Extradiol Cleavage of 3-Substituted Catechols by an Intradiol Dioxygenase, Pyrocatechase, from a Pseudomonad*

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MOTOKAZU FUJIWARA,† L. A. GOLOVLEVA,§ YUKIKAZU SAEKI, MITSUHIRO NOZAKI, AND OSAMU HAYAISHI
From the Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto, Japan

SUMMARY

Pyrocatechase (catechol 1,2-oxidoreductase (decyclizing), EC 1.13.11.1), a ferric ion-containing dioxygenase from Pseudomonas arvilla C-1, catalyzes the intradiol cleavage of catechol with insertion of 2 atoms of molecular oxygen to form cis,cis-muconic acid. The enzyme also catalyzed the oxidation of various catechol derivatives, including 4-methylcatechol, 4-chlorocatechol, 4-formylcatechol (protocatechualdehyde), 4,5-dichlorocatechol, 3,5-dichlorocatechol, 3-methylcatechol, 3-methoxycatechol, and 3-hydroxyacetophenone (pyrogallol). All of these substrates gave products having an absorption maximum at around 260 nm, which is characteristic of cis,cis-muconic acid derivatives. However, when 3-methylcatechol was used as substrate, the product formed showed two absorption maxima at 390 and 260 nm. These two absorption maxima were found to be attributable to two different products, 2-hydroxy-6-oxo-2,4-heptadienoic acid and 5-carboxy-2-methyl-2,4-pentadienoic acid (2-methylmuconic acid). The former was produced by the extradiol cleavage between the carbon atom carrying the hydroxyl group and the carbon atom carrying the methyl group; the latter by an intradiol cleavage between two hydroxyl groups. Since these products were unstable, they were converted to and identified as 6-methylpyridine-2-carboxylic acid and 2-methylmuconic acid, respectively. Similarly, 3-methoxycatechol gave two products, namely, 2-hydroxy-5-methoxy-2,4-pentadienoic acid and 5-carboxy-2-methoxy-2,4-pentadienoic acid (2-methoxymuconic acid).

With 3-methylcatechol as substrate, the ratio of intradiol and extradiol cleavage activities of Pseudomonas pyrocatechase during purification was almost constant and was about 17. The final preparation of the enzyme was homogeneous when examined by disc gel electrophoresis and catalyzed both reactions simultaneously with the same ratio as during purification. All attempts to resolve the enzyme into two components with separate activities, including inactivation of the enzyme with urea or heat, treatment with sulphydryl-blocking reagents or chelating agents, and inhibition of the enzyme with various inhibitors, proved unsuccessful.

These results strongly suggest that Pseudomonas pyrocatechase is a single enzyme, which catalyzes simultaneously both intradiol and extradiol cleavages of some 3-substituted catechols.

Dioxygenases are a group of enzymes that catalyze the incorporation of 2 atoms of molecular oxygen into their substrates. The cleavage of the aromatic ring in nature is a function that depends largely or entirely upon this type of enzyme. When o-dihydroxylphenyl compounds (catechols) are cleaved by an individual dioxygenase, three modes of ring fission have been demonstrated so far: (a) cleavage of the bond between carbon atoms bearing the hydroxyl groups (intradiol cleavage), (b) cleavage of the bond between the carbon atoms of positions 2 and 3 (extradiol cleavage, proximal), and (c) that of positions 1 and 6 (extradiol cleavage, distal). Pyrocatechase (catechol: oxygen 1,2-oxidoreductase (decyclizing) EC 1.13.11.1), which catalyzes the conversion of catechol to cis,cis-5-carboxy-2,4-pentadienoic acid (cis,cis-muconic acid) (Equation 1), is an enzyme that catalyzes a typical intradiol cleavage (3).

\[
\text{HO}_2 + \text{O}_2 \rightarrow \text{COOH} \quad \text{(1)}
\]

Metapyrocatechase (catechol: oxygen 2,3-oxidoreductase (decyclizing) EC 1.13.11.2), which catalyzes the conversion of catechol to 2-hydroxy-6-oxo-2,4-hexadienoic acid (α-hydroxymuconic α-semialdehyde) (Equation 2), is an enzyme that catalyzes a typical extradiol cleavage (4).

\[
\text{HO}_2 + \text{O}_2 \rightarrow \text{COOH} \quad \text{(2)}
\]

When substituted catechol derivatives are used as substrate they are cleaved exclusively at the proximal site by the action of metapyrocatechase (Equation 3) (2).
Although both enzymes contain nonheme iron as a sole cofactor, the intradiol type of enzyme contains the ferric form of iron, whereas the extradiol type contains the ferrous form of iron (5, 6). It has been believed previously that the site of cleavage of an aromatic ring is strictly specific for each enzyme, namely, the ferric iron-containing dioxygenases would exclusively cleave the catechol ring in the intradiol manner, whereas the ferrous iron-containing dioxygenases would cleave it in the extradiol manner (7, 8).

In this paper, however, we present some evidence indicating that a ferric iron-containing dioxygenase, pyrocatechase, from a pseudomonad catalyzes not only an intradiol cleavage, but also an extradiol proximal cleavage when 3-substituted catechols are used as substrates (Scheme 1).

**Materials and Methods**

Two different pyrocatechase samples were prepared in the present experiments: one purified from a pseudomonad designated as P-pyrocatechase and the other from *Brevispora* designated as B-pyrocatechase.

P-Pyrocatechase was prepared as previously described (9) from extracts of *Pseudomonas arvilla* C-1 (ATCC 23974) grown with benzoyl as the sole carbon source with the following modifications. All subsequent manipulations were carried out at 4°C and centrifugations were carried out at 8,000 rpm for 15 min with a Sorvall RC2 centrifuge unless otherwise specified. One hundred grams of packed cells, wet weight, were suspended in 400 ml of 50 mM Tris-acetate buffer, pH 8.5, and disrupted by sonication with a Sonifier model W185D cell disruptor for 12 min. The residue was removed by centrifugation. To the supernatant solution, solid ammonium sulfate was then added to give 55% saturation. After stirring the mixture for 20 min, the resulting precipitate was collected by centrifugation and was suspended in a minimum volume of the Tris buffer. The suspension was dialyzed overnight against the Tris buffer. The dialyzed solution was applied on a Bio-Gel A-0.5m column (3 × 50 cm), previously equilibrated with the Tris buffer. The enzyme was eluted with the Tris buffer and the active fractions (84 ml) were combined. The enzyme solution was then charged on a DEAE-cellulose column (5 × 40 cm), equilibrated with the Tris buffer. The column was washed with 1 liter of the buffer containing 1% ammonium sulfate and the enzyme was eluted with a linear gradient established with 500 ml of the buffer containing 15% ammonium sulfate in the mixing chamber and 500 ml of the buffer containing 5% ammonium sulfate in the reservoir. The enzyme fractions with a specific activity higher than 16 were combined and concentrated to 10 ml with a Diaflo apparatus, using Diaflo membrane XM-50. The combined enzyme solution was centrifuged to remove insoluble materials and the clear supernatant solution was applied on a Sephadex G-200 column (4 × 90 cm), equilibrated with the Tris buffer. The enzyme was eluted with the same buffer, concentrated as described above and stored in a refrigerator with little loss of activity for about 1 month.

B-Pyrocatechase with a specific activity of about 15, prepared as previously described from *Brevispora fuscum* P-13 grown with phenol as the sole carbon source (10), was kindly donated by Doctors S. Senoh and K. Nagami of the Central Research Institute, Suntory Ltd.

Crystalline metapyrocatechase with a specific activity of about 300 was obtained from *P. arvilla* (ATCC 23973) by the method described previously (5, 11).

3-Methylcatechol was obtained from Koch-Light Laboratories Ltd. and purified by distillation under reduced pressure. 3-Methoxycatechol, 3-carboxycatechol, and 3-methoxycarboxylic acid were gifts of Dr. S. Senoh of the Central Research Institute, Suntory Ltd. Streptomycin sulfate was obtained from Meiji-Seika Co., Ltd., Tokyo. 6-Methylpyri dine-2-carboxylic acid (6-methylpicolinic acid) was synthesized from 2,6-lutidine (12). Purity of these compounds was established by conventional procedures. Diazomethane was prepared from N-nitrosomethyl urea by the method of Arndt (13). All other chemicals were of analytical grade and purchased from commercial sources.

**High Voltage Electrophoresis and Thin Layer Chromatography**—High voltage electrophoresis was performed with a Phorograph Original Franklin model 64, Weisloch. Samples were applied on Whatman No. 3MM, which was moistened with pyridine/acetic acid/H₂O (100/4/86) at pH 6.4. Electrophoresis was carried out at 2,200 volts and 80 mA for 90 min with the same buffer. The product formed by the intradiol cleavage was detected by an ultraviolet lamp, and that formed by the extradiol cleavage and unreacted substrate were visualized by yellow and dark brown spots, respectively, after spraying with a 14% aqueous ammonia solution.

Thin layer chromatography was carried out with cellulose or silica gel plate of 0.25 mm thickness (Merck). Solvent systems used were pyridine/isooamyl alcohol/acetic acid/water (4/2/1/2) for the products of 3-methyl catechol and chloroform/methanol/triethylamine (50/15/1) for those of 3-methoxy catechol. Spots were detected as described above for electrophoresis.

**Instrumental Spectrophotometric Measurements**—were carried out with a Union Giken model SM401 recording spectrophotometer. Infrared spectra were measured on a Hitachi model EPS32 spectrophotometer in potassium bromide disc or in chloroform. The nuclear magnetic resonance spectra were taken on a Varian T-60 spectrometer in deuteriochloroform with tetramethylsilane as an internal standard. The mass spectra were determined on a JMS-OlSG double focusing spectrometer operating at 75 e.v. Melting points were determined on a Yanagimoto micro-hot stage apparatus and were uncorrected.

**Enzyme Assay and Protein Determination**—The standard assay of P-pyrocatechase was performed spectrophotometrically by monitoring the increase in the absorbance at 293 nm (9). The reaction mixture contained in a final volume of 3 ml, 60 mM Tris-acetate buffer, pH 7.5, 0.1 mL of 10 mM catechol, and the enzyme. When 3-methylcatechol was used as substrate, catechol was replaced by the same concentration of 3-methyl catechol, and the intradiol and the extradiol cleavage activities were followed at 260 and 390 nm, respectively. B-Pyrocatechase was assayed spectrophotometrically.
Finally at 260 nm using the same reaction mixture as described above for P-pyrocatechase. Metapyrocatechase was assayed spectrophotometrically at 375 nm when catechol was used as substrate and at 390 nm when 3-methylcatechol was used as substrate, with the same reaction mixture described above. Activity of these enzymes in the presence of various substrates were also assayed polarographically by measuring oxygen consumption (14). One unit of enzyme was defined as the amount that catalyzes the utilization of 1 µmol of substrate per min at 24°C under the conditions specified above. Specific activity was defined as the number of enzyme units per mg of protein.

Protein determination for P-pyrocatechase and B-pyrocatechase was performed spectrophotometrically by measuring absorbances at 280 and 260 nm (15). The protein concentration of metapyrocatechase was determined by the method of Lowry et al. (16).

RESULTS

Substrate Specificity

Among a number of catechol derivatives tested, the following compounds were oxidized by P-pyrocatechase with the relative rates of oxygen consumption shown in parentheses: catechol (100), 4-methylcatechol (90), 3-methylcatechol (80), 4-chlorocatechol (3.6), 3-methoxycatechol (0.8), pyrogallol (0.6), 4,5-dichlorocatechol (0.3), 3,5-dichlorocatechol (0.02), and protocatechualdehyde (0.02). The following compounds were either not oxidized or oxidized at rates less than 0.01% that of catechol: 3-carboxycatechol, 3-methoxycarboxylatechol, protocatechueic acid, 4-ethoxycarboxylatechol, 4-nitrocatechol, dopamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylmandelic acid, caffeic acid, o-eresol, and guaiacol.

Spectra of Reaction Products

Among the above-mentioned substrates, catechol, 4-substituted catechol derivatives, and disubstituted catechol derivatives gave products having an absorption peak at around 260 nm. However, when P-pyrocatechase was incubated with 3-methylcatechol, a yellow colored reaction product (or products) was obtained by the action of B-pyrocatechase and metapyrocatechase, respectively. One of the products with an RF value of each spot were 0.9 and 0.75, respectively.

Fig. 2. Absorption spectra of the reaction product with 3-methylcatechol as substrate. Reaction mixture contained in a final volume of 1 ml, 50 mM Tris-acetate buffer, pH 7.5, and either 100 µM 3-methylcatechol and 0.67 mg of P-pyrocatechase (a) or 50 µM 3-methylcatechol and 0.21 mg of metapyrocatechase (b). After completion of the reaction, absorption spectra were measured before (%) and after (%) addition of 10 µl of 5 N NaOH, using the same reaction mixture without 3-methylcatechol as a blank.

Further, when P-pyrocatechase was incubated with 3-methoxy catechol, a product having an absorption peak at around 280 nm was obtained (Fig. 2b). In contrast, B-pyrocatechase gave a yellow product having two absorption peaks at 280 and 360 nm at pH 7.5 when reacted with 3-methylcatechol. The peak at 390 nm increased upon addition of an alkaline solution with simultaneous disappearance of the peak at 320 nm (Fig. 2b). The molar extinction coefficients (ε) of the products by B-pyrocatechase and metapyrocatechase were 18,000 at 260 nm (pH 7.5) and 44,600 at 390 nm (pH 12.0), respectively.

Further, when P-pyrocatechase was incubated with 3 methoxycatechol, a product having an absorption peak at around 280 nm was obtained (Fig. 3a). On the addition of an alkaline solution, a new absorption peak appeared at 370 nm accompanied by a decrease in absorption at around 300 nm. When metapyrocatechase was incubated with 3-methoxy catechol, a product was obtained with an absorption peak at 305 nm, which shifted to 370 nm upon the addition of an alkaline solution (Fig. 3b). Molar extinction coefficient of the product at 370 nm was about 21,400, at pH 12. The reaction product of 3-methoxystechol with B-pyrocatechase showed an absorption peak at 285 nm (ε = 17,800). The spectral shift to the longer wavelength of alkalization is a characteristic property of the α-hydroxymuconate derivatives (17).

Thin Layer Chromatography and Paper Electrophoresis of Reaction Products

In order to determine whether the above-mentioned spectra are attributable to a single product or two products, each formed by intradiol and extradiol cleavages, respectively, thin layer chromatography and high voltage electrophoresis of the products were carried out.

When 3-methylecathol was used as substrate, each product obtained by the action of B-pyrocatechase and metapyrocatechase gave only a single spot on cellulose thin layer chromatogram. RF values of each spot were 0.9 and 0.75, respectively. However, P-pyrocatechase gave two spots which coincided in RF values with those obtained with B-pyrocatechase and metapyrocatechase, respectively. One of the products with an RF value

Fig. 3. Absorption spectra of the reaction product with 3-methoxystechol as substrate. Reaction mixture contained in a final volume of 1 ml, 50 mM Tris-acetate buffer, pH 7.5, 100 µM 3-methoxystechol and either 0.67 mg of P-pyrocatechase (a) or 0.21 mg of metapyrocatechase (b). After completion of the reaction, absorption spectra were measured before (%) and after (%) addition of 10 µl of 5 N NaOH, using the same reaction mixture without 3-methoxystechol as a blank.
of 0.75 as well as the product by metapyrocatechase gave a red-
brown spot with 2,4-dinitrophenylhydrazine, suggesting that
both products have a carbonyl moiety. Similarly, the reaction
products of P-pyrocatechase with 3-methoxycatechol yielded two
spots. Essentially the same results were obtained on high voltage
paper electrophoresis.

Identification of Reaction Products of 3-Methylecatechol

For the identification of the reaction products, they were
converted to their stable derivatives according to the following
scheme (Scheme 2).

\[
\begin{align*}
\text{(I)} & \quad \xrightarrow{\text{metabolic cleavage}} \quad \text{(II)} \quad \xrightarrow{\text{P-pyrocatechase}} \quad \text{(III)} \\
\text{(IV)} & \quad \xrightarrow{\text{metapyrocatechase}} \quad \text{(V)} \quad \xrightarrow{\text{metapyrocatechase}} \quad \text{(VI)}
\end{align*}
\]

Isolation and Identification of Reaction Product Formed by
Intradiol Cleavage. Incubation was carried out at 25\(^\circ\)C in a final
volume of 60 ml containing 50 mM potassium phosphate buffer,
pH 7.5, 70 mg of P-pyrocatechase, and 100 mg (0.8 mmol) of
3-methylecatechol (I). The reaction was performed by stepwise
additions (0.5 ml each) of 80 mM 3-methylecatechol in order to
prevent the inhibition by excess substrate. During the reaction,
the pH was maintained at 7.5 by occasional additions of 5 N
NaOH. After completion of the reaction, pH was lowered to 1.5
by the addition of 6 N HCl. The reaction product was extracted
five times with 100 ml each of ethyl acetate. The ethyl acetate
extracts were combined, dried over anhydrous Na\(_2\)SO\(_4\), and evap-
orated to dryness to give crystals (6.4 mg). Recrystallization from
methanol/methanol (9/1) gave needles, m.p. 127\(^\circ\)C.

The product (II) was then methylated with diazomethane in
methanol. After evaporation of the solvent, the residue was sub-
jected to preparative thin layer chromatography on silica gel F\(_{254}\)
(Merck) using chloroform as a developing solvent. The band of
the main band was cut out from the plate, and extracted with
methanol. The extract was evaporated to dryness and the product
was extracted with 100 ml each of ethyl acetate. The ethyl acetate
extracts were combined, dried over anhydrous Na\(_2\)SO\(_4\), and evap-
orated to dryness. The residue was dissolved in a small amount of
methanol and applied zonally on a silica gel thin layer plate (0.5
mm thickness). The plate was developed with a solvent system
consisting of chloroform-methanol-acetic acid (9:3:1). One main
band with an RF value of 0.48 was detected with an ultraviolet
lamp and a brown band was visible near the origin with an
RF value of 0.1. The silica gel with the main band was cut out
from the plate, and extracted with methanol. The extract was
evaporated to dryness (41.5 mg). The product thus obtained
(II) showed an absorption peak at 260 nm in 0.05 M Tris-acetate
buffer, pH 7.5.

The product (II) was then methylated with diazomethane in
methanol. After evaporation of the solvent, the residue was sub-
jected to preparative thin layer chromatography on silica gel F\(_{254}\)
(Merck) using chloroform as a developing solvent. The band of
an RF value of 0.53, which was visualized under ultraviolet light,
was scraped and extracted with chloroform. After evaporation of
the solvent, the product (IV) was crystallized from chloroform
(m.p. 57-59\(^\circ\)C). Its infrared spectrum had an ester carbonyl absorp-
tion band at 1,708 cm\(^{-1}\) in chloroform. The electron impact mass
ion. Furthermore, the fragment ion peaks, suggesting the presence
peak at m/e 184 which corresponded to a molecular formula CsH\(_{12}\)O\(_4\), and a base ion
m/e 125 resulted from the loss of COOCH\(_3\) from parent ion. Furthermore, the fragment ion peaks, suggesting the presence
of methyl and methoxycarbonyl groups in the molecule, were
assigned to this product. The structure (IV) was tentatively
assigned to this product.

Isolation and Identification of Reaction Product Formed by
Extradiol Cleavage—Since the expected product (V) of extradiol
cleavage was extremely unstable, the product was converted to its
 corresponding picolinic acid derivative (VI) for final identification.
Incubation was carried out in essentially the same manner as
above using 250 mg (2.0 mmol) of 3-methylecatechol (I) and 140
mg of P-pyrocatechase in a total volume of 200 ml. After com-
pletion of the reaction, the enzyme was separated from the
product by filtering the mixture through a Diaflo membrane
(PM-10), using a Diaflo apparatus. To the filtrate, 600 ml of a
28% aqueous ammonia solution were added and the mixture was
allowed to stand for 40 hours at 25\(^\circ\)C to convert the product (V) to
the corresponding picolinic acid derivative (VI). The mixture
was evaporated to dryness and the product was extracted with
methanol. The extract was applied in a zone to a silica gel thin
layer plate (0.5 mm thickness) and developed with a mixture of
chloroform/methanol (9/1). One thick zone with an
RF value of 0.48 and another with an RF value of 0.24 were
detected by the addition of 6 N HCl. Moreover, the pattern of spin-spin couplings among
three olefinic protons and the presence of long range coupling
between methyl (2.05 ppm) and one of the olefinic protons (6.47
ppm) was assigned to this product.

The nuclear magnetic resonance spectrum (Fig. 4) showed a
broad methyl singlet at 2.05 ppm, two methoxyls at 3.77 ppm
(singlet), and three signals assignable to olefinic protons at 5.97
ppm (doublet, J = 16 Hz), 6.47 ppm (broad doublet, J = 12 and 16 Hz), and 8.10 ppm (double doublet,
J = 12 and 16 Hz). The pattern of spin-spin couplings among
three olefinic protons behaved in a trans geometry to each other (18). However,
the geometry of the double bond between C\(_2\) and C\(_3\) has not
been determined. Thus, the structure (IV) was tentatively
assigned to this product.
FIQ.
5. Infrared spectra of the picolinic acid derivative (VI) (lower tracing) from the extradiol cleavage product of α-methyl-catechol (I) and authentic 6-methylpyridine-2-carboxylic acid (upper tracing) in KBr pellet.

parison with the authentic sample, prepared from 9,6-lutidine, by infrared spectroscopy as shown in Fig. 5.

Isolation and Identification of Reaction Products of 3-Methoxycatechol

Conversion of the reaction products to their stable derivatives was carried out according to the following scheme (Scheme 3).

The reaction mixture contained in a final volume of 60 ml, 50 mM potassium phosphate buffer, pH 7.5, 140 mg of P-pyrocatechase, and 100 mg (0.7 mmol) of 3-methoxycatechol (VII). Incubation of the mixture and extraction of the reaction products by ethyl acetate were carried out in essentially the same manner as those for the reaction product of 3-methylcatechol formed by the intradiol cleavage. The dried extract was dissolved in 20 ml of ethyl acetate and applied on a Sephadex LH-20 column (3 × 70 cm) equilibrated with ethyl acetate. The products were eluted in separate fractions when the column was developed by ethyl acetate. The first fraction showed an absorption peak at 305 nm, which shifted to 370 nm upon alkalization, whereas the second fraction had an absorption peak at 285 nm which did not shift on alkalization. Each fraction was pooled separately and evaporated to dryness to give crystals. The first fraction (8.0 mg) gave Compound IX (m.p. 144-146°) and the second fraction (46 mg) Compound VIII (m.p. 176-177°). Each compound showed a single spot on a silica gel thin layer chromatogram with a solvent system of chloroform/methanol/triethylamine (50/15/1) having Rf values of 0.38 (IX) and 0.27 (VIII), respectively. Judging from the absorption spectra of the products (Fig. 3) and their molar extinction coefficients mentioned above, the ratio of the products formed by the intradiol and extradiol cleavage of 3-methoxycatechol was about 5.

Mass spectra of these products were shown in Fig. 6, a and b. Both compounds showed an identical molecular ion peak at m/e 172 corresponding to C14H10O6, whereas fragmentation patterns were different from each other as shown in the figure. Through methylation with diazomethane, the product VIII gave crystalline methyl ester (X), m.p. 48-49°, whereas the product IX
afforded another methyl ester (XI), m.p. 52-53°. Each compound showed a single spot on a silica gel thin layer chromatogram using chloroform as a developing solvent with Rf values of 0.37 (X) and 0.27 (XI), respectively. The mass spectra of both compounds showed the same molecular ion peak at m/e 200 (M+, C₇H₁₄O₅) with a different fragmentation pattern as shown in Fig. 6, c and d. However, the ester X was gradually converted into the ester XI in chloroform at 60°. Thus, it was suggested that the ester X should be a geometric isomer of the ester XI.

Nuclear magnetic resonance spectrum of ester X (Fig. 7) showed olefinic proton on C₅ at 5.72 ppm as a doublet with a coupling constant of 9.5 Hz, in addition to three methoxyl singlets at 3.73, 3.78, and 3.87 ppm. From the value of the coupling constant, the geometry of the double bond between C₄ and C₅ appeared to be in the cis configuration (18). On the other hand, C₅ proton of the XI appeared at 6.08 ppm as a doublet with coupling constant of 16 Hz. Thus, the geometry of the double bond between C₄ and C₅ in the Compound XI was assigned to be the trans configuration (18). However, the stereochemistry of the double bond between C₃ and C₄ has not been determined. Consequently, the structures of these methylated compounds derived from VIII and IX were tentatively assigned as X and XI, respectively.

**Purification of Enzyme**

The above-mentioned results indicated that two products were produced from 3-methylethanol by the action of P-pyrocatechase. One with an absorption peak at 260 nm was formed by the intradiol cleavage and the other with a peak at 390 nm formed by the extradiol cleavage. The question remained as to whether the extradiol cleavage was due to contamination by an extradiol enzyme or whether a single enzyme catalyzed both intradiol and extradiol cleavages simultaneously. In order to answer this question, ratios of the intradiol and extradiol cleavage activities were determined at each step during the purification. As shown in Table I, the ratios were almost constant throughout the purification. The final preparation obtained with Sephadex G-200 was homogeneous on ultracentrifugation and disc gel electrophoresis.

Since the possibility existed that the extradiol cleavage activity was due to contamination or to mutation, the bacteria were isolated by a plating out technique and the enzyme was purified from several different single colonies. In all cases, the

![Figure 7. Nuclear magnetic resonance spectrum of the methyl ester (X) from the intradiol cleavage product of 3-methoxycatechol in deuteriochloroform. TMS, tetramethylsilane.](image)

**Table I**

| Purification of pyrocatechase from Pseudomonas arvilla C-1 |
|-----------------|-------|------|------|--------|
| Volume | Units | Protein (mg) | Specific activity (µmol/min/mg) | Yield (%) | Intradiol to extradiol ratio |
|---------|-------|---------------|-------------------------------|----------|-----------------------------|
| Crude extracts | 475 | 17,600 | 21,300 | 0.8 | 100 | 17.0 |
| Streptomycin | 600 | 10,900 | 7,800 | 2.2 | 96 | 18.1 |
| Ammonium sulfate | 58 | 13,700 | 2,900 | 4.7 | 78 | 16.8 |
| Bio-Gel A-0.5m | 98 | 12,600 | 1,050 | 7.0 | 72 | 17.0 |
| DEAE-cellulose | 200 | 9,200 | 350 | 26.3 | 52 | 17.7 |
| Sephadex G-200 | 46 | 7,300 | 190 | 38.4 | 41 | 17.2 |

**Fig. 8. Stability of P-pyrocatechase in various concentrations of urea. Reaction mixture contained in a final volume of 1 ml, 50 mM Tris-acetate buffer, pH 8.5, 4 mg of P-pyrocatechase, and various concentrations of urea as indicated. The reaction was carried out at 24°. At each indicated time, 50- and 10-µl portions of the mixture were removed and assayed for extradiol (○-○) and intradiol (□-□) cleavage activities with 3-methylethanol as substrate.**

**Inactivation and Inhibition Studies**

**Stability**—The time course of the inactivation of these two activities was studied with various concentrations of urea (Fig. 8). As the urea concentration increased, the rate of inactivation increased. The time course of inactivation at various concentrations of urea were essentially identical for both activities. Likewise, inactivation on heating at various temperatures or on treating with a proteinase, Nagase, paralleled each other for both activities, pH profiles for stability of both activities (45°, 10 min) were also superimposed and the optimal pH was about 8.5.

**Effect of Inhibitors**—As shown in Table II, when the enzyme was pretreated with various inhibitors including oxidizing agents, sulphydryl inhibitors, and metal-chelating agents, both activities decreased to about the same extent. Metapyrocatechase was completely inactivated by 10⁻⁴ M H₂O₂ (5), but this concentration of H₂O₂ did not show any effect on the extradiol cleavage activity of P-pyrocatechase. The inhibition of the enzyme with 5 × 10⁻⁴ M Tiron (4, 5-dihydroxy-m-benzenedisulfonic acid disodium salt), a specific chelator for ferric ion, caused complete inactivation of both activities (Table II). As shown in Fig. 9, however, the absorbance at 475 nm due to the formation of a ferric ion-Tiron complex increased with concomitant loss of both activities.

All other attempts to separate the components responsible for the two activities have been unsuccessful, indicating that a single enzyme is involved in catalyzing both activities simultaneously. The next question studied was whether or not the active sites for the two activities were identical. To answer this question, the
Table II 
Effects of various inhibitors

Preincubation mixture contained in a final volume of 1 ml, 50 mM Tris-acetate buffer, pH 8.5, 0.4 mg of the enzyme and inhibitor. After incubation at 24°C for 30 min, 200- and 50-pl aliquots were removed and assayed for extradiol and intradiol cleavage activities, respectively.

| Inhibitors            | Concentration | Inhibition | |  
|-----------------------|---------------|------------| |  
|                       |               | Intradiol  | Extradiol |
| H2O2                  | 10^-3         | 0          | 0          |
| Mersalyl              | 10^-3         | 14         | 8          |
| HgCl2                 | 10^-3         | 41         | 37         |
| ApNO3                 | 10^-3         | 21         | 16         |
| N-Ethylmaleimide      | 10^-4         | 0          | 0          |
| Iodoacetamide         | 10^-4         | 0          | 0          |
| p-Chloromercuribenzoate | 2 x 10^-3    | 40         | 48         |
| EDTA                  | 10^-3         | 0          | 0          |
| o-Phenanthroline      | 10^-3         | 0          | 0          |
| α,α'-Dipyridyl        | 10^-3         | 0          | 0          |
| Tiron                 | 5 x 10^-2     | 100        | 100        |
| KCN                   | 10^-4         | 0          | 0          |

Fig. 9. Correlation between the increase in the absorbance of the chelate complex and the decrease in the enzyme activity. Reaction mixture contained in a final volume of 3 ml, 50 mM Tris-acetate buffer, pH 8.5, 12 mg of P-pyrocatechase, and 5 mM Tiron. The reaction was carried out at 24°C. At the indicated time, the absorbance at 475 nm (X—X) which indicates the formation of a chelate complex between the ferric ion and Tiron, and extradiol (●—●) and intradiol (○—○) cleavage activities were measured as described in Fig. 8.

The nature of the inhibition by the substrate analogue, o-nitrophenol, was examined. This compound was not metabolized by the enzyme, but acted as a competitive inhibitor for both activities. The Km values of 3-methylcatechol for intradiol and extradiol activities were almost the same, being 9.5 and 8.8 μM, respectively. The K values of o-nitrophenol for the two activities were also of the same order of magnitude, being 20 and 34 μM, respectively. These results seem to suggest that only a single active site is responsible for both activities.

On the other hand, the Km values for the other substrate, oxygen, were found to be 47 and 200 μM for intradiol and extradiol activities, respectively.

Discussion

P-Pyrocatechase, which has long been believed to cleave the catechol ring exclusively in an intradiol manner, appears to act on 3-methylcatechol to produce two products: a yellow one with an absorption maximum at 390 nm and a second with an absorption maximum at 260 nm. A yellow product is normally produced by the extradiol cleavage of catechol (8), and in fact, the yellow product produced by the action of P-pyrocatechase on 3-methylcatechol was found to be identical with that produced by meta-pyrocatechase, an extradiol dioxygenase. The product absorbing at 260 nm was found to be identical with that produced by Bpyrocatechase. All attempts to separate the two activities of P-pyrocatechase by such means as purification of the enzyme, inactivation under various conditions and inhibition by substrate analogues, have so far been unsuccessful. Dioxygenase reactions are believed to be highly specific with respect to the cleavage site in the catechol ring, with the ferric ion-containing dioxygenases catalyzing an intradiol cleavage and the ferrous ion-containing dioxygenases catalyzing an extradiol cleavage. However, the data presented in this paper indicate that P-pyrocatechase, a single enzyme, catalyzes two different cleavage reactions of 3-methylcatechol simultaneously.

The physiological substrate, catechol, and the 4 substituted catechol derivatives were cleaved exclusively by the enzyme in the intradiol manner. However, some 3-substituted catechol derivatives, including 3-methylcatechol and 3-methoxycatechol, were cleaved not only in the intradiol but also in the extradiol, proximal manner, but not in the extradiol, distal manner. It is of interest that 3- or 4-substituted catechol derivatives were cleaved exclusively by metapyrocatechase in the extradiol, proximal manner, but not in the distal manner (2). The ratios of intradiol and extradiol cleavages for 3-methylcatechol and 3-methoxycatechol were 17 and 5, respectively. Whether differences in the specificity of the cleavage site and in the ratio of two cleavages for different substrates are due to the electron distribution in the molecule or steric properties of these substrates needs further investigation.

The fact that the Km value for 3-methylcatechol as well as the K values for a competitive inhibitor, o-nitrophenol, was the same order of magnitude for intradiol and extradiol activities indicates that a single substrate binding site is probably responsible for both activities. However, the Km value for oxygen was different for the two activities. This is consistent with the reaction sequence proposed previously for dioxygenases (8), in which the organic substrate reacts first with the enzyme and then with oxygen. These results suggest that the initial step for both reactions may be identical, but that the reaction with oxygen is different for each. Whether or not a nonenzymatic reaction is involved in the latter step remains unsolved.

There are several other examples of enzymes that catalyze more than one reaction with a single substrate. Peroxidase is known to catalyze a variety of reactions, including peroxidation and hydroxylation of various substrates. A mixture of reaction products is formed from a single substrate (19, 20). The exact nature of the reaction has not been elucidated fully, but the formation of a free radical from the substrate appears to occur during the reaction (21). Lysine monooxygenase, a flavoprotein, which catalyzes the monooxygenation of lysine to form L-amino-norvaleronamide, catalyzes both monooxygenation and oxidation reactions simultaneously with certain substrates. Poor fit of the substrate analogues with the active site of the enzyme appears to affect the activation mechanism of oxygen by this.

1 S. Yamamoto, T. Yamauchi, T. Ohnishi, K. Maruyama, and O. Hayaishi, manuscript in preparation.

2 T. Yamauchi, S. Yamamoto, and O. Hayaishi, manuscript in preparation.
oxygenase, so that some of the substrate molecules are oxidized rather than oxygenated. Likewise, another flavoprotein mono-oxygenase, lactate oxidase (decarboxylating) catalyzes the formation of two different products, chloroacetate and pyruvate, from a novel substrate, β-chlorolactate (22).

It is also well known that several vitamin B6 enzymes can catalyze more than a single reaction (23, 24). α-Aspartate-β-decarboxylase was one of the first enzymes which was shown to catalyze additional reactions. In the presence of pyruvate, the enzyme catalyzes the β-decarboxylation and transamination of aspartate (25). With β-chloralanine as substrate, the enzyme catalyzes the formation of pyruvate, ammonia, and Cl-, as well as an inactive alkylated form of the enzyme (26).

Thus, a number of enzymes are not as specific in terms of substrate and reaction pathway as originally thought. The data presented in this paper are the first to show that a nonheme iron-containing enzyme can catalyze more than a single reaction. These results may provide a clue to better understanding of the reaction mechanism of dioxygenases.

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