The Mechanism-based Inactivation of 2,3-Dihydroxybiphenyl 1,2-Dioxygenase by Catecholic Substrates*

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2,3-Dihydroxybiphenyl 1,2-dioxygenase (EC 1.13.11.39), the extradiol dioxygenase of the biphenyl biodegradation pathway, is subject to inactivation during the steady-state cleavage of catechols. Detailed analysis revealed that this inactivation was similar to the O₂-dependent inactivation of the enzyme in the absence of catecholic substrate, resulting in oxidation of the active site Fe(II) to Fe(III). Interestingly, the catecholic substrate not only increased the reactivity of the enzyme with O₂ to promote ring cleavage but also increased the rate of O₂-dependent inactivation. Thus, in air-saturated buffer, the apparent rate constant of inactivation of the free enzyme was (0.7 ± 0.1) × 10⁻³ s⁻¹ versus (0.7 ± 0.4) × 10⁻³ s⁻¹ for 2,3-dihydroxybiphenyl, the preferred catecholic substrate of the enzyme, and (501 ± 19) × 10⁻³ s⁻¹ for 3-chlorocatechol, a potent inactivator of 2,3-dihydroxybiphenyl 1,2-dioxygenase (partition coefficient = 8 ± 2, Kające = 4.8 ± 0.7 μM). The 2,3-dihydroxybiphenyl 1,2-dioxygenase-catalyzed cleavage of 3-chlorocatechol yielded predominantly 2-pyrene-6-carboxylic acid and 2-hydroxy-6-phenylhexa-2,4-dienoic acid, consistent with the transient formation of an acyl chloride. However, the enzyme was not covalently modified by this acyl chloride in vitro or in vivo. The study suggests a general mechanism for the inactivation of extradiol dioxygenases during catalytic turnover involving the dissociation of superoxide from the enzyme-catecholic-dioxygen ternary complex and is consistent with the catalytic mechanism.

Extradiol dioxygenases play a key role in the metabolism of aromatic compounds. These enzymes utilize non-heme ferrous iron to cleave the aromatic nucleus of catechols meta (adjacent) to the hydroxyl substituents, incorporating both atoms of dioxygen into the product (1–3). In microorganisms, extradiol dioxygenases are involved in the aerobic catabolism of a variety of aromatic compounds including toluene, naphthalene, and biphenyl (4). In humans, homogentisate dioxygenase (EC 1.13.11.1) and 3-hydroxyanthranilate dioxygenase (EC 1.13.11.6), two extradiol-type enzymes, have been associated with the genetic disorders alkaptonuria and Huntington’s disease, respectively (5, 6). Extradiol-type dioxygenases are, thus, of considerable interest due to their general metabolic significance, their potential utility in the degradation of environmental pollutants such as polychlorinated biphenyls (PCBs),¹ and as potential targets in the treatment of genetic disorders.

Sequence and structural data indicate the existence of at least two evolutionarily independent types of extradiol dioxygenases (7, 8). The catalytic strategy utilized by these different enzymes appears to be similar, and mechanisms have been proposed based on studies of members of each family (1–3). Spectroscopic and biochemical studies (9–17) support a mechanism in which the catechol first binds to the active site Fe(II) as a monoaion in a bidentate manner. Subsequent O₂ binding to the Fe(II) followed by the iron-mediated electron transfer from the catechol to O₂ yields a semiquinone-Fe(II)-superoxide intermediate. This species reacts to give an iron-alkylperoxo intermediate, which undergoes alkyl migration, Criegee rearrangement, and O-O bond cleavage to give an unsaturated lactone intermediate and an Fe(III)-bound hydroxide anion. The latter hydrolyzes the lactone 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.

It has long been recognized that extradiol-type dioxygenases are susceptible to mechanism-based inactivation by their aromatic substrates (18, 19). This phenomenon has been studied in the xylE-encoded catechol 2,3-dioxygenase (C23O; EC 1.13.11.2) of Pseudomonas putida mt-2 of the TOL pathway and in mammalian 3-hydroxyanthranilate dioxygenase. Different catechols inactivate C23O to different extents, and several mechanisms of inactivation have been proposed. The inactivation of C23O by 3-chlorocatechol has been suggested to occur either through reversible chelation of the active site iron (19) or irreversible covalent modification by an acyl chloride species generated by the ring cleavage reaction (20). However, no evidence for either mechanism has been presented. In contrast, the inactivation of C23O by alkyl catechols appears to involve the accidental oxidation of the active site Fe(II) to Fe(III) during turnover (21). Indeed, several pathways have recruited a 2Fe-2S ferredoxin to maintain the dioxygenase active site iron in the reduced state (22, 23). It has also been proposed that the inactivation of C23O by 3-methylcatechol involves alternate

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¹ The abbreviations used are: PCB, polychlorinated biphenyl; C23O, catechol 2,3-dioxygenase; DHB, 2,3-Dihydroxybiphenyl DHBD, DHB 1,2-dioxygenase; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; HOPDA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; HEPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; DTT, dithiothreitol; HPLC, high performance liquid chromatography.
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binding modes of the catecholic substrate (24). An early report suggested that the mechanism-based inactivation of 3-hydroxyanthranilate dioxygenase also involves oxidation of the active site Fe(II) (18). However, this was refuted in a subsequent study (25). Interestingly, a halogenated substrate analogue, 4-chloro-3-hydroxyanthranilate, had been suggested to inhibit 3-hydroxyanthranilate dioxygenase via covalent modification by an acyl halide (26), although it was subsequently shown that this analogue inhibits the enzyme reversibly in vivo (27). Clearly, many aspects of the inactivation of extradiol-type dioxygenases and the relationship of this inactivation to productive catalysis remain to be clarified.

2,3-Dihydroxybiphenyl 1,2-dioxygenase (DHBD; EC 1.13.11.30) catalyzes the extradiol cleavage of 2,3-dihydroxybiphenyl (DHB; Fig. 1). DHBD is the third enzyme of the microbial biphenyl (bph) pathway. This pathway has been studied for its potential to remediate PCB-contaminated soils. The ability of the bph pathway to degrade PCBs is limited in part by DHBD, which is incapable of transforming certain chlorinated DHBs (28, 29) and is inhibited by 3-chlorocatechol (30–33). The availability of highly active preparations of DHBD from Burkholderia sp. LB400, a good PCB degrader, accelerated the development of structural data (34) and enabled kinetic studies that established that the enzyme is subject to reversible substrate inhibition and mechanism-based inactivation (35). This enzyme is thus an attractive system for experiments to further our understanding of extradiol-type dioxygenase function and mechanism-based inactivation in particular.

Herein, the inactivation of DHBD by different catecholic substrates, including 3-chlorocatechol, was studied. An experimental design based on the theoretical approach of Duggleby (36) and the steady-state mechanism of the enzyme (Fig. 2) was utilized to investigate which forms of the enzyme are subject to inactivation. A variety of biophysical experiments were conducted to substantiate the mechanism of inactivation. The results are discussed in terms of the proposed catalytic mechanism of extradiol dioxygenases and have implications for the engineering of pathways to degrade environmental pollutants. The approach developed in this study is critical to correctly analyze the steady-state cleavage of PCB metabolites by DHBD as well as the reactivity of extradiol-type dioxygenases in general.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth**—DHBD was hyperexpressed in P. putida KT2442 freshly transformed with pLEBD4 (37) as previously described (35). Burkholderia sp. LB400 was cultured at 30°C and 200 rpm in M9 minimal media (36) supplemented with an HCl-solubilized solution of minerals that did not contain thiamine and CaCl₂ (35) with a resistivity of greater than 17 megaohms-cm. All manipulations involving DHBD were performed under an inert atmosphere unless otherwise specified, usually in a Mbraun Labmaster 100 glovebox (Newburyport, MA) maintained at 2 ppm O₂ or less. DHBD was purified and flash-frozen in liquid nitrogen for long term storage as described previously (35). Aliquots of DHBD were thawed immediately before use and were exchanged into 20 mM HEPPS, 80 mM NaCl (J = 0.1), pH 8.0, by gel filtration chromatography (35) unless otherwise stated. Samples of DHBD were further diluted using the same buffer as required. Protein concentrations were determined using the Bradford method (43). Iron concentrations were determined colorimetrically using Ferene S (44).

**Kinetic Measurements**—Enzymatic activity was routinely measured by following the consumption of dioxygen using a Clark-type polarographic O₂ electrode (Yellow Springs Instruments model 5301, Yellow Springs, OH) as previously described (35). All experiments were performed using 20 mM HEPPS, 80 mM NaCl, pH 8.0, 25°C ± 0.1°C (290 μM dissolved O₂) unless otherwise stated. The standard activity assay was performed using 80 μM DHB. Concentrations of active DHBD in the assay were defined by the iron content of the injected purified enzyme solution and were used in calculating specificity, catalytic, and inactivation constants. Steady-state rate equations were fit to data using the least squares and dynamic weighting options of LEONORA (45). One unit of enzymatic activity was defined as the quantity of enzyme required to consume 1 μmol of O₂/min.

For inactivation studies in which progress curves were integrated, DHBD activity was followed spectrophotometrically by following the appearance of product with a Varian Cary 1E spectrophotometer equipped with a thermostatted cuvette holder (Varian Canada, Mississauga, Ontario, Canada) and interfaced to a microcomputer equipped with Cary WinUV software version 2.00. Experiments involving catechol, 3-methylcatechol, and DHB were monitored at 376, 389, and 434 nm, respectively, using molar extinction coefficients of 38.1, 22.0, and 25.7 μM cm⁻¹ for the corresponding ring-cleaved products. These values were determined as described previously (46).

**Reportor Substrate Studies**—The steady-state cleavage of 3-chlorocatechol by DHBD was studied using DHB as a reporter substrate. The concentration of DHB was varied from 5 to 85 μM (i.e., at concentrations below those at which substrate inhibition is observed), and the concentration of 3-chlorocatechol was varied from 2.4 to 7.6 μM (i.e., 0.5 times the apparent Kₘ for the new concentration possible without enzy-matic inactivation affecting the initial velocities). An equation identical in form to that for competitive inhibition was fit to the data (45). In this equation, the K₂₀₀ of 3-chlorocatechol replaces the competitive inhibition constant, Kᵢ⁻⁻. **Reversible Inhibition Studies**—Inhibition experiments with HOPDA were performed using air-saturated buffer. The concentration of DHB

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**FIG. 1. Reaction catalyzed by DHBD.**

**FIG. 2. General mechanism of DHBD inactivation during steady-state turnover. Asterisks denote inactivated forms of the enzyme. The rate constants j₁ to j₉ are associated with reactions that lead to the formation of inactive enzyme species.**
was varied from 5 to 85 μM, and the concentration of HOPDA was varied from 240 μM to 4.8 mM. Equations for competitive, uncompetitive, and mixed inhibition were fit to the data (45).

**Inactivation Kinetics**—The respective stabilities of the EA, AEA, and EP complexes (Fig. 2) were studied by anaerobically incubating DHBD with appropriate amounts of substrates or product, withdrawing aliquots at timed intervals, and determining the remaining DHBD activity using the standard assay. In these experiments, a solution of ~1.2 μM DHBD was incubated with either 0.08, 0.8, or 6 mM DHB, 5 mM catechol, and 2.5 or 5 mM HOPDA.

The stability of the DHBD-3-chlorocatechol complex was evaluated by incubating a 150 μM solution of the enzyme under anaerobic conditions in the presence of either 1 or 5 mM 3-chlorocatechol for 30 min. The EA complex was then desalted in 20 mM HEPPS, 80 mM NaCl pH 8.0 by gel filtration as described previously (35), and the remaining DHBD activity was determined using the standard assay.

The stability of the free enzyme in the presence of O₂ was studied by incubating DHBD in the oxygraph cuvette under standard assay conditions, and monitoring Aₜ, the activity remaining after different time intervals, by adding 80 μM DHB to the cuvette. The apparent first-order rate constant of inactivation, jₓpp (Fig. 2), was evaluated using Equation 1, in which Aₜₐₐₚₜ is the activity observed in the absence of pre-incubation.

\[ Aₜ = Aₜₒᵤₜ₋ₑᵦₕᵦ \]  
\[ (Eₐ q₁) \]  
\[ (Eq. 1) \]

**Mechanism-based Inactivation Studies**—Partition ratios for each substrate were determined using an oxygraph assay in which limiting amounts of DHBD were added to defined amounts of catecholic substrate (2–10 times the Kₘ). The amount of DHBD added to the reaction cuvette was such that the enzyme was completely inactivated before 10% of either the catecholic substrate or O₂ was consumed in the reaction mixture. The partition ratio was calculated by dividing the amount of O₂ consumed to the amount of active DHBD added to the assay (Equation 2). For 3-chlorocatechol, the partition ratio was also calculated from the amount of substrate remaining in an HPLC-based assay.

**Detection of Reactive Oxygen Species**—Three different methods were used to detect superoxide: spin-trapping using DMPO, fluorescence detection of the reduction of hydroethidine, and spectrophotometric detection of the reduction of XT.T. The reduction of hydroethidine to ethidium (49, 50) was followed using a model LS 50B spectrophotofluorometer (PerkinElmer Life Sciences). The excitation and emission wavelengths were 470 and 595 nm, respectively, with slit widths of 4 and 20 nm, respectively. Samples were placed in a 5-mm quartz cell at room temperature. The reduction of XT.T at 470 nm (21,600 m⁻¹ cm⁻¹ (51)) was followed on the Varian Cary 1E spectrophotometer described above.

For spin-trapping studies, DHBD was prepared anaerobically in potassium phosphate buffer, pH 7.5 (l = 0.1). Mixtures with substrates and DMPO were prepared using air-saturated potassium phosphate buffer.
buffer, pH 7.5 (I = 0.1). The reaction of 3-chlorocatechol with superoxide was investigated using xanthine oxidase (0.04 units/ml) and 50 μM xanthine to generate superoxide. All reactions were performed in 100 μl and transferred into capillary tubes with a glass pipette. The capillary was then placed into a quartz EPR tube and transferred to the cavity for EPR analysis. Spectra were recorded at 293 K as described above. The time between placing the sample in the EPR tube and tuning the spectrometer was less than 60 s.

Hydrogen peroxide was detected by monitoring the production of O2⁻. This was followed using a modification of the oxygraph assay described in Experiment 2. Reaction was initiated by the addition of DHBD and incubated for 30 s at 23°C. An aliquot was then withdrawn and immediately analyzed by HPLC as described below. Experiments designed to determine the partition coefficient of DHBD for 3-chlorocatechol were performed in a similar fashion, except that reaction mixtures initially contained 25 or 30 μM DHBD (1.5 mM HEPPS, 80 mM NaCl, pH 8.0). The activity of DHBD was inhibited using 400 μM 3-chlorocatechol to obtain k_APP.

RESULTS

Identification of Steady-state Species Susceptible to Inactivation—It had previously been observed that DHBD is susceptible to inactivation during the steady-state cleavage of catechols (35). Even in the presence of the preferred substrate of the enzyme, DHBD, inactivation occurs within 10 min. As described by Duggleby (36), any form of an enzyme that occurs during steady-state turnover can be susceptible to inactivation. DHBD utilizes a compulsory order, ternary complex mechanism subject to substrate inhibition (35). The general approach described by Duggleby was adapted to this steady-state mechanism as shown in Fig. 2, and the susceptibility of various forms of DHBD that occur during catalytic turnover was investigated.

Anaerobic incubation of DHBD with saturating quantities of various substrates including DHB, catechol, and 3-chlorocatechol for up to 2 h resulted in no significant change in specific activity. Similar results were obtained when amounts of substrate sufficient to cause substrate inhibition (35) were used. These results indicate that the corresponding rate constants of inactivation are negligible during the steady-state reaction (i.e., j_s and j_i are essentially equal to zero). Moreover, the anaerobic incubation of DHBD with 3-chlorocatechol did not affect the iron content of the enzyme.

In the presence of 2.5 and 5 mM HOPDA, DHBD lost ~10% of its activity after 30 min. Although these concentrations of HOPDA reversibly inhibit DHBD cleavage (see below), such concentrations never occurred in experiments in which inactivation was observed. More importantly, the HOPDA-induced inactivation cannot account for the relatively rapid inactivation that occurs during catalysis. Thus, the value of j_s (Fig. 2) was concluded to be essentially zero.

In contrast to anaerobic preparations of EA and EP complexes, free DHBD was subject to significant inactivation in air-saturated buffer. Thus, the pseudo-first order rate constant of inactivation in air-saturated buffer, j_APP, was (0.7 ± 0.1) × 10⁻² s⁻¹. This corresponds to a half-life of 16 ± 2 min.

The apparent rate constant of inactivation of DHBD by various catecholic substrates in air-saturated buffer, j_APP, was significantly faster than j_APP (Table 1). Even for DHB, j_APP, determined using spectrophotometrically derived progress curves, was 5-times larger than j_APP. Values of j_APP for two poorer substrates, 3-methylcatechol and catechol, were approximately an order of magnitude larger. The general agreement of j_APP and j_APP, determined using independent experimental approaches, validates the determined values (Table 1).

Studies of 3-Chlorocatechol Cleavage Using a Reporter Substrate—3-Chlorocatechol inactivated DHBD too efficiently for the steady-state cleavage of this compound to be directly monitored using the oxygraph or spectrophotometric assays. For this reason, the K_APP of DHBD for this substrate was deter-

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<td>DHB⁺</td>
<td>12 (1)</td>
<td>2.7 (0.6)</td>
<td>251 (6)</td>
<td>21 (1)</td>
<td>84,900 (1400)</td>
<td>3.0 (0.1)</td>
<td>3.7 (0.4)</td>
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<td>3-Mc catechol⁺</td>
<td>530 (30)</td>
<td>97 (3)</td>
<td>0.18 (0.10)</td>
<td>5,300 (300)</td>
<td>18.3 (1.6)</td>
<td>23 (3)</td>
<td>0.043 (0.08)</td>
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<td>Catechol⁺</td>
<td>860 (150)</td>
<td>51 (6)</td>
<td>0.060 (0.004)</td>
<td>1,230 (70)</td>
<td>41.5 (7.2)</td>
<td>56 (2)</td>
<td>0.065 (0.014)</td>
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<td>4.8 (0.7)</td>
<td>4.0 (1.2)</td>
<td>0.8 (0.4)</td>
<td>8 (2)</td>
<td>501 (19)</td>
<td>104 (19)</td>
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Inactivation of Extradiol Dioxygenases

Fig. 3. The DHBD-catalyzed cleavage of DHB in the presence of 3-chlorocatechol. The rate of DHB cleavage was determined using 4.8 μM (■), 8.3 μM (△), 15.2 μM (○), 48.5 μM (□), and 82.9 μM (○) DHB (20 mM HEPPS, 80 mM NaCl, pH 8.0, 25 °C). Best-fit lines were obtained using an equation similar in form to that describing competitive inhibition where $K_{iapp}$ replaces the competitive inhibition constant, $K_i$. The fit of the equation to the data using the least squares, dynamic weighting options of LEONORA yielded the following parameters: $K_{iapp}^{3CC} = 4.8 \pm 0.7$ μM, $K_{iapp}^{3CH} = 18.6 \pm 1.7$ μM, and $V = 142 \pm 6$ μM/min.

Inactivation studies, which also used DHB as a reporter substrate. The quality of the data was good, even though the concentration of 3-chlorocatechol could only be varied over a limited range (Fig. 3). Apparent catalytic and specificity constants for 3-chlorocatechol were calculated using the partition ratio and $V/K_{m}'$ (Equation 2). The results demonstrate that DHBD has good specificity for 3-chlorocatechol; the apparent specificity constant of the enzyme for 3-chlorocatechol was only 20-fold less than that for DHB, and the $K_{iapp}^{m3CC}$ was half that for DHB (Table 1).

Reversible Inhibition of DHBD by HOPDA—Product inhibition studies indicated that the mode of DHBD inhibition by HOPDA is mixed. Thus, when an equation describing mixed inhibition was fit to the data, random trends in the residuals were observed, and the residuals were smaller than when equations describing competitive or uncompetitive inhibition were fit to the data (45). The competitive inhibition constant ($K_c$) and the apparent uncompetitive inhibition constant ($K_{iapp}$) were $3.7 \pm 0.9$ and $3.3 \pm 0.3$ μM, respectively. The uncompetitive inhibition constant ($K_{iapp}$), calculated as described previously (35), was $2.7 \pm 0.2$ μM. In ordered, ternary complex mechanisms such as that utilized by DHBD, products usually act as competitive inhibitors. The observation of mixed inhibition of DHBD by HOPDA may be due to the binding of the latter to a site in the DHBD:DHB complex similar to that occupied by t-butanol, which is in contact with the distal phenyl ring of DHB (35). Inactivation-induced Changes in DHBD—To elucidate inactivation-induced changes in DHBD, the enzyme was inactivated using several different techniques, and the properties of the different preparations of DHBD were investigated. Preparations of DHBD inactivated with 1,10-phenanthroline, O$_2$, catechol, and 3-chlorocatechol could each be partially reactivated through anaerobic incubation with a reducing agent (Table II). However, incubation with Fe(II) and DTT was necessary to restore most of the activity. Thus, the O$_2$-dependent inactivation of DHBD both in the absence and presence of catecholic substrate results in the loss of the active site iron.

Preparations of DHBD inactivated with 1,10-phenanthroline, O$_2$, catechol, and 3-chlorocatechol each had a molecular mass of 32,350 ± 4 Da, identical to active DHBD as determined by ion spray mass spectroscopy. Nanospray mass spectral analyses of DHBD inactivated with 1,10-phenanthroline and 3-chlorocatechol gave essentially identical results. These data indicate that DHBD is not covalently modified during mechanism-based inactivation.

Further evidence for the oxidation of active site Fe(II) during inactivation by 3-chlorocatechol was obtained by EPR. Thus, anaerobically prepared complexes of DHBD (0.34 mM iron) and 10 mM 3-chlorocatechol had no detectable EPR signal at 77 K. An aliquot of the same sample yielded signals at $g = 5.75$ and $g = 4.28$ (Fig. 5) upon exposure to air for 5 min before flash-freezing. The signal at 4.28 is typical of high spin ferric iron in a rhombic environment and is identical to that of a solution of ferric chloride and an excess 3-chlorocatechol. Based on the relative peak areas of the $g = 4.28$ species in samples of inactivated enzyme and a known mixture of ferric chloride and
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3-chlorocatechol, it was estimated that this protein-free species accounted for 55% of the total iron in the sample of inactivated enzyme. In the same inactivation experiments, the formation of a purple complex with a broad absorption band (λ_{max} = 489 nm) was observed. This spectrum is typical of Fe(III)-catecholate complexes (54) and is similar to that of a solution of ferric iron and excess 3-chlorocatechol (λ_{max} = 494 nm; ε_{494} = 4.6 mM^{-1} cm^{-1}). Based on this extinction coefficient, more than 90% of the ferric iron in the sample of inactivated enzyme was complexed to 3-chlorocatechol. However, the small difference in λ_{max} suggests the presence of multiple Fe(III)-catecholate complexes in the sample of inactivated enzyme. It is thus likely that the g = 5.75 species in the latter sample, which accounts for up to 45% of the ferric iron, represents a DHBD-3-chlorocatechol complex containing ferric iron. This interpretation is consistent with partial occupancy of the active site of DHBD in a crystalline complex of ferric DHBD:DHB (55). Regardless of the exact nature of the Fe(III) species, these results together with the reactivation studies suggest that the mechanism-based inactivation of DHBD by 3-chlorocatechol results in the oxidation of the active site Fe(II) to Fe(III) and that the released ferric iron is then chelated by the excess 3-chlorocatechol in solution.

Detection of Reactive Oxygen Species—To investigate whether superoxide was produced during the inactivation of DHBD, inactivation reactions were performed in the presence of various superoxide-trapping agents. When 95 μM DHBD was stirred with XTT in air-saturated buffer, 16.1 ± 0.4 μM reduced XTT were detected after 100 min. Superoxide dismutase inhibited the reduction of XTT by ~70%, demonstrating that superoxide is produced during the inactivation of the free enzyme.

When DHBD (10–500 μM) was inactivated using different concentrations of 3-chlorocatechol (0.1–5 mM), no superoxide was detected using either DMPO, hydroethidine, or XTT. Moreover, in enzymatic reactions monitored with the oxygen electrode, no additional O_{2} production was observed in the presence of superoxide dismutase and/or catalase, indicating that H_{2}O_{2} was not formed.

To investigate whether 3-chlorocatechol or one of its cleavage products inhibits the reaction of superoxide with the trapping agents, the effect of the former on the detection of superoxide production by xanthine oxidase was studied. In spin-trapping experiments performed using 50 mM DMPO, the production of the EPR signal was inhibited by 83 ± 3 and 100% by 0.1 mM and 1 mM 3-chlorocatechol, respectively. Similarly, 0.1 and 1 mM 3-chlorocatechol inhibited the detection of superoxide using XTT by 27 ± 7 and 52 ± 12%, respectively, and 0.5 mM 3-chlorocatechol inhibited the detection of superoxide using hydroethidine by 45 ± 8%. In contrast, the 3-chlorocatechol-cleaved products, 2-hydroxymuconic acid and 2-pyrone-6-carboxylic acid, did not detectably inhibit the detection of superoxide using hydroethidine. Finally, 3-chlorocatechol did not inhibit the production of urate by xanthine oxidase. Thus, 3-chlorocatechol inhibited the reaction of superoxide with DMPO, XTT, and HE, presumably by reacting with superoxide directly.

The Inactivation of DHBD in Vivo—The in vivo inactivation of DHBD was studied using the native strain of the enzyme *Burkholderia* sp. LB400 and a heterologous expression host, *E. coli* DH5α. The activity of DHBD in biphenyl-grown *Burkholderia* sp. LB400 and LB-grown *E. coli* DH5α was 0.3 and 0.2 units/A_{600}, respectively. The addition of 400 μM 3-chlorocatechol to the assay completely inhibited the DHBD activity in both strains. Upon removal of 3-chlorocatechol from the cells, DHBD activity recovered to almost pre-inhibition levels within 12 min (Table III). This recovery occurred in the presence of chloramphenicol, indicating that protein synthesis is not required for the recovery of DHBD activity.

Ring-cleaved Products of 3-Chlorocatechol—Because of the instability of the extradiol cleavage products of 3-chlorocatechol (53), the products and partition coefficient of the DHBD-catalyzed cleavage of 3-chlorocatechol were investigated in re-action mixture that was incubated for a short period of time. Thus, mixtures containing 50 μM 3-chlorocatechol and 30–60 μM DHBD were incubated for 30 s. Analysis of the reaction mixture by HPLC using solvent A indicated that ~20% of the 3-chlorocatechol (t_{R} = 8.15 min, λ_{max} = 199.9 nm) was uncleaved. Using solvent B, two reaction products were eluted. These were identified as 2-pyrene-6-carboxylic acid (t_{R} = 5.4 min, λ_{max} = 298.8 nm) and 2-hydroxymuconic acid (t_{R} = 11.4 min, λ_{max} = 304.7 nm) based on the retention times and spectra of the authentic compounds. 2-Pyrene-6-carboxylic acid and 2-hydroxymuconic acid, both of which arise from the proximal 2,3-cleavage of 3-chlorocatechol (41), accounted for 18.5 ± 0.3 and 19.2 ± 0.6%, respectively, of the initial 3-chlorocatechol.

Analysis of the same reaction mixtures using solvent C permitted the detection of a third product (t_{R} = 6.8 min, λ_{max} = 375 nm). Based on retention times and absorption spectra (53), this was identified as 3-chloro-2-hydroxymuconic semialdehyde, resulting from the distal cleavage (1,6 cleavage) of 3-chlorocatechol. Considering the respective extinction coefficients of 2-pyrene-6-carboxylic acid (ε_{489} = 8.3 mM^{-1} cm^{-1} in solvent B) and 3-chloro-2-hydroxymuconic semialdehyde (ε_{375} = 54 mM^{-1} cm^{-1} at pH 7.5 (53)) and that the absorbance of the latter is similar at pH 7.5 and 10.0 (56), it was estimated that the distal ring-cleaved product accounted for 1.8 ± 0.1% of the initial amount of 3-chlorocatechol in the reaction mixture. Based on the amount of 3-chlorocatechol remaining in the reaction mixture, the partition coefficient of DHBD for this compound was 5.2 ± 0.5 and 5.4 ± 0.5 in experiments using 25 and 50 μM
3-chlorocatechol, respectively. In contrast, oxygraph assays yielded a partition coefficient of $11 \pm 2$.

**DISCUSSION**

DHBD is typical of extradiol-type dioxygenases in that it is subject to inactivation during the steady-state cleavage reaction (35). The present analysis indicates that this inactivation in DHBD requires the formation of the EAO$_2$ ternary complex. In particular, the rates of inactivation of EA, AEA, and EP ($f_5$, $f_4$, and $f_5$ in Fig. 2) are negligible with respect to the rate of inactivation during steady-state turnover. Thus, DHBD is not inactivated by chelation of the active site Fe(II) by catecholic substrates (19). Although free DHBD is subject to significant inactivation during steady-state turnover, DHBD is not inactivated by chelation of the active site Fe(II) by catecholic substrates (19). Although free DHBD is subject to significant inactivation by O$_2$, the apparent rate constant of this inactivation during steady-state turnover. Thus, DHBD is not inactivated by chelation of the active site Fe(II) by catecholic substrates (19). Although free DHBD is subject to significant inactivation by O$_2$, the apparent rate constant of this inactivation during steady-state turnover. Thus, DHBD is not inactivated by chelation of the active site Fe(II) by catecholic substrates (19).

The present study does not rule out the possibility that the AEA and EP forms are unstable in the presence of O$_2$. However, given the DHBD high $K_m$ value for O$_2$ (1.3 mM (35)), high $K_m$ for DHBD (3 mM (35)), and high $K_m$ values for HOPDA ($\sim 3$ mM), such inactivation seems unlikely to be significant under the conditions studied.

Further analysis of the mechanism-based inactivation of DHBD revealed that it is similar in nature to the O$_2$-dependent inactivation of DHBD in the absence of catecholic substrate, arising principally from the oxidation of the active site Fe(II) to Fe(III). Thus, EPR and absorption spectroscopy data demonstrate the formation of Fe(III) in samples of inactivated enzyme, and anaerobic incubation of the inactivated enzyme with Fe(II) and DTT restored the activity. The activity was partially restored upon incubation of desalted samples of inactivated DHBD with DTT alone, indicating that part of the oxidized Fe(III) remained bound to the protein. Although no association constants of an extradiol enzyme for Fe(III) and Fe(II) have been reported, these data are consistent with previous results with the catalytic data of DHBD from *Pseudomonas* sp. KKS102, in which a more intense electron density was observed in the active site when the iron was reduced (55). Moreover, the oxidation of the active site Fe(II) of C23O by H$_2$O$_2$ resulted in the immediate release of Fe(III) (57).

The present studies suggest that the mechanism-based inactivation of DHBD does not involve covalent modification, as judged by a lack of change to the molecular mass of DHBD inactivated in a number of ways. Moreover, DHBD was readily reactivated in cells in the absence of protein synthesis. Thus, inactivation does not involve hydroxylation of an active site residue as observed in the O$_2$-dependent inactivation of an $\alpha$-ketoglutarate-dependent oxygenase (58), which like DHBD has a catalytically essential mononuclear iron bound to the enzyme by a 2-histidine 1-carboxylate facial triad (59). These results also demonstrate that although 3-chlorocatechol is a very potent mechanism-based inactivator and that the DHBD-catalyzed cleavage of 3-chlorocatechol produces an acyl halide, the inactivation does not involve covalent modification by the acyl chloride as has been proposed for C23O (20). Indeed, one study reported that the inactivation of C23O by 3-chlorocatechol also involves oxidation of the active site Fe(II) (60).

A straightforward explanation of the mechanism-based inactivation of DHBD involves the dissociation of superoxide from the EAO$_2$ ternary complex. In a proposed catalytic mechanism, the formation of the EAO$_2$ ternary complex is followed by successive electron transfer steps from the Fe(II) to the bound O$_2$ and from the bound catecholate to the iron. C-O bond formation at C-2 in the resulting semiquinone-Fe(II)-superoxide intermediate yields an iron-alkylperoxo intermediate that undergoes a Criegee rearrangement (3). Mechanism-based inactivation could arise from dissociation of the bound superoxide before electron transfer from the catecholate to the iron or before C-O bond formation between the bound superoxide and semiquinone. Thus, catecholic substrates that slow either step either through sterical or electronic factors would be good mechanism-based inactivators. For example, electron transfer between 3-chlorocatechol and Fe(III) might be slower than between 3-methylcatechol and Fe(III) due to the expected higher reduction potential of a catechol with an electron-withdrawing substituent. Consistent with this hypothesis, catechols with electron-withdrawing substituents were not cleaved in a model extradiol cleavage reaction (61).

Failure to detect superoxide in the inactivation of DHBD by 3-chlorocatechol seems to be due to the rapid reaction of superoxide with the catechol, possibly before their diffusion from the active site channel of the enzyme. The current studies with xanthine oxidase demonstrate that 3-chlorocatechol is highly reactive with superoxide, consistent with the known role of catechols as superoxide scavengers (62, 63). Moreover, ferric
iron accelerates the reaction between catechols and superoxide (62) and complicates the detection of superoxide by DMPO (64). The reaction between superoxide and 3-chlorocatechol is expected to produce a mixture of multimeric species and o-quinones (62, 65), which would be difficult to detect given their low concentrations. Finally, it is noted that the inactivation of DHBD with 3-chlorocatechol was rapid (<10 s) and would thus produce a burst of superoxide. Such bursts are harder to detect because the efficiency of trapping agents decreases as the rate of superoxide production increases (50, 64). Nevertheless, the results strongly imply that inactivation of DHBD during catalytic turnover involves the dissociation of superoxide from the EAO₂ ternary complex. Thus, the O₂-dependent inactivation of DHBD in the absence and presence of catecholic substrate both result in the oxidation of active site Fe(II) and the concomitant production of superoxide. Indeed, it is possible that the DHBD high Kₘ for O₂ reflects the low affinity of the free enzyme for O₂, which may have evolved as a protective adaptation against oxidative inactivation. Interestingly, C23O, which is less susceptible to O₂-dependent inactivation (66), has a much lower Kₘ for O₂ (9).

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**REFERENCES**


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The Mechanism-based Inactivation of 2,3-Dihydroxybiphenyl 1,2-Dioxygenase by Catecholic Substrates
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