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AROMATIC RING HYDROXYLATING DIOXYGENASES

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Abbreviations: NDO, Naphthalene dioxygenase; TDO, toluene dioxygenase; NBDO, nitrobenzene dioxygenase; BPDO, biphenyl dioxygenase; PCB, polychlorinated biphenyl; TCE, trichloroethylene; PAH, polycyclic aromatic hydrocarbon

1. INTRODUCTION

The initial oxidation of the aromatic ring is the most difficult catalytic step in the aerobic degradation of aromatic compounds. Bacterial aromatic ring hydroxylating dioxygenases (also known as Rieske non-heme iron dioxygenases) catalyze the addition of hydroxyl groups to the highly stable aromatic ring, setting the stage for further oxidation, and eventual ring cleavage. Aromatic ring hydroxylating dioxygenases are known to catalyze the initial reaction in the bacterial biodegradation of a diverse array of aromatic and polycyclic aromatic hydrocarbons (PAHs), chlorinated aromatic, nitroaromatic, aminoaromatic, and heterocyclic aromatic compounds, and aromatic acids. Aromatic ring hydroxylating dioxygenases use molecular oxygen as a substrate, adding both atoms

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of O_2 to the aromatic ring of the substrate. To date, over 100 aromatic ring hydroxylating dioxygenases have been identified based on biological activity or nucleotide sequence identity. These enzymes are distributed among a variety of Gram-negative and Gram-positive bacteria and are important for the catabolism of a wide range of environmental pollutants.

Aromatic ring hydroxylating dioxygenases are multicomponent enzyme systems (E.C. 1.14.12.-) that catalyze reductive dihydroxylation of their substrates, and are distinct from aromatic ring cleavage (or ring fission) dioxygenases (E.C. 1.13.11.-), which act on the downstream catechol intermediates in many of the same catabolic pathways. Many of these enzymes are typically quite promiscuous, catalyzing the oxidation of a wide range of compounds in addition to their native substrates. At the same time, however, many of these enzyme systems are highly enantioselective, producing chiral *cis*-dihydrodiols or other chiral products in high enantiomeric purity. These properties have made aromatic ring hydroxylating dioxygenases attractive as biocatalysts.

This chapter will focus primarily on the aromatic ring hydroxylating dioxygenases present in pseudomonads and related proteobacteria, but it is important to keep in mind that closely related enzyme systems have been identified in a variety of other bacterial genera, including *Rhodococcus*, *Nocardia*, *Mycobacterium*, etc. Aspects described in this chapter include dioxygenase classification, enzymology, structure and mechanism, and applications in biotechnology. Much of our understanding of the structure and function of aromatic hydrocarbon dioxygenases comes from studies of naphthalene dioxygenase (NDO), and characteristics of this enzyme system will be described in detail and used as a basis for comparisons with other dioxygenases.

2. DISTRIBUTION OF RING HYDROXYLATING DIOXYGENASES IN CATABOLIC PATHWAYS

Bacterial pathways for the degradation of numerous aromatic hydrocarbons, PAHs, chlorinated aromatic compounds, nitroaromatic compounds, aminoaromatic compounds, aromatic acids, and heterocyclic aromatic compounds are initiated by aromatic ring hydroxylating dioxygenases (Figure 1). Many of these compounds are toxic environmental pollutants. Some, including various chlorinated and nitroarene compounds, are man-made, while many others are naturally occurring biological products or components of petroleum. Regardless of the source, bacterial pathways for the degradation of this group of chemicals are important both in the recycling of carbon on earth and in the removal of toxic pollutants at contaminated sites.

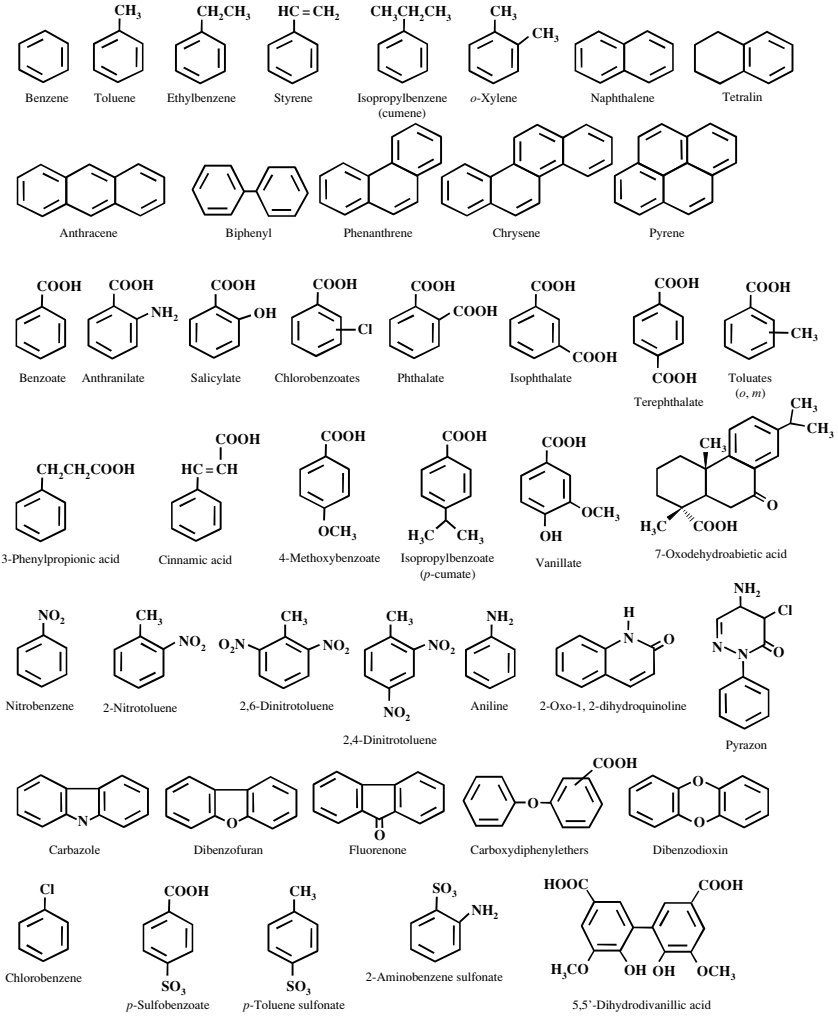
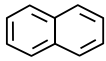
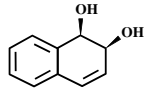
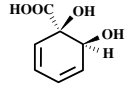
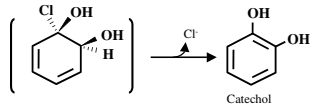
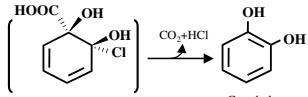
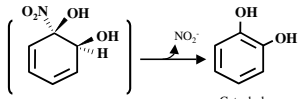
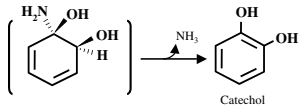
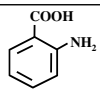
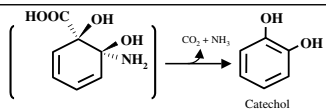
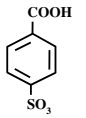
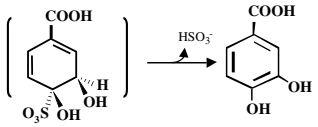
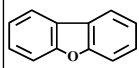
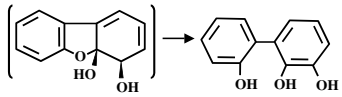


Figure 1. Aromatic compounds degraded by bacterial pathways that are initiated by aromatic ring hydroxylating dioxygenases.

2.1. Types of Reactions Catalyzed

The initial reaction catalyzed by aromatic ring hydroxylating dioxygenases on aromatic hydrocarbons and certain other substrates is a *cis*-dihydroxylation of the carbon–carbon double bond of adjacent unsubstituted carbon atoms. This reaction typically generates a chiral *cis*-dihydrodiol as seen with the reaction on naphthalene (Reaction A, Table 1). Oxidation of aromatic

Table 1. Representative types of *cis*-dihydroxylation reactions catalyzed by aromatic ring hydroxylating dioxygenases.

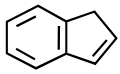
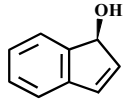
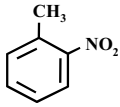
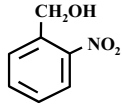
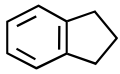
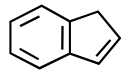
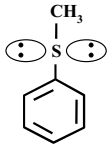
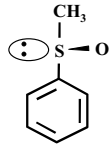
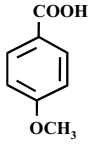
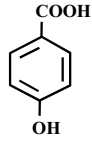
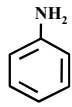
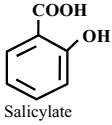
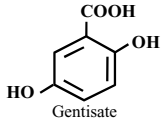
Reaction Type	Substrate	Enzyme	Product
A. *cis*-dihydroxylation	 Naphthalene	Naphthalene dioxygenase, O_2	 Naphthalene *cis* 1,2-dihydrodiol
B. *cis*-dihydroxylation	 Benzoate	Benzoate dioxygenase, O_2	 Benzoate *cis* 1,2-dihydrodiol
C. *cis*-dihydroxylation and dehalogenation	 Chlorobenzene	Chlorobenzene dioxygenase, O_2	 Catechol
D. *cis*-dihydroxylation, dehalogenation and decarboxylation	 2-Chlorobenzoate	Chlorobenzoate dioxygenase, O_2	 Catechol
E. *cis*-dihydroxylation and nitrite elimination	 Nitrobenzene	Nitrobenzene dioxygenase, O_2	 Catechol
F. *cis*-dihydroxylation and deamination	 Aniline	Aniline dioxygenase, O_2	 Catechol
G. *cis*-dihydroxylation, deamination and decarboxylation	 Anthranilate	Anthranilate dioxygenase, O_2	 Catechol
H. *cis*-dihydroxylation, and desufonation	 *p*-Sulfobenzoate	*p*-Sulfobenzoate dioxygenase, O_2	 Protocatechuate
I. Angular dihydroxylation	 Dibenzofuran	Dibenzofuran dioxygenase, O_2	 2, 2', 3-trihydroxy biphenyl

acids such as benzoate occurs at a carboxylated carbon and an adjacent unsubstituted carbon, and results in the formation of chiral *cis*-dihydroxylated cyclohexadiene carboxylic acids (Reaction B, Table 1). Dioxygenase-catalyzed dechlorination has also been demonstrated for chlorinated benzoates, benzenes, and biphenyls (Reactions C and D, Table 1). Dioxygenation at a chlorine-substituted carbon results in concomitant elimination of chloride.[88,113,193] Similar reactions have been shown with nitroaromatic, aminoaromatic, and sulfoaromatic substrates (Reactions E–H, Table 1), resulting in nitrite, ammonia, or sulfite elimination.[4,47,94,177,199,259] In these cases, the displacement reactions result in the formation of dihydroxylated (catecholic) products that are activated for further metabolism. A specialized group of ring hydroxylating dioxygenases catalyzes angular dioxygenation, as seen with dibenzofuran[48,81] (Reaction I, Table 1). Other substrates that are oxidized by angular dioxygenation include carbazole, diphenylethers, and dibenzo-*p*-dioxin.[80,239,297] Two recent reviews provide excellent summaries of angular dioxygenases and the pathways in which they operate.[200,202] Finally, certain members of this large family of multicomponent enzymes function as monooxygenases, as exemplified by methoxybenzoate monooxygenase[26] and salicylate 5-hydroxylase (Reactions N and P, Table 2).[305] Very recently, multicomponent salicylate 1-hydroxylases have been identified in three different sphingomonads.[55,68,220]

2.2. Classification of Ring Hydroxylating Dioxygenases

Historically, Rieske non-heme iron oxygenases were classified using the Batie system, which was based on the electron transfer components present in the 10 Rieske non-heme iron oxygenase systems known at that time.[20] Two-component (reductase and oxygenase; Class I) and three-component (reductase, ferredoxin, and oxygenase; Class II, III) enzyme systems were represented, and the classes were further subdivided based on the type of flavin cofactor (FAD or FMN) in the reductase, the presence or absence of an iron–sulfur center in the reductase, the number of proteins in the oxygenase, and if a ferredoxin was involved, the type of iron–sulfur center (plant or Rieske) in the ferredoxin. This classification system worked well with a small number of known enzymes, but as more enzyme systems with diverse properties were identified, not all of the new enzymes fit into this classification system. A second classification system based on amino acid sequence alignments of the available oxygenase α subunits was proposed by Werlen *et al.*, and it identified four dioxygenase families (naphthalene, toluene/benzene, biphenyl, and benzoate/toluate).[293] This classification system was based on the catalytic activity of the enzymes because the α subunit of the oxygenase plays a major role in determining substrate specificity.[17,24,186,209,212,275,307] The results of the Werlen *et al.* study demonstrated that, in general, related dioxygenases coded for enzymes with similar substrates. Nakatsu *et al.*[192] subsequently built upon this classification system,

Table 2. Other types of oxidation reactions catalyzed by aromatic ring hydroxylating dioxygenases.

Reaction Type	Substrate	Enzyme	Product
J. Benzylic hydroxylation	 Indene	Naphthalene dioxygenase, O_2	 Indenol
K. Methyl group hydroxylation	 2-Nitrotoluene	Naphthalene dioxygenase, O_2	 2-Nitrobenzyl alcohol
L. Oxygen-dependent desaturation	 Indan	Naphthalene dioxygenase, O_2	 Indene
M. Sulfoxidation	 Methyl phenyl sulfide	Naphthalene dioxygenase, O_2	 Methyl phenyl sulfoxide
N. *O*-Dealkylation	 4-Methoxybenzoate	Naphthalene dioxygenase, O_2	 4-Hydroxybenzoate
O. *N*-Dealkylation	 N-Methylaniline	Naphthalene dioxygenase, O_2	 Aniline
P. Net aromatic ring hydroxylation	 Salicylate	Salicylate 5- hydroxylase, O_2	 Gentisate

adding the α_n dioxygenases (those in the Batie system Class IA) to the Werlen system and demonstrating that these α_n oxygenases formed a separate lineage.

Two modified classification systems, also based on the phylogeny of the oxygenase α subunits have been proposed, each of which divided the known enzymes into four families or groups (Groups I–IV, or phthalate, benzoate, naphthalene, and toluene/biphenyl families respectively[106,196]). From current phylogenetic analyses, additional families or groups are apparent, as well as several enzymes that do not cluster tightly with any of the distinct families (Figure 2). In some of the families, enzymes with similar native substrates cluster together. The benzoate family (Figure 2) includes enzymes with activities toward various aromatic acids and aminoaromatic compounds. Monocyclic aromatic hydrocarbon dioxygenases, biphenyl dioxygenases (BPDOs), and chlorinated aromatic hydrocarbon dioxygenases from both Gram-negative and Gram-positive bacteria are tightly clustered in the toluene/biphenyl family. Enzymes for naphthalene and PAH degradation from Gram-negative and Gram-positive bacteria form separate clusters (naphthalene and Gram-positive PAH families, respectively), and nitroarene dioxygenases cluster tightly with NDO from Gram-negative bacteria. The phthalate family (Figure 2), which consists of all of the Rieske non-heme iron oxygenases that contain only α subunits, is a very diverse group of enzymes that catalyze the oxidation of a variety of structurally unrelated aromatic compounds. Substrates for the phthalate family include aromatic acids such as phthalate, *p*-toluene sulfonate, and phenoxybenzoate, as well as carbazole and 2-oxo-1,2-dihydroquinoline. Two phthalate dioxygenases from Gram-positive bacteria were recently identified.[76,260] These enzymes do not cluster with the phthalate family but with the Gram-positive PAH dioxygenases (Figure 2), and in addition, are comprised of both α and β subunits. A new family has recently emerged, and is designated here as the salicylate family (Figure 2). This family consists of enzymes that catalyze oxidation at either the 1- or 5-position of salicylate,[55,68,220,305] as well as other substrates. There are now a number of enzymes that are quite distantly related to those found in the core of these groups or families (Figure 2), and thus, none of the classification systems can completely describe the diversity of enzymes that have been identified to date.

2.3. Diversity of NDO and Related Nitroarene Dioxygenases

Based on sequence alignments of NDO α subunit genes, the genes from Gram-negative bacteria were found to fall into three distinct groups that have been designated *nah*-like, *dnt/ntd*-like (sometimes called *nag*-like), and *phn*-like.[165,176] An additional distantly related naphthalene/phenanthrene dioxygenase (A5 PhnA1, Figure 2) was identified from a marine *Cycloclasticus* isolate (strain A5) that grows on several PAHs.[144] Tetralin dioxygenase (TFA ThnA1,

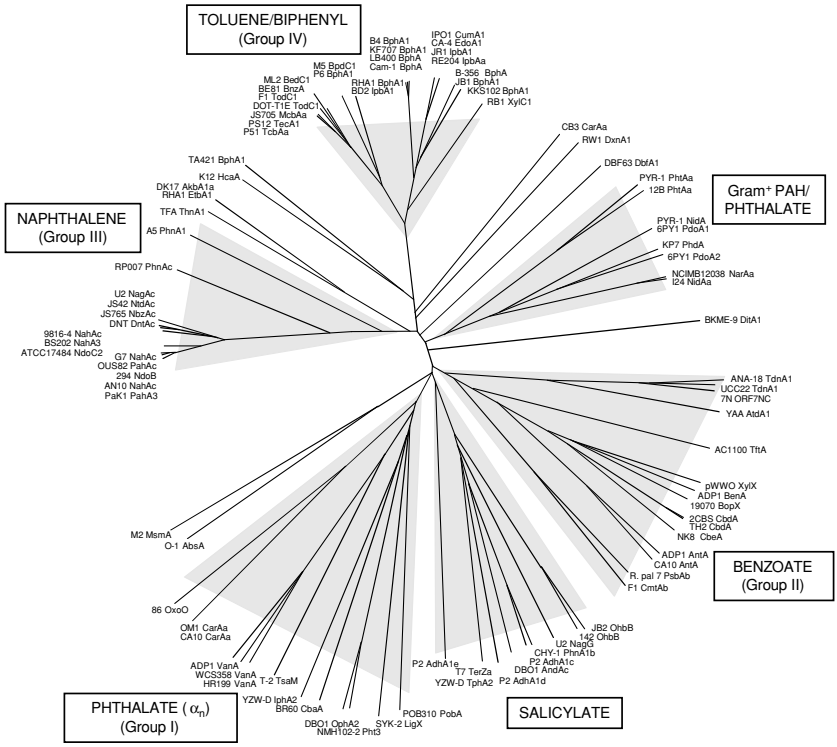


Figure 2. Phylogenetic tree of α subunits of aromatic ring hydroxylating dioxygenases. Sequences were aligned and the tree was generated using the Clustal W package. [277]. α subunit sequences are from the following organisms/dioxygenase systems. PaK1 PahA3, NDO: *P. aeruginosa* PaK1 (D84146); AN10 NahAc, NDO: *P. stutzeri* AN10 (AF039533); 294 NdoB, NDO: *P. fluorescens* 294 (AF004283); OUS82 PahAc, NDO: *P. putida* OUS82 (D16629); G7 NahAc, NDO: *P. putida* G7 (M86949); ATCC17484 NdoC2, NDO: *P. putida* ATCC17484 (AF004284); BS202 NahA3, NDO: *P. putida* BS202 (AF010471); 9816-4 NahAc, NDO: *Pseudomonas* sp. NCIB 9816-4 (U49496); U2 NagAc, NDO: *Ralstonia* sp. U2 (AF036940); JS42 NtdAc, 2-nitrotoluene dioxygenase: *Acidovorax* sp. JS42 (U49504); JS765 NbzAc, NBDO: *Comamonas* sp. JS765 (AF379638); DNT DntAc, 2,4-dinitrotoluene dioxygenase: *Burkholderia* sp. DNT (U62430); RP007 PhnAc, PAH dioxygenase: *Burkholderia* sp. RP007 (AF061751); A5 PhnA1, PAH dioxygenase: *Cycloclasticus* sp. A5 (AB102786); TFA ThnA1, tetralin dioxygenase: *Sphingopyxis macrogoltabida* TFA (AF157565); RHA1 EtbA1, ethylbenzene dioxygenase: *Rhocococcus* sp. RHA1 (AB120955); DK17 AkbA1a, alkylbenzene dioxygenase: *Rhodococcus* sp. DK17 (AY502075); K-12 HcaA, 3-phenylpropionate dioxygenase: *E. coli* K-12 (NC000913); TA421 BphA1, BPDO: *R. erythopolis* TA421 (D88020); P51 TcbAa, chlorobenzene dioxygenase: *Pseudomonas* sp. P51 (U15298); PS12 TecA1, chlorobenzene dioxygenase: *Burkholderia* sp. PS12 (U78099); JS705 McbAa, chlorobenzene dioxygenase: *Ralstonia* sp. JS705 (AJ006307); F1 TodC1, TDO: *P. putida* F1 (J04996); DOT-T1E TodC1, TDO: *P. putida* DOT-T1E (Y18245); BE81 BnzA, benzene dioxygenase *P. putida* BE81 (M17904); ML2 BedC1, benzene dioxygenase: *P. putida* ML2 (AF148496); M5 BpdC1, BPDO: *Rhodococcus* sp. M5 (U27591); P6 BphA1, BPDO: *Rhodococcus globerulus* P6 (X80041); RHA1 BphA1, BPDO: *Rhodococcus* sp. RHA1 (D32142); BD2 IpbA1, isopropylbenzene dioxygenase: *R. erythropolis* BD2 (U24277);

Figure 2 (*continued*). B4 BphA1, BPDO: *Pseudomonas* sp. B4 (AJ251217); 28, KF707 BphA1, BPDO: *P. pseudoalcaligenes* KF707 (AF049345); LB400 BphA, BPDO: *B. xenovorans* LB400 (M86348); Cam-1 BphA, BPDO: *Pseudomonas* sp. Cam-1 (AY027651); IPO1 CumA1, isopropylbenzene dioxygenase: *P. fluorescens* IPO1 (D37828); CA-4 EdoA1, ethylbenzene dioxygenase: *P. fluorescens* CA-4 (AF049851); JR1 IpbA1, isopropylbenzene dioxygenase: *Pseudomonas* sp. JR1 (U53507); RE204 IpbAa, isopropylbenzene dioxygenase: *P. putida* RE204 (AF006691); B-356 BphA, BPDO: *C. testosteroni* B-356 (U47637); JB1 BphA1, BPDO: *Burkholderia* sp. JB1 (AJ010057); KKS102 BphA1, BPDO: *Pseudomonas* sp. KKS102 (D17319); RB1 XylC1, aromatic hydrocarbon dioxygenase: *Cycloclasticus oligotrophicus* RB1 (U51165); CB3 CarAa, carbazole dioxygenase: *Sphingomonas* sp. CB3 (AF060489); RW1 DxnA1, dioxin dioxygenase: *Sphingomonas* sp. RW1 (X72850); DBF63 DbfA1, dibenzofuran dioxygenase: *Terrabacter* sp. DBF63 (AB054975); PYR-1 PhtAa, phthalate dioxygenase: *Mycobacterium vanbaalenii* PYR-1 (AY365117); 12B PhtAa, phthalate dioxygenase: *Arthrobacter keyseri* 12B (AF331043); PYR-1 NidA, naphthalene-inducible dioxygenase: *M. vanbaalenii* PYR-1 (AF249301); 6PY1 PdoA1, PAH dioxygenase: *Mycobacterium* sp. 6PY1 (AJ494745); KP7 PhdA, phenanthrene dioxygenase: *Nocardioides* sp. KP7 (AB017794); 6PY1 PdoA2, PAH dioxygenase: *Mycobacterium* sp. 6PY1 (AJ494743); NCIMB12038 NarAa, NDO: *Rhodococcus* sp. NCIMB12038 (AF082663); I24 NidAa, naphthalene-inducible dioxygenase: *Rhodococcus* sp. I24 (AF121905); BKME-9 DitA1, diterpenoid dioxygenase: *P. abietaniphila* BKME-9 (AF119621); ANA-18 TdnA1, aniline dioxygenase: *Fraturia* sp. ANA-18 (AB089795); UCC22 TdnA1, aniline dioxygenase: *P. putida* UCC22 (D85415); 7N ORF7NC, aniline dioxygenase: *Delftia acidovorans* 7N (AB177545); YAA AtdA1, aniline dioxygenase: *Acinetobacter* sp. YAA (D86080); AC1100 TftA, 2,4,5-trichlorophenoxyacetic acid oxygenase: *B. cepacia* AC1100 (U11420); pWWO XylX, toluate dioxygenase: *P. putida* pWWO (AJ344068); ADP1 BenA, benzoate dioxygenase: *Acinetobacter* sp. ADP1 (AF009224); 19070 BopX, benzoate dioxygenase: *Rhodococcus* sp. 19070 (AF279141); 2CBS CbdC, 2-halobenzoate dioxyegnase: *B. cepacia* 2CBS (X79076); TH2 CbdA, 2-halobenzoate dioxyegnase: *Burkholderia* sp. TH2 (AB035324); NK8 CbeA, chlorobenzoate dioxygenase: *Burkholderia* sp. NK8 (AB024746); ADP1 AntA, anthranilate dioxygenase: *Acinetobacter* sp. ADP1 (AF071556); AntA, CA10, anthranilate dioxygenase: *P. resinovorans* CA10 (NC_004444); *R. pal* 7 PsbAb, cumate dioxygenase: *R. palustris* 7 (AB022919); F1 CmtAb, cumate dioxygenase: *P. putida* F1 (AB042508); JB2 OhbB, *o*-halobenzoate dioxygenase: *P. aeruginosa* JB2 (AF422937); 142 OhbB, *o*-halobenzoate dioxygenase: *P. aeruginosa* 142 (AF121970); U2 NagG, salicylate 5-hydroxylase: *Ralstonia* sp. U2 (AF036940); CHY-1 PhnA1b, salicylate 1-hydroxylase: *Sphingomonas* sp. CHY-1 (AJ633552); P2 AdhA1c, salicylate 1-hydroxylase: *Sphingobium* sp. P2 (AB091693); DBO1 AndAc, anthranilate dioxygenase: *B. cepacia* DBO1 (AY223539); P2 AdhA1d, salicylate 1-hydroxylase: *Sphingobium* sp. P2 (AB091692); T7 TerZa, terephthalate dioxygenase: *Delftia* sp. T7 (AB081091); YZW-D TphA2, terephthalate dioxygenase: *C. testosteroni* YZW-D (AY923836); AdhA1e, salicylate 1-hydroxylase: *Sphingobium* sp. P2 (AB091692); POB310 PobA, phenoxybenzoate dioxygenase: *P. pseudoalcaligenes* POB310 (X78823); SYK-2 LigX, 5,5′-dehydrodivanillic acid *O*-demethylase: *S. paucimobilis* SYK-2 (AB021319); NMH102-2 Pht3, phthalate dioxygenase: *P. putida* NMH102-2 (D13229); DBO1 OphA2, phthalate dioxygenase: *B. cepacia* DBO1 (AF095748); BR60 CbaA, 3-chlorobenzoate dioxygenase: *C. testosteroni* BR60 (U18133.2); YZW-D IphA2, iosphthalate dioxygenase: *C. testosteroni* YZW-D (AY923836); T-2 TsaM, *p*-toluenesulfonate monooxygenase: C. *testosteroni* T-2 (AF311437); HR199 VanA, vanillate *O*-demethylase: *Pseudomonas* sp. HR199 (Y11521); WCS358 VanA, vanillate *O*-demethylase: *P. putida* WCS358 (Y14759); ADP1 VanA, vanillate *O*-demethylase: *Acinetobacter* sp. ADP1 (AF009672); OM1 CarAa, carbazole dioxygenase: *P. stutzeri* OM1 (AB088757); CA10 CarAa, carbazole dioxygenase: *P. resinovorans* CA10 (AB088420); 86 OxoO, 1-oxo-1,2-dihydroquinoline 8-monooxygenase: *P. putida* 86 (Y12655); O-1 AbsA, 2-aminobenzene sulfonate dioxygenase: *Alcaligenes* sp. O-1 (AF109074); M2 MsmA, methanesulfonic acid monooxygenase: *Methylsulfomonas methylovora* M2 (AF091716).

Figure 2) from *Sphingopyxis macrogoltabida* TFA is also distantly related to members of this family.[187]

The *nah*-like group contains enzymes (Figure 2) from various *Pseudomonas* species (*P. putida*, *P. fluorescens*, *P. aeruginosa*, *P. stutzeri*[34,69,274]) and includes the well-studied NDO from *Pseudomonas* sp. NCIB 9816-4 and *P. putida* G7.[256] Sequence and gene order in this group appears to be conserved. Based on further sequence analysis, the *nah*-like group has been divided into two subgroups, and a few strains were shown to contain copies of both types of NDO genes.[87]

The *dnt/ntd* group (Figure 2) is represented by dioxygenases for naphthalene and nitroarene compounds (nitrobenzene and nitrotoluenes) and these enzymes are from members of the β-proteobacteria, such as *Ralstonia*, *Burkholderia*, *Comamonas*, and *Acidovorax*, rather than the γ-proteobacteria. Recent studies have suggested that NDO in the *dnt/ntd* group may be more common in nature than previously thought.[73,295] *Comamonas* sp. strain JS765, *Acidovorax* sp. strain JS42, and *Burkholderia* sp. strain DNT contain nitroarene dioxygenases that are capable of attack at the nitro-substituted carbons of nitrobenzene, 2-nitrotoluene, or 2,4-dinitrotoluene, respectively, resulting in the removal of the nitro group as nitrite and rearomatization of the aromatic ring to form a catechol.[173,208,265] These enzyme systems are very closely related to NDO, in particular that from *Ralstonia* sp. strain U2 (*nagA*; 94% DNA sequence identity[92]), and are believed to have evolved from an ancestral NDO.[92,210]

The *phn*-like group is represented by the naphthalene/phenanthrene dioxygenase from *Burkholderia* sp. RP007 (Figure 2).[165] Additional *phn*-like dioxygenases have recently been identified from several other *Burkholderia* species.[296] The gene order of the cluster from RP007 is very different from that of the *nah*-like or *dnt/ntd* group of NDO. Genes encoding electron transport components were not found within the 11-kb sequenced region.[165] In addition, the presence of genes encoding a LysR-type regulator that is very distantly related to NahR from *P. putida* G7 (21% similarity) and a regulator of the NtrC family suggests that the genes may be controlled differently from those of the other two groups of NDO genes, possibly in a manner reminiscent of the regulation of the TOL plasmid genes.[165,226]

A series of naphthalene and/or phenanthrene and pyrene dioxygenases (Gram-positive PAH family, Figure 2) have been identified in Gram-positive organisms, including *Rhodococcus*,[5,157,164,281] *Nocardioides*,[238] and *Mycobacterium*.[149,156] The sequences of the α subunit genes from Gram-positive bacteria cluster together, and it is of note that they are quite distantly related to those from Gram-negative bacteria (Figure 2). The order of the naphthalene pathway genes differs from those of the γ- and β-proteobacteria, and the ferredoxin and reductase genes have only been identified in *Nocardioides* sp. strain KP7. In this strain, the ferredoxin and reductase genes are located approximately 3-kb downstream of the dioxygenase α and β subunit genes,[238]

but the ferredoxin and reductase genes do not appear to be co-localized with the dioxygenase genes in *Rhodococcus* or *Mycobacterium*.[149,157,281] The ferredoxin from strain KP7 has features of a [3Fe–4S] or a [4Fe–4S] type ferredoxin rather than a [2Fe–2S] type ferredoxin.[238] Another example of a [3Fe–4S] or a [4Fe–4S] type ferredoxin participating in electron transfer to a Rieske non-heme iron oxygenase is in the diterpenoid dioxygenases from *Pseudomonas abietaniphila* BKME-9 (Figure 2).[182] These two enzyme systems represent examples of oxygenases that do not fit the Batie classification system.

2.4. Diversity of Toluene/Biphenyl Family Dioxygenases

In contrast to enzymes for naphthalene and PAH degradation, the toluene/biphenyl family of dioxygenases is a cohesive group with enzymes from both Gram-positive and Gram-negative organisms represented. This family is comprised of three-component enzymes from pathways for the degradation of benzene, alkylbenzenes, chlorobenzenes, and biphenyl. Dioxygenases that initiate pathways for the degradation of benzene in *P. putida* ML2 and BE81,[131,276] and benzene, toluene, and ethylbenzene degradation in *P. putida* F1[308,309] and *P. putida* DOT-T1E,[189] are members of this family (Figure 2). However, *P. putida* ML2 uses an *ortho*-cleavage pathway,[13] *P. putida* BE-81 can use either an *ortho* or a *meta* pathway,[254] and *P. putida* F1 and DOT-T1E use a *meta* pathway for completing the conversion of these substrates to TCA cycle intermediates. Chlorobenzene dioxygenases[23,190,293] also fall into this family, and those that have been sequenced thus far cluster with benzene and toluene dioxygenases. These enzymes catalyze *cis*-dihydroxylation and dechlorination of their substrates (Reaction C, Table 1). The chlorobenzene dioxygenases from *Pseudomonas* sp. P51[293] and *Ralstonia* (formerly *Pseudomonas*) sp. PS12[23] allow these strains to grow with multiply chlorinated benzenes. Both enzymes have broad substrate specificities.[221,227] The ethylbenzene-degrading isolate *P. fluorescens* CA-4[59,60] apparently uses one pathway for the degradation of toluene, ethylbenzene, propylbenzene, and *sec*-butylbenzene, and the dioxygenase that initiates degradation of these compounds is most closely related to the isopropylbenzene dioxygenase from *Pseudomonas* sp. JR1 (see below).

Numerous biphenyl degradation pathways in both Gram-negative and Gram-positive bacteria are initiated by BPDOs. BPDOs from *Pseudomonas pseudoalcaligenes* KF707,[96] *Burkholderia xenovorans* LB400,[85] BPDO *Comamonas testosteroni* B-356,[271] and *Rhodococcus* sp. RHA1[184] have been studied in some detail. Closely related enzyme systems can be found in *Rhodococcus globerulus* P6,[12] and *P. putida* KF715,[119] and many of these enzymes have the ability to attack various polychlorinated biphenyls (PCB) congeners (see Section 4.5). One BPDO, from *Rhodococcus erythropolis* TA421,[7] does not cluster tightly with this family (Figure 2).

Isopropylbenzene (cumene) dioxygenases (Figure 2) have been identified from several bacteria. *P. putida* RE204 and *P. fluorescens* IP01 were isolated by selective enrichment for growth with isopropylbenzene as sole carbon and energy source.[6,77] *P. putida* RE204 also grew with toluene, ethylbenzene and *n*-butylbenzene, but not benzene. *P. fluorescens* IP01 also grew with toluene, ethylbenzene, *sec*-butylbenzene, and *tert*-butylbenzene, but not biphenyl or benzene.[6] *Pseudomonas* sp. JR1 was isolated from an isopropylbenzene enrichment and was able to grow with toluene. Several Gram-positive isopropylbenzene degraders have been characterized. *Rhodococcus erythropolis* BD2 was isolated from a toluene- and TCE-contaminated soil sample with isopropylbenzene as sole carbon source.[63] BD2 utilizes a linear conjugative plasmid-encoded isopropylbenzene degradation pathway identical to that described for *P. putida* RE204.[62] The isopropylbenzene dioxygenase proteins in *R. erythropolis* BD2 are most closely related to the corresponding proteins from the biphenyl pathway in *Rhodococcus* sp. RHA1, ranging from 87 to 99% amino acid sequence identity.[148]

Compared to other alkylbenzenes, there have been few reports of bacterial isolates capable of growth with *o*-xylene, and most characterized strains utilize a monooxygenase-mediated pathway.[14,29,31] Recently, however, *Rhodococcus* sp. strain DK17 was isolated for its ability to grow with *o*-xylene.[152] DK17 also grows with benzene, toluene, ethylbenzene, and isopropylbenzene, and a single mutation eliminated the ability to grow with all of these substrates as well as the ability to convert indole to indigo. These results suggested that a single oxygenase carries out the initial oxidation of all substrates, but two different pathways are used to complete the degradation of benzene and alkylbenzenes. Results of catechol dioxygenase assays demonstrated that the alkybenzenes were degraded through a *meta* pathway, but growth with benzene induced an *ortho*-cleavage dioxygenase.[152] Surprisingly, the genes encoding the oxygenase subunits, ferredoxin and reductase components (*akbA1A2A3A4)* were identical in sequence to the ethylbenzene dioxygenase components from *Rhodococcus* sp. RHA1 and the α subunits do not cluster with the toluene/biphenyl family (RHA1 EtbA1; DK17 AkbA1a, Figure 2).[151]

2.5. Diversity of Benzoate Family Dioxygenases

The benzoate family is thus far represented by two-component enzymes (reductase and dioxygenase) where the dioxygenase is composed of both α and β subunits. Substrates for these enzymes include various aromatic acids and aniline. Enzymes that have been characterized include benzoate dioxygenases from *Acinetobacter* sp. ADP1,[197] *P. putida* mt-2,[132] and *Rhodococcus* sp. strain 19070,[112] and toluate dioxygenase from *P. putida* pWWO[116] (Reaction B, Table 1). Closely related enzymes for anthranilate degradation have been identified in several organisms, including *Pseudomonas resinovorans* CA10[203] and

Acinetobacter sp. ADP1.[47,79] Interestingly, the anthranilate dioxygenase from *Burkholderia cepacia* DBO1[52] is a three-component enzyme whose α subunit sequence clusters with the salicylate family (DBO1 AndAc, Figure 2). In each case, however, these enzymes catalyze *cis*-dihydroxylation, deamination, and decarboxylation to form catechol (Reaction G, Table 1). In addition, cumate (isopropylbenzoate) dioxygenase and 2,4,5-trichlorophenoxyacetate oxygenase have been characterized from *P. putida* F1[75] and *B. cepacia* AC1100,[64] respectively. The 2-halobenzoate-1,2-dioxygenases from *Burkholderia* (formerly *Pseudomonas*) *cepacia* 2CBS and *Burkholderia* sp. TH2 and chlorobenzoate dioxygenase from *Burkholderia* sp. NK8 also fall into this family.[90,109,270] These enzymes catalyze *cis*-dihydroxylation, decarboxylation, and dehalogenation of *o*-halogenated aromatic acids (Reaction D, Table 1), although they differ in structure and sequence from enzymes catalyzing similar reactions (see Section 2.6). Finally, aniline dioxygenases from *P. putida* UCC22 (pTDN1),[94] *Acinetobacter* sp. YAA aniline dioxygenase,[93] *Delftia acidovorans* 7N,[284] and *Fraturia* sp. ANA-18[191] also group with this family. These enzymes catalyze *cis*-dihydroxylation and deamination of aniline to form catechol (Reaction F, Table 1).

2.6. Diversity of Salicylate Family Dioxygenases

Salicylate 5-hydroxylase (Figure 2) from *Ralstonia* sp. strain U2[305] catalyzes the conversion of salicylate to gentisate (Reaction P, Table 2), and is a key enzyme in the naphthalene degradation pathway in this strain (Figure 3).[92,137] Interestingly, the ferredoxin and reductase components of salicylate 5-hydroxylase are shared with NDO.[305] Five multicomponent salicylate 1-hydroxylases were recently identified in three different sphingomonads.[55,68,220] These enzymes catalyze the conversion of salicylate to catechol, but are unrelated to the well-characterized single polypeptide flavoprotein monooxygenases that are known to catalyze the same reaction in other organisms. The salicylate 1-hydroxylase from *Sphingomonas yanoikuyae* B1 was shown to share its ferredoxin and reductase components with a NDO/BPDO and toluate dioxygenase,[55] and this also appears to be the case in the other two sphingomonads.[68,220] It is not clear at this time, however, whether these multicomponent salicylate 1-hydroxylases catalyze an initial dioxygenation of the aromatic ring of salicylate with the formation of an unstable compound that rearranges, releasing water and CO_2 (analogous to Reaction G, Table 1), or if they actually function as monooxygenases that attack directly at the carboxyl-substituted carbon.

P. aeruginosa strains 142 and JB2 utilize halobenzoates using an interesting three-component dioxygenase that is related to the salicylate hydroxylases (Figure 2). The enzyme catalyzes the oxygenolytic *ortho*-dehalogenation

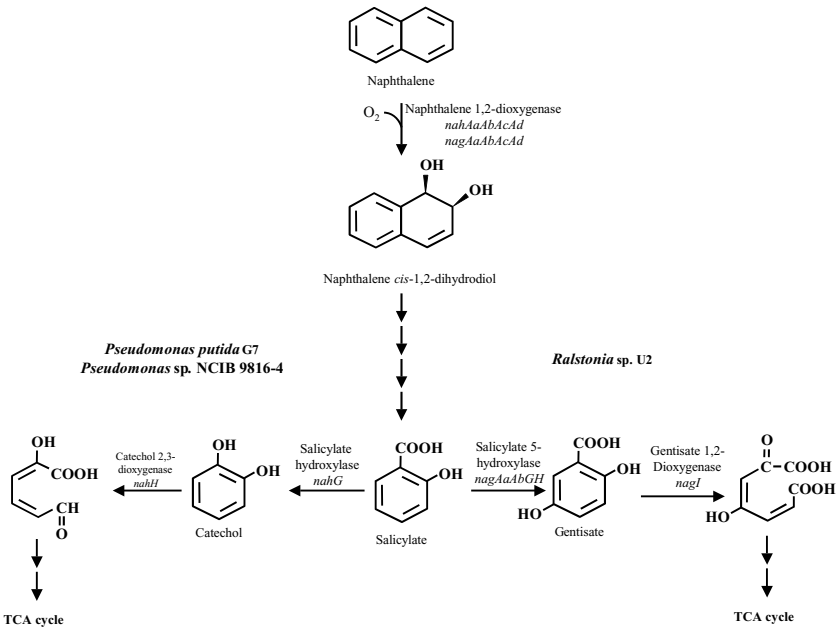


Figure 3. Pathways for the aerobic degradation of naphthalene in pseudomonads and *Ralstonia* sp. U2.

of 2-chlorobenzoate to form catechol (Reaction D, Table 1).[283] The electron transport protein-encoding genes are not co-localized with the oxygenase α and β subunit genes in either strain.[120,283] Terephthalate dioxygenase is also related to these enzymes[252,291] and is different from phthalate dioxygenases in structure and sequence (see Section 2.7). Like other members of the salicylate family, terephthalate dioxygenase has two components, reductase and oxygenase, and the oxygenase is composed of α and β subunits.[241,252,291] As mentioned in Section 2.5, the unusual anthranilate dioxygenase from *B. cepacia* DBO1 clusters with this family.[52]

2.7. Diversity of Phthalate Family Dioxygenases

Most current members of the phthalate family of Rieske non-heme iron oxygenases are two-component enzymes, each consisting of an α_n oxygenase component (lacking β subunits) and a reductase component. Phthalate dioxygenase, the namesake of this family, has been identified in a number of different Gram-positive and Gram-negative bacteria.[76,111,204,260] The well-studied phthalate dioxygenase from *Burkholderia cepacia* DBO1[19,20,61] is encoded by

the *ophA1* and *ophA2* genes, which are separated by the genes encoding the next two steps in phthalate degradation [dihydrodiol dehydrogenase (*ophB*) and decarboxylase (*ophC*)].[53] Genes encoding the oxidation of isophthalate have recently been identified from *C. testosteroni* YZW-D.[291] Like phthalate dioxygenase, isophthalate dioxygenase is a two-component enzyme with a homomultimeric oxygenase. A related enzyme is the chlorobenzoate 3,4-dioxygenase (CbaAB) from *Alcaligenes* sp. (BR60), which catalyzes the *cis*-dihydroxylation and dechlorination of 3-chlorobenzoate (Reaction C, Table 1).[193]

The phenoxybenzoate dioxygenase from *P. pseudoalcaligenes* POB310 catalyzes the angular dioxygenation (Reaction I, Table 1) of 3- and 4-carboxydiphenyl ether.[66] The unstable product of the reaction spontaneously rearranges to release phenol and protocatechuate. Like other members of this family, the enzyme has two components, PobA and PobB and the oxygenase (PobA) lacks a β subunit.

Some enzymes in this family do not appear to function as dioxygenases with their native substrates, but as monooxygenases. 2-Oxo-1,2-dihydroquinoline-8-monooxygenase from *P. putida* 86[236] is involved in the degradation of quinoline. This two-component enzyme is encoded by *oxoO* (oxygenase component) and *oxoR* (reductase component), which are separated by 15 kb of DNA.[237] The reaction results in net monooxygenation (Reaction P, Table 2) of the aromatic ring of 2-oxo-1,2-dihydroquinoline. Toluene sulfonate methyl-monooxygenase from *C. testosteroni* T-2 oxidizes the methyl group of toluene sulfonate, converting it to 4-sulfobenzyl alcohol (Reaction K, Table 2). The *tsaMB* genes encode the two components (oxygenase and reductase) of the enzyme.[139] The same strain, *C. testosteroni* T-2, also has a sulfobenzoate 3,4-dioxygenase that catalyzes the dioxygenation and desulfonation of sulfobenzoate (Reaction H, Table 1) to form protocatechuate and sulfite.[177] Vanillate demethylase, encoded by the *vanAB* genes, has been identified in *P. putida* WCS358,[286] *Acinetobacter* sp. ADP1,[245] *Pseudomonas* sp. ATCC 19151,[46] *P. fluorescens* BF13,[56]and *Pseudomonas* sp. HR199.[223] This enzyme catalyzes the oxygenative demethylation (Reaction N, Table 2) of vanillate to protocatechuate, and plays an important role in the degradation of the common plant metabolite ferulate. The product of the *ligX* gene from *Sphingomonas paucimobilis* SYK-6 encodes a 5,5′-dehydrodivanillic acid *O*-demethylase that participates in the complex pathway of lignin metabolism in this strain.[258]

Unlike other members of this family, carbazole dioxygenases from *P. resinovorans* CA10 and *P. stutzeri* OM1 are three-component enzymes, consisting of oxygenase (α_3), ferredoxin (Rieske [2Fe–2S]), and reductase (plant-type [2Fe–2S] and FAD).[207,239] Interestingly, both strains carry adjacent duplicate dioxygenase genes (CarAa). Carbazole dioxygenase catalyzes an angular dioxygenation (Reaction I, Table 1) to form 2′-aminobiphenyl-2,3-diol.

2.8. Other Ring Hydroxylating Dioxygenases

Several additional multicomponent Rieske non-heme iron oxygenases do not fall into any of the major families. The diterpenoid dioxygenase from *P. abietaniphila* BKME-9 catalyzes the dioxygenation of 7-oxo-dehydroabietic acid (Figure 1). A large gene cluster encoding several steps in the abietane diterpenoid degradation pathway has been characterized.[183,182,257] The *ditA1A2* genes encode the dioxygenase, which is not closely related to other dioxygenases (Figure 2); and the *ditA3* gene encodes an atypical [4Fe–4S] or [3Fe–4S] ferredoxin.[182] Apparently, the gene encoding the reductase has not yet been identified.

The 3-phenylpropionate dioxygenase from *Escherichia coli* K-12 catalyzes the initial dioxygenation of 3-phenylpropionate and cinnamate to the corresponding *cis*-2,3-dihydrodiols.[71] This three-component enzyme is most closely related to members of the toluene/biphenyl family of enzymes, but does not cluster with the family (Figure 2).

Several enzymes that catalyze angular dioxygenation (Reaction I, Table 1) on their respective substrates do not cluster tightly with the defined dioxygenase families (Figure 2). *Terrabacter* sp. strain DBF63 has an interesting angular dioxygenase that catalyzes the initial oxidation of dibenzofuran. The *dbfA1A2* genes encode dibenzofuran 4,4a-dioxygenase, and these proteins do not cluster with their counterparts in any of the dioxygenase families.[145] The ferredoxin component is encoded by the *dbfA3* gene, which is located 2.5 kb downstream of the *dbfA1A2* genes.[111] The ferredoxin contains a [3Fe–4S] center rather than a typical [2Fe–2S] center,[145,272] and the reductase component has not yet been identified. In contrast, a tightly clustered set of four genes encoding dibenzofuran dioxygenase was cloned from *Terrabacter* sp. YK3.[129] Several differences were noted between the *Terrabacter* sp. YK3 and *Terrabacter* sp. strain DBF63 dibenzofuran dioxygenases. The dioxygenase α and β subunits from YK3 were quite distantly related to those from DBF63, the YK3 ferredoxin carried a [2Fe–2S] cluster rather than a [3Fe–4S] center as in DBF63, and the gene encoding the YK3 FAD-containing reductase was located just downstream of the ferredoxin gene. The dibenzofuran/dibenzo-*p*-dioxin dioxygenase from *Sphingomonas wittichii* RW1[300] also catalyzes angular dioxygenation on these substrates.[48] The α and β subunits of the RW1 dioxygenase are only distantly related to those from DBF63 and YK.[39,129,145] Two isofunctional putidaredoxin-type (rather than the more common Rieske type) ferredoxins and two isofunctional flavin-containing reductases were shown to have the ability to transfer electrons to the oxygenase, and the genes encoding these components are apparently dispersed on the chromosome.[8,10,11] The carbazole dioxygenase from *Sphingomonas* sp. CB3 also does not group with any of the main families.[251] This three-component carbazole dioxygenase is very different from that from *P. resinovorans* CA10. The oxygenase has both α and β subunits unlike that

from CA10, and the α subunits from these two proteins only share 13% amino acid sequence identity. At the time it was identified, the CB3 carbazole dioxygenase was actually most closely related to isopropylbenzene dioxygenases and BPDOs from Gram-positive bacteria, but it does not cluster with the toluene/biphenyl family. More recently, it was found to be more closely related to the dioxin dioxygenase from *Sphingomonas wittichii* RW1.[200] Nojiri *et al*.[200] have argued that the enzyme from *Sphingomonas* sp. CB3 may not actually be a carbazole dioxygenase, because the only evidence for its role in carbazole degradation is the fact that the genes are induced during growth with carbazole. However, it is unlikely to be a BPDO, because biphenyl is not a growth substrate for CB3 and carbazole-grown CB3 cells do not oxidize biphenyl.[251]

2-Aminobenzene sulfonate dioxygenase is a two-component enzyme system comprised of an oxygenase with both α and β subunits, and a reductase. The oxygenase has been purified and characterized.[181] The enzyme (O-1 AbsA, Figure 2) from *Alcaligenes* sp. O-1 initiates the pathway for 2-aminobenzoate degradation by catalyzing *cis*-dihydroxylation and deamination to form 3-sulfocatechol (Reaction F, Table 1). The most closely related enzyme to AbsA, surprisingly, is methanesulfonic acid monooxygenase (M2 MsmA, Figure 2) from *Methylosulfonomonas methylovora* M2, an enzyme that oxidizes a non-aromatic substrate.[65] The enzyme has three components. The oxygenase is composed of α and β subunits and has an unusual iron–sulfur center-binding motif: while most α subunits of Rieske centers have 15–17 residues between the two Cys-His pairs, this enzyme has 26. The ferredoxin and reductase components are more like those from diiron center-containing monooxygenases such as toluene 4-monooxygenase from *P. mendocina* KR1, than from aromatic ring hydroxylating dioxygenase systems, making this quite an unusual enzyme.[65]

3. RING HYDROXYLATING DIOXYGENASE STRUCTURE AND FUNCTION

To date, protein components from several dioxygenase systems have been purified and studied in detail. In this chapter, we will focus on NDO as a model system and as a basis for comparison to other related enzyme systems.

3.1. Enzymology of NDO from *Pseudomonas* sp. NCIB 9816-4

Two pathways for the aerobic degradation of naphthalene have been described (Figure 3). Both pathways utilize naphthalene 1,2-dioxygenase (E.C. 1.14.12.12) to convert naphthalene to (+)-naphthalene *cis*-(1*R*,2*S*)-dihydrodiol (naphthalene *cis*-dihydrodiol). Naphthalene *cis*-dihydrodiol

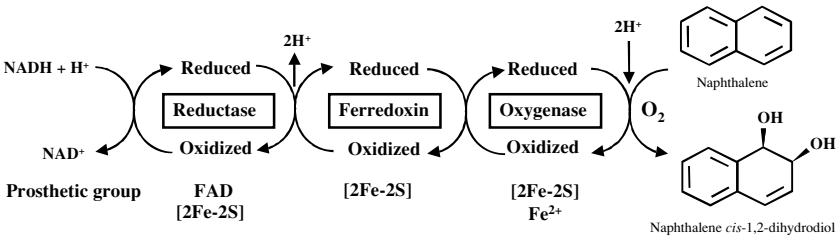


Figure 4. Reaction catalyzed by the three-component naphthalene dioxygenase (NDO) system. Electrons from NADH are transferred by the iron–sulfur flavoprotein reductase to the Rieske [2Fe–2S] ferredoxin. An electron is then transferred from the ferredoxin to one of the Rieske centers in the oxygenase. Reduced oxygenase catalyzes the addition of both atoms of O_2 to naphthalene, forming enantiomerically pure (+)-naphthalene *cis*-(1*R*, 2*S*)-dihydrodiol.

dehydrogenase (E.C. 1.3.1.29) then oxidizes naphthalene *cis*-dihydrodiol to 1,2-dihydroxynaphthalene. The oxidized ring is then cleaved and further degraded to form salicylate (Figure 3). Pseudomonads and some Gram-positive organisms convert salicylate to catechol, which is degraded by a standard *meta* cleavage pathway,[34,50,158,301] although variants that use the *ortho* pathway have been reported.[50,89] In contrast, *Ralstonia* sp. strain U2 and some strains of *Rhodococcus* convert salicylate to gentisate and utilize a gentisate dioxygenase pathway to complete naphthalene degradation (Figure 3).[3,70,305,306]

NDO is a three-component enzyme system that catalyzes the addition of both atoms of oxygen to the aromatic ring of naphthalene (Figure 4). All three protein components of NDO have been purified from *Pseudomonas* sp. NCIB 9816-4.[82,114,115] The reductase is a 35 kDa monomer that contains one molecule of FAD and a plant-type iron–sulfur center. It can accept electrons from either NADH or NADPH.[115,256] The ferredoxin is a Rieske [2Fe–2S] center-containing monomer of approximately 11.4 kDa.[114,256] The catalytic oxygenase component is an $\alpha_3\beta_3$ hexamer consisting of large (α) and small (β) subunits.[147] Each α subunit contains two redox centers, a Rieske [2Fe–2S] center and mononuclear Fe^{2+}at the active site. Individually purified α and β subunits of the oxygenase were reconstituted,[263,264] demonstrating that both subunits are essential for activity, a result consistent with those obtained with BPDO[127] and toluene dioxygenase.[135] As purified, the Rieske center of the oxygenase is oxidized, and the iron at the active site is reduced. Electrons are transferred sequentially from NAD(P)H to the reductase, to the ferredoxin, to the Rieske center of the oxygenase, and finally to the iron at the active site of the oxygenase. The reduced oxygenase catalyzes the addition of both atoms of O_2 to the aromatic ring. Two electrons are necessary to complete the reaction cycle.

Among the aromatic ring hydroxylating dioxygenases that have been identified to date, two types of oxygenase structures are known: those with both α and β subunits, such as NDO, and those consisting of only α subunits,

such as phthalate dioxygenase. Based on studies of hybrid dioxygenases, in which the individual α and β subunits from different enzymes were substituted, it appears that the α subunits of NDO and the closely related enzymes 2-nitrotoluene dioxygenase and 2,4-dinitrotoluene dioxygenase control substrate specificity.[209,212] Similar results were reported with BPDO hybrids, toluene dioxygenase-tetrachlorobenzene dioxygenase hybrids, and benzene-BPDO hybrids.[17,24,186,275,307] These results are consistent with the crystal structures of NDO and related dioxygenases (see Section 3.2), which showed that no β subunit residues are near the active site.[91,98,147] Other studies have suggested, however, that β subunits may play a role determining substrate specificity in toluene, toluate, and other BPDOs.[54,117,121,128] Therefore, it seems as though the β subunit has a structural function in most dioxygenases, but in some cases, the β subunit may be capable of modulating substrate specificity.

3.2. Dioxygenase Structure and Mechanism

Crystal structures of the oxygenase component of NDO have been determined in the presence and absence of various substrates.[51,143,147] NDO is an $\alpha_3\beta_3$ hexamer. The β subunits contain no redox centers and based on the currently available crystal structures of NDO, nitrobenzene dioxygenase (NBDO) from *Comamonas* sp. strain JS765, cumene dioxygenase from *P. fluorescens* IP01, and BPDO from *Rhodococcus* sp. strain RHA1, the β subunit residues are distant from the active sites in each enzyme.[91,98,147] The $\alpha_3\beta_3$ hexamer of NDO contains three active sites, which are located at the junctions of adjacent α subunits. Each α subunit contains a Rieske [2Fe–2S] center, which is coordinated by two histidines (His83; His104) and two cysteines (Cys81; Cys101), and mononuclear non-heme iron. This iron at the active site is in a distorted octahedral conformation coordinated by His208, His213, Asp362 and a water molecule (Figure 5).[147] These two redox center-binding motifs (C-X-H-X_{15-26}-C-X_2-H and H-X_{4-5}-H-X_n-D) are conserved in all sequenced Rieske non-heme iron oxygenases. The 2-His-1-carboxylate mode of mononuclear iron coordination has also been identified in a variety of non-heme iron-containing enzymes that catalyze a wide range of reactions, including not only Rieske non-heme iron oxygenases, but *meta*-cleavage dioxygenases, pterin-dependent hydroxylases such as tyrosine hydroxyase, and a family of β-lactam biosynthesis enzymes.[163,224] The recently published crystal structures of the *Comamonas* sp. strain JS765 NBDO, *Rhodococcus* sp. RHA1 BPDO, and *P. fluorescens* IP01 cumene dioxygenase showed very similar overall structures compared to NDO, including similar relative positions of the redox centers.[74,98] One difference was apparent between NDO and the two members of the toluene/biphenyl family of Rieske non-heme iron oxygenases. The conserved Asp residue that coordinates the active site iron in NDO (Asp 362) does so in a bidentate fashion with both coordinating carboxyl oxygen atoms approximately equidistant from the

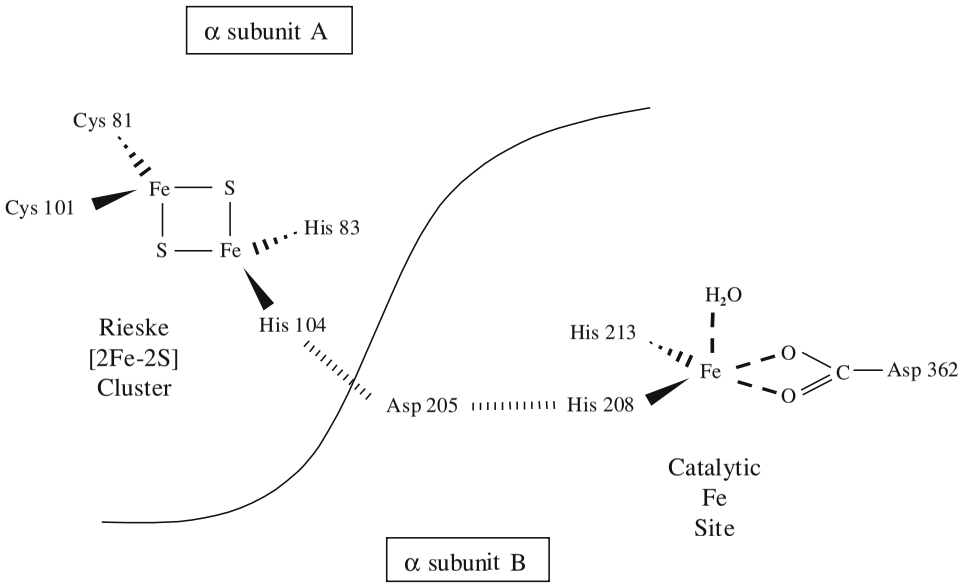


Figure 5. The junction between two α subunits in NDO based on the crystal structure of the enzyme. Shown are the Rieske [2Fe–2S] center and mononuclear iron at the active site. Amino acids Cys81, His83, Cys101, and His104 coordinate the Rieske center; His208, His213, and Asp362 coordinate mononuclear iron at the active site. See text for additional details.

iron.[147] In contrast, the corresponding aspartate residues in BPDO and cumene dioxygenase (Asp 378 and 388, respectively), are coordinated to the iron via a single carboxyl oxygen; in both cases, the other carboxyl oxygen is apparently too far away to coordinate the iron. Therefore, the coordination geometry of the mononuclear iron in both BPDO and cumene dioxygenase is distorted tetrahedral.[74,98]

Substitution of Asp362 with an alanine inactivated NDO, demonstrating that this iron-coordinating residue is essential.[214] In an earlier study, site directed mutagenesis of toluene dioxygenase (TDO) demonstrated that the conserved residues Glu214, Asp219, His222, and His228 (corresponding to Glu200, Asp205, His208, and His213 in NDO) were essential for enzyme activity, and were suggested to be mononuclear iron ligands.[136] Substitution of the corresponding histidines in benzene dioxygenase also resulted in loss of enzyme activity.[49] Of the four potential iron ligands, only the histidines were found to coordinate iron based on the NDO structure.[147] The loss of activity in the other two TDO mutants can be explained using the NDO structure. The structure revealed that Glu200 provides an important contact between adjacent α subunits by forming a salt link with Arg84.[146] Asp205 in NDO is hydrogen bonded to the Rieske center ligand His104 and mononuclear iron ligand His208. Based on this structural conformation, Asp205 appeared to participate in the electron transfer pathway between the NDO Rieske center and active site iron in adjacent α subunits in (Figure 5).[147] This hypothesis was supported by the analysis of mutant forms of NDO in which Asp205 was replaced by Ala, Glu, Gln, or Asn.[215] The modified proteins had little or no dioxygenase activity, although they appeared to have no major structural defects. Additional support for this role for Asp205 was provided when benzene, an efficient uncoupling agent for wild-type NDO,[168] was unable to stimulate oxygen uptake by the purified Gln205-containing enzyme. This result suggests that electrons cannot be passed from the Rieske center to the active site iron in the mutant form of the enzyme.[215] An alternative role for this conserved aspartate was proposed in studies of anthranilate dioxygenase, an enzyme that catalyzes the first step in anthranilate catabolism in *Acinetobacter* sp. strain ADP. In anthranilate dioxygenase, substitution of the aspartate corresponding to Asp205 in NDO with Ala, Glu, or Asn resulted in completely inactive enzymes. Analysis of wild-type and mutant forms of anthranilate dioxygenase at various pH values suggested that this aspartate may play a role in maintaining the reduction potential and the protonation state of the Rieske center of the oxygenase.[22]

As purified, NDO contains an oxidized Rieske center and ferrous iron at the active site. Spectroscopic studies with the *Burkholderia cepacia* DBO1 phthalate dioxygenase, which catalyzes the *cis*-dihydroxylation of phthalate, demonstrated that the iron is in a six-coordinate octahedral configuration in the absence of substrate, but a five-coordinate configuration results when substrate

is bound.[101,217,282] This result is in contrast to crystal structure data for NDO, which indicates that the coordination of iron does not change upon substrate binding, and in fact no significant conformational changes occur anywhere in the active site when substrate binds.[51,143] The crystal structure of the *Rhodococcus* sp. RHA1 BPDO revealed that when biphenyl was bound, significant conformational changes occurred at the active site.[98] These conformational changes in BPDO resulted in the formation of an entryway and an expansion of the substrate-binding pocket to allow biphenyl to enter and bind the active site pocket. The active site pockets of the *Rhodococcus* sp. RHA1 BPDO and the *P. fluorescens* IP01 cumene dioxygenase are strikingly similar,[74] which is consistent with their overall level of primary amino acid sequence identity (67%).

The nature of the iron-oxygen species involved in catalysis is not known. Studies of 4-methoxybenzoate monooxygenase suggested an attack by a ferric-peroxo intermediate in the monooxygenation and *cis*-dihydroxylation reactions,[27] and recent work with NDO supports this possibility.[51,143,168] The participation of a high valent iron-oxo species, as in the methane monooxygenase[175] and cytochrome $P450^{242}_{cam}$ reaction cycles, has also been proposed.[225,298,299] Single turnover studies with NDO demonstrated that 0.85 units of product were formed per electron added, and both the Rieske center and mononuclear iron were oxidized during the reaction,[299] and peroxide-mediated turnover with NDO was recently reported.[298] The observation that the reaction stopped after one turnover is consistent with the idea that the release of product requires the input of a second electron. NDO is known to catalyze other reaction types in addition to *cis*-dihydroxylation (dioxygenation), including benzylic monohydroxylation (monooxygenation[234,288]), *O*- and *N*-dealkylation,[229] sulfoxidation,[169] and desaturation[107,280] reactions (Table 2). However, the *cis*-dihydroxylation reaction is unique to bacterial aromatic ring-hydroxylating dioxygenases, and it has implications for the mechanism of the enzyme. Recent crystal structure data demonstrated that oxygen binds side-on to the iron at the active site of NDO, and the substrates naphthalene or indole bind in a long cleft in the active site approximately 4 Å from the iron atom.[51,143] Alone, dioxygen binds side-on approximately 2.2 Å from the iron, but measurably closer to the iron when substrate is present.[143] This series of NDO crystal structures with substrate, oxygen, both substrate and oxygen, or product (naphthalene *cis*-dihydrodiol) bound suggest a concerted reaction mechanism in which both atoms of molecular oxygen react with the carbon atoms of the substrate double bond in a way that would explain the *cis*-specific dioxygenation catalyzed by this class of enzymes. Based on the structures of NDO alone and in complex with substrate, oxygen, and product, the reaction cycle shown in Figure 6 was proposed.[143]

Three ferredoxin reductase structures from three different dioxygenase systems are currently available. The phthalate dioxygenase reductase

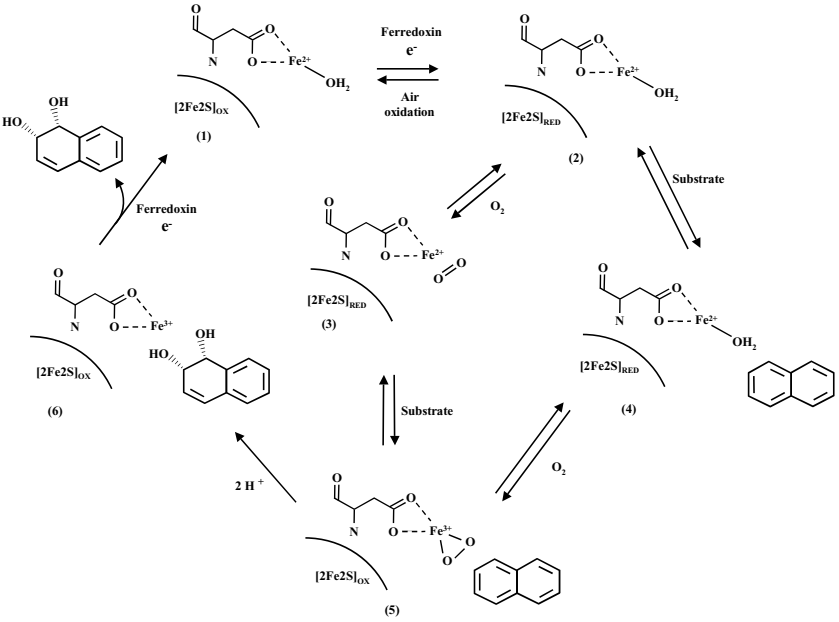


Figure 6. Proposed mechanism of NDO based on crystal structures of the enzyme alone, with bound substrate (naphthalene or indole), with bound O_2, with bound substrate and O_2, or with bound product (naphthalene *cis*-dihydrodiol). (1) As purified, the enzyme has an oxidized Rieske site. (2) One electron is transferred from the ferredoxin. Either oxygen (3) or substrate (naphthalene) (4) can bind at the active site. (5) Ternary complex with naphthalene and oxygen bound at the active site. (6) Product formation; release of product requires input of an additional electron from the ferredoxin. Reproduced wth permission from Karlsson *et al*. [143].

(PDR) from *Burkholderia* (formerly *Pseudomonas*) *cepacia* DB01[61] and the benzoate dioxygenase reductase (BenC) from *Acinetobacter* sp. strain ADP1[142]are members of the $NADP^+$-ferredoxin reductase superfamily. PDR is the most well-studied of the electron transfer partners from this family of dioxygenases.[19,61,99,100,102] Benzoate and phthalate dioxygenases are two-component systems consisting of an oxygenase and a ferredoxin reductase.[20] The ferredoxin reductases are both composed of three distinct domains, for binding a plant-type [2Fe–2S] center, NAD, and flavin (FMN in PDR; FAD in BenC), but the arrangement of the domains differs in the two proteins.[61] The BPDO reductase (BphA4) from the three-component enzyme in *Pseudomonas* sp. strain KKS102 has very low sequence identity with other aromatic ring-hydroxylating dioxygenase reductase components.[150] It contains NAD- and FAD-binding domains, but no iron–sulfur cluster; electrons are transferred from BphA4 to the [2Fe–2S] center in an intermediary ferredoxin protein. The

protein fold of BphA4 is actually similar to that of glutathione reductases[248] and putidaredoxin reductase from *P. putida* cytochrome $P450_{cam}$.[250] Although these reductases belong to two distinct families, the overall structures of the NAD- and FAD-binding domains are quite similar.[142] On the basis of modeling studies, the NDO reductase structure is predicted to be similar to that of benzoate dioxygenase reductase.[142]

The structure of the BPDO ferredoxin component (BphF) from *Burkholderia* sp. strain LB400 was determined at 1.6 Å resolution.[57] The structure of the carbazole dioxygenase ferredoxin component (CarAc) from *P. resinovorans* CA10 was solved by molecular replacement with the BphF structure.[194] Although the sequences of the two proteins are only 34% identical, their structures were very similar, and the location of the Rieske center at the surface of the proteins may have implications for docking with the reductase and oxygenase components to allow electron transfer. One notable difference was in the surface charges of the two proteins, which probably determine the ability to interact productively (and specifically) with the respective oxygenases of each enzyme system.[194] To our knowledge, no reports have yet been published demonstrating co-crystallization of non-covalent complexes of ferredoxins with their corresponding oxygenases or ferredoxin reductases for any of the three-component aromatic ring hydroxylating dioxygenase systems, and information regarding specific protein–protein interactions is limited. However, cross-linking studies with the three toluene dioxygenase components suggested the involvement of electrostatic interactions between the proteins, and argued against the formation of a ternary complex.[167] These results imply that the ferredoxin in three-component enzyme systems alternately binds to the reductase and oxygenase components using the same surface near the exposed Rieske center in order to carry out electron transfer.

3.3. Substrate Specificity of NDO and Other Dioxygenases

In general, aromatic ring hydroxylating dioxygenases are capable of initiating oxidative attack on a very wide range of substrates.[41,232] Many of these enzymes display remarkable diversity in the number of substrates oxidized and the types of reactions catalyzed. Our understanding of the mechanism of substrate oxidation has been based on studies with blocked mutant strains that accumulate the products of dioxygenase-catalyzed reactions, recombinant strains expressing dioxygenase genes, and in some cases with purified enzymes. NDO from *Pseudomonas* sp. NCIB 9816-4 is known to catalyze the oxidation of more than 75 different substrates by reactions including *cis*-dihydroxylation, monooxygenation,[234,288] desaturation,[107,280] *O*- and *N*-dealkylation,[229] and sulfoxidation[169] (Tables 2 and 3). The complete list of substrates oxidized and products formed by NDO is summarized in a useful

Table 3. Representative reactions catalyzed by Rieske non-heme iron oxygenases for specific pathway substrates.

	Type of oxidation reaction[a,b]																
	Dihydroxylations									Monohydroxylations							
Substrate	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	References
Aromatic hydrocarbons																	
Naphthalene	X																33,69,83,133,164,256,274
Benzene	X																103,130,276
Toluene	X										T						171,189,262,309
Ethylbenzene	X									T		T					59,171
Styrene	X/T																141,140,170,292
Isopropylbenzene	X																6,63,77
o-Xylene	X										T						152,171
Biphenyl	X																7,12,85,96,119,184,271
Anthracene	X/T																1,134,201
Phenanthrene	X/T																134,165,238
9,10-Dihydrophenanthrene	T									T							40,230
Chrysene	T																35,39,104
Pyrene	X																149,156,290
Fluoranthene	X																201,261
Acenaphthene										T							246
Fluorene	X/T									T							108,231,247
Substituted aromatic compounds																	
Aniline						X											93,94,284
Anthranilate							X										47,52,191
Benzoate		X															112,132,197
Salicylate																X	55,68,220,305
o-Halobenzoates			X	X													90,109,270,283
Chlorobenzoates	T	X	X														90,192,193

(*Continued*)

Table 3. (*Continued*)

Substrate	Type of oxidation reaction[a,b]																References
	Dihydroxylations									Monohydroxylations							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	
Isopropylbenzoate	X																75
3-Phenylpropionate	X																71
Cinnamate	X																71
Chlorobenzenes	T		X														23,126,285
(poly)Chlorobiphenyls	T		T														15,21,105,206,249
Phthalate (*o*-)	X																53,76,111,195,207
Isophthalate		X															291
Terephthalate		X															241,252,291
Toluates (*o*-, *m*-)		X															116
4-Methoxybenzoate														X			25
Vanillate														X			46,223,245
p-Toluene sulfonate											X						139,178
Aminobenzene sulfonate						X											181
p-Sulfobenzoate								X									177
Nitrobenzene					X												173
2-Nitrotoluene	T				X						T						4,208,212,235
3-Nitrotoluene					T						T						173,235
4-Nitrotoluene	T				T						T						173,235
2,4-Dinitrotoluene					X						T						173,266
2,6-Dinitrotoluene					X												198
2,2′-Dinitrobiphenyl					T												244
1,2,4-Trimethylbenzene											T						246
1- and 2-substituted naphthalenes	T	X															30,44,67,154,294
Dimethylnaphthalenes											T						30,246
7-Oxodehydroabietic acid	X																182
5,5′-Dihydrodivanillic acid														X			258
Heterocyclic aromatic compounds																	
Carbazole	T								X								110,201,207,233,239,251
Dibenzofuran	T								X								48,129,145,231,244,272
Dibenzodioxin	T								X								8–11,244

Table 3. (*Continued*)

Substrate	Type of oxidation reaction[a,b]																References
	Dihydroxylations									Monohydroxylations							
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	
Dibenzothiophene	X/T												T				155,201,231
Indole	T																84
Fluorenone	T								X								246,247
Quinolines	T																38,41
2-Oxo-1,2-dihydroquinoline																X	236,237
Pyrazon	X																240
3-Methyl benzothiophene											T		T				246
2-Methylbenzo-1,3-thiole													T				2
Carbocyclic, alkyl-aryl ether, thioether or N-alkyl substrates																	
Indan										T		T					43,107,288
Indene	T									T							58,107,281
1,2-Dihydronaphthalene	T									T		T					78,280
Tetralin	X																187,255
Methyl phenyl sulfide													T				2,169
Ethyl phenyl sulfide													T				2,169
Methyl *p*-tolyl sulfide													T				169
p-Methoxyphenyl methyl sulfide													T				169
Methyl *p*-nitrophenyl sulfide													T				169
Anisole	T													T			229
Phenetole	T											T		T			229
Carboxydiphenylethers									X								66
N-Methylindole															T		232
N-Methylaniline															T		166
N,*N*-Dimethylaniline															T		166

[a] The types of reactions are shown in Tables 1 and 2: A, *cis*-dihydroxylation (C=C); B, *cis*-dihydroxylation (at and adjacent to a carboxyl bearing carbon); C, *cis*-dihydroxylation and dehalogenation; D, *cis*-dihydroxylation, decarboxylation, and dehalogenation; E, *cis*-dihydroxylation and nitrite elimination; F, *cis*-dihydroxylation and deamination; G, *cis*-dihydroxylation, deamination, and decarboxylation; H, dihydroxylation and desulfonation; I, angular dihydroxylation; J, benzylic hydroxylation; K, methyl group hydroxylation; L, oxygen-dependent desaturation; M, sulfoxidation; N, *O*-dealkylation; O, *N*-dealkylation; P, net aromatic ring monohydroxylation.

[b] "*X*" denotes reactions catalyzed for growth substrates; "*T*" indicates a transformation reaction catalyzed for a non-growth substrate. This list is not exhaustive and additional information, particularly on transformation substrates, can be found in recent reviews.[36,42,125,232]

review.[232] TDO from *P. putida* F1 is even more impressive in its substrate range, with the ability to catalyze over 100 oxidation reactions on monocyclic aromatic compounds, fused and linked aromatic compounds, heterocyclic aromatic compounds, and a variety of halogenated and non-halogenated aliphatic olefins.[42,125,161] TDO has overlapping but frequently distinct specificity from NDO, and as with NDO, multiple reaction types are catalyzed by TDO [dioxygenation, monooxygenation,[288] desaturation,[280] and sulfoxidation[2,169] (Tables 2 and 3)]. For example, TDO oxidizes indan to (1*R*)-indanol and converts indene to *cis*-(1*S*, 2*R*)-indandiol and (1*R*)-indenol[288] while NDO oxidizes indan to (1*S*)-indanol and oxidizes indene to *cis*-(1*R*, 2*S*)-indandiol and (1*S*)-indenol.[107] Similar trends in enantioselectivity are typically observed for the *cis*-dihydroxylation, benzylic monohydroxylation, and sulfoxidation reactions catalyzed by NDO.[232] The range of multi-ring substrates oxidized by the angular dioxygenase carbazole 1,9a-dioxygenase from *P. resinovorans* CA10 has been reported and these results were compared with the products formed by the *Terrabacter* sp. dibenzofuran dioxygenase.[201,273] Both enzymes are capable of standard *cis*-dihydroxylation, angular dioxygenation, and monooxygenation depending on the substrate, suggesting that the specificity is determined by the position of the substrate in the active site relative to the active site iron atom.

3.4. Critical Amino Acids at the Active Site of NDO and Other Dioxygenases

The active site iron of NDO is located in a hydrophobic pocket that accommodates the predominantly hydrophobic substrates for the enzyme. The amino acids located near the active site were identified from the crystal structure of NDO.[51,143] Site-directed mutagenesis of residues near the active site iron has identified those that are important in determining the substrate specificity and enantioselectivity of NDO. These studies demonstrated that NDO was able to tolerate a wide range of single amino acid substitutions near the active site.[214,216] Enzymes with substitutions at position 352 of the oxygenase α subunit (phenylalanine in the wild-type enzyme) had the most striking changes in substrate specificity. These enzymes had altered enantioselectivity with naphthalene, biphenyl, phenanthrene, and anthracene, and changes in the regioselectivity were seen when biphenyl and phenanthrene were provided as substrates.[214,216] Replacement of Phe352 with smaller amino acids (Gly, Ala, Val, Ile, Leu, Thr) resulted in enzymes that produced increased amounts of biphenyl *cis*-3,4-dihydrodiol relative to biphenyl *cis*-2,3-dihydrodiol, and the stereochemistry of the biphenyl *cis*-3,4-dihydrodiol was altered. The NDO-F352V and NDO-F352T enzymes formed significant amounts of (−)-biphenyl *cis*-(3*S*,4*R*)-dihydrodiol, a compound not produced by wild-type NDO. A major shift in the regioselectivity was also seen with enzymes carrying substitutions

at Phe352 with phenanthrene as a substrate. In addition, enzymes with substitutions at position 206 (NDO-A206I and NDO-A206I/L253T) formed significantly more phenanthrene *cis*-1,2-dihydrodiol than wild type.[302] Several of the enzymes formed phenanthrene *cis*-9,10-dihydrodiol, a new product not formed by the wild type.

An attempt to engineer NDO into an enzyme with the substrate specificity of 2-nitrotoluene dioxygenase by making the corresponding amino acid substitutions in the active site was only partially successful. The α subunits of the two enzymes are 84% identical in primary amino acid sequence, with only five amino acid differences at the active site. The substrate specificities of the two enzymes are significantly different.[209] Only two amino acid substitutions at the active site of NDO (F352I, A206I) were necessary to change the enantioselectivity with naphthalene to that of 2-nitrotoluene dioxygenase, which forms 70% (+)-(1*R*,2*S*)-*cis*-naphthalene dihydrodiol.[302] Wild-type NDO makes >99% (+)-(1*R*,2*S*)-*cis*-naphthalene dihydrodiol. None of the mutant forms of NDO was capable of oxidizing the aromatic ring of 2-nitrotoluene with release of nitrite. In fact, enzymes with four or more amino acid substitutions were inactive,[302] and all of the mutant enzymes had lower overall product formation rates with all tested substrates.[214,216,302]

The nitrobenzene and 2-nitrotoluene dioxygenase components have been purified[213] and the crystal structure of the oxygenase component of NBDO has been solved.[91] The overall structure of the $\alpha_3\beta_3$ hexamer of NBDO looks strikingly similar to that of NDO,[147] and an overlay of the $\alpha\beta$ heterodimers from the two enzymes highlights the similarity (Figure 7a). However, several amino acid differences at the active site can account for the observed differences in substrate specificity. In particular, an asparagine at position 258 in the α subunit of NBDO (corresponding to Val260 in NDO) was shown to form a hydrogen bond with the nitro groups of nitro-substituted aromatic compounds (Figure 7b). This hydrogen bond positions the nitroarene substrates for oxidation at the nitro-substituted carbon.[91] Replacement of this asparagine with a valine in NBDO resulted in an enzyme with a significantly reduced ability to oxidize the aromatic ring of nitro-substituted aromatics.[138] A similar result was obtained with the same substitution in 2-nitrotoluene dioxygenase.[172] It therefore appears that the orientation of the nitroarene substrate in the active site governs the regioselectivity of the enzyme.

Recent studies have used the crystal structure of the NDO active site to predict active site residues in related dioxygenases. Based on such a model, the active site of the tetrachlorobenzene dioxygenase from *Ralstonia* sp. PC12 (TecA) was modified to identify specific amino acids involved in controlling both regioselectivity and product formation rate.[222] Substitution of Phe366 in the α subunit of TecA (corresponds to Phe352 in NDO) with tyrosine or tryptophan resulted in inactive enzymes,[222] a result similar to that obtained with the corresponding NDO mutants.[216] Substitution of smaller residues at this position

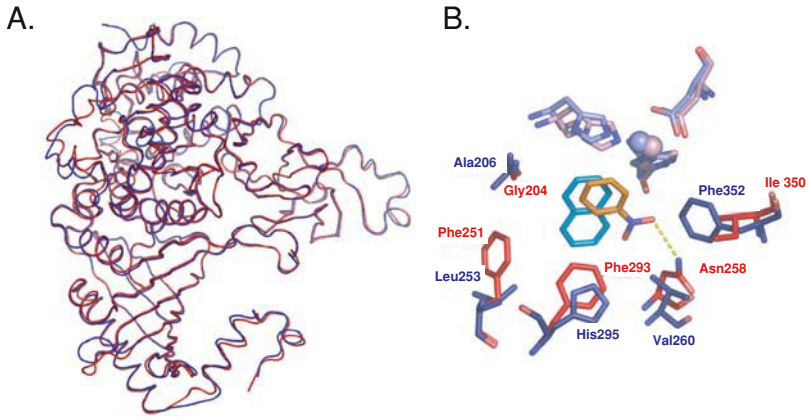


Figure 7. Comparison of the NDO and NBDO structures. (a) Overlay of αβ heterodimers (NDO in blue; NBDO in red). The α subunits are toward the top and β subunits toward the bottom of the figure. The active site iron and coordinating histidine side chains are visible in the α subunit. (b) Overlay of active site structures of NDO and NBDO (NDO in blue; NBDO in red). Histidine and aspartate residues coordinating iron at the active site are in light blue and pink for NDO and NBDO, respectively. Five residues at the active that differ between the two enzymes are shown. Bound naphthalene in NDO is aqua; bound nitrobenzene in NBDO is yellow.

in TecA resulted in a shift from dioxygenation of the aromatic ring to monooxygenation of the methyl group of mono- and dichlorotoluenes. Replacement of Leu272 with tryptophan or phenylalanine resulted in enzymes with improved rates of dichlorotoluene transformation but little or no change in regioselectivity. This residue corresponds to Val260 in NDO, and Asn258 in NBDO and 2-nitrotoluene dioxygenase. Therefore, based on substrates tested to date, the residue at this position plays a role in controlling regioselectivity in NBDO and 2-nitrotoluene dioxygenase, but not NDO or TecA.[91,210,214,222] In a similar study, the α subunit of BPDO from *P. pseudoalcaligenes* KF707 was subjected to site-directed mutagenesis based on modeling with the NDO coordinates. This study demonstrated that Phe227, Ile335, Thr376, and Phe377 were important for determining the position of oxidation with various PCB congeners.[269] Thr376 had been previously identified as playing an important role in determining substrate specificity based on sequence comparisons of BPDOs with different specificities.[153,186] In contrast, changing the corresponding residue in NDO (Thr351) did not seem to affect substrate specificity.[214] Similarly, substitutions in NDO at Phe202 (corresponds to Phe227 in $BPDO_{KF707}$) had little or no effect on substrate specificity. Phe377 corresponds to Phe352 in NDO, however, so the amino acid at this position has now been shown to be critical in TecA, NDO, and $BPDO_{KF707}$. From these studies, it appears that certain residues, such as Phe352 in NDO, may be important in determining the specificity in many

(or all) dioxygenase active sites, while others may play a specific role in just a small subset of enzymes. With more crystal structures becoming available, other key residues effecting substrate catalysis and dioxygenase selectivity may be identified.

4. BIOTECHNOLOGY APPLICATIONS

A number of biotechnology applications have been enabled by the high regioselectivity and enantioselectivity of aromatic hydrocarbon dioxygenases. Several examples include: (1) dioxygenase catalyzed synthesis of a wide range of chiral intermediates, some which have been subsequently used in the preparation of natural products, polyfuntionalized metabolites, or pharmaceutical intermediates, (2) recombinant expression of NDO in a multi-step pathway engineered for the fermentation based production of indigo from glucose, and (3) target-specific agents for biodegradation of environmental pollutants.

4.1. Production of Chiral Synthons

As noted in Section 3.3, several well-characterized aromatic hydrocarbon dioxygenases have been shown to catalyze diverse types of oxidation reactions for a wide range of substrates.[36,125,232] TDOs from *P. putida* F1 and UV4 have extremely broad specificity with respect to the monocyclic, substituted aromatic substrates, which are typically oxidized to arene-*cis*-dihydrodiols of high enantiopurity. Similarly, NDO,[232] BPDO,[36] and carbazole dioxygenase[201] catalyze *cis*-dihydroxyation, as well as other oxidations on a range of polycyclic, heterocyclic, and substituted aromatic compounds. Interested readers are referred to recent reviews detailing the chiral *cis*-dihydrodiols obtained to date, rational approaches for expanding asymmetric (synthetic) methodology, and their versatile application in synthesis of natural products.[34,36,125] The range of available metabolites resulting mainly from *cis*-dihydroxylation have been utilized in numerous syntheses for the preparation of fine chemicals, natural products, pharmaceutical intermediates, and compounds with biological activity (Table 4). It should be noted that many, but not all, metabolites described are amenable to large-scale production using whole cell biocatalysis. This whole-cell or resting-cell biotransformation approach has facilitated the production of multi-kilogram quantities of chiral metabolites and relies on the integrity of multicomponent dioxygenases activity, typically expressed in recombinant hosts with reduced cofactors supplied through the metabolism of exogenous carbon substrates (e.g. glucose). Alternatively, the use of purified oxygenases can be facilitated by the inclusion systems for enzymatic[243] or electrochemical[122,123] regeneration of reduced NAD(P)H cofactor.

Table 4. Examples of synthetic reactions and compounds accessible from *cis*-dihydrodiols.

Compounds that can be synthesized from chiral cis-dihydrodiols[a]
Azasugars, aminosugars, fluorodeoxysugars, perdeuterosugars
Conduritols, conduramines
Deoxysugar analogs
Erythroses
Inositols, inositol oligimers
Kifunensine
Lycoricidine
Narcliclasine
Nojirimycin analogs
Pancreastatin, (+) and (−)-7-deoxypancreastatin
Prostaglandin intermediates ($PGE_{2\alpha}$)
Pinitols
Pyrrolizidine alkaloids
Shikimic acid, methyl shikimate
Specionin
Zeylena
Synthetic reactions conducted utilizing chiral cis-dihydrodiols[a]
Cycloadditions of dienediols and their acetonides
Diels–Alder reactions
Oxidative cleavage-reductive cyclization
Cyclopropanation

[a] See Hudlicky *et al.* [125] for a detailed review of chiral synthons, product structures, and primary literature.

4.2. Indigo Production

The oxidation of indole to indigo was initially shown in *E. coli* strains expressing NDO from *Pseudomonas* sp. NCIB 9816-4.[84] NDO was shown to oxidize indole to an unstable *cis*-dihydroindolediol, which dehydrates to indoxyl, and undergoes spontaneous oxidation to indigo. Since the reaction is catalyzed by many related dioxygenases, this discovery has been widely utilized for detection and isolation of strains expressing mono- and dioxygenases. Commercial interest in the reaction led Genencor International to genetically engineer recombinant *E. coli* for the cost-competitive, multi-step production of indigo from glucose.[28] The fermentation process was based on a single strain expressing a modified tryptophan pathway (allowing high level indole production) and NDO from *P. putida*.[28] Several strategies were employed (e.g. gene dosage, gene amplification, gene inactivation) to improve metabolite flux, enzymatically eliminate the formation of isatin byproduct, and ultimately increase production of indigo to levels exceeding 18 g/l. Despite technical successes, the process for indigo production has not been implemented at an industrial scale.

4.3. Indinavir Production

Interest in enantiopure (−)-*cis*-(1*S*, 2*R*)-indandiol is based on its direct conversion to *cis*-(1*S*)-amino-(2*R*)-indanol, a key intermediate in the chemical synthesis of Merck's HIV-1 protease inhibitor Indinivir Sulfate (Crixivan®).[58] Biotransformations conducted with *P. putida* F39/D (a mutant strain lacking *cis*-dihydrodiol dehydrogenase activity) or a recombinant TDO expressed in *E. coli* indicated that wild-type TDO oxidized indene to (−)-*cis*-(1*S*, 2*R*)-indandiol (∼30% e.e.) and (1*R*)-indenol as main products, with traces of 1-indenone.[228,288] The (−)-*cis*-(1*S*, 2*R*)-indandiol could be obtained in >98% e.e. in late stages of indene conversion with wild-type *P. putida* F1[58] or by co-expression of *cis*-dihydrodiol dehydrogenase (*todD*) with recombinant TDO in *E. coli*.[228] The upgraded *cis*-(1*S*, 2*R*)-indandiol enantiopurity occurs at the expense of total indandiol yield as a result of kinetic resolution catalyzed by the *cis*-dihydrodiol dehydrogenase, which is selective for the unwanted (+)-*cis*-(1*R*, 2*S*)-indandiol.[228] Directed evolution of TDO was conducted to select for reduced levels of the indene by-products, 1-indenol and 1-indenone, while maintaining the highest (−)-*cis*-(1*S*, 2*R*)-indandiol enantiopurity.[303] After three rounds of mutagenesis, variants were obtained that produced significantly more *cis*-indandiol relative to the undesired by-product indenol. However, the stereoselectivity was changed to favor the production of the undesired (+)-*cis*-indandiol enantiomer.[303] These strategies, as well as the application of *Rhodococcus*-derived oxygenases,[205] were unable to alleviate formation of indene by-products and limited the maximum yields to <60% (−)-*cis*-(1*S*, 2*R*)-indandiol. The TDO-catalyzed enantioselective monohydroxylation of 2-indanol to (−)-*cis*-(1*S*, 2*R*)-indandiol in high e.e. represents an alternative route to the vicinal aminoindanol and served as the basis for a process to prepare chiral 1-hydroxy-2-substituted indan intermediates.[32] TDO expressed in *P. putida* strain UV-4[37] and strain *P. putida* F39/D oxidized 2-indenol to (−)-*cis*-(1*S*, 2*R*)-indandiol in >98% e.e. and in >85% yield, while minor products included *trans*-1,2-indandiol (<15%) and 2-hydroxy-1-indanone (<2%).[160]

4.4. Bioremediation of TCE Contamination

Trichloroethylene (TCE) is widely used as a solvent and is classified as an Environmental Protection Agency priority pollutant. It is difficult to degrade and no bacteria are known to utilize it as a carbon and energy source. TDO and other dioxygenases are capable of oxidizing TCE.[148,180,287] TDO converts TCE into non-toxic products: formic acid and glyoxylic acid,[174] but the host strain does not obtain energy from the reaction. TCE is actually capable of inducing the genes encoding TDO,[253] but *P. putida* F1 cells in resting cultures exposed to TCE rapidly lost TCE oxidation ability.[287] A recent study demonstrated that the addition of benzene or toluene restored TCE-degrading activity to *P. putida*

F1,[188] suggesting TCE degradation by this strain could be optimized using this strategy. In another study, a hybrid TDO–BPDO was found to have an enhanced ability to degrade TCE.[97] Characterization of the purified hybrid protein showed that it had higher catalytic efficiency and a lower K_m for TCE than wild-type TDO.[180] A cooxidation strategy for bioremediation of TCE in the field has been reported.[124] In this study, toluene or phenol and oxygen or hydrogen peroxide were added as co-substrates to stimulate *in situ* TCE degradation.

4.5. Improved Biodegradation of PCBs

Genetic engineering has been used to improve a variety of bacterial enzymes and pathways for more efficient degradation of environmental pollutants.[185,211,218,219,278,279] Engineered forms of various dioxygenases have been generated with a variety of methods, and have led to the development of enzymes with novel or enhanced activities, and have provided insights into the control of substrate specificity. Various BPDOs have very different abilities to oxidize PCBs.[105] Using sequence alignments of BPDOs from *B. xenovorans* LB400 and *P. pseudoalcaligenes* KF707 as a guide, Mondello and coworkers generated hybrid and site-directed mutant forms of BPDO. This work resulted in the identification of regions of the protein and specific amino acid residues in the α subunit that controlled substrate specificity.[86,186] These and other studies[153] produced engineered enzymes with the ability to oxidize a wider range of PCB congeners. In particular, this work identified the important role of the residue at position 377 in the *B. xenovorans* LB400 enzyme in the oxidation of 2,5,2′,5′-tetrachlorobiphenyl. Using DNA shuffling,[304] variant forms of BPDO with enhanced abilities to degrade single-ring aromatic hydrocarbons, PCBs, and heterocyclic aromatic compounds have been generated.[16,95,159,267,268] Recently, Barriault *et al*.[18] used random mutagenesis of the *B. xenovorans* LB400 BPDO *bphA* gene to improve enzymatic activity with PCB congeners. Their results showed that amino acid substitutions at Thr335 and Phe336 in BphA caused changes in regiospecificity toward 2,2′-dichlorobiphenyl.

4.6. Construction of New Biodegradation Pathways

Ring hydroxylating dioxygenases have been used to construct new pathways for the degradation of particularly recalcitrant compounds. An attempt was made to assemble a pathway for 2-chlorotoluene degradation by expressing the *todC1C2BA* genes encoding TDO from *P. putida* F1 (to convert 2-chlorotoluene to 2-chlorobenzylalcohol) with the upper TOL pathway genes from pWWO of *P. putida* strain mt-2 encoding benzylalcohol dehydrogenase and benzaldehyde dehydrogenase (to convert 2-chlorobenzylalcohol to 2-chlorobenzoate).[118] These genes were expressed in two different host strains carrying either the *ortho*

or modified *ortho* pathways. Unfortunately, due to the production of dead-end products and unfavorable metabolic flux, the pathway was not functional – even though each individual part of the pathway was functional.[118]

In a more successful pathway construction strategy, expression of genes encoding TDO from *P. putida* F1 and cytochrome $P450_{cam}$ monooxygenase resulted in an engineered *Pseudomonas* strain capable of metabolizing polyhalogenated compounds through sequential reductive and oxidative reactions. In this constructed pathway, cytochrome $P450_{cam}$ catalyzed the conversion of polyhalogenated ethanes, such as pentachloroethane, to TCE under low oxygen tension, and TDO oxidized TCE to glyoxalate and formate.[289]

Many bacterial strains degrade only a limited number of toxic aromatic compounds, and genetic engineering has been used to increase the substrate range of specific organisms. A constructed cassette carrying genes for the conversion of styrene to phenylacetate was introduced into phenylacetate-degrading bacteria to generate strains capable of using styrene as the sole source of carbon and energy.[179] When the plasmid-borne styrene cassette was introduced into *P. putida* F1 carrying the TOL plasmid, the new strain was capable of growth on an increased number of aromatic hydrocarbons, including benzene, toluene, ethylbenzene, *m*-xylene, *p*-xylene, and styrene. To reduce the possibility of horizontal transfer of genetically engineered DNA among bacteria, the cassette was inserted into a minitransposon carrying an engineered gene containment system.[72]

The *tod* genes encoding TDO have also been cloned into the chromosome of *Deinococcus radiodurans*, a bacterium that is highly resistant to radiation. This new strain was capable of degrading toluene and related aromatic hydrocarbons in the presence of high levels of radiation.[162] Expression of the mercury resistance gene (*merA*) together with the toluene dioxygenase genes in *D. radiodurans* resulted the integration of multiple remediation functions in a single engineered strain, which could be used for amelioration of mixed radioactive waste containing aromatic hydrocarbon pollutants and the heavy metal mercury.[45]

5. CONCLUSIONS

Aromatic ring hydroxylating dioxygenases play a key role in the biodegradation of numerous environmental pollutants, both in the natural environment (via natural attenuation) and in the engineered bioremediation systems. Recent structural and mechanistic information, together with enzyme engineering and strain construction strategies should allow the development of engineered microorganisms with new and/or optimized degradation abilities. The continued application of these approaches should also facilitate the development of Rieske non-heme iron dioxygenases with requisite selectivities for specific opportunities in target direct biocatalysis or metabolic engineering.

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