CHARACTERIZATION OF 2,3-DIHYDROXYBIPHENYL 1,2-DIOXYGENASE, A KEY ENZYME IN POLYCHLORINATED BIPHENYLS (PCBs) BIODEGRADATION

by

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B.Sc., Université Laval, 1996

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
THE DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

We accept this thesis as conforming To the required standard

The University of British Columbia

September 2002

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ABSTRACT

2,3-Dihydroxybiphenyl 1,2-dioxygenase (DHBD_{LB400}, EC 1.13.11.39) from Burkholderia sp. LB400, the extradiol dioxygenase of the biphenyl biodegradation pathway, was investigated using a highly active, anaerobically purified preparation of enzyme. The steady-state kinetic data obtained using 2,3-dihydroxybiphenyl (DHB) fit a compulsory-order ternary-complex mechanism in which substrate inhibition occurs. DHBD_{LB400} was also stabilized by small organic molecules. The molecules that stabilized DHBD_{LB400} most effectively also inhibited the cleavage reaction most strongly. DHBD_{LB400} was subject to reversible substrate inhibition and mechanism-based inactivation. Analysis of the mechanism-based inactivation revealed that it was similar to the O_2-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 O_2 to promote ring-cleavage, but also increased the rate of O_2-dependent inactivation. The study suggests a general mechanism for the inactivation of extradiol dioxygenases during catalytic turnover involving the dissociation of superoxide from the enzyme:catecholic:O_2 ternary complex.

To evaluate the role of DHBDs in the degradation of polychlorinated biphenyls (PCBs), the ability of DHBD_{LB400} and two evolutionarily divergent isozymes (DHBD_{P6-I} and DHBD_{P6-III}) from Rhodococcus globerulus P6 to cleave each of the six monochlorinated DHBs was studied. DHBD_{LB400} cleaved these compounds with specificities between 0.06 and 0.3 times that of unchlorinated DHB. In contrast to DHBD_{LB400}, both rhodococcal enzymes had higher apparent specificity constants for some chlorinated DHBs. Interestingly, DHBD_{LB400} and DHBD_{P6-I} had a very poor reactivity towards 2'-Cl DHB, and were more susceptible to mechanism-based inactivation in its presence. Subsequent work with 2',6'-diCl DHB revealed that this compound was cleaved extremely slowly relative to DHB. It was found that 2',6'-diCl DHB competitively inhibited the cleavage of DHB with a $K_{ic}^{app} = 7 \pm 1$ nM; $0.14 \pm 0.01$ μM and $2.6 \pm 0.2$ μM for
DHBD_{LB400}, DHBD_{P6}-I and DHBD_{P6}-III respectively. These data were shown to be in good agreement with the crystal structures of the DHBD_{LB400:2'-Cl DHB} and DHBD_{LB400:2',6'-diCl DHB} complexes (Dai et al. 2002, In preparation) that show that the chlorinated DHBs fit the active site very well and that the ortho chloro substituents partially occlude the putative O₂-binding pocket, thereby inhibiting O₂-binding as well as the reaction between the activated oxygen and catecholic species in the enzyme ternary complex.

Finally, UV/vis spectroscopy was used to probe the nature of the binding of DHB and 3-nitrocatechol to DHBD_{LB400}. The electronic absorption data demonstrate that DHBD_{LB400} binds its catecholic substrate as a monoanion, confirming this feature of the proposed mechanism of extradiol dioxygenases. This conclusion is supported by UV resonance Raman spectroscopic data and a crystal structure of the DHBD_{LB400:DHB} complex at 2.0 Å resolution (Vaillancourt et al. 2002, J. Am. Chem. Soc. 124, 2485-2496), which suggests that the substrate’s 2-hydroxyl substituent, and not the 3-hydroxyl group, deprotonates upon binding.
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ABBREVIATIONS

C12O  catechol 1,2-dioxygenase (EC 1.13.11.1)
C23O  catechol 2,3-dioxygenase (EC 1.13.11.2)
DHB  2,3-dihydroxybiphenyl
DHBD  2,3-dihydroxybiphenyl 1,2-dioxygenase (EC 1.13.11.39)
DHBD_{LB400}  DHBD from *Burkholderia* sp. LB400
DHBD_{KKS102}  DHBD from *Pseudomonas* sp. KKS102
DHBD_{P6-I}  DHBD I from *Rhodococcus globerulus* P6
DHBD_{P6-II}  DHBD II from *Rhodococcus globerulus* P6
DHBD_{P6-III}  DHBD III from *Rhodococcus globerulus* P6
DMPO  5,5-dimethyl-1-pyrroline-N-oxide
DTT  dithiothreitol
EPR  electron paramagnetic resonance
GO  gentisate 1,2-dioxygenase (EC 1.13.11.4)
HAD  3-hydroxyanthranilate 3,4-dioxygenase (EC 1.13.11.6)
HEPPS  4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid
HGO  homogentisate 1,2-dioxygenase (EC 1.13.11.5)
HOPDA  2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate
HPLC  high performance liquid chromatography
NADH  $\beta$-nicotinamide adenine dinucleotide, reduced form
PCB  polychlorinated biphenyl
3,4-PCD  protocatechuate 3,4-dioxygenase (EC 1.13.11.3)
3,5-PCD  protocatechuate 4,5-dioxygenase (EC 1.13.11.8)
UVRR  ultraviolet resonance Raman
XTT  2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2$H$-tetrazolium-5-carboxanilide
ACKNOWLEDGEMENTS

I would like to acknowledge the many people who have helped and supported me throughout my studies. I would especially like to thank my supervisor Lindsay Eltis, for his patience, guidance and support. Thank you for everything.

Thank must also go to all the members of the Eltis lab, both past and present for your support and friendship. Especially Nathalie Drouin, Geneviève Labbé, Pascal Fortin, Halim Maaroufi, Amparo Haro and Zamil Karim without whom such an extensive kinetic study would not have been possible. A special thanks to Chris Barbosa, Michael Blades, Robin Turner and Thomas Spiro for such a fun and challenging collaboration.

Many thanks to my committee members, Grant Mauk and Ross MacGillivray, for looking after me and for reviewing my thesis. I would also like to thank Jacques Lapointe and Rock Breton for looking after me as my committee members at the beginning of the Ph.D. at Université Laval.

Many thanks to Seungil Han, Shaodong Dai and Jeffrey Bolin for providing very good quality crystal structures of DHBD_{LB400} and to Victor Snieckus and his group for providing most of the substrates used in this study. The project would not have been the same without your help.

I would also like to acknowledge the Natural Sciences and Engineering Research Council of Canada and the Li Tze Fong Memorial Fund for postgraduate scholarships. Shouming He for performing the mass spectrometry analysis, Paul Witting for his expert assistance in the EPR experiments and John D. Lipscomb for generously providing purified 3,4-PCD.

On a more personal level, j’aimerais remercier toute ma famille et mes amis pour leur support et compréhension malgré le fait que mes études m’aient menées à plusieurs
milliers de kilomètres de la maison. Vous êtes toujours là pour moi et je l’apprécie grandement.

Pour finir, j’aimerais remercier mon amoureuse et meilleure amie, Annie, pour son support, sa patience, sa compréhension et par-dessus tout, son amour. Je ne te serai jamais assez reconnaissant.
FOREWORD

Various parts of this thesis have appeared in refereed journals. The description of the anaerobic purification of DHBD_{LB400} and the explanation of the molecular basis for the stabilization and inhibition of DHBD_{LB400} appeared in the Journal of Biological Chemistry (Vaillancourt, F.H., Han, S., Fortin, P.D., Bolin, J.T., and Eltis, L.D. (1998) J. Biol. Chem. 273, 34887-34895). In this publication, I was responsible for the purification and kinetic characterization of DHBD_{LB400} with the assistance of one undergraduate student in the kinetic characterization.

The description of the mechanism-based inactivation of DHBD_{LB400} by catecholic substrates appeared in the Journal of Biological Chemistry (Vaillancourt, F.H., Labbé, G., Drouin, N.M., Fortin, P.D., and Eltis, L.D. (2002a) J. Biol. Chem. 277, 2019-2027). In this publication, I was responsible for every aspect of the work with the assistance of one undergraduate student and two research assistants.

Definitive evidence for monoanionic binding of DHB to DHBD_{LB400} from UV/Vis absorption spectroscopy, UV resonance Raman spectroscopy, and crystallography was published in the Journal of the American Chemical Society (Vaillancourt, F.H.*, Barbosa, C.J.*, Spiro, T.G., Bolin, J.T., Blades, M.W., Turner, R.F.B.; and Eltis, L.D. (2002b) J. Am. Chem. Soc. 124, 2485-2496 (*shared first authorship)). In this truly collaborative contribution, I shared first authorship with Christopher J. Barbosa and I was responsible for the evaluation of the different pK_a values of catechol and DHB. I was also responsible for the UV/Vis portion of the study and sample preparation for the UVRR portion of the study. This part of the study, together with the UVRR and crystallography section permitted a very detailed analysis of one step of the catalytic mechanism of extradiol dioxygenases.

The characterization of the specificity of DHBD_{LB400} for chlorinated DHBs, which led to the identification and analysis of a bottleneck in PCB degradation will be published in Nature Structural Biology (Dai, S.*, Vaillancourt, F.H.*, Maaroufi, H., Drouin, N.M., Neau,
The characterization of the specificity of DHBD_{P6}-I and DHBD_{P6}-III for chlorinated DHBs, which led to the identification of extradiol dioxygenases with higher specificities for chlorinated metabolites has been submitted to the Journal of Bacteriology (Vaillancourt, F.H., Haro, M.-A., Drouin, N.M., Karim, Z., Maaroufi, H., and Eltis, L.D. *J. Bacteriol.* Submitted).
1. INTRODUCTION

1.1 Aromatic compounds

Aromatic compounds are organic chemicals that have a special stability and properties due to a closed loop of electrons in bonding orbitals. These molecules are planar and fully conjugated with \((4n + 2)\) \(\pi\) electrons where \(n\) is a non-negative integer (Hückel’s rule, McMurry 1992). Their great stability is due to stabilization of the \(\pi\) electrons through delocalisation in the \(\pi\) orbitals (resonance structure). A prototypical aromatic compound is benzene \((C_6H_6)\), a highly stable, planar and symmetrical molecule.

Aromatic compounds are widely distributed in nature. They comprise a wide variety of low-molecular-mass compounds, e.g. the aromatic amino acids (tryptophan, phenylalanine and tyrosine), quinones, flavonoids, phenolic compounds and tannins. They are also found in biopolymers, e.g. lignin and melanin. In fact, the structural polymer lignin comprises about 30% of plant material and is the second most abundant polymer in nature after cellulose (Alder 1977).

Xenobiotic compounds are synthetic compounds that are not normally found in nature, e.g. herbicides, insecticides, fungicides, detergents, plastic and other synthetic polymers. Xenobiotic compounds are often aromatics and frequently have chloro substitutents, e.g. polychlorinated biphenyls and 2,4-D, an herbicide. These compounds are often very stable and tend to accumulate in the environment.

1.1.1 Polychlorinated biphenyls (PCBs)

PCBs were manufactured by the direct chlorination of biphenyl to produce complex mixtures of up to 209 different congeners containing up to 10 chlorines. Common commercial PCB mixtures typically contain tri- to hexachlorinated PCBs. Due to their chemical and physical stability, they were widely used in industrial applications as organic diluents, plasticizers, cutting oils, flame retardants, sealants and, especially, as dielectrics in
transformers. They were used in massive quantities from the 1930s to the 1960s, but their production was banned in the 1970s because of their toxicity (vide infra). The total amount of PCBs produced is estimated at 1.2 million tons (World Health Organization 1993). Due to their stability and lipophilicity, they tend to accumulate in the biosphere (McFarland and Clarke 1989, Safe 1994).

PCBs and their hydroxylated metabolites affect human health in a number of ways. They have been implicated or related to cancer in populations of humans and animals exposed to high concentrations of PCBs (Silberhorn et al. 1990, Cogliano 1998). Exposure to ortho-substituted (non-planar) congeners causes neurological problems (Jacobson and Jacobson 1996, Kodavanti and Tilson 2000). Hydroxylated metabolites also adversely affect sexual development and reproductive function by interfering with the hormonal metabolism (Kester et al. 2000).

1.2 Microbial catabolism of aromatic compounds

The microbial degradation of aromatic compounds constitutes an essential link in the global carbon cycle. Several pathways for the degradation of aromatic compounds exist in anaerobic and aerobic microorganisms. Two key steps in these pathways are the activation of the benzene ring followed by its cleavage. The benzene ring must be activated because it is thermodynamically stable and relatively resistant toward enzymatic attack due to its large resonance energy. In anaerobic microbial degradation, the benzene ring is first activated using different strategies to yield one of three intermediates: benzoyl-CoA, resorcinol, and phloroglucinol (Heider and Fuchs 1997, Schink et al. 2000). The aromatic ring is then dearomatized through a series of reductive steps followed by cleavage by a hydrolase to yield aliphatic acids for cell growth. In aerobic microbial degradation, oxygenases activate the benzene ring by catalyzing the incorporation of oxygen-containing substituents. Ring-cleaving dioxygenases then catalyze ring fission (Dagley 1978, Dagley 1986, Harayama et al. 1992).
Microorganisms utilize different aerobic pathways to degrade the different types of aromatic compounds. However, degradation usually proceeds via one of three common intermediates: catechol, protocatechuate or gentisate (Figure 1). Compounds containing more than one aromatic ring are degraded via iterations of the strategies used to degrade monocyclic compounds (Figure 1). The ring cleavage of catechol and protocatechuate is performed by enzymes from one of two distinct classes (Figure 2, Harayama and Rekik 1989). Intradiol dioxygenases utilize non-heme Fe(III) to cleave the aromatic nucleus ortho to (between) the hydroxyl substituents. In contrast, extradiol dioxygenases utilize non-heme Fe(II) to cleave the aromatic nucleus meta (adjacent) to the hydroxyl substituents. Interestingly, a few Mn(II)-dependent extradiol dioxygenases with strong sequence similarity to the Fe(II) counterpart have also been reported (Que and Reynolds 2000). Although these distinctions between intradiol and extradiol dioxygenases may appear to be minor, they are in fact a manifestation of enzymes that have completely different structures and utilize different catalytic mechanisms (for recent reviews see Que and Ho 1996, Solomon et al. 2000, Bugg and Lin 2001). The ring cleavage of gentisate is catalyzed by gentisate 1,2-dioxygenase (GO; Crawford et al. 1975, Harpel and Lipscomb 1990), an extradiol-type dioxygenase. The products of these reactions are further transformed to intermediates of the tricarboxylic acid cycle as shown in Figure 2.

In addition to GO, several other Fe(II)-dependent extradiol-type dioxygenases involved in the metabolism of aromatic compounds have been identified in microorganisms. These include 1-hydroxy-2-naphthoate dioxygenase (Iwabuchi and Harayama 1998), 2-aminophenol 1,6-dioxygenase (Lendenmann and Spain 1996), and chlorohydroquinone dioxygenase (Miyauchi et al. 1999, Xu et al. 1999), that are involved in the metabolism of phenanthrene, nitrobenzene, and polychlorinated phenols, respectively. In comparison to the substrate of typical extradiol dioxygenases, the substrate of these enzymes have either a carboxylate, an amino or even a chloro group in place of the second hydroxyl group.
Figure 1. Pathways for the degradation of polyaromatic compounds. Reactions designated by an (a) are catalyzed by ring-hydroxylating dioxygenases. Reactions designated by a (b) are catalyzed by ring-cleaving dioxygenases. The intermediate obtained after the hydroxylation of benzoic acid depends on the substituent present on its aromatic ring.

Figure 2. Catabolism of ring-cleavage intermediates to metabolites of the tricarboxylic cycle. Fumarate formed from gentisate enters the cycle directly. Acetaldehyde enters the cycle as acetyl-CoA. Succinate is formed from succinic semialdehyde (adapted from Dagley 1978, Dagley 1986).
Aromatic compounds are also metabolized in mammals. The different pathways for the degradation of aromatic amino acids involve ring-cleavage reactions catalyzed by extradiol-type enzymes. In humans, these enzymes are homogentisate dioxygenase (HGO), an enzyme involved in the catabolism of phenylalanine and tyrosine, and 3-hydroxyanthranilate dioxygenase (HAD), an enzyme involved in the catabolism of tryptophan in the kynurenine pathway. HGO and HAD are associated with the genetic disorders alkaptonuria and Huntington's disease, respectively (La Du et al. 1958, Schwarcz et al. 1988).

Some microbial pathways are also able to transform xenobiotic compounds as the structures of such compounds often resemble naturally occurring ones. However, in most cases, the degradation of xenobiotics is not complete due to blockage in one of the degradation steps. Several strategies to improve the degradation of such recalcitrants compounds have been described (Timmis et al. 1994).

In summary, ring-cleaving dioxygenases are of considerable interest due to their general metabolic significance, their potential utility in the degradation of environmental pollutants such as PCBs, and as potential targets in the treatment of genetic disorders.

1.2.1 The bph pathway

The aerobic microbial degradation of PCBs has been intensively studied as a possible means to destroy these toxic, persistent environment pollutants. Particular attention has focused on the bph pathway, which is responsible for the aerobic degradation of biphenyl in a wide range of microorganisms (Abramowicz 1990, Furukawa 2000). The upper bph pathway (Figure 3) consists of four enzymatic activities which together transform biphenyl to benzoate and 2-hydroxypenta-2,4-dienoate. The first enzyme of the pathway, biphenyl dioxygenase, a typical ring-hydroxylating dioxygenase, utilizes \( \text{O}_2 \) and electrons originating from the reduced form of \( \beta \)-nicotinamide adenine dinucleotide (NADH) to transform biphenyl to \( \text{cis}-2,3\)-dihydro-2,3-dihydroxybiphenyl. The enzyme comprises three
components: an FAD-containing reductase, a Rieske-type Fe$_2$S$_2$ ferredoxin, and a two-subunit oxygenase that contains a Rieske cluster and a mononuclear iron centre. The second enzyme of the pathway, cis-2,3-dihydro-2,3-dihydroxybiphenyl dehydrogenase, is a member of the short-chain alcohol dehydrogenase/reductase family and catalyzes the NAD$^+$-dependent dehydrogenation of cis-2,3-dihydro-2,3-dihydroxybiphenyl to 2,3-dihydroxybiphenyl (DHB). The third enzyme of the pathway, 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD), is an extradiol dioxygenase that utilizes a mononuclear non-heme iron(II) centre to catalyze the cleavage of the catecholic ring of DHB. This reaction results in the incorporation of O$_2$ into the product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA; see section 1.5 and 1.6 for structural and mechanistic details of DHBD). The fourth enzyme of the pathway, HOPDA hydrolase, catalyses the cleavage of HOPDA at a carbon-carbon bond, with the incorporation of H$_2$O to yield benzoate and 2-hydroxypenta-2,4-dienoate.

The bph pathway enzymes also transform some of the 208 different congeners of chlorinated biphenyl that can occur in commercial mixtures of PCBs. The range of PCBs that is transformed by the bph pathway is highly dependent upon the bacterial strain. Some strains are not able to transform PCBs that contain more than 3 chlorines whereas other strains, such as Burkholderia sp. LB400 (Bopp 1986, Bedard et al. 1986, Bedard and Haberl 1990, Gibson et al. 1993, Billingsley et al. 1997) and Rhodococcus globerulus P6 (Bedard et al. 1986, Bedard and Haberl 1990, Furukawa et al. 1978, Furukawa et al. 1979, Furukawa et al. 1983, Kohler et al. 1988), transform up to hexachlorinated biphenyls.
The optimization of microbial catabolic activities for the biodegradation of PCBs necessitates the determination of the specificity of bph pathway enzymes for chlorinated metabolites. PCB transformation has been studied largely as a function of the first enzyme of the bph pathway, biphenyl dioxygenase (Seeger et al. 1995b, Haddock et al. 1995, McKay et al. 1997, Hurtubise et al. 1998, Chebrou et al. 1999, Seeger et al. 1999, Arnett et al. 2000, Imbeault et al. 2000). A few studies have focussed on the second enzyme of the pathway, cis-2,3-dihydro-2,3-DHB dehydrogenase (Barriault et al. 1999, Seeger et al. 1995b, Bruhlmann and Chen 1999). However, its role in PCB degradation remains to be fully elucidated as only a few congeners were tested. More recently, the fourth enzyme of the pathway, HOPDA hydrolase was identified as an important determinant of PCB degradation, being competitively inhibited by some chlorinated HOPDAs (Seah et al. 2000, Seah et al. 2001).

DHBD, the third enzyme of the bph degradation pathway, is of particular significance to the degradation of PCBs as it is incapable of transforming certain chlorinated DHBs (Furukawa et al. 1979, Seeger et al. 1995a), including 2’6’-diCl DHB. DHBD is also inhibited by 3-chlorocatechol (Sondossi et al. 1992, Adams et al. 1992, Asturia and Timmis 1993, Arensdorf and Focht 1994), a compound that is formed during the degradation of PCBs. The specificity of this enzyme towards chlorinated metabolites and the nature of the inhibition remain to be elucidated. Interestingly, Burkholderia sp. LB400 contains one DHBD while R. globerulus P6 contains 3 different DHBDs (Asturia and Timmis 1993, Asturias et al. 1994a) of which a minimum of two were shown to be expressed (Asturias et al. 1994a). Several other Gram-positive actinomycetes were also found to possess multiple DHBD isozymes (Schmid et al. 1997, Kosono et al. 1997, Kulakov et al. 1998, Shimizu et al. 2001). Most of these organisms are able to transform a wide range of PCBs and other aromatic compounds. However, the relationship between these catabolic capabilities and the occurrence of multiple DHBDs is unclear.
1.3 Classification of extradiol and extradiol-type dioxygenases

As described in section 1.2, extradiol and extradiol-type dioxygenases are involved in the aerobic catabolism of a variety of aromatic compounds. Phylogenetic analyses indicate that these enzymes belong to at least three evolutionarily independent superfamilies (Elitis and Bolin 1996, Dunwell et al. 2001). Type I extradiol dioxygenases belong to the vicinal oxygen chelate superfamily (Armstrong 2000, Gerlt and Babbitt 2001). This type of extradiol dioxygenase includes two-domain enzymes, exemplified by DHBD from *Burkholderia* sp. LB400 (DHBD\textsubscript{LB400}) and DHBD I from *R. globerulus* P6 (DHBD\textsubscript{P6-I}), and one-domain enzymes, such as DHBD II from *R. globerulus* P6 (DHBD\textsubscript{P6-II}) and DHBD III from *R. globerulus* P6 (DHBD\textsubscript{P6-III}). Each domain contains two copies of a $\beta\alpha\beta\beta\beta$ module. Phylogenetic analyses indicate that these enzymes share a common one-domain ancestor; the evolution of type I extradiol dioxygenases therefore appears to be the result of two duplication events followed by the divergence of one- and two-domain enzymes. Subsequent divergence among the two-domain dioxygenases has resulted in several families, at least two of which are based on substrate preference. DHBD\textsubscript{LB400} and DHBD\textsubscript{P6-I} belong to a family with a preference for bicyclic substrates. In contrast, catechol 2,3-dioxygenase (C23O) from *Pseudomonas putida* mt-2 belongs to a family with a preference for monocyclic substrates.

Type II extradiol dioxygenases include enzymes consisting of one (e.g. 2,3-dihydroxyphenylpropionate 1,2-dioxygenase, Spence et al. 1996a) or two (e.g. protocatechuate 4,5-dioxygenase (4,5-PCD), Sugimoto et al. 1999) different subunits. Sequence alignment revealed that the sequence of the one-subunit enzyme is similar to the $\beta$ subunit of the two-subunit enzyme. The crystal structure of 4,5-PCD revealed that the small $\alpha$ subunit is composed of 10 $\alpha$-helices and that it covers the active site that is located in the $\beta$ subunit. The latter has an $\alpha/\beta$ structure composed of 11 $\beta$ stands, nine $\alpha$ helices and one 3$_{10}$ helix. To date, type II extradiol dioxygenases have not been classified in a superfamily. Interestingly, chlorohydroquinone dioxygenase (Miyauchi et al. 1999, Xu et al. 1999) and 2-
aminophenol 1,6-dioxygenase (Takenaka et al. 1997, Davis et al. 1999), two extradiol-type dioxygenases, are related to the type I and type II extradiol dioxygenases respectively. Other extradiol-type dioxygenases belong to the cupin superfamily (Dunwell et al. 2001). Dioxygenases belonging to this superfamily include GO, 1-hydroxy-2-naphthoate dioxygenase, HGO and HAD.

1.3.1 The vicinal oxygen chelate superfamily

As described in the previous section, DHBD is a member of the vicinal-oxygen-chelate superfamily (Armstrong 2000, Gerlt and Babbitt 2001). The common structural fold of this superfamily is the $\beta\alpha\beta\beta$ motif, and most members of the superfamily utilize a divalent metal ion to catalyze a reaction involving direct metal ion chelation by vicinal oxygens of the substrate or an intermediate in the reaction. The members of the superfamily identified to date are: (1) type I extradiol dioxygenases, (2) 4-hydroxyphenylpyruvate dioxygenase (Serre et al. 1999, EC 1.13.11.27), (3) glyoxalases I (Cameron et al. 1997, EC 4.4.1.5), (4) fosfomycin resistance proteins (Bernat et al. 1997), and (5) methylmalonyl-CoA epimerases (McCarthy et al. 2001, EC 5.1.99.1). The metal ion requirement of these enzymes, and the reactions they catalyze, are summarized in Figure 4. To date, the one exception in the superfamily are the (6) bleomycin resistance proteins (Dumas et al. 1994) that do not bind any metal ion and sequester bleomycin and related compounds without degrading or transforming them. It is thought that during the evolution of this protein, the divalent metal ion may have been shed in favour of a more hydrophobic cavity to accommodate the antibiotic.

The proteins in this superfamily probably evolved from a common $\beta\alpha\beta\beta$ module ancestor through gene duplications and fusions (Bergdoll et al. 1998, McCarthy et al. 2001) that led to the formation of the two and four-module proteins that form the vicinal oxygen chelate superfamily. The use of the pseudosymmetric structure provided by the pair of
\( \beta_3\alpha_3\beta_3\beta_3 \) modules offers a versatile template and structural flexibility when compared to the well-known barrel folds.

![Figure 4](adapted from Gerlt and Babbitt 2001).

1.3.2 The cupin superfamily

As described in section 1.3, GO, 1-hydroxy-2-naphthoate dioxygenase, HGO and HAD are part of the cupin superfamily (Dunwell et al. 2001). This superfamily is composed of proteins containing at least one domain with six antiparallel \( \beta \)-sheets that form a \( \beta \)-barrel structure. Within this \( \beta \)-barrel are two distinct motifs. The first motif is composed of the first two \( \beta \)-sheets and the second, of the last two \( \beta \)-sheets. One of the major differences between the various classes of cupins involves variations of the two middle \( \beta \)-strands and the less conserved loop of variable length that separates them. Some cupins exemplified by HAD, germin and germin-like proteins involved in the response of plants to pathogens and
stresses are composed of a single domain. Other cupins are composed of two copies of the
domain that probably arose from gene duplication. The group of two-domain cupins is
exemplified by GO, 1-hydroxy-2-naphthoate dioxygenase, HGO, oxalate decarboxylase and
seed storage proteins. A special class of cupins exemplified by AraC/XylS-type
transcription factors and some helix-turn-helix transcription factors are composed of a cupin
domain linked to a DNA binding domain. In these protein, the cupin domain binds the
effector molecule (Dunwell et al. 2000, Dunwell et al. 2001).

In cupins with a catalytic function, a metal ion is often present at the active site
centre. When present, the metal ion ligands are found in each of the two motifs. Cupins
utilise as diverse a range of metal ions as the vicinal oxygen chelate superfamily: iron (GO
(Harpel and Lipscomb 1990), 1-hydroxy-2-naphthoate dioxygenase (Iwabuchi and
Harayama 1998), HGO (Titus et al. 2000), HAD (Vescia and Di Prisco 1962), and cysteine
dioxygenase (Yamaguchi et al. 1978)); manganese (germin and germin-like proteins (Woo
et al. 2000)); zinc (phosphomannose isomerase (Cleasby et al. 1996)); copper (quercetin
2,3-dioxygenase (Fusetti et al. 2002)); and nickel or cobalt (enolase-type enzymes (Dai et al.
1999)).

1.4 The 2-His-1-carboxylate structural motif

Although three different families of extradiol-type dioxygenases have been identified
to date, they all contain a 2-His-1-carboxylate structural motif (Hegg and Que 1997, Que
2000). Interestingly, this structural motif is found in many unrelated non-heme Fe(II)
enzymes, illustrating its importance in providing a basis for the catalysis of a variety of
reactions. The active site of these enzymes contains an Fe(II) ligated by two histidines and
one carboxylate all located on one face of the Fe(II) coordination sphere. This motif
therefore provides up to three sites to ligate solvent species or substrate molecules on the
other side of the coordination sphere. In addition to the three families of extradiol-type
dioxygenases mentioned above, at least three additional families of enzymes have been
identified to utilise this metal ion site. These families are: ring-hydroxylating dioxygenases, pterin-dependent hydroxylases, and \( \alpha \)-ketoglutarate dependent enzymes and related enzymes that do not need \( \alpha \)-ketoglutarate as a cosubstrate. Ring-hydroxylating dioxygenases, exemplified by naphthalene and biphenyl dioxygenases (Kauppi et al. 1998, Imbeault et al. 2000), catalyze the NADH-mediated cis-dihydroxylation of an arene double bond yielding a cis-diol. The Fe(II) of these enzymes has two available sites as it is ligated by the two oxygens of the carboxylate group. Pterin-dependent hydroxylases, such as mamalian tyrosine hydroxylase (Flatmark and Stevens 1999), use tetrahydrobiopterin as a cofactor to hydroxylate the ring of aromatic amino acids residue in the synthesis of brain signalling agents. Examples of \( \alpha \)-ketoglutarate-dependent enzymes and related enzymes include enzymes involved in the synthesis of \( \beta \)-lactam antibiotics. Among these enzymes, isopenicillin N synthase (Roach et al. 1997) requires no cofactor, whereas deacetoxycephalosporin C synthase (Valegard et al. 1998) and clavaminic acid synthase (Zhang et al. 2000) both require \( \alpha \)-ketoglutarate as a cosubstrate to facilitate their respective reactions. Another member of this family that does not require \( \alpha \)-ketoglutarate as a cosubstrate is 1-aminocyclopropane-1-carboxylic acid oxidase (Hamilton et al. 1990), an enzyme involved in the formation of ethylene, a plant signalling molecule, that requires ascorbate as a cofactor and \( \text{CO}_2 \) as an activator for continuous turnover. Interestingly, the enzymes in this family have recently been suggested to be members of the cupin superfamily based on their similar distorted jelly roll \( \beta \)-barrel structure (Hewitson et al. 2002).

The remarkable range of reaction catalyzed by this type of Fe(II) centre can be explained by the mechanism of these enzymes that always involves binding of oxygen atoms to the open sites of the Fe(II) centre. This motif can be seen as the counterpart of the heme cofactor where only one site is available for endogenous ligand compared to three for this motif. The close proximity of the three open sites allows the juxtaposition of the two reactants to promote catalysis.
In addition to the enzymes described here, iron superoxide dismutase (Lah et al. 1995) and lipoxygenase (Minor et al. 1996) also have this structural motif. However, they have an additional histidine ligand, which alters the role of the iron as it shuttles between the Fe(III) and Fe(II) oxidation states during catalysis. The contrast between these two enzymes and the Fe(II) oxygen activating enzymes shows the flexibility of the 2-His 1-carboxylate motif.

### 1.5 Structural aspects of extradiol dioxygenases

Several crystal structures of Fe(II)-dependent extradiol and extradiol-type dioxygenases are now available in the ferrous (active) form for DHBD<sub>LB400</sub> (Han et al. 1995b), DHBD from *Pseudomonas* sp. KKS102 (DHBD<sub>KKS102</sub>; Uragami et al. 2001), C23O from *P. putida* mt-2 (Kita et al. 1999) and in the ferric (inactive) form for DHBD<sub>KKS102</sub> (Senda et al. 1996), 4,5-PCD from *Sphingomonas paucimobilis* SYK-6 (Sugimoto et al. 1999) and human HGO (Titus et al. 2000). Even though the structures are from members of the three families of extradiol and extradiol-type dioxygenases, they all share similar active sites and all have the same iron ligands, 2 histidines and one glutamate, that constitute the 2-His 1-carboxylate structural motif. Several other conserved residues identified through sequence alignments of each type of dioxygenases were observed at their respective active sites (Han et al. 1995b, Sugimoto et al. 1999, Titus et al. 2000). Further investigation is required to probe the respective roles of these residues in the catalytic mechanism.

The crystal structures of the ferric form of extradiol and extradiol-type dioxygenases will not be described in detail as these are of inactive enzymes. The ferrous structure of C23O from *P. putida* mt-2 was solved at a resolution of 2.8 Å, therefore details of the active site were not extremely well resolved. Only the crystal structures of DHBD<sub>LB400</sub> and DHBD<sub>KKS102</sub> were solved at resolutions of 2.0 Å or less. Not surprisingly, the crystal structures of DHBD<sub>LB400</sub> and DHBD<sub>KKS102</sub> are very similar as their amino acid sequences share 66% identity and 80% similarity. The following detailed structural description is
based on the crystal structure of DHBD\textsubscript{LB400} as it is one of the enzymes under study during this Ph.D. but differences with the structure of ferrous DHBD\textsubscript{KS102} will be mentioned.

The crystal structure of substrate-free DHBD\textsubscript{LB400} was determined at a resolution of 1.9 Å (Han 1995a, Han et al. 1995b). DHBD\textsubscript{LB400} was shown to be a homooctamer of 32.5 kDa subunits, thus confirming the results of a previous electron micrograph study (Eltis et al. 1993). Each monomer comprises one chain of 297 residues, the N-terminal methionine being excised. Each monomer possesses two domains of very similar structure (Figure 5), the ferrous iron located in the C-terminal domain (C-domain). Each domain is made-up of two $\beta\alpha\beta\beta\beta$ modules as described in section 1.3. A large, funnel-shaped space lies entirely within the domain where the active site ferrous iron is ligated deep within this space in the C-domain. The C-domain possesses two additional $\beta$-strands after the common core structure and the central funnel is slightly larger than that of the N-domain. Therefore, evolutionary adaptation of the two-domain enzymes seems to have resulted in the loss of a second active site within the N-domain.

Figure 5. 3D structure schematic representation of the monomer of DHBD\textsubscript{LB400}. The course of the polypeptide backbone is colour-ramped from blue (N-terminus) to red and the ferrous ion is plotted as a magenta sphere (from Bolin and Eltis 2001).
The ferrous iron active site is located midway in the 20 Å long funnel of the C-terminal domain. This funnel is opened at both ends, the large opening is 10 Å wide and the smaller opening is 6 Å wide. Thus the iron is probably only accessible to catecholic substrates from the wide opening, but water or \( \text{O}_2 \) can have access to the iron through both ends. The coordination geometry of the iron is that of a well-defined square pyramid, with His146 as the axial ligand, and His210, Glu260 and two waters as equatorial ligands in the basal plane (Figure 6, Han 1995a, Han et al. 1995b).

**Figure 6.** Structure of the active site of DHBD\(_{1, B400}\). Carbon, oxygen, nitrogen, and iron atoms are coloured orange, red, cyan, and green, respectively. Hydrogen bonds are indicated by yellow dotted lines (adapted from Han et al. 1995b; figure made using PyMol (DeLano 2002)).

The crystal structures of the DHBD\(_{1, B400}\) complexes formed with DHB, 3-methylcatechol and catechol were also solved and refined against diffraction data extending to 2.0, 1.9, and 1.9 Å resolution, respectively (Han 1995a, Vaillancourt et al. 1998, Vaillancourt et al. 2002b). The substrate was shown to bind to the iron inside the funnel-shaped cavity. The mode of catecholic substrate binding is similar in all three complexes. One hydroxyl group of the substrate binds in the site trans to His146, whereas the other binds trans to His210, displacing the two ordered water ligands (Figure 7). Interestingly, a hydrogen bonded water bridges Asp244 and the 3-hydroxyl group. A water also remains in a position between His195 and the Fe, but the Fe-water distance increases from 2.1 to 2.4 Å,
whereas the distance to Ne2 of His195, 2.7 Å, does not change. This water site overlaps the likely site for O2 binding and is observed at lower apparent occupancies in structures obtained from crystals prepared by different protocols (Vaillancourt et al. 2002b). In contrast, this water site is not occupied in the structure of the enzyme:DHB complex of ferrous DHBD\textsubscript{KKS102}, and a hydrogen bond of length 3.1 Å is inferred between the substrates 3-hydroxyl and Ne2 of the equivalent His residue (Uragami et al. 2001). Such a hydrogen bond is less consistent with the structure of DHBD\textsubscript{L84G} inasmuch as the corresponding distance is 3.5 Å and the donor-acceptor geometry is less appropriate. However, minor fluctuations in structure and loss of the water could allow such an interaction to form.

**Figure 7.** Hydrogen bonding in the active site of the DHBD\textsubscript{L84G}:DHB complex. Carbon atoms are coloured orange in protein residues and yellow in DHB. Oxygen, nitrogen, and iron atoms are coloured red, cyan, and green, respectively. Fe-ligand bonds are indicated by grey sticks, and hydrogen bonds are indicated by green dotted lines (from Vaillancourt et al. 2002b).

The geometry of the substrate-free structure is clearly square pyramidal. In the enzyme:DHB complex, the geometric features allow only equivocal classification. One possible description is that of a distorted octahedron, with both the 3-OH group of the substrate and the water near His195 included as weak ligands. Prior crystallographic and
spectroscopic studies of homologues (Mabrouk et al. 1991, Uragami et al. 2001), suggest a
five-coordinate site with the three protein side chains and the two hydroxyl groups of the
substrate as ligands. A recent study on DHBĐLB400 (Davis et al. 2002) also found a five
coordinate site in the different enzyme:substrate complexes. For this five coordinate ligand
set, the geometry observed is intermediate between square planar and trigonal bipyramidal,
but strongly biased toward the former. This was demonstrated by Dr. Jeffrey Bolin
(Vaillancourt et al. 2002b) by calculation of the parameter τ, which has been used in the
context of inorganic complexes to characterize five-coordinate systems (Addison et al.
1984). τ is defined by the equation \( \tau = (\beta - \alpha)/60 \), where \( \beta \) and \( \alpha \) are the largest and second
largest ligand-metal ion-ligand angles. For an ideal trigonal bipyramid \( \tau = 1 \), whereas \( \tau = 0 \)
for a square pyramid. For the free-enzyme, \( \tau = 0.05 \), whereas \( \tau = 0.25 \) for the
DHBĐLB400:DHB complex, as compared to 0.27 for the DHB complex of ferrous
DHBĐKKS102 (Uragami et al. 2001).

1.6 Mechanism of extradiol dioxygenases

As described in section 1.3, sequence and structural data indicate the existence of at
least two evolutionarily independent types of extradiol dioxygenases (Eltis and Bolin 1996,
Sugimoto et al. 1999). 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

In the first step of the proposed mechanism (Figure 8), bidentate binding of the
catecholic substrate displaces the two solvent ligands of the ferrous iron (Mabrouk et al.
1991, Arciero et al. 1985, Arciero et al. 1986, Uragami et al. 2001, Han 1995a) and
activates the latter for \( \text{O}_2 \) binding (Mabrouk et al. 1991, Arciero et al. 1985, Shu et al.
1995). Subsequent steps in the catalytic mechanism are less well substantiated.
Biochemical studies provide some support for a mechanism involving iron-mediated transfer
of an electron from the catechol to the \( \text{O}_2 \), yielding a semiquinone-Fe(II)-superoxide
intermediate (Spence et al. 1996b). This species is proposed to react to give an iron-alkylperoxo intermediate (Winfield et al. 2000), which undergoes alkenyl migration, Criegee rearrangement and O-O bond cleavage to give an unsaturated lactone intermediate and an Fe(II)-bound hydroxide ion. The latter hydrolyses the lactone to yield the reaction product (Sanvoisin et al. 1995).

Figure 8. Proposed mechanism of extradiol dioxygenases (adapted from Shu et al. 1995, Bugg and Lin 2001). For clarity, the displacement of solvent species from the ferrous centre is not depicted explicitly.

1.7 Structural and mechanistic comparison of extradiol and intradiol dioxygenases

The intradiol family of dioxygenases include protocatechuate 3,4-dioxygenase (3,4-PCD), which cleave hydroxybenzoates, and catechol 1,2-dioxygenase (C12O), which cleave catechols and hydroxyquinols. The ferric iron of substrate-free intradiol dioxygenase has a distorted trigonal bipyramid geometry, with a tyrosine, a histidine and a solvent species coordinated in the equatorial plane and a tyrosine and a histidine coordinated in the axial positions (Figure 9, panel A; Ohlendorf et al. 1988). As in extradiol dioxygenases, the intradiol enzymes utilize an ordered mechanism in which substrate binding precedes O$_2$ reactivity (Hori et al. 1973, Bull et al. 1981, Walsh et al. 1983). Substrate-binding is a multi-step process that ultimately results in displacement of an axial tyrosine and an
equatorial hydroxide ion to yield a bidentate bound catecholate (Figure 9, panel B; True et al. 1990, Orville et al. 1997b, Frazee et al. 1998, Vetting et al. 2000a, Vetting et al. 2000b).

In the proposed mechanism (Figure 10), O₂ attacks the bound substrate directly, before coordinating to the iron and yielding an iron-alkylperoxo intermediate (Que et al. 1977). Although recent evidence indicates that this intermediate is similar in structure to that of the extradiol reaction (Winfield et al. 2000), in the case of intradiol enzymes, the Criegee rearrangement and O-O bond cleavage involve acyl migration to yield the cyclic anhydride and an iron-bound oxide or hydroxide. The latter functions as a nucleophile to hydrolyse the anhydride and yield the ring-opened product. The stereo-electronic factors that determine extradiol versus intradiol cleavage from the common intermediate have been proposed to involve the orientation of the iron-alkylperoxo moiety relative to the organic substrate. In particular, the extradiol dioxygenases are proposed to form a pseudo-axial iron-alkylperoxo species that would favour alkenyl migration and the intradiol dioxygenases are proposed to form a pseudo-equatorial iron-alkylperoxo species that would favour acyl migration (Bugg and Lin 2001).

Figure 9. Structure of the active site of substrate-free (A) and protocatechuate-bound (B) 3,4-PCD. Carbon atoms are coloured orange in amino acids and yellow in protocatechuate. Oxygen, nitrogen, and iron atoms are coloured red, cyan, and brown, respectively. (adapted from Ohlendorf et al. 1994 and Orville et al. 1997b; figure made using PyMol (DeLano 2002)).
An important difference in the initial stages of the proposed extradiol and intradiol mechanisms is the protonation state of the bidentate-bound catechol in the enzyme:substrate complex (Que and Ho 1996). Thus, in extradiol dioxygenases, a monoanionic Fe(II)-bound catecholate activates the ferrous centre for $O_2$-binding (Shu et al. 1995). By contrast, in intradiol dioxygenases, a dianionic Fe(III)-bound catecholate promotes direct electrophilic attack of the substrate by $O_2$, a reaction that is further favoured by ketonization of the catecholate (Que et al. 1977). Despite the proposed significance of the different protonation states of the substrate in the two enzymes, the evidence for these respective states is not definitive. Thus, structural and EXAFS data demonstrate that in both enzymes, the substrate is asymmetrically bound: one Fe-O bond is shorter than the other. This has been interpreted as a ketonized dianion in the case of intradiol dioxygenases (Orville et al. 1997b, Vetting et al. 2000a, Elgren et al. 1997) and as a monoanion in the case of extradiol dioxygenases (Han 1995a, Shu et al. 1995, Uragami et al. 2001). Electronic absorption and resonance Raman data supporting these interpretations were obtained using poor substrates or inhibitors (Tyson 1975, Que and Epstein 1981, Elgren et al. 1997) which may not bind in the same
manner as preferred substrates. Additional evidence for monoanionic binding in extradiol dioxygenases is provided by a model reaction using a ferrous complex of 1,4,7-triazacyclononane in which extradiol cleavage apparently required a monoanionic catecholate substrate (Lin et al. 2001).

1.8 Inactivation of extradiol dioxygenases

Mechanism-based inactivation has long been recognized in the cleavage of the aromatic substrate of extradiol-type dioxygenases (Mitchell et al. 1963, Klecka and Gibson 1981). This phenomenon has been studied in the xylE-encoded C23O of P. putida mt-2 of the TOL pathway and in mammalian HAD. 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 (Klecka and Gibson 1981) or irreversible covalent modification by an acyl chloride species generated by the ring cleavage reaction (Battels et al. 1984). 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 (Cerdan et al. 1994). Indeed, several pathways have recruited a 2Fe-2S ferredoxin to maintain the dioxygenase’s active site iron in the reduced state (Polissi and Harayama 1993, Hugo et al. 2000, Tropel et al. 2002). It has also been proposed that the inactivation of C23O by 3-methylcatechol involves alternate binding modes of the catecholic substrate (Cerdan et al. 1995). An early report suggested that the mechanism-based inactivation of HAD also involves oxidation of the active site Fe(II) (Mitchell et al. 1963). However, this was refuted in a subsequent study (Koontz and Shiman 1976). Interestingly, a halogenated substrate analogue, 4-chloro-3-hydroxyanthranilate, had been suggested to inhibit HAD via covalent modification by an acyl halide (Parli et al. 1980), although it was subsequently shown that this analogue inhibits the enzyme reversibly in vivo (Walsh et al. 1994). Clearly, many aspects of the inactivation of extradiol-type
dioxygenases and the relationship of this inactivation to productive catalysis remain to be clarified.

1.9 Stabilization and inhibition of extradiol dioxygenases by organic additives

The stability of a number of extradiol dioxygenase preparations has been improved through the inclusion of organic additives in solutions of the enzymes (Nozaki et al. 1963, Eltis et al. 1993, Kobayashi et al. 1995). Acetone, a good stabilizing agent of C23O from \textit{P. putida} mt-2, was also used in crystallographic studies to stabilize the enzyme (Kita et al. 1999). It was found that the acetone molecule could occupy the active site close to the catalytic iron centre. In good agreement with the binding at the active site, acetone was found to inhibit the cleavage of catechol (Bertini et al. 1994, Kobayashi et al. 1995). It was even proposed that acetone was directly binding to the iron (Bertini et al. 1994, Kita et al. 1999). However, direct binding to the iron still remains to be clarified as the precision of the respective experiments was limited.

Organic additives were also used in the purification of DHBD_{LB400} but even with this precaution, the best reported preparations of DHBD_{LB400} contain at most 50\% of their complement of active site Fe(II) (Eltis et al. 1993). This variability in preparations of extradiol dioxygenases complicates spectroscopic studies and the determination of steady-state kinetic parameters. For example, the \( k_{\text{cat}} \) of the \textit{xylE}-encoded C23O of \textit{P. putida} mt-2 has been variously reported as 930 \( \text{s}^{-1} \) (100 mM phosphate, pH 7.5, 25°C; Cerdan et al. 1995) and 278 \( \text{s}^{-1} \) (50 mM phosphate, pH 7.5, 25°C; Kobayashi et al. 1995).

1.10 Aim of this study

With the development of structural data, DHBD_{LB400} 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 first goal of this project was to improve the techniques for purifying and manipulating
DHBD_{LB400} and to perform steady-state kinetic studies with the highly active preparation 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 DHB, 3-methylcatechol and catechol complexes.

Another goal of this project was to study the inactivation of DHBD_{LB400} by different catecholic substrates, including 3-chlorocatechol. An experimental design based on the theoretical approach of Duggleby (Duggleby 1986) and the steady-state mechanism of the enzyme 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.

As described in section 1.2, the specificity of DHBDs for different chlorinated DHBs is not well characterized. For this reason, the reactivity of three evolutionarily divergent DHBDs (DHBD_{LB400}, DHBD_{P6-I} and DHBD_{P6-III}) towards each of the six monochlorinated DHBs was investigated. The two organisms from which these three isozymes originate have different PCB-degrading characteristics. Steady-state kinetic studies were conducted to determine the specificity of the enzyme for the DHBs and O_2. These results led to the study of the reactivity of DHBDs with one dichlorinated DHB. In addition, the suicide inhibition of the enzyme by each of these compounds was investigated, and the physiological significance of this inhibition was investigated with one of the organisms. The results are discussed in term of the crystallographic structures of the 2'-Cl DHB:DHBD_{LB400} and 2',6'-diCl DHB:DHBD_{LB400} binary complexes.

As described in section 1.7, an important difference in the initial stages of the proposed extradiol and intradiol mechanisms is the protonation state of the bidentate-bound
catechol in the enzyme:substrate complex. However, no direct evidence for the anionic state of a good substrate with either type of enzymes is available. For this reason, the binary complexes \( \text{DHBD}_{\text{LB400}}:\text{DHB} \) and \( \text{DHBD}_{\text{LB400}}:3\text{-nitrocatechol} \) were studied using UV/Vis absorption spectroscopy. Spectra of the enzyme:substrate (ES) complexes were analyzed through comparison with the spectra of free DHB and free 3-nitrocatechol in their three different ionization states. The spectroscopic results are discussed in terms of the proposed catalytic mechanism of extradiol dioxygenases and of the crystal structure of the ES complex with DHB.
2. MATERIALS AND METHODS

2.1 Chemicals

Catechol, 3-methylcatechol, protocatechuate, biphenyl, sodium methoxide, sodium t-butoxide, sodium hydrosulfite, t-butanol, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), xanthine, xanthine oxidase (EC 1.1.3.22) from buttermilk, and bovine hepatic catalase (EC 1.11.1.6) were from Sigma-Aldrich (Mississauga, Ontario). 3-Methylcatechol was further purified by sublimation and DMPO was further purified as previously described (Witting et al. 2000). DHB, 4-Cl DHB, 5-Cl DHB, 6-Cl DHB, 2'-Cl DHB, 3'-Cl DHB, 4'-Cl DHB, 2',6'-diCl DHB, 2,6-diCl biphenyl, 3-ethylcatechol, 3-chlorocatechol and 3-nitrocatechol were synthesized according to standard procedures (Nerdinger et al. 1999, Nerdinger et al. 2002) and were gifts from Victor Snieckus (Department of Chemistry, Queens University, Kingston, Ontario). 2-Pyrone-6-carboxylic acid was a gift from Walter Reineke (Chemische Mikrobiologie, Bergische Universität, Wuppertal, Germany). Ferene S and bovine erythrocytic 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)-2//-tetrazolium-5-carboxanilide (XTT) were purchased from Molecular Probes Inc. (Eugene, OR). 2-Hydroxymuconic acid was prepared by the alkaline hydrolysis of 2-pyrone-6-carboxylic acid (Kaschabek et al. 1998). HOPDA was prepared enzymatically from DHB (Seah et al. 2000). All other chemicals were of analytical grade and used without further purification.

2.2 Bacterial strains, media and growth conditions

DHBD_{LB400} was overexpressed in Pseudomonas putida KT2442 (Herrero et al. 1990) freshly transformed with pLEBD4, a broad host range expression plasmid containing the structural gene encoding the dioxygenase, as previously described (de Lorenzo et al. 1993). For DHBD_{LB400} expression, the strain was grown in Luria Broth containing a potassium phosphate buffer described for Terrific Broth (Ausubel et al. 2000) and supplemented (10 ml per litre) with an HCl-solubilized solution of minerals containing 14.1 mM MgCl₂·6H₂O,
2.5 mM CaCO₃, 4.27 mM FeSO₄·7H₂O, 0.625 mM ZnSO₄·7H₂O, 0.625 mM MnSO₄·H₂O, 0.125 mM CuSO₄·5H₂O, 0.125 mM CoCl₂·6H₂O, 0.125 mM H₃BO₃, 262.5 mM MgSO₄, 10 mM CaCl₂, and 0.1 mM thiamine. Cultures were grown at 30°C and 250 rpm. One litre of media in a 2-litre flask was inoculated with 4 ml of an overnight culture. When the O.D. at 600 nm of the culture reached 0.5, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.25 mM and the culture was incubated for an additional 18 hours before harvesting. *Burkholderia* sp. LB400 was cultured at 30°C and 200 rpm in M9 minimal media (Ausubel *et al.* 2000) supplemented with the HCl-solubilized solution of minerals that did not contain thiamine and CaCl₂ with 2% biphenyl 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. DHBD₉-I and DHBD₉-III were overexpressed and purified according to established protocols (Vaillancourt *et al.* 2002c).

### 2.3 Protein analysis

SDS-PAGE was performed on a Bio-Rad MiniPROTEAN II apparatus and stained with Coomassie Blue according to established procedures (Ausubel *et al.* 2000). Protein concentrations were determined by the Bradford method (Bradford 1976). Iron concentrations were determined colorimetrically using Ferene S (Haigler and Gibson 1990).

### 2.4 Purification of DHBD₉-LB400

The protein was purified following a modified protocol (Eltis *et al.* 1993). After disruption of the cells, all procedures were performed under an inert atmosphere unless otherwise specified. Chromatography was performed on an ÄKTA Explorer (Amersham Biosciences, Montreal, Quebec). This instrument was installed next to an MBraun Unilab glovebox (MBraun, Stratham, NH) equipped with 1/16" and 3/8" Swagelok feedthroughs as well as a potted RS232 feedthrough. In this configuration, anaerobic buffers could be delivered to the ÄKTA Explorer and the column eluate could be directed to a fraction collector in the glovebox. Buffers were made from water purified on a Barnstead
NANOpure UV apparatus to a resistivity of greater than 17 MΩ•cm. Buffer A was 10 mM Tris, pH 7.5 containing 10% 2-butanol, 2 mM dithiothreitol (DTT) and 0.25 mM ferrous ammonium sulphate. Buffer B was buffer A containing 1.0 M NaCl. Buffer C was 10 mM Tris, pH 7.5 containing 10% 2-butanol, 150 mM NaCl, 2 mM DTT and 0.25 mM ferrous ammonium sulphate. Buffers were sparged with N₂ and equilibrated in the glovebox for at least 24 hours prior to the addition of 2-butanol, DTT and ferrous ammonium sulphate.

Cells from 3 L of culture were harvested by centrifugation. The cell pellet was resuspended in 15 ml of 10 mM Tris, pH 8.0 containing 20% 2-butanol, 1 mM MgCl₂, 1 mM CaCl₂ and 0.1 mg/ml DNase I. The cells were disrupted by two successive passages through a French Press (Spectronic Instruments Inc., Rochester, NY) using an operating pressure of 20,000 psi. The cell debris was removed by ultracentrifugation in gas-tight tubes at 170,000 × g for 60 minutes. The clear supernatant fluid was carefully decanted and referred to as the raw extract.

The raw extract (approximately 20 ml) was divided into four equal portions, each of which was loaded onto a HR10/10 Mono Q anion exchange column equilibrated with buffer A. The column was operated at a flow rate of 3.0 ml/min. The enzyme activity was eluted with a 66 ml linear gradient of 8 to 19% buffer B. Fractions of 2.5 ml were collected. Fractions containing activity from the four runs were concentrated to 4 ml with a stirred cell concentrator equipped with a YM10 membrane (Amicon, Oakville, Ontario) and loaded onto a HiLoad 26/60 Superdex 200HR column equilibrated with buffer C. The column was eluted with buffer C at a flow rate of 2.0 ml/min. Fractions (5 ml) exhibiting activities of more than 1500 U/ml were combined, concentrated to 25 to 35 mg/ml protein, and flash frozen as beads in liquid N₂. Purified DHBDL400 was stored in buffer C at -80°C for several months without significant loss of activity.

For protein samples that were to be used in crystallographic and magnetic circular dichroism spectroscopy studies, hydrophobic chromatography was performed between the
anion-exchange and gel filtration steps to remove minor contaminants. This additional step was performed using a Source-15-phenyl column (Amersham Biosciences; 2 X 9 cm) equilibrated with buffer D (10 mM potassium phosphate, pH 7.0, 2 mM DTT) containing 0.5 M (NH₄)₂SO₄. Prior to loading, the protein sample was exchanged into this buffer using an Amicon ultrafiltration cell (Millipore Corporation, Bedford, MA). The column was operated at a flow rate of 15 ml/min, and DHBD_{LB400} eluted at approximately 0.3 M (NH₄)₂SO₄ using a linear gradient of 0.5 to 0 M (NH₄)₂SO₄ in 210 ml. The protein sample was exchanged into 10 mM Tris, 150 mM NaCl, pH 7.5 and the standard procedure on Superdex column was performed in buffer C. Using this procedure, preparations had high specific activity (400-500 U/mg).

2.5 Handling of DHBD samples

Buffers were prepared using water purified on a Barnstead NANOpure UV apparatus to a resistivity of greater than 17 MΩ·cm. All manipulations involving DHBDs were performed under an inert atmosphere unless otherwise specified, usually in a Mbraun Unilab glovebox (Stratham, NH) maintained at 2 ppm O₂ or less. Aliquots of DHBDs were thawed immediately prior to use and were exchanged into 20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (HEPPS), 80 mM NaCl (I = 0.1), pH 8.0 by gel filtration chromatography to remove exogenous iron unless otherwise stated. Typically, 100 to 200 µl of purified DHBD was thawed in the glovebox and applied to a 0.7 cm x 5 cm column of Biogel P6 DG (BioRad, Mississauga, Ontario) equilibrated with the appropriate buffer. Samples of DHBD were further diluted using the same buffer as required.

2.6 Stabilization of DHBD_{LB400} samples

The ability of different anaerobic buffers to stabilize the DHB-cleaving activity of DHBD_{LB400} was investigated by monitoring the activity of a 25 to 50 µg/ml solution of the enzyme over 24 hours. Parameters that were varied include pH and the concentrations of
ferrous ammonium sulphate, DTT, and BSA. Acetone, ethanol, glycerol, isopropanol, and t-
butanol were also tested for their ability to stabilize the activity of DHBDLB.

2.7 Oxygen electrode assay

Enzymatic activity was measured by following the consumption of O₂ using a Clark-
type polarographic O₂ electrode (Yellow Springs Instruments Model 5301, Yellow Springs, OH). Reactions were performed in a thermojacketted Cameron Instrument Company 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 and recorded on a microcomputer 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 each consecutive 6 s intervals using the full set of 61 data points collected over that interval. The calculated slopes used for further calculation had correlation coefficients of at least 0.998.

Reaction buffers containing different concentrations of dissolved O₂ were prepared by vigorously bubbling them with humidified mixtures of O₂ and N₂ gases for at least 15 minutes 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. During this operation, the reaction chamber was flushed continuously with the humidified gas mixture. Ultra high purity O₂ (100% O₂, or 5-10% O₂ in N₂) and prepurified N₂ 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 x 10 cm column of water. The concentration of dissolved O₂ in the reaction mixture was verified using the O₂ electrode.

The standard activity assay was performed in a total volume of 1.3-1.45 ml of air-
saturated 20 mM HEPPS, 80 mM NaCl (I = 0.1 M), pH 8.0, 25.0 ± 0.1°C (290 μM dissolved
O₂) containing 80 µM DHB. The reaction was initiated by injecting between 2 and 10 µl of an appropriate dilution of enzyme preparation into the reaction chamber. Buffers were used within 24 hours of preparation. Stock solutions were prepared fresh daily and stored under argon on ice. On each day kinetic assays were performed, the zero of the O₂ electrode was established by adding an excess of sodium hydrosulfite to the buffer in the reaction chamber. The O₂ electrode was calibrated using standard concentrations of 3,4-PCD and protocatechuate (Whittaker et al. 1990) or DHB and an excess of DHBD (DHBDLB400 or DHBDp6-I or DHBDp6-III). One unit of enzymatic activity was defined as the quantity of enzyme required to consume 1 µmol of O₂ per minute.

2.8 DHBD spectrophotometric assay

Enzymatic activity was measured spectrophotometrically when a rapid determination of activity with DHB was needed and in inactivation experiments where progress curves were integrated. The spectrophotometric assay was also used to follow the activity of DHBDs with 2',6'-diCl DHB. The activity was measured spectrophotometrically by following the rate of appearance of the yellow-coloured ring-cleaved product with a Varian Cary 1E spectrophotometer equipped with a thermojacketed cuvette holder (Varian Canada, Mississauga, Ontario) and interfaced to a microcomputer equipped with Cary WinUV software version 2.00. Initial velocities were determined from progress curves by analyzing the data using the Cary WinUV software. The slope of the progress curve and the correlation coefficient of the slope were calculated for the first 6 s of the assay. Experiments involving catechol, 3-methylcatechol and DHB were performed in 20 mM HEPPS, 80 mM NaCl (I = 0.1 M), pH 8.0, 25.0 ± 0.1°C and monitored at 376 nm, 389 nm, and 434 nm, respectively, using molar extinction coefficients of 38.1 mM⁻¹cm⁻¹, 22.0 mM⁻¹cm⁻¹ and 25.7 mM⁻¹cm⁻¹ for the corresponding ring-cleaved products. Experiments involving 2',6'-diCl DHB were performed in potassium phosphate buffer pH 7.0, (I = 0.1), and monitored at 391 nm using a molar extinction coefficient of 36.5 mM⁻¹cm⁻¹. The molar extinction coefficients were determined as described previously (Seah et al. 1998). The standard activity assay was
performed in a total volume of 1.0 mL of air-saturated 20 mM HEPPS, 80 mM NaCl (I = 0.1 M), pH 8.0, 25.0 ± 0.1°C containing 80 μM DHB. All reactions were initiated by injecting between 2 and 10 μl of an appropriate dilution of enzyme preparation into the cuvette.

2.9 Details of steady-state kinetic experiment

In all experiments, 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.

2.9.1 Coupling and specificity experiments

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 coupling experiments, whereas a limiting amount of DHBD was used in specificity experiments.

In specificity experiments with catechol, 3-methylcatechol, 3-ethylcatechol, DHB and monochlorinated DHBs, concentrations of the aromatic substrates were varied from 0.2 times the determined $K_m$ to the maximum concentration possible without suicide inhibition affecting the initial velocities. When the specificity for O₂ was investigated, dissolved O₂ concentrations were varied from approximately 60 to 1300 μM. For specificity experiments in which only the concentration of O₂ was varied at a fixed concentration of catecholic substrate, an excess of catecholic substrate (≥ 6 X $K_{ma}^{app}$) was used.

Using the spectrophotometric assay, the apparent catalytic constant, $k_{cat}^{app}$, for 2',6'-diCl DHB could be determined for all three DHBDs. The $K_{ma}^{app}$ of DHBD$_{po-III}$ for 2',6'-diCl DHB was also determined using this assay.
2.9.2 Inhibition and reporter substrate experiments

Inhibition experiments of DHBD\textsubscript{LB400} with \textit{t}-butanol, isopropanol, acetone, ethanol and glycerol were performed in air-saturated buffer using concentrations of the inhibitor up to 3 M unless otherwise noted. In these experiments, the catecholic substrate was used at concentrations below those at which substrate inhibition or suicide inhibition were observed.

Inhibition experiments of DHBD\textsubscript{LB400} with HOPDA were performed using air-saturated buffer. The concentration of DHB was varied from 5 \( \mu \text{M} \) to 85 \( \mu \text{M} \) and the concentration of HOPDA was varied from 240 \( \mu \text{M} \) to 4.8 mM.

The steady-state cleavage of 3-chlorocatechol and 3-nitrocatechol by DHBD\textsubscript{LB400} was studied using DHB as a reporter substrate. The concentration of DHB was varied from 5 \( \mu \text{M} \) to \( \sim 90 \mu \text{M} \) (\textit{i.e.} at concentrations below those at which substrate inhibition is observed) and the concentrations of 3-chlorocatechol and 3-nitrocatechol were varied from 2.4 to 7.6 \( \mu \text{M} \) and from 11 to 93 \( \mu \text{M} \), respectively (\textit{i.e.} \( \sim 0.5 \) times the apparent \( K_m \) to the maximum concentration possible without enzyme inactivation affecting the initial velocities).

The steady-state cleavage of 2',6'-diCl DHB for DHBD\textsubscript{LB400} and DHBD\textsubscript{P6-I} was also studied using DHB as a reporter substrate in the oxygen electrode assay using the same buffer as the spectrophotometric assay. The concentration of DHB was varied from 3-5 \( \mu \text{M} \) to 85 \( \mu \text{M} \) (\textit{i.e.} at concentrations below those at which substrate inhibition is observed) and the concentration of 2',6'-diCl DHB was varied from 2.1 to 20.9 nM for DHBD\textsubscript{LB400} and from 0.02 to 1.3 \( \mu \text{M} \) for DHBD\textsubscript{P6-I}.

2.10 Analysis of steady-state kinetic data

Steady-state rate equations to determine specificity and inhibition parameters were fitted to data using the least squares and dynamic weighting options of LEONORA (Cornish-Bowden 1995).
2.10.1 Kinetic mechanism

C23O utilizes a compulsory-order ternary-complex mechanism in which the binding of catecholic substrate, \( A \), precedes that of \( O_2 \) (Hori \textit{et al}. 1973). \( \text{DHBD}_{LB400}, \text{DHBD}_{P6-I} \) and \( \text{DHBD}_{P6-III} \) utilize essentially the same mechanism. However, \( \text{DHBD}_{LB400} \) and \( \text{DHBD}_{P6-I} \) are subject to substrate inhibition at high concentrations of catecholic substrate. A mechanism accounting for these observations is shown in Figure 11.

\[
\begin{align*}
\text{AEA} & \\
\text{E} + \text{A} + \text{O}_2 & \xrightleftharpoons[k_1]{k_4} \text{EA} + \text{O}_2 \\
\text{EA} + \text{O}_2 & \xrightarrow[k_2]{k_3} \text{EAO}_2 \\
\text{EAO}_2 & \xrightarrow[k_4]{k_3} \text{E} + \text{P}
\end{align*}
\]

\textbf{Figure 11.} Compulsory-order ternary-complex mechanism with substrate inhibition

The steady-state rate equation derived from this mechanism using the King-Altman method (King and Altman 1956) is given in Equation 1.

\[
v = \frac{v[A][O_2]}{K_{dA}K_{mO2} + K_{mA}[O_2] + K_{mO2}[A] + [A][O_2] + K_{mO2}[A]^2/K_{iA}} \quad \text{Equation 1}
\]

In Equation 1, \( K_{mA} \) represents the \( K_m \) for the catecholic substrate; \( K_{mO2} \), the \( K_m \) for \( O_2 \); \( K_{dA} \), the dissociation constant for the catecholic substrate; and \( K_{iA} \), the inhibition constant for the catecholic substrate. \( K_{mA} \) and \( K_{mO2} \) 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, \( K_{mA} \) and \( K_{mO2} \) 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 (\textit{e.g.} \( K_{mO2} = (k_{-2} + k_3)/k_2 \)). Furthermore, \( K_{mA} \) and \( K_{mO2} \) are the ratios between \( k_{\text{cat}} \) and the specificity constants \( k_A \) and \( k_{O2} \), respectively (\textit{i.e.} \( k_A = k_{\text{cat}}/K_{mA} \) and \( k_{O2} = k_{\text{cat}}/K_{mO2} \)).
For specificity experiments in which the concentrations of substrates A and O2 were varied, the initial velocities were fitted to Equation 1 or to the equivalent steady-state rate equation describing a compulsory-order ternary-complex mechanism without substrate inhibition. For specificity experiments in which the concentration of only one substrate was varied, the initial velocities obtained were fitted to the Michaelis-Menten equation or to the equivalent equation describing a mechanism in which substrate inhibition occurs (Equation 2).

\[
v = \frac{V[A]}{K_{mA}^{app} + [A] + [A]^2/K_{iA}^{app}} \quad \text{Equation 2}
\]

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

In every cases, the substrate concentration and reaction velocities after the initiation of the reaction (10-20 s) was monitored and compared to the calculated one in order to reject assays involving more than 15% inactivation of the enzyme.

2.10.2 Reversible inhibition and reporter substrate studies

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

\[
v = \frac{V[A]}{K_{mA}^{app}(1 + [I]/K_{ic} + [A](1 + [I]/K_{iu}^{app}))} \quad \text{Equation 3}
\]

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

\[
K_{iu}^{app} = \frac{K_{iu}(K_{mO2} + [O2])}{K_{mO2}} \quad \text{Equation 4}
\]
In studies using DHB as a reporter substrate, an equation identical in form to that for competitive inhibition was fitted to the data (Cornish-Bowden 1995). In this equation, the $K_{m}^{app}$ of 3-chlorocatechol, 3-nitrocatechol, or 2',6'-diCl DHB replaces the competitive inhibition constant, $K_{ic}$.

2.11 Analysis of the inactivation mechanism of DHBD$_{LB400}$

The inactivation of DHBD$_{LB400}$ by different catecholic substrates, including 3-chlorocatechol, was studied. An experimental design based on the theoretical approach of Duggleby (Duggleby 1986) and the steady-state mechanism of the enzyme with possible inactivation at every step (Figure 12) was utilized to investigate which forms of the enzyme are subject to inactivation.

- $E^*$
- $j_5$
- AEA
- $A + \uparrow$
- $E + A + O_2 \leftrightarrow EA + O_2 \leftrightarrow EAO_2 \leftrightarrow EP \leftrightarrow E + P$
- $+ O_2 \downarrow j_1$
- $E^*_1$
- $\downarrow j_2$
- $E^*_2$
- $\downarrow j_3$
- $E^*_3$
- $\downarrow j_4$
- $E^*_4$

**Figure 12.** General mechanism of DHBD$_{LB400}$ inactivation during steady-state turnover. Asterixes denote inactivated forms of the enzyme. The rate constants $j_1$ to $j_5$ are associated with reactions that lead to the formation of inactive enzyme species.

2.11.1 Stability of the different enzyme complexes

The respective stabilities of the EA, AEA and EP complexes were studied by anaerobically incubating DHBD$_{LB400}$ with appropriate amounts of substrates or product, withdrawing aliquots at timed intervals, and determining the remaining DHBD$_{LB400}$ activity.
using the standard assay. In these experiments, a solution of ~1.2 μM DHBD\textsubscript{LB400} was incubated with either 0.08, 0.8 or 6 mM DHB, 5 mM catechol, 2.5 or 5 mM HOPDA.

The stability of the DHBD\textsubscript{LB400}:3-chlorocatechol complex was evaluated by incubating a 150 μM solution of the enzyme under anaerobic conditions in the presence of either 1 mM or 5 mM 3-chlorocatechol for 30 minutes. The EA complex was then desalted in 20 mM HEPPS, 80 mM NaCl pH 8.0 by gel filtration as described in section 2.5 and the remaining DHBD\textsubscript{LB400} activity was determined using the standard assay.

### 2.11.2 Determination of partition ratio and inactivation constants

The inactivation of the free enzyme in the presence of O\textsubscript{2} was studied by incubating DHBDs in the oxygraph cuvette under standard assay conditions, and monitoring \( A_t \), the activity remaining after different time intervals, by adding 80 μM DHB to the cuvette. The apparent first-order rate constant of inactivation, \( j^{\text{app}}_1 \) (Figure 12), was evaluated using equation 5, in which \( A_{\text{max}} \) is the activity observed in the absence of pre-incubation.

\[
A_t = A_{\text{max}} e^{-j^{\text{app}}_1 t} \quad \text{Equation 5}
\]

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 \( K_m \)). For 2′,6′-diCl DHB, the partition ratio was determined spectrophotometrically using the assay described in section 2.8. The amount of DHBD added to the reaction cuvette was such that the enzyme was completely inactivated before 15% of either the catecholic substrate or O\textsubscript{2} was consumed in the reaction mixture. The partition ratio was calculated by dividing the amount of O\textsubscript{2} consumed or product formed to the amount of active DHBD added to the assay (Equation 6). For 3-chlorocatechol, the partition ratio was also calculated from the amount of substrate remaining in an high performance liquid chromatography (HPLC)-based assay (section 2.12). For 3-nitrocatechol, the HPLC-based assay was the only assay in which the partition ratio could be evaluated.
Partition ratio = \frac{\text{No. of substrate molecules consumed}}{\text{No. of enzyme molecules inactivated}} = \frac{k_{\text{cat}}}{\Sigma j_i} \quad \text{Equation 6}

The apparent rate constant of inactivation during catalytic turnover in air-saturated buffer, $j_3^{\text{app}}$ (Figure 12), was evaluated using one of two (or both) different experimental designs. In the first approach, $j_3^{\text{app}}$ was calculated from the partition ratio determined using the oxygraph or the spectrophotometric (only for 2',6'-diCl DHB) assay under saturating substrate conditions ($[S] \gg K_m$). Under such conditions, the concentration of free enzyme, [E], is negligible, and the partition ratio is equal to the ratio of the catalytic constant, $k_{\text{cat}}^{\text{app}}$, and the inactivation constant $j_3^{\text{app}}$ (i.e. $\Sigma j_i = j_3$). The rate constant evaluated using this method was termed $j_{3\text{sc}}^{\text{app}}$.

In the second experimental approach, $j_3^{\text{app}}$ 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, 3-chlorocatechol, and 3-nitrocatechol, the substrate concentration was varied from the determined $K_m^{\text{app}}$ to 5 times the determined $K_m^{\text{app}}$. In the case of DHB and 2'6'-diCl DHB, the substrate concentration was varied from 80 to 250 µM and from 25 to 200 µM respectively.

The rate constant of inactivation at each substrate concentration, $j_s$, was determined by fitting equation 7 (Tudela et al. 1987) to the corresponding progress curve using SCIENTIST version 2.01 (Micromath Scientific Software, Salt Lake City, UT).

\[ P_t = P_\infty (1 - e^{-j_s t}) + P_i \quad \text{Equation 7} \]

In this equation, $P_t$ is the concentration of product recorded at the start of the assay and $P_\infty$ is the concentration of product subsequently generated during the assay. To minimize the effect of substrate depletion 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 $j_{3E}^{app}$. For catechol, 3-methylcatechol, 2',6'-diCl DHB and DHB, $j_{3E}^{app}$ was evaluated from $j_s$ measured at different substrate concentrations, S, using equation 8 (Tudela et al. 1987) in which $K_m^{app}$ is the apparent $K_m$ of the catecholic substrate in air-saturated buffer.

$$j_s = \frac{j_{3E}^{app} [S]}{K_m^{app} + [S]}$$  \hspace{1cm} \text{Equation 8}

For 3-chlorocatechol and 3-nitrocatechol, DHB was used as a reporter substrate and $j_{3E}^{app}$ was obtained using equation 9 (Escribano et al. 1989).

$$j_s = \frac{j_{3E}^{app} [3-CC]}{K_m^{app} (1 + [DHB] / K_m^{DHB}) + [3-CC]}$$  \hspace{1cm} \text{Equation 9}

In this equation, $K_m^{app}$ and $K_m^{DHB}$ are the apparent $K_m$ for 3-chlorocatechol or 3-nitrocatechol, and DHB, respectively, in air-saturated buffer and $j_s$ at each concentration of 3-chlorocatechol or 3-nitrocatechol, and DHB was determined using equation 7.

2.11.3 

\textit{In vitro} inactivation and reactivation

DHBD\textsubscript{LB400} was inactivated \textit{in vitro} using three different methods, each performed at 23°C using 20 mM HEPPS, 80 mM NaCl, pH 8.0. First, DHBD\textsubscript{LB400} was inactivated anaerobically by incubating a 30 μM solution of the protein with 5 mM 1,10-phenanthroline for 20 hours. In a second experiment, DHBD\textsubscript{LB400} was inactivated with O\textsubscript{2} by exposing a solution of the enzyme to air for 20 hours. Finally, DHBD\textsubscript{LB400} was inactivated by incubating a 12-15 μM solution of the enzyme with 10 mM catechol, or 3-chlorocatechol, or 4-Cl DHB or 2'-Cl DHB. In this experiment, the reaction mixture was gently bubbled with air for 10 minutes, which was sufficient for complete inactivation. The activity of the preparations was monitored using the standard assay to verify inactivation.

Samples of DHBD\textsubscript{LB400} were reactivated under the inert atmosphere of the glovebox. Aerobically-inactivated samples were gently bubbled with argon for 15 minutes 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 and the second with 2 mM DTT and 1 mM FeCl₂•4H₂O. After 30-60 minutes of 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.

2.11.4 Mass spectrometry analysis

Mass spectra were recorded on a PE-Sciex API 300 triple quadrupole mass spectrometer (Sciex, Thornhill, Ontario) equipped with an ionspray ion source (Sciex) or nanospray ion source (Protana, Odense, Denmark). The protein samples were injected onto an Ultrafast Microprotein Analyzer UMA (Michrom Bioresources, Inc., Aubum, 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., 8 U, 300 Å, 1 mm X 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 minutes 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 to 2400 amu, with a step size of 0.5 amu and a dwell-time of 1.0 ms per step. The ionspray 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.

2.11.5 electron paramagnetic resonance spectroscopy

X-band electron paramagnetic resonance (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 DHBD₄₋₄₀ 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 at 77 K for EPR analysis. EPR spectra were obtained as an average of two scans with a sweep time of 336 s, a microwave power of 2 mW, a modulation amplitude of 1.027 mT and a modulation frequency of 100 kHz.

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 0.103 mT and a scan range of 343 to 353 mT. The peak area was estimated by integration using standard WINEPR software.

2.11.6 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 (Bindokas et al. 1996, Benov et al. 1998) was followed using a Model LS 50B spectrofluorimeter (Perkin Elmer Ltd., Markham, Ontario). The excitation and emission wavelengths were 470 nm and 595 nm, respectively, with slit widths of 4 nm 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$^{-1}$; Sutherland and Learmonth 1997) was followed on the Varian Cary 1E spectrophotometer described above.

For spin-trapping studies, DHBD$_{18400}$ was prepared anaerobically in potassium phosphate buffer, pH 7.5 (I = 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 U/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 $O_2$ in the presence of catalase using an oxygen electrode. Typically, 1500 U/ml of catalase was used in the assay. The effect of superoxide dismutase (200 U/ml) on the reaction of $\text{DHBD}_{\text{LB400}}$ 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-hydroxymuconic acid 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 from xanthine by xanthine oxidase was followed at 292 nm (11 mM$^{-1}$cm$^{-1}$; Rubbo et al. 1991). In the hydroethidine and XTT assays, superoxide was produced by incubating 200 $\mu$M xanthine with 0.08 U/ml xanthine oxidase.

2.11.7 In vivo inactivation

Assays were performed using biphenyl-grown *Burkholderia* sp. LB400 and LB-grown *E. coli* DH5$\alpha$ containing the plasmid pLEBD4 (de Lorenzo et al. 1993). Cells were grown to stationary phase (OD$_{600}$ 1.6-2.0), harvested by centrifugation, and washed twice with potassium phosphate buffer pH 7.0 (I = 0.1) containing 100 $\mu$g/ml chloramphenicol to prevent protein synthesis. The activity of $\text{DHBD}_{\text{LB400}}$ was followed using a modification of the oxygraph assay described in section 2.7. Whole cells were injected into a reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.0, 100 $\mu$g/ml chloramphenicol and 400 $\mu$M DHB. The $\text{DHBD}_{\text{LB400}}$ activity was inhibited using 400 $\mu$M 3-chlorocatechol. $\text{DHBD}_{\text{LB400}}$-inactivated cells were harvested from the reaction mixture by centrifugation, resuspended in 1 ml of potassium phosphate buffer, pH 7.0 containing 100 $\mu$g/ml chloramphenicol, recentrifuged, then re-assayed for $\text{DHBD}_{\text{LB400}}$. The loss of cells during this manipulation was corrected by monitoring the OD$_{600}$. 
2.12 HPLC analyses for the identification of 3-chlorocatechol and 3-nitrocatechol ring-cleaved products and partition ratios

HPLC measurements were performed using a Waters Alliance HPLC system equipped with a Waters 996 or 2996 Photodiode Array Detector (Mississauga, Ontario) and a Phenomenex Prodigy 10 μ ODS-PREP column (4.6 × 250 mm, Torrance, CA). The HPLC was interfaced to a microcomputer and controlled by the Waters Millenium²² Software. Samples of 50 μl were injected and eluted at a flow of 1 ml/min for the 3-chlorocatechol study and at 2 ml/min for the 3-nitrocatechol study. Enzymatic reactions were diluted 1:5 with the elution solvent immediately prior to injection to prevent peak tailing.

3-Chlorocatechol and 3-nitrocatechol were eluted using a mixture of 35% acetonitrile, 64.7% H₂O, and 0.3% H₃PO₄ (solvent A). 2-Pyrone-6-carboxylic acid and 2-hydroxymuconic acid were eluted using a mixture of 20% methanol, 79.7% H₂O and 0.3% H₃PO₄ (solvent B). Standard calibration curves for 3-chlorocatechol, 3-nitrocatechol, 2-pyrone-6-carboxylic acid and 2-hydroxymuconic acid were prepared by injecting solutions containing known amounts of the pure chemicals. The distal ring-cleaved product of 3-chlorocatechol was eluted 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 sulphate as an ion pairing reagent (solvent C; Riegert et al. 2001). The distal ring-cleaved product of 3-nitrocatechol was eluted using a solution of 15% methanol and 85% of the same aqueous buffer (solvent D).

Reaction products were identified in mixtures containing 50 μM 3-chlorocatechol or 100 μM 3-nitrocatechol and 30-60 μM DHBD₄₄₀₀ (20 mM HEPPS, 80 mM NaCl, pH 8.0). The reaction was initiated by the addition of DHBD₄₄₀₀ and incubated for 30 s and 20 min. at 23°C for 3-chlorocatechol and 3-nitrocatechol respectively. The reaction was then diluted with the appropriate solvent and immediately analyzed by HPLC. Experiments designed to
determine the partition coefficient of DHBD\textsubscript{LB400} for 3-chlorocatechol and 3-nitrocatechol were performed in a similar fashion, except that reaction mixtures initially contained 25 \( \mu \text{M} \) or 50 \( \mu \text{M} \) 3-chlorocatechol or 100 \( \mu \text{M} \) 3-nitrocatechol. The reactions were initiated using a limiting amount of DHBD\textsubscript{LB400} (1.5-12 \( \mu \text{M} \)). The partition coefficient was calculated from the amounts of remaining 3-chlorocatechol or 3-nitrocatechol and added DHBD\textsubscript{LB400}. 

2.13 Anionic state of substrates in solution and bound to DHBD\textsubscript{LB400}

2.13.1 Preparation of samples

Samples for spectroscopy and titrations were prepared under the inert atmosphere of the glovebox and maintained at less than 1 ppm O\textsubscript{2}. Catecholic compounds were weighed in small glass vials (Wheaton, Millville, NJ) and transferred to the glovebox for sample preparation. The ES complex was prepared by combining DHB or 3-nitrocatechol with a slight molar excess of DHBD\textsubscript{LB400} (ratios between 1:1.3 and 1:1.7; protein prepared as described in section 2.5). Samples for spectroscopy were transferred to the appropriate sealed cuvettes and removed from the glovebox.

2.13.2 Titration of 3-substituted catechols

The first pK\textsubscript{a} values for the different catecholic compounds were determined using a model PHM93 Reference pH Meter (Radiometer, Copenhagen, Denmark) mounted in the glovebox. Each catecholic compound was dissolved in deionized water to a final concentration of 50 mM, except DHB, which was dissolved at a final concentration of 25 mM. Each compound was titrated by adding microlitre volumes of an equimolar solution of NaOH.

2.13.3 UV/Vis absorption spectroscopy

Spectra were recorded using the spectrophotometer equipped with a thermostated cuvette holder set at 25°C. The spectrophotometer was interfaced to a microcomputer as
described in section 2.8. A 1-ml gas-tight cuvette (Hellma, Concord, Ontario) was used for anaerobic samples of catecholic compounds prepared in the absence and presence of DHBD$_{LB400}$. 
3. RESULTS

3.1 Purification of DHBD\textsubscript{LB400}

Relevant details of the purification are shown in Table 1. The enzyme was estimated to be greater than 95% pure as judged by SDS polyacrylamide gel electrophoresis followed by Coomassie Blue staining. This is comparable to the purity obtained previously (Eltis \textit{et al.} 1993). Anaerobically purified DHBD\textsubscript{LB400} had a specific activity of 430 U/mg, which was over twice that reported previously (Eltis \textit{et al.} 1993).

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Purification step & total protein & total activity & specific activity & Yield \\
& mg & U & U/mg & \%
\hline
raw extract & 1,150 & 116,900 & 101 & 100
\hline
MonoQ & 125 & 52,800 & 422 & 45
\hline
Superdex 200HR & 90 & 39,000 & 431 & 33
\hline
\end{tabular}
\caption{Purification details of DHBD\textsubscript{LB400}.}
\end{table}

Purification from 3 L of culture. Activity units (U) are defined in section 2.7.

3.2 Stabilization of DHBD\textsubscript{LB400}

The activity of a 25 to 50 \textmu g/ml anaerobic solution of DHBD\textsubscript{LB400} was stabilized by the presence of DTT and BSA but not by ferrous ammonium sulphate. The activity was most stable at pH 8.0; deviations of 0.25 pH units decreased this stability. In the presence of 1 M \textit{t}-butanol or isopropanol, approximately 24% of the activity remained after 24 hours, as compared to 19% in the presence of 1 M ethanol or glycerol and 13% in the absence of organic compounds. Acetone caused a rapid loss of activity when added to the solution. For steady-state kinetic assays, it was determined that DHBD\textsubscript{LB400} is most stable if diluted in 20 mM HEPPS, 80 mM NaCl, pH 8.0, 10% \textit{t}-butanol, 2 mM DTT, 0.1 mg/ml BSA and stored on ice under an inert atmosphere. Under these conditions, more than 90% of the initial activity of the diluted preparation remained after 6 hours. Therefore, DHBD\textsubscript{LB400} was
diluted in this buffer for most steady-state experiments. However, in the inactivation study (including the measurement of partition ratios), DHBD_{LB400} was only diluted in 20 mM HEPPS, 80 mM NaCl, pH 8.0 to prevent any effect due to DTT. As DHBD_{LB400} was less stable in this buffer, fresh dilutions were prepared in the glovebox every 1-2 hours. In every kinetic experiment, the activity was monitored so that only assays performed with fully active DHBD_{LB400} were used in data analysis. The respective activities of DHBD_{P6-I} and DHBD_{P6-III} were also monitored during kinetic experiments, and dilutions of these enzymes were also freshly prepared at regular intervals as necessary.

3.3 Specificity of DHBD_{LB400} for 3-substituted catechols and dioxygen

3.3.1 Steady-state kinetics with DHB, 3-alkyl catechols and dioxygen

The coupling of catecholic substrate and O_{2} utilization by DHBD_{LB400} was investigated using 20 mM HEPPS, 80 mM NaCl, pH 8.0, 25°C, using an O_{2} electrode that had been calibrated with protocatechuate and 3,4-PCD (Whittaker et al. 1990). Within experimental error, the amount of O_{2} consumed corresponded to the amount of catechol or DHB added to the reaction mixture. It was concluded that the utilization of catecholic substrate and O_{2} by DHBD_{LB400} were tightly coupled under these conditions. In subsequent experiments, the O_{2} electrode was calibrated with DHB and DHBD_{LB400}.

In experiments performed in air-saturated buffer using low concentrations of DHBD_{LB400}, the velocity of the reaction decreased to zero prior to the complete consumption of catecholic substrate or O_{2}. This inactivation of the enzyme was faster in the presence of catechol than DHB. These observations indicated that DHBD_{LB400} was subject to irreversible, suicide inhibition as has been described for C230 (Cerdan et al. 1994, 1995).

High concentrations of catecholic substrate were observed to reduce the initial velocity of O_{2} consumption. To assess whether this decrease in the initial velocity was due to inactivation of DHBD_{LB400} via suicide inhibition, the substrate concentrations and
reaction velocities were calculated 10 to 30 s after the initiation of the reaction. Reaction velocities were calculated from 61 data points over a 6 s interval. Substrate concentrations were based on initial concentrations and the depletion of O₂. Measured reaction velocities were compared to those calculated using steady-state equations, best-fit parameters, and the estimated substrate concentrations. In experiments in which the catechol or 3-methylcatechol concentration exceeded 750 μM or 1400 μM, respectively, the measured velocity after 10 s was less than 90% the calculated velocity. The difference between the measured and calculated velocities increased as the reaction progressed. These observations indicated that suicide inhibition significantly affected the initial velocity under these conditions and no initial velocities obtained from such experiments were further analyzed.

In contrast, suicide inhibition affected the initial velocities of reactions performed with DHB and 3-ethylcatechol to a much lesser extent. Using 4 mM 3-ethylcatechol, the measured velocity after 10 s was within 90% of the calculated velocity. Using 800 μM DHB, the measured velocity after 30 s was within 95% of the calculated velocity.

When Equation 1 was fitted to the initial rates of O₂ consumption obtained over a range of concentrations of DHB (1 to 800 μM) and O₂ (55 to 1300 μM), random trends in the residuals were observed. Examples of the data and the quality of the fit are shown in Figure 13. The values for the steady-state kinetic parameters obtained from this fit are provided in Table 2. Similar values for \( K_mA \), \( K_{mO₂} \), \( K_dA \), and \( k_{cat} \) were obtained by fitting a subset of these data, corresponding to 1 to 90 μM DHB, to an equation describing a compulsory-order ternary-complex mechanism without substrate inhibition. The data obtained using 3-ethylcatechol (10 to 4200 μM) and O₂ (55 to 1300 μM) also yielded random trends in the residuals when fitted to Equation 1. DHBD₄₄₀ was approximately 2.5 times more specific for DHB than 3-ethylcatechol. In the presence of either substrate, the \( K_{mO₂} \) was approximately equal to the concentration of dissolved O₂ in buffer equilibrated with 100% O₂ at atmospheric pressure (Table 2).
Figure 13. Steady-state cleavage of DHB by DHBD\textsubscript{LB400}. A. The dependence of initial velocity on the concentration of DHB in air-saturated buffer. The line represents a best fit of equation 2 to the data. The fitted parameters are $K_{m}^{\text{app}} = 12 \pm 1$ μM, $K_{i}^{\text{app}} = 2.7 \pm 0.6$ mM and $V = 79 \pm 2$ μM/min. B. The dependence of initial velocity on the concentration of O\textsubscript{2} using 82 μM DHB. The line represents a best fit of the Michaelis-Menten equation to the data. The fitted parameters are $K_{mO_2}^{\text{app}} = 1,200 \pm 200$ μM and $V = 260 \pm 30$ μM/min. Initial velocities obtained on different days were normalized according to the amount of enzyme used in the assay. All experiments were performed using 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25°C.
Table 2. Kinetic parameters of DHBD\(_{LB400}\) using DHB and 3-ethylcatechol.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(K_{dA})</th>
<th>(K_{mA})</th>
<th>(K_{mO2})</th>
<th>(k_{cat})</th>
<th>(K_{iA})</th>
<th>(k_A)</th>
<th>(k_{O2})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(s^{-1})</td>
<td>(mM)</td>
<td>(x 10^6 M^{-1}s^{-1})</td>
<td>(x 10^6 M^{-1}s^{-1})</td>
</tr>
<tr>
<td>DHB</td>
<td>8 (1)</td>
<td>22 (2)</td>
<td>1,280 (70)</td>
<td>1,300 (100)</td>
<td>3.0 (0.5)</td>
<td>62 (8)</td>
<td>1.0 (0.1)</td>
</tr>
<tr>
<td>3-ethylcatechol</td>
<td>77 (9)</td>
<td>53 (20)</td>
<td>1,100 (100)</td>
<td>1,300 (200)</td>
<td>11 (3)</td>
<td>24 (10)</td>
<td>1.2 (0.1)</td>
</tr>
</tbody>
</table>

Experiments were performed using 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25\(^\circ\)C. The values of the parameters and their standard errors (provided in parentheses) were calculated using the least squares and dynamic weighting options of LEONORA (Cornish-Bowden 1995). The data sets used to calculate the parameters for DHB and 3-ethylcatechol contain 189 and 96 data points, respectively.
When Equation 1 was fitted to initial velocities obtained using catechol (50 to 750 μM) at different O₂ concentrations, negative values for some of the steady-state kinetic parameters were obtained. Negative values were also obtained using the equivalent equation describing a mechanism in which substrate inhibition does not occur. Poor fits were presumably obtained because the concentrations of catechol and O₂ could not be varied over a sufficiently wide range. As discussed above, the useful range of concentration for 3-methylcatechol (50 to 1400 μM) was also limited by suicide inhibition. Apparent steady-state kinetic parameters for catechol and 3-methylcatechol were thus evaluated by fitting the Michaelis-Menten equation to the data obtained in air-saturated buffer (Table 3). Fits of Equation 2 (with substrate inhibition) to the same data yielded essentially the same values for $K_{\text{mA}}^{\text{app}}$ and $K_{\text{cat}}^{\text{app}}$, respectively. The values for DHB and 3-ethylcatechol in air-saturated buffer, evaluated by fitting the substrate inhibition equation to the data, are also provided in Table 3 and do not differ significantly from the values calculated from the parameters provided in Table 2. Consistent with previous studies (Eltis et al. 1993), the apparent specificity of DHBD$_{LB400}$ in air-saturated buffer is 350-fold higher for DHB than for catechol. Although the $K_{mO_2}$ of DHBD$_{LB400}$ in the presence of catechol could not be evaluated, the apparent constant, $K_{mO_2}^{\text{app}}$, was 1,000 ± 200 μM at a concentration of 1275 μM catechol.

3.3.2 Cleavage of 3-chlorocatechol and 3-nitrocatechol using a reporter substrate

3-Chlorocatechol inactivated DHBD$_{LB400}$ too efficiently and 3-nitrocatechol was cleaved too slowly for the steady-state cleavage of these compound to be directly monitored using the oxygraph assays. Moreover, it was not possible to directly monitor the steady-state cleavage of 3-nitrocatechol using a spectrophotometric assay as the reaction products absorb in the same region as 3-nitrocatechol and are not very stable. For this reason, the $K_{mA}^{\text{app}}$ of DHBD$_{LB400}$ for these substrates were determined using DHB as a reporter substrate. The quality of the data was good for both substrates (Figure 14), even though the concentration of 3-chlorocatechol could only be varied over a limited range due to strong
inactivation. Apparent catalytic and specificity constants were calculated using the partition ratio and $I_{3H}^{app}$ (equation 6). The results demonstrate that DHBD\textsubscript{LB400} 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 $K_{m3H}^{app}$ was half that for DHB (Table 3). In comparison, the specificity for 3-nitrocatechol was 2000-fold less than that for DHB due to a very slow rate of turnover.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure14}
\caption{The DHBD\textsubscript{LB400}-catalyzed cleavage of DHB in the presence of 3-chlorocatechol (A) and 3-nitrocatechol (B). A. The rate of DHB cleavage was determined using 4.8 μM (□), 8.3 μM (■), 15.2 μM (Δ), 48.5 μM (○) and 82.9 μM (●) DHB. The fit of the equation to the data yielded the following parameters: $K_{m3A}^{app} = 5 \pm 1 \mu M$, $K_{mDHB}^{app} = 19 \pm 2 \mu M$, and $V = 142 \pm 6 \mu M/min$. B. The rate of DHB cleavage was determined using 5.3 μM (□), 9.3 μM (■), 17 μM (Δ), 54 μM (○) and 93 μM (●) DHB. The fit of the equation to the data yielded the following parameters: $K_{m3A}^{app} = 19 \pm 2 \mu M$, $K_{mDHB}^{app} = 9 \pm 1 \mu M$, and $V = 75 \pm 3 \mu M/min$. All experiments were performed using 20 mM HEPPS, 80 mM NaCl, pH 8.0, 25°C. Best fits were obtained using an equation similar in form to that describing competitive inhibition as described in Materials and Methods.}
\end{figure}
3.4 Characterization of inactivation mechanism of DHBD\textsubscript{LB400}

3.4.1 Identification of steady-state species susceptible to inactivation

In the previous section, it was shown that DHBD\textsubscript{LB400} is susceptible to inactivation during the steady-state cleavage of catechols. Even in the presence of the preferred substrate (DHB) of the enzyme, inactivation occurs within 10 minutes. As described by Duggleby (Duggleby 1986), any form of an enzyme that occurs during steady-state turnover can be susceptible to inactivation. DHBD\textsubscript{LB400} utilizes a compulsory order, ternary complex mechanism subject to substrate inhibition. The general approach described by Duggleby was adapted to this steady-state mechanism as shown in Figure 12, and the susceptibility of various forms of DHBD\textsubscript{LB400} that occur during catalytic turnover was investigated.

Anaerobic incubation of DHBD\textsubscript{LB400} with saturating quantities of various substrates, including DHB, catechol, and 3-chlorocatechol for up to 2 hours, resulted in no significant change in specific activity. Similar results were obtained when amounts of substrate sufficient to cause substrate inhibition were used. These results indicate that the corresponding rate constants of inactivation are negligible during the steady-state reaction (\textit{i.e.} \( j_2 \) and \( j_5 \) are essentially equal to zero (Figure 12)). Moreover, the anaerobic incubation of DHBD\textsubscript{LB400} with 3-chlorocatechol did not affect the iron content of the enzyme.

In the presence of 2.5 mM and 5 mM HOPDA, DHBD\textsubscript{LB400} lost approximately 10% of its activity after 30 min. While these concentrations of HOPDA reversibly inhibit DHB-cleavage (see section 3.6), 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 \( j_4 \) (Figure 12) was concluded to be essentially zero. These experiments show that the inactivation during the steady-state cleavage of catechols is due to inefficient catalysis after formation of the ternary complex between the catecholic substrate and \( O_2 \). The apparent rate
constant of this inactivation, $j_{3\text{app}}$, can be evaluated using two different methods as described in section 2.11.2.

In contrast to anaerobic preparations of EA and EP complexes, free DHBD_{LB400} was subject to significant inactivation in air-saturated buffer. Thus, the pseudo-first order rate constant of inactivation in air-saturated buffer, $j_{1\text{app}}$, was $\left(0.7 \pm 0.1\right) \times 10^{-3}$ s\(^{-1}\). This corresponds to a half-life of $16 \pm 2$ min.

### 3.4.2 Inactivation of DHBD_{LB400} in presence of 3-substituted catechols

The apparent rate constants of inactivation of DHBD_{LB400} by various catecholic substrates in air-saturated buffer, $j_{3\text{app}}$, was significantly faster than $j_{1\text{app}}$ (Table 3). Even for DHB, $j_{3E\text{app}}$, determined using spectrophotometrically-derived progress curves, was 5-times larger than $j_{1\text{app}}$. The general agreement of $j_{3E\text{app}}$ and $j_{3C\text{app}}$, determined for DHB, 3-methylcatechol and catechol using independent experimental approaches, validates the determined values (Table 3). Therefore, in subsequent studies, only $j_{3E\text{app}}$ or $j_{3C\text{app}}$ was determined.

Inactivation studies with 3-chlorocatechol and 3-nitrocatechol were performed using DHB as a reporter substrate. These experiments confirmed that 3-chlorocatechol potently inactivates DHBD_{LB400}. Based on $j_{3E\text{app}} / K_{m\text{app}}$, 3-chlorocatechol inactivates the enzyme over 100-fold more efficiently than the next best mechanism-based inactivator, 3-nitrocatechol (Table 3). 3-Ethylcatechol, 3-methylcatechol and catechol inactivate DHBD_{LB400} less potently than DHB due to their higher $K_m$ for the enzyme. However, in the presence of high concentrations of substrates (i.e. 1-5 times $K_m$), the relative rates at which DHBD_{LB400} is inactivated are: 3-chlorocatechol > catechol > 3-nitrocatechol > 3-methylcatechol > 3-ethylcatechol > DHB. Figure 15 illustrates typical spectrophotometric inactivation assays for all substrates except 3-ethylcatechol as $j_{3E\text{app}}$ was not determined with this substrate.
Figure 15. The inactivation of DHBD_{LB400} during steady-state cleavage of catecholic substrates. A. DHBD_{LB400} (0.3 nM) was incubated with 80 μM DHB and the appearance of product was followed at 434 nm (O). The fitted parameters are $j_s = (3.131 \pm 0.005) \times 10^{-3}$ s$^{-1}$, $P_\infty = 25.36 \pm 0.03$ μM, $P_i = 1.338 \pm 0.001$ μM. B. DHBD_{LB400} (2 nM) was incubated with 200 μM 3-nitrocatechol and DHB (250 μM). The appearance of product was followed at 434 nm (◇). The fitted parameters are $j_s = (8.0 \pm 0.1) \times 10^{-3}$ s$^{-1}$, $P_\infty = 16.31 \pm 0.02$ μM, $P_i = 0.78 \pm 0.01$ μM. C. DHBD_{LB400} (3 nM) was incubated with 1 mM 3-methylcatechol and the appearance of product was followed at 389 nm (□). The fitted parameters are $j_s = (15.22 \pm 0.02) \times 10^{-3}$ s$^{-1}$, $P_\infty = 11.936 \pm 0.004$ μM, $P_i = 1.682 \pm 0.003$ μM. D. DHBD_{LB400} (10 nM) was incubated with 1 mM catechol and the appearance of product was followed at 376 nm (Δ). The fitted parameters are $j_s = (29.00 \pm 0.05) \times 10^{-3}$ s$^{-1}$, $P_\infty = 8.45 \pm 0.01$ μM, $P_i = 2.51 \pm 0.01$ μM. E. DHBD_{LB400} (4 nM) was incubated with 10 μM 3-chlorocatechol and DHB (80 μM). The appearance of product was followed at 434 nm (X). The fitted parameters are $j_s = (110.5 \pm 0.3) \times 10^{-3}$ s$^{-1}$, $P_\infty = 5.75 \pm 0.01$ μM, $P_i = 5.35 \pm 0.01$ μM. All experiments were performed using 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25°C. The parameters were calculated by fitting equation 3 to the data using the least squares fitting option of the Scientist software.
Table 3. Apparent steady-state kinetic parameters and inactivation parameters of DHBD_{LB400} for different substrates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{mA}^{app}$</th>
<th>$K_{IA}^{app}$</th>
<th>$k_{cat}^{app}$</th>
<th>$k_A^{app}$</th>
<th>Partition Ratio</th>
<th>$j_{j3C}^{app}$</th>
<th>$j_{j3E}^{app}$</th>
<th>$j_{j3E}^{app} / K_{mA}^{app}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu M$</td>
<td>mM</td>
<td>s$^{-1}$</td>
<td>$x 10^6 M^{-1}s^{-1}$</td>
<td>$x 10^{-3}$ s$^{-1}$</td>
<td>$x 10^{-3}$ s$^{-1}$</td>
<td>$x 10^3 M^{-1}s^{-1}$</td>
<td></td>
</tr>
<tr>
<td>DHB</td>
<td>12 (1)</td>
<td>2.7 (0.6)</td>
<td>251 (6)</td>
<td>21 (1)</td>
<td>85,000 (1,000)</td>
<td>3.0 (0.1)</td>
<td>3.7 (0.4)</td>
<td>0.31 (0.06)</td>
</tr>
<tr>
<td>3-Et catechol</td>
<td>61 (4)</td>
<td>17 (4)</td>
<td>281 (5)</td>
<td>4.6 (0.2)</td>
<td>29,000 (5,000)</td>
<td>9 (2)</td>
<td>0.16 (0.04)</td>
<td></td>
</tr>
<tr>
<td>3-Me catechol</td>
<td>530 (30)</td>
<td>97 (3)</td>
<td>0.2 (0.1)</td>
<td>5,300 (300)</td>
<td>18 (2)</td>
<td>23 (3)</td>
<td>0.043 (0.008)</td>
<td></td>
</tr>
<tr>
<td>Catechol</td>
<td>900 (100)</td>
<td>51 (6)</td>
<td>0.060 (0.004)</td>
<td>1,200 (100)</td>
<td>41 (7)</td>
<td>56 (2)</td>
<td>0.06 (0.01)</td>
<td></td>
</tr>
<tr>
<td>3-Cl catechol</td>
<td>4.8 (0.7)</td>
<td>4 (1)$^a$</td>
<td>0.8 (0.4)$^a$</td>
<td>8 (2)$^b$</td>
<td>500 (20)</td>
<td>100 (20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-NO$_2$ catechol</td>
<td>19 (2)</td>
<td>0.17 (0.03)$^a$</td>
<td>0.009 (0.003)$^a$</td>
<td>7 (1)$^c$</td>
<td>24.4 (0.3)</td>
<td>1.3 (0.2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Experiments were performed using air-saturated 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25°C. Values in parentheses represent standard errors. $^a$Values calculated by multiplying the partition ratio by $j_{j3E}^{app}$ to obtain $k_{cat}^{app}$ (cf. equation 6) and by dividing the calculated $k_{cat}^{app}$ by $K_{mA}^{app}$ to obtained $k_A^{app}$. $^b$Average of values obtained by oxygraph and HPLC assays. $^c$Value obtained by HPLC assay.
3.4.3 Inactivation-induced changes in DHBD<sub>LB400</sub>

To elucidate inactivation-induced changes in DHBD<sub>LB400</sub>, the enzyme was inactivated using several different methods and the properties of the different preparations of DHBD<sub>LB400</sub> were investigated. Preparations of DHBD<sub>LB400</sub> inactivated with 1,10-phenanthroline, O<sub>2</sub>, catechol and 3-chlorocatechol could each be partially reactivated through anaerobic incubation with a reducing agent (Table 4). However, incubation with Fe(II) and DTT was necessary to restore most of the activity. Thus, the O<sub>2</sub>-dependent inactivation of DHBD<sub>LB400</sub>, both in the absence and presence of catecholic substrate, results in the oxidation and loss of the active site iron.

<table>
<thead>
<tr>
<th>Inactivator</th>
<th>Reactivation with DTT</th>
<th>Reactivation with DTT and Fe(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,10-Phenanthroline</td>
<td>8 (1)</td>
<td>120 (10)</td>
</tr>
<tr>
<td>Air</td>
<td>38 (2)</td>
<td>99 (5)</td>
</tr>
<tr>
<td>Catechol</td>
<td>30 (6)</td>
<td>100 (10)</td>
</tr>
<tr>
<td>3-Chlorocatechol</td>
<td>21 (3)</td>
<td>75 (6)</td>
</tr>
</tbody>
</table>

Table 4. In vitro reactivation of DHBD<sub>LB400</sub>.

Experiments were performed using air-saturated 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25°C. DHBD<sub>LB400</sub> was inactivated using different protocols as described in section 2.11.3. Inactivated, desalted preparations were reactivated anaerobically using 2 mM DTT with and without 1 mM FeCl<sub>2</sub>•4H<sub>2</sub>O as described in section 2.11.3. Values in parentheses represent standard deviations.

Preparations of DHBD<sub>LB400</sub> inactivated with 1,10-phenanthroline, O<sub>2</sub>, catechol and 3-chlorocatechol each had a molecular mass of 32,350 ± 4 Da, identical to active DHBD<sub>LB400</sub> as determined by ionspray mass spectroscopy. Nanospray mass spectral analyses of DHBD<sub>LB400</sub> 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 spectroscopy. Thus, anaerobically prepared complexes of DHBD$_{LB400}$ (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 $g = 4.28$ (Figure 16) upon exposure to air for 5 minutes prior to 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 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 (Avdeef et al. 1978) and is similar to that of a solution of ferric iron and excess 3-chlorocatechol ($\lambda_{\text{max}} = 494$ nm; $\varepsilon_{494} = 4.6$ mM$^{-1}$cm$^{-1}$). Based on this extinction coefficient, over 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)-chlorocatecholate 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$_{LB400}$:3-chlorocatechol complex containing ferric iron. This interpretation is consistent with partial occupancy of the active site of DHBD$_{LB400}$ in a crystalline complex of ferric DHBD$_{LB400}$:DHB (Uragami et al. 2001). 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$_{LB400}$ 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.
Figure 16. Low temperature EPR spectra of DHBD_{LB400} incubated with 3-chlorocatechol. DHBD_{LB400} (0.34 mM iron) was prepared anaerobically in 20 mM HEPPS, 80 mM NaCl, pH 8.0 and incubated with 10 mM 3-chlorocatechol. A. The sample was flash frozen in liquid nitrogen and transferred to a finger Dewar insert to record spectra (77 K). B. An aliquot of the same sample was exposed to air for 5 minutes prior to flash freezing. Spectra represent the average of 2 scans and were obtained under the following conditions: microwave power, 2 mW; modulation amplitude, 1.027 mT; modulation frequency, 100 kHz; and sweep time, 336 s.

3.4.4 Detection of reactive oxygen species

To investigate whether superoxide was produced during the inactivation of DHBD_{LB400}, inactivation reactions were performed in the presence of various superoxide trapping agents. When 95 μM DHBD_{LB400} was stirred with XTT in air-saturated buffer, 16.1 ± 0.4 μM of reduced XTT were detected after 100 min. Superoxide dismutase inhibited the reduction of XTT by approximately 70%, demonstrating that superoxide is produced during the inactivation of the free enzyme.

When DHBD_{LB400} (10-500 μM) was inactivated using different concentrations of 3-chlorocatechol (0.1-5 mM), no superoxide was detected using DMPO, hydroethidine or XTT. Moreover, in enzymatic reactions monitored with the oxygen electrode, no additional
O₂ production was observed in the presence of superoxide dismutase and/or catalase, indicating that H₂O₂ 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 50 ± 10%, 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 hydroethidine, presumably by reacting with superoxide directly.

**3.4.5 The inactivation of DHBD<sub>LB400</sub> in vivo**

The *in vivo* inactivation of DHBD<sub>LB400</sub> was studied using the native strain of the enzyme *Burkholderia* sp. LB400, and a heterologous expression host, *E. coli* DH5α. The activity of DHBD<sub>LB400</sub> in biphenyl-grown *Burkholderia* sp. LB400 and LB-grown *E. coli* DH5α was 0.3 and 0.2 U/OD<sub>600</sub>, respectively. Addition of 400 μM 3-chlorocatechol to the assay completely inhibited the DHBD<sub>LB400</sub> activity in both strains. Upon removal of 3-chlorocatechol from the cells, DHBD<sub>LB400</sub> activity recovered to almost pre-inhibition levels within 12 minutes (Table 5). This recovery occurred in the presence of chloramphenicol, indicating that protein synthesis is not required for the recovery of DHBD<sub>LB400</sub> activity.
Table 5. *In vivo* reactivation of 3-chlorocatechol-inactivated DHBD<sub>LB400</sub>

<table>
<thead>
<tr>
<th>Strain / plasmid</th>
<th>Reactivation time</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>%</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α / pLEBD4</td>
<td>5</td>
<td>42 (5)</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α / pLEBD4</td>
<td>20</td>
<td>86 (4)</td>
</tr>
<tr>
<td><em>E. coli</em> DH5α / pLEBD4</td>
<td>60</td>
<td>99 (8)</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. LB400</td>
<td>12</td>
<td>82 (9)</td>
</tr>
<tr>
<td><em>Burkholderia</em> sp. LB400</td>
<td>20</td>
<td>89 (2)</td>
</tr>
</tbody>
</table>

Reactivation time refers to the period of incubation following removal of 3-chlorocatechol. Additional experimental details are provided in section 2.11.7. Values in parentheses represent standard deviations.

3.5 HPLC analyses of the ring-cleaved products and partition ratios of 3-chlorocatechol and 3-nitrocatechol

Previous studies have demonstrated that 3-chlorocatechol can be cleaved in distal (1,6-) and proximal (2,3-) manners. Accordingly, the products of the DHBD<sub>LB400</sub>-catalyzed ring cleavage of 3-chlorocatechol and 3-nitrocatechol were analyzed using HPLC. Four different solvents systems (A to D) were used to resolve the components of the reaction mixtures. Using solvent A, it was determined that approximately 20% of the 3-chlorocatechol (t<sub>R</sub> = 8.15 min, λ<sub>max</sub> = 199.9 nm at 1 ml/min) or 3-nitrocatechol (t<sub>R</sub> = 4.36 min, λ<sub>max</sub> = 300 nm at 2 ml/min) were uncleaved (20 mM HEPPS, 80 mM NaCl, pH 8.0, 23°C). Using solvent B, two products were eluted (see Figure 17 for product description) from reactions with 3-chlorocatechol and 3-nitrocatechol. Based on the retention times and spectra of the authentic compounds, these were identified as 2-pyrone-6-carboxylic acid (t<sub>R</sub> = 5.4 min, λ<sub>max</sub> = 298.8 nm at 1 ml/min) and 2-hydroxymuconic acid (t<sub>R</sub> = 11.4 min, λ<sub>max</sub> = 304.7 nm at 1 ml/min). 2-Pyrone-6-carboxylic acid and 2-hydroxymuconic acid, both of which arise from the proximal (2,3-) cleavage of 3-chlorocatechol (Kaschabek *et al.* 1998) or 3-nitrocatechol, accounted for 18.5 ± 0.3% and 19.2 ± 0.6%, respectively of the initial 3-
chlorocatechol and for $14.2 \pm 0.4\%$ and $32.9 \pm 0.4\%$, respectively of the initial 3-nitrocatechol. Analysis of the same reaction mixtures using solvent C (3-chlorocatechol) and solvent D (3-nitrocatechol) permitted the detection of a third product in the cleavage of 3-chlorocatechol ($t_R = 6.8$ min, $\lambda_{\text{max}} = 375$ nm at 1 ml/min) and 3-nitrocatechol ($t_R = 4.06$ min, $\lambda_{\text{max}} = 325$ nm at 2 ml/min). Based on retention times and absorption spectra, the 3-chlorocatechol product was identified as 3-chloro-2-hydroxymuconic semialdehyde (Riegert et al. 2001), resulting from the distal cleavage (1,6-cleavage) of 3-chlorocatechol. According to the similarities with the 3-chlorocatechol product, the 3-nitrocatechol product is tentatively assigned to 3-nitro-2-hydroxymuconic semialdehyde. Considering the respective extinction coefficients of 2-pyrone-6-carboxylic acid ($\varepsilon_{298.8} = 8.3$ mM$^{-1}$cm$^{-1}$ in solvent B) and 3-chloro-2-hydroxymuconic semialdehyde ($\varepsilon_{378} = 54$ mM$^{-1}$cm$^{-1}$ at pH 7.5; Riegert et al. 2001) and that the absorbance of the latter is similar at pH 7.5 and pH 10.0 (Riegert et al. 1998), it was estimated that the distal ring-cleaved product of 3-chlorocatechol accounted for $1.8 \pm 0.1\%$ of the initial amount of 3-chlorocatechol in the reaction mixture. Assuming a similar extinction coefficient for the distal ring-cleaved product of 3-nitrocatechol, this product formation can be estimated to range from 1.5 to 4%.

**Figure 17.** Alternative reactions occurring when DHBD$_{LB400}$ cleaves 3-chlorocatechol and 3-nitrocatechol.
Based on the amount of 3-chlorocatechol remaining in the reaction mixture, the partition coefficient of DHBD_{LB400} for this compound was 5.2 ± 0.5 and 5.4 ± 0.5 in experiments using 25 μM and 50 μM 3-chlorocatechol, respectively. In contrast, oxygraph assays yielded a partition coefficient of 11 ± 2 for 3-chlorocatechol. Based on the amount of 3-nitrocatechol remaining in the reaction mixture, the partition coefficient of DHBD_{LB400} for this compound was 7 ± 1 in experiments using 100 μM 3-nitrocatechol.

The cleavage products of 3-methylcatechol (solvent D) and DHB (solvent D supplemented with 30% methanol) were also analyzed and assigned based on published retention times and spectra (Riegert et al. 2001). In both cases a major peak corresponding to proximal cleavage was observed (t_R = 7.83 min, λ_max = 389.9 nm and t_R = 5.71 min, λ_max = 439 nm for the 3-methylcatechol and the DHB products at 2 ml/min). For 3-methylcatechol, a minor peak (t_R = 4.7 min, λ_max = 374 nm at 2 ml/min) corresponding to the distal product was found to account for less than 0.5% of the observed products. In comparison, the analysis of the cleavage product of DHB did not reveal any distal product formation.

### 3.6 Inhibition of DHBD_{LB400} with organic additives and HOPDA

In section 3.2, DHBD_{LB400} was shown to be stabilized in the presence of organic additives. All the additives tested except glycerol were shown to inhibit the enzyme to different extent (Table 6). When an equation describing mixed inhibition (Equation 3) was fitted to the steady-state kinetic data obtained from the DHBD_{LB400}-catalyzed cleavage of DHB with the different additives present as inhibitors, random trends in the residuals were obtained. 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 even when the limiting concentration of O_2 is considered (Table 6). Steady-state kinetic data obtained from the DHBD_{LB400}-catalyzed cleavage of DHB with t-butanol present as an inhibitor are presented in Figure 18. The relative ability
of the tested organic molecules to inhibit the DHBD\textsubscript{LB400}-catalyzed cleavage of DHB was \textit{t}-butanol > isopropanol ~ acetone > ethanol >> glycerol (Table 6).

Table 6. Inhibition of the DHBD\textsubscript{LB400}-catalyzed cleavage of different substrates by organic additives.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>$K_{ic}$</th>
<th>$K_{iu}^{app}$</th>
<th>$K_{iu}^{a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>DHB</td>
<td>\textit{t}-butanol</td>
<td>700 (100)</td>
<td>1,400 (100)</td>
<td>1,200 (100)</td>
</tr>
<tr>
<td>DHB</td>
<td>isopropanol</td>
<td>1,000 (100)</td>
<td>3,700 (600)</td>
<td>3,000 (500)</td>
</tr>
<tr>
<td>DHB</td>
<td>acetone</td>
<td>1,300 (200)</td>
<td>2,600 (200)</td>
<td>2,200 (200)</td>
</tr>
<tr>
<td>DHB</td>
<td>ethanol</td>
<td>3,500 (800)</td>
<td>6,500 (800)</td>
<td>5,300 (700)</td>
</tr>
<tr>
<td>DHB</td>
<td>glycerol</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3-methylcatechol</td>
<td>\textit{t}-butanol</td>
<td>990 (90)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>catechol</td>
<td>\textit{t}-butanol</td>
<td>520 (40)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Inhibition experiments were performed using air-saturated 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25°C. Values in parentheses represent standard errors of the mean. \textsuperscript{a}Calculated from $K_{iu}^{app}$ using Equation 4.

Inspection of the quality of fits to equations describing competitive, uncompetitive and mixed inhibition, respectively, indicated that the DHBD\textsubscript{LB400}-catalyzed cleavage of catechol and 3-methylcatechol were inhibited by \textit{t}-butanol in a competitive fashion. The inhibition constants were similar in magnitude to the competitive inhibition constants of the DHBD\textsubscript{LB400}-catalyzed cleavage of DHB (Table 6). Nevertheless, weak uncompetitive inhibition of the DHBD\textsubscript{LB400} cleavage of catechol and 3-methylcatechol could not be ruled out as the maximum concentration of \textit{t}-butanol used in these experiments was 2 M and the maximum substrate concentration did not exceed three times their respective $K_{mA}$ values.

Product inhibition studies indicated that the mode of DHBD\textsubscript{LB400} inhibition by HOPDA is mixed when DHB is used as a substrate in air-saturated buffer. Thus, when an
The equation describing mixed inhibition was fitted to the data, random trends in the residuals were observed and the residuals were smaller than when equations describing competitive or uncompetitive inhibition were fitted to the data. The competitive inhibition constant ($K_{ic}$) and the apparent uncompetitive inhibition constant ($K_{aupp}^{app}$) were 3.7 ± 0.9 mM and 3.3 ± 0.3 mM, respectively. The uncompetitive inhibition constant ($K_{iu}$) was 2.7 ± 0.2 mM.

**Figure 18.** Inhibition of the DHBD$_{L400}$-catalyzed cleavage of DHB by t-butanol. A. Dixon plot. B. Cornish-Bowden plot. Experiments were performed using 4.8 μM (□), 8.3 μM (■), 15.1 μM (X), 48.4 μM (○) and 82.8 μM (●) DHB. All experiments were performed using air-saturated 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25°C. The lines represent a best fit of an equation describing mixed inhibition to the data. The fitted parameters are $K_{ic} = 700 ± 100$ mM, $K_{aupp}^{app} = 1,400 ± 100$ mM, $K_{aupp}^{app} = 6.5 ± 0.5$ μM, and $V = 45 ± 1$ μM/min.
3.7 Reactivity of DHBD_{LB400}, DHBD_{P6-I} and DHBD_{P6-III} with chlorinated DHBs

3.7.1 Reactivity of DHBD_{LB400} with chlorinated DHBs

3.7.1.1 Steady-state kinetic analysis

The reactivity of DHBD_{LB400} with chlorinated DHBs that might arise during the metabolism of PCBs was studied using the approaches described in the preceding sections. Kinetic experiments performed by varying the concentration of monochlorinated DHBs and O_2 (Table 7) showed that DHBD_{LB400} cleaved these compounds between 0.06 and 0.3 times that of unchlorinated DHB in the following order of specificity: DHB > 4'-Cl > 6-Cl > 4-Cl > 3'-Cl > 5-Cl ~ 2'-Cl. Interestingly, the \( K_m \) of DHBD for O_2 was lower in the presence of 4'-Cl and 3'-Cl DHB than unchlorinated DHB, and the specificity constant for O_2 varied in the presence of the DHBs by more than 100-fold in the following order: 4'-Cl > 3'-Cl > DHB > 6-Cl > 5-Cl > 4-Cl > 2'-Cl. Interestingly, the slowest catalyzed monochlorinated DHB, 2'-Cl DHB, also had a high affinity for DHBD_{LB400} (low \( K_d \)) as well as a 50-fold decrease in specificity for O_2 when compared to DHB.

The reactivity of DHBD_{LB400} with 2'-Cl DHB led to the synthesis of 2',6'-diCl DHB to study the enzymatic cleavage of a di-ortho chlorinated DHB. The steady-state cleavage of 2',6'-diCl DHB by DHBD was too slow for the oxygraph assay. Moreover, the appearance of the yellow-colored ring-cleaved product could only be observed spectrophotometrically at saturating concentrations of 2',6'-diCl DHB. However, by using DHB as a reporter substrate in the oxygraph assay, it was shown that DHBD had a \( K_{m,A}^{app} = 7 \pm 1 \) nM for 2',6'-diCl DHB in air-saturated buffer, significantly lower than for 2'-Cl DHB (Table 8, Figure 19).
Table 7. Steady-state kinetic parameters of DHBD<sub>LB400</sub> for monochlorinated DHBs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(K_{dA})</th>
<th>(K_{mA})</th>
<th>(K_{iA})</th>
<th>(K_{mb})</th>
<th>(k_{cat})</th>
<th>(k_A)</th>
<th>(k_B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\mu M)</td>
<td>(\mu M)</td>
<td>(mM)</td>
<td>(\mu M)</td>
<td>(s^{-1})</td>
<td>(x 10^6 M^{-1} s^{-1})</td>
<td>(x 10^6 M^{-1} s^{-1})</td>
</tr>
<tr>
<td>DHB&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 (1)</td>
<td>22 (2)</td>
<td>3.0 (0.5)</td>
<td>1,280 (70)</td>
<td>1,300 (100)</td>
<td>62 (8)</td>
<td>1.0 (0.1)</td>
</tr>
<tr>
<td>2'-Cl DHB</td>
<td>0.8 (0.6)</td>
<td>9 (2)</td>
<td>2.7 (0.6)</td>
<td>1,500 (200)</td>
<td>33 (5)</td>
<td>3.7 (0.8)</td>
<td>0.021 (0.001)</td>
</tr>
<tr>
<td>3'-Cl DHB</td>
<td>8 (5)</td>
<td>130 (20)</td>
<td>0.270 (0.005)</td>
<td>510 (80)</td>
<td>800 (100)</td>
<td>6.3 (0.5)</td>
<td>1.5 (0.2)</td>
</tr>
<tr>
<td>4'-Cl DHB</td>
<td>53 (6)</td>
<td>47 (6)</td>
<td>0.224 (0.002)</td>
<td>330 (50)</td>
<td>800 (100)</td>
<td>18 (3)</td>
<td>2.6 (0.6)</td>
</tr>
<tr>
<td>4-Cl DHB</td>
<td>9 (2)</td>
<td>40 (10)</td>
<td>---</td>
<td>1,200 (300)</td>
<td>280 (60)</td>
<td>7 (1)</td>
<td>0.24 (0.02)</td>
</tr>
<tr>
<td>5-Cl DHB</td>
<td>9 (3)</td>
<td>45 (4)</td>
<td>1.5 (0.2)</td>
<td>470 (30)</td>
<td>160 (10)</td>
<td>3.7 (0.4)</td>
<td>0.35 (0.03)</td>
</tr>
<tr>
<td>6-Cl DHB</td>
<td>9 (1)</td>
<td>6.1 (0.6)</td>
<td>0.29 (0.03)</td>
<td>100 (10)</td>
<td>53 (2)</td>
<td>9 (1)</td>
<td>0.52 (0.07)</td>
</tr>
</tbody>
</table>

Experiments were performed using air-saturated 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25°C. Values in parentheses represent standard errors. <sup>a</sup>Data taken from Table 2.
Table 8. Apparent steady-state kinetic and inactivation parameters of DHBD_{LB400} for chlorinated DHBs.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_{\text{mA}}^{\text{app}}$</th>
<th>$K_{\text{IA}}^{\text{app}}$</th>
<th>$k_{\text{cat}}^{\text{app}}$</th>
<th>$k_{5}^{\text{app}}$</th>
<th>Partition Ratio</th>
<th>$j_{3}^{\text{app}}$</th>
<th>$j_{3}^{\text{app}} / K_{\text{mA}}^{\text{app}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu M$</td>
<td>$mM$</td>
<td>$s^{-1}$</td>
<td>$x 10^6 M^{-1} s^{-1}$</td>
<td>$x 10^3 s^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHB\textsuperscript{a}</td>
<td>12 (1)</td>
<td>2.7 (0.6)</td>
<td>251 (6)</td>
<td>21 (1)</td>
<td>85,000 (1,000)</td>
<td>3.0 (0.1)</td>
<td>0.2 (0.1)</td>
</tr>
<tr>
<td>2'-Cl DHB</td>
<td>2.3 (0.2)</td>
<td>2.7 (0.6)</td>
<td>5.0 (0.7)</td>
<td>2.1 (0.2)</td>
<td>1,400 (100)</td>
<td>3.6 (0.9)</td>
<td>1.6 (0.5)</td>
</tr>
<tr>
<td>3'-Cl DHB</td>
<td>58 (5)</td>
<td>0.40 (0.05)</td>
<td>300 (20)</td>
<td>5.3 (0.2)</td>
<td>40,000 (10,000)</td>
<td>7 (3)</td>
<td>0.13 (0.06)</td>
</tr>
<tr>
<td>4'-Cl DHB</td>
<td>70 (10)</td>
<td>0.38 (0.07)</td>
<td>430 (50)</td>
<td>6.4 (0.4)</td>
<td>40,000 (5,000)</td>
<td>11 (3)</td>
<td>0.16 (0.07)</td>
</tr>
<tr>
<td>4-Cl DHB</td>
<td>23 (1)</td>
<td>---</td>
<td>72 (1)</td>
<td>3.1 (0.1)</td>
<td>10,800 (400)</td>
<td>6.6 (0.3)</td>
<td>0.29 (0.06)</td>
</tr>
<tr>
<td>5-Cl DHB</td>
<td>26 (3)</td>
<td>2.5 (0.7)</td>
<td>63 (3)</td>
<td>2.4 (0.2)</td>
<td>12,000 (1000)</td>
<td>5.2 (0.9)</td>
<td>0.20 (0.06)</td>
</tr>
<tr>
<td>6-Cl DHB</td>
<td>6.5 (0.6)</td>
<td>1.3 (0.1)</td>
<td>38.3 (0.9)</td>
<td>5.9 (0.4)</td>
<td>5,500 (500)</td>
<td>7.0 (0.8)</td>
<td>1.1 (0.2)</td>
</tr>
<tr>
<td>2',6'-diCl DHB</td>
<td>0.007 (0.001)</td>
<td>---</td>
<td>0.036 (0.001)</td>
<td>5.1 (0.9)</td>
<td>50 (2)</td>
<td>0.69 (0.01)</td>
<td>100 (20)</td>
</tr>
</tbody>
</table>

Experiments were performed using air-saturated 20 mM HEPPS, 80 mM NaCl, pH 8.0 at 25°C except for 2',6'-diCl DHB where the experiment was performed in air-saturated potassium phosphate buffer, pH 7.0 (I=0.1) at 25°C. Values in parentheses represent standard errors. \textsuperscript{a} Taken from Table 3. \textsuperscript{b} $j_{3}^{\text{app}}$ was calculated by dividing $k_{\text{cat}}^{\text{app}}$ by the partition ratio (cf. equation 6) except for 2',6'-diCl DHB where $j_{3}^{\text{app}}$ was determined spectrophotometrically (cf. equation 7 and 8).
Figure 19. Steady-state cleavage of 2'-Cl DHB (A) and DHB in the presence of 2',6'-diCl DHB (B) by DHBD\textsubscript{LB400}. A. The DHBD\textsubscript{LB400}-catalyzed cleavage of 2'-Cl DHB. The experiment was performed using air-saturated 20 mM HEPES, 80 mM NaCl, pH 8.0 at 25°C. The line represents a best fit of the substrate inhibition equation to the data. The fitted parameters are \( K_{mA}^{app} = 2.3 \pm 0.2 \text{ \mu M} \), \( K_{iA}^{app} = 2.7 \pm 0.6 \text{ mM} \) and \( V = 46 \pm 1 \text{ \mu M/min} \). The inset shows the initial portion of the graph (0-50 \text{ \mu M} 2'-Cl DHB) in more detail. B. The DHBD\textsubscript{LB400}-catalyzed cleavage of DHB in presence of 2',6'-diCl DHB. The rate of DHB cleavage was determined using 4.8 \text{ \mu M} (\square), 8.3 \text{ \mu M} (■), 15.2 \text{ \mu M} (△), 48.5 \text{ \mu M} (○) and 82.9 \text{ \mu M} (●) DHB (potassium phosphate buffer, pH 7.0, I = 0.1, 25°C). Best fits were obtained using an equation similar in form to that describing competitive inhibition as described in Materials and Methods. The fit of the equation to the data yielded the following parameters: \( K_{mA}^{app} = 7 \pm 1 \text{ nM} \), \( K_{mDHBD}^{app} = 5.6 \pm 0.6 \text{ \mu M} \), and \( V = 61 \pm 1 \text{ \mu M/min} \).
Overall, the apparent specificity constant of DHBD for each of the chlorinated DHBs in air-saturated buffer was between 0.1 and 0.3 times that of unchlorinated DHB in the following order of specificity: DHB > 4'-Cl > 6-Cl > 3'-Cl > 2',6'-diCl > 4-Cl > 5-Cl > 2'-Cl (Table 8). In contrast, the $k_{\text{cat}}^{\text{app}}$ values of DHBD for 2',6'-diCl and 2'-Cl DHB were respectively 7000 times and 50 times slower than DHB.

Based on $j_{\text{act}}^{\text{app}} / K_{\text{mact}}^{\text{app}}$, the studied DHBs inactivated DHBD in the following order of efficiency: 2',6'-diCl > 2'-Cl > 6-Cl > 4-Cl > DHB > 5-Cl > 4'-Cl > 3'-Cl. 2',6'-diCl DHB and 2'-Cl DHB inactivated DHBD about 400 and 6 times more efficiently than DHB respectively, (Table 8) due to their low $K_{\text{mact}}^{\text{app}}$. However, in the presence of saturating concentrations of individual substrates, DHBD$_{400}$ would be inactivated in the following order: 4'-Cl > 3'-Cl > 6-Cl > 4-Cl > 5-Cl > 2'-Cl > DHB > 2',6'-diCl.

### 3.7.1.2 Inactivation-induced changes in DHBD$_{400}$ with 4-Cl DHB and 2'-Cl DHB

The inactivation-induced changes in DHBD$_{400}$ were investigated using 4-Cl and 2'-Cl DHB. Preparations of DHBD$_{400}$ inactivated with these substrates could be partially reactivated to 10 ± 1 and 12 ± 1% of its initial activity for 4-Cl and 2'-Cl DHB respectively through anaerobic incubation with a reducing agent (DTT). However, incubation with Fe(II) and DTT was necessary to restore 84 ± 6 and 71 ± 7% of the initial activity for 4-Cl and 2'-Cl DHB respectively. As determined by ion spray mass spectroscopy, preparations of DHBD$_{400}$ inactivated with 4-Cl and 2'-Cl DHB had molecular masses of 32,352 ± 4 and 32,350 ± 4 Da, respectively, identical to active DHBD$_{400}$. These data indicate that DHBD$_{400}$ was not covalently modified during mechanism-based inactivation. Thus, the O$_2$-dependent inactivation of DHBD$_{400}$ in presence of chlorinated DHBs, results in the oxidation and loss of the active site iron as described for other catecholic substrates in section 3.4.3.
3.7.1.3 In vivo inhibition of DHBD_{LB400}

The in vivo inhibition of DHBD_{LB400} was studied using the native host of the enzyme, Burkholderia sp. LB400, and a heterologous expression host, E. coli DH5α as described in section 2.11.7, using 80 μM 2',6'-diCl DHB instead of 3-chlorocatechol. The activity of DHBD_{LB400} in biphenyl-grown Burkholderia sp. LB400 and LB-grown E. coli DH5α was 0.3 and 0.2 U/OD_{600}, respectively. Addition of 80 μM 2',6'-diCl DHB to the assay completely inhibited the DHBD_{LB400} activity in both strains. Upon removal of 2',6'-diCl DHB from the cells, the activity of DHBD_{LB400} in Burkholderia sp. LB400 and in E. coli DH5α was 123 ± 1 and 87.5 ± 0.5% respectively when compared to the activity of controls where the cells were incubated with DHB but not inhibited with 2',6'-diCl DHB. This recovery occurred in the presence of chloramphenicol, indicating that protein synthesis is not required for the recovery of DHBD_{LB400} activity.

3.7.1.4 In vivo inhibition of biphenyl metabolism

Growth rates of Burkholderia sp. LB400 were evaluated at different stages during growth on biphenyl in the presence and absence of 2,6-diCl biphenyl. During the early log phase of growth, cells grew on biphenyl at a rate of 0.061 ± 0.005 ΔOD_{600}/hour. At the corresponding culture time, cells growing on a mixture of biphenyl and 2,6-diCl biphenyl did so at a rate of 0.010 ± 0.001 ΔOD_{600}/hour. At the mid log phase of growth, cells grew on biphenyl at a rate of 0.205 ± 0.004 ΔOD_{600}/hour. At the corresponding culture time, cells growing on the mixture did so at a rate of 0.041 ± 0.004 ΔOD_{600}/hour. Thus, 2,6-diCl biphenyl inhibited the growth of Burkholderia sp. LB400 on biphenyl 5-6 fold.

3.7.2 Reactivity of DHBD_{Pc-I} and DHBD_{Pc-III} with chlorinated DHBs

To investigate whether the reactivity of DHBD_{LB400} with DHB and PCB metabolites is representative of that of other DHBDs, two evolutionarily divergent DHBDs from another good PCB-degrader were studied.
3.7.2.1 Coupling of the reaction and stability in presence of \( \text{O}_2 \)

The coupling of DHB and \( \text{O}_2 \)-utilization in DHBD\(_{P6}\)-I and DHBD\(_{P6}\)-III was investigated using an \( \text{O}_2 \) electrode that had been calibrated using DHB and DHBD\(_{LB400}\), which constitutes a well-coupled system (see section 3.3.1). For each P6 isozyme, the amount of \( \text{O}_2 \) consumed corresponded to the amount of DHB added to the reaction mixture, demonstrating that the utilization of DHB and \( \text{O}_2 \) was tightly coupled in both enzymes.

The stability of each enzyme in the presence of \( \text{O}_2 \) was evaluated by determining \( j_1^{\text{app}} \), the pseudo-first order rate constant of inactivation in air-saturated buffer. The respective \( j_1^{\text{app}} \) of DHBD\(_{P6}\)-I and DHBD\(_{P6}\)-III was \((0.7 \pm 0.1) \times 10^{-3} \text{s}^{-1}\) and \((4.4 \pm 0.5) \times 10^{-3} \text{s}^{-1}\), which correspond to half-lives of 16 ± 2 min and 2.6 ± 0.3 min, respectively.

3.7.2.2 Steady-state kinetic analysis

Steady-state kinetic analyses in which DHB and \( \text{O}_2 \) concentrations were varied as described for DHBD\(_{LB400}\) (section 2.9.1) demonstrated that DHBD\(_{P6}\)-I and DHBD\(_{P6}\)-III utilize a compulsory order, ternary complex mechanism (Vaillancourt et al. 2002c). DHBD\(_{P6}\)-I, like DHBD\(_{LB400}\), was subject to substrate inhibition. In contrast, DHBD\(_{P6}\)-III was not. Both P6 isozymes, like DHBD\(_{LB400}\), were susceptible to inactivation during the steady-state turnover. Accordingly, the specificity of DHBD\(_{P6}\)-I and DHBD\(_{P6}\)-III for chloro DHBs, and their inactivation by these compounds were studied essentially as described for DHBD\(_{LB400}\) (see section 2.10.1 and 2.11.2).

The results of typical steady-state kinetic experiments performed by varying the concentration of monochlorinated DHBs in air-saturated buffer are shown in Figure 20. Interestingly, DHBD\(_{P6}\)-I and DHBD\(_{P6}\)-III cleaved some chloro DHBs more specifically than DHB (Table 9). Thus, DHBD\(_{P6}\)-I cleaved 6-Cl DHB 3 times more specifically than DHB, and cleaved 3'-Cl DHB, 4'-Cl DHB and unchlorinated DHB with similar specificity. Overall, DHBD\(_{P6}\)-I cleaved monochlorinated DHBs in the following order of specificity: 6-
CI > 3'-Cl ~ DHB ~ 4'-Cl > 2'-Cl > 4-Cl > 5-Cl. Similarly, DHBD\textsubscript{P6-III} cleaved a total of 4 monochlorinated DHBs more specifically than DHB, showing the highest apparent specificity for 4-Cl DHB in air-saturated buffer. DHBD\textsubscript{P6-III} cleaved the DHBs in the following order of specificity: 4-Cl > 5-Cl ~ 6-Cl ~ 3'-Cl > DHB > 2'-Cl ~ 4'-Cl. By comparison, DHBD\textsubscript{LB400} cleaved monochlorinated DHBs between 0.10 and 0.3 times that of unchlorinated DHB in the following order of specificity: DHB > 4'-Cl > 6-Cl >> 3'-Cl > 4-Cl > 5-Cl > 2'-Cl (section 3.7.1.1).

The apparent specificities of the two P6 isozymes for O\textsubscript{2} were studied at saturating concentrations of DHB and each of the six monochlorinated DHBs (Table 10). The apparent specificity constant of both DHBD\textsubscript{P6-I} and DHBD\textsubscript{P6-III} for O\textsubscript{2} ($k_{\text{app}}^B$) was highest in the presence of DHB. In contrast, the $k_B$ of DHBD\textsubscript{LB400} was highest in the presence of 3'-Cl and 4'-Cl DHB (Table 7). Moreover, chlorinated DHBs influenced the respective reactivities of DHBD\textsubscript{P6-I} and DHBD\textsubscript{P6-III} with O\textsubscript{2} in different ways. For example, the $K_{mB}^{\text{app}}$ of DHBD\textsubscript{P6-I} was lowest in the presence of 4'-Cl DHB, whereas that of DHBD\textsubscript{P6-III} was highest in the presence of this same compound. Nevertheless, the lowest $k_{\text{app}}^B$ of both isozymes was observed in the presence of 2'-Cl DHB, the most slowly cleaved substrate. However, the decrease in specificity versus that observed in the presence of DHB was more drastic for DHBD\textsubscript{P6-I} (80-fold) than for DHBD\textsubscript{P6-III} (3.6-fold). In comparison, the $k_B$ of DHBD\textsubscript{LB400} was 50-fold lower in the presence of 2'-Cl DHB than in the presence of DHB (Table 7).

As with DHBD\textsubscript{LB400}, the cleavage of 2',6'-diCl DHB by DHBD\textsubscript{P6-I} and DHBD\textsubscript{P6-III} was too slow to follow directly using the oxygen electrodes. Using a spectrophotometric assay to follow product formation, the $K_{mA}^{\text{app}}$ of DHBD\textsubscript{P6-III} for 2',6'-diCl DHB was 3.9 ± 0.4 µM (Figure 21B). However, the $K_{mA}^{\text{app}}$ of DHBD\textsubscript{P6-I} for 2',6'-diCl DHB could not be determined using this assay due to the very low $K_{mA}^{\text{app}}$ of the enzyme for this compound. Using DHB as a reporter substrate in the oxygen electrode assay, 2',6'-diCl DHB competitively inhibited the cleavage of DHB by DHBD\textsubscript{P6-I} with a $K_{i}^{\text{app}} = 0.14 ± 0.01$ µM in
air-saturated buffer (Figure 21A). Using the same assay, 2',6'-diCl DHB competitively inhibited the cleavage of DHB by DHBD<sub>P6</sub>-III with a \( K_{ic}^{app} = 2.6 \pm 0.2 \, \mu M \), confirming the spectrophotometrically determined value. Under saturating substrate conditions, 2',6'-diCl DHB was cleaved 900 times and 250 times slower than DHB by DHBD<sub>P6</sub>-I and DHBD<sub>P6</sub>-III respectively (Table 9).

The DHBD isozymes had different susceptibilities to inactivation \( (j_{3E}^{app} / K_{mA}^{app}) \) by the chlorinated substrates. Thus, DHBD<sub>P6</sub>-I was inactivated by these compounds in the following order: 2',6'-diCl > 6-Cl > 3'-Cl > 2'-Cl > 4'-Cl > 5-Cl > DHB > 4-Cl. In contrast, DHBD<sub>P6</sub>-III was inactivated by these compounds in the following order: 5-Cl > 4-Cl > 6-Cl > 3'-Cl > 2',6'-diCl > 4'-Cl ~ DHB > 2'-Cl. These patterns differ to that of DHBD<sub>LB400</sub> (2',6'-diCl > 2'-Cl > 6-Cl > 4-Cl > DHB ~ 5-Cl > 4'-Cl > 3'-Cl (Table 8)). In the presence of saturating concentrations of individual substrates, DHBD<sub>P6</sub>-I would be inactivated by these compounds in the following order: 6-Cl > 3'-Cl > 5-Cl > 4'-Cl > 4-Cl > DHB > 2',6'-diCl > 2'-Cl. In contrast, DHBD<sub>P6</sub>-III would be inactivated by these compounds in the following order: 5-Cl > 4'-Cl > 6-Cl > DHB > 3'-Cl ~ 4-Cl > 2',6'-diCl > 2'-Cl. Again, these patterns differ to that of DHBD<sub>LB400</sub> (4'-Cl > 3'-Cl ~ 6-Cl > 4-Cl > 5-Cl > 2'-Cl > DHB > 2',6'-diCl (Table 8)).
Table 9. Apparent steady-state kinetic and inactivation parameters of DHBD<sub>P6-I</sub> and DHBD<sub>P6-III</sub> for chlorinated DHBs.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Compound</th>
<th>$K_{\text{mA}}^{\text{app}}$</th>
<th>$K_{\text{Ia}}^{\text{app}}$</th>
<th>$k_{\text{cat}}^{\text{app}}$</th>
<th>$k_{\text{A}}^{\text{app}}$</th>
<th>Partition Ratio</th>
<th>$j_3^{\text{app}}$</th>
<th>$j_3^{\text{app}} / K_{\text{mA}}^{\text{app}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\mu M$</td>
<td>$mM$</td>
<td>$s^{-1}$</td>
<td>$x 10^6 M^{-1}s^{-1}$</td>
<td>$x 10^{-3} s^{-1}$</td>
<td>$x 10^3 M^{-1}s^{-1}$</td>
<td></td>
</tr>
<tr>
<td>DHBD&lt;sub&gt;P6-I&lt;/sub&gt;</td>
<td>DHB</td>
<td>6 (1)</td>
<td>2.2 (0.7)</td>
<td>77 (3)</td>
<td>13 (2)</td>
<td>31,000 (3,000)</td>
<td>2.5 (0.4)</td>
<td>0.4 (0.1)</td>
</tr>
<tr>
<td></td>
<td>2'-Cl DHB</td>
<td>0.22 (0.06)</td>
<td>---</td>
<td>2.02 (0.04)</td>
<td>9 (2)</td>
<td>1,650 (80)</td>
<td>1.2 (0.1)</td>
<td>5 (2)</td>
</tr>
<tr>
<td></td>
<td>3'-Cl DHB</td>
<td>1.9 (0.3)</td>
<td>1.5 (0.3)</td>
<td>27.1 (0.8)</td>
<td>14 (2)</td>
<td>2,300 (200)</td>
<td>12 (1)</td>
<td>6 (2)</td>
</tr>
<tr>
<td></td>
<td>4'-Cl DHB</td>
<td>2.6 (0.3)</td>
<td>7 (3)</td>
<td>29.1 (0.7)</td>
<td>11 (1)</td>
<td>3,300 (100)</td>
<td>8.9 (0.6)</td>
<td>3.5 (0.6)</td>
</tr>
<tr>
<td></td>
<td>4-Cl DHB</td>
<td>22 (3)</td>
<td>2.6 (0.7)</td>
<td>109 (5)</td>
<td>4.9 (0.4)</td>
<td>27,800 (200)</td>
<td>3.9 (0.2)</td>
<td>0.18 (0.03)</td>
</tr>
<tr>
<td></td>
<td>5-Cl DHB</td>
<td>5.3 (0.6)</td>
<td>---</td>
<td>7.2 (0.2)</td>
<td>1.4 (0.1)</td>
<td>660 (30)</td>
<td>11.0 (0.8)</td>
<td>2.1 (0.4)</td>
</tr>
<tr>
<td></td>
<td>6-Cl DHB</td>
<td>1.9 (0.3)</td>
<td>3.0 (0.9)</td>
<td>72 (2)</td>
<td>39 (5)</td>
<td>4,080 (20)</td>
<td>17.7 (0.6)</td>
<td>9 (2)</td>
</tr>
<tr>
<td></td>
<td>2'6'-diCl DHB</td>
<td>0.14 (0.01)</td>
<td>---</td>
<td>0.088 (0.004)</td>
<td>0.61 (0.09)</td>
<td>50 (1)</td>
<td>1.7 (0.1)</td>
<td>12 (2)</td>
</tr>
<tr>
<td>DHBD&lt;sub&gt;P6-III&lt;/sub&gt;</td>
<td>DHB</td>
<td>17 (2)</td>
<td>---</td>
<td>17.0 (0.6)</td>
<td>1.0 (0.1)</td>
<td>895 (60)</td>
<td>19.0 (2.0)</td>
<td>1.1 (0.2)</td>
</tr>
<tr>
<td></td>
<td>2'-Cl DHB</td>
<td>10 (1)</td>
<td>---</td>
<td>5.3 (0.1)</td>
<td>0.51 (0.04)</td>
<td>2,000 (200)</td>
<td>2.7 (0.3)</td>
<td>0.25 (0.05)</td>
</tr>
<tr>
<td></td>
<td>3'-Cl DHB</td>
<td>10.4 (0.7)</td>
<td>---</td>
<td>14.2 (0.3)</td>
<td>1.36 (0.08)</td>
<td>870 (70)</td>
<td>16 (2)</td>
<td>1.6 (0.3)</td>
</tr>
<tr>
<td></td>
<td>4'-Cl DHB</td>
<td>27 (3)</td>
<td>---</td>
<td>12.7 (0.4)</td>
<td>0.47 (0.04)</td>
<td>410 (60)</td>
<td>31 (5)</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td></td>
<td>4-Cl DHB</td>
<td>1.8 (0.2)</td>
<td>---</td>
<td>4.0 (0.1)</td>
<td>2.2 (0.2)</td>
<td>260 (40)</td>
<td>15 (3)</td>
<td>9 (3)</td>
</tr>
<tr>
<td></td>
<td>5-Cl DHB</td>
<td>4.3 (0.6)</td>
<td>---</td>
<td>6.6 (0.3)</td>
<td>1.5 (0.2)</td>
<td>97 (20)</td>
<td>70.0 (20)</td>
<td>16 (6)</td>
</tr>
<tr>
<td></td>
<td>6-Cl DHB</td>
<td>3.9 (0.8)</td>
<td>---</td>
<td>5.5 (0.3)</td>
<td>1.4 (0.2)</td>
<td>200 (10)</td>
<td>28 (3)</td>
<td>7 (2)</td>
</tr>
<tr>
<td></td>
<td>2'6'-diCl DHB</td>
<td>3.9 (0.4)</td>
<td>---</td>
<td>0.069 (0.002)</td>
<td>0.017 (0.001)</td>
<td>14.8 (0.3)</td>
<td>5.4 (0.2)</td>
<td>1.4 (0.1)</td>
</tr>
</tbody>
</table>

Experiments were performed using air-saturated potassium phosphate buffer, pH 7.0 (I=0.1) at 25°C. Values in parentheses represent standard errors.
Figure 20. Steady-state cleavage of 6-Cl DHB by DHBD\textsubscript{P6-I} and 4-Cl DHB by DHBD\textsubscript{P6-III}. The experiments were performed using air-saturated potassium phosphate buffer, pH 7.0, I = 0.1 at 25°C. A. The DHBD\textsubscript{P6-I} catalyzed cleavage of 6-Cl DHB. The line represents a best fit of the substrate inhibition equation to the data. The fitted parameters are $K_{mA}^{app} = 1.9 \pm 0.3$ µM, $K_{IA}^{app} = 3.0 \pm 0.9$ µM and $V = 41 \pm 1$ µM/min. The inset shows the initial portion of the graph (0-30 µM 6-Cl DHB) in more detail. B. The DHBD\textsubscript{P6-III} catalyzed cleavage of 4-Cl DHB. The line represents a best fit of the Michaelis-Menten equation to the data. The fitted parameters are $K_{mA}^{app} = 1.8 \pm 0.2$ µM and $V = 53 \pm 1$ µM/min. The inset shows the initial portion of the graph (0-30 µM 4-Cl DHB) in more detail.
Table 10. Apparent steady-state kinetic parameters of DHBD<sub>p6</sub>-I and DHBD<sub>p6</sub>-III for O<sub>2</sub> in the presence of monochlorinated DHBs.

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Compound</th>
<th>( K_{n83}^{\text{app}} ) (( \mu M ))</th>
<th>( k_{\text{cat}}^{\text{app}} ) (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>( k_B^{\text{app}} ) (x 10&lt;sup&gt;6&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt;s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHBD&lt;sub&gt;p6&lt;/sub&gt;-I</td>
<td>DHB</td>
<td>250 (20)</td>
<td>121 (4)</td>
<td>0.48 (0.02)</td>
</tr>
<tr>
<td></td>
<td>2'-Cl DHB</td>
<td>8,000 (2,000)</td>
<td>50 (10)</td>
<td>0.0055 (0.0001)</td>
</tr>
<tr>
<td></td>
<td>3'-Cl DHB</td>
<td>240 (20)</td>
<td>32 (1)</td>
<td>0.134 (0.005)</td>
</tr>
<tr>
<td></td>
<td>4'-Cl DHB</td>
<td>167 (4)</td>
<td>32.1 (0.4)</td>
<td>0.193 (0.003)</td>
</tr>
<tr>
<td></td>
<td>4-Cl DHB</td>
<td>1,030 (30)</td>
<td>303 (5)</td>
<td>0.294 (0.005)</td>
</tr>
<tr>
<td></td>
<td>5-Cl DHB</td>
<td>330 (50)</td>
<td>10.7 (0.8)</td>
<td>0.032 (0.003)</td>
</tr>
<tr>
<td></td>
<td>6-Cl DHB</td>
<td>420 (30)</td>
<td>103 (4)</td>
<td>0.243 (0.008)</td>
</tr>
<tr>
<td>DHBD&lt;sub&gt;p6&lt;/sub&gt;-III</td>
<td>DHB</td>
<td>480 (80)</td>
<td>46 (3)</td>
<td>0.095 (0.009)</td>
</tr>
<tr>
<td></td>
<td>2'-Cl DHB</td>
<td>700 (100)</td>
<td>18 (2)</td>
<td>0.026 (0.003)</td>
</tr>
<tr>
<td></td>
<td>3'-Cl DHB</td>
<td>390 (50)</td>
<td>26 (1)</td>
<td>0.067 (0.006)</td>
</tr>
<tr>
<td></td>
<td>4'-Cl DHB</td>
<td>730 (80)</td>
<td>43 (3)</td>
<td>0.060 (0.003)</td>
</tr>
<tr>
<td></td>
<td>4-Cl DHB</td>
<td>120 (20)</td>
<td>5.0 (0.2)</td>
<td>0.044 (0.005)</td>
</tr>
<tr>
<td></td>
<td>5-Cl DHB</td>
<td>600 (100)</td>
<td>19 (1)</td>
<td>0.031 (0.003)</td>
</tr>
<tr>
<td></td>
<td>6-Cl DHB</td>
<td>400 (100)</td>
<td>16 (2)</td>
<td>0.036 (0.006)</td>
</tr>
</tbody>
</table>

Experiments were performed using potassium phosphate buffer, pH 7.0 (I=0.1) at 25°C with saturating concentrations of monochlorinated DHBs. Values in parentheses represent standard errors.
Figure 21. Steady-state utilization of 2’,6’-diCl DHB by DHBD_{P6}-I and DHBD_{P6}-III. The experiments were performed using air-saturated potassium phosphate buffer, pH 7.0, I = 0.1 at 25°C. A. The DHBD_{P6}-I-catalyzed cleavage of DHB in presence of 2’,6’-diCl DHB. The rate of DHB cleavage was determined using 3.1 μM (□), 5.4 μM (■), 9.3 μM (▲), 13.1 μM (○), 16.9 μM (+), 54 μM (●), and 82.9 μM (×) DHB. Fits were obtained using an equation similar in form to that describing competitive inhibition as described in Materials and Methods. The fit of the equation to the data yielded the following parameters: $K_m^{app} = 0.14 \pm 0.01$ μM, $K_{mDHB}^{app} = 3.4 \pm 0.3$ μM, and $V = 60 \pm 1$ μM/min. B. The DHBD_{P6}-III-catalyzed cleavage of 2’,6’-diCl DHB. The line represents a best fit of the Michaelis-Menten equation to the data. The fitted parameters are $K_m^{app} = 3.9 \pm 0.4$ μM and $V = 3.0 \pm 0.1$ μM/min. The inset shows the initial portion of the graph (0-50 μM 2’,6’-diCl DHB) in more detail.
3.8 Protonation state of substrates in solution and bound to DHBD$_{LB400}$

The protonation state of DHBD-bound DHB and 3-nitrocatechol were studied using UV/vis absorption spectroscopy. To facilitate the interpretation of the spectra of ES species, the $pK_a$ values of the free catechols were determined, and the spectra of the compounds in their different protonation states were recorded.

3.8.1 $pK_a$ values of 3-substituted catechols

In handling solutions of catechols, it was noted that these compounds were $O_2$-labile, particularly in their monoanionic and dianionic forms. Exposure of catecholic solutions to air caused a rapid change in their colour followed by the formation of a precipitate, most probably corresponding to the formation of quinones and their subsequent polymerization (Raff and Ettling 1963-1970). The $O_2$-lability of the catechols, even in the absence of DHBD$_{LB400}$, required all measurements to be made under strictly anaerobic conditions as described in section 2.13.1.

Catechols can exist in three different formal charge states depending on the ionization state of the two hydroxyl groups of the catechol moiety (Figure 22). Table 11 describes the first $pK_a$ value that was determined for different 3-substituted catechols by titration. From these values, it can be seen that removal of the first proton is almost insensitive to the presence of a 3-methyl or 3-phenyl substituent whereas the $pK_a$ decreases with the presence of a 3-chloro or 3-nitro substituent, two electron withdrawing groups that stabilize the formed anion. The lower $pK_a$ of 3-nitrocatechol compared to 3-chlorocatechol can be explained by the stronger electron withdrawing capacity of the nitro substituent compared to the chloro (Exner 1978). The determined values are in good agreement with the published value of 9.45 for catechol and 6.48 for 3-nitrocatechol (Smith and Martell 1989). The $pK_a$ for removal of the second proton from the different compounds could not be determined from titration data in aqueous solution. The second $pK_a$ values of catechol, 3-methylcatechol and DHB are higher than the second $pK_a$ values of 1,3-dihydroxybenzene.
and 1,4-dihydroxybenzene (−11.0−11.4; Smith and Martell 1989), presumably due to the intramolecular hydrogen bond between the remaining oxygen-bound proton and the neighbouring ionized oxygen atom (Gerhards et al. 1996, Ramirez and Lopez-Navarrete 1993).

\[
\begin{align*}
R & \quad \text{OH} & \quad \text{OH} & \quad R \\
& \quad \text{OH} & \quad \text{O} & \quad \text{H} & \quad \text{OH} & \quad \text{O} & \quad \text{O} \\
\end{align*}
\]

**Figure 22.** The protonation states of catechols. \( pK_a^1 \) can be evaluated based on a titration curves and \( pK_a^2 \) can be estimated using UV/vis absorption spectra recorded in 0.05% NaOH (−pH 12), 0.5% NaOH (−pH 13), and 10% NaOH (−pH 14).

<table>
<thead>
<tr>
<th>Compound</th>
<th>( pK_a^1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>9.40 (0.05)</td>
</tr>
<tr>
<td>3-Methylcatechol</td>
<td>9.5 (0.1)</td>
</tr>
<tr>
<td>DHB</td>
<td>9.4 (0.05)</td>
</tr>
<tr>
<td>3-Chlorocatechol</td>
<td>8.1 (0.1)</td>
</tr>
<tr>
<td>3-Nitroocatechol</td>
<td>6.6 (0.1)</td>
</tr>
</tbody>
</table>

Values in parentheses represent standard deviations.

UV/vis absorption spectroscopy was used to identify the predominant forms of DHB, catechol, in 0.5% NaOH (−pH 13), 10% NaOH (−pH 14), methanol containing an excess of sodium methoxide \( (pK_a = 15.54; \) McMurry 1992) and in t-butanol containing an excess of sodium t-butoxide \( (pK_a = 18.00; \) McMurry 1992).

The UV/vis absorption spectra of DHB in 0.5% NaOH were essentially identical to those in pH 11 buffer solution, consistent with the presence of the monoanionic species.
alone. In contrast, the UV/vis absorption spectra recorded using 10% NaOH solution, were intermediate between the monoanionic and dianionic species. This is in good agreement with a ultraviolet resonance Raman (UVRR) study (Barbosa 2002, Vaillancourt et al. 2002b) that showed that the two species were present at similar concentrations in 10% NaOH, indicating that the $pK_a^2$ of DHB is approximately 14. Interestingly, UV/vis absorption and UVRR (Barbosa 2002, Vaillancourt et al. 2002b) spectra indicated that DHB was predominantly monoanionic in a solution of methanol containing an excess of sodium methoxide ($pK_a = 15.54$), and thus that the $pK_a^2$ of DHB is higher in the lower dielectric solvent. However, a solution of DHB in t-butanol containing an excess of sodium t-butoxide ($pK_a = 18.00$) produced a strongly red-shifted UV/vis absorption spectrum and a new UVRR spectrum (Barbosa 2002, Vaillancourt et al. 2002b) that was unchanged in t-butanol-OD, as expected for the dianionic species.

The UV/vis and UVRR (Barbosa 2002, Vaillancourt et al. 2002b) spectra of catechol were also recorded in t-butanol / t-butoxide solution and in methanol / methoxide solution and produced similar results, identifying the monoanion in methanol / methoxide solution and the dianion in t-butanol / t-butoxide solution. In contrast with DHB, the UV/vis spectrum of catechol in 0.5% NaOH was intermediate between the monoanionic and dianionic species and the UVRR study (Barbosa 2002, Vaillancourt et al. 2002b) showed a superposition of the monoanionic and dianionic spectral features, consistent with the published $pK_a^2$ of catechol (13.3; Smith and Martell 1989). As in the case of DHB, these data indicate that the $pK_a^2$ of catechol is higher in the lower dielectric solvent. This is consistent with the observation that the $pK_a^2$ of catechol increases with methanol concentration (Tyson and Martell 1968).

UV/vis absorption spectroscopy was used to identify the predominant forms of 3-nitrocatechol in 0.05% NaOH (~pH 12), 0.5% NaOH (~pH 13) and in 10% NaOH (~pH 14). The spectra observed in 0.5% and 10% NaOH were assigned to the dianion by comparison with the spectra obtained in 0.05% NaOH (~pH 12), which was intermediate between the
spectra that were assigned to the monoanion (recorded at pH 8-11) and the spectra recorded in 0.5-10% NaOH. Even though no unequivocal assignment was performed with UVRR, the UV/vis absorption spectra are consistent with the published $pK_a^2$ of 3-nitrocatechol (11.8; Smith and Martell 1989).

3.8.2 UV/Vis absorption spectroscopy of DHB, catechol and 3-nitrocatechol

The electronic absorption spectra of biphenyl, DHB and catechol were recorded to compare the different transitions in these molecules. Spectra of biphenyl and the neutral species of catechol and DHB are compared in Figure 23A. The assigned maxima at the shortest wavelengths (< 210 nm) are to be regarded with caution due to the high background absorption of the solvent in this region. Interestingly, the spectrum of DHB can be approximated by the superposition of the spectra of catechol and biphenyl, which permitted the assignment of the different transitions using the composite molecule model (Barbosa 2002, Vaillancourt et al. 2002b). This approach was successfully applied to biphenyl (Dick and Hohlneicher 1985, Rubio et al. 1995) in which the two subsystems were two benzene molecules.

Deprotonation of the hydroxyl groups of DHB induced dramatic changes in the absorption spectrum (Figure 23B). In general, the removal of each proton resulted in successive red shifts of the lowest lying transitions and an increase in the extinction coefficients. In DHB, the most obvious changes occur in the shift of the lowest energy transition from 283 nm for the neutral species in aqueous solution (pH 8) to 305 nm for the monoanionic species (pH 11) and to 348 nm for the dianionic species in t-butanol / t-butoxide. The next lowest transition undergoes a similar red shift from 243 in the neutral species to 261 and 280 nm in the monoanion and dianion, respectively. The same pattern of decreased energy and increased intensity was observed in the transitions of catechol as each hydroxyl proton was removed (Table 12). The band maxima and extinction coefficients for each of the three species of both catechol and DHB, as well as the biphenyl transitions, are
given in Table 12. Note that impurities in the t-butoxide absorbed at the shorter wavelengths, limiting the reliable measurement of the dianionic catechols to wavelengths longer than approximately 245 nm.

In contrast to biphenyl, DHB and catechol, which absorbed only in the UV region, 3-nitrocatechol absorbed strongly in the UV and visible regions, due to the presence of a nitro substituent at position C3. Deprotonation of the hydroxyl groups of 3-nitrocatechol also induced dramatic changes in the absorption spectrum (Table 13), particularly for the lowest energy transition where removal of each proton resulted in successive red shifts of 69 and 87 nm.

![Figure 23. Electronic absorption spectra of DHB, biphenyl and catechol. Panel A depicts: (a) 100 μM DHB in 20 mM Tris pH 8.0; (b) 100 μM biphenyl in methanol; and (c) 100 μM catechol in 20 mM Tris pH 8.0. Panel B depicts: (a) 100 μM neutral DHB in t-butanol; (b) 100 μM neutral DHB in 20 mM Tris pH 8.0; (c) 100 μM monoanionic DHB in potassium phosphate buffer pH 11 (I = 0.1); and (d) 100 μM dianionic DHB in 250 mM sodium t-butoxide/t-butanol solution. For clarity, each spectrum was offset by one absorbance unit with respect to the spectra below.](image)
<table>
<thead>
<tr>
<th></th>
<th>Biphenyl</th>
<th>DHB</th>
<th>DHB⁻</th>
<th>DHB²⁻</th>
<th>Catechol</th>
<th>Catechol⁻</th>
<th>Catechol²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>nm, M⁻¹cm⁻¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>207 (24,900)</td>
<td>~210 (&lt;30,000)</td>
<td>&lt;206</td>
<td>&lt;250</td>
<td>&lt;200 (&lt;16,000)</td>
<td>&lt;220</td>
<td>&lt;240</td>
<td></td>
</tr>
<tr>
<td>218 (22,700)</td>
<td>231 (22,100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>247 (17,200)</td>
<td>243 (11,800)</td>
<td>261 (8,700)</td>
<td>280 (8,000)</td>
<td>215 (6,300)</td>
<td>237 (7,700)</td>
<td>256 (8,700)</td>
<td></td>
</tr>
<tr>
<td>283 (2,500)</td>
<td>305 (3,200)</td>
<td>348 (4,000)</td>
<td>275 (2,300)</td>
<td>289 (4,300)</td>
<td>308 (7,600)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The spectra of catechol and DHB were taken in 20 mM Tris pH 8.0. The spectra of catechol⁻ and DHB⁻ were taken in potassium phosphate buffer pH 11 (I = 0.1). The spectra of catechol²⁻ and DHB²⁻ were taken in 250 mM sodium t-butoxide / t-butanol solution. Values in parentheses represent extinction coefficients in M⁻¹cm⁻¹.
Table 13. Electronic absorption bands of 3-nitrocatechol

<table>
<thead>
<tr>
<th></th>
<th>3-Nitrocatechol</th>
<th>3-Nitrocatechol(^+)</th>
<th>3-Nitrocatechol(^{2-})</th>
</tr>
</thead>
<tbody>
<tr>
<td>nm, M(^{-1})cm(^{-1})</td>
<td>nm, M(^{-1})cm(^{-1})</td>
<td>nm, M(^{-1})cm(^{-1})</td>
<td></td>
</tr>
<tr>
<td>215 (8,100)</td>
<td>222 (7,900)</td>
<td>225 (5,600)</td>
<td></td>
</tr>
<tr>
<td>~237 (3,900)</td>
<td>~255 (4,400)</td>
<td>~272 (5,000)</td>
<td></td>
</tr>
<tr>
<td>298 (5,100)</td>
<td>307 (3,500)</td>
<td>358 (4,900)</td>
<td></td>
</tr>
<tr>
<td>364 (1,300)</td>
<td>435 (2,600)</td>
<td>520 (2,000)</td>
<td></td>
</tr>
</tbody>
</table>

The spectra of 3-nitrocatechol, 3-nitrocatechol\(^+\), and 3-nitrocatechol\(^{2-}\) were recorded using potassium phosphate buffer (I= 0.1) pH 3.0, 20 mM Tris pH 8.0, and 10% NaOH solution, respectively. Values in parentheses represent extinction coefficients in M\(^{-1}\)cm\(^{-1}\).

3.8.3 Solvent induced UV/Vis absorption spectral changes

The absorption spectrum of DHB in a given ionization state was unaffected by the identity of the buffer in aqueous solutions (phosphate, CAPS or Tris). However, the spectra of neutral and monoanionic DHB differed in aqueous and alcoholic solutions. The low lying transitions of neutral DHB in \(t\)-butanol (Figure 23B, spectrum a) or methanol were red-shifted from that in aqueous solution at pH 8.0 (Figure 23B, spectrum b). Solvent-induced band shifts of similar magnitude were observed for monoanionic DHB (i.e. a 7 nm red shift of the 305 nm transition in methanol/methoxide vs. aqueous solution). Similar shifts in the electronic transitions of biphenyl have been attributed to the twist angle of the two rings (Suzuki 1959 and Rubio et al. 1995). The transition of biphenyl at 247 nm shifts to shorter wavelengths as the aromatic rings of the biphenyl molecule twist away from co-planarity. It is therefore concluded that DHB is more planar in \(t\)-butanol and methanol than in water, regardless of its ionization state. In agreement with this interpretation, there were no significant solvent-induced shifts in the transitions of catechol.

The absorption spectrum of 3-nitrocatechol in a given ionization state was slightly affected by the identity of the buffer in aqueous solutions (5-10 nm). The spectra of 3-
nitrocatechol also differed in aqueous and alcoholic solutions. The shifts of the absorption peaks of nitrophenols in different solvents is a well documented phenomenon (Abboud et al. 1982, Kamlet and Taft 1982, Schmid et al. 1986) and is attributed to differences in the hydrogen bonding environment of the nitro group.

While the solvent-induced shifts in the DHB and 3-nitrocatechol transitions are significant and easily measurable, their magnitude is not sufficient to explain those induced by deprotonation, which shift the lowest energy transition of DHB and 3-nitrocatechol by up to 40 and 70 nm, respectively. Moreover, due to the lower pKₐ values of 3-nitrocatechol, the three ionic species could be studied in water. Thus, the effects of non-aqueous solvents on this compound need not be considered.

3.8.4 UV/Vis absorption of DHBD_{LB400}-bound DHB and 3-nitrocatechol

The spectra of DHBD_{LB400}:DHB and DHBD_{LB400}:3-nitrocatechol were recorded at pH 8.0. Both spectra are dominated by absorption due to peptide bonds and aromatic residues of the enzyme at wavelengths lower than ~315nm. Thus, the spectra of bound DHB and bound 3-nitrocatechol were obtained by subtracting the spectrum of DHBD_{LB400} from those of the DHBD_{LB400}:DHB and DHBD_{LB400}:3-nitrocatechol complexes, respectively (Figure 24). The lower affinity of DHBD_{LB400} for 3-nitrocatechol necessitated the use of high protein concentrations. For this reason, the difference spectrum of bound 3-nitrocatechol is not accurate at wavelengths below 315 nm. The lowest energy transition of 3-nitrocatechol was nevertheless clearly observable as it occurs at longer wavelengths.
Figure 24. Electronic absorption spectra of DHBD_{LB400}-bound DHB and DHBD_{LB400}-bound 3-nitrocatechol. A. DHBD_{LB400}-bound DHB (a) Spectrum of 43 μM DHBD_{LB400} with 25 μM DHB. (b) Spectrum of 43 μM DHBD_{LB400}. (c) Difference spectrum (a - b). (d) Spectrum of 100 μM monoanionic DHB (potassium phosphate buffer pH 11 (I = 0.1), 25°C). B. DHBD_{LB400}-bound 3-nitrocatechol (a) Spectrum of 95 μM DHBD_{LB400} with 71 μM 3-nitrocatechol. (b) Spectrum of 95 μM DHBD_{LB400}. (c) Difference spectrum (a - b). (d) Spectrum of 75 μM monoanionic 3-nitrocatechol. All spectra except spectrum d, panel A, were recorded in 20 mM Tris pH 8.0, 25°C. For clarity, spectra a and b were overlaid and the others were offset by 1 (panel A) and 0.25 (panel B) absorbance unit with respect to the one below it.

Table 14. Electronic absorption bands of DHBD_{LB400}-bound DHB and DHBD_{LB400}-bound 3-nitrocatechol

<table>
<thead>
<tr>
<th>DHBD_{LB400}-bound DHB</th>
<th>DHBD_{LB400}-bound 3-nitrocatechol</th>
</tr>
</thead>
<tbody>
<tr>
<td>nm, M⁻¹ cm⁻¹</td>
<td>nm, M⁻¹ cm⁻¹</td>
</tr>
<tr>
<td>~261 (6000)</td>
<td>299 (2300)</td>
</tr>
<tr>
<td></td>
<td>439 (1900)</td>
</tr>
</tbody>
</table>

The spectra of DHBD_{LB400}-bound DHB and DHBD_{LB400}-bound 3-nitrocatechol were taken in 20 mM Tris, pH 8.0. Values in parentheses represent extinction coefficients in M⁻¹ cm⁻¹.
It is possible that substrate-induced changes in the environment of Tyr250, a conserved tyrosine in close proximity of the active site iron, might affect the spectrum of DHBD$_{LB400}$. However, it was shown that such changes mostly affect the absorption spectrum around 278 nm (Pace et al. 1995), clearly not in the region where significant maxima are observed at 299 nm and 439 nm for bound DHB and bound 3-nitrocatechol (Table 14). Through comparison with spectra of free DHB and free 3-nitrocatechol, these bands were respectively assigned to the lowest transition of bound DHB and bound 3-nitrocatechol. Addition of DHB to a concentration twice that of the enzyme concentration resulted in an absorption peak corresponding to neutral DHB in buffer superimposed upon the spectrum of the bound species. In contrast, addition of 3-nitrocatechol to a concentration twice that of the enzyme concentration resulted in an absorption peak corresponding to monoanionic 3-nitrocatechol. Both results are in good agreement with the determined pK$_a$ values.

The maximum for the lowest transition of DHBD$_{LB400}$-bound DHB is most similar to that of monoanionic DHB in aqueous solution (305 nm), indicating that the bound substrate is monoanionic. Moreover, the 6 nm blue shift of the transition of bound DHB with respect to that of monoanionic DHB in aqueous solution suggests that the twist angle of DHB increases upon binding to the enzyme. This conclusion is consistent with the structural data, which show that the two rings of DHB are orthogonal to within 6 degrees in the ES complex (Figure 6).

The maximum for the lowest transition of DHBD$_{LB400}$-bound 3-nitrocatechol is most similar to that of monoanionic 3-nitrocatechol in aqueous solution (435 nm), indicating that the bound substrate is monoanionic. Moreover, the 4 nm red shift of the transition of bound 3-nitrocatechol with respect to that of monoanionic 3-nitrocatechol in aqueous solution suggests that the hydrogen bonding environment of the nitro group changes upon binding to the enzyme. It is also possible that the red shift reflects the binding of part (< 5%) of the 3-nitrocatechol as a dianion. Significantly, the blue-shift of bound DHB with respect to
monoanionic DHB and the red-shift of bound 3-nitrocatechol with respect to monoanionic 3-nitrocatechol suggest that these shifts do not share the same chemical origin, such as hydrogen bonding of the catecholic hydroxyls in the enzyme active site or the binding of the catechol to the iron.
4. DISCUSSION

4.1 Purification of DHBD<sub>LB400</sub>

The described rapid, anaerobic purification of DHBD<sub>LB400</sub> yielded a preparation whose specific activity is at least twice that of aerobically purified preparations of the same enzyme (Eltis et al. 1993) or a highly similar one (Furukawa and Arimura 1987). 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. EPR, magnetic circular dichroic and X-ray absorption spectra indicated that DHBD<sub>LB400</sub> 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 (Davis et al. 2002).

4.2 Specificity of DHBD<sub>LB400</sub> for 3-substituted catechols

The steady-state kinetic analyses revealed that the specificity and rate of catalysis depend on at least two factors: geometric and electronic. The fit of the substrate in the active site influences the geometry when bound to the iron in the ternary complex and can therefore affect the specificity and rate of catalysis. For example, when comparing the values obtained for the substrates with alkyl/phenyl substituents in C-3, it can be seen that in most cases the $K_{\text{mA}}^{\text{app}}$ value increases and the $k_{\text{cat}}^{\text{app}}$ value decreases with decreasing C-3 substituent size. This would seem to be mainly due to geometric factors as the electronic properties of the alkyl and phenyl substituents are fairly similar (Exner 1978). However, by comparing the ES complexes of DHBD with DHB, 3-methyl catechol and catechol, it can be seen that the catechol ring binds in a restricted pocket that is highly complementary in size and shape and that no significant geometric differences are observed in the different complexes. If it is assumed that the crystallographically observed binding mode is the one that leads to productive catalysis, then the increase in $K_{\text{mA}}^{\text{app}}$ and decrease in $k_{\text{cat}}^{\text{app}}$ values may be due to the fact that the optimal geometry required for cleavage in the ternary complex is not present as.
often in catechol complexes with smaller substituents as movement in the substrate pocket can occur more readily in the absence of the distal ring.

The electronic factor can be seen by comparing the values obtained with 3-chlorocatechol and 3-nitrocatechol with the ones obtained with the alkyl substituted catechols: the rate of catalysis decreases dramatically as the electron withdrawing capacity of the catechol increases. This could be due to a slower electron transfer from the catecholate to the iron and/or a lower reactivity of the semiquinone-Fe(II)-superoxide complex. Consistent with this hypothesis, catechols with electron-withdrawing substituents were not cleaved in a model extradiol cleavage reaction (Lin et al. 2001).

The steady-state kinetic analysis also revealed that the ability of DHBD_{LB400} to utilize O_2 is strikingly different from that of evolutionarily related C23Os. Although the steady-state utilization of O_2 has not been investigated in many extradiol dioxygenases, the K_{mO_2} of DHBD_{LB400} is two to three orders of magnitude higher than those of a number of C23Os determined using catechol (0.7 to 10 μM; Hori et al. 1973, Kobayashi et al. 1995, Kukor and Olsen 1996). Significantly, the nature of the substituent at C-3 (alkyl/phenyl) of the catechol does not appear to appreciably affect the K_{mO_2} of DHBD_{LB400}. The K_{mO_2} of an extradiol-type 2-aminophenol dioxygenase was reported to be 710 μM (Lendenmann and Spain 1996). Intradiol dioxygenases also have a wide range of K_{mO_2}. For example, 3,4-PCD from P. putida has a K_{mO_2} of 43 μM (50 mM Tris-acetate, pH 7.5, 24°C; Hori et al. 1973) while that of Brevibacterium brevis has a K_{mO_2} of 800 μM (50 mM MOPS, 100 mM Na_2SO_4, pH 7.0, 23°C; Whittaker et al. 1990). As noted by Fersht (Fersht 1985), catalytically efficient enzymes evolve to maximize their specificity constants while increasing K_m. Nonetheless, the specificity constant of DHBD_{LB400} for O_2, 1 x 10^6 M^{-1}s^{-1}, is significantly lower than the value of 37 x 10^6 M^{-1}s^{-1} reported for the xylE-encoded C23O of P. putida mt-2 (50 mM sodium phosphate, pH 7.5, 25°C; Kobayashi et al. 1995). Moreover, the growth rate of P. putida mt-2 on benzoate at different pO_2 is limited by the K_{mO_2} of this
C23O (Arras et al. 1998). The physiological significance of the high \( K_{mO2} \) of DHBD\(_{LB400} \) remains to be clarified.

The kinetic analysis further establishes that DHBD\(_{LB400} \) is subject to two forms of substrate inhibition: reversible substrate inhibition, and a mechanism-based inactivation. The latter, also known as suicide inhibition, is discussed in section 4.5. The reversible substrate inhibition observed in DHBD\(_{LB400} \) has been reported for a number of other DHB-cleaving extradiol dioxygenases (Adams et al. 1992, Eltis et al. 1993, Happe et al. 1993, Asturias et al. 1994a, Heiss et al. 1995) as well as for a 2,3-dihydroxyphenyl-propionate-cleaving enzyme (Spence et al. 1996a) although it has only rarely been reported for C23O (Pascal and Huang 1987). 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 (Heiss et al. 1995). DHBD\(_{LB400} \) 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\(_{LB400} \), 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\(_{LB400} \) as it has also been reported for a monomeric enzyme (Happe et al. 1993). It is possible that DHB could occupy the auxiliary \( t \)-butanol binding site observed in the DHBD\(_{LB400}: \)DHB complex (see section 4.5), thereby inhibiting the cleavage reaction in a similar manner.

4.3 Cleavage products of 3-chlorocatechol and 3-nitrocatechol

Analysis of the reaction products demonstrates that DHBD\(_{LB400} \) catalyzes the proximal cleavage of 3-chlorocatechol and 3-nitrocatechol. This implies that 3-chlorocatechol and 3-nitrocatechol bind to DHBD\(_{LB400} \) in the same manner as DHB and 3-
methylcatechol, which are also proximally cleaved. It is not clear whether the small amount of distal cleavage produced in the cleavage of 3-chlorocatechol, 3-nitrocatehol and 3-methylcatechol arises from the binding of these substrates in a flipped configuration or from a different position of attack of the superoxide species in the ternary complex. However, the reactivity of 3-chlorocatechol with DHBD_{LB400} suggests that appropriate adaptation of the stereoelectronic environment of the active site could give rise to an extradiol dioxygenase that efficiently cleaves 3-chlorocatechol, as is the case for C23O from \textit{P. putida} GJ31 (Kaschabek \textit{et al.} 1998) and as implied by mutagenesis of other extradiol enzymes (Wasserfallen 1991, Riegert \textit{et al.} 2001).

4.4 Inactivation mechanism of DHBD_{LB400}

As discussed in section 4.2, the kinetic analysis revealed that DHBD_{LB400} is subject to two forms of substrate inhibition: reversible substrate inhibition (discussed in section 4.2), and a mechanism-based inactivation or suicide inhibition. For both DHBD_{LB400} and C23O, 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 mechanism-based inactivation during the cleavage of that substrate. Interestingly, the partition ratio of C23O for catechol is 1,400,000 (Cerdan \textit{et al.} 1994), indicating that DHBD_{LB400} is much more susceptible than is C23O to suicide inactivation by its putative preferred substrate.

The present analysis indicates that this inactivation in DHBD_{LB400} 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 Figure 12) are negligible with respect to the rate of inactivation during steady-state turnover. Thus, DHBD_{LB400} is not inactivated by chelation of the active site Fe(II) by catecholic substrates (Klecka and Gibson 1981). Although free DHBD_{LB400} 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 O₂. However, given DHBD_{LB400} high K_m value for O₂ (1.3 mM), high K_{iA} value for DHB (3 mM), and high K_i values for HOPDA (~3 mM), such inactivation seems unlikely to be significant under the conditions studied.

Further analysis of the mechanism-based inactivation of DHBD_{LB400} revealed that it is similar in nature to the O₂-dependent inactivation of DHBD_{LB400} 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_{LB400} 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_{LB400} for Fe(II) than for Fe(III) is consistent with the crystallographic data of DHBD_{KK102}, in which a more intense electron density was observed at the active site when the iron was reduced (Uragami et al. 2001). Moreover, the oxidation of the active site Fe(II) of C23O by H₂O₂ resulted in the immediate release of Fe(III) (Nozaki et al. 1968).

The present studies suggest that the mechanism-based inactivation of DHBD_{LB400} does not involve covalent modification, as judged by a lack of change to the molecular mass of DHBD_{LB400} inactivated in a number of ways. Moreover, DHBD_{LB400} 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 O₂-dependent inactivation of an α-ketoglutarate dependant oxygenase (Liu et al. 2001), which, like DHBD_{LB400}, has a catalytically essential mononuclear iron bound to the enzyme by a 2-histidine 1-carboxylate structural motif. These results also demonstrate that although 3-chlorocatechol is a very potent mechanism-based inactivator, and that the DHBD_{LB400}-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 (Bartels et al. 1984). Indeed, one study reported that the inactivation of C23O by 3-chlorocatechol also involves oxidation of the active site Fe(II) (Wasserfallen 1989).

A straightforward explanation of the mechanism-based inactivation of DHBD_{LB400} 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 (Figure 25, Bugg and Lin 2001). Mechanism-based inactivation could arise from dissociation of the bound superoxide prior to electron transfer from the catecholate to the iron or prior to 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. Explanation of the slower inactivation observed with 3-nitrocatechol compared to 3-chlorocatechol requires further investigation.

Failure to detect superoxide in the inactivation of DHBD_{LB400} by 3-chlorocatechol seems to be due to the rapid reaction of superoxide with the catechol, possibly prior to 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 (Zhao et al. 1998, Macarthur et al. 2000). Moreover, ferric iron accelerates the reaction between catechols and superoxide (Zhao et al. 1998) and complicates the detection of superoxide by DMPO (Buettner 1993). The reaction between superoxide and 3-chlorocatechol is expected to produce a mixture of multimeric species and o-quinones (Zhao et al. 1998, Raff and Ettling 1963-1970) which
would be difficult to detect given their low concentrations. Finally, it is noted that the inactivation of DHBD$_{LB400}$ with 3-chlorocatechol was rapid (< 10 s) and would thus produce a burst of superoxide. Such bursts are harder to detect as the efficiency of trapping agents decreases as the rate of superoxide production increases (Benov et al. 1998, Buettner 1993). Nevertheless, the results strongly imply that inactivation of DHBD$_{LB400}$ during catalytic turnover involves the dissociation of superoxide from the EAO$_2$ ternary complex. Thus, the O$_2$-dependent inactivation of DHBD$_{LB400}$ 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 DHBD$_{LB400}$ high $K_m$ value 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, C230, which is less susceptible to O$_2$-dependent inactivation (Nozaki et al. 1963), has a much lower $K_m$ for O$_2$ (Hori et al. 1973).

A general mechanism for the turnover-dependent inactivation of extradiol dioxygenases is presented in Figure 25. This mechanism is consistent with the proposed catalytic mechanism. The oxidative inactivation of extradiol dioxygenases is clearly of physiological significance as a number of catabolic pathways have recruited XylT-like ferredoxins to reactivate these enzymes (Polissi and Harayama 1993, Hugo et al. 2000, Tropel et al. 2002). Despite the higher susceptibility of DHBD$_{LB400}$ to suicide inactivation, no such ferredoxin has been associated with the bph pathway. However, the in vivo reactivation of 3-chlorocatechol-inactivated DHBD$_{LB400}$ in Burkholderia sp. LB400 and E. coli suggests that a non-specific electron transfer protein can play this role. This process is nevertheless slow. Thus, the PCB-transforming properties of biphenyl-degrading strains may be improved by recruiting a XylT-like ferredoxin. Consideration of mechanism-based inactivation, and the approach developed in this study, provides a basis for studying and understanding the reactivity of DHBD$_{LB400}$ with chlorinated DHBs produced during the catabolism of PCBs. More generally, this approach should facilitate the characterization of other extradiol-type dioxygenases, such as HAD. For example, halogenated substrate
analogues of HAD that reversibly inhibit this enzyme in vivo (Walsh et al. 1994, Fornstedt-Wallin et al. 1999), may function in a manner analogous to 3-chlorocatechol, which effectively acts as a reversible inhibitor of DHBD_{LB400} in vivo.

Figure 25. General mechanism of inactivation of extradiol dioxygenases. The exact step at which superoxide dissociates from the ternary complex has not been determined. The ligands in the ferric form of the enzyme are unknown (adapted from Shu et al. 1995, Bugg and Lin 2001). For clarity, the displacement of solvent species from the ferrous centre is not depicted explicitly.

4.5 Stabilization and inhibition of DHBD_{LB400}

Previous studies have established that organic additives such as isopropanol and acetone stabilize the active site iron of extradiol dioxygenases (Nozaki et al. 1963, Mabrouk et al. 1991, Kobayashi et al. 1995). These additives were also used in crystallographic studies to stabilize the enzymes. The crystallographic data from DHBD_{LB400} (Han 1995a, Han et al. 1995b, Vaillancourt et al. 1998) and C23O (Kita et al. 1999) indicate that such small organic molecules can occupy the active site, close to the catalytic iron centre. In
C23O, it was even proposed that acetone binds directly to the iron (Bertini et al. 1994, Kita et al. 1999). However, this direct binding remains to be clarified as the precision of the respective experiments was limited. Inspection of the t-butanol binding site in DHBD_{LB400} and of the acetone binding site in C23O reveals that it is partly formed by non-conserved residues, suggesting that the best organic stabilizer, if any, will be isozyme-specific.

In the DHBD_{LB400} substrate-free structure, the distance from the central carbon of t-butanol, C-1, to the Fe atom is 6.0 Å. Binding of the substrate to the iron displaces the two ordered water ligands (section 1.3) and the t-butanol molecule that is in close proximity of the iron. The crystal structures of DHB, 3-Me catechol and catechol DHBD_{LB400}:substrate complexes demonstrate the binding of t-butanol in a location distinct from the site occupied in the substrate-free structure: the position of C1 is shifted by more than 4.8 Å and it is more than 8.4 Å distant from the Fe. 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.

In contrast to DHB, 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 Figure 26. 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 centre of the nonhydroxylated ring of DHB in the respective DHBD_{LB400}:substrate complexes.

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\textsubscript{LB400}. Moreover, \textit{t}-butanol and isopropanol, which inhibit DHBD\textsubscript{LB400} more effectively than ethanol and glycerol, also stabilize the enzyme more effectively (Table 6). Interestingly, acetone competitively inhibits C23O with \textit{K}_\text{i} of 13 mM (Nozaki \textit{et al.} 1963, Kobayashi \textit{et al.} 1995). This suggests that this enzyme has a much higher affinity for acetone than DHBD\textsubscript{LB400} has for \textit{t}-butanol, and may explain why the former is so much more stable in acetone-containing buffers (Kobayashi \textit{et al.} 1995) than is DHBD\textsubscript{LB400} in \textit{t}-butanol-containing buffers.

\textbf{Figure 26.} Electron density maps and models illustrating the structure of the DHBD\textsubscript{LB400}:3-methylcatechol-bound and substrate free forms of DHBD\textsubscript{LB400}. Both forms of enzymes were shown to be present in the crystal (Han 1995a). In the models, the carbon atoms are more darkly shaded than the nitrogen and oxygen atoms. A. |Fo|-[Fc] electron density representing the Fe, 3-methylcatechol, and two water ligands. The |Fc|’s and phases are from the structure of the substrate-free enzyme (Han 1995a, Han \textit{et al.} 1995b). The model is the initial fit to this density. B. Residual |Fo|-|Fc| electron density following refinement of a model that included the Fe, 3-methylcatechol, two water ligands, and a \textit{t}-butanol bound in the auxiliary site, as shown. The |Fc|’s and phases are from this model. The density features arise from the fraction of the crystal that is in the substrate free-form, as demonstrated by C., which shows the refined model of this form (Han 1995a, Han \textit{et al.} 1995b) in conjunction with the same density. Note that the \textit{t}-butanol binds in the substrate binding site in this form of the enzyme (adapted from Vaillancourt \textit{et al.} 1998).

The structural results are remarkably concordant with the specific types of inhibition manifested by \textit{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 a 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 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 (Li and Nussinov 1998). 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.

Finally, it was found that HOPDA inhibited the cleavage of DHB in a mixed fashion. In ordered, ternary complex mechanisms such as that utilized by DHBD$_{LB400}$, products usually act as competitive inhibitors. The observation of mixed inhibition of DHBD$_{LB400}$ by HOPDA may be due to the binding of the latter to a site in the DHBD$_{LB400}$:DHB complex similar to that occupied by t-butanol, which is in contact with the distal phenyl ring of DHB (Han 1995a, Vaillancourt et al. 1998).

### 4.6 Reactivity of DHBD$_{LB400}$ with chlorinated DHBs

Although DHBD$_{LB400}$ was able to cleave all chlorinated DHBs under study, the efficiency of transformation varied considerably according to the position of the chloro substituents. In particular, DHBD$_{LB400}$ catalyzed the cleavage of two high affinity substrates, 2'-Cl and 2',6'-diCl DHB, at very slow rates. In addition, the specificity constant for O$_2$ in the presence of 2'-Cl DHB was about 50-fold lower than in the presence of the enzyme’s preferred substrate, DHB. Finally, compared to DHB and all mono-Cl-DHBs, the slow transformation of 2',6'-diCl DHB greatly increased potential for irreversible
inactivation of DHBD by a mechanism known to require formation of the E:S:O₂ ternary complex (Vaillancourt et al. 2002a).

The crystal structures of the complexes of DHBD₂B₄₀₀ with 2'-Cl DHB and 2',6'-diCl DHB (Figure 27) were determined at a resolution of 1.7 Å (Dai et al. 2002) and rationalize the distinguishing properties of these substrates. The active site structure and positioning of the catecholic ring of 2'-Cl DHB and 2',6'-diCl DHB was essentially the same as the one described for DHB (section 1.5). A notable difference could be observed for the torsion angle of the two rings that varied by ~25° for both complexes. This change is due to the position of the chloro substituents in the active site pocket. In both cases, the 2'-Cl substituent binds in direct contact with conserved side chains that are believed to define the O₂ binding site and with a water molecule that occupies a major portion of that site in the absence of O₂. These interactions are a likely cause of the reduced K_d observed for 2'-Cl DHB. In addition, for both compounds, the 2'-Cl substituent has the potential to partially inhibit binding of O₂ to the binary complex and/or affect the orientation of the bound O₂ relative to the Fe and the point of attack on the hydroxylated ring of the substrate. One likely consequence is inefficient catalysis as reflected in the appreciably reduced values of k_cat. Moreover, contact between O₂ and the Cl substituent could bias the orientations available to the O₂ away from those competent for the cleavage reaction without prohibiting reduction of O₂ or dissociation of superoxide. This scenario would explain why the Cl substituent does not reduce the rate of inactivation within the ternary complex to the same extent that it reduces k_cat.

For 2',6'-diCl DHB, k_cat and K_mₐₐₐ are reduced by factors of 7000 and 1700 relative to DHB, such that the specificity constant is only four-fold lower. These nearly parallel effects on k_cat and K_mₐₐₐ suggest non-productive binding of 2',6'-diCl DHB. In the case of 2'-diCl DHB, k_cat and K_mₐₐₐ are reduced relative to DHB by smaller factors of 50 and 5.2, respectively, such that the specificity constant is ten-fold lower. If it is assumed that the crystallographically observed binding mode is non-productive, then the differences between
$k_{\text{cat}}^{\text{app}}, K_{\text{m}}^{\text{app}}$ and $k_{\text{cat}}^{\text{app}}/K_{\text{m}}^{\text{app}}$ for these two compounds suggest that 2'-Cl DHB can more readily assume the productive binding mode. It is reasonable to expect that the site occupied by the 6'-Cl substituent of the dichlorinated compound would be at least partially occupied by the Cl substituent of 2'-Cl DHB. However, throughout the analysis of the 2'-Cl DHB complex, difference electron density maps provided no conclusive evidence of this second binding mode (Dai et al. 2002). The analysis concluded that the occupancy of the 2'-Cl substituent of 2'-Cl DHB in the alternative site near His241 is expected to be less than 25%. Even though not occurring frequently, the alternative binding mode is sufficient to explain the relative differences between the catalysis of 2'-Cl DHB and 2',6'-diCl DHB. This is also consistent with the crystal structure of the 2',6'-diCl DHB complex inasmuch as the orientation and conformation of this substrate are more restricted because of contacts between the 6'-Cl substituent and the enzyme.

Figure 27. Stereoscopic drawing of 2',6'-diCl DHB bound to the active site of DHBDLB400. For 2',6'-diCl DHB, carbon atoms and bonds are light gray, chlorine atoms are green, and oxygen atoms are red. The 2'-Cl atom is near the center of the field. For DHBDLB400, carbon atoms are yellow or orange, nitrogen atoms are cyan, and oxygen atoms are red. The side chains with darker carbon atoms are ligands of the Fe atom, which is the larger magenta sphere. Isolated red spheres represent water molecules (From Dai et al. 2002).
2',6'-diCl DHBs are generated from ortho-chlorinated PCB congeners. Historically, health concerns have focused on the non-ortho substituted, or "dioxin-like" congeners, which were shown to be carcinogenic in part via the aryl hydrocarbon receptor-mediated activity. More recent studies have demonstrated that the ortho substituted, or "non-dioxin-like" congeners elicit a wide range of toxic responses including neurotoxicity (Chauhan et al. 2000), tumour promotion (van der Plas et al. 2000), and endocrine changes (Arcaro et al. 1999). Moreover, these ortho-chlorinated congeners are among the most recalcitrant to chemical and biological remediation. Thus, various chemical treatments, including palladized iron (Korte et al. 2002) and fly ash (Weber et al. 2002), preferentially dechlorinate meta- and para-substituted PCBs. Biological treatments include the use of consortia of anaerobic bacteria to reductively dechlorinate highly chlorinated congeners (reviewed in Wiegel and Wu 2000) to less chlorinated ones, and the use of strains containing the bph pathway to aerobically degrade lightly chlorinated congeners. Indeed, one of the most promising PCB bioremediation strategies consists of sequential anaerobic-aerobic treatment (Abramowicz 1990). Unfortunately, most consortia of anaerobic bacteria preferentially catalyze the meta- and para-dehalogenation of PCBs, yielding mixtures of predominantly ortho-chlorinated congeners (Wiegel and Wu 2000). Moreover, such congeners are poorly transformed by aerobic PCB degrading bacteria (Furukawa et al. 1979, Abramowicz, 1990, Maltseva et al. 1999, Master et al. 2002). Burkholderia sp. LB400, which transform ortho-chlorinated congeners relatively well, transformed less than 5% of 2,6-diCl biphenyl to the corresponding benzoate compared to more than 70% for other congeners (Maltseva et al. 1999). Two in vivo studies strongly suggest that catabolism of 2,6-diCl biphenyl is blocked at the ring-cleavage step. First, no ring-cleavage product was observed when Burkholderia sp. LB400 was incubated in the presence of 2,6-diCl biphenyl (Seeger et al. 1995a). Second, a diCl DHB accumulated when a PCB-degrading strain was incubated in the presence of 2,6-diCl biphenyl (Furukawa et al. 1979). These studies are consistent with our observation that 2',6'-diCl DHB effectively inhibits DHBD, and the growth of Burkholderia sp. LB400 on biphenyl.
Strains that aerobically degrade ortho-chlorinated congeners would be useful whether used alone to remediate environments contaminated with lightly chlorinated congeners or in conjunction with other treatments to remediate environments contaminated with highly chlorinated congeners. Identification of 2',6'-diCl DHB as a metabolite with a high affinity for DHBD that inactivates the enzyme not only identifies a metabolic block, but also suggests strategies to overcome it. In particular, the engineering of a DHBD variant possessing either an improved ability to cleave 2',6'-diCl DHB, or a decreased affinity for this compound should enhance the utility of bacterial strains for the aerobic biodegradation of PCBs.

4.7 Reactivity of DHBD<sub>P6</sub>-I, DHBD<sub>P6</sub>-III with chlorinated DHBs

*R. globerulus* P6 contains three extradiol ring-cleavage enzymes that have been identified as DHBDs. The properties of the highly active preparations of DHBD<sub>P6</sub>-I and DHBD<sub>P6</sub>-III described differed slightly from those previously reported for the DHBD<sub>P6</sub> isozymes. First, DHBD<sub>P6</sub>-I and DHBD<sub>P6</sub>-III in raw extracts of *P. putida* KT2442 displayed strong substrate inhibition for DHB (Asturias and Timmis 1993). In contrast, these isozymes were subject to minor or no substrate inhibition by DHB in the current study. It is possible that the previous analyses did not adequately account for the rapid inactivation of the enzymes observed at high substrate concentrations. The substrate inhibition observed in DHBD<sub>P6</sub>-I is similar in magnitude as that observed in DHBD<sub>LB400</sub> with most substrates and presumably is due to the binding of a second substrate molecule in the active site of the enzyme, as proposed for DHBD<sub>LB400</sub>. Given the high sequence identity between the enzymes (52%), it is likely that as in DHBD<sub>LB400</sub>, the inhibition in DHBD<sub>P6</sub>-I is due to the binding of a second molecule of substrate to the enzyme. In contrast, the fact that no substrate inhibition is observed in DHBD<sub>P6</sub>-III, a single-domain enzyme (Eltis and Bolin 1996) that shares 16 and 17% sequence identity with the C-domains of DHBD<sub>P6</sub>-I and DHBD<sub>LB400</sub> respectively, could reflect differences in its active site.
It is unclear whether all of the enzymes identified as DHBDs in *R. globerulus* P6 are involved in biphenyl or PCB degradation. Indeed, the apparent specificity of each of the P6 isozymes for DHB is significantly lower than that of DHBD\(_{LB400}\). Thus, the respective apparent specificities of DHBD\(_{P6-I}\) and DHBD\(_{P6-III}\) for DHB were 1.6 and 21 times lower than that of DHBD\(_{LB400}\). Interestingly, the specificity of DHBD\(_{P6-II}\) for DHB estimated from the reported specific activity and \(K^{app}_{mA}\) (Asturias *et al.* 1994a) is very similar to that of DHBD\(_{P6-III}\) reported here. It is unclear whether the low specificities of DHBD\(_{P6-II}\) and DHBD\(_{P6-III}\) in particular reflect their adaptation to the degradation of other compounds such as PCBs (see below). Interestingly, the apparent \(K_m\) for oxygen, \(K^{app}_{mO_2}\), of DHBD\(_{P6-I}\) and DHBD\(_{P6-III}\) in the presence of DHB are significantly lower than that of DHBD\(_{LB400}\), so the differences in specificity for DHB between the P6 isozymes and DHBD\(_{LB400}\) would not be as great at lower \(O_2\) concentrations.

The most striking result of the current study is that DHBD\(_{P6-I}\) and DHBD\(_{P6-III}\) cleaved certain chlorinated DHBs with higher apparent specificity than DHB. This is in clear contrast to DHBD\(_{LB400}\), whose best substrate is DHB. Moreover, the respective specificities of the two P6 isozymes for chlorinated DHBs appear to be quite complementary. Thus, the best substrates for DHBD\(_{P6-I}\) (6-Cl, 3′-Cl and 4′-Cl DHB) were the worst substrates for DHBD\(_{P6-III}\), and the best substrates for DHBD\(_{P6-III}\) (4-Cl and 5-Cl DHB) where the worst substrates for DHBD\(_{P6-I}\). It is likely that the differences in the specificities of the enzymes for polychlorinated DHBs would be even larger. Overall, these data suggest that the existence of multiple DHBD isozymes in a single bacterial strain improves the PCB-degrading capabilities of that strain, and suggest that these enzymes may have been recruited or adapted to improve the PCB-degrading capabilities of *R. globerulus* P6.

The different reactivities of DHBD\(_{P6-I}\), DHBD\(_{P6-III}\) and DHBD\(_{LB400}\) towards PCB metabolites is further illustrated by their respective reactivities towards 2′,6′-diCl DHB. All three isozymes turned over this compound very slowly, indicating that the chlorine
substituents probably partially occlude the accessibility for O₂, as observed in DHBD₄₀₀. However, the \( K_{\text{m}}^{\text{app}} \) of the isozymes for 2',6'-diCl DHB differed by over 500-fold. Moreover, the \( K_{\text{m}}^{\text{app}} \) for 2',6'-diCl DHB was 1700 times lower than for DHB in DHBD₄₀₀, 40 times lower in DHBD₆-I, and only 4 times lower in DHBD₆-III. Thus, the P6 isoizymes, particularly DHBD₆-III, are not as susceptible to competitive inhibition by 2',6'-diCl DHB. As this inhibition would prevent the degradation of other chlorinated DHBs, the different \( K_{\text{m}}^{\text{app}} \) values of DHBD₆-I and DHBD₆-III for 2',6'-diCl DHB further demonstrates how the existence of multiple DHBD isozymes in the same strain is advantageous to PCB-degradation.

There appears to be no correlation between substrate specificity and susceptibility to inactivation in DHBD₆-I and DHBD₆-III: in general, the best substrates were not those that inactivated the enzymes more slowly. Similarly, both P6 isoizymes were least susceptible to inactivation by DHB although this was not the best substrate of either enzyme. This lack of correlation between specificity and susceptibility to inactivation might reflect the recent evolution of these enzymes for the degradation of PCB metabolites as PCBs were only recently introduced into the environment in significant amounts. The tuning of the enzyme against inactivation versus optimization of specificity could be more difficult to evolve. Alternatively, \( R. \) globerulus P6 might possess a XylT-like ferredoxin (Polissi and Harayama 1993, Hugo et al. 2000, Tropel et al. 2002) to reduce oxidized iron in DHBD₆-I and DHBD₆-III. The existence of such a ferredoxin would prevent prolonged inactivation of the enzymes \textit{in vivo}, and might reduce the evolutionary pressure to minimize susceptibility to inactivation. In section 3.4.5, it was shown that the activity of DHBD₄₀₀ in \textit{Burkholderia} sp. LB400 inactivated with 3-Cl catechol recovered \textit{in vivo} in the absence of protein synthesis, indicating the presence of an agent capable of reducing the enzyme in this organism.

Commercial mixtures of PCBs typically contain up to 60 different congeners. The effective microbial degradation of these environmental pollutants thus requires enzymes of
broad or complementary specificities. The present study of the specificities of DHBD_{P6-I} and DHBD_{P6-III} points to several similarities as well as significant structural or electronic differences in the respective active sites of DHBD_{P6-I}, DHBD_{P6-III} and DHBD_{LB400}. Structural and spectroscopic studies of DHBD_{P6-I} and DHBD_{P6-III} may provide insights into the molecular basis of these differences. The poor cleavage of 2',6'-diCl DHB by DHBD_{P6-I} and DHBD_{P6-III} further suggests that directed evolution of DHBD might be worthwhile to improve the cleavage of this recalcitrant metabolite and, ultimately, the microbial degradation of PCBs.

4.8 Anionic state of DHB and 3-nitrocatechol bound to DHBD_{LB400}

The spectroscopic data presented in this study provide evidence that DHBD_{LB400} binds its preferred substrate, DHB, as a monoanion. Further evidence supporting this same conclusion was provided by a UVRR study (Barbosa 2002, Vaillancourt et al. 2002b) and the observed asymmetric binding of DHB to DHBD_{LB400} (r_{Fe-O} = 2.0 Å and 2.4 Å; Han 1995a, Vaillancourt et al. 2002b), which indicates that O-2 is deprotonated, but not O-3. These results substantiate spectroscopic data that had been interpreted to indicate this state of protonation of the substrate in the ES complex. Thus, XAS data of C230:catechol complex indicated that there are 5 N/O scatters coordinated to the Fe(II): four at 2.10 Å and one at 1.93 Å (Shu et al. 1995). The short distance was attributed to the asymmetric binding of catechol, suggesting that the latter is bound as a monoanion, as observed in [Fe(II)(6TLA)(DBCH)](ClO_4), a well characterized mononuclear Fe(II) catechol model compound (Chiou and Que 1995). Interestingly, the asymmetry of DHB binding in a complex with a reactivated homologue does not appear to be as significant (r_{Fe-O} = 2.02 Å and 2.25 Å; Uragami et al. 2001). UV/vis absorption spectroscopy also showed that 3-nitrocatechol, a poor substrate, was binding to DHBD_{LB400} as a monoanion. A similar experiment with 4-nitrocatechol and C230 also showed monoanionic binding (Tyson 1975).
For intradiol dioxygenases, it is generally accepted that the substrate binds as a dianion, and that the displaced tyrosyl and hydroxide ligands accept the two hydroxyl protons (Orville and Lipscomb 1997a). The protonation state of the substrate is largely based on crystallographically determined bond lengths, which indicate that the substrate is asymmetrically bound: the long Fe-O bond is \textit{trans} to a tyrosinate ligand and the short Fe-O bond is \textit{trans} to a neutral histidine ligand (Orville \textit{et al.} 1997b, Vetting \textit{et al.} 2000a, Vetting and Ohlendorf 2000b). The asymmetry is proposed to reflect ketonization of the bond \textit{trans} to the tyrosine. A survey of the structures in the PDB database (3PCA, 1EOB, 1DLT) reveals that the Fe-O bond lengths are similar to those observed in ES complexes of extradiol enzymes ($r_{\text{Fe-O}} = 2.0 \pm 0.1$ Å and $2.4 \pm 0.2$ Å). Visible resonance Raman studies using the ligand-to-metal ion charge transfer bands show that 4-nitrocatechol and 3,4-dihydroxyphenylacetate bind to 3,4-PCD as dianions (Que and Epstein 1981, Elgren \textit{et al.} 1997). UV/Vis absorption spectroscopy corroborates dianionic binding of 4-nitrocatechol to 3,4-PCD and C12O (Tyson 1975, Vetting \textit{et al.} 2000a). 4-Nitrocatechol is an inhibitor of 3,4-PCD and 3,4-dihydroxyphenylacetate is a very poor substrate. Thus, these analogues may not bind in the same manner as the preferred substrate of the enzyme, PCA. However, structural data indicate that 3,4-PCD binds 3,4-dihydroxyphenylacetate and PCA in a similar manner. Considering the proposed importance of dianionic binding of the substrate to substrate activation in the catalytic mechanism of intradiol enzymes (Orville \textit{et al.} 1997b), it would be useful to obtain direct evidence for the protonation state of the bound substrate. This can probably be achieved using UVRRS (Barbosa 2002).

4.9 Role of conserved active site residues in the mechanism of extradiol dioxygenase

The spectroscopic data presented in this study and the availability of the crystal structures of DHBD$_{LB400}$ in the absence and presence of substrate (Figure 28; Han 1995a, Vaillancourt \textit{et al.} 2002b), suggest roles for the different conserved active site residues in the mechanism of extradiol dioxygenases. The spectroscopic and crystallographic data substantiate the initial step of the proposed catalytic mechanism (Figure 8): the bidentate binding of the catecholic
substrate to the ferrous centre as a monoanion (Que and Ho 1996, Solomon et al. 2000, Bugg and Lin 2001). The Lewis acidity of the Fe(II) should assist in the deprotonation of the substrate. Nevertheless, a recent model reaction study suggested that a base was required for substrate monodeprotonation and an acid was required for the subsequent Criegee rearrangement (Lin et al. 2001). The structural data strongly suggest that the conserved active site His241 of DHBD_{LB400} assists in the deprotonation of the catechol in the enzyme-catalyzed reaction (Figure 29). In the substrate-free enzyme, His241 is presumed to be in the imidazole (neutral) state because of its proximity to the Fe(II) atom and its hydrogen bonding interactions. The substrate-induced structural changes (Figure 28) observed for His241 are consistent with its protonation. In the enzyme:DHB complex, the side chain of His241 is stacked with the catechol ring and His241 and Glu260 have rotated toward each other forming a new hydrogen bond. This change implies that His241 has acquired a proton during formation of the ES complex. Tyr250 forms a new hydrogen bond with the 2-hydroxyl oxygen of the substrate. Tyr250 could act as a proton shuttle between the catecholic substrate and His241.

Figure 28. Displacements of active site atoms/residues associated with DHB binding. This is a (divergent) stereoscopic diagram. Atoms and bonds in the substrate-free structure are represented by smaller spheres, thinner sticks, and lighter shades. Carbon atoms are coloured orange in protein residues and yellow in DHB. Nitrogen and iron atoms are cyan and green, respectively. Protein and DHB oxygen atoms are red, whereas water oxygens are magenta (From Vaillancourt et al. 2002b).
In the subsequent step of the proposed mechanism, O₂ binds to the ferrous iron. As shown in Figure 29, the dominant form of this species would be Fe(III)-O₂⁻. Formation of Fe(III) would induce deprotonation of the 3-hydroxyl, forming the dianion chelate, as proposed in intradiol enzymes (Orville et al. 1997b, Vetting et al. 2000a, Elgren et al. 1997). The proton could be picked up by the iron-bound superoxide. Interestingly, Ne2 of the conserved His195 is positioned within 3 Å of the proximal O atom in the modeled DHBD₁₉₄₀:DHB:O₂ ternary complex (Bolin and Eltis 2001), and could thus stabilize protonation of that particular O atom of the superoxide species. Electron transfer from the bound catechol would produce an Fe(II)-semiquinone. Attack of the activated oxygen species in a pseudo-axial position at C-2 satisfies the orbital steering requirements proposed by Bugg to be critical for extradiol cleavage (Bugg and Lin 2001). In the subsequent step, the proton originating from the 3-hydroxyl group would assist in the heterolysis of the O-O bond in the proposed Criegee rearrangement that results in the lactone and the Fe-hydroxide. Finally, hydrolysis of the lactone by the Fe-bound hydroxide, and release of the proton on His241 complete the catalytic cycle. This mechanism, which is summarized in Figure 29, does not rule out alternate roles for His195 and His241. However, it illustrates the importance of the Fe(II)-bound monoanion in coordinating electron and proton transfer upon O₂ binding. The recent crystal structure of a DHBD₁₉₄₀₂₁:DHB:NO ternary complex is in good agreement with this proposal (Sato et al. 2002). In this study, it was suggested that His195 deprotonates the 3-hydroxyl group of the substrate and that the protonated His then stabilizes the superoxide species.
Figure 29. Proposed mechanism of extradiol dioxygenases with the role of conserved active site residues (adapted from Shu et al. 1995, Bugg and Lin 2001). For clarity, the displacement of solvent species from the ferrous centre is not depicted explicitly.

4.10 Concluding Remarks

This thesis describes the characterization of highly active anaerobically purified DHBDs. The study of the kinetic and inactivation mechanisms of DHBD$_{LB400}$ provides a good framework to study other extradiol-type dioxygenase. Indeed, the studies on DHBD$_{LB400}$ facilitated the characterization of DHBD$_{P6}$-I and DHBD$_{P6}$-III in this thesis, as well as the characterization of HGO in the Eltis group.

The characterization of the specificity of DHBDs for some chlorinated DHBs identified the molecular basis of the inhibition of the biodegradation of PCB mixtures containing di-ortho substituted chlorinated biphenyls. This lead to the design of directed evolution strategies to develop enzymes able to cleave 2',6'-diCl DHBs. The different specificities of DHBD$_{P6}$-I and DHBD$_{P6}$-III for monochlorinated DHBs undoubtedly contribute to the superior PCB-degrading properties of $R$. globerulus P6, demonstrating the
specific advantages that multiple isozymes can confer to their host. These results, together with the 3-chlorocatechol inactivation studies, have implications for the engineering of bacterial pathways to degrade PCBs.

UV/vis and UVRR (Barbosa 2002) studies elucidated the anionic state of the preferred substrate of an extradiol dioxygenase as a monoanion. This substantiates an important step in the proposed catalytic mechanism for this large family of dioxygenases. Analysis of the crystallographic data indicated which hydroxyl group is deprotonated, and allowed the suggestion of the catalytic roles for conserved active site residues. The methodologies developed in this study should prove useful in the study of the interaction of other enzymes with aromatic substrates, particularly the extradiol-type and intradiol dioxygenases.

Future studies of extradiol dioxygenases should include the substantiation of the catalytic roles of key active site residues. This could be achieved through the design of active site variants and their study by spectroscopic techniques and stopped-flow kinetics using a slowly cleaved, coloured substrate, such as 3-nitrocatechol.
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