Molecular Basis for the Stabilization and Inhibition of 2,3-Dihydroxybiphenyl 1,2-Dioxygenase by \textit{t}-Butanol*

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The steady-state cleavage of catechols by 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD), the extradiol dioxygenase of the biphenyl biodegradation pathway, was investigated using a highly active, anaerobically purified preparation of enzyme. The kinetic data obtained using 2,3-dihydroxybiphenyl (DHB) fit a compulsory ternary complex mechanism in which substrate inhibition occurs. The $K_m$ for dioxygen was 1280 ± 70 μM, which is at least 2 orders of magnitude higher than that reported for catechol 2,3-dioxygenases. $K_m$ and $K_d$ for DHB were 22 ± 2 and 8 ± 1 μM, respectively. DHBD was subject to reversible substrate inhibition and mechanism-based inactivation. In air-saturated buffer, the partition ratios of catecholic substrates substituted at C-3 were inversely related to their apparent specificity constants. Small organic molecules that stabilized DHBD most effectively also inhibited the cleavage reaction most strongly. The steady-state kinetic data and crystallographic results suggest that the stabilization and inhibition are due to specific interactions between the organic molecule and the active site of the enzyme. \textit{t}-Butanol stabilized the enzyme and inhibited the cleavage of DHB in a mixed fashion, consistent with the distinct binding sites occupied by \textit{t}-butanol in the crystal structures of the substrate-free form of the enzyme and the enzyme-DHB complex. In contrast, crystal structures of complexes with catechol and 3-methylcatechol revealed relationships between the binding of these smaller substrates and \textit{t}-butanol that are consistent with the observed competitive inhibition.

The microbial degradation of aromatic compounds constitutes an essential link in the global carbon cycle. The aerobic degradation of aromatic compounds such as tolue, naphthalene, and biphenyl generally proceeds via a catechol catabolite with hydroxyl substituents on two adjacent carbon atoms. This catecholic compound is cleaved by a dioxygenase from one of two very different classes. Intradiol dioxygenases utilize non-heme ferrous iron to cleave the aromatic nucleus \textit{ortho} to (between) the hydroxyl substituents whereas extradiol dioxygenases utilize non-heme ferrous iron to cleave the aromatic nucleus \textit{meta} (adjacent) to the hydroxyl substituents. The mechanism of intradiol dioxygenases is better understood due to their greater stability, favorable properties for spectroscopic examination, and the accessibility of catalytic intermediates (1, 2). Interest in extradiol dioxygenases is nonetheless considerable, not only because of their general metabolic significance and catalytic properties, but also because of the potential exploitation of these enzymes in the degradation of environmental pollutants such as polychlorinated biphenyls.

2,3-Dihydroxybiphenyl 1,2-dioxygenase (DHBD)* is a component of the aerobic biphenyl degradation pathway of a number of microorganisms and cleaves 2,3-dihydroxybiphenyl (DHB) in an extradiol fashion as shown in Scheme 1. Crystallographic studies of DHBD from \textit{Burkholderia cepacia} LB400 (3) and \textit{Pseudomonas} sp. strain KKS102 (4) have provided details of the active site Fe(II) environment and conserved active site residues, and have contributed to our understanding of the evolution of these enzymes (5, 6). Phylogenetic analyses indicate the existence of two evolutionarily independent types of extradiol dioxygenases (5). However, the catalytic strategy utilized by these two types of enzymes appears to be very similar and the emerging mechanism has been proposed based on studies of both types of enzymes. Spectroscopic and biochemical studies (7–11) suggest a mechanism in which the catechol first binds to the active site Fe(II) in a bidentate manner as a monoanion. Subsequent O₆ binding to the Fe(II) with charge transfer from the bound catecholic monoanion results in the generation of an iron-bound superoxide-like moiety and an iron-bound, activated catechol. It has been proposed that reaction of these two species forms a bridged peroxy intermediate, which undergoes Criegee rearrangement to give an unsaturated lactone intermediate. This lactone is finally hydrolyzed to yield the reaction product. Several steps in this mechanism have yet to be substantiated, and the catalytic roles of conserved active site residues remain to be fully elucidated.

With the development of structural data, DHBD has become an attractive system for experiments that will further our understanding of extradiol dioxygenase function. Such studies depend upon the availability of homogeneous, active preparations of the enzyme. The stability of a number of extradiol dioxygenase preparations has been improved through the inclusion of organic additives in solutions of the enzymes. While this strategy has been in use for at least 30 years (12), the mechanism of stabilization has yet to be reported. Even with this precaution, the best reported preparations of DHBD contain at most 50% of their complement of active site Fe(II) (13).

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1 The abbreviations used are: DHBD, 2,3-dihydroxybiphenyl 1,2-dioxygenase; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; DHB, 2,3-dihydroxybiphenyl.
This variability in preparations of extradiol dioxygenases complicates spectroscopic studies and the determination of steady-state kinetic parameters. For example, the $k_{cat}$ of the xylem-encoded catechol 2,3-dioxygenase of *Pseudomonas putida* MT-2 has been variously reported as 950 s$^{-1}$ (100 mM phosphate, pH 7.5, 25°C; Ref. 14) and 278 s$^{-1}$ (50 mM phosphate, pH 7.5, 25°C; Ref. 15).

We report improved techniques for purifying and manipulating DHBD of *B. cepacia* LB400. Steady-state kinetic studies were conducted to determine the specificity of the enzyme for different aromatic substrates and $O_2$. In addition, the inhibition of the enzyme by different organic solvents was investigated. The results of the specificity and inhibition studies were evaluated in terms of crystallographically determined structures of catechol, 3-methylcatechol, and DHB complexes.

MATERIALS AND METHODS

**Strains, Media and Growth—**DHBD was hypoxpressed in *P. putida* KT2442 freshly transformed with pLEBD4, a broad host range plasmid containing the structural gene encoding the dioxygenase, as described previously (16). For DHBD expression, the strain was grown in Luria broth containing a potassium phosphate buffer described for Terrific Broth (17) and supplemented (10 ml per liter) with an HCl-solubilized solution of minerals containing 14.1 mM MgCl$_2$·6H$_2$O, 2.5 mM CaCl$_2$, 4.27 mM FeSO$_4$·7H$_2$O, 0.625 mM ZnSO$_4$·7H$_2$O, 0.625 mM MnSO$_4$·H$_2$O, 0.125 mM CuSO$_4$·5H$_2$O, 0.125 mM CoCl$_2$·6H$_2$O, 0.125 mM Mg$_2$BO$_3$·5H$_2$O, 262.5 mM MgSO$_4$, 10 mM CaCl$_2$, and 0.1 mM thiamine. Cultures were grown at 30°C and 250 rpm. One liter of media in a 2-liter flask was inoculated with 4 ml of an overnight culture. The standard activity assay was performed in a total volume of 1.45 ml of an appropriate dilution of enzyme preparation into the reaction chamber. Buffers were used within 24 h of preparation. Stock solutions were prepared fresh daily and stored unrefrigerated. Protein concentrations were determined from progress curves by analyzing the data using Microsoft Excel (Redmond, WA). The slope of the progress curve and the correlation coefficient of the slope were calculated for all consecutive 6-s intervals using the full set of 61 data points. The calculated slopes had a correlation coefficient of at least 0.998.

**Protein Purification—**The protein was purified following a modified protocol (13). After disruption of the cells, all procedures were performed under an inert atmosphere unless otherwise specified. Chromatography was performed on an AKTA Explorer (Amersham Pharmacia Biotech, Baie d’Urfé, P.Q., Canada). A stainless steel column (0.7×20 cm) of Bio-Gel P-100 was prepared by vigorously bubbling them with humidified mixtures of argon on ice. The gas-tight syringe and the stopper was inserted into the reaction chamber. During this operation, the reaction chamber was flushed continuously with argon with a gas-tight syringe. This mixture was humidified by bubbling it through a 5×10-cm column of water. The concentration of dissolved $O_2$ in the reaction mixture was verified using the $O_2$ electrode.

**Reaction buffers containing different concentrations of dissolved $O_2$ were prepared by vigorously bubbling them with humidified mixtures of $O_2$ and $N_2$ gases for at least 15 min prior to the experiment. The equilibrated buffer was transferred to the reaction chamber using a gas-tight syringe and the stopper was inserted into the reaction chamber.** Buffer solutions prepared for gas-tight syringes were flushed continuously with the humidified gas mixture. Ultra high purity $O_2$ (100% $O_2$ or 10% $O_2$ in $N_2$) and prepurified $N_2$ were mixed in the desired proportions with a stainless steel Concoa Model 561 gas proportioner. This mixture was humidified by bubbling it through a 5×10-cm column of water. The concentration of dissolved $O_2$ in the reaction mixture was verified using the $O_2$ electrode.

**Stabilization and Inhibition of an Extradiol Dioxygenase**

**Handling of DHBD Samples—**Exogenous iron was anaerobically removed from small samples of DHBD prior to kinetic experiments by gel filtration chromatography. Typically, 100–200 μl of purified DHBD was thawed in the glovebox and applied to a 0.7×5-cm column of Bio-Gel P-6 DG (Bio-Rad, Mississauga, Ont., Canada) equilibrated with stabilization buffer lacking bovine serum albumin (see below). The sample of DHBD was further diluted with stabilization buffer as appropriate.

The ability of different anaerobic buffers to stabilize the DHBD cleavage activity of DHBD was investigated by monitoring the activity of a 25–50 μg/ml solution of the enzyme over 24 h. Parameters that were varied include pH and the concentrations of ferrous ammonium sulfate, dithiothreitol, and bovine serum albumin. Acetone, ethanol, glycerol, isopropyl alcohol, and t-butanol were tested for their ability to stabilize the activity of DHBD.

**SDS-polyacrylamide gel electrophoresis was performed on a Bio-Rad MiniPROTEAN II apparatus and stained with Coomassie Blue according to established procedures (17). Protein concentrations were determined by the Bradford method (18). Iron concentrations were determined colorimetrically using Ferene-S (19).**

**Kinetic Measurements—**Enzymatic activity was measured by following the consumption of oxygen using a Clark-type polarographic $O_2$ electrode (Yellow Springs Instruments Model 5301 (Yellow Springs, OH)). Reactions were performed in a thermostatted Cameron Instrument Co. model RC1 respiration chamber (Port Aransas, TX) equipped with a Lauda Model RM6 circulating water bath. The electrode signal was amplified using a Cameron Instrument model OM200 o2 meter equipped with a PC-LPM-16 multifunction board and Virtual Bench Data Logger (National Instruments, Austin, TX). Data were recorded every 0.1 s. Initial velocities were determined from progress curves by analyzing the data using Microsoft Excel (Redmond, WA). The slope of the progress curve and the correlation coefficient of the slope were calculated for all consecutive 6-s intervals using the full set of 61 data points. The calculated slopes had correlation coefficients of at least 0.999.

**Reaction buffers containing different concentrations of dissolved $O_2$ were prepared by vigorously bubbling them with humidified mixtures of $O_2$ and $N_2$ gases for at least 15 min prior to the experiment. The equilibrated buffer was transferred to the reaction chamber using a gas-tight syringe and the stopper was inserted into the reaction chamber.** Buffers were used within 24 h of preparation. Stock solutions were prepared fresh daily and stored under argon on ice. On each day kinetic assays were performed, the zero time was established by adding an excess of sodium hydrosulfite to the buffer in the reaction chamber. The $O_2$ electrode was calibrated using standard concentrations of protocatechuate (3,4-dioxygenase and protocatechuate (20) or DHBD and an excess of DHBD. The amount of active DHBD in the assay was defined by the iron content of the injected purified enzyme solution. This quantity was used in the calculation of the specificity and catalytic constants. One unit of enzyme...
matic activity was defined as the quantity of enzyme required to consume 1 μmol of O₂/min.

Specificity, coupling, inactivation and inhibition experiments were carried out in 20 mM HEPES, 80 mM NaCl, pH 8.0, 25.0 ± 0.1 °C. The coupling of catecholic substrate and O₂ consumption was investigated by monitoring the amount of O₂ consumed upon the addition of weighed amounts of catecholic substrate to the reaction mixture. An excess of DHBD was used in these experiments, whereas a limiting amount of DHBD was used in experiments designed to determine the partition ratio for a given catecholic substrate. The amount was chosen such that the enzyme was completely inactivated before the catecholic substrate or O₂ was completely consumed in the reaction mixture. The partition ratio was calculated from the ratio of the amount of O₂ consumed to the amount of active DHBD added to the assay.

In specificity experiments with catechol, 3-methylcatechol, 3-ethylcatechol, and DHB, concentrations of the aromatic substrates were varied from 0.2 times the determined catechol, and DHB, concentrations of the aromatic substrates were varied, the initial velocities were fit to Equation 1 or to the equivalent equation describing a mechanism in which the binding of substrate inhibition ([A] 2) is to make the rate approach zero at high concentrations of the substrate; and

\[
\text{Equation 1:} \quad v = \frac{V[A][O_2]}{K_{mA}K_{mO_2} + K_{mO_2}[O_2] + K_{mA}[A] + [A][O_2] + K_{mO_2}[A]}/K_{mA} \text{ (Eq. 1)}
\]

In Equation 1, \(K_{mA}\) represents the \(K_m\) for the catecholic substrate; \(K_{mO_2}\), the \(K_m\) for O₂; \(K_{mA}\), the dissociation constant for the catecholic substrate; and \(K_{mA}\), the inhibition constant for the catecholic substrate. \(K_{mA} \text{ and } K_{mO_2}\) are not classically defined Michaelis constants as the effect of substrate inhibition \([A]^2\) is to make the rate approach zero at high concentrations of A. However, \(K_{mA} \text{ and } K_{mO_2}\) are derived from the same first-order rate constants as the equivalent steady-state constants for a compulsory order ternary complex mechanism in which substrate inhibition does not occur (e.g. \(K_{mO_2} = (k_2 + k_4 y)k_2\)). Furthermore, \(K_{mA}\) and \(K_{mO_2}\) are the ratios between \(k_2\) and the specificity constants \(k_3\) and \(k_5\), respectively (i.e. \(k_2 = k_3/k_{mA}\) and \(k_5 = k_5/k_{mO_2}\)).

For specificity experiments in which the concentrations of the catecholic substrate A and O₂ were varied, the initial velocities were fit to Equation 1 or to the equivalent equation describing a mechanism in which substrate inhibition occurs (Equation 2).

\[
\text{Equation 2:} \quad v = \frac{V[A]}{K_{mA}' + [A]}/K_{mA}' \text{ (Eq. 2)}
\]

In this equation, \(K_{mA}'\) and \(K_{mA}'\) are the apparent \(K_{mA}\) and \(K_{mA}\) in air-saturated buffer.

Steady-state kinetic data obtained from experiments using organic additives as inhibitors, I, were fit to competitive, uncompetitive, and mixed inhibition equations (Equation 3).

\[
\text{Equation 3:} \quad v = \frac{V[A]}{K_{mI}'[1 + [I]/K_{mI}'] + [A]}/1 + [I]/K_{mI}' \text{ (Eq. 3)}
\]

In Equation 3, \(K_{mI}'\) represents the competitive inhibition constant and \(K_{mI}'\) the apparent uncompetitive inhibition constant. The latter is related to the true \(K_{mI}\) by Equation 4.

\[
\text{Equation 4:} \quad K_{mI}' = \frac{K_{mI}([O_2] + [I])}{K_{mO_2}} \text{ (Eq. 4)}
\]

Data were fit to steady-state rate equations using the least squares and dynamic weighting options of LEONORA (22).

Preparation of Crystalline Enzyme-Substrate Complexes and Diffraction Experiments—All crystals were prepared and mounted under an aerobic conditions within a N₂ atmosphere glove box (Innovative Technology, Newburyport, MA) maintained at 2 ppm O₂ or less. Crystals of DHBD were grown in the presence of t-butanol and absence of substrates as described previously (3). Binary complexes were prepared by incubating crystals in small volumes (about 1 ml) of anaerobic solutions containing the substrate, 22% (w/v) PEG-4000, 15% (v/v) t-butanol, and 100 mM HEPES buffer at pH 7.5; for some experiments, 1 mM Fe(II)NH₄(SO₄) and 0.5 mM Na₂S₂O₄ were also present. For catechol, 3-methylcatechol, and DHB, the concentrations used were 16, 10, and 1 mM, respectively. Crystals were incubated for 12–24 h and mounted in quartz capillaries sealed with epoxy cement. By these procedures, the enzyme was maintained in the active, ferrous form. Diffraction data were collected at room temperature (~20 °C) by the rotation method with the use of an RAXIS IIC imaging plate diffractometer. X-rays were provided by a Rigaku RU200 x-ray generator equipped with a copper anode and focusing mirror optics (Molecular Structure Corp.) and operated at 50 kV and 80–100 mA. The rotation angle and exposure time per image were typically 1° and 10 min, and the crystal-to-detector distance was 110 mm. The diffraction data were analyzed with the HKL package (23).

Structure Refinement and Other Crystallographic Procedures—The CCP4 package (24) was used for general crystallographic calculations, whereas the XPLOR system (25) was used for restrained (26) crystallographic refinement. Electron density analysis and model building were accomplished with the program O (27). Refinement of the 3-methylcatechol complex was initiated with a model derived from the refined structure of the substrate-free protein (2) by deleting the active site iron atom and its two water ligands as well as one additional water and a t-butanol molecule located within 6 Å of the iron. Following one cycle of XPLOR refinement (coordinates and individual B factors), Fcat/ Fobs and 2Fe~ Fobs were mapped, and the active site iron was added to the model. Protein-associated solvent atoms with B factors larger than 60 Å² were deleted, and a single solvent atom was added to account for a strong, positive feature ~9 Å from the iron. Following a second cycle of XPLOR refinement and map evaluation, 3-methylcatechol and two waters adjacent to the iron atom were added, and the previously added solvent atom was replaced with a t-butanol molecule. Four additional rounds of computational refinement and model building completed the refinement. Whenever the active site iron atom was included in the model, the iron-ligand bond distances were restrained to a target value of 2.2 Å with a weak force constant of 10 kcal mol⁻¹ Å⁻².

During the refinement, residual positive difference peaks in the vicinity of the active site were recognized as features representing the active site structure of the substrate-free state. That is, it was apparent that the crystal contained active sites in two states: the substrate-free form and the ES complex. The structure at the active site was therefore modeled by two sets of atoms at partial occupancy (50% each). Refinement of the enzyme-catechol complex followed a similar course and required similar modeling of the active site. The refinement of the enzyme-DHB complex is described elsewhere. The procedures were similar, but the substrate-binding site was fully occupied by DHB so that the use of two models of the active site was not necessary.

RESULTS

Relevant details of the purification are shown in Table I. The enzyme was estimated to be greater than 99% pure as judged by SDS-polyacrylamide gel electrophoresis followed by Coomassie Blue staining (results not shown). This is comparable to the purity obtained previously (13). Anaerobically purified DHBD had a specific activity of 430 units/mg, which was over twice that reported previously (13).

The activity of a 25–50 μg/ml solution of DHBD was stabilized by the presence of dithiothreitol and bovine serum albu-
min, but not ferrous ammonium sulfate. The activity was most stable at pH 8.0; deviations of 0.25 pH units decreased this stability. In the presence of 1 mM t-butanol or isopropyl alcohol, approximately 24% of the activity remained after 24 h, as compared with 19% in the presence of 1 mM ethanol or glycerol and 13% in the absence of organic compounds (see data on inhibition below). For steady-state kinetic assays, DHBD was diluted in 20 mM HEPPS, 80 mM NaCl, pH 8.0 (25 °C), using an O₂ electrode that had been calibrated with protocatechuate and protocatechuate 3,4-dioxygenase (20).

Within experimental error, the amount of O₂ consumed corresponded to the amount of catechol or DHB added to the reaction mixture (results not shown). It was concluded that the utilization of catecholic substrate and O₂ by DHBD were tightly coupled under these conditions. In subsequent experiments, the O₂ electrode was calibrated with DHB and DHBD.

In experiments performed in air-saturated buffer using low concentrations of DHBD, the velocity of the reaction decreased to zero prior to the complete consumption of catecholic substrate or O₂. This inactivation of the enzyme was faster in the presence of catechol than DHB. These observations indicated that DHBD was subject to irreversible, suicide inhibition as has been described for catechol 2,3-dioxygenase (14, 28). In the presence of catechol than DHB. These observations indicated that DHBD was subject to irreversible, suicide inhibition as has been described for catechol 2,3-dioxygenase (14, 28).

High concentrations of catecholic substrate were observed to reduce the initial velocity of O₂ consumption. To assess whether this decrease in the initial velocity was due to inactivation of DHBD via suicide inhibition, the substrate concentrations and reaction velocities were calculated 10–30 s after the initiation of the reaction. Reaction velocities were calculated from 1,230 for catechol to 84,900 for DHB (Table III).

The coupling of catecholic substrate and O₂ utilization by DHBD was investigated in 20 mM HEPPS, 80 mM NaCl, pH 8.0 (25 °C), using an O₂ electrode that had been calibrated with protocatechuate and protocatechuate 3,4-dioxygenase (20). Within experimental error, the amount of O₂ consumed corresponded to the amount of catechol or DHB added to the reaction mixture (results not shown). It was concluded that the utilization of catecholic substrate and O₂ by DHBD were tightly coupled under these conditions. In subsequent experiments, the O₂ electrode was calibrated with DHB and DHBD.

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not be varied over a sufficiently wide range. As discussed above, the useful range of concentration for 3-methylcatechol (50–1400 \mu M) was also limited by suicide inhibition. Apparent steady-state kinetic parameters for catechol and 3-methylcatechol were thus evaluated by fitting the data obtained in air-saturated buffer to the Michaelis-Menten equation (Table III). Fits of the same data to Equation 2 (with substrate inhibition) yielded essentially the same values for \( K_{\text{m,app}} \) and \( K_{\text{cat}} \), respectively. The values for DHB and 3-ethylcatechol in air-saturated buffer, evaluated by fitting the data to the substrate inhibition equation, are also provided in Table III and do not differ significantly from the values calculated from the parameters provided in Table II. Consistent with previous studies (13), the apparent specificity of DHBD in air-saturated buffer is 350-fold higher for DHB than for catechol. Although the \( K_{\text{m,app}} \) of DHB in the presence of catechol could not be evaluated, at a concentration of 1275 \mu M catechol, the apparent constant, \( K_{\text{m,app}} \), was 1000 ± 160 \mu M.

Steady-state kinetic data obtained from the DHBD-catalyzed cleavage of DHB with \( t \)-butanol present as an inhibitor showed random trends in the residuals when fit to an equation describing mixed inhibition (Equation 3). When equations describing competitive or uncompetitive inhibition were used, larger residuals were obtained. The competitive component of this inhibition was stronger than the uncompetitive component (Fig. 2), even when the limiting concentration of \( O_2 \) is considered (Table IV). The relative ability of the tested organic molecules to inhibit the DHBD-catalyzed cleavage of DHB was \( t \)-butanol > isopropyl alcohol > acetone > ethanol > glycerol (Table IV).

Inspection of the quality of fits to equations describing competitive, uncompetitive, and mixed inhibition, respectively, indicated that the DHBD-catalyzed cleavage of catechol and 3-methylcatechol were inhibited by \( t \)-butanol in a competitive fashion. The inhibition constants were similar in magnitude to the competitive inhibition constants of the DHBD-catalyzed cleavage of DHB (Table IV). Nevertheless, weak uncompetitive inhibition of the DHBD cleavage of catechol and 3-methylcatechol could not be ruled out as the maximum concentration of \( t \)-butanol used in these experiments was 2 M and the maximum substrate concentration did not exceed three times their respective \( K_{\text{m,app}} \) values.

Table V documents the extent and quality of the diffraction data. Models for the catechol, 3-methylcatechol, and DHB complexes were refined against diffraction data extending to 1.9-, 1.9-, and 2.0-Å resolution, respectively. Statistics pertinent to the quality of the final models are provided in Table VI. Fig. 3 illustrates the essential features of the active site in the substrate-free and substrate bound states of the enzyme. The iron is bound to the protein by the side chains of His-146, His-210, and Glu-260 in both states. In the substrate-free state, the iron ligation is five-coordinate, with sites trans to His-146, His-210, and Glu-260 occupied by water; the site trans to His-146 is unoccupied. A \( t \)-butanol molecule binds at the bottom of a channel that leads from the surface to the iron atom. Although the electron density for the \( t \)-butanol is strong, its features suggest rotationally disordered binding; van der Waals contacts with seven side chains are potentially involved in different orientations. The distance from the central carbon of \( t \)-butanol, C-1, to the iron atom is 6.0 Å.

The mode of (catecholic) substrate binding is similar in all three complexes and is consistent with the results reported for complexes of the ferric form of a homologous DHBD (4). One hydroxyl group of the substrate binds in the site trans to His-146, whereas the other binds trans to His-210, displacing a water ligand. The water trans to Glu-260 shifts by approximately 1 Å, but remains weakly associated with the iron in all complexes of the active, ferrous form of the enzyme (this work), but is not observed in complexes of the inactive, ferric form (4).

The refinements of the catechol and 3-methylcatechol complexes established that the crystals in each case contained molecules in both the substrate-free and substrate-bound states, as demonstrated by the electron density maps shown for the 3-methylcatechol complex in Fig. 4. This phenomenon was not observed for the preferred substrate DHB.2 The maps very clearly demonstrate the significant overlap between the substrate-binding site and the site occupied by \( t \)-butanol in the substrate-free form of the enzyme. In fact, the refined position of the central atom of the \( t \)-butanol in the substrate-free form is within 1.1 Å of the position of the 3-methyl group of 3-methylcatechol and within 0.7 Å of the position at the center of the non-hydroxylated ring of DHB in the respective DHBD-substrate complexes.

The crystal structures of all three DHBD-substrate complexes demonstrate the binding of \( t \)-butanol in a location distinct from the site occupied in the substrate-free structure: the position of C-1 is shifted by more than 4.8 Å and it is more than 8.4 Å distant from the iron. In this auxiliary site \( t \)-butanol is in van der Waals contact with the non-hydroxylated ring of DHB, but not with any atom of catechol or 3-methylcatechol. For DHB, three (C to C) contacts at distances of 3.3, 3.8, and 4.2 Å are observed, whereas the shortest distances for catechol and 3-methylcatechol are 4.5 Å.

DISCUSSION

The described rapid, anaerobic purification of DHBD of \( B. \) cepacia LB400 yielded a preparation whose specific activity is at least twice that of aerobically purified preparations of the same enzyme (13) or a highly similar one (29). Furthermore,
the anaerobic removal of exogenous iron from preparations used for steady-state kinetic studies enabled the calculation of catalytic and specificity constants based on the iron content of these preparations. Electron paramagnetic resonance, magnetic circular dichroic, and x-ray absorption spectra indicated that DHBD samples prepared in this manner contain a single species of iron and that the properties of this species are those expected for a high spin Fe(II) in the active site of the enzyme (7).\(^3\)

The steady-state kinetic analysis revealed that the ability of DHBD from \textit{B. cepacia} LB400 to utilize O\(_2\) is strikingly different from that of evolutionarily related catechol 2,3-dioxygenases. Although the steady-state utilization of O\(_2\) has not been investigated in many extradiol dioxygenases, the \(K_{m,O_2}\) of DHBD is 2–3 orders of magnitude higher than those of a number of catechol 2,3-dioxygenases determined using catechol (0.7–10 \(\mu M\)) (9, 15, 30). Interestingly, the \(K_{m,O_2}\) of an extradiol-type 2-aminophenol dioxygenase was reported to be 710 \(\mu M\) (31). Significantly, the nature of the substituent at C-3 of the catechol does not appear to appreciably affect the \(K_{m,O_2}\) of DHBD. Intradiol dioxygenases also have a wide range of \(K_{m,O_2}\).

For example, protocatechuic 3,4-dioxygenase from \textit{P. putida} has a \(K_{m,O_2}\) of 43 \(\mu M\) (50 mM Tris acetate, pH 7.5, 24 °C; Ref. 9) while that of \textit{Brevibacterium brevis} has a \(K_{m,O_2}\) of 800 \(\mu M\) (50 mM MOPS, 100 mM Na\(_2\)SO\(_4\), pH 7.0, 23 °C; Ref. 20). As noted by Fersht (32), catalytically efficient enzymes evolve to maximize their specificity constants while increasing \(K_{m}\). Nonetheless, the specificity constant of DHBD for O\(_2\), \(1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\), is significantly lower than the value of \(37 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}\) reported for the \(xyl/E\)-encoded catechol 2,3-dioxygenase of \textit{P. putida} MT-2 (50 mM sodium phosphate, pH 7.5, 25 °C; Ref. 15). Moreover, the growth rate of \textit{P. putida} MT-2 on benzoate at different pO\(_2\) is limited by the \(K_{m,O_2}\) of this catechol 2,3-dioxygenase (33). The physiological significance of the high \(K_{m,O_2}\) of DHBD remains to be determined.

The kinetic analysis further establishes that DHBD is subject to two forms of substrate inhibition: reversible substrate inhibition, and a mechanism-based inactivation or suicide inhibition. The latter has also been reported for \(xyl/E\)-encoded catechol 2,3-dioxygenase and seems to involve the oxidation of the active site iron to the catalytically incompetent ferric state (14, 28). For both DHBD and catechol 2,3-dioxygenase, suicide inhibition is more marked for poorer substrates, suggesting that the substrate-binding pocket of these enzymes is tuned both to maximize specificity for a particular substrate and minimize unproductive oxidation of the iron during the cleavage of that substrate. Interestingly, the partition ratio of catechol 2,3-dioxygenase for catechol is 1,400,000 (28), indicating that DHBD is much more susceptible than is catechol 2,3-dioxygenase to suicide inactivation by its putative preferred substrate. Despite the higher susceptibility of DHBD to suicide inactivation, the bph pathway apparently does not contain a homologue to XylT, the small ferredoxin of the TOL pathway, which serves to maintain the active site iron of catechol 2,3-dioxygenase in the ferrous state (34).

The reversible substrate inhibition observed in DHBD has been reported for a number of other DHB-cleaving extradiol dioxygenases (13, 35–38) as well as for a 2,3-dihydroxyphenyl propionate cleaving enzyme (39) although it has only rarely been reported for catechol 2,3-dioxygenases (40). However, for the other DHB cleaving enzymes, it is not clear what proportion of the decrease in the initial rate of DHB cleavage at high concentrations of DHB is due to reversible substrate inhibition and irreversible suicide inhibition, respectively. Notably, the initial rates of cleavage of substituted catechols by a DHBD of strain BN6 could not be fitted to substrate inhibition Equation 2 (38). DHBD of \textit{B. cepacia} LB400 is clearly subject to both modes of inhibition by both DHB and 3-ethylcatechol. Because catechol and 3-methylcatechol are such potent suicide inhibitors of DHBD, it was not possible to determine whether these substrates also reversibly inhibit the enzyme. While the mechanism of reversible substrate inhibition is not clear, it is unlikely to involve negative cooperativity between the subunits of DHBD as it has also been reported for a monomeric enzyme (37). It is possible that DHB could occupy the auxiliary \(t\)-butanol-binding site observed in the DHBD-DHB complex, thereby inhibiting the enzyme in a similar manner (see below).

Previous studies have established that organic additives such as isopropyl alcohol and acetone stabilize the active site iron of extradiol dioxygenases (12, 13). The crystallographic data from DHBD indicate that such small organic molecules can occupy the active site, close to the catalytic iron center (3). In this position, the organic molecule could stabilize the active site and/or protect the iron from direct access by oxidants or substrates. Consistent with this notion, \(t\)-butanol competitively inhibited the cleavage of DHB, 3-methylcatechol, and catechol by DHBD. Moreover, \(t\)-butanol and isopropyl alcohol, which

\(^{3}\)M. I. Davis, E. Wasinger, and E. I. Solomon, personal communication.
root mean square deviations versus restraints

Waters

Bonds (Å)

Angles (°)

A

B

FIG. 3. Relationship of the two t-butanol-binding sites to groups at the active site. A, in the free enzyme, t-butanol occupies the distal portion of the substrate-binding site. B, in the enzyme-substrate complexes, t-butanol occupies an auxiliary site adjacent to the distal portion of the substrate-binding site, further removed from the iron.

inhibit DHBD more effectively than ethanol and glycerol, also stabilize the enzyme more effectively (Table IV). Interestingly, acetone competitively inhibits catechol 2,3-dioxygenase with $K_{ic}$ of 13 mM (12, 15). This suggests that this enzyme has a much higher affinity for acetone than DHBD has for t-butanol, and may explain why the former is so much more stable in acetone-containing buffers (15) than is DHBD in t-butanol-containing buffers. Inspection of the t-butanol-binding site in DHBD reveals that it is partly formed by nonconserved residues, suggesting that the best organic stabilizer will be dioxygenase-specific.

Finally, we note that the structural results are remarkably concordant with the specific types of inhibition manifest by t-butanol with the three substrates DHB, 3-methylcatechol, and catechol. The uncompetitive component of mixed inhibition observed with DHB as substrate correlates with the close interactions between DHB and t-butanol molecule that binds at an auxiliary site in the enzyme-DHB complex. By binding in this position, t-butanol could uncompetitively inhibit DHB cleavage by decreasing the access of O$_2$ to the active site, perturbing the substrate orientation or conformation so as to lower the rate of ring cleavage, and/or interfering with product release. The absence of an uncompetitive component for the
FIG. 4. Electron density maps and models illustrating the structure of the DHBD:3-methyl catechol-bound and substrate-free forms of DHBD. Each part is a (divergent) stereo drawing prepared with the program MolView (42). The identical observed structure factors were used in all maps, which demonstrates the presence of both forms of the enzyme in the same crystal. All maps are at 1.9-Å resolution and are contoured at two standard deviations above the mean of the map. In the models, the carbon atoms are more darkly shaded than the nitrogen and oxygen atoms. A, $F_o - F_c$ electron density representing the iron, 3-methyl catechol, and two water ligands. The $F_o$'s and phases are from the structure of the substrate-free enzyme (3). The model is the initial fit to this density. B, residual $F_o - F_c$ electron density following refinement of a model that included the iron, 3-methyl catechol, two water ligands, and a t-butanol bound in the auxiliary site, as shown. The $F_o$'s and phases
smaller substrates correlates with the larger catechol to t-butanol (auxiliary site) distances: the closest contact distance in either complex is 4.5 Å, which is much larger than the expected van der Waals contact distance, 3.8 Å (41). The crystallographic data thus suggest that the manifestation of uncompetitive inhibition with DHB as substrate, whatever the mechanism, depends on the close contact between t-butanol and DHB.

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REFERENCES
1. Que, L., and Ho, R. Y. N. (1996) Chem. Rev. 96, 2607–2624
2. Elgren, T. E., Orville, A. M., Kelly, K. A., Lipscomb, J. D., Ohlendorf, D. H., and Que, L. (1997) Biochemistry 36, 11504–11513
3. Han, S., Eltis, L. D., Timmis, K. N., Muchmore, S. W., and Bolin, J. T. (1995) Science 270, 976–980
4. Senda, T., Sugiyama, K., Narita, H., Yamamoto, T., Kimbara, K., Fukuda, M., Sato, M., Yano, K., and Mitsui, Y. (1996) J. Biochem. 119, 188–196
5. Eltis, L. D., and Bolin, J. T. (1996) J. Bacteriol. 178, 5807–5817
6. Bergdoll, M., Eltis, L. D., Cameron A. D., Dumas P., and Bolin, J. T. (1998) Protein Sci. 7, 1661–1670
7. Mahouk, P. A., Orville, A. M., Lipscomb, J. D., and Solomon, E. I. (1991) J. Am. Chem. Soc. 113, 4053–4061
8. Shu, L., Chiu, Y.-M., Orville, A. M., Miller, M. A., Lipscomb, J. D., and Que, L. (1995) Biochemistry 34, 6649–6659
9. Heri, K., Hashimoto, T., and Nozaki, M. (1973) J. Biochem. 74, 375–384
10. Spence, E. L., Langley, G. J., and Bugg, T. D. H. (1996) J. Am. Chem. Soc. 118, 8336–8343
11. Sanvoisin, J., Langley, G. J., and Bugg, T. D. H. (1995) J. Am. Chem. Soc. 117, 7836–7837
12. Nozaki, M., Kagamiyama, H., and Hayashi, O. (1963) Biochem. Z. 338, 582–590
13. Ellis, L. D., Hofmann, B., Hecht, H.-J., Lünsdorf, H., and Timmis, K. N. (1993) J. Biol. Chem. 268, 2727–2732
14. Cerdan, P., Rekik, M., and Harayama, S. (1995) Eur. J. Biochem. 229, 113–118
15. Kobayashi, T., Ishida, T., Horike, K., Takahara, Y., Numao, N., Nakazawa, A., Nakazawa, T., and Nozaki, M. (1995) J. Biochem. (Tokyo) 117, 614–622
16. de Lorenzo, V., Eltis, L. D., Kessler, B., and Timmis, K. N. (1995) Gene (Amst.) 123, 17–24
17. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. T., Seidman, J. G., Smith, J. A., and Struhl, K. (1997) Current Protocols in Molecular Biology, J. Wiley & Sons Inc., New York
18. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
19. Haigler, B. E., and Gibson, D. T. (1990) J. Bacteriol. 172, 457–464
20. Whitaker, J. W., Orville, A. M., and Lipscomb, J. D. (1990) Methods Enzymol. 188, 82–88
21. King, E. L., and Altman, C. (1956) J. Phys. Chem. 60, 1375–1378
22. Cornish-Bowden, A. (1995) Analysis of Enzyme Kinetic Data, Oxford University Press, New York
23. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
24. Collaborative Computing Project, Number 4 (1994) Acta Crystallogr. Sect. D 50, 760–763
25. Brunger, A. T. (1992) X-PLOR: A System for X-ray Crystallography and NMR, Version 3.1, Yale University Press, New Haven, CT
26. Engbl, R. A., and Huber, R. (1991) Acta Crystallogr. Sect. A 47, 24–36
27. Jones, T. A., Kou, J. Y., Cowan, S. W., and Kjeldgaard, M. (1991) Acta Crystallogr. Sect. A 47, 110–119
28. Cerdan, P., Wasserfallen, A., Rekik, M., Timmis, K. N., and Harayama, S. (1994) J. Bacteriol. 176, 6074–6081
29. Furukawa, K., and Arimura, N. (1987) J. Bacteriol. 169, 924–927
30. Kikor, J. J., and Olsen, R. H. (1996) Appl. Environ. Microbiol. 62, 1728–1740
31. Lendenmann, U., and Spain, J. C. (1996) J. Bacteriol. 178, 6227–6332
32. Fersht, A. (1985) Enzyme Structure and Mechanism, p. 325, W. H. Freeman and Co., New York
33. Arras, T., Schirawski, J., and Unden, G. (1998) J. Bacteriol. 180, 2133–2136
34. Polissi, A., and Harayama, S. (1993) EMBO J. 12, 3339–3347
35. Adams, R. H., Huang, C.-M., Higson, F. K., Brenner, V., and Poch, D. D. (1992) Appl. Environ. Microbiol. 58, 647–654
36. Asturias, J. A., Eltis, L. D., Prucha, M., and Timmis, K. N. (1994) J. Biol. Chem. 269, 7807–7815
37. Happe, B., Eltis, L. D., Poth, H., Hedderich, R., and Timmis, K. N. (1993) J. Bacteriol. 175, 7313–7320
38. Spence, E. L., Kawamura, M., Sanvoisin, J., Braven, H., and Bugg, T. D. H. (1996) J. Bacteriol. 178, 5249–5256
39. Pascual, R. A., and Huang, D.-S. (1987) J. Am. Chem. Soc. 109, 2854–2855
40. Li, A.-J., and Nussinov, R. (1998) Proteins Struct. Funct. Genet. 32, 111–127
41. Smith, T. J. (1995) J. Mol. Graph. 13, 122–125
Molecular Basis for the Stabilization and Inhibition of 2,3-Dihydroxybiphenyl 1,2-Dioxygenase by \textit{t}-Butanol

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