Purification and Characterization of Catechol 1,2-Dioxygenase from Aniline-assimilating *Rhodococcus erythropolis* AN-13[†]

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Catechol 1,2-dioxygenase (EC 1.13.11.1) was purified to homogeneity from a cell-free extract of aniline-grown *Rhodococcus erythropolis* AN-13. The purified enzyme was stable between pH 7.0 and 10.5 and up to 40°C. The optimal pH was 7.5 for catechol as a substrate. The molecular weight of the enzyme was 37,000 by gel filtration and 36,000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This enzyme contained 3 mol of sulfhydryl groups per mol of protein, but not disulfide bonds of cystine residues. The enzymatic activity was inhibited by AgNO₃, HgCl₂, iodoacetic acid and *p*-chloromercuribenzoic acid. FeSO₄ and FeCl₃ also inhibited the activity, although the enzyme contained 1.3 g atoms of iron per mol of protein. This enzyme catalyzed the intradiol cleavage of 3- and 4-methylcatechol, but not the extradiol cleavage of 3-methylcatechol.

Aniline is metabolized by some bacterial strains through catechol and β -ketoadipic acid to inorganic end products, ammonia, carbon dioxide and water.^{1,2)} An aniline-assimilating bacterium, *Rhodococcus erythropolis* AN-13, degrades aniline at pH 5.0 to 9.0.³⁾ Aniline-grown cells of this strain show rapid oxygen uptake with catechol besides aniline,³⁾ and a high catechol 1,2-dioxygenase (EC 1.13.11.1) activity is seen in a cell-free extract of the strain.¹⁾

Catechol 1,2-dioxygenase is the initial enzyme of the β -ketoadipic acid pathway for the degradation of aromatic compounds in microorganisms. There have been several reports about the purification and characterization of this enzyme produced by bacterial^{4~7)} and yeast^{8,9)} strains. Kojima *et al.*⁵⁾ purified the catechol 1,2-dioxygenase from a cell-free extract of benzoate-grown *Pseudomonas arvilla* C-1 and reported the spectral properties associated with its catalytic activity. Varga and Neujahr⁸⁾ purified the catechol 1,2-dioxygenase of a *Trichosporon cutaneum* strain grown

on phenol and stated the molecular and catalytic properties of the purified enzyme.

Most of the catechol 1,2-dioxygenases purified and characterized, were from strains that grew on benzoate or phenol; a catechol 1,2-dioxygenase from an aniline-assimilating bacterium has not been reported. In addition, the enzymes from *Rhodococcus* strains grown on benzoate or phenol were not purified or characterized.⁶⁾ We thus attempted to purify the catechol 1,2-dioxygenase produced in aniline-grown cells of *R. erythropolis* AN-13 to characterize it. This enzyme was stable in cell-free preparations, compared with catechol 1,2-dioxygenases from other microbial sources.^{5~9)} Therefore, the purification procedure was readily established. In this paper, we report the enzyme purification, and molecular, chemical and catalytic properties of the purified enzyme.

MATERIALS AND METHODS

Chemicals. Catechol, pyrogallol and 5,5'-dithiobis(2-

[†] Microbial Metabolism of Aromatic Amines. Part V. For Part IV, see ref. 2.

nitrobenzoic acid) (DTNB) were purchased from Wako Pure Chem., Osaka. 3-Methylcatechol was from Tokyo Kasei Kogyo, Tokyo. 4-Methylcatechol and protocatechuic acid were from Nakarai Chem., Kyoto. DE-52 (DEAE-cellulose) was a product of Whatman Chem. Sepn., Clifton. DEAE-Toyopearl 650S (DEAE-cellulose) and Toyopearl HW-55 superfine were obtained from Toyo Soda MFG, Tokyo. Pharmalyte (pH 3~10), isoelectric focusing pI calibration kit (pH 3~10) and electrophoresis calibration kits HMW and LMW were from Pharmacia Fine Chem., Uppsala. Bovine serum albumin, ovalbumin and Taka-amylase A were obtained from Seikagaku Kogyo, Tokyo. Pepsin was from P-L Biochem., Milwaukee. Chymotrypsinogen A and myoglobin were products of Schwarz/Mann, Orangeburg. *cis,cis*-Muconic acid was prepared from catechol following the reaction with the cell-free extract of *R. erythropolis* AN-13.¹⁾

Strain and its growth. *R. erythropolis* AN-13 was cultured at pH 6.0 in this experiment, instead of at pH 6.8 as described in the previous report³⁾. In solution A, 3.0 g of KH_2PO_4 was replaced by 15.8 g of KH_2PO_4 per 700 ml to adjust the pH to 6.0; the pH of solution C was adjusted to 6.0. The bacterium was precultured in 21 ml of medium in 3 tubes (1.8 × 18 cm) at 30°C for 2 days with shaking and then transferred to 400 ml of the medium in a 3-liter flask. It was cultured at 30°C for 40 hr on a reciprocating shaker. The cells were harvested by centrifugation at $10,000 \times g$ for 30 min and then washed twice with 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.0). The washed cells were stored at -20°C.

Enzyme assay. The activity of catechol 1,2-dioxygenase was determined spectrophotometrically by measuring an increase in absorbance at 260 nm.¹⁰⁾ The reaction mixture contained 2.5 ml of 0.1 M potassium-sodium (K-Na) phosphate buffer (pH 7.5) and 0.1 ml of 10 mM catechol. The reaction was started at 24°C by adding 0.1 ml of enzyme solution. After incubation for 10 min, 0.5 ml of 1 N HCl was added to stop the reaction (resulting pH 1.5). Absorbance at 260 nm was read. A control sample contained 0.1 ml of the buffer used for enzyme preparations, instead of enzyme. Standard curves were linear in the range of zero to 0.6 μg of the purified enzyme (optical density of zero to 1.3 at 260 nm). The molar extinction coefficient of *cis,cis*-muconic acid, a product of the enzyme reaction, was calculated to be 19,000 under these conditions using an authentic sample. The value was larger than that at pH 7.5 reported before,¹⁰⁾ because the protonated form of *cis,cis*-muconic acid is predominant at low pH caused by the addition of HCl; an absorption peak of the product at pH 1.5 was seen at 260 nm as well as at pH 7.5. One unit of enzyme activity was defined as the amount of enzyme that formed 1 μmol of *cis,cis*-muconic acid per min. Specific activity was defined as units per mg of protein. Protein concentrations were determined by the method of Lowry *et al.*¹¹⁾

Molecular weight determination. The molecular weight of catechol 1,2-dioxygenase was determined by gel filtration on Toyopearl HW-55 superfine, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and polyacrylamide gel electrophoresis using a gel concentration gradient.¹²⁾ Detailed experimental conditions are described in figure legends.

Isoelectric focusing. The isoelectric point (pI) was determined by gel isoelectric focusing with a Pharmacia FBE 3000 flat bed apparatus (Pharmacia Fine Chem.). The purified enzyme (1.8 μg) was applied to a 5% polyacrylamide gel (15 × 230 × 1 mm) containing 3% Pharmalyte (pH 3~10). Electrophoresis was performed at the constant current of 30 mA for 13 min and then at the constant voltage of 1000 V for 87 min at 13°C. An isoelectric focusing pI calibration kit was used as standard markers. The gel was stained with 0.2% Coomassie brilliant blue R (Sigma Chem., St. Louis) in a solvent of methanol-acetic acid-water (9:2:9) for 45 min at room temperature and then destained in this solvent omitting the dye.

Iron analysis. Iron in the enzyme was determined with a Hitachi 170-10 atomic absorption spectrophotometer; 0.54 mg of enzyme in 2 ml of buffer A containing 0.02 M NaCl was used. A standard curve was made by using iron(II) ammonium sulfate (Mohr's salt).

Amino acid analysis. The enzyme (0.90 mg) was hydrolyzed with 6 N HCl at 110°C for 24, 41 and 73 hr. Amino acid analysis was carried out with a Hitachi KLA-5 amino acid analyzer. The tryptophan content of the enzyme was determined spectrophotometrically on the basis of the measurement of absorbance at 280 and 288 nm in a 6 M guanidine hydrochloride-0.02 M K-Na phosphate buffer (pH 6.5).¹³⁾ Sulfhydryl groups were measured by a spectrophotometric method at 412 nm using DTNB.¹⁴⁾ Half cystine residues were determined by measuring total sulfhydryl groups, after the reduction of disulfide bonds with dithiothreitol in 0.3% SDS.

Inhibitory tests. The enzyme (0.14 μg) was incubated with 0.1 or 1 mM inhibitor in 3 ml of 0.1 M Tris-HCl buffer (pH 7.5) at 24°C for 10 min. To the incubation mixture, 0.1 ml of 10 mM catechol was added. After 10-min incubation at 24°C, the mixture was left for 10 min in a water bath at 50°C to stop the enzymatic reaction. Absorbance at 260 nm was read in contrast to the incubation mixture omitting enzyme. For AgNO_3 , 0.1 M Tris-acetic acid buffer (pH 7.5) was used.

Substrate specificity. Intradiol cleavage activities for 3- and 4-methylcatechol and pyrogallol were assayed spectrophotometrically under the reaction conditions described above using these catechol analogues instead of catechol. Absorbance of the reaction mixture containing 3- or 4- methylcatechol as a substrate was measured im-

mediately after 10-min reaction without addition of 1 N HCl. Molar extinction coefficients of 18,000 at 260 nm for 2-methylmuconic acid and 14,000 at 255 nm for 3-methylmuconic acid were used.¹⁵⁾ A numerical value of the activity for pyrogallol could not be determined, because the molar extinction coefficient of a product derived from this substrate was not obtained.⁴⁾ The intradiol cleavage activity for protocatechuic acid was assayed by the method of Fujisawa.¹⁶⁾ The extradiol cleavage activity for 3-methylcatechol was measured spectrophotometrically at 390 nm by the method of Fujiwara *et al.*¹⁷⁾

RESULTS

Cultivation at pH 6.0

R. erythropolis AN-13 grew faster at pH 6.0 than at pH 6.8 as shown in the previous report.³⁾ We obtained about 7 g of wet cells per liter of medium on 40-hr incubation. The cells grown at pH 6.0 had higher enzymatic activity for catechol than those cultured at pH 6.8¹⁾ (Table I). Catechol 1,2-dioxygenase of *R. erythropolis* AN-13 is probably induced faster at pH 6.0 than at pH 6.8, as seen in *Frateruia* species ANA-18.²⁾

Purification of enzyme

Step 1. Cell-free extract. All operations for enzyme purification were performed at zero to 6°C. Frozen cells (14 g, wet weight) were thawed, suspended in 150 ml of 20 mM Tris-HCl buffer, pH 8.0 (buffer A) and then disrupted with a Kubota 200 M ultrasonic oscillator at 180 W for 20 min in a 30-ml batch. Intact cells and cell debris were removed by centrifugation at 20,000 × *g* for 40 min. The cell-free extract was obtained in the supernatant fluid (Fraction 1, 172 ml).

Step 2. Streptomycin sulfate treatment. To Fraction 1, 9.1 ml of 20% streptomycin sulfate was added at a final concentration of 1% with stirring. The solution was stirred for 30 min and centrifuged at 20,000 × *g* for 20 min; the precipitated supernatant was discarded and then 180 ml of supernatant fluid was obtained (Fraction 2).

Step 3. Ammonium sulfate fractionation. To Fraction 2, 31.7 g of ground ammonium sulfate was added to 30% saturation with stirring. The mixture was stirred for 30 min

and centrifuged at 20,000 × *g* for 20 min; the precipitate was discarded. To the supernatant, 31.5 g of ammonium sulfate was added to 55% of saturation with stirring. After 30-min stirring, the sample was centrifuged at 20,000 × *g* for 20 min. The precipitate was dissolved in 18 ml of buffer A; the solution was dialyzed against 1 liter of buffer A with two changes of the buffer. The dialyzed solution was 30 ml (Fraction 3).

Step 4. DE-52 chromatography. Fraction 3 was applied to a column (2 × 27 cm) of DE-52 that had been equilibrated with buffer A. The column was washed with 400 ml of buffer A. Enzymes were eluted with a linear gradient starting with 600 ml of buffer A in the mixing chamber and 600 ml of buffer A containing 0.35 M NaCl in the reservoir. Fractions (6.6 ml/tube) were collected at a flow rate of 70 ml per hr, and then protein and enzymatic activity were assayed. Two distinct enzymatic activities appeared on the chromatogram (Fig. 1). The highest peak of one activity was eluted with 0.23 M NaCl in buffer A, and that of the other with 0.30 M NaCl. The total activity of the enzyme eluted with low concentrations of NaCl was about one-twentieth that eluted with high concentrations. Therefore, only the latter effluent was subjected to further purification;

FIG. 1. Column Chromatography of Catechol 1,2-Dioxygenase on DE-52.

The enzyme solution (Fraction 3, 30 ml) was applied to a column (2 × 27 cm) of DE-52 equilibrated with buffer A. The column was washed with 400 ml of buffer A and then enzymes were eluted with a linear gradient between zero and 0.35 M NaCl in buffer A at a flow rate of 70 ml/hr. ●—●, enzymatic activity; △—△, protein; and ----, NaCl.

TABLE I. SUMMARY OF PURIFICATION OF CATECHOL 1,2-DIOXYGENASE

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Recovery (%)
1: Cell-free extract	700	1300	0.54	100
2: Streptomycin sulfate	660	1300	0.51	94
3: Ammonium sulfate	500	420	1.2	71
4: DE-52	520	64	8.1	74
5: DEAE-Toyopearl 650S	320	16	20	46
6: Toyopearl HW-55	290	12	24	41

fractions with a specific activity higher than 6.9 were pooled (Fraction 4, 92 ml).

Step 5. DEAE-Toyopearl chromatography. Fraction 4 was dialyzed against 1 liter of buffer A, and the dialyzed solution was applied to a column (2×14 cm) of DEAE-Toyopearl 650S equilibrated with buffer A. The column was washed with 250 ml of buffer A. The enzyme was eluted with a linear gradient between zero and 0.35 M NaCl in 700 ml of Buffer A. The effluent was collected in 5.2-ml fractions at a flow rate of 60 ml per hr. The highest peak of enzymatic activity was eluted with 0.24 M NaCl in buffer A. Fractions with a specific activity of 12 or more were pooled (Fraction 5, 31 ml).

Step 6. Gel filtration. Fraction 5 was concentrated to 1.5 ml with a Collodion bag, model 25 (Sartorius GmbH, Goettingen). The concentrated enzyme solution was placed on the top of a column (3×58 cm) of Toyopearl HW-55 superfine that had been washed with buffer A containing 0.2 M NaCl. The enzyme was eluted with the same buffer. The effluent was collected in 2-ml fractions at a flow rate of 26 ml per hr. The enzyme in each fraction was tested by polyacrylamide gel electrophoresis. Fractions showing a single protein band on the gel were pooled (Fraction 6, 16 ml).

The purification procedure is summarized in Table I. The specific activity of the final enzyme preparation was 24 with an overall recovery of 41%; it was increased to 44-fold of that of the cell-free extract.

Purity of enzyme

The purified enzyme showed a single protein band on the polyacrylamide gel (Fig. 2(A)).

FIG. 2. Polyacrylamide (A) and SDS-Polyacrylamide (B) Gel Electrophoresis of Purified Catechol 1,2-Dioxygenase.

(A) Polyacrylamide gel electrophoresis. The purified enzyme ($4 \mu\text{g}$) was run on a 7.5% gel of pH 8.9 at 2.5 mA/tube for 2.5 hr in a running buffer (pH 8.3) of Tris-glycine. The gel was stained with 1% amido black 10B in 7% acetic acid for 2 hr and destained in 7% acetic acid.

(B) SDS-Polyacrylamide gel electrophoresis. The enzyme ($3 \mu\text{g}$) denatured with SDS was run on a 7.5% gel containing 0.1% SDS at 6 mA/tube for 4 hr in a running buffer (pH 7.2) of 0.1% SDS-0.1 M sodium phosphate. The gel was stained with 0.5% amido black 10B in 20% acetic acid overnight and destained in 7% acetic acid.

The native enzyme was red-brown on the gel. Consequently, the location of enzyme was readily recognized, and coincided with the protein band stained with amido black 10B. In addition, the enzymatic activity for catechol was recovered from sliced gels with the red-brown color. The purified enzyme preparation also showed a single protein band on a SDS-polyacrylamide gel (Fig. 2(B)).

Stability of enzyme

The activity of the purified enzyme did not

FIG. 3. Determination of Molecular Weight by Gel Filtration.

The purified enzyme (2 mg) and markers were placed on the top of a column (3×58 cm) of Toyopearl HW-55 superfine and eluted with buffer A containing 0.2 M NaCl (A) and with buffer A omitting NaCl (B). 1, bovine serum albumin (molecular weight, 67,000); 2, ovalbumin (43,000); 3, chymotrypsinogen A (25,000); 4, myoglobin (17,800); 5, pepsin (tetramer, 144,000); 6, bovine serum albumin (dimer, 134,000); 7, ovalbumin (dimer, 86,000); and CD, catechol 1,2-dioxygenase ((A), 37,000; (B), 150,000).

decrease at all for 50 days at 6°C in buffer A containing 0.2 M NaCl without any protective agents for enzymes such as 2-mercaptoethanol or dithiothreitol. The enzyme of *R. erythropolis* AN-13 was more stable than those of *P. arvilla* C-1,⁵ *Acinetobacter calcoaceticus* ADP-96,⁶ *Pseudomonas* sp. B13,⁷ and yeast strains of *T. cutaneum*.^{8,9}

Molecular weight

The molecular weight of catechol 1,2-dioxygenase of *R. erythropolis* AN-13 was calculated to be 37,000 by gel filtration on Toyopearl HW-55 superfine, when the enzyme was eluted with buffer A containing 0.2 M NaCl (Fig. 3(A)). However, when buffer A omitting NaCl was used, it was 150,000 (Fig. 3(B)), indicating that this enzyme was readily polymerized to a tetramer in a buffer with low ionic strength. In the presence of SDS, the molecular weight of the enzyme was 36,000 (Fig. 4). In polyacrylamide gel electrophoresis at pH 8.9, the enzyme retained its activity in the gel as described above. The molecular weight of the native enzyme with its activity

FIG. 4. Determination of Molecular Weight by SDS-Polyacrylamide Gel Electrophoresis.

Electrophoresis was performed under the same conditions as described in Fig. 2(B). Electrophoresis calibration kit LMW was used as markers: 1, phosphorylase b (94,000); 2, bovine serum albumin (67,000); 3, ovalbumin (43,000). 4, carbonic anhydrase (30,000); 5, trypsin inhibitor (20,100); 6, α -lactalbumin (144,000); and CD, catechol 1,2-dioxygenase (36,000).

FIG. 5. Determination of Molecular Weight by Polyacrylamide Gel Electrophoresis Using Gel Concentration Gradient.¹²⁾

The purified enzyme (6 μ g) and markers were run on gels (6, 7.5, 8.5, 10, 12.5 and 15%) of pH 8.9 at 3 mA/tube in a running buffer (pH 8.3) of Tris-glycine, until the band of BPB was 10 mm from the bottom. The staining and destaining were carried out as described in Fig. 2(A). An electrophoresis calibration kit HMW and three other proteins were used as markers: 1, catalase (232,000); 2, lactate dehydrogenase (140,000); 3, bovine serum albumin (dimer, 134,000). 4, bovine serum albumin (67,000); 5, Taka-amylase A (51,000); 6, ovalbumin (43,000); 7, pepsin (36,000), and CD, Catechol 1,2-dioxygenase (40,000).

can be measured by this electrophoresis using a gel concentration gradient¹²⁾; the value of 40,000 was obtained for the enzyme of *R. erythropolis* AN-13 (Fig. 5). The result indicates that this enzyme migrated in the gel of pH 8.9 as a monomer without polymerization.

FIG. 6. Absorption Spectra of Catechol 1,2-Dioxygenase.

The concentrations of the enzyme were 0.33 mg/ml in curve A and 3.3 mg/ml in curve B in buffer A containing 0.2 M NaCl.

Absorption spectrum

The concentrated enzyme was red-brown, as seen on the polyacrylamide gel. Figure 6 shows the absorption spectra of the purified enzyme in the visible and ultraviolet ranges. In the visible, the enzyme had a broad absorption band in the range of 370 to 640 nm. The absorption maximum was at about 440 nm: $E_{1\text{ cm}}^{1\%} = 0.87$ at 440 nm. In the ultraviolet, the enzyme exhibited an absorption peak at 280 nm and a small shoulder at 290 nm based on the high content of tryptophan residues in the protein (Table II): $E_{1\text{ cm}}^{1\%} = 20$ at 280 nm. The E_{280}/E_{260} ratio of the enzyme was 1.8.

pI

The pI of the enzyme was determined to be 5.8 by gel isoelectric focusing.

Iron content

The enzyme of *R. erythropolis* AN-13 contained 1.1 μg of iron per 0.54 mg of protein. The result shows that it retained 1.3 g atoms of iron per mol of protein on the basis of a molecular weight of 36,000. That of *A. calcoaceticus* ADP-96 has been reported to contain 2 g atoms of iron per mol of protein on the

TABLE II. AMINO ACID COMPOSITION OF CATECHOL 1,2-DIOXYGENASE

The enzyme was hydrolyzed with 6 N HCl for 24, 41 and 73 hr. All numbers of amino acid residues except half cystine and tryptophan were obtained by extrapolation to zero time, or on the average or maximum of the three values.

Amino acid	Number of residues per molecule ^a
Aspartic acid	35
Threonine	23
Serine	10
Glutamic acid	31
Proline	29
Glycine	26
Alanine	29
Half cystine ^b	3
Valine	25
Methionine	3
Isoleucine	12
Leucine	20
Tyrosine	12
Phenylalanine	9
Lysine	6
Histidine	9
Arginine	16
Tryptophan ^c	17
Total	315

^a The values were expressed on the basis of a molecular weight of 36,000.

^b Determined by using DTNB after the reduction of disulfide bonds with dithiothreitol in 0.3% SDS.

^c Determined spectrophotometrically.¹³⁾

basis of a molecular weight of 81,000.⁶⁾

Amino acid composition

Table II shows the amino acid composition of the enzyme. The tryptophan content of the enzyme of *R. erythropolis* AN-13 was higher than those of other strains.^{6,18)} This finding was responsible for the higher value of $E_{1\text{ cm}}^{1\%}$ at 280 nm than that of *P. arvilla* enzyme,⁵⁾ and for the small shoulder at 290 nm (Fig. 6). This enzyme contained 3 mol of half cystine residues per mol of enzyme; they were all cysteine residues. Table III shows the reactivity of sulfhydryl groups in the protein molecule with DTNB in the absence or in the presence of SDS and ethylenediaminetetraacetic acid (EDTA). One sulfhydryl group reacted with

TABLE III. REACTIVITY OF SULFHYDRYL GROUPS WITH DTNB

Sulfhydryl groups were determined after 30 min of incubation at room temperature with 66 μ M DTNB in the absence or in the presence of SDS and EDTA.

	Native enzyme	Enzyme + 0.3% SDS	Enzyme + 1% SDS 10 mM EDTA
SH (mol/mol·enzyme)	0.8 (1) ^a	2.6 (3)	2.5 (3)

^a Nearest integer.

TABLE IV. EFFECTS OF VARIOUS COMPOUNDS ON ENZYMATIC ACTIVITY FOR CATECHOL

Compound	Concentration (mM)	Remaining activity (%)
None	—	100
CuSO ₄	0.1	94
FeSO ₄	0.1	44
FeCl ₃	0.1	44
AgNO ₃	0.1	0
	1	0
HgCl ₂	0.1	51
	1	0
<i>o</i> -Phenanthroline	0.1	43
<i>N</i> -Ethylmaleimide	0.1	100
	1	83
Iodoacetic acid	0.1	94
	1	36
PCMB	0.1	83
	0.5	51

The enzymatic activity was not influenced by Tiron (0.1 mM), α,α' -dipyridyl (0.1 mM), EDTA (0.1, 1 mM), NaN₃ (0.1, 1 mM) or DTNB (0.1 mM).

DTNB without the denaturant or chelating agent. The other two were modified by DTNB in the presence of 0.3% SDS. In a mixture containing 1% SDS and 10 mM EDTA, no increase of sulfhydryl groups was seen.

Optimal pH

The enzyme showed maximal activity for catechol at pH 7.5.

pH-Stability

The enzyme was incubated in buffers of pH 5.0 to 10.5 at 5°C for 24 hr, and then remaining activity was assayed. This enzyme was stable in

TABLE V. INTRADIOL CLEAVAGE ACTIVITIES FOR CATECHOL ANALOGUES

Substrate	Relative activity (%)
Catechol	100
3-Methylcatechol	127
4-Methylcatechol	75
Pyrogallol	+ ^a
Protocatechuic acid	0

^a The numerical value was not determined. See the text for details.

the range of pH 7.0 to 10.5.

Thermostability

The enzymatic activity was not decreased by heating at 40°C for 10 min. When the enzyme was heated at 45°C for 10 min, its remaining activity was 75% that of intact enzyme. The activity was lost by heating at 50°C for 10 min.

Inhibition

The effects of metal salts, and chelating and sulfhydryl agents on the activity of catechol 1,2-dioxygenase of *R. erythropolis* AN-13 were tested. Table IV shows that the activity was inhibited by AgNO₃, HgCl₂, *o*-phenanthroline, iodoacetic acid and *p*-chloromercuribenzoic acid (PCMB). The activity was also inhibited by FeSO₄ and FeCl₃, although the enzyme contained about one gram atom of iron per mol of protein. The inhibition by AgNO₃, HgCl₂, iodoacetic acid and PCMB was probably dependent on the fact that this enzyme contained 3 mol of sulfhydryl groups per mol of protein, which react with these inhibitors. The enzyme of *R. erythropolis* AN-13 was not inhibited by Tiron under conditions for these inhibitory tests, although this chelating agent inhibits the activity of *P. arvilla* enzyme.

Substrate specificity

Catechol 1,2-dioxygenases have been reported to catalyze the intradiol and extradiol cleavage of some analogues of catechol.^{4~9)} The enzyme of *R. erythropolis* AN-13 showed high activities for the intradiol cleavage of 3-

and 4-methylcatechol (Table V). The intradiol oxidation activity for 3-methylcatechol was more than that for catechol. It also oxidized pyrogallol. This enzyme did not catalyze the extradiol cleavage of 3-methylcatechol nor did it oxidize protocatechuic acid.

DISCUSSION

Catechol 1,2-dioxygenase was purified to homogeneity from the cell-free extract of aniline-grown *R. erythropolis* AN-13. The purified enzyme was markedly stable compared with those from other microorganisms grown on benzoate or phenol: No decrease of the activity was seen for 50-days storage at 6°C in buffer A containing 0.2 M NaCl; the activity also did not decrease in buffer A without NaCl. As the enzyme was stable, it was purified with the high recovery of 41% and was available for the subsequent experiments in this report.

The molecular weight of catechol 1,2-dioxygenase of *R. erythropolis* AN-13 was about 40,000 by gel filtration with buffer A containing 0.2 M NaCl, SDS-polyacrylamide gel electrophoresis and polyacrylamide gel electrophoresis using a gel concentration gradient. The molecular weights of the enzymes from other microbial strains have been reported to be 63,000 to 105,000 by gel filtration and ultracentrifugation,^{5-10,18)} although they were 30,000 to 40,000 by SDS-polyacrylamide gel electrophoresis. This discrepancy in the molecular weight of catechol 1,2-dioxygenase between *R. erythropolis* AN-13 and other strains probably arose from the buffers used for gel filtration and ultracentrifugation: The molecular weight of the enzyme of *A. calcoaceticus* ADP-96 was calculated to be 85,000 by gel filtration on Bio-Gel A-1.5 and 81,000 by ultracentrifugation using 50 mM Tris-HCl buffer (pH 8.0);⁶⁾ that of *T. cutaneum* YW 2-2 was 105,000 by gel filtration on Sephadex G-200 using 50 mM Tris-HCl buffer (pH 8.0). On the other hand, we determined the molecular weight of 37,000 for the enzyme of *R. erythropolis* AN-13 by gel filtration on

Toyopearl HW-55 using 20 mM Tris-HCl buffer, pH 8.0 (buffer A) containing 0.2 M NaCl (Fig. 3(A)). However, this enzyme was readily polymerized to a tetramer in buffer A without NaCl and gave a value of 150,000 in this buffer (Fig. 3(B)). It is concluded that the minimal molecular weight of native enzyme of *R. erythropolis* AN-13 is about 40,000.

The enzyme of *R. erythropolis* AN-13 contained 3 mol of sulfhydryl groups per mol of protein (Table III). One sulfhydryl group reacted with DTNB in the native enzyme, indicating that this sulfhydryl exists on the surface of the protein molecule, to which the reagent is capable of approaching. However, the sulfhydryl is probably not responsible for the enzymatic activity, because DTNB did not inhibit the activity of the native enzyme (Table IV). When SDS was added to the incubation mixture, two other sulfhydryl groups reacted with DTNB. These sulfhydryls should take part in the enzymatic activity since AgNO₃, HgCl₂, iodoacetic acid and PCMB inhibited the activity; these compounds probably reacted with the sulfhydryls in the protein molecule without SDS.

Catechol 1,2-dioxygenases of various bacterial strains exhibit characteristic patterns of enzymatic activities for catechol analogues.¹⁹⁾ The enzyme of *R. erythropolis* AN-13 catalyzed the intradiol cleavage of 3- and 4-methylcatechol; the activity for 3-methylcatechol was higher than that for catechol (Table V). This enzyme oxidized pyrogallol, but did not catalyze the extradiol cleavage of 3-methylcatechol nor did it oxidize protocatechuic acid. This pattern of enzymatic activities for catechol analogues by the enzyme of *R. erythropolis* AN-13 is similar to those of the gram-positive bacteria *Arthrobacter crystallopoietes*, *Arthrobacter poeppigii* and *Brevibacterium flavum* strains,¹⁹⁾ but not to those of the gram-negative strains *P. arvilla* C-1¹⁷⁾ and *A. calcoaceticus* ADP-96,¹⁹⁾ that catalyze the extradiol cleavage of 3-methylcatechol, although the intradiol oxidation activity for this substrate is low.

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