Two Catechol 1,2-Dioxygenases from an Aniline-assimilating Bacterium, *Frateuria* species ANA-18[†]

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When *Frateuria* species ANA-18 was grown on aniline, two catechol 1,2-dioxygenases (CD I and CD II, EC 1.13.11.1) were found in a cell-free extract of the strain. CD I and CD II were separated from each other by DE-52 chromatography and purified to homogeneity by successive column chromatography. The molecular weights of CD I and CD II were 38,000 and 36,000, respectively, by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. CD I contained 2 mol of sulfhydryl groups per mol of protein, but CD II did not have cysteine or cystine residues. CD I was more stable on the acidic side and against heating than CD II. The activity of CD I was inhibited by AgNO₃, HgCl₂, iodoacetic acid and *p*-chloromercuribenzoic acid, but that of CD II was not inhibited or was less affected by them because of the lack of sulfhydryl groups. CD I exhibited a high activity for the extradiol cleavage of 3-methylcatechol, and the ratio of intradiol to extradiol activity was 100:120.

In the previous report,¹⁾ we have reported the purification and characterization of catechol 1,2-dioxygenase (CD, EC 1.13.11.1) from an aniline-assimilating bacterium, *Rhodococcus erythropolis* AN-13. The other aniline-assimilating bacterium *Frateuria* species ANA-18 grows on aniline at pH 4.0 to 7.0 and degrades it more rapidly than *R. erythropolis* AN-13.²⁾ Resting cells of *Frateuria* sp. ANA-18 grown on aniline also show a faster rate of oxygen uptake with catechol than those of *R. erythropolis* AN-13. In addition, the former strain is gram-negative, and the latter gram-positive. We thus attempted to purify the CD of *Frateuria* sp. ANA-18 and characterize it compared with the CD of *R. erythropolis* AN-13.

In purification procedures, we found two comparable CD (CD I and CD II) in a cell-free extract of aniline-grown *Frateuria* sp. ANA-18, although in *R. erythropolis* AN-13, a small amount of CD other than the purified CD was seen. Dorn and Knackmuss³⁾ have also found

two CD, pyrocatechase I and pyrocatechase II, in 3-chlorobenzoic acid-grown cells of *Pseudomonas* sp. B13. Pyrocatechase II shows high relative activities with 3- and 4-chlorocatechol compared with catechol. Pyrocatechase I was purified 29-fold, but the specific activity of pyrocatechase II is not increased by the purification procedure since it is unstable on chromatography and dialysis. Consequently, the molecular and chemical properties of pyrocatechase II are not clarified completely.

In the present study, however, we could separate CD I and CD II of *Frateuria* sp. ANA-18 from each other by chromatography on DE-52 and purify them to homogeneity. The two purified enzymes were stable and therefore good materials for various tests as to enzyme chemistry. This report describes the separation and purification of CD I and CD II, and molecular, chemical and catalytic differences between the two enzymes in comparison with the CD of *R. erythropolis* AN-13.¹⁾

[†] Microbial Metabolism of Aromatic Amines. Part VI. For Part V, see ref. 1.

MATERIALS AND METHODS

Materials. Cellex E (ECTEOLA-cellulose) was a product of Bio-Rad Lab., Richmond. Other chemicals had been described in the previous paper.¹⁾

Strain and culture. *Frateruia* sp. ANA-18 was pre-cultured in 18 ml of aniline medium²⁾ at 30°C for 2 days with shaking and then transferred to 450 ml of the medium in a 3-liter flask. The bacterium was cultured at 30°C for 24 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). About 6 g of wet cells was obtained per liter of medium. The washed cells were stored at -20°C.

Enzyme assays and analytical methods. These were carried out by the methods reported in the previous paper.¹⁾

RESULTS

Purification of enzymes

Steps 1 to 3. All operations for purification of enzymes were performed at 0~6°C. Frozen cells (16.4 g, wet weight) of *Frateruia* sp. ANA-18 were used for the enzyme preparation. The preparation of a cell-free extract (Step 1, Fraction 1, 110 ml), streptomycin sulfate treatment (Step 2, Fraction 2, 113 ml), and ammonium sulfate fractionation (Step 3, Fraction 3, 30 ml) were carried out essentially by the same methods as described in the previous report.¹⁾

Step 4. DE-52 chromatography. Fraction 3 was dialyzed against 1 liter of 20 mM Tris-HCl buffer, pH 8.0 (buffer A) with two changes of the buffer. The dialyzed solution was applied to a column (2 × 25 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 0.3 M NaCl in the reservoir. Fractions of 6.6 ml were collected at a flow rate of 70 ml per hr, and then protein and enzymatic activity were measured. Figure 1 shows that two enzymatic activities were separated from each other by DE-52 chromatography. The highest peak of the activity of CD I was eluted with 0.16 M

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

The enzyme solution (Fraction 3, 45 ml) was applied to a column (2 × 25 cm) of DE-52 equilibrated with buffer A. The column was washed with 400 ml of buffer A. Enzymes were eluted with a linear gradient between zero and 0.3 M NaCl in 1200 ml of buffer A at a flow rate of 70 ml/hr. ●—●, enzymatic activity; △—△, protein; -----, NaCl.

NaCl in buffer A, and that of CD II with 0.22 M NaCl. Fractions with specific activities higher than 3.2 for CD I (Fraction 4-I, 79 ml) and 6.1 for CD II (fraction 4-II, 79 ml) were pooled separately.

Step 5. DEAE-Toyopearl chromatography. Fractions 4-I and 4-II were dialyzed against 1 liter of buffer A without changes of the buffer. The dialyzed samples were applied separately to columns (2.2 × 13 mm for the two enzymes) of DEAE-Toyopearl 650S equilibrated previously with buffer A. Each column was washed with 250 ml of buffer A. CD I and CD II were eluted with a linear gradient between zero and 0.3 M NaCl in 700 ml of buffer A. Fractions of 5.2 ml were collected for the two enzymes at a flow rate of 70 ml per hr. The highest peaks of enzymatic activity of CD I and CD II appeared at 0.16 and 0.20 M NaCl, respectively, in buffer A. Fractions with specific activities higher than 7.2 for CD I (Fraction 5-I, 42 ml) and 17 for CD II (Fraction 5-II, 42 ml) were pooled.

Step 6. Gel filtration. Fractions 5-I and 5-II were concentrated to 1.5 ml separately with a Collodiol bag, model 25 (Sartorius GmbH,

TABLE I. SUMMARY OF PURIFICATION OF CD I AND CD II

Fraction	Total activity (units)		Total protein (mg)		Specific activity (units/mg)		Recovery (%)	
	CD I	CD II	CD I	CD II	CD I	CD II	CD I	CD II
1: Cell-free extract	1100		1500		0.73		100	
2: Streptomycin sulfate	1100		1500		0.73		100	
3: Ammonium sulfate	810		670		1.2		74	
4: DE-52	530	350	120	39	4.4	9.0	48	32
5: DEAE-Toyopearl	350	230	30	9.9	12	23	32	21
6: Toyopearl HW-55	300	160	20	6.8	15	24	27	15
7: Cellex E	170	120	9.9	4.8	17	25	15	11

Goettingen). The concentrated samples were placed separately on top of columns (3×58 cm) of Toyopearl HW-55 superfine equilibrated with buffer A containing 0.2 M NaCl. Each enzyme was eluted with buffer A containing 0.2 M NaCl at a flow rate of 24 ml per hr with a 2-ml sample per tube. Fractions with specific activities higher than 13 for CD I (Fraction 6-I, 12 ml) and 23 for CD II (Fraction 6-II, 10 ml) were pooled.

Step 7. Cellex E chromatography. Fractions 6-I and 6-II were dialyzed separately against 1 liter of buffer A with one change of the buffer. Each dialyzed solution was applied to a column (1.5×17 cm) of Cellex E equilibrated with buffer A. The columns were washed with 250 ml of buffer A. CD I and CD II were eluted with a linear gradient between zero and 0.2 M NaCl, and zero and 0.25 M NaCl, respectively, in 500 ml of buffer A. Fractions of 5.2 ml were collected for the two enzymes at a flow rate of 60 ml per hr. The highest peaks of the activity of CD I and CD II appeared at 0.07 and 0.11 M NaCl, respectively, in buffer A. Each fraction was subjected to analytical polyacrylamide gel electrophoresis to examine the enzyme purity. Fractions showing a single protein band were pooled (Fraction 7-I for CD I, 73 ml; Fraction 7-II for CD II, 78 ml). The pooled samples were dialyzed separately against 1 liter of buffer A containing 0.1 M NaCl.

The purification procedures are summarized in Table I. The specific activities of CD I and CD II were 17 and 25 units per mg of protein,

FIG. 2. Polyacrylamide Gel Electrophoresis of Purified CD I and CD II.

The purified CD I ($7 \mu\text{g}$) and CD II ($6 \mu\text{g}$) were run on 7.5% gels of pH 8.9 at 2.5 mA/tube for 110 min in a running buffer (pH 8.3) of Tris-glycine. The gels were stained with 1% amido black 10B in 7% acetic acid for 2 hr and destained in 7% acetic acid.

respectively, with an overall recovery of 26% as CD I plus CD II.

Purity

Final preparations of CD I and CD II showed single protein bands on the polyacrylamide gels (Fig. 2). As native CD I and CD II were red-brown on the gels, the locations of these enzymes were recognized, and they coincided with the protein bands stained with amido black 10B. In addition, CD activity was recovered from sliced gels with the red-brown color. CD II migrated a little faster than CD I on the gel (Fig. 2). When the mixture of CD I and CD II was applied to the gel, however, they showed only a single broad protein band without separation. The results

indicate that total charges on the protein molecules of CD I and CD II were similar to each other under the conditions used for this electrophoresis. The final preparations of CD I and CD II also showed single protein bands on sodium dodecyl sulfate (SDS)-polyacrylamide gels.

Stability of enzymes

No decrease of the activities of purified CD I and CD II was seen for 50 days of storage at 6°C in buffer A containing 0.1 M NaCl without any additions.

Molecular weight

The molecular weights of CD I and CD II were calculated to be 37,000 by gel filtration on Toyopearl HW-55 superfine, when buffer A containing 0.2 M NaCl was used as an effluent. When buffer A omitting NaCl was used, however, the molecular weights of the two enzymes were increased to 140,000, as seen in CD of *R. erythropolis* AN-13.¹⁾ In the presence of SDS, the molecular weights of CD I and CD II were 38,000 and 36,000, respectively (Fig. 3). Polyacrylamide gel electrophoresis using a gel concentration gradient gave the molecular weight of 42,000 to both CD I and CD II. These results shows that CD I and CD II have the molecular weight of about 40,000 and that they were readily polymerized to a tetramer in a buffer with low ionic strength, like buffer A.

Absorption spectrum

In the visible range, the two enzymes exhibited broad absorption bands in the range of 360 to 640 nm. The absorption maximum was at about 440 nm for the two: $E_{1\text{ cm}}^{1\%} = 0.78$ at 440 nm for CD I; $E_{1\text{ cm}}^{1\%} = 0.83$ for CD II. The two enzymes also showed absorption peaks at 280 nm: $E_{1\text{ cm}}^{1\%} = 10$ at 280 nm for CD I; $E_{1\text{ cm}}^{1\%} = 11$ for CD II. The values at 280 nm were about half that of CD of *R. erythropolis* AN-13, because the content of tyrosine and tryptophan residues in CD I and CD II was much less than that in the *R. erythropolis* enzyme¹⁾ (Table II). The E_{280}/E_{260} ratios of

FIG. 3. Determination of Molecular Weights of CD I and CD II by SDS-Polyacrylamide Gel Electrophoresis.

CD I (4 μg), CD II (4 μg) and markers denatured with SDS were run on 7.5% gels 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 gels were stained with 0.5% amido black 10B in 20% acetic acid overnight and destained in 7% acetic acid. Electrophoresis calibration kit LMW was used as markers: 1, phosphorylase *b* (molecular weight, 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 (14,400); CD I (38,000); and CD II (36,000).

CD I and CD II were 1.7 and 1.5, respectively.

Isoelectric point (pI)

The pIs of CD I and CD II were 5.2 and 5.0, respectively, by gel isoelectric focusing.

Iron content

CD I contained 1.0 μg of iron per 0.55 mg of protein; there was 0.90 μg of iron per 0.50 mg of CD II. These results show that both CD I and CD II contained 1.2 g atoms of iron per mol of protein on the basis of molecular weights of 38,000 and 36,000 for CD I and CD II, respectively.

Amino acid composition

The amino acid compositions of CD I and CD II are listed in Table II that shows numbers of amino acid residues per molecule and per 1000 residues. The compositions of hydrophobic amino acid residues of the two enzymes were similar to each other. Those of basic amino acid residues, however, were distinctly different between them: CD I contained more lysine and histidine residues than CD II, but CD I more arginine residues than CD

II, with the result that the pIs of the two enzymes were not very distinct. In addition, CD I had more threonine residues than CD II,

and CD II more methionine and phenylalanine than CD I.

TABLE II. AMINO ACID COMPOSITIONS OF CD I AND CD II

CD I (0.88 mg) and CD II (1.0 mg) were hydrolyzed with 6N HCl at 110°C 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		Number of residues per 1000 residues	
	CD I	CD II	CD I	CD II
Aspartic acid	43	41	126	130
Threonine	21	13	62	41
Serine	11	9	32	29
Glutamic acid	31	34	91	108
Proline	19	19	56	60
Glycine	37	33	109	105
Alanine	35	30	103	95
Half cystine ^b	2	0	6	0
Valine	24	20	70	64
Methionine	1	2	3	6
Isoleucine	12	11	35	35
Leucine	33	30	97	95
Tyrosine	8	9	23	29
Phenylalanine	12	17	35	54
Lysine	21	10	62	32
Histidine	11	8	32	25
Arginine	10	19	29	60
Tryptophan ^c	10	10	29	32
Total	341	315	1000	1000

^a The values were expressed on the basis of molecular weights of 38,000 for CD I and 36,000 for CD II.

^b Determined by using DTNB after the reduction of disulfide bonds with dithiothreitol in the presence of 0.3% SDS.⁽⁶⁾

^c Determined spectrophotometrically.⁽⁷⁾

CD I contained 2 mol of sulfhydryl groups of cysteine per mol of enzyme, but we could not find cysteine or cystine residues in the protein of CD II (Tables II and III). The sulfhydryl groups of native CD I did not react with DTNB (Table III). When the protein was denatured by the addition of 0.3% SDS, two sulfhydryl groups in the protein reacted with the reagent. An increase of sulfhydryl groups was not seen for the two enzymes in the incubation mixtures containing 1% SDS and 10 mM ethylenediaminetetraacetic acid (EDTA). These results indicate that the sulfhydryl groups of native CD I are buried in the protein molecule and therefore did not react with DTNB without SDS, and that those of the denatured enzyme are probably free to react with DTNB, instead of binding to the iron contained in the protein.

Optimal pH

CD I and CD II showed maximal enzymatic activity for catechol at pH 7.5. In the range of pH 6.5 to 10, the enzymes had the activity higher than 80% of the maximum at pH 7.5.

pH-Stability

CD I and CD II were incubated in buffers of pH 5.0 to 10.5 at 5°C for 24 hr, and then the remaining activity was assayed. The remaining activity of the two enzymes did not decrease in the range of pH 7.5 to 10.5. At pH 7.0, the remaining activity of CD I was 97% of that of intact enzyme; that of CD II was 85%. At pH 6.5, the activity of CD I was 59% of that of the

TABLE III. REACTIVITY OF SULFHYDRYL GROUPS IN CD I AND CD II WITH DTNB

Sulfhydryl groups were determined after 30 min of incubation at room temperature with 66 μ M 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the absence or in the presence of SDS and EDTA.

SH (mol/mol enzyme)	Native enzyme		Enzyme + 0.3% SDS	Enzyme + 1% SDS 10 mM EDTA
	CD I	0 (0) ^a	1.6 (2)	1.6 (2)
	CD II	0 (0)	0.2 (0)	0.2 (0)

^a Nearest integer.

intact enzyme; that of CD II was 26%. These results show that CD I was more stable on the acidic side than CD II.

Thermostability

The activity of CD I was not influenced by heating at 35°C for 10 min, but that of CD II was decreased to 89% of that of intact enzyme. When CD I was heated at 45°C for 10 min, the remaining activity was 18% of that of intact enzyme. On the other hand, that of CD II was only 2% of that of intact enzyme. These results show that CD I had higher thermostability than CD II.

Inhibitory effects of compounds

The effects of metal salts and chelating and sulfhydryl agents on the enzymatic activity of CD I and CD II were tested. The activity of CD I for catechol was inhibited by AgNO₃, HgCl₂, iodoacetic acid and *p*-chloromercuribenzoic acid (PCMB), but that of CD II was not inhibited or was less affected by these compounds (Table IV). CD II did not contain any sulfhydryl groups in the protein as described above, which is presumably why the activity of CD II was less influenced by these metal salts and sulfhydryl agents than that of CD I. *o*-Phenanthroline inhibited the activity of both enzymes. The activity of CD II was increased to 130% of that of the intact enzyme by the addition of FeSO₄ or FeCl₃; that of CD I was not affected by these iron salts. The reason that the activity of CD II was increased by them, was not further investigated in this report.

Substrate specificity

Table V shows the differences in substrate specificity for catechol analogues between CD I and CD II. CD II catalyzed the intradiol cleavage of 3- and 4-methylcatechol at rates of 13 and 24%, respectively, of that of catechol; CD I, on the other hand, catalyzed the intradiol oxidation of 3-methylcatechol at a rate less than 0.9% of that of catechol, although it oxidized 4-methylcatechol at a rate of 36%. CD I and CD II did not oxidize pyrogallol or

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

Compound	Concentration (mM)	Remaining activity (%)	
		CD I	CD II
None	—	100	100
CuSO ₄	0.1	94	108
FeSO ₄	0.1	103	130
FeCl ₃	0.1	99	130
AgNO ₃	0.1	0	92
	1	0	86
HgCl ₂	0.1	64	101
	1	14	82
<i>o</i> -Phenanthroline	0.1	42	51
Iodoacetic acid	0.1	100	100
	1	18	100
PCMB	0.1	54	108
	0.5	39	106

The enzymatic activity of CD I and CD II was not influenced by Tiron (0.1 mM), α, α' -dipyridyl (0.1 mM), EDTA (0.1, 1 mM), NaN₃ (0.1, 1 mM), *N*-ethylmaleimide (0.1, 1 mM) or DTNB (0.1 mM).

TABLE V. INTRADIOL CLEAVAGE ACTIVITIES FOR CATECHOL ANALOGUES

Substrate	Relative activity (%)	
	CD I	CD II
Catechol	100	100
3-Methylcatechol	<0.9	13
4-Methylcatechol	36	24
Pyrogallol	0	0
Protocatechuic acid	0	0

protocatechuic acid.

The extradiol cleavage activities for 3-substituted catechols by CD have been reported in some bacterial strains.^{3~5)} CD I and CD II catalyzed the extradiol oxidation of 3-methylcatechol; the activities of CD I and CD II were 0.18 and 0.093 units per mg of enzyme, respectively (Table VI). However, the ratios of intradiol to extradiol activity of CD I and CD II were 100:120 and 100:3, respectively; CD I had higher activity for the extradiol cleavage of 3-methylcatechol than that for the intradiol. Spectra of the intradiol and extradiol cleavage activities for this substrate by the two enzymes

TABLE VI. RATIOS OF INTRADIOL TO EXTRADIOL CLEAVAGE ACTIVITY FOR 3-METHYLCATECHOL

The enzymatic activities of CD I and CD II were assayed by the method described in the legend of Fig. 4.

	Intradiol cleavage activity (units/mg· enzyme) ^a	Extradiol cleavage activity (units/mg· enzyme) ^b	Ratio (intradiol: extradiol)
CD I	0.15	0.18	100:120
CD II	3.0	0.093	100:3

^a Molar extinction coefficient of 18,000 at 260 nm was used.⁸⁾

^b Molar extinction coefficient at 390 nm was calculated to be 15,000 at pH 7.5 from Fig. 2 in ref. 4.

FIG. 4. Absorption Spectra of Reaction Products of 3-Methylcatechol Reacting with CD I and CD II.

CD I (50 μ g) and CD I (3.8 μ g) were incubated at 24°C in 3.3 ml of mixtures consisting of the following: 0.1 M K-Na phosphate buffer (pH 7.5), 2.5 ml; 10 mM 3-methylcatechol, 0.1 ml; enzyme, 0.2 ml; and water, 0.5 ml. After 25 min of incubation, absorption spectra of the mixtures were measured in contrast to the same incubation mixture omitting enzyme. Curve A, CD I; curve B, CD II.

are shown in Fig. 4.

DISCUSSION

The aniline-assimilating bacterium *Frateruia* sp. ANA-18 produced two distinct CD, CD I and CD II, in its cells. CD I and CD II were separated from each other by chromatography on DE-52 and purified to homogeneity on

polyacrylamide gels. Pyrocatechase I, found in 3-chlorobenzoic acid-grown cells of *Pseudomonas* sp. B13, was purified 29-fold with a few minor contaminating proteins, but the specific activity of pyrocatechase II was not increased because of its instability.³⁾ But CD I and CD II of *Frateruia* sp. ANA-18 were stable during the purification procedures, and the activity of the purified enzymes did not decrease at all for 50 days of storage at 6°C without any additions. These characteristics of the two enzymes were favorable for investigations on the molecular, chemical and catalytic differences between CD I and CD II.

The two enzymes were similar in molecular weight, isoelectric point, iron content and absorption spectra in the ultraviolet and visible ranges. Although the amino acid compositions of the two enzymes were similar in the composition of hydrophobic amino acid residues, the contents of basic and sulfur-containing amino acid residues were different.

CD I had 2 mol of sulfhydryl groups per mol of enzyme that reacted with DTNB in the presence of SDS. The CD of *R. erythropolis* AN-13¹⁾ also contains 2 mol of the sulfhydryl groups per mol of protein that reacted with DTNB in the presence of SDS, besides one mol of the sulfhydryl reacting with the reagent without SDS. AgNO₃, HgCl₂, iodoacetic acid and PCMB which inhibit the activity of CD of *R. erythropolis* AN-13, likewise inhibited that of CD I of *Frateruia* sp. ANA-18. The activity of CD II, however, was not inhibited or was less affected by these compounds; CD II did not have cysteine residues in its protein. It seems to be due to the lack of sulfhydryl groups in CD II that the activity of CD II was less influenced by these compounds than that of CD I and CD of *R. erythropolis* AN-13. No CD that is free from cysteine or cystine residues has been reported, other than CD II.

The specific activity of CD II for catechol was 1.5-fold that of CD I. CD I and CD II catalyzed the intradiol cleavage of 3- and 4-methylcatechol, but the activity for 3-methylcatechol by CD I was markedly low (less than 0.9% that for catechol) (Table V). Higher

activity of CD I than that of CD II was found for the extradiol cleavage of 3-methylcatechol (Table VI). In addition, the ratios of intradiol to extradiol cleavage activity for it by CD I and CD II were 100:120 and 100:3, respectively. CD I is the first enzyme that has been reported to exhibit higher activity for the extradiol oxidation of 3-methylcatechol than that for the intradiol.

CD I was more stable on the acidic side than CD II; CD I also showed more thermostability against heating than CD II. On the other hand, the activity of CD II for catechol was not inhibited by the compounds given above or less influenced than that of CD I. Furthermore, CD II showed higher activity for the intradiol cleavage of 3-methylcatechol than CD I, and lower activity for the extradiol oxidation than CD I. These relationships between CD I and CD II in chemical and catalytic characteristics seem "complementary": CD I would compensate for the instability of CD II on the acidic side and to heating; CD II, on the other hand, would compensate for the weakness of CD I to inhibitors of AgNO_3 , HgCl_2 , iodoacetic acid and PCMB; and in catalysis for 3-methylcatechol, CD I would mainly take part in the extradiol

cleavage and CD II in the intradiol.

CD I and CD II of aniline-assimilating *Frateruria* sp. ANA-18 thus are good materials for investigation on the role of isofunctional enzymes in organisms. Further investigations on active sites of the two enzymes as to substrate specificity and inductive and regulatory mechanisms of the enzymes in aniline degradation should be carried out.

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