 345</td>
<td>Ortho</td>
</tr>
</tbody>
</table>

**Fig. 4.** Kinetic study of the disappearance of caffeic acid with the concomitant accumulation of ring fission product with an absorption maximum at 272 nm.

The formation of _β_-carboxy _cis, cis-_muconic acid from protocatechuc acid was demonstrated with cells grown with both _p_-hydroxy-benzoate and _p_-hydroxy-trans-cinnamic acid by following the procedure of Ornston and Stanier (12). A 1.0-μmole amount of protocatechuc acid was allowed to react with 0.5 to 1.0 mg of crude extract in a total volume of 1.0 ml of 0.1 M phosphate buffer (pH 7.0). The disappearance of protocatechuc acid which absorbs at 290 nm and the appearance of the ring fission product which absorbs at 257 nm were observed by repeatedly scanning the reaction mixture in a Cary model 14 spectrophotometer. Spectral characteristics of the product at pH 7.0 and in 2 N acid are presented in Table 1. The above procedure was used to follow the cleavage of caffeic acid, except that fractionated extracts were required in this experiment (Fig. 4). This ring fission product showed no keto-enol tautomrism, having no functional keto, aldehyde, or hydroxyl groups (7, 14). Thin-layer chromatography of the ring fission product in the benzene-dioxan-acetic acid solvent system gave one spot (Rf = 0.23), which was more polar and readily distinguishable from caffeic acid (Rf = 0.43). Elution of the compound with an Rf value of 0.23 followed by spectral analysis gave absorption maxima at 272 and 242 nm in acid and at 345 and 235 nm in alkali (Fig. 5). When the ring fission product was allowed to stand in 5 N HCl overnight, a product having the infrared spectrum of a γ,-β-unsaturated γ-lactone (1,750 cm⁻¹) was isolated. Also, further infrared analysis of this compound showed a carbonyl absorption band at 1,720 cm⁻¹ as well as -C—O—C stretch at 1,090 cm⁻¹. These data indicate that caffeic acid is degraded via an ortho cleavage mechanism to form a tricarboxylic acid which may lactonize to form a substituted muconolactone (Fig. 6).

**Fig. 5.** Spectral properties of the ring fission product from caffeic acid.

The specific activity of caffeic acid:3,4-oxygenase was determined as the change in absorbancy at 320 nm in 5 min per milligram of protein.
Caffeic acid: 3,4-oxygenase, a new oxygenase, has been fractionated and a suitable assay to follow its activity has been developed. This enzyme may be useful for studying the mechanism of O₂ insertion by a dioxygenase; binding studies could be fruitful because caffeic acid is a fluorescent substrate but its ring fission product does

Fig. 6. Degradation of caffeic acid via an ortho cleavage mechanism to form a tricarboxylic acid.

Fig. 7. Linear dependence of the rate of disappearance of caffeic acid on substrate concentration.

With this assay, this oxygenase was shown to have a linear dependence on substrate concentration (Fig. 7) and a pH optimum of 7.0 (Fig. 8). Further studies are in progress on the complete degradative sequence for the metabolism of p-hydroxy-trans-cinnamic acid.

DISCUSSION

The strain of P. fluorescens used in this study is unique in its ability to derepress the synthesis of enzymes which cleave substituted catechols either by ortho or by meta mechanisms. The versatility of this organism is demonstrated in Fig. 9. Both ortho- and meta-cleaving enzymes are not derepressed by growth on any one substrate. Thus far, this study has been limited to four alternative pathways; however, on the basis of these four substrates, a pattern of cleavage influenced by the nephelauxetic effect of side-chain substituents is apparent. A broader survey of substrates is required before this hypothesis can be adopted as a general rule. However, it is of interest to note that studies with different organisms grown with cresols and with other substrates containing good electron-donating, side-chain substituents invariably show a derepression of meta-cleaving enzymes (1, 2, 13).

Fig. 8. pH optimum of caffeic acid: 3, 4-oxygenase. The assays at pH 6.0 and 6.5 were done in 0.05 M phosphate buffer, and assays at pH 7.0, 7.5, 8.0, and 8.5 were done in 0.05 M Tris buffer.

Fig. 9. Versatility of the strain of P. fluorescens used in this study as shown by its ability to derepress the synthesis of enzymes which cleave substituted catechols either by ortho or by meta mechanisms.
not fluoresce. Studies are in progress on the purification and mechanism of caffeic acid:3,4-oxygenase.

ACKNOWLEDGMENTS

We thank Francis Engle for technical assistance. This investigation was supported by National Science Foundation grant GB 5813.

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