NOTES

Extradiol Cleavage of 3-Methylcatechol by Catechol 1,2-Dioxygenase from Various Microorganisms

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Received for publication 1 October 1976

The isofunctional enzymes of catechol 1,2-dioxygenase from species of Acinetobacter, Pseudomonas, Nocardia, Alcaligenes, and Corynebacterium oxidize 3-methylcatechol according to both the intradiol and extradiol cleavage patterns. However, the enzyme preparations from Brevibacterium and Arthrobacter have only the intradiol cleavage activity. Comparison of substrate specificity among these isofunctional dioxygenases shows striking differences in the oxidation of 3-methylcatechol, 4-methylcatechol and pyrogallol.

Catechol 1,2-dioxygenase (catechol:oxygen 1,2-oxidoreductase, EC 1.13.11.1; pyrocatechase), an enzyme catalyzing the cleavage of the aromatic ring of catechol to cis,cis-muconic acid with the consumption of 1 mol of oxygen, has been intensively studied (7, 9, 11). Two of these enzymes, one from Pseudomonas arvilla (7) and the other from Brevibacterium fuscum (10), have been purified to homogeneity.

When o-dihydroxyphenol compounds are cleaved by a dioxygenase, three modes of enzymatic ring fission have been demonstrated (10): (i) intradiol cleavage, in which the bond between carbon atoms bearing the hydroxy groups is cleaved, i.e., pyrocatechase and protocatechuate 3,4-dioxygenase; (ii) proximal extradiol cleavage, in which the bond between the carbon atoms of positions 2 and 3 is cleaved, i.e., catechol 2,3-dioxygenase and 3,4-dihydroxyphenylacetate 2,3-oxygenase; and (iii) distal extradiol cleavage, in which the bond of positions 4 and 5 is cleaved, i.e., protocatechuate 4,5-dioxygenase. It has been believed that the sites of cleavage of an aromatic ring is strictly specific for each enzyme; i.e., trivalent iron-containing dioxygenases would exclusively act on the catechol ring by intradiol cleavage, whereas the divalent iron-containing dioxygenases would act on it by extradiol cleavage (12).

Recently, Fujiwara et al. (1) reported extradiol cleavage of 3-substituted catechol by the pyrocatechase of P. arvilla C-1. The pyrocatechase from B. fuscum P-13, however, does not oxidize 3-substituted catechol by extradiol cleavage (1).

In a continuing effort to understand the nature and the active site conformation of non-heme trivalent iron-containing dioxygenases (3–5, 14; Hou et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K156, p. 173, and RT1, p. 275, 1975), we have purified pyrocatechase from Acinetobacter calcoaceticus ADP-96 (13). We found that the pyrocatechase of A. calcoaceticus is very similar to the isofunctional dioxygenase from P. arvilla (7) and differs from that of B. fuscum (10). In this paper, we describe the findings on extradiol (proximal) cleavage activity of pyrocatechase among various species of bacteria and the ratio of intradiol to extradiol cleavage of 3-methylcatechol. We also report differences between these isofunctional dioxygenases in the oxidation of substituted catechols.

Cultures of various microorganisms were grown in shake flasks containing 100 ml of mineral medium with 10 mM sodium benzoate as the sole carbon source (13). Cells, 10 g (wet weight) harvested by centrifugation were suspended in 30 ml of 50 mM tris(hydroxymethyl)aminomethane (Tris-hydrochloride buffer, pH 8.0, and disrupted by three 1-min sonications with a Megason ultrasonic disintegrator at its full power at 4°C. The supernatant solution obtained by centrifugation at 12,000 × g for 30 min was used as crude enzyme preparation throughout the experiments. Pure Acinetobacter pyrocatechase was prepared from a cell-free extract of A. calcoaceticus ADP-96 (13). The reaction mixture contained, in a final volume of 1 ml (or 3 ml when monitored polarographically), 50 mM Tris-acetate buffer (pH 7.5), 0.2 mM substrate, and the enzyme preparation.

When pure Acinetobacter pyrocatechase was incubated with 3-methylcatechol, a yellow-col-
ored reaction product(s) was obtained which showed two absorption peaks, one at 260 nm and the other at 390 nm. The latter peak increased in absorbance upon addition of an alkaline solution (Fig. 1). The spectral shift to a longer wavelength or increased intensity upon alkalization is a characteristic property of α-hydroxymuconate derivatives (6). The products were chromatographed on a precoated, 0.1-mm-thick cellulose thin-layer plate (E. Merck AG, Darmstadt, Germany), with pyridine-isomyl alcohol-acetic acid-water (4:2:1:2, vol/vol) as the solvent system. The intradiol cleavage product was detected under ultraviolet radiation, and the extradiol cleavage product and unreacted substrate were visualized, as yellow and dark brown spots, respectively, after spraying with 14% aqueous ammonium hydroxide. The $R_f$ values of the products are identical to intradiol ($R_f$ 0.90) and proximal extradiol ($R_f$ 0.75) cleavage products of 3-methylcatechol by the pyrocatechase of $P$. arvilla (1). The time courses of the optical density changes are also shown in Fig 1. The extradiol cleavage reaction was completed after about 30 min under the assay conditions.

To determine whether the differences in abilities and, possibly, in the ratio of intradiol and extradiol cleavage activities of these isofunctional dioxygenases are genus dependent or strain dependent, a survey was conducted using 3-methylcatechol as substrate. Some of the microorganisms metabolize benzoate and catechol by an alternative pathway, involving, for example, a ferrous-containing catechol 2,3-dioxygenase, and do not have pyrocatechase activity (2). Therefore, only those crude enzyme preparations having pyrocatechase activity were compared for their intradiol and extradiol cleavage activities. Results were expressed as reaction rates (nanomoles of product formed per minute per milligram of protein) and are shown in Table 1. The extinction coefficients for the products of 3-methylcatechol were 18,000 at 260 nm (pH 7.5) and 44,600 at 390 nm (pH 12.0), respectively (1). Protein concentrations were determined spectrophotometrically at 260 and 390 nm, respectively (1, 7).

![Fig. 1. Absorption spectra of the reaction product of Acinetobacter pyrocatechase with 3-methylcatechol as substrate. The reaction mixture contained, in a final volume of 1 ml, 50 mM Tris-acetate buffer (pH 7.5), 0.1 mM 3-methylcatechol, and 125 µg of Acinetobacter pyrocatechase. The spectral changes were scanned at various times, as shown in the inset; O.D., optical density. After completion of the reaction (30 min), the absorption spectra were recorded before (solid line) and after (broken line) addition of 10 µl of 5 N NaOH, using the same reaction mixture, but without 3-methylcatechol as a blank.](https://apm.asm.org/)

### Table 1. Ratio of intradiol and extradiol cleavage activities of 3-methylcatechol among isofunctional dioxygenases from various microorganisms

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Catechol</th>
<th>Intradiol cleavage</th>
<th>Extradiol cleavage</th>
<th>Ratio (intradiol/ extradiol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter calcoaceticus ATCC 14987</td>
<td>230</td>
<td>10.7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Acinetobacter calcoaceticus ADP-96</td>
<td>490</td>
<td>55</td>
<td>4.5</td>
<td>12</td>
</tr>
<tr>
<td>Pure enzyme preparation</td>
<td>20,000</td>
<td>1,086</td>
<td>90</td>
<td>12</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 23975</td>
<td>690</td>
<td>42</td>
<td>2.4</td>
<td>17</td>
</tr>
<tr>
<td>Pseudomonas oleovorans POIR</td>
<td>370</td>
<td>12</td>
<td>1.6</td>
<td>8</td>
</tr>
<tr>
<td>Nocardia opaca</td>
<td>310</td>
<td>21</td>
<td>1.1</td>
<td>19</td>
</tr>
<tr>
<td>Alcaligenes eutrophus</td>
<td>270</td>
<td>15.8</td>
<td>1</td>
<td>16</td>
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<tr>
<td>Arthrobacter crystallopoietes</td>
<td>10</td>
<td>12</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Arthrobacter poons</td>
<td>30</td>
<td>32</td>
<td>0</td>
<td></td>
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<tr>
<td>Brevibacterium flavum</td>
<td>110</td>
<td>74</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Corynebacterium sp. ATCC 21235</td>
<td>670</td>
<td>21</td>
<td>5</td>
<td>4</td>
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</tbody>
</table>

* Enzyme activity was expressed as nanomoles of product formed per minute per milligram of protein. For assay, cell-free extract (150 – 500 µg of protein) was used in a final volume of 1 ml of reaction mixture containing 0.2 mM substrate and 50 mM Tris-acetate buffer, pH 7.5. In the case of the pure enzyme preparation, 9 µg of enzyme protein was used. Enzyme activities for intradiol and extradiol cleavages were determined spectrophotometrically at 260 and 390 nm, respectively (1, 7).
determined by the method of Lowry et al. (8). Among the microorganisms tested, strains of both Arthrobacter and Brevibacterium showed no extradiol cleavage activity. Among those iso-functional dioxygenases having both activities, the reaction ratio of intradiol and extradiol cleavage activities varied between 3 and 19. As had been reported for Pseudomonas pyrocatechase (intradiol/extradiol = 17) (1), it was also found for Acinetobacter pyrocatechase (intradiol/extradiol = 12) that the ratio of intradiol and extradiol cleavage activities is the same in both crude and pure enzyme preparations.

Crude enzyme preparations from various microorganisms were studied for their substrate specificities by using a monitor for dissolved oxygen. Results are shown in Table 2. The iso-functional dioxygenases from Arthrobacter and Brevibacterium, the enzymes with no extradiol cleavage activity, oxidized both 3-methylcatechol and 4-methylcatechol at almost the same rate as catechol. The pyrocatechase from the other microorganisms, except A. calcoaceticus ADP-96, oxidized 4-methylcatechol at a rate about one order greater than 3-methylcatechol. This may be due to the fact that strain ADP-96 is a regulatory gene mutant that produces the pyrocatechase constitutively in the absence of its inducer (13). Only the crude enzyme preparation from Brevibacterium flavum shows pyrogallol oxidation.

**LITERATURE CITED**


