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

Received for publication, July 20, 2001, and in revised form, October 12, 2001
Published, JBC Papers in Press, November 13, 2001, DOI 10.1074/jbc.M106890200

Frédéric H. Vaillancourt†‡§, Geneviève Labbé‡§, Nathalie M. Drouin‡§, Pascal D. Fortin‡§, and Lindsay D. Eltis‡§**

From the ‡Departments of Microbiology and Biochemistry, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada and §Department of Biochemistry, Pavillon Marchand, Université Laval, Quebec City, Quebec G1K 7P4, Canada

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 catechol 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 (3.7 ± 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, K_{app} = 4.8 ± 0.7 μM). The 2,3-dihydroxybiphenyl 1,2-dioxygenase-catalyzed cleavage of 3-chlorocatechol yielded predominantly 2-pyrones and 2-carboxylic acid and 2-hydroxymuconic 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) 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 1.13.11.5) 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 monoanion 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-alkyloxepeno intermediate, which undergoes alkyl migration, Criegee rearrangement, and O-O bond cleavage to give an unsaturated lactone intermediate and an Fe(II)-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 1.13.11.12) 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 ferrodox 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

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.
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 1.15.11.39) 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 hyperepressed 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 (38) supplemented with an HCl-solubilized solution of minerals that did not contain thiamine and CaCl₂ (35) with 2% biphemyl as sole carbon source. *Escherichia coli* DH5α containing the plasmid pLEBD4 was cultured at 37 °C and 200 rpm in Luria-Bertani broth containing 20 μg/ml tetracycline.

**Chemicals**—Catechol, 3-methylcatechol, 5,5-dimethyl-1-pyrrole-N-oxide (DMPO), xanthine, xanthine oxidase (EC 1.1.3.22) from butter-milk, and bovine hepatic catalase (EC 1.11.1.6) were from Sigma-Aldrich. 3-Methylcatechol was further purified by sublimation, and DMPO was further purified as previously described (39). Ferene S and bovine erythrocyte superoxide dismutase (EC 1.15.1.1) were from ICN Biomedicals Inc. (Costa Mesa, CA). Hydroethidine and 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) were purchased from Molecular Probes Inc. (Eugene, OR). DHBD (40) and 3-chlorocatechol were gifts from Victor Snieckus (Department of Chemistry, Queens University, Kingston, Ontario, Canada). 2-Pyrene-6-carboxylic acid was a gift from Walter Reinke (Chemische Mikrobiologie, Bergische Universität, Wuppertal, Germany). 2-Hydroxymuconic semicarbazone acid was prepared using the alkaline hydrolysis of 2-pyrene-6-carboxylic acid (41). 2-Hydroxy-6-oxo-6-phenylhexa-2,4-dienolic acid (HOPDA) was prepared enzymatically from DHBD (42). All other chemicals were of analytical grade.

**Purification and Handling of DHBD Samples**—Buffers were prepared using water purified on a Barnstead NANOpure UV apparatus to a resistivity of greater than 17 megohms-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 (pH 8.0, µg/ml tetracycline. Ferric concentrations were determined colorimetrically using Fe-3.7 mM, respectively, using molar extinction coefficients of 38.1, 22.0, and 58.6 for O₂, O₂, and Fe(II), respectively, with Cary WinUV software version 2.00. Experiments involving catecholic reagents were performed using air-saturated buffer. The concentration of DHB was varied from 5 to 85 mM. DHBD activity was followed spectrophotometrically 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 ± 0.1 °C (290 ± 1 °C; dissolved O₂) unless otherwise stated. The standard activity assay was performed using 80 µM DHBD. 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 fitted 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 thermostated 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 DHBD were monitored at 376, 389, and 434 nm, respectively, using molar extinction coefficients of 38.1, 22.0, and 25.7 mM⁻¹ cm⁻¹ for the corresponding ring-cleaved products. These values were determined as described previously (46).

**Reversible Inhibition Studies**—Inhibition experiments with HOPDA were performed using air-saturated buffer. The concentration of DHBD...
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 A, the activity remaining after different time intervals, by adding 80 μM DHBD to the cuvette. The apparent first-order rate constant of inactivation, j0app (Fig. 2), was evaluated using Equation 1, in which Amax is the activity observed in the absence of pre-incubation.

\[ A_t = A_{\text{max}}e^{-j_t} \]  
(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 catechol substrate (2–10 times the K_m). 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.

Partition ratio = No. of substrate molecules consumed No. of enzyme molecules inactivated = \( \frac{k_{\text{cat}}}{j_i} \)  
(Eq. 2) 

The apparent rate constant of inactivation during catalytic turnover in air-saturated buffer, j0app (Fig. 2), was independently evaluated using two different experimental designs. In the first approach, j0app was calculated from the partition ratio determined using the oxygraph assay under saturating substrate conditions ([S] >> K_m). Under such conditions, the concentration of free enzyme, [E], is negligible, and the partition ratio is equal to the ratio of the catalytic constant, k_{cat}, and the inactivation constant j (i.e. \( \frac{K_{\text{cat}}}{j_i} \)). The rate constant evaluated using this approach is termed j0app. In the second experimental approach, j0app was determined from progress curves obtained from reactions performed at different substrate concentrations. In these experiments, the spectrophotometric assay was utilized. In the case of catechol, 3-methylcatechol, and 3-chlorocatechol, the substrate concentration was varied from the determined K_m to 5 times the determined K_m. In the case of DHBD, the substrate concentration was varied from 80 to 250 μM.

The rate constant of inactivation at each substrate concentration, j0, was determined by fitting Equation 3 (47) to the corresponding progress curve using SCIENTIST version 2.01 (Micromath Scientific Software, Salt Lake City, UT).

\[ P_r = P_i(1 - e^{-j_0S}) + P_c \]  
(Eq. 3) 

In this equation, P_r is the concentration of product recorded at the start of the assay, and P_c is the concentration of product subsequently generated during the assay. To minimize the effect of substrate deple- tion on the rate of reaction, the assays were performed using minimal amounts of enzyme (i.e. substrate consumption was less than 15%). The apparent rate constant of inactivation evaluated using this method was termed j0. For catechol, 3-methylcatechol and DHB, j0app was evaluated from j0 measured at different substrate concentrations, S, using Equation 4 (47) in which K_app is the apparent K_m of the catecholic substrate in air-saturated buffer.

\[ j_0 = \frac{j_0 \text{app} [S]}{K_\text{app} + [S]} \]  
(Eq. 4) 

For 3-chlorocatechol, DHB was used as a reporter substrate, and j0app was obtained using Equation 5 (48).

\[ j_0 = \frac{j_0 \text{app} [3-CC]}{K_\text{app} + [3-CC]} \]  
(Eq. 5) 

In this equation, K_3Capp and j0_3Capp are the apparent K_m values for 3-chlorocatechol and DHB, respectively, in air-saturated buffer, and j0 is the rate constant for the reaction at each concentration of 3-chlorocatechol and DHB.

In Vivo Inactivation and Reactivation of DHBD—DHBD was inactivated in vitro using three different methods, each performed at 23 °C using 20 mM HEPPS, 80 mM NaCl, pH 8.0. First, DHBD was inactivated anaerobically by incubating a 30 μM solution of the protein with 5 mM 1,2-dichloroethane for 20 h. In a second experiment, DHBD was inactivated with O2 by exposing a solution of the enzyme to air for 20 h. Finally, DHBD was inactivated by incubating a 12–15 μM solution of the enzyme with 10 mM catechol or 3-chlorocatechol. In this experiment, the reaction mixture was gently bubbled with air for 10 min, which was sufficient for complete inactivation. The activity of the preparations was monitored using the standard assay to verify inactivation.

Samples of DHBD were reactivated under the inert atmosphere of the glovebox. Aerobically inactivated samples were gently bubbled with argon for 15 min before being transferred to the glovebox. All samples were exchanged into 20 mM HEPPS, 80 mM NaCl, pH 8.0, by gel filtration. Samples of inactivated protein were then divided into two aliquots. The first aliquot was incubated with 2 mM DTT. After anaerobic incubation, the protein was exchanged into fresh 20 mM HEPPS, 80 mM NaCl, pH 8.0, and the specific activity of the preparation was determined using the standard assay.

Mass Spectrometry Analysis—Mass spectra were recorded on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada) equipped with an ion spray ion source (Sciex) or nanospray ion source (Protana, Odense, Denmark). The protein samples were injected onto an Ultrafast Microprotein Analyzer UMA-UML (Michrom Bioreources, Inc., Auburn, CA) directly interfaced with the mass spectrometer. In each experiment, the protein was loaded onto a polymeric reversed phase column for protein (Michrom Bioresources Inc., 81, 300 Å, 1 mm × 50 mm) equilibrated with 0.05% trifluoroacetic acid, 2% acetonitrile in water and then eluted at a flow rate of 50 μl/min over 5 min with a 20–90% gradient of solvent containing 0.045% trifluoroacetic acid and 90% acetonitrile in water. Spectra were obtained in the single quadrupole scan mode, and the quadrupole mass analyzer was scanned over a mass to charge ratio (m/z) range of 600–2400 atomic mass units, with a step size of 0.5 atomic mass units and a dwell-time of 1.0 ms/step. The ion spray ion source voltage was set at 5.0 kV, and the nanospray ion source voltage was set at 0.9 kV. The orifice energy was set at 45 or 50 V.

EPR Spectroscopy—X-band EPR spectroscopy was carried out using a Bruker model ESP 300e spectrometer equipped with a Hewlett Packard microwave frequency counter. For low temperature studies, samples of DHBD were prepared anaerobically in 20 mM HEPPS, 80 mM NaCl, pH 8.0, transferred to a 3-mm quartz cell (Wilmad, Buena, NJ), and flash-frozen in liquid nitrogen within 10 s of removal from the glovebox. Samples were then placed in a finger Dewar (Wilmad) insert and swept with argon for 77 K for EPR analysis. EPR spectra were obtained as an average of two scans with a sweep time of 336 s. Other parameters are indicated in the legend of Fig. 5.

For spin-trapping studies, EPR spectra were recorded at 293 K as an average of 3 scans with a modulation frequency of 100 kHz, a sweep time of 42 s, a microwave power of 10 mW, a modulation amplitude of 0.105 millitesla, and a scan range of 545 to 553 millitesla. The peak area was estimated by integration using standard WINEPR software.

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 XTT. The reduction of hydroethidine to ethidium (49, 50) was followed using a model LS 60B spectrophotometer (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 XTT 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 (t = 0.1). Mixtures with substrates and DMPO were prepared using air-saturated potassium phosphate...
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⁻ in the presence of catalase using an oxygen electrode. Typically, 1500 units/ml catalase was used in the assay. The effect of superoxide dismutase (200 units/ml) on the reaction of DHBD with 3-chlorocatechol was also investigated. In all cases, experiments were performed in potassium phosphate buffer, pH 7.5 (I = 0.1). The reaction of 3-chlorocatechol, 2-hydroxyxymalonate, and 2-pyrone-6-carboxylic acid with superoxide was also studied using the xanthine oxidase system with xanthine as substrate to generate superoxide. The production of urate during catalysis of xanthine by xanthine oxidase was followed at 292 nm (11 mM⁻¹ cm⁻¹ (52)). In the hydroethidine and XTT assay, the production of superoxide was achieved by incubating 200 μM xanthine with 0.08 units/ml xanthine oxidase.

To study the reaction of DHBD in the presence of superoxide, the reaction was performed with non-superoxide forms of DHBD, as well as catalase production of superoxide was achieved by incubating 200 μM xanthine (50 mM sodium carbonate, pH 10.0, and 5 mM tetrabutylammonium hydrogen sulfate as an ion pairing reagent (solvent C (53)).

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 DHBD, 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. Jᵣ and Jₛ 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 Jₛ (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, 0.7 (0.1) × 10⁻¹ s⁻¹. This corresponds to a half-life of 16 ± 2 min. The apparent rate constant of inactivation of DHBD by various catechol substrates in air-saturated buffer, was significantly faster than (Table 1). Even for DHBD, determined using spectrophotometrically derived progress curves, was 5-times larger than (Table 1). Values of for two poorer substrates, 3-methylcatechol and catechol, were approximately an order of magnitude larger. The general agreement of and for two substrates, mycrocatechol and catechol, validated 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 Kᵣᵣᵣᵣ of DHBD for this substrate was determined using a solution of 20% methanol and 80% of an aqueous buffer containing 50 mM sodium carbonate, pH 10.0, and 5 mM tetrabutylammonium hydrogen sulfate as an ion pairing reagent (solvent C (53)).
were fit to the data (45). The competitive inhibition constant studies indicated that the mode of DHBD inhibition by Fig. 4.

rocatechol; the apparent specificity constant of the enzyme for results demonstrate that DHBD has good specificity for 3-chlorocatechol, and 3-chlorocatechol could each be partially reacti-
tivated through anaerobic incubation with a reducing agent (Ta-
ble I). However, incubation with Fe(II) and DTT was neces-
mixed using DHB as a reporter substrate. The quality of the data was good, even though the concentration of 3-chloro-
catechol could only be varied over a limited range (Fig. 3). Appar-
ent catalytic and specificity constants for 3-chlorocatechol were calculated using the partition ratio and $K_{app}$ (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 $K_{app}$ was half that for DHB (Table I).

Inactivation studies, which also used DHB as a reporter substrate, confirmed that 3-chlorocatechol potently inactivates DHBD. Based on $j_{app}/P_{app}$, 3-chlorocatechol is more than 2 orders of magnitude more efficient than the next best mecha-
nism-based inactivator, DHB (Table I). 3-Methylcatechol and catechol inactivate DHBD less potently than DHB due to their high $K_m$ for the enzyme. However, in the presence of high concentrations of substrates (i.e. 1–5 times $K_m$), DHBD will be inactivated faster with the following substrates: 3-chloro-
catechol > catechol > 3-methylcatechol > DHB as illustrated in Fig. 4.

**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 ($K_{app}$) were $3.7 \pm 0.9$ and $3.3 \pm 0.3$ mM, respectively. The uncom-
petitive inhibition constant ($K_{app}$), calculated as described previ-
ously (35), was $2.7 \pm 0.2$ mM. In ordered, ternary complex mechanisms such as that utilized by DHBD, products usually act as competitive inhibitors. The observation of mixed inhi-
bition 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 inacti-
vated using several different techniques, and the properties of the different preparations of DHBD were investigated. Prepara-
ations of DHBD inactivated with 1,10-phenanthroline, O$_2$, catechol, and 3-chlorocatechol could each be partially reacti-
vated through anaerobic incubation with a reducing agent (Ta-
ble II). However, incubation with Fe(II) and DTT was neces-
sary 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-chloro-
catechol gave essentially identical results. These data indicate that DHBD is not covalently modified during mechanism-based inactiva-
tion.

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 rel-
ative peak areas of the $g = 4.28$ species in samples of inacti-
vated enzyme and a known mixture of ferric chloride and
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 ($\lambda_{\text{max}} = 489$ 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 ($\lambda_{\text{max}} = 494$ nm; $\epsilon_{494} = 4.6 \text{ M}^{-1} \text{cm}^{-1}$). 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 $\lambda_{\text{max}}$ 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 $\mu$M DHBD was stirred with XTT in air-saturated buffer, 16.1 ± 0.4 $\mu$M 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 $\mu$M) 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 ring-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 HR, presumably by reacting with superoxide directly.

The Inactivation of DHDB 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 DH5a. The activity of DHBD in biphenyl-grown Burkholderia sp. LB400 and LB-grown E. coli DH5a was 0.3 and 0.2 units/A$_{600}$, respectively. The addition of 400 $\mu$M 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 reactivation mixture that was incubated for a short period of time. Thus, mixtures containing 50 $\mu$M 3-chlorocatechol and 30–60 $\mu$M 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, $\lambda_{\text{max}} = 199.9$ nm) was un-cleaved. Using solvent B, two reaction products were eluted. These were identified as 2-pyrene-6-carboxylic acid ($t_R = 5.4$ min, $\lambda_{\text{max}} = 298.8$ nm) and 2-hydroxymuconic acid ($t_R = 11.4$ min, $\lambda_{\text{max}} = 304.7$ 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, $\lambda_{\text{max}} = 375$ 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 ($\epsilon_{305} = 8.3 \text{ M}^{-1} \text{cm}^{-1}$ in solvent B) and 3-chloro-2-hydroxymuconic semialdehyde ($\epsilon_{294} = 54 \text{ M}^{-1} \text{cm}^{-1}$ 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 $\mu$M
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 (\(j_2\), \(j_4\), and \(j_5\) 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 is significantly lower than the rate constant of inactivation by the preferred catecholic substrate of the enzyme, DHB. The current 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 \(K_a\) for DHBD (3 mM (35)), and high \(K_v\) 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, the apparently higher affinity of DHBD for Fe(II) than for Fe(III) is consistent with the crystallographic data of DHBD from *Pseudomonas* sp. KKS102, in which a more intense electron density was observed at the active site when the iron was reduced (55). Moreover, the oxidation of the active site Fe(II) of C23O by \(H_2O_2\) 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, 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 steric 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 K_m for O_2 reflects the low affinity of the free enzyme for O_2, which may have evolved as a protective adaptation against oxidative inactivation. Interestingly, C230O, which is less susceptible to O_2-dependent inactivation (66), has a much lower K_m for O_2 (9).

For the reaction products described above, the inactivation of DHBD by 3-chlorocatechol, which effectively acts as a reversible inhibitor of DHBD in vivo.

Acknowledgments—We thank Prof. A. Grant Mauk (Department of Biochemistry and Molecular Biology, University of British Columbia) for access to the EPR spectrometer, Dr. Shouming He (Department of Chemistry, University of British Columbia) for performing the mass spectrometry analysis, Dr. Paul K. Witting for technical support in the EPR experiments, Dr. Witting and Prof. Jeffrey T. Bolin for helpful discussion of the manuscript, and Dr. Johan Janzen (Department of Pathology, University of British Columbia) for technical support in the HPLC experiments.

REFERENCES


Inactivation of Extradiol Dioxygenases

[References]

The Mechanism-based Inactivation of 2,3-Dihydroxybiphenyl 1,2-Dioxygenase by Catecholic Substrates
Frédéric H. Vaillancourt, Geneviève Labbé, Nathalie M. Drouin, Pascal D. Fortin and Lindsay D. Eltis

doi: 10.1074/jbc.M106890200 originally published online November 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M106890200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 32 of which can be accessed free at http://www.jbc.org/content/277/3/2019.full.html#ref-list-1