A Series of Crystal Structures of a meta-Cleavage Product Hydrolase from *Pseudomonas fluorescens* IP01 (CumD) Complexed with Various Cleavage Products

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Meta-cleavage product hydrolase (MCP-hydrolase) is one of the key enzymes in the microbial degradation of aromatic compounds. MCP-hydrolase produces 2-hydroxypenta-2,4-dienoate and various organic acids, according to the C6 substituent of the substrate. Comprehensive analysis of the substrate specificity of the MCP-hydrolase from *Pseudomonas fluorescens* IP01 (CumD) was carried out by determining the kinetic parameters for nine substrates and crystal structures complexed with eight cleavage products. CumD preferred substrates with long non-branched C6 substituents, but did not effectively hydrolyze a substrate with a phenyl group. Superimposition of the complex structures indicated that benzoate was bound in a significantly different direction than other aliphatic cleavage products. The directions of the bound organic acids appeared to be related with the $k_{cat}$ values of the corresponding substrates. The Ile139 and Trp143 residues on helix α4 appeared to cause steric hindrance with the aromatic ring of the substrate, which hampers base-catalyzed attack by water.

Key words: α/β-hydrolase; cumene degradation; meta-cleavage product hydrolase; polychlorinated biphenyl degradation; *Pseudomonas fluorescens*

Aromatic compounds such as polychlorinated biphenyls (PCBs) and monoalkylnlbenzenes are known or suspected to be toxic, mutagenic, or carcinogenic.1 One of the most attractive means of removing them from the environment is the use of microorganisms. Hence, the bacterial degradation of aromatic compounds has been studied extensively.2-4 In the pathways for the bacterial degradation of aromatic compounds, catechol derivatives are cleaved into meta- or ortho-ring fission products.5 Meta-Cleavage occurs during the degradation of various aromatic compounds (Fig. 1) such as biphenyl, toluene, ethylbenzene, and cumene (isopropylbenzene). The meta-cleavage product is hydrolyzed by meta-cleavage product hydrolase (MCP-hydrolase), producing 2-hydroxypenta-2,4-dienoate and various organic acids, according to the C6 substituent of the substrate. The substrate specificities of MCP-hydrolases are exclusively strict for substrates with large-aromatic or short-alkyl C6 substituents.6,7 Most known MCP-hydrolases are classified into two major groups, groups I and III (the biphenyl and monoalkylbenzene groups respectively).7 The substrate specificities and catalytic rates of MCP-hydrolases often determine total degradation ability as to aromatic compounds.8-14 Cumene is an aromatic compound that is intermediate in size between ethylbenzene and biphenyl. *Pseudomonas fluorescens* IP01 can grow on cumene and toluene as the sole source of carbon,15,16 but it cannot grow on biphenyl, because the meta-cleavage pathway is blocked at the step of MCP-hydrolase (CumD).17 A biochemical study of CumD indicated that the enzyme can hydrolyze 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate (isopropyl-HODA) effectively, but can only slightly hydrolyze 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (phenyl-HODA).18 (Substrates are designated by the C6 substituents of the 2-hydroxy-6-oxo-hexa-2,4-dienoate (HODA) group.) However, the substrate specificity of CumD covers larger C6 substituents than that of another monoalkylbenzene-type MCP-hydrolase, TodF from *P. putida* F1.

The crystal structures of MCP-hydrolases are known for BphD from *Rhodococcus* sp. strain RHA1 (RHA1 BphD), CumD, and CarC from *Janthinobacterium* sp.
Materials and Methods

Kinetic measurement. Propylbenzene, n-butylbenzene, isobutylbenzene (2-methyl-1-phenylpropane), s-butylbenzene, and t-butylbenzene were purchased from Wako Pure Chemical Industries (Osaka). The sources of other chemicals, preparation of the substrate by the resting cell method, expression and purification of the native and S103A mutant of CumD, and kinetic measurement of the activity of the CumD enzyme were described previously. An extinction coefficient of 8.0 mM cm$^{-1}$ at 324.5 nm was employed for kinetic measurement of t-butyl-HODA (2-hydroxy-6-oxo-7,7-dimethylocta-2,4-dienoate). The absorption maxima of propyl-HODA (2-hydroxy-6-oxo-nona-2,4-dienoate), n-butyl-HODA (2-hydroxy-6-oxo-deca-2,4-dienoate), isobutyl-HODA (2-hydroxy-6-oxo-octylmethylene-2,4-dienoate), and (±)-s-butyl-HODA (2-hydroxy-6-oxo-7-methyleneocta-2,4-dienoate) were found to be 390, 390, 391, and 394 nm respectively. An estimated extinction coefficient of 10.5 mM$^{-1}$ cm$^{-1}$ was uniformly employed for these substrates, according to the measured coefficients of alkyl-HODAs (10.2 for 6-ethyl-HODA and 10.7 for 6-isopropyl-HODA).

Crystallography. Crystallization and X-ray data collection were performed basically as described previously. A reservoir solution comprising 10–20% PEG 4000, 0.08 M NH$_3$, 0.167 M organic acid + NaOH, pH 5.1, was used for the growth of crystals complexed with propionate, n-butylate, n-valerate, isovalerate, and (S)-2-methylbutyrate. A reservoir solution comprising 10–20% PEG 4000, 0.08 M NH$_3$, 0.05 M benzoate + NaOH, pH 5.1, was used for the growth of crystals complexed with benzoate. All datasets were collected on the BL6A and BL18B stations at the Photon Factory (PF) of the High Energy Accelerator Research Organization (KEK), Tsukuba, Japan. Data processing, refinement, and model correction were performed with the programs DPS/MOSFLM, CNS1.1, and XtalView. Least-square fitting of CumD structures was performed using the C$_\alpha$ atoms from Asn3 to Ala273. The figures were generated with XFIT in the XtalView program suite, MOLSCRIPT, and RASTER3D. The coordinates and structure factors have been deposited in a Protein Data Bank (accession nos. 1UK6, 1UK7, 1UK8, 1UK9, 1UKA, and 1UKB).
Results

Kinetic parameters of CumD for various substrates

In addition to the previously determined kinetic parameters for four substrates, here we determined those for five other substrates (propyl-, n-butyl-, isobutyl-, (±)-s-butyl-, and t-butyl-HODAs; Table 1). These substrates were prepared from propylbenzene, n-
butylbenzene, isobutylbenzene, s-butylbenzene, and t-
butylbenzene respectively by the resting cell method. CumD can effectively hydrolyze isopropyl-HODA, whose C6 substituent is branched, but it has been found that CumD prefers substrates with non-branched C6 substituents longer than the ethyl group. As a general trend, the $K_m$ value decreased as the C6 substituent increased in size. The hydrophobicity of the C6 substituent appears to be correlated with the affinity to

<table>
<thead>
<tr>
<th>HODA C6 substituent</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m \times 10^{-5}$</th>
<th>Corresponding organic acids</th>
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<tr>
<td>Methyl-</td>
<td>9.0 (0.9)</td>
<td>18 (1.8)</td>
<td>19</td>
<td>Acetate</td>
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<tr>
<td>Ethyl-</td>
<td>9.5 (0.7)</td>
<td>41 (1.4)</td>
<td>43</td>
<td>Propionate</td>
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<td>Propyl-</td>
<td>3.7 (0.4)</td>
<td>18 (0.6)</td>
<td>48</td>
<td>n-Butyrate</td>
</tr>
<tr>
<td>Isopropyl-</td>
<td>7.3 (0.7)</td>
<td>21 (1.0)</td>
<td>29</td>
<td>Isobutyrate</td>
</tr>
<tr>
<td>n-Butyl-</td>
<td>5.7 (0.4)</td>
<td>28 (0.8)</td>
<td>50</td>
<td>n-Valerate</td>
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<tr>
<td>Isobutyl-</td>
<td>4.3 (0.6)</td>
<td>7.6 (0.3)</td>
<td>18</td>
<td>Isovalerate</td>
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<tr>
<td>(±)-s-Butyl-</td>
<td>1.1 (0.2)</td>
<td>1.9 (0.06)</td>
<td>18</td>
<td>2-Methylbutyrate</td>
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<td>t-Butyl-</td>
<td>12 (3.1)</td>
<td>0.58 (0.08)</td>
<td>0.49</td>
<td>Trimethylacetate</td>
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<tr>
<td>Phenyl-</td>
<td>0.74 (0.07)</td>
<td>0.030 (0.0008)</td>
<td>0.40</td>
<td>Benzoate</td>
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*Values taken from reference. Values in parentheses indicate standard errors calculated from curve fitting.

Table 2. X-ray Crystallography Statistics

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<tr>
<th>Complex</th>
<th>Propionate</th>
<th>n-Butyrate</th>
<th>n-Valerate</th>
<th>Isovalerate</th>
<th>(S)-2-Methylbutyrate</th>
<th>Benzoate</th>
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<tr>
<td>PDB code</td>
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<td>1UK7</td>
<td>1UK8</td>
<td>1UK9</td>
<td>1UKA</td>
<td>1UKB</td>
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<td>Beamline</td>
<td>BL6A</td>
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<td>BL6A</td>
<td>BL18B</td>
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<td>BL18B</td>
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<td>Wavelength (Å)</td>
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<td>0.978</td>
<td>0.978</td>
<td>0.978</td>
<td>1.000</td>
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<td>Unit cell (Å)</td>
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<td>78.5</td>
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<td>78.7</td>
<td>78.6</td>
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<td>78.8</td>
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<td>Resolution limit (Å)</td>
<td>1.95</td>
<td>1.70</td>
<td>1.60</td>
<td>1.80</td>
<td>1.70</td>
<td>1.80</td>
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<td>Last shell (Å)</td>
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<td>1.79–1.70</td>
<td>1.69–1.60</td>
<td>1.90–1.80</td>
<td>1.79–1.70</td>
<td>1.90–1.80</td>
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<td>No. of total reflections</td>
<td>92,542</td>
<td>139,516</td>
<td>167,782</td>
<td>119,868</td>
<td>112,482</td>
<td>117,690</td>
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<tr>
<td>No. of unique reflections</td>
<td>26,075</td>
<td>38,090</td>
<td>45,682</td>
<td>32,978</td>
<td>39,097</td>
<td>32,962</td>
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<td>7.6</td>
<td>6.7</td>
<td>6.8</td>
<td>7.6</td>
<td>6.8</td>
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<tr>
<td>of last shell</td>
<td>34.8</td>
<td>29.9</td>
<td>27.1</td>
<td>23.6</td>
<td>27.6</td>
<td>25.9</td>
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<tr>
<td>Average (I/σ(I))</td>
<td>6.3</td>
<td>8.3</td>
<td>9.7</td>
<td>9.2</td>
<td>8.4</td>
<td>10.1</td>
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<tr>
<td>of last shell</td>
<td>2.1</td>
<td>2.4</td>
<td>2.7</td>
<td>3.1</td>
<td>2.7</td>
<td>3.0</td>
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<tr>
<td>Completeness (%)</td>
<td>99.9</td>
<td>97.8</td>
<td>97.8</td>
<td>99.5</td>
<td>99.8</td>
<td>100.0</td>
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<tr>
<td>of last shell</td>
<td>100.0</td>
<td>96.1</td>
<td>96.3</td>
<td>98.8</td>
<td>99.9</td>
<td>99.9</td>
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</table>

Additional notes:

- Calculated using a test data set; 5% of total data randomly selected from the observed reflections.
- Estimated coordinate error from Luzzati plot.
the hydrophobic D-part of the substrate-binding pocket.20) On the other hand, the $k_{\text{cat}}$ value appears not to be correlated with the size of the C6 substituent. As for the substrates with branched C6 substituents, CumD showed $K_m$ values toward isopropyl- and isobutyl-HODA comparable to those toward non-branched substrates. (±)-s-Butyl-HODA exhibited the lowest $K_m$ value among the substrates with aliphatic C6 substituents. The methyl group of one of the two optical isomers, (S)-s-butyl-HODA, fit into the vacant space ahead of the isobutyrate observed in the crystal structure.20) t-Butyl-HODA exhibited the lowest activity among the substrates with aliphatic C6 substituents because of the large $K_m$ and $k_{\text{cat}}$ values. The substrate-binding pocket of CumD is narrow at the entrance of the D-part, the t-butyl group is sterically hindered by the side chain of Val226. Phenyl-HODA exhibited smaller $K_m$ and $k_{\text{cat}}$ values than those for any substrates with aliphatic C6 substituents. Since the substrate-binding pocket of CumD is narrow at the entrance of the D-part, the t-butyl group is sterically hindered by the side chain of Val226. Phenyl-HODA exhibited smaller $K_m$ and $k_{\text{cat}}$ values than those for any substrates with aliphatic C6 substituents. In contrast to t-butyl-HODA, phenyl-HODA cannot efficiently be hydrolyzed, primarily due to the low catalytic efficiency. The general trends of the $K_m$ and $K_i$ values20) for corresponding substrates and organic acid inhibitors are similar, although the orders of the values are very different ($\mu$M for $K_m$ and mM for $K_i$).

**Complex structures of CumD with various organic acids**

In addition to the two previously determined crystal structures of the inactive CumD S103A mutant complexed with acetate and isobutyrate (PDB code 1IUO and 1IUP), in this study we determined the complex structures of the CumD S103A mutant with six organic acids (propionate, n-butyrate, n-valerate, isovalerate, (S)-2-methylbutyrate, and benzoate) at 1.95 to 1.60 Å resolution (Table 2). These complex structures were almost identical, except for several points mentioned below. The root mean square deviations of Cα atoms between the isobutyrate complex and the six complex structures reported here were within the range of 0.14 to 0.22 Å. These complex structures were easily obtained by replacing the organic acids in the crystallization buffer. But, when we tried to prepare crystals complexed with n-hexanoate, most of the protein in the crystallization drops precipitated. A very small crystal grown in the precipitate diffracted up to 2.4 Å resolution, but electron density in the shape of n-butyrate, which is probably a trace contaminant of the n-hexanoate reagent, was observed at the active site (data not shown). Although n-hexanoate exhibits the smallest $K_i$ value (0.22 mM), complex formation can induce structural changes in CumD and thereby hinder crystal formation.

The complex structure with propionate was determined at 1.95 Å resolution, but the electron density at the ethyl group was somewhat ambiguous (Fig. 2A). The complex structures with other five organic acids were determined at 1.80 to 1.60 Å resolution, and their

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**Fig. 2.** Fo–Fc Omit Electron Density Maps around the Active Site of the Crystal Structures Complexed with (A) Propionate, (B) n-Butyrate, (C) n-Valerate, (D) Isovalerate, (E) (S)-2-Methylbutyrate, and (F, G) Benzoate. The benzoate bound at the active site (BEZ300) is shown from two different viewpoints.
electron density maps were clear (Fig. 2B–G). In the complex structures with propionate, \(n\)-valerate, and benzoate, one or two additional molecules were found at positions outside the active site (Table 2B). The electron densities for these additional molecules were also clear (data not shown). One of the two additional benzoates (BEZ301) was bound at the entrance of the substrate-binding pocket near Trp253 (Fig. 3A), like the ISB301 molecule in the isobutyrate complex structure.\(^{20)\}

The electron density map of the side chain of Trp253 was ambiguous, and several alternative conformations were observed in several cases (Fig. 4A). Other additional molecules (propionate, \(n\)-valerate, and an additional molecule of benzoate) were located on the surface of the CumD molecule and were involved in crystal packing.

As for the two optical isomers of 2-methylbutyrate, \((S)\)-2-methylbutyrate showed higher affinity compared with the racemic mixture.\(^{20)\}

In the complex structure with \((S)\)-2-methylbutyrate, clear electron density for the chiral molecule was observed (Fig. 2E). The side chain of the Ile139 residue was rotated in this structure (Fig. 3B). The \(\chi^2\) value of Ile139 was 150°, in contrast to those of the seven other structures ranging from 49° to 56°. The four carbon atoms of the bound \((S)\)-2-methylbutyrate were aligned to form a flat face, and the side chain of Ile139 appears to have rotated according to it.

**Discussion**

Superimposition of the eight complex structures of CumD is shown in Fig. 4A and B. As can be seen at a glance, benzoate was bound in a significantly different direction compared with the seven other aliphatic organic acids. The aromatic ring of the benzoate molecule is tilted in the direction of Ala129, in order to circumvent steric hindrance of the Leu139 and Trp143 residues (Fig. 4A). In the complex structure with acetate only, the side chain of Ala129 pointed in a different direction, accompanied by deviation of the main chain around it. In contrast to benzoate, the seven aliphatic organic acids were bound in basically similar conformations. Isobutyrate and \((S)\)-2-methylbutyrate, whose alkyl chains are branched at the C2 position, were slightly moved in the direction of the C4 atom and Val142 (Fig. 4B). \(n\)-Butyrate, \(n\)-valerate, \((S)\)-2-methylbutyrate, and isovalerate extend their long alkyl chains to the hydrophobic pocket ahead of the C3 atom, which is formed by Ala129, Phe133, Ile199, and Val227. The alkyl chain of \(n\)-valerate, whose \(K_i\) value (0.47 mM) is the smallest among the eight organic acids, appears to fit the hydrophobic pocket of CumD.

As for the two oxygen atoms of the carboxyl group, the positions of the O1 atoms were dispersed, in contrast with the clustered O2 atoms (Fig. 4B). The standard deviations for the O1 and O2 atoms of the eight structures were 0.48 and 0.15 Å respectively. The O2 atom is situated in an oxyanion hole, which is formed by the main-chain nitrogen atoms of Ser34 and Phe104. On the other hand, the O1 atom is hydrogen-bonded with a water molecule and His252. The Ser103, His252, and Asp224 residues are the strictly conserved ‘‘catalytic triad’’ of MCP-hydrolases.\(^{29)\} MCP-hydrolases belong to a larger group of enzymes showing a unique catalytic reaction, viz. the \(\beta\)-ketolases, which cleave the carbon-carbon bonds of 1,3-diketones and 1,5-dioxovinyls.\(^{30)\} The catalytic mechanism of MCP-hydrolase has been shown to proceed through a base-catalyzed attack on the ketonized substrate by water, rather than a nucleophilic attack by the active site serine.\(^ {31–33)\) Previously, we proposed that the positions of the O1 and O2 atoms correspond to those of the attacking water and the negatively charged oxygen atom of the gem-diol intermediate.\(^ {20)\} Accordingly, when the substrate was bound in an improper direction, the base-catalyzed attack by the water becomes ineffective.

Based on these results, we concluded that improper binding of the 6-phenyl group causes inefficient hydrolysis ability of CumD as to phenyl-HODA. Figure 4C

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**Fig. 3. Structural Differences in the Complex Structures.**

A wireframe model of the isobutyrate complex structure (dark gray) is superimposed. Water molecules are shown as spheres. (A) Wireframe model (light gray) and 2\(F_o\)–\(F_c\) electron density map of the structure complexed with benzoate (BEZ301), around the Trp253 residue. The isobutyrate molecule is indicated as ISB301. (B) Wireframe model (light gray) and 2\(F_o\)–\(F_c\) electron density map of the structure complexed with \((S)\)-2-methylbutyrate, around the Ile139 residue.
shows the relationship between the directions of the bound organic acids, represented by the angle of the Ala103-C/C12-C1-C2 atoms, and the $k_{\text{cat}}$ values of the corresponding substrates. The $k_{\text{cat}}$ values of the substrates corresponding to benzoate, isovalerate, and (S)-2-methylbutyrate (phenyl-, isobutyl- and (±)-s-butyl-HODA), whose Ala103-C/C12-C1-C2 angles are outside of 100°–120°, were below 10 s$^{-1}$. The Leu139 and Trp143 residues in the N-terminal half of helix $\alpha$4 directly influence the direction of the bound benzoate, and thus this region is supposed to be the most important region determining the substrate specificities of MCP-hydrolases. For example, J3 CarC preferentially hydrolyzes an aromatic substrate, despite the fact that it belongs to the monoalkylbenzene group. The overall structure of J3 CarC resembled that of CumD rather than that of RHA1 BphD, but helix $\alpha$4 was completely disordered. Probably because of the flexibility of this region, J3 CarC can accept a large substrate.

The hydrophobic pocket, into which the long alkyl chains of $n$-valerate and isovalerate penetrate, is formed by the side chains of the Ala129, Phe133, Ile199, and Val227 residues (Fig. 4A). As for the TodF enzyme, which cannot effectively hydrolyze isopropyl-HODA,
Ala129, Ile199, and Val227 of CumD are substituted by valine, valine, and isoleucine respectively. Replacement of these residues of CumD with TodF-type ones caused a change in the substrate specificity to the TodF-type (S.-Y. Jun, unpublished data).

Interestingly, it is evident from superimposition that the side chain of the Trp253 residue can take on at least three conformations (Fig. 4A). The neighboring His252 residue also moves slightly. Moreover, the Arg185 three conformations (Fig. 4A). The neighboring His252 group of the HODA substrate. Thus the residues in the P-part possibly undergo large conformational changes in the catalytic cycle, and available crystal structures are insufficient fully to understand the interactions between a substrate and this unique enzyme.

Acknowledgments

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