Hydroquinone Dioxygenase from *Pseudomonas fluorescens* ACB: a Novel Member of the Family of Nonheme-Iron(II)-Dependent Dioxygenases

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Hydroquinone 1,2-dioxygenase (HQDO), an enzyme involved in the catabolism of 4-hydroxyacetophenone in *Pseudomonas fluorescens* ACB, was purified to apparent homogeneity. Ligandation with 4-hydroxybenzoate prevented the enzyme from irreversible inactivation. HQDO was activated by iron(II) ions and catalyzed the ring fission of a wide range of hydroquinones to the corresponding 4-hydroxymuconic semialdehydes. HQDO was inactivated by 2,2'-dipyridyl, o-phenanthroline, and hydrogen peroxide and inhibited by phenolic compounds. The inhibition with 4-hydroxybenzoate (Kₐ = 14 μM) was competitive with hydroquinone. Online size-exclusion chromatography-mass spectrometry revealed that HQDO is an α2β2 heterotetramer of 112.4 kDa, which is composed of an α-subunit of 17.8 kDa and a β-subunit of 38.3 kDa. Each β-subunit binds one molecule of 4-hydroxybenzoate and one iron(II) ion. N-terminal sequencing and peptide mapping and sequencing based on matrix-assisted laser desorption ionization—two-stage time of flight analysis established that the HQDO subunits are encoded by neighboring open reading frames (*hapC* and *hapD*) of a gene cluster, implicated to be involved in 4-hydroxyacetophenone degradation. HQDO is a novel member of the family of nonheme-iron(II)-dependent dioxygenases. The enzyme shows insignificant sequence identity with known dioxygenases.

Several aerobic microorganisms are capable of utilizing acetophenones for their growth (16, 17, 30–32). However, relatively little is known about the oxidative enzymes involved in acetophenones for their growth (16, 17, 30–32). However, related genes involved in the catabolism of other aromatic compounds. The function of the *hapC* and *hapD* genes is linked to the conversion of hydroquinone to 4-hydroxymuconic semialdehyde.

Several ring cleavage enzymes acting on substituted hydroquinones have been described. These include intradiol dioxygenases acting on hydroxyhydroquinone (4, 20, 22, 35, 39, 42, 55, 66) and extradiol dioxygenases that are active with (homono)genitrate (3, 28) or chlorohydroquinone (10, 44, 51). However, enzymes that use hydroquinone as the physiological ring cleavage substrate have not been characterized. Here we report on the purification and properties of hydroquinone dioxygenase (HQDO) from *P. fluorescens* ACB. It is shown that the heterotetrameric enzyme, encoded by the *hapC* and *hapD* genes, is a novel member of the family of nonheme-iron(II)-dependent dioxygenases. The present results confirm that the *hapG* gene, encoding an intradiol dioxygenase (46), is not involved in 4-hydroxyacetophenone degradation. This finding has important implications for the function of related genes involved in the catabolism of other aromatic compounds.

**MATERIALS AND METHODS**

*Materials.* Phenolic compounds were purchased from Aldrich or Acros. NADPH, NADH, NAD⁺, dithiothreitol (DTT), DNase, and RNase were from Boehringer. Phenylmethanesulfonyl fluoride was from Merck. N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), bis(2-hydroxyethyl)iminom-tris(2-hydroxymethyl)-methane (BisTris), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), bovine serum albumin (BSA), and 2,2'-dipyridyl and protamine sulfate were from Sigma. Phenanthroline was from Fluka. All other chemicals were of analytical grade.

*Bacterial strains and enzymes.* *P. fluorescens* ACB (31) was grown on 4-hydroxyacetophenone as described before (37). p-Hydroxybenzoate 1-hydroxylase from *Candida parapsilosis* CBS640 (21), p-hydroxybenzoate 3-hydroxylase from

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P. fluorescens (73), lipomamide dehydrogenase from Azotobacter vinelandii (77), α-amino acid oxidase from pig kidney (18), and 4-hydroxyacetonephycogenon-oxide (HAPMO) from P. fluorescens ACB (37) were purified as described previously.

**Purification of HQDO from P. fluorescens ACB.** Purification steps were performed at 4°C, unless stated otherwise. P. fluorescens ACB cells (5 g, wet weight) were suspended in 5 ml of 20 mM Bis-Tris chloride, pH 7.0, containing 1 mM 4-hydroxy-benzoylaldehyde (HAPAO) from P. fluorescens ACB (37) were purified as described previously.

The metal ion specificity of HQDO was determined by measuring the activity of HQDO, whereas the other part was used as a control. Immediately after the incubation, both samples were frozen in liquid nitrogen and stored at −80°C.

HQDO was therefore reconstituted in the presence of 4-hydroxyphenylacetate (HAPAO) from P. fluorescens ACB (37) were purified as described previously.

**Enzyme activity.** Enzyme activity measurements were performed at 25°C using air-saturated buffer. The activity of HQDO was determined as described before (37). For activity studies with HQDO, the stock enzyme solution (1 ml) was freshly incubated for at least 1 min with each substrate (14.4 kDa) in 20 mM Bis-Tris, pH 7.0, containing 10% glycerol.

HQDO activity was routinely determined spectrophotometrically by monitoring the formation of 4-hydroxymuconic semialdehyde at 320 nm (ε₃₂₀ = 11.0 mM⁻¹ cm⁻¹) (63). The assay mixture (1.0 ml) typically contained 50 to 200 nM enzyme and 10% (wt/vol) glycerol in 20 mM BES, pH 7.0. Reactions were started by the addition of 10 µl of 50 mM hydroquinone in dimethylformamide (DMF). One unit of HQDO activity is defined as the formation of 1 µmol of semialdehyde product per minute.

Potential atmospheric substrates (freshly dissolved in DMF) were tested at concentrations ranging from 10 µM to 10 mM with the total amount of DMF never exceeding 1%. Conversion of 2-fluorohydroquinone was studied by ²⁹F nuclear magnetic resonance (NMR). For this purpose, 0.3 mM 2-fluoro-4-hydroxybenzylalcohol was incubated with 0.45 µM NADPH and 0.5 µM HQDO in 50 mM Bis-Tris-Cl, pH 7.0, at 30°C. After NADPH consumption had ceased, the reaction mixture was divided in two parts. One part was incubated for 10 min with 1 mM HQDO, whereas the other part was used as a control. Immediately after the incubation, both samples were frozen in liquid nitrogen and stored at −20°C.

Potential phenolic inhibitors (freshly dissolved in DMF) were tested at concentrations ranging from 1 µM to 10 mM with the total amount of DMF never exceeding 1%. Conversion of 2-fluoro-4-hydroxybenzylalcohol was studied by ²⁹F nuclear magnetic resonance (NMR). For this purpose, 0.3 mM 2-fluoro-4-hydroxybenzylalcohol was incubated with 0.45 µM NADPH and 0.5 µM HQDO in 50 mM Bis-Tris-Cl, pH 7.0, at 30°C. After NADPH consumption had ceased, the reaction mixture was divided in two parts. One part was incubated for 10 min with 1 mM HQDO, whereas the other part was used as a control. Immediately after the incubation, both samples were frozen in liquid nitrogen and stored at −20°C.

Potential phenolic inhibitors (freshly dissolved in DMF) were tested at concentrations ranging from 10 µM to 100 mM with the total amount of DMF never exceeding 1%. Conversion of 2-fluoro-4-hydroxybenzylalcohol was studied by ²⁹F nuclear magnetic resonance (NMR). For this purpose, 0.3 mM 2-fluoro-4-hydroxybenzylalcohol was incubated with 0.45 µM NADPH and 0.5 µM HQDO in 50 mM Bis-Tris-Cl, pH 7.0, at 30°C. After NADPH consumption had ceased, the reaction mixture was divided in two parts. One part was incubated for 10 min with 1 mM HQDO, whereas the other part was used as a control. Immediately after the incubation, both samples were frozen in liquid nitrogen and stored at −20°C.

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trypsin, and keratin. GPMAW, version 6.10 (Lighthouse Data), was used to identify the peaks with known sequences.

For peptide sequencing an identical approach was followed, but in these experiments the MS and MS/MS measurements were performed on a MALDI-TOF-TOF instrument (AB 4700 Proteomics Analyzer, Applied Biosystems) in a reflection positive-ion mode using delayed extraction. The instrument is equipped with a 200 Hz neodymium-doped yttrium-aluminum-garnet laser operating at 355 nm. Typically 2,000 shots/spectrum were acquired in the MS mode, and 15,000 shots/spectrum were acquired in the MS/MS mode. During MS/MS analysis air was used as the collision gas. All spectra were calibrated externally using a peptide mixture that consists of 100 fmol of des-Arg bradykinin, angiotensin I, Glu-fibrinopeptide, adrenocorticotropic hormone (ACTH) clip 1 to 17, ACTH clip 7 to 38, and ACTH clip 18 to 39. Mass accuracy is within 50 ppm. The data were analyzed using de novo sequencing by GPS software (Applied Biosystems) and was confirmed by manual examination.

N-terminal sequencing. The N-terminal sequence of HQDO was determined by Edman degradation. The contents of a 15% SDS-PAGE gel (140 by 120 by 1.5 mm) loaded with 9.2 and 18.4 μg of purified enzyme were blotted onto a polyvinylidene difluoride Immobilon-P support (Millipore) in 10 mM CAPS, pH 11.0, containing 10% ethanol. After the membrane was stained with 0.1% Coomasie R-250 in 50% methanol, the main bands corresponding to relative molecular masses of 38, 32, and 18 kDa were excised. Gas phase sequencing of the polypeptide on the Immobilon support was carried out at the sequencing facility of Leiden University, The Netherlands.

Sequence comparison. As reported in the accompanying paper (46), the nucleotide and amino acid sequences of HQDO have been deposited in the DDBJ/EMBL/GenBank databases (accession number AF355751: gi 182374631 and gi 182374632). For sequence alignment studies, a PSI-BLAST (1) search was performed at the National Centre for Biotechnology Information (NCBI). Multiple sequence alignments and an unrooted tree were made with the CLUSTAL W program at the European Bioinformatics Institute (www.ebi.ac.uk/clustalw) (69). BioEdit (26) was used to calculate the pairwise identity and similarity scores (PAM250 matrix) from the aligned sequences and for the display of the alignment. Treeview was used for visualization of the dendrogram (52).

RESULTS

Purification of HQDO from P. fluorescens ACB. Purification of HQDO was not straightforward. Initial purification attempts, performed either in the absence or presence of 1 mM DTT, resulted in rapid loss of enzyme activity. In these procedures, part of the activity could be restored by incubation of the partially purified enzyme with iron(II) salts. Addition of ascorbate had almost no effect. A dramatic improvement in the yield of active enzyme was obtained after the finding that the enzyme could be stabilized by ligandation with the inhibitor 4-hydroxybenzoate. Table 1 summarizes a typical purification from 5 g of 4-hydroxycacetophenone-grown cells. Purification by four steps resulted in a yield of 38%, a purification factor of 22.5, and a specific activity of 5.9 U mg⁻¹. Purified enzyme, not treated with iron(II) ions, has 10 to 20% of the activity of iron(II)-complexed enzyme.

SDS/PAGE analysis of the purified enzyme revealed the presence of one main protein band, corresponding to a molecular mass of about 38 kDa, and several minor bands (Fig. 1). The purified HQDO eluted from an analytical Superdex 200 column in one symmetrical peak with an apparent molecular mass of 105 ± 5 kDa.

Spectral properties. HQDO from P. fluorescens ACB showed an absorption maximum at 279 nm and very weak absorbances around 350 and 420 nm (Fig. 2). A similar feature was described for extradiol dioxygenases (68, 80). For comparison, intradiol dioxygenases display a maximum in the visible region around 450 nm (41, 50) due to tyrosine ligandation (54).

Catalytic properties. HQDO from P. fluorescens ACB catalyzed the ring cleavage of hydroquinone to 4-hydroxymuconic acid. The UV-visible light absorption spectrum of 16 μM enzyme as isolated (2).

![FIG. 1. SDS-PAGE of HQDO from P. fluorescens ACB.](http://jb.asm.org/)

![FIG. 2. Spectral properties of HQDO from P. fluorescens ACB.](http://jb.asm.org/)
Addition of Fe$^{2+}$ in nonliganded form. Most of the activity could be restored by the competitive inhibitor 4-hydroxybenzoate strongly increased the thermal stability of HQDO. Using the same incubation conditions, ACB was rather unstable. At 30°C and pH 7.0, more than 90% of activity was lost in a time-dependent manner by the oxidizing agent hydrogen peroxide (Table 4). This suggests that the enzyme contains a nonheme iron(II) ion in the active site (13, 14, 27, 63). In line with this, HQDO was inactivated by the oxidizing agent hydrogen peroxide (Table 4).

**TABLE 2. Substrate specificity of HQDO from P. fluorescens ACB**

<table>
<thead>
<tr>
<th>Substrate (200 μM)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroquinone</td>
<td>100</td>
</tr>
<tr>
<td>Methylhydroquinone</td>
<td>120</td>
</tr>
<tr>
<td>Methoxyhydroquinone</td>
<td>50</td>
</tr>
<tr>
<td>Chlorohydroquinone</td>
<td>70</td>
</tr>
<tr>
<td>Bromohydroquinone</td>
<td>30</td>
</tr>
<tr>
<td>2,3-Difluorohydroquinone</td>
<td>80</td>
</tr>
<tr>
<td>2,5-Difluorohydroquinone</td>
<td>75</td>
</tr>
<tr>
<td>3,5-Difluorohydroquinone</td>
<td>90</td>
</tr>
</tbody>
</table>

*Activity expressed as relative absorbance increase at 320 nm.*

**TABLE 3. Phenolic inhibitors of HQDO**

<table>
<thead>
<tr>
<th>Inhibitor name</th>
<th>Activity (%)</th>
<th>Inhibitor concn (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxybenzoate</td>
<td>12</td>
<td>200</td>
</tr>
<tr>
<td>2,4-Dihydroxybenzoate</td>
<td>13</td>
<td>200</td>
</tr>
<tr>
<td>2,3,4-Trihydroxybenzoate</td>
<td>81</td>
<td>200</td>
</tr>
<tr>
<td>2,4,6-Trihydroxybenzoate</td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>2-Fluoro-4-hydroxybenzoate</td>
<td>12</td>
<td>200</td>
</tr>
<tr>
<td>3-Fluoro-4-hydroxybenzoate</td>
<td>19</td>
<td>200</td>
</tr>
<tr>
<td>2-Chloro-4-hydroxybenzoate</td>
<td>23</td>
<td>200</td>
</tr>
<tr>
<td>4-Methoxybenzoate</td>
<td>76</td>
<td>200</td>
</tr>
<tr>
<td>4-Hydroxybenzonitrile</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>4-4-Hydroxyphenylacetate</td>
<td>3</td>
<td>200</td>
</tr>
<tr>
<td>4-Hydroxybenzylalcohol</td>
<td>8</td>
<td>200</td>
</tr>
<tr>
<td>4-Hydroxybenzaldehyde</td>
<td>12</td>
<td>200</td>
</tr>
<tr>
<td>4-Hydroxybenzylycanide</td>
<td>24</td>
<td>200</td>
</tr>
<tr>
<td>Vanillin</td>
<td>33</td>
<td>200</td>
</tr>
<tr>
<td>4-Hydroxycinnamate</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>59</td>
<td>80</td>
</tr>
<tr>
<td>Tetrafluorohydroquinone</td>
<td>68</td>
<td>40</td>
</tr>
<tr>
<td>Hydroxyhydroquinone</td>
<td>64</td>
<td>20</td>
</tr>
<tr>
<td>Phenol</td>
<td>83</td>
<td>200</td>
</tr>
<tr>
<td>Hydroxyacectophenone</td>
<td>64</td>
<td>20</td>
</tr>
<tr>
<td>4-Hydroxycinnamate</td>
<td>35</td>
<td>40</td>
</tr>
<tr>
<td>Catechol</td>
<td>86</td>
<td>200</td>
</tr>
<tr>
<td>4-Nitrophenol</td>
<td>77</td>
<td>20</td>
</tr>
<tr>
<td>Isoeugenol</td>
<td>94</td>
<td>200</td>
</tr>
<tr>
<td>Less than 20%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Activity expressed as relative absorbance increase at 320 nm.*

**FIG. 3. Thermal stability of HQDO from P. fluorescens ACB.**

Time-dependent loss of activity of a 6 μM concentration of purified enzyme at 30°C in 20 mM BisTris-Cl–10% glycerol, pH 7.0, in the absence (○) and presence (★) of 1 mM 4-hydroxybenzoate.
TABLE 4. Time-dependent inactivation of HQDO by iron(II) modifying agents

<table>
<thead>
<tr>
<th>Inactivation agent</th>
<th>Conc (mM)</th>
<th>Half-time of inactivation at 30°C (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2′-Dipyridyl</td>
<td>0.1</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>20</td>
</tr>
<tr>
<td>o-Phenanthroline</td>
<td>0.1</td>
<td>40</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0.1</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Regioselectivity of dioxygenation. In the accompanying paper we showed that 2,3-difluorohydroquinone and 2,5-difluorohydroquinone undergo ring fission in between C-1 and C-6, yielding the corresponding difluorinated 4-hydroxymuconic semialdehydes (46). Absorption spectral analysis indicated that the regioselectivity of dioxygenation of monohalogenated hydroquinones by HQDO differs with the type and position of the halogen substituents. Activity measurements with chloro- and bromohydroquinone resulted in an increase in absorption at 320 nm, pointing at the formation of a halogenated 4-hydroxymuconic semialdehyde product. In contrast, incubation of fluorohydroquinone with HQDO did not result in an absorption increase at 320 nm. 19F NMR confirmed that in the latter reaction no semialdehyde was formed but that the fluorohydroquinone substrate was enzymatically defluorinated in a time-dependent manner compared to the absence of fluoride anion production in the control experiment. This suggests that the ring cleavage of the fluorinated substrate takes place in between the hydroxyl and fluorine substituent. Such ring cleavage will result in an unstable acyl halide which readily reacts with water, yielding nonfluorinated maleylacetate and halide anion, as described for the conversion of chlorohydroquinones by chlorohydroquinone dioxygenase (9, 51, 81).

N-terminal sequence analysis. The N-terminal sequence of the protein corresponding to the major band in SDS-PAGE (Fig. 1, lane f) was determined by Edman degradation. The N-terminal sequence of this 38-kDa protein was found to be AMLEAVETEN. Except for the starting methionine, this sequence corresponds with the N-terminal sequence of HapD derived from the DNA sequence (46). The 32- and 18-kDa protein bands observed in lane f of Fig. 1 were also analyzed by Edman degradation. The 32-kDa band was selected because its mass corresponds to that of HapG, a putative hydroxyhydroquinone 1,2-dioxygenase (46). The 18-kDa band was selected because during purification it always coelutes with the 38-kDa protein. The N-terminal sequence of the 32-kDa protein was determined to be AAITAALVKE. This sequence is specific for the translation elongation factor Ts present in many Pseudomonas organisms and suggests that the 32-kDa protein represents an impurity in the HQDO preparation. The N-terminal sequence of the 18-kDa protein was found to be STQPAPFK TVF. Except for the starting methionine, this sequence corresponds with the N-terminal sequence of HapC derived from the DNA sequence (46), suggesting that HapC is a distinct subunit of the HQDO protein.

Hydrodynamic properties of HQDO. Initial analysis of partially purified HQDO by size exclusion chromatography (SEC) suggested a protein complex with a size corresponding to a molecular mass of about 105 kDa. Electrospray MS analysis of HQDO in the absence of iron(II) sulfate and 4-hydroxybenzoate revealed two series of protein ions having a different number of charges and molecular masses of 30,293 ± 5 Da and 112,376 ± 50 Da (Fig. 4). The 30-kDa protein is likely to be the translation elongation factor Ts impurity already identified by Edman degradation.

The subunit composition of the HQDO protein assembly was studied in more detail by coupling SEC to MS. The ion chromatography of HQDO in the absence of iron(II) sulfate and 4-hydroxybenzoate showed two protein peaks eluting around 27 and 31 min (Fig. 5A, top). The late-eluting compounds around 41 min are smaller molecules, and the peak around 47 min corresponds with the total volume of the column. Peak 1, eluting after 27 min, resulted in three ion series in the mass spectra in between m/z 1,300 and m/z 8,500 (Fig. 5A, bottom). The corresponding molecular masses for these three ion series were 17,769 ± 1 Da, 38,288 ± 10 Da, and 94,477 ± 45 Da. As the elution volume of this peak suggests a hydrodynamic volume corresponding to a mass of about 100 kDa, the observed proteins in the mass spectrum are likely to be fragments of the larger protein assembly. The masses of these fragments correspond with the subunit molecular masses observed in SDS-PAGE and with the masses calculated from the HapC and HapD sequences, suggesting that they are part of the HQDO assembly. The peak eluting at 31 min contained a protein with a mass of 30.3 kDa, corresponding to the size of the elongation factor (mass spectrum not shown).

In a subsequent online SEC-MS experiment, we added iron(II) sulfate and 4-hydroxybenzoate to the protein sample before injection onto the column. This protein sample yielded a similar elution chromatogram as the experiment without iron(II) sulfate and 4-hydroxybenzoate (data not shown). However, the accumulated mass spectra of the peak around 27 min

FIG. 4. Nanoelectrospray MS spectrum of HQDO directly infused from capillaries using gentle conditions. This spectrum shows two charge envelopes. The first charge distribution at m/z 1,000 to 3,000 with charges from +10 to +20 corresponds to a protein of 30 kDa, which is likely the translation elongation factor Ts. The second charge distribution at m/z 5,000 to 6,000 with charges between +19 to +22 reveals a mass of 112 kDa and corresponds to the intact HQDO protein complex. The instrument settings were chosen such that no fragmentation of the protein assembly occurs. The transformation of the charge envelopes to their molecular masses is shown.
were clearly different (Fig. 5B). Mass determination of the ion series centered around 5,400 \(m/z\) revealed a mass of 112,387 ± 50 Da. This mass spectrum also showed ion series in which the determined protein mass was 17,769 ± 5 Da and 38,283 ± 10 Da and mass of the trimeric subcomplex was 94,488 ± 45 Da (Fig. 5B). These fragments were generated from the 112-kDa protein assembly by gas phase dissociation.

The calculated mass based on the primary sequence is 38,268.98 without the methionine at position 1. The observed mass by online SEC-MS is 38,283 ± 10 Da in the absence of iron(II) sulfate and 4-hydroxybenzoate.

These results unambiguously show that native HQDO has a mass of 112 kDa and is composed of two α-subunits of 17.8 kDa and two β-subunits of 38.3 kDa. The addition of iron(II) sulfate resulted in a new ion peak near the original ion peaks of the monomeric β-subunit at \(m/z\) 3,190, but not near the ion peaks of the monomeric α-subunit at \(m/z\) 1,975 (Fig. 6A). Mass determination revealed a mass difference of 55 Da, strongly indicating the interaction between the β-subunit with one molecule of iron(II). This interaction was likely to be specific, as we did not observe any interaction between the α-subunit and iron(II). In dissociation experiments the HQDO heterotetramer was fragmented into an α-subunit and a αβ-β-trimer. Upon the addition of 4-hydroxybenzoate to HQDO, a peak shift in the trimeric ions of HQDO (αβ-β) corresponding to a mass shift of 270 Da was observed (Fig. 6B), which may suggest that, under the experimental conditions applied, one 4-hydroxybenzoate molecule (molecular mass of 138 Da) binds to one β-subunit. Again, no changes were observed in the α-subunits ions.

**Peptide mapping and sequencing.** In-gel tryptic digestion of HapA (Fig. 1, 67-kDa band in lane e) and subsequent MALDI-TOF analysis yielded 56 peptides. Comparison of these peptides with the known sequence of HapA (37) gave 66% sequence overlap with 42 out of 56 peptides.

MALDI-TOF analysis of the tryptic digests of the 38-kDa band of HQDO (Fig. 1, lane f) revealed 22 peptide fragments (\(m/z\) 1,239.6, 1,557.7, 1,868.7, 1,884.8, 1,979.8, 1,985.8, 2,007.2, 2,021.8, 2,033.9, 2,135.9, 2,231.9, 2,247.9, 2,296.9, 2,326.9, 2,424.9, 2,476.9, 2,750.6, 2,756.3, 3,209.0, 3,248.1, 3,368.1, and 3,408.1). Database mass analysis of these peptides yielded no significant mapping with any protein in the Swissprot database. However, 14 peptides corresponded with the sequence of HapD (46) and covered 55% of the protein sequence (Fig. 7).

Analysis of the 18-kDa band (Fig. 1, lane f) showed that this band corresponds to the HapC protein (46). MALDI-TOF-TOF analysis of tryptic fragments yielded 59% of the total amino acid sequence (Fig. 7) by mapping 7 of 16 expected peptides (\(m/z\) 1,214.7, 1,413.8, 1,583.9, 1,645.1, 1,712.0, 1,861.1, and 2667.3).

**DISCUSSION**

This paper describes the purification and initial characterization of HQDO, an unusual ring cleavage enzyme involved in the catabolism of 4-hydroxyacetophenone in *P. aeruginosa* ACB. HQDOs have been described in degradation pathways of *Sphingobium chlorophenolicum* (9), *Pseudomonas* sp. strain HH35 (60), *Anthraxsubacter protonphormae* (14), *Burkholderia cepacia* (15), *Pseudomonas putida* JD1 (19), and *Moraxella* (63). However, purification procedures or amino acid sequence data were not reported for any of these enzymes.
views of the oxide and the iron chelators 2,2-purification of this enzyme in its active form. Hydrogen per-
charge state of the /H9251/monomer after the addition of 4-hydroxybenzoate . Overlay and zoom in to the
charge state of the /H9251/-subunit and of the +12 charge state of the /H9251/-subunit remains unchanged. The adduct peaks in the /H9251/-subunit correspond to a mass of 55 units, strongly indicating that one iron molecule binds to the /H9251/-monomer. (B) HQDO monomer after the addition of 4-hydroxybenzoate. Overlay and zoom views of the +9 charge state of the /H9251/-subunit and of the +12 charge state of the trimeric subcomplex. Traces represent HQDO with and without 4-hydroxybenzoate. The +12 charge state of the trimeric subcomplex shows a considerable mass shift to higher m/z after the addition of 4-hydroxybenzoate, whereas the +9 charge state of the /H9251/-subunit remains unchanged. The mass shift corresponds to a mass of 270 units, strongly indicating that two molecules of 4-hydroxybenzoate are bound to the trimeric subcomplex. pOHB, 4-hydroxybenzoate. α1β2 trimer.

Binding of the competitive inhibitor 4-hydroxybenzoate increased the stability of HQDO and allowed, for the first time, purification of this enzyme in its active form. Hydrogen peroxide and the iron chelators 2,2′-dipyridyl and 1,2-phenanthroline inactivated HQDO. From this and its catalytic features, we concluded that HQDO belongs to the family of nonheme-iron(II)-dependent dioxygenases.

HQDO from P. fluorescens ACB catalyzed the ring fission of a wide range of hydroquinones to the corresponding 4-hydroxymuconic semialdehydes. Substrate profiling showed that both para-hydroxyl groups of hydroquinone are crucial for enzyme activity. Hydroquinones with an electron-donating methyl or methoxy group were readily converted. Hydroquinones containing electron-withdrawing substituents were also converted, albeit at lower rates. The number and position of fluorne substituents determined both the reaction rate and the regioselectivity of dioxygenation. With 2-fluorohydroquinone, ring splitting occurred in between C-1 (bonded to an OH) and C-2 (bonded to a F). 2,3-Difluorohydroquinone was cleaved in between C-1 (bonded to an OH) and C-6 (bonded to a H) (46), and tetrafluorohydroquinone turned out to be a strong inhibitor. Introduction of a hydroxyl (hydroxyhydroquinone) or carboxyl (gentisate) group at the ortho-position resulted in strong or weak enzyme inhibition, respectively. These results suggest that the activity of HQDO is determined by both electronic and steric constraints and that substrates may become differently oriented in the active site.

HQDO was reversibly inhibited by a large number of nolic compounds. 4-Hydroxybenzylic compounds and 4-hydroxycinnamates were strong inhibitors. From this and the fact that the inhibition by 4-hydroxybenzoate was found to be competitive with hydroquinone, it is concluded that the 4-hydroxyl group serves as the iron ligand and that the para-substituent of the phenol is an important determinant for discriminating between weak and tight binding.

HQDO from P. fluorescens ACB appeared to be a heterotetramer built up by subunits of 18 and 38 kDa. Online SEC-MS was used to elucidate the components and stoichiometry of the protein assembly. This is one of the first examples in which SEC-MS was used to elucidate the stoichiometry and oligomeric state of a protein complex. Cavanagh et al. (12) used the coupling of SEC to establish the oligomeric state of the transition state regulator AbrB and the stoichiometry of its interaction with DNA. Moreover SEC-MS has been shown to be a valuable method for online separation of products in a reaction mixture with subsequent MS analysis (72) The mass of the /H9251/-subunit agrees well with the theoretical mass based on the primary sequence of protein HapD encoded by the hapD gene (46).

Using MS/MS analysis 7 peptides of the HQDO /H9252/-subunit and 14 peptides of the HQDO /H9252/-subunit were sequenced. These data confirmed that HQDO is encoded by the hapC and hapD genes. Except for nine noncharacterized orthologous hapCD clusters present in the sequenced genomes of Pseudomonas aeruginosa PA7, P. luminescens subsp. laumondii TT01, and seven Burkholderia strains, HQDO showed no significant sequence similarity with any other protein. The heterotetrameric character of HQDO clearly discriminates the enzyme from most other nonheme-iron(II)-dependent dioxygenases. Except for protocatechuate 4,5-dioxygenase and 2-aminophenol 1,6-dioxygenase, they all consist of one type of subunit (6, 68). From the sequence alignment it appears that HQDO is to some extent related to 3-hydroxyanthranilate 3,4-dioxygenase but that HQDO constitutes a new type of enzyme in the iron(II)-dependent dioxygenase family.

The size and subunit composition of HQDO suggest that the enzyme contains two active sites per heterotetramer. As for protocatechuate 4,5-dioxygenase (65) and 2-aminophenol 1,6-dioxygenase (40), these active sites are likely positioned in the larger subunits. Our MS analysis showed that each /H9252/-subunit binds one molecule of the competitive inhibitor 4-hydroxybenzoate. Moreover, the binding of one iron atom to the monomer /H9252/-subunit was observed. This is reminiscent of other heteromeric extradiol dioxygenases that have one iron atom per αβ tetramer (2, 68), although protocatechuate 4,5-dioxygenase from Pseudomonas pseudoalcaligenes JS45 was shown to contain two iron atoms per αβ tetramer (40). Whether...
native HQDO contains one or two iron atoms per heterotetramer cannot be deduced from the charge envelope of the complex as the peak resolution decreases compared to the monomeric species.

The active site of iron(II)-dependent dioxygenases generally harbors two histidines and a glutamate that are involved in binding the iron (58, 70). Most of these enzymes use a catechol as a substrate. When a catecholic substrate binds, the two hydroxyl groups of the substrate replace two water molecules in iron ligandation (58). The strong iron-chelating properties of a catecholic compound are nicely illustrated by the action of enterochelin, a catecholate siderophore that scavenges iron during bacterial infections (23). In the case of HQDO, the question arises as to how the interaction between the phenolic substrate and the iron cofactor occurs. Here, the situation has to be different because the hydroquinone substrate does not contain an \textit{ortho}-hydroxyl group. The same is true for (homo)-gentisate dioxygenases. Based on the crystal structure of sub-

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Multiple sequence alignment of the \(\alpha\)- and \(\beta\)-subunits of HQDO from \textit{P. fluorescens} ACB. CLUSTAL W alignment of the amino acid sequences of HapC and HapD from \textit{P. fluorescens} ACB and hypothetical proteins from \textit{Pseudomonas aeruginosa} PA7 (gi 152988009 and gi 152987326), \textit{Burkholderia} sp. strain 383 (gi 78063587 and gi 78063586), and \textit{Photorhabdus luminescens} subspp. \textit{laumondii} TTO1 (gi 37524165 and gi 37524166). Conserved residues are in dark boxes, and similar residues are in gray boxes. The amino acid sequences predicted by MALDI-TOF-TOF peptide mapping and sequencing are depicted on top of the CLUSTAL W alignment by asterisks. Related sequences of other \textit{Burkholderia} species (\textit{B. cepacia} AMD [gi 115359956 and gi 115359957], \textit{Burkholderia ambifaria} MC40-6 [gi 11869769 and gi 118697689], \textit{Burkholderia cenocepacia} PC184 [gi 84353671 and gi 84353670], \textit{Burkholderia multivorans} ATCC 17616 [gi 118718179 and gi 118718178], \textit{Burkholderia phymatum} STM815 [gi 118032827 and gi 118032828], and \textit{Burkholderia cenocepacia} HI2424 [gi 116691528 and gi 116691529]) are not shown in the alignment but are very similar to the \textit{Burkholderia} sp. strain 383 sequences.}
\end{figure}
strate-free human homogentisate dioxygenase (70) and electron paramagnetic resonance studies (29), it was proposed that in this enzyme the carboxylate anion of (homo)gentisate serves as an iron ligand as does the phenolate group of catechol in catechol dioxygenases. For HQDO, the active-site residues involved in binding the iron could not simply be predicted from sequence alignments. Based on the sequence similarity with 3-hydroxyanthranilate 3,4-dioxygenases, His258 and His305 could serve as iron ligands in HapD. In addition to the two histidines and an acidic residue, another residue might be involved in binding the metal cofactor (8, 43, 54, 56, 64). Another possibility is that upon hydroquinone binding, the iron atom remains bound to one or more water molecules.

The identification of the two subunits of HQDO confirms our earlier proposal (46) that the HQDO encoding the hapC and hapD genes is part of the hapCDEFGHIB4 gene cluster. The HapC sequence shows significant homology with only nine other protein sequences (49 to 78% sequence identity) that are present in the microbial genome database (Fig. 7). All of these bacterial genes are neighbored by a hapD ortholog (Fig. 7). This conserved gene clustering is in line with the tight interaction between HapC and HapD. We suspect that HapC is also homologous to the open reading frame found downstream of pnpS in P. fluorescens ENV2030 (5, 82). Unfortunately, the genome sequence of the latter organism has not yet been published (5, 82). The α- and β-subunits of the (putative) HQDOs mutually have little sequence similarity, suggesting that they have a different ancestor. Furthermore, HapD has no significant sequence identity with known nonheme-iron(II)-dependent dioxygenases.

Interestingly, amino acids 258 to 323 of HapD show 25% sequence identity with amino acids 50 to 116 of 3-hydroxyanthranilate 3,4-dioxygenase from Saccharomyces cerevisiae. This monomeric enzyme, involved in the kynurenine pathway (11), is also an iron(II)-dependent dioxygenase. Hydroxyanthranilate 3,4-dioxygenases contain two conserved histidines, which are thought to be involved in iron ligandation (49). Selective replacement of these residues by alanine resulted in no detectable activity (49). The conserved histidines of 3-hydroxyanthranilate 3,4-dioxygenases correspond to His258 and His305 in HapD. Therefore, these histidines might serve as iron ligands in HapD.

With the elucidation of the function of the hapC and hapD genes and the observed instability of the HQDO protein, it is tempting to speculate on the function of the ferredoxin encoded by the hapI gene (46). For catechol 2,3-dioxygenases it was reported that in some degradation pathways, these proteins are needed for the reductive reactivation of the iron cofactor (25, 33, 34, 53, 71). A similar in vivo protection of HQDO can be envisioned for HapI.

In summary, this paper describes the first purification and characterization of an HQDO. The enzyme is a heterotrimer composed of 18- and 38-kDa subunits and is the prototype of a novel subclass of the family of iron(II)-dependent dioxygenases.

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