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Purification and Characterization of *meta*-Cleavage Compound Hydrolase from a Carbazole Degrader *Pseudomonas resinovorans* Strain CA10

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2-Hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoic acid [6-(2′-aminophenyl)-HODA] hydrolase, involved in carbazole degradation by *Pseudomonas resinovorans* strain CA10, was purified to near homogeneity from an overexpressing *Escherichia coli* strain. The enzyme was dimeric, and its optimum pH was 7.0–7.5. Phylogenetic analysis showed the close relationship of this enzyme to other hydrolases involved in the degradation of monocyclic aromatic compounds, and this enzyme was specific for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (6-phenyl-HODA), having little activity toward 2-hydroxy-6-oxohepta-2,4-dienoic acid and 2-hydroxymuconic semialdehyde. The enzyme had a K_m of 2.51 μM and k_{cat} of 2.14 (s^{-1}) for 6-phenyl-HODA (50 mM sodium phosphate, pH 7.5, 25°C). The effect of the presence of an amino group or hydroxyl group at the 2′-position of phenyl moiety of 6-phenyl-HODA on the enzyme activity was found to be small; the activity decreased only in the order of 6-(2′-aminophenyl)-HODA (2.44 U/mg) > 6-phenyl-HODA (1.99 U/mg) > 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl)-hexa-2,4-dienoic acid (1.05 U/mg). The effects of 2′-substitution on the activity were in accordance with the predicted reactivity based on the calculated lowest unoccupied molecular orbital energy for these substrates.

Key words: *meta*-cleavage compound hydrolase; carbazole; dibenzofuran; *Pseudomonas resinovorans* strain CA10

Carbazole is a toxic *N*-heterocyclic aromatic compound containing dibenzopyrrole system, and is derived from coal tar and shale oil.[1,2] Efforts have been made to isolate the carbazole-degrading bacteria and to analyze the microbial metabolism of this compound as reviewed previously.[3–5] *Pseudomonas resinovorans* strain CA10 was isolated from a sample of activated sludge as a bacterium that can use carbazole as the sole source of carbon, nitrogen, and energy.[6] The initial step of carbazole degradation by strain CA10 is dioxygenation at the angular position adjacent to the nitrogen atom to give the dihydroxylated intermediate, which is spontaneously converted to 2′-aminobiphenyl-2,3-diol. This process is called angular dioxygenation. Then, 2′-aminobiphenyl-2,3-diol is converted to anthranilate and 2-hydroxypenta-2,4-dienoate *via meta*-cleavage and subsequent hydrolysis. 2-Hydroxypenta-2,4-dienoate is metabolized to TCA cycle intermediates through the *meta*-cleavage pathway.

By shotgun cloning and gene walking, we earlier isolated the *carAaAaBaBbCAcAdDFE* gene cluster involved in the conversion of carbazole to anthranilate and 2-hydroxypenta-2,4-dienoate, and of 2-hydroxypenta-2,4-dienoate to TCA cycle intermediates.[7–9] The terminal oxygenase component, CarAa, of the angular dioxygenase system has less than 10% identity to the catalytic subunits of oxygenase components of known dioxygenase systems, although identity was 39% with OxoO protein in the 2-oxo-1,2-dihydroquinoline 8-monooxygenase system,[10] as reported by Nam *et al.*[11] The *meta*-cleavage pathway enzymes, CarD, CarE, and CarF, had about 70–80% identity with reported isofunctional enzymes.[9] CarBaBb (the *meta*-cleavage enzyme) and CarC [the *meta*-cleavage compound hydrolase; 2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoic acid (2′-aminophenyl-HODA) hydrolase] had about 30% identities to known isofunctional enzymes.[8] These results suggest that the *meta*-cleavage enzyme and subsequent metabolic enzymes, CarBaBbCDEF, may be more closely related to the corresponding enzymes

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Abbreviations: 6-(2′-aminophenyl)-HODA, 2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoic acid; HODA, 2-hydroxymuconic semialdehyde; 6-(2′-hydroxyphenyl)-HODA, 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl)-hexa-2,4-dienoic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; LUMO, lowest unoccupied molecular orbital; 6-methyl-HODA, 2-hydroxy-6-oxohepta-2,4-dienoic acid; 6-phenyl-HODA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid

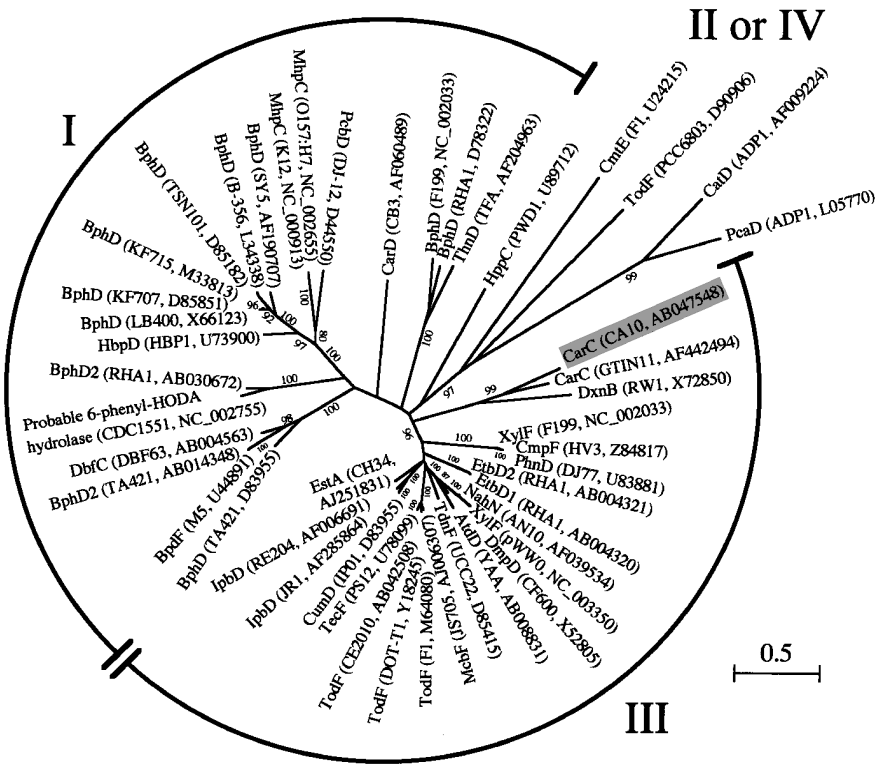

Fig. 1. Phylogenetic Tree of the *meta*-Cleavage Compound Hydrolases.

The tree was constructed by the neighbor-joining method with the PHYLIP package (version 3.573; obtained from J. Felsenstein, University of Washington). The bootstrap values of 80 or more from 100 resamplings are shown for each node. The scale bar denotes 0.5 substitution per site. The name of bacterial strain or plasmid and the DDBJ/EMBL/GenBank accession number are shown in parentheses after the enzyme name. Grouping reported by Hernáez *et al.*[22] are shown by the term, I, II, III, and IV. CarC from *P. resinovorans* strain CA10 is shaded.

in degradation pathways for other aromatic compounds than CarAa. Dibenzofuran and dibenzo-*p*-dioxin, the parental compounds of dioxin, which causes environmental problems, were degraded through a pathway similar to that of carbazole by strain CA10.[3,5,12] This similarity may reflect the relatedness of these two degradation pathways. In fact, carbazole-degrading enzymes degrade dibenzofuran, dibenzo-*p*-dioxin, and their chlorinated derivatives.[7,13,14]

The substrate specificity of *meta*-cleavage compound hydrolase helps to govern the overall selectivity of the pathway with respect to the degradation of aromatic compounds.[15–20] It has been proposed that CarC is one of the key determinants of substrate specificity of the carbazole degradation system of strain CA10 for chlorinated dibenzofuran and chlorinated dibenzo-*p*-dioxin.[14] Sequence comparisons of such hydrolases indicate that they belong to the family of proteins that have the α/β hydrolase fold,[21] and that the hydrolases can be divided into several groups as indicated by Hernáez *et al.*[22] (Fig. 1). Generally this phylogeny-based grouping well corresponds to the grouping based on the preferred substrate, especially for the size of the functional group at C6 of the substrate. Most hydrolases involved in the degradation of monocyclic and bicyclic aromatic compounds are in groups III and I, respectively,[22] although there are several hydrolases with intermediate features. The substrate specificity of CarC was similar to that of hydrolases classified into group I, but a closer phylogenetic relation was observed with those in group III. Although classified into group III, CarC of strain CA10 and related proteins (CarC of *Sphingomonas* sp. strain GTIN11[23] and DxnB of *S. wittichii* strain RW1[24,25]) have intermediate features and comprise a highly divergent branch. With their different substrate specificities, these enzymes might be classified into a new group apart from group III. These facts suggest that CarC of strain CA10 is a novel hydrolase. Thus, it is interesting to discover the structure and function of CarC, and to compare them with those of other *meta*-cleavage compound hydrolases involved in the degradation pathways of monocyclic, bicyclic, and heterocyclic aromatic compounds. With these considerations, we purified and characterized CarC. On the basis of enzymatic features, we discuss the relationship between CarC and other hydrolases.

Materials and Methods

Bacterial strains, plasmids, media, and culture conditions. Eshericia coli strain JM109[26] was used as the host of the plasmids pUC118,[26] pUC119,[26] pSTV28 (Takara Shuzo Co., Ltd., Kyoto, Japan), or their derivatives. The pSTV28-based plasmid pIP301 containing a 2.4-kb *Sal*I-*Eco*RI fragment encoding CumC (*meta*-cleavage enzyme involved in isopropylbenzene degradation in *Pseudomonas fluorescens* strain IP01) was prepared from pIP103.[27] *E. coli* strains were grown on 2xYT medium[28] with ampicillin (50 μg/ml) or chloramphenicol (50 μg/ml) at 37°C. For plate cultures, media solidified with 1.6% agar (wt/vol) were used. An appropriate amount of isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium to induce the expression of the enzymes as described below.

DNA manipulation. Plasmid DNA was prepared from the *E. coli* host strain by alkaline lysis.[29] Restriction endonuclease and a DNA Ligation Kit version 2 (Takara Shuzo) were used according to the manufacturer's instructions. DNA fragments were fractionated by electrophoresis with 0.9% Agarose S (Nippon Gene Co., Ltd., Tokyo, Japan) in TAE buffer,[28] and were extracted from the gel with Concert Rapid Gel Extraction System (Invitrogen Corp., Carlsbad, CA) by the manufacturer's instructions. Other DNA manipulations were done by standard methods.[28]

Expression and purification of CarC. E. coli strain JM109 harboring pUCA191[8] containing a 1.3-kb *Hin*dIII-*Sph*I fragment encoding CarC was grown on 100 ml of 2xYT medium containing ampicillin and IPTG (final concentration, 0.5 mM). After 18 h of incubation at 37°C, the cells were harvested, washed twice with 50 mM sodium phosphate buffer (pH 7.5; buffer A), and resuspended in 30 ml of the same buffer. The cell suspensions were sonicated and centrifuged at 21,600 $\times g$ at 4°C for 60 min, and the supernatants were used as crude cell extracts. Purification was done at 4°C throughout. The crude cell extract was brought to 40% saturation with solid ammonium sulfate. The mixture was stirred for 30 min, and the precipitate was discarded. The resultant supernatant was dialyzed against buffer A. From the solution after the dialysis, CarC was separated on a column of DEAE-Sephadex A-50 (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire) with a linear gradient of 0.0 to 0.5 M NaCl. CarC was further purified on a column of Sephacryl S-200 (Amersham Pharmacia Biotech UK) equilibrated with buffer A containing 0.1 M NaCl. The relative molecular weight of the native protein was estimated by calibrated gel filtration chromatography on a Sephacryl S-200 column. The purity of CarC was checked by SDS-PAGE[30] with a 13% polyacrylamide gel. The gels were stained for proteins with Coomassie Brilliant Blue R-250. The protein concentration was measured by the method of Bradford[31] with bovine serum albumin as the standard and with a Protein Assay Kit II (Bio-Rad Laboratories, Richmond, CA).

Preparation of substrates for assays of activity of CarC. 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (6-phenyl-HODA; a *meta*-cleavage compound in the biphenyl degradation pathway) was prepared from 2,3-dihydroxybiphenyl with *E. coli* cells harboring pUCA122 as described previously.[8] 2-Hydroxy-6-oxohepta-2,4-dienoic acid (6-methyl-HODA; a *meta*-cleavage compound in the toluene degradation pathway) and 2-hydroxymuconic semialdehyde (HODA; a *meta*-cleavage compound in the catechol degradation pathway) were similarly prepared from 3-methylcatechol and catechol, respectively, with *E. coli* cells harboring pIP301.

Assay of activity of purified CarC. The hydrolase activity of the purified CarC was assayed by monitoring of changes in A_{434}, A_{388}, and A_{375} with the substrates, 6-phenyl-HODA, 6-methyl-HODA, and HODA, respectively, with a Beckman DU-7400 spectrophotometer equipped with a thermojacketed cuvette holder and a circulating water bath. The molar extinction coefficients of 6-phenyl-HODA, 6-methyl-HODA, and HODA under the assay conditions used (including a pH of 7.5) were taken to be 13,200,[32] 32,000,[33] and 36,000 $M^{-1}\,cm^{-1}$,[34] respectively. The standard assay was done in a total volume of 1 ml of 50 mM sodium phosphate buffer (pH 7.5) at 25°C. To estimate the relative activity of CarC toward 6-phenyl-HODA, 6-methyl-HODA, and HODA, a substrate was added to the final concentration of 40 μM to the reaction mixture. For calculation of K_m, 6-phenyl-HODA was added to the final concentration of 1 to 80 μM. The reaction was started by the addition of 10 μg of purified enzyme if not mentioned otherwise. One unit of activity was defined as the amount of the enzyme that degraded 1 μmol of substrate per minute at 25°C. Relative activities are expressed as percentages of that measured with 6-phenyl-HODA as the substrate, taken to be 100%.

To discover the effects of pH on the enzyme activity, we assayed the hydrolase activity toward 6-phenyl-HODA in the following buffers at 50 mM: sodium citrate buffer (pH 5.0–6.0), sodium-potassium phosphate buffer (pH 5.0–7.0), sodium phosphate buffer (pH 6.0–8.0), and Tris-HCl buffer (pH 7.5–9.0). As molar extinction coefficients at various pHs, the values reported by Hatta *et al.*[35] were used. To discover the effects of temperature, after CarC was incubated at various temperatures for 1 to 10 min, the remaining hydrolase activity for 6-

phenyl-HODA was assayed at 25°C as described above.

Preparation of 2′-substituted 2,3-dihydroxybiphenyl. 2′-Aminobiphenyl-2,3-diol and 2,2′,3-trihydroxybiphenyl were prepared from carbazole and dibenzofuran, respectively, by a resting cell reaction with *E. coli* cells harboring pUCARA expressing angular dioxygenase (CarAaAcAd) as described previously.[7] After the conversion of the substrate to 2′-substituted-2,3-dihydroxybiphenyl, the reaction mixtures were extracted with an equal volume of ethyl acetate. The ethyl acetate layers were dried over Na_2SO_4 and then evaporated to dryness at room temperature under the reduced pressure. The residues were dissolved in a small amount of ethyl acetate and put on a silica gel column for chromatography as described previously.[13] The purified 2′-aminobiphenyl-2,3-diol and 2,2′,3-trihydroxybiphenyl were dissolved in a small amount of ethanol. The concentrations of these compounds in the resultant solution were measured by monitoring of the oxygen consumption during complete *meta*-cleavage with an excess of the purified CarB[36] with a Clark-type oxygen electrode (Iijima Electronics Co., Aichi, Japan) as described previously.[8]

Measurment of molar extinction coefficient of meta*-cleavage compounds from 2′-substituted-2,3-dihydroxybiphenyl.* To 1 ml of 50 mM Tris-HCl buffer (pH 7.5), an excess of purified CarB and a known amount of 2′-aminobiphenyl-2,3-diol or 2,2′,3-trihydroxybiphenyl were added, resulting in the formation of 2-hydroxy-6-oxo-6-(2′-aminophenyl)-hexa-2,4-dienoic acid [6-(2′-aminophenyl)-HODA; a *meta*-cleavage compound in the carbazole degradation pathway] or 2-hydroxy-6-oxo-6-(2′-hydroxyphenyl)-hexa-2,4-dienoic acid [6-(2′-hydroxyphenyl)-HODA; a *meta*-cleavage compound in the dibenzofuran degradation pathway]. After complete *meta*-cleavage, the absorbance between 190 to 800 nm of the reaction mixture was monitored at 25°C as described above.

The stability of 6-phenyl-HODA and 6-(2′-substituted phenyl)-HODAs was measured by spectrophotometric monitoring of changes in the absorbance of a solution of these compounds (50 mM Tris-HCl buffer, pH 7.5, 25°C) at the wavelength of maximum absorbance. Half-lives were calculated by fitting of the data to first-order decay.

Assay of hydrolase activity for 6-(2′-substituted phenyl)-HODAs. The hydrolase activity of CarC for 6-(2′-substituted phenyl)-HODAs was measured by monitoring of the conversion of 2′-substituted-2,3-dihydroxybiphenyl to 2′-substituted benzoic acid and 2-hydroxypenta-2,4-dienoate with purified CarB and CarC. For comparison, 2,3-dihydroxybiphenyl was used as a substrate. To 1 ml of 50 mM Tris-HCl buffer (pH 7.5), the 10 to 20 μl of an ethanol stock solution of 2′-substituted-2,3-dihydroxybiphenyl or 2,3-dihydroxybiphenyl was added to the final concentration of 100 μM. Two micrograms of the purified CarB was added to this solution, and the mixture was stirred for 30 s, resulting in the complete conversion of the starting compounds to the corresponding 6-(2′-substituted phenyl)-HODA. Then, 40 μg of CarC was added to the reaction mixture, and changes in specific absorption (see Results section) were monitored. The molar extinction coefficients found in this study were used. The measurements were done at 25°C, with the equipment mentioned above. One unit of activity was defined as the amount of the enzyme that degraded 1 μmol of substrate per minute at 25°C.

To analyze the enzymatic and nonenzymatic conversions of HODA-related compounds, we extracted reaction mixtures containing CarB with or without CarC with equal volumes of ethyl acetate under acidic conditions (pH 2–3). The organic phase was dried over Na_2SO_4, and then evaporated to dryness under reduced pressure. The resultant extract was analyzed by GC-MS after the trimethylsilylation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (70°C for 20 min) as described previously.[13]

Theoretical calculations. Theoretical calculations were done by the semiempirical quantum-mechanical AM1 method[37] in the MOPAC 2000 program (Fujitsu Ltd., Tokyo, Japan). In the description of the solvent, we used the COSMO method.[38] All structures were optimized with the eigenvector by the method of Baker.[39]

Results

Expression and purification of CarC

CarC was sufficiently expressed for purification and enzymatic characterization from *E. coli* cells harboring the pUC119-based plasmid pUCA191[8] (Fig. 2). IPTG was added to the final concentration of 0.5 mM at the same time as the seeding of an overnight first culture, because no difference was detected when IPTG was added when the cell growth reached an OD_{550} of 1.0. Eventually, 34.6 mg of CarC was purified from a 200-ml culture of these *E. coli* cells (Table 1). CarC was apparently purified to near homogeneity, as indicated by SDS-PAGE (Fig. 2), and its relative molecular mass was estimated to be 31,000 Da, close to the value deduced from the amino acid sequence (31,330 Da). The relative molecular weight of the native protein was estimated by gel filtration chromatography, to be 70,000.

Table 1. Purification of CarC Protein from 200-ml Culture of *E. coli* Harboring pUCA191

Enzymatic activity for 6-phenyl-HODA was monitored throughout purification. Hydrolase activity was assayed at 25°C in 50 mM sodium phosphate buffer (pH 7.5). One unit of enzyme activity is the activity that converts 1 μmol of 6-phenyl-HODA.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)
Crude extract	136	233	1.71	1.00
Supernatant after precipitation	73.3	202	2.76	1.61
DEAE-Sephadex A-50	49.8	175	3.52	2.06
Sephacryl S-200	34.6	133	3.84	2.25

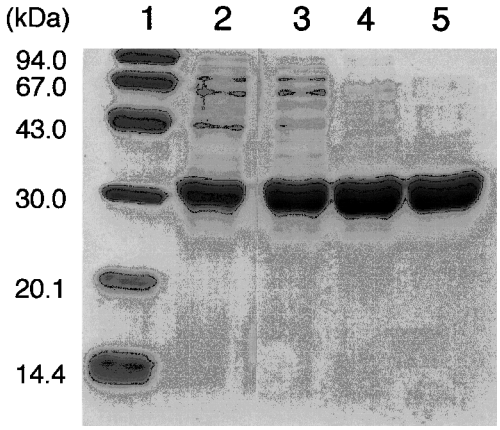

Fig. 2. SDS-PAGE of Samples from the Subsequent Purification Steps.

Lane 1, molecular mass standards; 2, crude cell extract; 3, supernatant after precipitation with 40% (wt/vol) $(NH_4)_2SO_4$; 4, preparation after DEAE-Sephadex A-50; 5, preparation after Sephacryl S-200. Proteins were stained with Coomassie Brilliant Blue R-250. Ten micrograms of protein was put on in all lanes.

Effects of pH and temperature for activity and stability

The optimum pH for the enzyme activity was at 7.0 to 7.5. Hydrolase activity was relatively high when the sodium citrate buffer was used (data not shown). CarC retained about 75% activity after a 10-min incubation at 55°C or less, although incubation at 65°C inactivated the enzyme.

Steady-state kinetic analysis

CarC obeyed Michaelis-Menten kinetics for 6-phenyl-HODA, 6-methyl-HODA, and HODA. CarC had only 0.38% and 0.54% activity toward 6-methyl-HODA and HODA, respectively, with that toward 6-phenyl-HODA taken to be 100%, indicating that CarC was specific for 6-phenyl-HODA. The K_m of CarC for 6-phenyl-HODA was 2.51 μM. The catalytic activity, k_{cat}, and specificity constant, k_{cat}/K_m, were 2.14 s^{-1} and 8.5×10^5 M^{-1} s^{-1}, respectively. We could not measure the K_m of CarC for other substrates because of its low activity.

Characterization of 6-(2′-substituted phenyl)-HODAs

The absorption spectra of the solutions of 6-(2′-aminophenyl)-HODA and 6-(2′-hydroxyphenyl)-HODA had wavelengths of maximum absorbance at 403 and 438 nm, respectively. The extinction coefficients at the appropriate wavelength for freshly prepared 6-(2′-aminophenyl)-HODA and 6-(2′-hydroxyphenyl)-HODA were 9,140 and 22,100 M^{-1} cm^{-1}, respectively. Half-lives of 6-(2′-aminophenyl)-HODA and 6-(2′-hydroxyphenyl)-HODA under the conditions we used were 6.1 and 7.3 min, respectively; that for 6-phenyl-HODA is 58 h.[19]

Effects of substitution at 2′-position of phenyl moiety on hydrolase activity of CarC

Because of the too-ready degradation of 6-(2′-substituted phenyl)-HODAs, we measured the hydrolase activity of CarC for these compounds by a combination assay in which the substrates, 6-(2′-substituted phenyl)-HODAs, were produced by the *meta*-cleavage enzyme from the corresponding catecholic compounds, followed by measurement of the hydrolase reaction in a single tube. Depletion of the substrates depended on the amount of CarC, and the hydrolytic activity of CarC decreased in the substrate order of 6-(2′-aminophenyl)-HODA (2.44 U/mg) > 6-phenyl-HODA (1.99 U/mg) > 6-(2′-hydroxyphenyl)-HODA (1.05 U/mg). The activity for 6-(2′-hydroxyphenyl)-HODA was much higher than that for 6-methyl-HODA and HODA.

To confirm whether the hydrolysis was actually caused by CarC, we analyzed the products of the enzymatic and nonenzymatic conversions of these 6-(2′-substituted phenyl)-HODAs. From the reaction mixtures with CarB, the respective *meta*-cleavage compounds were detected (Table 2). The cyclization product of 6-(2′-hydroxyphenyl)-HODA[40] was detected. When CarC was added, anthranilate and salycilate, which are the hydrolysis products of 6-(2′-aminophenyl)-HODA and 6-(2′-hydroxyphenyl)-HODA, respectively, were detected.

Theoretical calculations

The physical features of 2′-substitution in the phenyl moiety might affect the substrate specificity of CarC. In C-C bond hydrolysis, an nucleophilic attack of water catalyzed by active site serine has been proposed to occur.[41] The lowest unoccupied molecular orbital (LUMO) could act as an electron acceptor,

Table 2. Identification of Transformation Products of 2,3-Dihydroxybiphenyl and Its Derivatives by CarB and CarC

Substrate	Enzyme used	GC-MS data[a]		Identification
		Retention time (min)	Principal ions and relative abundance (% base peak)	
2,3-Dihydroxybiphenyl	CarB	12.0	347(M-15$^+$, trace), 257(2), 245(28), 147(16), 73(100)	6-Phenyl-HODA[d]
	CarB + CarC	4.6	194(M$^+$, 6), 179(100), 135(89), 105(87), 77(69)	Benzoate[e]
2′-Aminobiphenyl-2,3-diol	CarB	12.6	377(M$^+$, 3), 362(3), 260(26), 147(23), 73(100)	6-(2′-Aminophenyl)-HODA[d]
	CarB + CarC	6.7[b]	209 (M$^+$, 34), 194(76), 176(27), 150(54), 120(100)	Anthranilate[b,e]
		7.8[c]	281(M$^+$, 1), 266(38), 218(12), 147(18), 120(100)	Anthranilate[c,e]
		12.6	377(M$^+$, 3), 362(2), 260(21), 147(25), 73(100)	6-(2′-Aminophenyl)-HODA[d]
2,2′,3-Trihydroxybiphenyl	CarB	11.6	378(M$^+$, 6), 293(10), 261(81), 193(20), 83(100)	3-(Chroman-4-on-2-yl)pyruvate[f]
		12.7	435(M-15$^+$, 4), 407(3), 333(100), 257(22), 73(81)	6-(2′-Hydroxyphenyl)-HODA[f]
	CarB + CarC	7.0	267(M-15$^+$, 20), 209(6), 193(2), 149(8), 73(100)	Salycilate[e]
		11.6	378(M$^+$, 5), 293(6), 261(92), 193(48), 83(100)	3-(Chroman-4-on-2-yl)pyruvate[f]
		12.7	435(M-15$^+$, 4), 407(2), 333(100), 257(20), 147(44)	6-(2′-Hydroxyphenyl)-HODA[f]

[a] Results of GC-MS of trimethylsilyl derivatives.
[b] Only the carboxyl group of anthranilate was trimethylsilylated.
[c] Both the carboxyl and amino groups of anthranilate were trimethylsilylated.
[d] The tentative identification was based on the results of GC-MS for the trimethylsilyl derivative of 6-(2′-hydroxyphenyl)-HODA.[40]
[e] The identification was based on comparison with the results of GC-MS of authentic samples.
[f] The identification was based on comparison with the results of Kohler *et al.*[40]

and the LUMO energy is equivalent to the electron affinity of the molecule, according to Koopmans theorem.[42] On the basis of frontier orbital theory,[43] when the energy difference of the highest occupied molecular orbital of a nucleophile and the LUMO of an electrophile is small, the reactivity of nucleophilic attack is high. We calculated the LUMO energies of the substrates to evaluate the reactivity of nucleophilic attack by water. The calculated LUMO energy was lowest for 6-(2′-aminophenyl)-HODA (−0.754), higher for 6-phenyl-HODA (−0.718), and highest for 6-(2′-hydroxyphenyl)-HODA (−0.671). This predicted reactivity was in agreement with the experimental results.

Discussion

The relative molecular weight of the native CarC was estimated by chromatography on a Sephacryl S-200 column, to be 70,000. CarC exhibited a single band on SDS-PAGE, which corresponded to 31,000 Da, suggesting that the CarC enzyme is composed of two identical subunits. Variation in the oligomeric structures (monomer to homooctamer) has been reported for *meta*-cleavage compound hydrolases, and the oligomeric structure seems not to be correlated with the substrate specificity.[18,35] However, there may be an interface structure that allows for specific oligomeric structures of enzymes. To date, the three-dimensional structures of BphD (a homooctamer) from *Rhodococcus* sp. strain RHA1[35,44] and CumD (a homodimer) from *P. fluorescens* strain IP01[45,46] has been identified. Comparison between the interface structures among the hydrolases will provide information on the construction of the oligomeric structure.

The optimum pH of *meta*-cleavage compound hydrolases from *Rhodococcus* sp. strain RHA1 has been reported to be 6.0 and 6.5,[35] but the optimum pH of hydrolases from pseudomonads has been reported to be 7.4,[47] 8.6,[48] and 7.0–7.5.[49] The optimum pH of MhpC from *E. coli* has been reported to be 8.5–9.0.[50] The optimum pH for CarC, 7.0–7.5, was similar to that for other *meta*-cleavage compound hydrolases originating from pseudomonads. Incubation at 65°C rapidly inactivated CarC, but the enzyme retained about 75% activity after 10 min of incubation at 55°C. At 80°C or less, hydrolase activity was higher when the reaction temperature was increased, and we could not detect a temperature for maximum hydrolase activity (data not shown). Considering that the optimum temperatures for EtbD1 and BphD from *Rhodococcus* sp. strain RHA1 are 65° and 45°C, respectively,[35] CarC seems to be a thermostable. It has been predicted that the *car* gene cluster was constructed from several ancestral genes through gene rearrangement,[9] and we cannot clarify the origin of the *carC* gene on the basis of the information we have about the genetic structure of this gene cluster. However, the characteristics of CarC might reflect conditions in the cytosol of the bacterium originally having the *carC* gene.

Almost all of the hydrolases involved in degradation of monocyclic aromatic compounds has been classified into group III. Phylogenetic analysis showed close relationships with group III hydrolases of CarCs of strain CA10 and *Sphingomonas* sp. strain GTIN11,[23] and DxnB of *S. wittichii* strain RW1.[24,25] Like DxnB of strain RW1[24,25] and other hydrolases found in bacteria that degrade biphenyl

and polychlorinated biphenyl,[18,35] CarC showed a strict preference for 6-phenyl-HODA. Accordingly, the substrate specificity was not in accordance with the results of phylogenetic analysis, indicating the divergence of the structures and functions of the C-C bond hydrolases involved in aromatic compound degradation. Examination of the structure of the 6-phenyl-HODA hydrolase, BphD, of *Rhodococcus* sp. strain RHA1, showed that the subunit protein can be divided into core and lid domains, and that the substrate binding pocket was between the two domains.[44] The substrate binding pocket of the BphD enzyme is composed of a hydrophilic P (proximal) part and a hydrophobic D (distal) part. The amino acid residues on the surface of the D part were not well conserved among similar enzymes, suggesting that the difference in this part contributes to the substrate specificities of *meta*-cleavage compound hydrolases. Part of the surface of the D part was composed of the amino acid residues on the lid domain.[44] Amino acid sequence alignment showed that the amino acid residues in the lid domains of group I or III hydrolases were well conserved within their groups; similarity was not found in the regions between groups (not shown). In contrast, many amino acid residues of the core domain were conserved in almost all of the *meta*-cleavage compound hydrolases tested. The phylogeny observed in Fig. 1 probably originated in the main from divergence in the amino acid sequence of the lid domain. If so, the three-dimensional structure of the lid domain is diverse, and CarC has a structure similar to the structures of the hydrolases of monocyclic compound degradation pathways. However, the CarC substrate specificity, which is unusual for group III hydrolases, indicates that there is some difference in the D part of the substrate binding pocket of CarC with other group III hydrolases involved in this kind of degradation. Recently, the three-dimensional structure of CumD, a hydrolase of the isopropylbenzene degradation pathway in *P. fluorescens* strain IP01 and classified into group III, has been identified as a complex form with a hydrolysis product.[46] For comparison, analysis of the three-dimensional structure of CarC is in progress.

Several *meta*-cleavage compound hydrolases involved in the dibenzofuran and carbazole degradation pathways have been characterized.[8,24,51] In those studies, 6-phenyl-HODA was used as a model substrate to discover the enzymatic features of the hydrolases, because of the unstability of 6-(2′-substituted phenyl)-HODAs.[52] We compared the hydrolase activity of CarC for the primary substrate, 6-(2′-aminophenyl)-HODA, with that for 6-(2′-hydroxyphenyl)-HODA and 6-phenyl-HODA. To overcome bias arising from the short half-lives of the substrates, we used a combination assay in which the substrate was produced by the *meta*-cleavage enzyme, and subsequently monitored substrate depletion by CarC. The results showed that CarC catalyze the hydrolysis of the *meta*-cleavage compounds involved in the degradation pathways of both carbazole and dibenzofuran. Compared with the effect of the volume of the C6 side chain, the effect of the presence of the amino group and hydroxyl group at the 2′-position of the phenyl moiety of substrate on the hydrolase activity was found to be small. However, as shown above, the CarC enzyme catalyzed the hydrolysis of 6-(2′-aminophenyl)-HODA more effectively than that of either 6-phenyl-HODA or 6-(2′-hydroxyphenyl)-HODA. CarC hydrolyzes 6-phenyl-HODA more than 6-(2′-hydroxyphenyl)-HODA. That the *car* gene cluster of strain CA10 was induced in cells cultivated on carbazole or anthranilate (Urata *et al.*, unpublished) indicates that at least a part of the *car* gene cluster is originally related to nitrogen metabolism. This possibility is in agreement with 6-(2′-aminophenyl)-HODA being a substrate for CarC of strain CA10. The pattern of substrate specificity was in accordance with theoretical calculations based on the LUMO energy. However, there may be various structures with local energy minimum, because part of the carbon chain is flexible. In addition, the effects of interaction with CarC would be large. Therefore, after analyzing the structure of CarC, it is expected that more exact analysis can be done on the state of a substrate-enzyme complex.

Compared with the half-life of 6-phenyl-HODA (58 h),[19] 6-(2′-aminophenyl)-HODA and 6-(2′-hydroxyphenyl)-HODA were unstable under the conditions used (6.1 and 7.3 min, respectively). 6-Phenyl-HODA is spontaneously converted to acetophenone.[19] The hour-scale half-life of 6-phenyl-HODA possibly originated from this spontaneous transformation. On the other hand, intermolecular cyclizations by the Michael-type addition of amino and hydroxyl groups at the 2′-position of *meta*-cleavage compounds in the carbazole and dibenzofuran degradation pathways to the double bond of the aliphatic side chain occurred mainly as a spontaneous transformation.[40,53,54] This reaction may cause the *meta*-cleavage compounds in carbazole and dibenzofuran degradation pathways to be unstable. When strain CA10 is cultivated on carbazole as the sole carbon, nitrogen, and energy source, anthranilate accumulates in the culture supernatant.[6] This finding indicates that angular dioxygenase, *meta*-cleavage enzyme, and hydrolase can catalyze the conversion of carbazole to anthranilate more effectively than the subsequent conversion of anthranilate. This phenomenon might indicate the presence of an unknown mechanism to allow the effective sequential metabolism of aromatic compounds to avoid the spontaneous degradation of the unstable intermediate, 6-(2′-aminophenyl)-HODA.

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