Crystal Structure of the Hydroxyquinol 1,2-Dioxygenase from Nocardioides simplex 3E, a Key Enzyme Involved in Polychlorinated Aromatics Biodegradation*

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Hydroxyquinol 1,2-dioxygenase (1,2-HQD) catalyzes the ring cleavage of hydroxyquinol (1,2,4-trihydroxybenzene), a central intermediate in the degradation of aromatic compounds including a variety of particularly recalcitrant polychloro- and nitroaromatic pollutants. We report here the primary sequence determination and the analysis of the crystal structure of the 1,2-HQD from Nocardioides simplex 3E solved at 1.75 Å resolution using the multiple wavelength anomalous dispersion of the two catalytic iron (1 Fe/293 amino acids). The catalytic Fe(III) coordination polyhedron composed by the side chains of Tyr164, Tyr197, His221, and His223 resembles that of the other known intradiol-cleaving dioxygenases, but several of the tertiary structure features are notably different. One of the most distinctive characteristics of the present structure is the extensive openings and consequent exposure to solvent of the upper part of the catalytic cavity arranged to favor the binding of hydroxyquinols but not catechols. A co-crystallized benzofuroxane-like molecule is also found bound to the metal center forming a distinctive hydrogen bond network as observed previously also in 4-chlorocatechol 1,2-dioxygenase from Rhodococcus opacus 1CP. This is the first structure of an intradiol dioxygenase specialized in hydroxyquinol ring cleavage to be investigated in detail.

Hydroxyquinol (1,2,4-trihydroxybenzene) (HQ)1 is one of the central intermediates in the degradation of a large variety of aromatic compounds. It has been detected in the breakdown of 4-hydroxybenzoate, resorcinol, salicylate, vanillic, benzoate, protocatechuic, and gentisate, by fungi such as Trichosporon cutaneum or Phanerochaete chrysosporium (1–3). In bacteria HQ has been identified as an intermediate in the degradation of mononuclear hydroxyaromatic compounds such as resorcinol and 2,4-dihydroxybenzoate (4, 5) or amino-hydroxyaromatic compounds such as 4-aminophenol (6) as well as in the degradation of hydroxylated biaryl ethers such as 2-hydroxydibenzop-dioxin and 3-hydroxydibenzo furan (7). HQ also occurs in the catabolic pathways of aromatic compounds carrying nitro groups such as in 3- and 4-nitrophenol or as 4-nitrocatechol (8–12).

HQ and its chloro-substituted derivatives 5-chlorohydroxyquinol (5CHQ) and 6-chlorohydroxyquinol (6CHQ) play an especially important role in the bacterial degradation of phenols or phenoxyacetates carrying a chloro-substituent in para position to the OH or OCH2COO group, respectively. Thus, pentachlorophenol by rhodococci and mycobacteria has been reported to be degraded via HQ (13–16), whereas in Sphingobium chlorophenolicum (Sphingomonas chlorophenolica) already the 2,6-dichloroquinol appears to be subject to ring cleavage and, in contrast to earlier reports, no 6CHQ is formed (17). On the contrary, for 2,4,6-trichlorophenol breakdown 6CHQ has been suggested as an intermediate for Streptomycetes rochei 303 as well as several Gram-negative bacteria (18–24). 2,6-Dichlorophenol can also be degraded via 6CHQ, whereas 2,4-dichlorophenol, 4- and 2-chlorophenol by 2,4,6-trichlorophenol-induced cells may be transformed on the same pathway, but yielding HQ as a ring cleavage substrate (18, 19, 24). In 2,4,5-trichlorophenoxyacetate degradation by Burkholderia (Pseudomonas) cepacia AC1100 5CHQ and HQ are formed sequentially as intermediates (25). Although 3,5-dichlorohydroxyquinol was found to be an intermediate of 2,4-dichlorophenoxyacetate degradation by Nocardioides simplex 3E, HQ may also be involved as a ring cleavage substrate (26, 27).

HQs are degraded aerobically by specialized intradiol ring-cleaving dioxygenases; the most studied enzymes from this family are the protocatechuic 3,4-dioxygenases (3,4-PCDs), the catechol 1,2-dioxygenases (1,2-CTDs), and the chlorocatechol 1,2-dioxygenases (1,2-CCDs) which generally possess distinctive substrate specificities (28). The hydroxyquinol 1,2-dioxygenases (1,2-HQDs hereafter) catalyze the intradiol cleavage of hydroxyquinolins to form 3-hydroxy-cis,cis-muconates, which occur in solution in the keto form, i.e. as maleylacetate (Scheme 1) (29).

Several 1,2-HQDs have been purified and characterized from...
conserved regions of the 1,2-HQDs of R. picketti (hadC), Arthrobacter sp. strain BA-5-17, Sphingomonas wittickii RW1 (dxtB), B. cepacia (gltF), and Agrobacterium tumefaciens C58 (7, 31, 32, 43). Primer HQD- 

fev1 (5′-CCG GAT TRS ATC CTG-3′) targets the bases corresponding to amino acid positions 82–87 in the alignment (see Fig. 4), whereas primer HQD-ref1 (5′-CCR TCR KNM GGN ATS GGR TA-3′) is expected to bind to the bases corresponding to positions 219–224 in the alignment (see Fig. 4). Thus, the PCR products had an expected length of about 400 bp.

The PCR mixture (50 μl) contained 30 pmol of each primer, 0.5 μg of genomic template DNA, 20 μl each deoxynucleotide triphosphate, 1 × PCR buffer (MBI Fermentas), 1.0 unit of DNA Taq polymerase (MBI Fermentas), 1.5 mM MgCl2, 5% dimethyl sulfoxide, and 0.5% bovine serum albumin. The PCR was performed with a touchdown thermocycle program: an initial denaturation (95 °C, 5 min); 10 cycles with decreasing annealing temperature (60–50 °C, 30 s), polymerization (72 °C, 1 min), and denaturation (95 °C, 30 s); 20 more cycles with 50 °C as the annealing temperature; and an additional 5 min of polymerization during the last cycle.

After cloning of the 400-bp PCR product into a T vector, giving rise to plasmid pNocSi01, sequencing of the fragment proved to be homologous to the corresponding segments of other 1,2-HQD genes. Labeling of the 400-bp fragment by a DIG DNA Labeling and Detection Kit Non radioactive (Roche) was performed as described in the Roche manual. The probe was then used to detect the corresponding fragment on a Southern blot of 0.64 μg of N. simplex 3E DNA digested with BamHI, PstI, SacI, and XhoI, respectively, and run on a 1% agarose gel with 1× TAE buffer (47). From a second gel, an area that corresponded in size to the hybridization signal (3 kbp, SacI) was excised, and the included DNA was eluted and ligated into the dephosphorylated SacI site of pBluescript II SK (+). After transformation of the ligation mixture into E. coli DH5α, the labeled insert of pNocSi01 was used to identify clone pNocSi89 by colony hybridization.

The nucleotide sequence of the 1,2-HQD was determined by preparing subclones of pNocSi89 with the restriction enzymes SacII and XhoI. Two different 650-bp SacII restriction fragments and a 1,2-kb XhoI fragment, respectively, encode the complete sequence of the gene. For sequencing, DNA Fermentas CycleSequencing Kit was used, with subsequent electrophoresis with a Li-cor sequencing Kit was used, with subsequent electrophoresis with a Li-cor 4200 IR2 sequencer and analyzer with the e-seq program (version 1.2). Sequences were assembled using Staden Package version 2002. The sequence is available under GenBank/EMBL/DDBJ accession number AY822041. Comparisons with data-base entries were performed by using BLASTX (50). Multiple sequence alignments were created using ClustalX (version 1.8) (51).

**Crystallization and Data Collection**—The enzyme was crystallized at 293 K using the sitting drop vapor diffusion method from a solution containing 2.0 mM ammonium sulfate, 4% polyethylene glycol 400, 100 mM Hepes pH 7.5 (52). The drops consisted of 4 μl of 20 mg/ml protein solution and 6 μl of reservoir solution equilibrated against 30 μl of reservoir solution (Crystal Clear Strips from Molecular Dimension, Inc.).

A native data set extending to a maximum resolution of 1.75 Å was collected at the X11 beamline, EMBL, DESY, Hamburg. Data were collected using a MAR CCD165 detector at a wavelength of 0.908 Å. Crystals belong to the primitive monoclinic space group P21, with unit cell dimensions a = 46.28, b = 84.98, c = 83.92 Å, β = 92.84°. For all data collections crystals of the native enzyme were cooled at 100 K adding 17% ethylene glycol to the mother liquor solution as cryoprotectant. Crystals suffered from damage if they were transferred in solution different from their mother solution unless they were previously cross-linked adding glutaraldehyde to the drops up to a final concentration of roughly 2% (v/v).

**Metal Content Analysis**—Analysis of the protein metal content was performed by using a PerkinElmer Optima 2000 Inductively Coupled Plasma AES (Atomic Emission Spectrometry) Dual Vision. The metal content analysis revealed the presence of 2 equivalents of iron ions and 1 equivalent of copper ions/mol of protein.

**Structure Determination and Refinement**—All molecular replacements attempts, using coordinates of known intradiol dioxygenases as a model, failed to provide a solution for Ns 1,2-HQD.

The structure of the enzyme was, therefore, solved by multiple wave-length anomalous dispersion (MAD) using the anomalous signal of the two catalytic iron cores. MAD data were collected at the BM14 beamline, ESRF, Grenoble. The data collected at three wavelengths (inflection, peak, remote) were processed and integrated with DENOZO and scaled by SCALEPACK, from the HKL program suite (53).

The program SOLVE (54) was used to identify the two iron sites and
for phase calculation. The 2.6 Å MAD phases were improved and extended to 2.2 Å by solvent flattening and histogram mapping using the program DM from the CCP4 program suite (55). Automatic tracing was performed initially with the program RESOLVE (56) and extended using ARP/WARP version 6.0 (57). After 200 cycles of refinement and 20 cycles of autobuilding, 532 amino acids of 586 were found and placed in 16 chains with a global connectivity index of 0.94. After this process manual intervention was required to complete the model. The model was initially refined against 2.2 Å resolution (MAD remote wavelength data set) and finally against 1.75 Å data, using the program Refmac 5.1.24 from the CCP4 program suite (55). Manual rebuilding of the model was performed using the program QUANTA (58). Solvent molecules were introduced automatically using ARP (57). Refinement resulted in R factor and Rfree values of 19.2 and 24.6%, respectively. Data processing and refinement statistics are summarized in Table I. The overall mean B factor of the structure after refinement was 25.68 Å² for chain A, 28.23 Å² for chain B, and 29.15 Å² for all atoms.

Protein coordinates have been deposited in the Protein Data Bank (accession number 1TMX).

The final model is composed of residues 2–293 for chain A and 4–293 for chain B, two Fe(III) ions, two benzoate ions, two phospholipids (C13/C17), two sulfate ions, one copper ion, one chloride ion, and 837 water molecules.

There are two disordered regions in chain B corresponding to residues N. simplex (IESGGDI), 1,2-HQD-61/63 (IEVW/HSFDATADPRH), 1,2-HQD-27 (S/F), 1,2-HQD-51 (A/E IT/DSFP), and 1,2-HQD-77 (RQEFILL). All of these sequences were obtained by amino acid sequencing of the N. simplex (IESGGDI), 1,2-HQD-55 (IESGGDI), 1,2-HQD-61/63 (IEVW X EAXDDGDFY DX QVYDD DX), 1,2-HQD-70 (AL T/H/E/L A/L ES), 1,2-HQD-72 (TLTVXIF M/F), 1,2-HQD-77 (RQEPILL). All of these peptides, except 1,2-HQD-27 (for which the sequence was of low quality), occur in the sequence predicted from the cloned N. simplex 3E DNA into E. coli. Sequencing of two 650-bp and a 1.2-kbp region from several subclones resulted in the complete sequence of the presumed 1,2-HQD gene.

RESULTS AND DISCUSSION

Gene and Protein Sequence—Using primers directed toward regions conserved in other 1,2-HQDs, it was possible to amplify a segment of the expected size (400-bp) from genomic DNA of N. simplex 3E. The use of this fragment as a probe allowed us to clone a 3-kbp fragment of N. simplex 3E DNA into E. coli. Sequencing of two 650-bp and a 1.2-kbp region from several subclones resulted in the complete sequence of the presumed 1,2-HQD gene.

Independently from the genetic approach, the following sequences were obtained by amino acid sequencing of the N. terminus and of nine tryptic peptides of the purified protein (amino acids separated by slashes or X indicating uncertain positions): 1,2-HQD-27 (S/X A/D/S/X L N S/X), 1,2-HQD-36 (SFDATADPR X/R), 1,2-HQD-51 (A/E I T/D/P/G T/P), 1,2-HQD-55 (IESGGDI), 1,2-HQD-61/63 (IEVW X EAXDDGDFY DX QVYDD DX), 1,2-HQD-70 (AL T/H/E/L A/L ES), 1,2-HQD-72 (TLTVXIF M/F), 1,2-HQD-77 (RQEPILL). All of these peptides, except 1,2-HQD-27 (for which the sequence was of low quality), occur in the sequence predicted from the cloned N. simplex 3E gene (as well as in those from the tfh, hadC, dxnF genes), thus proving that, in fact, the gene of a 1,2-HQD was cloned and sequenced.

The most similar sequence in the data base was that of...
1,2-HQD from Arthrobacter sp. strain BA-5-17 (32) (73% identical positions in the alignment of Fig. 4). The similarity to other 1,2-HQDs ranged from 42 to 69% identical positions (see alignment of Fig. 4). In contrast, the similarity to the representatives of catechol and chlorocatechol 1,2-dioxygenases given in Fig. 4 was between 22 and 30% identical positions.

Overall Structure and Linker Domain—The 1,2-HQD from the Gram-positive bacterium N. simplex 3E is a homodimeric protein with overall dimensions 110 × 50 × 50 Å. The statistics for data collection, phasing, and structure refinement are summarized in Table I. The final model is composed of residues 2–293 for chain A and 4–293 for chain B, two Fe(III) ions, one copper ion, two benzoate ions, two phospholipids (C13/C17), two sulfate ions, one chloride ion and 837 water molecules.

The general topology of Ns 1,2-HQD resembles that of Ac 1,2-CTD and comprises two catalytic domains separated by a common “α-helical zipper” motif that consists of six N-terminal helices from each subunit.

Two phospholipid molecules are located inside a large hydrophobic channel formed by the two protein monomers at the interface between the two subunits and in the center of the linker domain, with the head group directed outward into the solvent and the tail moieties pointing inward, toward each other (Fig. 1). A phosphatidylcholine molecule with two C12–13/C17 hydrophobic tails was used as a model because the absence of the electron density of the head groups did not allow determination of their precise identity, and the length of each tail was based on the length of the electron density and on the stereochemistry of known phospholipids. The presence of such phospholipids appears to be distinctive for this class of enzymes, although their possible role has still to be clarified (41, 42).

The linker domain is mainly composed of three long (H1–H3) and three short (H4–H6) α-helices supplied by each subunit; five helices from the N terminus of each monomer are interacting with the equivalent motif from the other subunit and with the sixth helix, which elongates from the catalytic domain (Figs. 1 and 2). A metal ion bound to both His42 (at 2.01 and 2.12 Å) from helices H2 of both subunits and to a chloride ion (at 2.57 Å) is shown in Fig. 1. The protein metal content analysis (see “Experimental Procedures”) and the trigonal coordination geometry observed suggest that such metal ion is copper in an oxidation state I. Its location suggests a possible structural role in stabilization of the enzyme quaternary assembly for such metal ion.

Fig. 2 shows the three-dimensional structural least squares
superposition of a single subunit of Ns 1,2-HQD and Ac 1,2-CTD. The first N-terminal $\alpha$-helix H1 and the random coil region preceding the $\alpha$-helix H4 extending from the central domain are longer in Ns 1,2-HQD compared with Ac 1,2-CTD. About one-half of the fifth helix (H5) is missing compared with the corresponding one from Ac 1,2-CTD. The first two short $\beta$-sheets in Ac 1,2-CTD are also missing in Ns 1,2-HQD, being substituted by random coil regions. The secondary structure of the central section of Ns 1,2-HQD thoroughly resembles that of the 1,2-CTD family. Finally, the C-terminal region of Ns 1,2-HQD, as observed also in the Rho 1,2-CCD, the only representative of chlorocatechol cleaving dioxygenases for which the three-dimensional structure is...
known, lacks the seventh helix, the last long random coil, and the final β-sheet present in Ac 1,2-CTD (42).

The Catalytic Domain—Each subunit contains one catalytic pocket accessible to the substrate from the hollow side of the dimer. The central part of the catalytic domain is made up of several β-sheets arranged in a β-sandwich conformation and by a number of random coils positioned between the linker domain and the β-sheets assembly (see Figs. 1 and 2). The active site

![Fig. 3. A and B, representations of the two active site entrances of Ns 1,2-HQD. The surface is color-coded on the basis of the calculated electrostatic potential. The blue color corresponds to positive and red to negative potentials. The benzoate-like molecule is depicted in yellow. A and B show 90° vertically rotated views of the enzyme, and the two active site entrances are indicated by yellow arrows. C, Fo – Fc electron density map for the active site of Ns 1,2-HQD. The electron density is contoured at the 3σ level. D, stereo representation of the superposed active site structures of HQ docked to Ns 1,2-HQD and of the catechol complex of Ac 1,2-CTD (orange colored, PDB code 1DLT). Designations of amino acids positions are first given for Ns 1,2-HQD and second (in parentheses) for Ac 1,2-CTD.](image-url)
metal center is located in the random coils region flanked on one side by the β-sandwich motif of each monomer and on the other side by the α-helices of the linker domain.

Each catalytic pocket of Ns 1,2-HQD is bordered by Leu80 and Asp83 from helix H4; Val107, Phe108, Pro110, and Phe111 from a first random coil; Trp156 from sheet S3; Tyr164, the mobile iron ligand, from a second random coil; Tyr197 from sheet S6; Ile199 and Pro200 from a third random coil; Arg218 from sheet S7; His221 and His223 from sheet S8; His237 from sheet S9; and Val251 from a fourth random coil.

As shown in Fig. 3, A and B, a distinctive feature of the present structure is that each active site presents two openings. The first one is located as the one observed previously in the Ac 1,2-CTD and Rho 1,2-CCD structures and delimited by residues Leu80, Pro110, Phe111, Pro198, Ile199, Pro200 plus the backbones of the Tyr164 and Tyr197 iron ligands; the second placed at about 90° on the right side of the first one and bordered by residues Leu80, Asp83, Val107, Phe108, Gly109, and Val251. A number of water molecules are present in the openings and in the upper part of the active cavity even though a benzoate-like molecule, bound to the active site iron, occupies a large part of the cavity.

Some of the active site residues, with the corresponding F− density overlaid are depicted in Fig. 3C. The mononuclear Fe(III) ion shows a His221,Tyr164 coordination (Tyr164, Tyr197, His221, and His223), typical of all intradiol ring cleaving dioxygenases (28). X-ray absorption spectroscopy data collected for the same Ns 1,2-HQD indicate that generally the native enzyme is pentacoordinated with two spheres of atoms: either two at 1.90 Å and three at 2.06 Å, or three at 1.92 Å and two at 2.08 Å (33). In the present crystal structure, a benzoate-like ion is coordinated to the iron ion in a bidentate asymmetric mode substituting the metal bound water molecule/hydroxide ion, observed in the native 1,2-CTDs, increasing the iron coordination number to 6 (Fig. 3C). An equivalent molecule has also been observed recently in the active site of Rho 1,2-CCD. In Ns 1,2-HQD the benzoate ion is stabilized by a hydrogen bond network that connects the benzoate O1 atom to Arg218 NH1 (hydrogen-bonded further to Asp249) and the benzoate O2 atom to a well ordered W16 active site water molecule (B factor = 20.63) (hydrogen-bonded further to Phe108, Pro110, Phe111, and Trp156). As observed in Rho 1,2-CCD the benzoate binding does not trigger the dissociation of Tyr197, although causing a conformational orientation of Arg218 observed when substrates bind to intradiol dioxygenases, but contrarily to what observed in all 1,2-CTDs, 1,2-CCDs and 3,4-PCDs, Arg218 supposed to promote the substrate positioning and deprotonation, is not stabilized by a strong hydrogen bond to a Glu because this residue is replaced by His237, which is positioned a bit further away (35, 37, 38, 40, 41).

No convincing hypotheses can be made, at the moment, on the possible reasons for the presence of a benzoate-like molecule bound to the catalytic metal ion, although exogenous ligands have been often found bound to metal sites acting as stabilizers of the active enzyme by hampering metal ion dissociation. The molecule resembles benzoate or benzamide, which actually act as very weak competitive inhibitors for these enzymes, easily displaced by catechols or HQs.2

In Table II the distances of the iron ligands are reported: Tyr164 and Tyr197 exhibit shorter bonds (1.95 and 2.01 Å, respectively) than His221 and His223 (2.11 and 2.24 Å, respectively), and the iron coordination sphere is completed by the benzoate-like molecule asymmetrically bound with O1 (~2.13 Å) and O2 (~2.59 Å).

**Substrate Specificity**—The catalytic mechanism of intradiol ring-cleaving dioxygenases has been proposed to operate via Fe(III) activation of the diol substrate first generating an Fe(II) semiquinone, which reacts directly with dioxygen to give a hydroperoxide intermediate, a Criegee rearrangement via acyl migration would finally generate the corresponding muconic anhydride (Scheme 2) (68). An alternative mechanism for migration of the electron-deficient acyl group, via a benzene-oxide-oxepin interconversion, has also been proposed (Scheme 2) (69).

Substrate selection and conversion are expected to be controlled mainly by the ring substituents effects on the electron density of the carbon atoms exposed to the molecular oxygen attack as well as by the interactions of ring substituents with the surrounding active site amino acidic residues.

Although a number of studies on inhibitors, substrates, and substrate analog adducts of 3,4-PCDs and Ac 1,2-CTD have revealed several important features of the mechanism of exogenous ligands binding to their active site, and the structure of

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2 M. Ferraroni, A. Scozzafava, and F. Briganti, unpublished results.
1,2-HQDs have some light on the substrate selectivity of chlorocatechol-cleaving enzymes, no conclusive rationalization of the observed substrate specificities for intradiol-cleaving dioxygenases has been achieved so far.

1,2-HQDs structurally belong to the group of intradiol dioxygenases comprising 1,2-CTDs. 1,2-CTDs are generally divided into two types, I and II (70). Type I dioxygenases (1,2-CTDs) are relatively specific enzymes that primarily have catechol and often also a methylcatechol as substrate. Chlorinated catechols are not used or are used only at negligible rates. Type II enzymes (better known as 1,2-CCDs) are relatively nonspecific with a wider substrate range being able to convert chlorinated catechols more rapidly than catechol and to additionally accommodate a wide range of methyl- or methoxy-substituted catechols. Regarding substrate specificity, 1,2-HQDs seem to be more closely related to type I than to the type II enzymes (Table III). Unfortunately, HQ was not tested as a potential substrate for most of the catechol and chlorocatechol 1,2-dioxygenases; a catechol 1,2-dioxygenase able to oxidize HQ at about half the rate (51%) of catechol was described only for _T. cutaneum_ (71).

The 1,2-HQD, subject of the present study, is remarkably substrate-selective. The conversions of HQ (K_m = 1.2 μM, k_cat = 29 s⁻¹, and k_cat/K_m = 24.2 μM⁻¹ s⁻¹), 5CHQ (2.4% relative to HQ activity), and 6CHQ (5.0% relative to HQ activity) are catalyzed (30). A range of catechols and variously substituted quinols were also tested as substrates but found to only act as inhibitors for HQ turnover (K_I ~ 10 μM for catechols).

Table III presents the comparison of the known substrate specificity data for 1,2-HQDs and some representatives of type I and II catechol-cleaving enzymes. The 1,2-HQDs from _B. cepacia_ AC1100, _Azotobacter_ sp. strain GP1, and _T. cutaneum_ as well as the 1,2-CHQD from _S. rochei_ 303, with respect to catechol, 3-methylcatechol, or pyrogallol conversion, show relatively high substrate specificities as the enzyme from _N. simplex_. HQ is the main substrate for the first four enzymes, whereas 6-CHQD is the best substrate for the _S. rochei_ dioxygenase. 6-CHQD was also a relatively good substrate for the 1,2-HQDs of _Azotobacter_ sp. GP1, _N. simplex_ 3E, _Wautersia eutropha_ JMP134, _R. pectietii_ DTP0602, but was not tested with the fungal enzymes from _T. cutaneum_ and _P. chrysosporium_.

The enzyme from _P. chrysosporium_ was found unable to convert 5-CHQ, but accepted catechol with 20% of the activity shown toward HQ. Also, the 1,2-HQD isolated from _R. pectietii_ DTP0602 presents relatively low substrate specificity, being able to catalyze the oxidation of 3-methylcatechol and pyrogallol in addition to HQ and 6-CHQ, but it is inactive toward catechol, 3- and 4-chlorocatechol, 4-methylcatechol, protocatechuic, and 2,3-dihydroxybiphenyl (31). Furthermore, the 1,2-HQD of _S. wittichii_ RW1 showed a high activity with catechol (7), and that of _Arthrobacter_ sp. strain BA-5-17 was shown to catalyze both the intradiol and extradiol cleavage of catechol, although the activity toward HQ was 6.8-fold higher than that toward catechol (32).

These results evidence that substrate selectivity is a very heterogeneous issue even inside the 1,2-HQD group. It appears challenging to attempt the rationalization, at the molecular level, of the structural factors responsible for the differential substrate selectivity.

The structural alignment of the active site residues of the catecholate complex of _Ac_ 1,2-CTD and the HQ docked in _Ns_ 1,2-HQD is shown in Fig. 3D. The main interactions of _Ac_ 1,2-CTD with the substrate involve the following residues (respective positions in the alignment of Fig. 4 given in parentheses): Leu_237_ (87), Pro_216_ (90), Ile_219_ (119), Pro_230_ (122), Leu_219_ (123), Arg_221_ (239), Phe_235_ (272), and Ala_254_ (273). Some of these residues and some additional ones in the cavity appear to be crucial in the correct positioning of the aromatic substrate in _Ns_ 1,2-HQD (respective positions in the alignment of Fig. 4 given in parentheses): Leu_186_ (87), Asp_68_ (90), Val_170_ (119), Phe_188_ (120), Gly_189_ (121), Pro_216_ (122), Phe_211_ (123), Ile_199_ (219), Pro_230_ (220), Arg_235_ (239), and Val_251_ (273). We noticed substantial changes in some of these residues with respect to the corresponding amino acids in the representative structures of 1,2-CTDs and 1,2-CCDs and specifically: Asp_68_ (position 90 in Fig. 4, Pro_216_ in _Ac_ 1,2-CTD and Ala_219_ in _Rho_ 1,2-CCD), Val_170_ (position 119 in Fig. 4, Ile_219_ in _Ac_ 1,2-CTD and Ile_274_ in _Rho_ 1,2-CCD), Phe_188_ (position 120 in Fig. 4, Glu_196_ in _Ac_ 1,2-CTD and Glu_276_ in _Rho_ 1,2-CCD), Phe_211_ (position 123 in Fig. 4, Leu_219_ in _Ac_ 1,2-CTD and Phe_278_ in _Rho_ 1,2-CCD), His_237_ (position 258 in Fig. 4, Glu_249_ in _Ac_ 1,2-CTD and Glu_250_ in _Rho_ 1,2-CCD), Val_251_ (position 273 in Fig. 4, Ala_254_ in _Ac_ 1,2-CTD and Cys_254_ in _Rho_ 1,2-CCD).

To understand which of these residues could be mainly responsible for substrate recognition, HQ was docked into the active site of _Ns_ 1,2-HQD. Two different orientations are likely: if the 4-OH substituent is oriented toward the internal part of the cavity (Fig. 3D) it would settle into a pocket formed by Asp_68_ and Val_251_ (positions 90 and 273, respectively, in Fig. 4), but if the substituent is oriented outward, it would essentially
interact with Leu80 and Pro110 (positions 87 and 122 in Fig. 4, Leu73/Leu49 and Pro108/Pro77 in Ac in 1,2-CTD/Rho, respectively). This second hypothesis seems to be unlikely because identical amino acids would interact with the substrate in the different enzymes, thus not clearly accounting for their markedly different substrate specificity. Furthermore, the orientation of HQ with the 4-OH substituent toward the internal part of the cavity is equivalent to that of bound protocatechuate in 3,4-PCDs and of bound 4-methylcatechol in Ac1,2-CTD (40, 41).
A multiple sequence alignment of intradiol dioxygenases revealed several positions identical in all available 1,2-HQDs but different in one or more of the selected 1,2-CTDs or 1,2-CCDs (shaded dark gray in Fig. 4). Such positions may be conserved without a specific selection pressure, but they also may be of importance for the substrate specificity without a structural reason for it so far having been elucidated.

The presence of Asp$^{63}$ and Val$^{251}$ (positions 90 and 273 in Fig. 4), the residues expected to interact with substituents in position 4 (Pro$^{76}$/Ala$^{56}$ and Ala$^{299}$/Cys$^{224}$ in Ac 1,2-CTD/Rho 1,2CCD, respectively), based on structural comparisons should be mainly responsible for the selective preference for Hqs. Valine residues corresponding to Val$^{251}$ (position 273 in Fig. 4) occur in all available 1,2-HQDs, but not in the (chloro-)catechol dioxygenases, a distribution supporting the conclusions drawn from structural comparisons.

Also, 3,4-PCDs are selective for a substrate carrying a hydrophobic substituent in a distal position with respect to the diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protocatechuate forms a hydrogen bond with Tyr$^{324}$ diol: protocatechuate. It has been shown that the carboxyl drophilic substituent in a distal position with respect to the group of protcate
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