

博士論文 (要約)

**Structural insight into the mechanism of angular  
dioxygenation in carbazole 1,9a-dioxygenase**

(カルバゾール 1,9a-ジオキシゲナーゼにおける核間  
二水酸化反応の構造基盤)

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## **Abbreviations**

ABP-diol	2' -aminobiphenyl-2,3-diol
ALK	Alkylperoxo
BTEX	Benzene, toluene, ethylbenzene and xylene
BP-diol	Biphenyl-2,3-diol
BP-triol	Biphenyl-2,2',3-triol
BZDO	Benzoate 1,2-dioxygenase
CAR	Carbazole
CARDO	Carbazole 1,9a-dioxugenase
Da	Dalton
DBF	Dibenzofuran
DBT	Dibenzothiophene
DMSO	Dimethyl sulfoxide
DTN	Sodium dithionite
Fd	Ferredoxin
IPTG	Isopropyl- $\beta$ -D-thiogalactopyranoside
Km	Kanamycin
KshA	Oxygenase of 3-Ketosteroid 9 $\alpha$ -hydroxylase
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NDO	Naphthalene dioxygenase
Oxy	Terminal oxygenase
PAHs	Polycyclic aromatic hydrocarbons
PEG	Polyethylene glycol
Red	Ferredoxin reductase
RMSD	Root mean square deviation
ROs	Rieske non-heme iron oxygenases
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid

WT

Wild-type

# Chapter 1

## Introduction

### 1-1 Aromatic compounds in the environment

Organic molecules containing one or more aromatic rings can be defined as aromatic compounds that are often not only common substrates for the growth of microorganism, but also core notorious pollutants in the environment (Fuchs et al., 2011). These compounds are abundant in nature with significant diversity in structure and are found in all organisms at least as three aromatic amino acids (nonpolar phenylalanine and tryptophan, polar tyrosine). Aromatic compounds are also produced by plants profusely as soluble secondary metabolites and lignin. In addition, aromatic chemicals such as BTEX (benzene, toluene, ethylbenzene and xylene), derived from petroleum and its derivatives, are very common environmental pollutants. Usually, various aromatic compounds co-exist as mixtures in the sites of distillation plants and petroleum refineries.

Polycyclic aromatic hydrocarbons (PAHs), heterocyclics and substituted aromatics are three main classes of aromatic compounds in the environment, among which PAHs are a large group of aromatics consisting of more than one aromatic rings fused in linear, angular or clustered arrangements (Cerniglia, 1992; Cheung and Kinkle, 2001). The number of rings and hence, the molecular weight, make a difference in the physical and chemical properties of PAHs, such as reactivity, aqueous solubility and volatility. Consequently, PAHs vary not only in their distribution, transportation and fate in nature, but also in terms of their influence on biosystem. 16 PAHs have been identified as priority pollutants by the US EPA, and some of them are considered to be of carcinogenic possibility to humans. Thus, much attention has been paid to the distribution of these PAHs in the environment and possible exposure to humans (Menzie et al., 1992; Seo et al., 2009), especially the recalcitrant PAHs with high molecular weight (Cheung and Kinkle, 2001). Compared with many other organic compounds, PAHs are more difficult to be degraded in nature due to their relative stability and recalcitrance. Therefore, they may accumulate in high concentrations in certain environments, such as coal gasification factory and tar oil distillation plants (Capotorti et al., 2004).

Heterocyclic compounds, such as dibenzothiophene (DBT) and carbazole (CAR), are main components of creosote, crude oil and shale oil, which often co-exist with PAHs and other aromatic compounds in the environment (Nestler, 1974). DBT is a tricyclic *S*-heterocyclic compound consisting of two benzene rings fused to a central thiophene ring. DBT is naturally occurring in petroleum and industrially produced from biphenyl and hydrogen sulfide under the conditions of catalytic oxidation. DBT is recalcitrant and is quite persistent in the environment. CAR is a recalcitrant *N*-heterocyclic aromatic compound with mutagenicity and toxicity which is predominant in coal tar creosote (Arcos and Argus, 1969; Tsuda et al., 1982). In addition, it is often used widely in the manufacture of diverse products, such as dyes, reagents, explosives, insecticides and lubricants, and is also used as color inhibitor in detergents (Nojiri, 2012). Known as environmental pollutant with mutagenicity and toxicity, the release of CAR has caused environmental concerns (Dutson et al., 1997; Jha and Bharti, 2002; Eisentraeger et al., 2008; Peddinghaus et al., 2012; Brinkmann et al., 2014; Mumbo et al., 2014; Salam et al., 2017). Dibenzofuran (DBF) is another heterocyclic aromatic compound with two benzene rings fused to a central furan ring. Environmental concerns to DBF are mostly related to its halogenated analogues, especially its chloro/bromo derivatives (Seo et al., 2009).

## **1-2 Bioremediation of aromatic pollutants**

Biodegradation is a productive and environmental friendly technology with xenobiotic-degrading bacteria for in situ bioremediation of recalcitrant pollutants in contaminated sites. It is well known that microorganisms own the ability of degrading environmental pollutants under various conditions. On the basis of the metabolic versatility of microorganisms, bioremediation has been the trend to degrade hazardous pollutants, with a goal of transforming pollutants into harmless metabolites or mineralizing the pollutants to carbon dioxide and water (Alexander, 1999). Requirement for a feasible remediation technology is that microorganisms are capable of adapting quickly to and utilizing the pollutants of interest efficiently in a particular case within a reasonable period. To this end, the isolation and characterization of bacterial biodegradation systems followed by application to bioremediation of contaminated environments have been investigated widely (Fuentes et al., 2014;



Ghosal et al., 2016; Varjani, 2017). Efforts have been made to reveal the molecular genetics and genomics, enzymology, ecology and evolution concerned with the novel metabolic capacities in xenobiotic degradation of environmental bacteria. Considering that many factors influence the ability of microorganisms to use pollutants as substrates, understanding the catabolic pathways and the mechanisms of the related enzymes plays a vital role in determining the key factors required for efficient cleanup of pollutants.

Many researches have been investigating the bioremediation of environmental pollutants, especially aromatic compounds that are most prevalent and persistent in the environment (Ghosal et al., 2016; Varjani, 2017). A critical point in the degradation pathway of these aromatics is the initial oxidation of the chemically inert aromatic ring. Take the degradation of CAR, the major *N*-heterocyclic aromatic xenobiotic in coal tar creosote as an example. Various CAR-degrading bacteria that use CAR as the sole source of nitrogen, carbon and energy, have been isolated from diverse niches (Salam et al., 2017). It has been reported that lateral dioxygenation, monohydroxylation and angular dioxygenation are three major pathways for CAR degradation (Grifoll et al., 1995; Lobastova et al., 2004; Nojiri, 2012), among which angular dioxygenation is most important by destroying the planar structure from which the toxicity derives, resulting in complete mineralization of CAR with the resultant catechol converted to tricarboxylic acid (TCA) cycle intermediate (Nojiri and Omori, 2002). Investigations on CAR-degrading bacteria have been carried out in our lab for a long time. *Pseudomonas* sp. strain CA10 was isolated as one of the CAR-utilizing bacteria from a sample of activated sludge which can utilize CAR as a sole source of carbon, nitrogen and energy (Ouchiyaama et al., 1993). Identification and characterization of genes involved in CAR degradation by *Pseudomonas* sp. strain CA10 revealed a Rieske non-heme iron oxygenase (RO), viz. carbazole-1,9a-dioxygenase (CARDO), is responsible for the initial oxygenation of the heterocyclic aromatic compounds by attacking the angular position adjacent to the hetero atom (Sato et al., 1997). In addition to CA10, CAR-degradative catabolic gene (*car*) clusters from *Janthinobacterium* sp. J3, *Novosphingobium* sp. KA1, *Nocardioides aromaticivoras* IC177 and *Erythrobacter* sp. Strain KY5 have also been characterized in our lab (Inoue et al., 2004, 2005, 2006; Urata et al., 2006; Shintani et al., 2007; Vejarano et al., 2018).

### 1-3 Rieske non-heme iron oxygenases (ROs)

Iron-dependent enzymes are prevalent in nature and participate in a wide range of biological redox activities. Rieske non-heme iron oxygenases (ROs) are a class of enzymes harboring non-heme iron as active site that catalyze the incorporation of both O atoms of molecular oxygen into the aromatic substrates and play a crucial role in the biodegradation of various recalcitrant environmental pollutants, as well as synthesis of bio-active compounds (Butler and Mason, 1996; Bertini et al., 1996; Timmis and Pieper, 1999; Lau and Lorenzo, 1999; Hudlicky et al., 1999; Gibson and Parales, 2000; Solomon et al., 2000). The multicomponent ROs usually consist of two or three sequential soluble proteins that form the electron-transport chain interactively: a reductase (Red) transfers electrons from NAD(P)H directly (in two-component systems) or via ferredoxin (Fd) (in three-component systems) to the terminal oxygenase (Oxy) which initiates the degradation of the aromatic compounds (Mason, 1992). The resulting arene *cis*-dihydrodiols with chirality are of interest in enantioselective synthesis (Hudlicky et al., 1999). Structures of RO-Oxy have been shown to be of doughnut-like  $\alpha_3$  or mushroom-like  $\alpha_3\beta_3$  quaternary structures with three-fold symmetry and each  $\alpha$  subunit can be divided into a Rieske [2Fe-2S] cluster domain and a mononuclear iron-containing catalytic domain (Wang et al., 2017) (Fig. 1-1). The Rieske cluster accepts electrons from Red or Fd and passes them on to the mononuclear iron for the catalysis reaction. A unique dioxygenation carried out by a large subset of ROs in this class is the *cis*-dihydroxylation of aromatic C=C bonds, which initiates the biodegradation of aromatic compounds (Ferraro et al., 2005), with incorporation of both O atoms of O<sub>2</sub> into the products.

Structures on the Oxy of several enzymes in this family have revealed occurrence of a mononuclear Fe(II) center coordinated by a recurring 2-His-1-carboxylate facial triad motif (Kauppi et al., 1998; Karlsson et al., 2003; Martins et al., 2005; D'Ordine et al., 2009; Dumitru et al., 2009; Daughtry et al., 2012; Penfield et al., 2014). Interestingly, the crystal structures of Oxys mentioned above show that the mononuclear center is always ~44 Å away from the Rieske cluster found in the same subunit, while ~12 Å away from the Rieske cluster of the adjacent subunit. Importantly, an aspartic or glutamic acid residue interacts with a His residue bound to the Rieske cluster of one subunit and a His residue on the mononuclear iron center of an adjacent subunit by hydrogen

bonding, providing a conduit for the electron transfer from the Rieske center to the mononuclear center (Kauppi et al., 1998; Nojiri et al., 2005; Martins et al., 2005). This presumption was supported by an observed decrease in the activity of naphthalene dioxygenase (NDO) when D205, connecting the Rieske cluster with the non-heme iron center, was replaced by other amino acids (Parales et al., 1999). These results illustrate that the non-heme iron and the Rieske cluster of adjacent subunit work together to form the functional unit to activate O<sub>2</sub>.

#### **1-4 Carbazole 1,9a-dioxygenase (CARDO)**

Carbazole 1,9a-dioxygenase (CARDO), a member of three-component ROs, consists of ferredoxin reductase (Red, 37 kDa), ferredoxin (Fd, 13 kDa) and terminal oxygenase (Oxy, 132 kDa) (Sato et al., 1997; Nam et al., 2002) (Fig. 1-2). CARDO owns the ability to catalyze diverse oxygenation reactions, viz, monooxygenation, lateral dioxygenation and angular dioxygenation, with a broad substrate range (Nojiri et al., 1999). Among these, angular dioxygenation is most attractive, since the ring cleavage results in destruction of the planar structure, which causes the toxicity of dioxins and its related compounds (Nojiri and Omori, 2002). CARDO catalyzes the angular dioxygenation of CAR by adding two hydroxy groups with *cis*-confirmation to the angular position (C9a) carbon bound to the imino nitrogen and its adjacent C1 carbon, to yield unstable *cis*-dihydrodiol, which subsequently gets converts to 2'-aminobiphenyl-2,3-diol (hereafter, ABP-diol) spontaneously (Nojiri et al., 1999, 2005) (Fig. 1-2). ROs have been classified into five groups (classes IA, IB, IIA, IIB and III) on the basis of the number of the constituents and the nature of the redox center (Batie et al., 1991) (Table 1-1). According to Batie's classification system, CARDO from strain CA10 (CARDO<sub>CA10</sub>), J3 (CARDO<sub>J3</sub>), KA1 (CARDO<sub>KA1</sub>) and IC177 (CARDO<sub>IC177</sub>) are categorized as class III, III, IIA and IIB ROs, respectively (Nojiri, 2012). To illuminate the mechanism of CARDO in angular dioxygenation of CAR, structures of Oxy from strain J3 and IC177, Fd from IC177 and CA10 have been solved (Nojiri et al., 2005; Inoue et al., 2009; Nam et al., 2005). Alignment of amino acid sequences shows that Oxy of strain J3 shares 381 identical residues with Oxy of strain CA10 in all 384-residue length, without obvious functional differences in the substrate specificity and inter-component electron transfer (Ashikawa et al., 2005, 2006). Based on high identity of Oxys

from strain J3 and CA10, the structures of Oxy:Fd binary complexes using Oxy of J3 and Fd of CA10 in non-reduced, reduced, and substrate-bound forms were determined at 1.9, 1.8 and 2.0 Å resolutions, respectively (Ashikawa et al., 2006). Furthermore, the reduced CAR-bound, dioxygen-bound and both CAR- and dioxygen-bound structures of Oxy:Fd complexes have also been solved (Ashikawa et al., 2012).

It has been demonstrated that Oxy alone is capable of catalyzing the dioxygenation of substrate in a rapid rate if both the Rieske cluster and mononuclear iron are reduced, and that the role of Fd is to transfer electrons from reductase to the Rieske cluster in Oxy (Wolfe et al., 2001, 2002). On the basis of the previous studies, it has been proposed in this study that in the case of CARDO, reduced Fd interacts with Oxy to transfer one electron to the Rieske center by forming the complex. After that, the oxidized Fd dissociates from the reduced Oxy, then the fully reduced Oxy initiates the reaction upon exposure to the substrate and O<sub>2</sub>. Therefore, previously determined structures of Oxy:Fd complexes, including Oxy:Fd<sup>rest</sup> (PDB: 2DE5), Oxy:Fd<sup>red</sup> (PDB: 2DE6), Oxy:Fd<sup>CAR</sup> (PDB: 2DE7), Oxy:Fd<sup>red-CAR</sup> (PDB: 3VMG), Oxy:Fd<sup>O<sub>2</sub></sup> (PDB: 3VMH) and Oxy:Fd<sup>CAR-O<sub>2</sub></sup> (PDB: 3VMI) (Ashikawa et al., 2006, 2012), would not exist in the actual catalytic cycle of CARDO.

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## **Chapter 2**

### **Substrate binding and product formation in the terminal oxygenase (Oxy) of CARDO during angular dioxygenation**

#### **2-1 Introduction**

#### **2-2 Results and Discussion**

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## **Chapter 3**

### **Product release from the active site of Oxy in CARDO**

#### **3-1 Introduction**

#### **3-2 Results and Discussion**

This part will be published in the related journal. According to the rule of magazine, it is forbidden to be public currently.

## **Chapter 4**

### **Summary and future prospects**

#### **4-1 Proposed reaction mechanism of angular dioxygenation in CARDO**

#### **4-2 Future prospects**

This part will be published in the related journal. According to the rule of journal, it is forbidden to be public currently.

## **Supplement**

### **Measurement of interactions between Oxy and Fd with the isothermal titration calorimetry (ITC)**

#### **S-1 Introduction**

#### **S-2 Interactions between Oxy and Fd with the isothermal titration calorimetry (ITC)**

This part will be published in the related journal. According to the rule of journal, it is forbidden to be public currently.



## **Materials and Methods**

This part will be published in the related journal along with the results and discussion above.

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