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MINI-REVIEW

Dioxygen activation responsible for oxidation of aliphatic and aromatic hydrocarbon compounds: current state and variants

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Abstract The most significant aspect in microbial metabolisms, especially those of bacteria and archaea, is their marvelously wide acceptability of substrate electron donors and acceptors. This feature makes them to be attractive catalysts for environmental biotechnology in terms of degradation of harmful recalcitrant compounds, including hydrocarbons. Transformation of highly reduced and inert hydrocarbon compounds is with no doubt a challenging biochemical reaction for a single enzyme. However, several multi-component enzyme systems enable microorganisms to utilize hydrocarbons as carbon and energy (electron) sources. Initial biological attack to hydrocarbons is, in most cases, the hydroxylation that requires molecular dioxygen as a co-substrate. Dioxygen also contributes to the ring cleavage reaction of homo- and hetero-cyclic aromatic hydrocarbons. Although the molecular dioxygen is omnipresent and highly soluble in water, activation and splitting this triplet ground-state molecule to wed with difficult hydrocarbons need special devices. Non-heme iron, heme iron, or flavin nucleotide was designated as a major hidden dagger for this purpose.

Keywords Oxygenase · Rieske dioxygenase · Naphthalene dioxygenase · Alkane monooxygenase · P450 · Hydrocarbon

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Metal or non-metal?

A group of metalloenzyme families, carrying an iron active site whichever heme or non-heme type, play crucial roles for the binding and activation of dioxygen in various oxidative transformations. These include hydroxylation of hydrocarbons and other xenobiotics, and also cleavage of aromatic rings. Dioxygen binds at these iron active sites and generates competent iron-peroxo and high valent iron-oxo intermediates. These reactive intermediate species allow the host metalloenzymes to cleave C–H or C–C bond in the substrate hydrocarbons. The molecular mechanisms of dioxygen activation have been subject to excellent reviews for both non-heme and heme ligand sets (Bugg and Ramaswamy 2008; Decker and Solomon 2005; Kovaleva and Lipscomb 2008; Vaillancourt et al. 2006). In either case, the initial oxygenases (C-H bond cleaving enzymes) often recruit redox partner proteins conjugate for reductive scission of dioxygen. They are, in most cases, a set of ferredoxin or rubredoxin and a specific reductase. On the other hand, flavin (nucleotide)-dependent monooxygenases are a representative non-metal oxygenase system that usually requires neither ferredoxin nor rubredoxin. This mini-review attempts to overlook briefly the diverse microbial dioxygen-activating systems relating to oxidative degradation of aliphatic and aromatic hydrocarbon compounds (Table 1). In order to facilitate easy understanding of the basic mechanisms, figures are partly schematic without experimental direct evidence.

Non-heme iron oxygenases

There are two major types in the non-heme iron oxygenases, one carries mononuclear iron and another carries

Table 1 Microbial dioxygenases and monooxygenases

Active site for activating O_2	Enzyme names	Subunits[a]	Co-factors[a]
[Non-heme]			
Mononuclear iron(II)	*Extradiol dioxygenases*		
	Catechol 2,3-dioxygenase (C23O)	α_4	None
	2,3-dihydroxybiphenyl 1,2-dioxygenase (HBDO)	α_8	None
	Homoprotocatechuate 2,3-dioxygenase (HPCD)	α_4	None
	Rieske dioxygenses		
	Naphthalene 2,3-dioxygense (NDO)	$(\alpha\beta)_3$	Ferredoxin, reductase
	Biphenyl 2,3-dioxygenase (BDO)	$(\alpha\beta)_3$	Ferredoxin, reductase
	Toluene 2,3-dioxygenase (TDO)	$(\alpha\beta)_3$	Ferredoxin, reductase
	Cupin family dioxygenses		
	Gentisate 1,2-dioxygenase (GDO)	α_4	None
	1-hydroxy 2-naphthoate dioxygenase (HNO)	α_6	None
	Homogentisate 1,2-dioxygenase (HGO/HDO)	$(\alpha_3)_2$	None
Mononuclear iron(III)	*Intradiol dioxygenases*		
	Catechol 1,2-dioxygenase (C12O)	α_2	None
	Protocatechuate 3,4-dioxygenase (PCD)	$(\alpha\beta)_{2\text{-}12}$	None
Dinuclear iron(II)	*Monooxygenases*		
	Soluble methane monooxygenase (sMMO)	$(\alpha\beta\gamma)_2$	Reductase, FAD, effector protein
	Alkane monooxygenases (AlkB/AlkM)		Rubredoxin, reductase, FAD
	Phenol hydroxylase (PHH)	$(\alpha\beta\gamma)_2$	Reductase, FAD, effector protein
	Toluene monooxygenase (TMO)	$(\alpha\beta\gamma)_2$	Ferredoxin, reductase, effector protein
Dinuclear copper(I)	Particulate methane monooxygenase (pMMO)	$(\alpha\beta\gamma)_3$	
[Heme]			
Mononuclear iron(II)	*Cytochrome P450 monooxygenase superfamily*	$\alpha_{1\text{-}3}$	(Ferredoxin, reductase, FAD/FMN)
[Flavin]	*Flavin dependent monooxygenases*		
	Alkane monooxygenases (LadA/AlmA)	α_2	Reductase, FMN
	4-hydroxyphenyl acetate 3-monooxygenase	α_2	Reductase, FAD
	Dibenzothiophene monooxygenase	α_2	Reductase, FMN

[a] Most major type

dinuclear irons at the catalytic site of terminal oxygenases. The mononuclear non-heme iron enzymes are further divided into two categories (Neidig and Solomon 2005). The first group utilizes ferrous iron, iron(II), resting site to bind and activate dioxygen for hydroxylation of substrate hydrocarbons. The second group adopts ferric iron, iron (III), center in the active site.

Mononuclear iron(II)/O_2-activating group

Ring-cleaving extradiol dioxygenases

The mononuclear non-heme iron(II) active sites carry highly conserved ligands that are composed of two histidines and one monodentate carboxylate, so called 2-His-1-carboxylate facial triad (Koehntop et al. 2005; Lipscomb 2008). This structure is formed by conserved sequence motif, $HX_{48\text{-}63}HX_{49\text{-}53}E$, in aromatic ring-cleaving extradiol dioxygenases such as catechol 2,3-dioxygenase ($H^{153}X_{60}H^{214}X_{50}E^{265}$ in C23O, P06622) (homo-tetramer), 2,3-dihydroxybiphenyl 1,2-dioxygenase ($H^{145}X_{63}H^{209}X_{49}E^{259}$ in HBDO, 1KND) (homo-octamer), and homoprotocatehuate 2,3-dioxygenase ($H^{155}X_{58}H^{214}X_{52}E^{267}$ in HPCD, AAB66502) (homo-tetramer), and $HX_{4\text{-}6}HX_{146\text{-}8}D$ in large subunit (α) of Rieske dioxygenases (trimer of hetero-dimer $\alpha_3\beta_3$) such as naphthalene 1,2-dioxygenase ($H^{208}X_4H^{213}X_{148}D^{362}$ in NahAc, AAO64274), biphenyl 2,3-dioxygenase ($H^{234}X_5H^{240}X_{146}D^{387}$ in BphA1, BAA04137), and toluene 2,3-dioxygenase ($H^{222}X_5H^{228}X_{147}D^{376}$ in TodC1, AAA26005). It is worth to note that the amino acid sequence similarity among the group enzymes is so low, except above iron ligand residues, enough to imply that the triad is formed as a result of typical convergent evolution of proteins.

At the catalytic site, one face of the octahedral coordination sphere of the iron(II) metal center is occupied by these three endogenous ligands, which leave other three coordination sites for binding of exogenous dioxygen, substrates, and cofactors. This flexible coordination chem-

istry would enable to catalyze diverse oxidative transformations. The binding of substrate inevitably activates the metal center for attack by dioxygen to form iron(II)-alkylperoxo intermediate. Another conserved two histidines and a tyrosine near the dioxygen binding site are also essential for ring cleavage reaction in the HPCD (H^{200}/H^{248}/Y^{257}, AAB66502) and C23O (H^{199}/H^{246}/Y^{255}, P06622) (Fig. 1a, Bugg and Ramaswamy 2008). These H^{200} and H^{199} might also serve as an active site base to deprotonate the entering substrate and/or OH group of the substrate carbon distal to the site of ring cleavage. The extradiol and intradiol dioxygenases utilize cathecholic substrates that carry all the reducing equivalents necessary for dioxygen activation, so they are called "intramolecular dioxygenases." However, several extradiol dioxygenases also require redox cofactors such as ferredoxin and reductase for their reactivation when inactivated by processing poor substrates such as chloro- or alkylcatechols (Tropel et al. 2002).

Rieske dioxygenases

Rieske dioxygenases catalyze the primary cis-dihydroxylation of arene (monocyclic and polycyclic aromatic hydrocarbon) substrates which is the initial step of many bacterial degradation pathways. Rieske protein was first reported by John Rieske et al. (Rieske et al. 1964) which has a characteristic [2Fe-2S] cluster with 2-His-2-Cys ligand. Besides a mononuclear iron active site, Rieske dioxygenases carry a dinuclear [2Fe-2S] cluster in which one iron (Fe1) is coordinated by two histidines while the other iron (Fe2) is coordinated by two cysteines (Fig. 1b). This bidentate ligand is described by a $CXHX_{15\text{-}17}CX_2H$ motif ($C^{81}XH^{83}X_{17}C^{101}X_2H^{104}$ in NahAc, AAO64274; $C^{101}XH^{103}X_{17}C^{121}X_2H^{124}$ in BphA1, BAA04137; $C^{96}XH^{98}X_{17}C^{116}XH^{119}$ in TodC1, AAA26005). Fe2 remains in a ferric state regardless of the reduction state of the cluster, while Fe1 is converted from a ferric state to a ferrous state when reduced during the reaction. The reaction of three-component type Rieske dioxygenase requires two electrons from a NAD(P)H and consecutively transferred to the terminal dioxygenase component through a ferredoxin (monomer) and a reductase (monomer) (Fig. 1c). Crystal structure of biphenyl 1,2-dioxygenase complex (BphA3/A4) has shown the butterfly-type motion of the flavin ring of FADH in the non-Rieske-type NADH/ferredoxin reductase (BphA4) and a direct transfer of the electron from the flavin ring in BphA4 through conserved tryptophan (W^{320} in BphA4) and histidine (H^{66} in BphA3; ferredoxin) to the Rieske-type cluster of BphA3 (Senda et al. 2007). Finally, cis-dihydroxylation takes place in the terminal dioxygenase component. In a single large subunit, the Rieske-type cluster and the mononuclear iron(II) center are too far apart to allow for electron transfer at a distance ~43 Å. However, the quaternary structure (trimer of hetero-dimer or homo-trimer type with three-fold symmetry) allows for electron transfer from a Rieske-type cluster to a mononuclear iron(II) center from a neighboring subunit which is only 5 Å apart (Fig. 1b) (Parales 2003). A key role in this electron transfer has been ascribed to an absolutely conserved aspartic acid residue (D^{205} in NahAc, AAO64274; D^{231} in BphA1, BAA04137; D^{219} in TodC1, AAA26005) that bridges between the two metal sites. Structural studies also implicate a side-on binding of a dioxygen to form catalytically active iron(III)-peroxide intermediate which is subsequently converted to a high-valent iron(IV)-oxo or iron(V)-oxo-hydroxo intermediate. After cis-dihydroxylation of the substrate, catalytic mononuclear iron will return to iron(II) resting-state configuration (Karlsson et al. 2003, Bugg et al. 2008). Another group of Rieske dioxygenases adopts a two-component enzyme system, including most anthranilate dioxygenases, benzoate dioxygenases, and toluate dioxygenases (Bundy et al. 1998; Yamaguchi and Fujisawa, 1982). It consists of a small NADH reductase component containing both flavin adenine dinucleotide (FAD) and chloroplast-type [2Fe-2S] cluster, and a large Rieske-type oxygenase component. The oxygenase component also constitutes $\alpha_3\beta_3$ structure similar to the three-component system as above described.

Bicupin family dioxygenases

Cupin superfamily dioxygenases are widely distributed in the living organisms from bacteria to human because some are responsible for aromatic amino acid metabolisms (Dunwell et al. 2000). A functional cupin 'small β-barrel' fold is characterized by two highly conserved sequence motifs of typically $GX_5HXHX_{3\text{-}4}EX_{6\text{-}7}G$ and $GX_5PXGX_2HX_3N$. The two motifs together form one functional metal-binding site with three histidines. Most of the subtypes contain iron (II) as the active site metal cofactor but other forms are also known, including copper or nickel. Characteristics of the reaction catalyzed by gentisate 1,2-dioxygenase (GDO, homo-tetramer), 1-hydroxy-2-naphthoate dioxygenase (HNO, homo-hexamer), and homogentisate 1,2-dioxygenase (HGO/HDO, dimer of homo trimer) appear to be similar to those of intramolecular extradiol dioxygenases, although their substrates do not have adjoining hydroxyl groups. For example, GDO catalyze the ring fission of gentisate between the carboxyl and proximal hydroxyl groups at positions 1 and 2 of the aromatic ring to form maleylpyruvate. An evolutionarily interesting protein group is bicupin dioxygenases. These bicupins usually contain single active site in one of the duplicated cupin domains, with the other domain remaining as a non-functional vestigial remnant. The ferrous iron ligand in bicupin or monocupin dioxygenases is usually

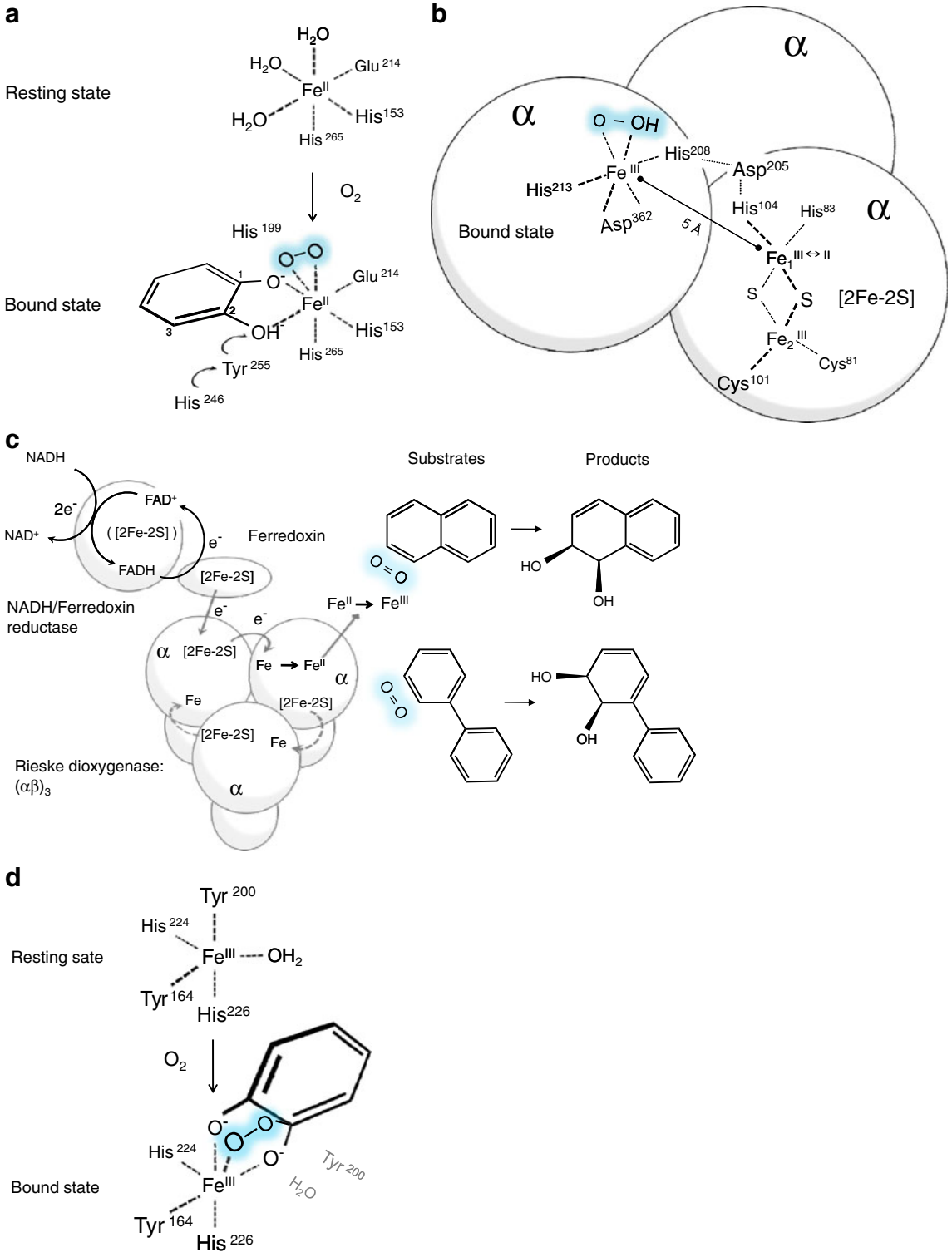

Fig. 1 **a** Ligand structure of iron in C23O from *P. putida*. **b** Ligand structure of irons in large subunits of NahAc from *P. putida*. **c** Schematic electron transfer system in three-component type Rieske dioxygenases. **d** Ligand structure of iron in C12O from *A. calcoaceticus*

composed of a three histidine motif $HXHX_{39}H$, such as $H^{104}/H^{106}/H^{145}$ in GDO from *E. coli* O157 (ABV16854), $H^{108}/H^{110}/H^{149}$ in GDO from *Xanthobacter polyaromaticivorans* (BAC98955, Hirano et al. 2007), and $H^{115}/H^{117}/H^{158}$ in HNO from *Nocardioides* sp. KP7 (BAA31235). It has been proposed that the conserved glutamate residue in the first signature motif is a fourth ligand to the metal (Steiner et al. 2002). The triple histidine motif is partly similar structure

to Rieske dioxygenases where the active site iron(II) coordinates near the interface between subunits of HGO trimer by 2-His-1-carboxylate ligand $HX_5EX_{29}H$, such as $H^{335}/E^{341}/H^{371}$ in HGO from *Homo sapiens* (NP_000178) and probably $H^{331}/E^{337}/H^{367}$ in *Pseudomonas putida* F1 (ABQ80601) and $H^{349}/E^{355}/H^{385}$ in *Sinorhizobium meliloti*, AAD29874) although electrons are not donated from extramolecular ferredoxin but substrate homogentisate itself.

Mononuclear iron(III)/substrate activating group

Ring cleaving intradiol dioxygenases

The intradiol dioxyenases catalyze the ring cleavage between the adjacent hydroxylated carbons (Brown et al. 2004). They utilize high-spin iron(III) sites to activate substrates for direct reaction with dioxygen. The resting structure is a distorted trigonal bipyramidal iron(III) center with histidine and tyrosine as the axial ligands, and histidine, tyrosine, and typically a water ligand in the equatorial plane: 2-Tyr-2-His coordinate, $YX_{35\text{-}8}YX_{12\text{-}23}HXH$ (Fig. 1d). The characteristic dark red color of intradiol dioxygenases is due to the tyrosine ligation and the resulting tyrosine ligand-to-metal charge transfer transitions. Active site structures and X-ray absorption spectrometries of protocatechuate 3,4-dioxygenase (PCD) from *P. putida* (3PCA)(dimer to dodecamer of hetero-dimer) $Y^{408}X_{38}Y^{447}X_{12}H^{460}XH^{462}$ and catechol 1,2-dioxygenase (C12O) from *Acinetobacter calcoaceticus* ADP1 (1DLM)(homo-dimer) $Y^{164}X_{35}Y^{200}X_{23}H^{224}XH^{226}$ have revealed that upon substrate binding to the active site both the axial tyrosine ligand and the equatorial water ligand are displaced to form a bidentate substrate complex. The axial tyrosine moves away to leave the iron(III) center with a vacant coordination site in a roughly octahedral geometry. Binding of the distal oxygen of the resulting peroxy-substrate intermediate to the vacant coordination site trans-axial to axial histidine subsequently promote acyl migration resulting in the O–O bond and intradiol ring cleavages.

Diiron coordinating monooxygenase group

Soluble/particulate methane monooxygenases, toluene 4-monooxygenases, and phenol hydroxylases

A major diiron-carboxylate protein group with large four-helix bundle structure have dinuclear metal ligand sites coordinated by four carboxylates and two histidines in two $E_{29\text{-}37}EX_2H$ motifs; such as $E^{100\text{-}114}X_{29}E^{130\text{-}144}X_2H^{133\text{-}147}$ and $E^{194\text{-}209}X_{33}E^{228\text{-}243}X_2H^{231\text{-}246}$ in soluble methane monooxygenases (sMMO), phenol hydroxylases (PHH), and toluene 2-, 3-, and 4-monooxygenases (TMO)(Fig. 2a, Nordlund and Eklund 1995; Leahy et al. 2003). PHH and sMMO are made up of three components: a monooxygenase/hydroxylase, a dinuclear iron NADH oxidoreductase, and a regulatory or cofactor less effecter protein. TMO and several PHH have a fourth, Rieske [2Fe-2S] ferredoxin. The monooxygenase/hydroxylase component of sMMO, TMO, and most PHH comprise a dimer of hetero-trimer $(\alpha\beta\gamma)_2$, with each α subunit housing the carboxylate bridged diiron center, the catalytic site of substrate hydroxylation. This structure of the catalytic site is shared with R2 component of ribonucleotide reductases and DesA1 component of stearoyl-ACP Δ-9 desaturases. The oxidoreductase component shuttles electrons from NADH through its bound FAD and [2Fe-2S] cofactors to the monooxygenase/hydroxylase either directly as in sMMO and PHH or indirectly via the Rieske ferredoxin component in TMO and several PHH. The assembly of protein complexes during the catalytic cycle is finely orchestrated to make electron consumption linked to substrate mono-hydroxylation. Reaction of the reduced diiron(II) state with dioxygen affords a peroxo-diiron(III) intermediate, which can evolve to a high-valent diiron(IV) species. It has been proposed that peroxo-diiron(III) and peroxo-diiron(IV) species are competent for alkene epoxidation and methane and norcarane hydroxylation, respectively (Bailey and Fox 2009). Upon product release, the active site assumes the resting-state diiron(III) configuration until effector protein binding and reduction begin another catalytic cycle.

Another type of methane monooxygenase is a particulate (integral membrane) methane monooxygenase (pMMO) that is composed of cylindrical trimer of hetero-trimer $(\alpha\beta\gamma)_3$ with a mononuclear and a catalytic dinuclear coppers in the β subunit and a zinc (presumably a mononuclear copper in vivo) between α and γ subunits (Balasubramanian and Rosenzweig 2007). Mononuclear and dinuclear copper ligands are two and three highly conserved histidines in β subunit, His^{48}/His^{72} and $His^{33}/His^{137}/His^{139}$ in pMMO from *Methylococcus capsulatus,* respectively (Fig. 2b). A zinc ion is coordinated by His^{160}, His^{173}, and Asp^{156} from γ subunit and Glu^{195} from α subunit. Due to difficulties of studying membraneous proteins and unavailability of co-crystal structure of the enzyme and substrate, catalytic mechanism of pMMO is still under debate (Rosenzweig 2008). While sMMO is capable of accepting various substrates including alkanes, alkenes, aromatics, and halogenated hydrocarbons, pMMO is more selective toward alkanes and alkenes that are five carbons or less.

Alkane monooxygenases

Alkane ω-hydroxylase is a three-component system comprising a soluble mononuclear iron and FAD containing NADH/rubredoxin reductase (AlkT), a [2Fe-2S] soluble rubredoxin

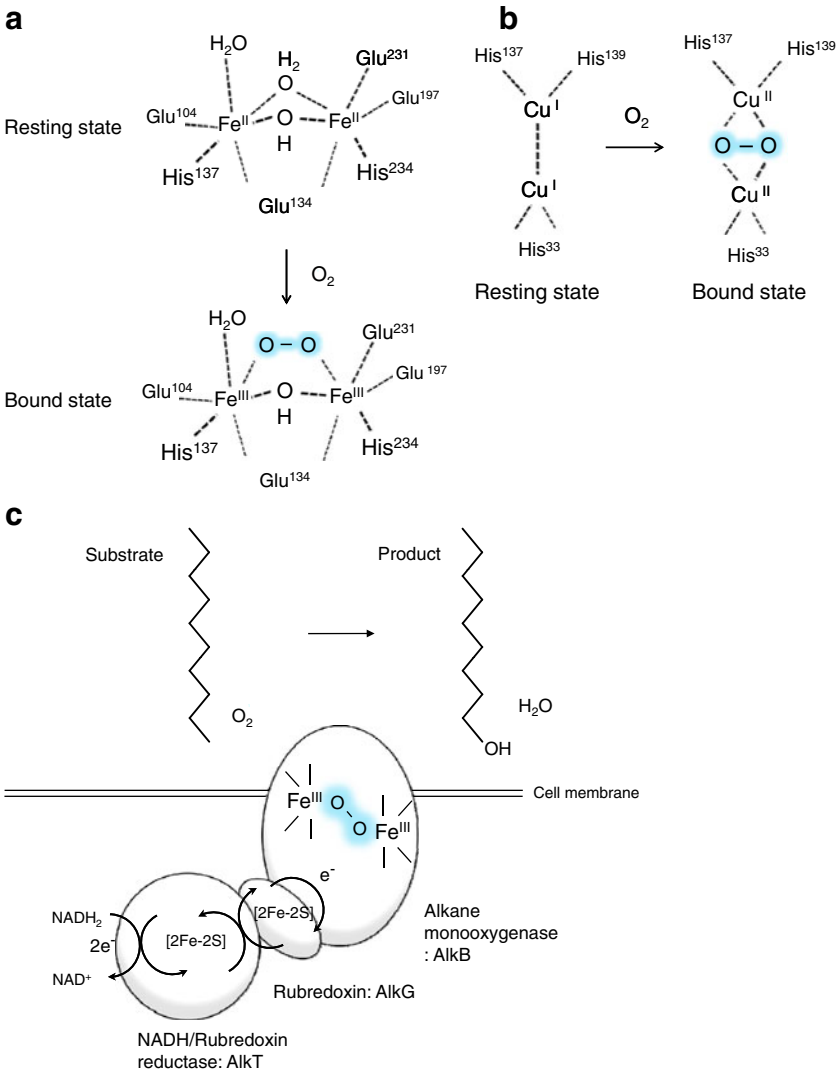

Fig. 2 **a** Ligand structure of dinuclear irons in TMO from *Pseudomonas stutzeri* (putative). **b** Ligand structure of dinuclear coppers in pMMO from *M. capsulatus* (putative). **c** Electron transfer system of Alkane monooxygenase (AlkB-type)

(AlkG), and an integral-membrane diiron monooxygenase (AlkB) (Fig. 2c). This type of multi-component enzyme system is widely distributed in bacteria (van Beilen and Funhoff 2007). Like sMMO and TMO, AlkB also adopts the oxygen rebound mechanism in order to hydroxylate alkanes but preferably from C5 to C16 alkanes. This mechanism involves homolytic cleavage of the C–H bond by an electrophilic oxo-iron intermediate to generate a substrate-based radical. Diiron ligand site of AlkB (probably monomer) is composed of eight histidine motifs $H^{138\text{-}172}X_3H^{142\text{-}176}$, $H^{167\text{-}202}X_3HH^{173\text{-}207}$, and $H^{312\text{-}346}XHH^{316\text{-}349}$ (van Beilen et al. 2005). There are $H^{138}EX_2H^{142}K$, $EH^{168}X_2GH^{172}H^{173}$, and $LQRH^{312}XDH^{315}H^{316}A$ in AlkB from *P. putida* GPo1 (CAB54050). These histidine residues are also potential ligands for the diiron atoms contained within the homolog, alkane monooxygenases (H^{150}/H^{154}, $H^{180}/H^{184}/H^{185}$, $H^{324}/H^{327}/H^{328}$ in AlkM, CAG68276), xylene monooxygenases (H^{113}/H^{117}, $H^{143}/H^{147}/H^{148}$, $H^{282}/H^{285}/H^{286}$ in XylM, AAA26026), *p*-cymene monooxygenases (H^{111}/H^{115}, $H^{141}/H^{145}/H^{146}$, $H^{280}/H^{283}/H^{284}$ in CymAa, AAB62299), stearoyl-CoA desaturases (H^{93}/H^{97}, $H^{128}/H^{132}/H^{133}$, $H^{305}/H^{309}/H^{310}$ in DesB, AAG08273), and other related proteins. This protein family has hydrophobic six helices that would be capable of spanning the membrane bilayer probably at six times. Unfortunately, active site structure of AlkB has not been solved yet; however, spectroscopic and genetic evidence suggests a nitrogen-rich coordination environment located near the inner surface of the cytoplasmic membrane with as many as eight histidines coordinating two irons and a carboxylate residue bridging these two metals. Recently, another histidine residue in a motif $NYXEH^{273}YG$ is also suggested to be essential for the activity of AlkB from *P. putida* GPo1 (van Beilen et al. 2005). A particular amino acid residue located in the middle of transmembrane helix-2 of AlkB has been shown to be important to determine the alkyl length of the substrate. When this amino acid has a bulky side chain like tryptophan (W^{55} in AlkB from *P.*

putida GPo1), the long-chain alkyl groups (C13<) are not acceptable in the substrate binding cleft.

Heme iron monooxygenases

The heme porphyrin ligand allows for delocalization of the iron *d*-orbitals into heme π cloud, and the iron sites in the iron(II) heme enzymes are often low spin as compare to non-heme enzymes which are generally high-spin. In addition, the presence of a planar tetradentate heme ligand imposes steric constraints on the iron site that are not present in the non-heme enzymes. The cytochrome P450s, heme protein superfamily, are widely distributed in archaea, bacteria, and eukaryotes, and are involved in numerous cellular functions including monooxygenation of hydrocarbon compounds. P450 reaction is initiated by introduction of an atom of oxygen to facilitate hydroxylation at an inactive carbon center on a molecule. To achieve this reaction, dioxygen is bound to iron(II) heme upon binding substrate compound at the center of a b-type heme bound to the P450 ultimately leading to formation of a highly reactive iron(IV)-oxo intermediate and the production of a molecule of water. The iron(IV)-oxo intermediate attacks a substrate molecule bound proximal to the heme iron to perform the oxygenation and to restore the resting state of the iron(III)-P450. Sequence conservation is relatively low within P450 superfamily; there are only three absolutely conserved residues—Glu, Arg, and Cys—but their general topography and structural fold are highly conserved constituting the heme-binding loop. The core region is composed of a coil termed the 'meander', a four-helix bundle, helices J and K, and two sets of β sheets. There is an absolutely conserved EXXR motif in helix K. A completely conserved cysteine residue proximal to heme, in FX_2GX_3CXG, provides a thiolate ligand to the heme iron that is essential for P450 catalysis, cysteine ligation (Fig. 3). The electrons for catalysis derive from NAD(P)H and are transferred from one or more redox partner proteins are those that are often fused to P450. It should be notable that all the known to date bacterial P450 monooxygenases are in soluble form whereas eukaryotic P450s are generally membraneous, attached to mitochondrial or microsomal membranes by an N-terminal peptide anchor. This would enable bacterial P450s to be more evolutionarily diverse. Also, bacterial P450 in a fused single component system is often observed. This might be due to evolutionary requirements for reducing the size of genome and transferring electrons most efficiently. Bacterial P450s for alkane monooxygenation belongs to soluble CYP153 oxygenase. CYP153 oxygenase also requires redox partners, FAD-binding iron–sulfur ferredoxin reductase (FR) and ferredoxin (Fd). Although there

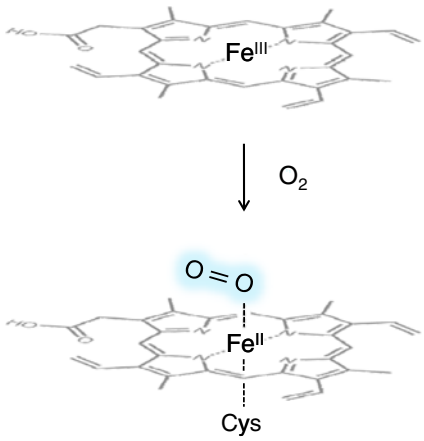

Fig. 3 Dioxygen binding to heme

are at least seven classes in the P450 and redox partner systems (Munro et al. 2007), only four major classes of them are described below.

1. FR/Fd/P450 electron transfer systems [class I/three components]: NADH → putidaredoxin reductase (FAD) → putidaredoxin (2Fe-2S) → P450:CYP101/CYP153 monooxygenase → O_2

This group is the majority of bacterial and mitochondrial P450s. CYP101/CYP153, and mitochondrial P450s take NAD(P)H electrons from a ferredoxin that shuttles electrons from a reductase. The *P. putida* camphor hydroxylase, P450cam (CYP101), is one of the best-studied P450 heme system. There is an increasing number of CYP153 alkane monooxygenases reported from both gram-positive and gram-negative bacteria, including *Mycobacterium* sp. HXN-1500, *Rhodococcus erythropolis* NRRL B-16531, *Alkanivorax borkumensis* AP1, and *Acinetobacter* sp. EB104 (van Beilen et al. 2006).

2. CPR/P450 electron transfer systems [class II/two components, class III/one component]: NADPH → Cytochrome P450 Reductase (FAD → FMN) → P450:CYP102 monooxygenase → O_2

This class II enzyme is the majority of eukaryotic microsomal P450s. The NADPH electrons are directly received from a reductase (CPR) containing both FAD and FMN. In *Bacillus megaterium* and *Bacillus subtilis*, CPR is fused in the C-terminal domain of CYP102 (P450BM3), a single polypeptide self-sufficient soluble fatty acid hydroxylase, and heme-monooxygenase resides in the N-terminal domain (class III).

3. FMN/Fd/P450 electron transfer systems [class IV/one component]: NADPH → P450:CYP116 monooxygenase (FMN → 2Fe-2S) → O_2

An interesting one-component P450 system was also found in *Rhodococcus* sp. NCIMB 9784 (CYP116B2) (Roberts et al. 2003). In this system, the C-terminal of heme-monooxygenase domain is fused to a reductase domain that shows sequence similarity to phthalate dioxygenase reductase containing FMN-binding domain and a plant-type ferredoxin domain. Similar P450 systems have been identified in the heavy-metal tolerant bacterium *Ralstonia metallidurans* (CYP116A1) and in several species of genus *Burkolderia*. There is a possible FAD-binding monooxygenase (AAM67415) encoded just upstream of CYP116B2. AAM67415 has significant amino acid sequence identity (72%) with pentachrolophenol monooxygenase from *Frankia* sp. EAN1pec (ABW13520).

Non-metal flavin-dependent monooxygenases

In this enzyme system, reduced $FADH_2$ or $FMNH_2$ binds to the apo-oxygenase component and reacts with oxygen to form a flavin-C4a-hydroperoxide structure, which then hydroxylates the substrate to yield product and the flavin-C4a-hydroxide adduct (Fig. 4). Rate-limiting reduction of the flavin ring by NAD(P)H in preparation for its reaction with O_2 occurs by hydride transfer stereospecifically with the pro-R hydrogen of the reduced pyridine nucleotide transferring to the N5 of isoalloxazine (flavin) ring (Ballou et al. 2005). Recently, a new luciferase family soluble FMN-binding alkane monooxygenase, LadA (homo-dimer), which is specific for long-chain substrates has been reported for a thermophilic *Geobacillus denitrificans* NG80-2 (Li et al. 2008). A histidine residue, His^{138}, has been postulated to be a catalytic residue that acts as a hydrogen bond donor to the proximal and/or distal oxygen atom of possible flavin-C4a-hydroperoxyde. This catalytic architecture is also found in flavin dependent aromatic monooxygenases, such as p-hydroxyphenyl acetate hydroxylases (HPAH), dibenzothiophene monooxygenases (DbzA), and HPAH-like PHH from thermophilic species of *Bacillus* and mesophilic actinobacteria (Alfieri et al. 2007; van Berkel et al. 2006). Another flavin-binding long-chain alkane monooxygenase, AlmA, has been reported for mesophilic *Acinetobacter* sp. DSM17874 (Throne-Holst et al. 2007, ABQ18224). Based on its hydropathy analysis, LadA and AlmA do not have membrane spanning region and seem to exist in a soluble form. Although LadA and AlmA are supposed to be two-component flavin dependent monooxygenases like flavin dependent aromatic monooxygenases, another redox partner protein presumably NADH/flavin reductase has not been identified yet. It seems like that alkane/aromatic monooxygenases have two different evolutionary lineages—one is iron-utilizing group, whatever non-heme or heme, and another is iron-independent flavin-dependent group regardless of free and integral form. Moreover, significant amino acid sequence similarity, 50% identical, between LadA (ABO68832) and DszA (YP_233462) strongly suggests that flavin-dependent alkane and aromatic hydrocarbon monooxygenase groups emerged after divergent evolution from a common ancestral protein. Several alkane-degrading thermophilic Geobacillus

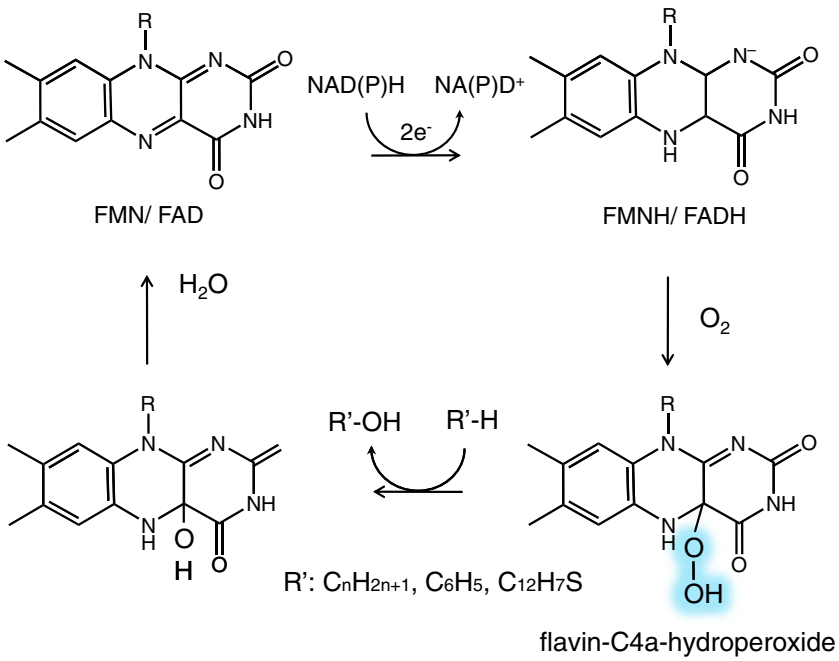

Fig. 4 Redox states of flavin ring during monooxygenase reaction

sp. have been reported to harbor multiple AlkB-type alkane monooxygenase genes (Liu et al. 2009). It remains to be elucidated whether flavin dependent monooxygenases are majority group of long-chain alkane monooxygenases.

References

Alfieri A, Fersini F, Ruangchan N, Prongjit M, Chaiyen P, Mattevi A (2007) Structure of the monooxygenase component of a two-component flavoprotein monooxygenase. Proc Natl Acad Sci U S A 104:1177–1182

Bailey LJ, Fox BG (2009) Crystallographic and catalytic studies of the peroxide-shunt reaction in a diiron hydroxylase. Biochemistry 48:8932–8939

Balasubramanian R, Rosenzweig AC (2007) Structural and mechanistic insights into methane oxidation by particulate methane monooxygenase. Acc Chem Res 40:573–580

Ballou DP, Entsch B, Cole LJ (2005) Dynamics involved in catalysis by single-component and two-component flavin-dependent aromatic hydroxylases. Biochem Biophys Res Commun 338:590–598

Brown CK, Vetting MW, Earhart CA, Ohlendorf DH (2004) Biophysical analyses of designed and selected mutants of protocatechuate 3, 4-dioxygenase1. Annu Rev Microbiol 58:555–585

Bugg TD, Ramaswamy S (2008) Non-heme iron-dependent dioxygenases: unravelling catalytic mechanisms for complex enzymatic oxidations. Curr Opin Chem Biol 12:134–140

Bundy BM, Campbell AL, Neidle EL (1998) Similarities between the antABC-encoded anthranilate dioxygenase and the benABC-encoded benzoate dioxygenase of Acinetobacter sp. strain ADP1. J Bacteriol 180:4466–4474

Decker A, Solomon EI (2005) Dioxygen activation by copper, heme and non-heme iron enzymes: comparison of electronic structures and reactivities. Curr Opin Chem Biol 9:152–163

Dunwell JM, Khuri S, Gane PJ (2000) Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily. Microbiol Mol Biol Rev 64:153–179

Hirano S, Morikawa M, Takano K, Imanaka T, Kanaya S (2007) Gentisate 1, 2-dioxygenase from Xanthobacter polyaromaticivorans 127 W. Biosci Biotechnol Biochem 71:192–9

Karlsson A, Parales JV, Parales RE, Gibson DT, Eklund H, Ramaswamy S (2003) Crystal structure of naphthalene dioxygenase: side-on binding of dioxygen to iron. Science 299:1039–1042

Koehntop KD, Emerson JP, Que L Jr (2005) The 2-His-1-carboxylate facial triad: a versatile platform for dioxygen activation by mononuclear non-heme iron(II) enzymes. J Biol Inorg Chem 10:87–93

Kovaleva EG, Lipscomb JD (2008) Versatility of biological non-heme Fe(II) centers in oxygen activation reactions. Nat Chem Biol 4:186–93

Leahy JG, Batchelor PJ, Morcomb SM (2003) Evolution of the soluble diiron monooxygenases. FEMS Microbiol Rev 27:449–479

Li L, Liu X, Yang W, Xu F, Wang W, Feng L, Bartlam M, Wang L, Rao Z (2008) Crystal structure of long-chain alkane monooxygenase (LadA) in complex with coenzyme FMN: unveiling the long-chain alkane hydroxylase. J Mol Biol 376:453–465

Lipscomb JD (2008) Mechanism of extradiol aromatic ring-cleaving dioxygenases. Curr Opin Struct Biol 18:644–649

Liu YC, Zhou TT, Zhang J, Xu L, Zhang ZH, Shen QR, Shen B (2009) Molecular characterization of the alkB gene in the thermophilic Geobacillus sp. strain MH-1. Res Microbiol 160:560–566

Munro AW, Girvan HM, McLean KJ (2007) Cytochrome P450-redox partner fusion enzymes. Biochim Biophys Acta 1770:345–359

Neidig ML, Solomon EI (2005) Structure–function correlations in oxygen activating non-heme iron enzymes. Chem Commun 47:5843–5863

Nordlund P, Eklund H (1995) Di-iron-carboxylate proteins. Curr Opin Struct Biol 5:758–766

Parales RE (2003) The role of active-site residues in naphthalene dioxygenase. J Ind Microbiol Biotechnol 30:271–278

Rieske JS, Maclennan DH, Coleman R (1964) Isolation and properties of an iron-protein from the (reduced coenzyme Q)-cytochrome C reductase complex of the respiratory chain. Biochem Biophys Res Commun 15:338–344

Roberts GA, Celik A, Hunter DJ, Ost TW, White JH, Chapman SK, Turner NJ, Flitsch SL (2003) A Self-sufficient Cytochrome P450 with a primary structural organization that includes a Flavin Domain and a [2Fe-2S] Redox Center. J Biol Chem 278:48914–48920

Rosenzweig AC (2008) The metal centres of particulate methane mono-oxygenase. Biochem Soc Trans 36:1134–1137

Senda M, Kishigami S, Kimura S, Fukuda M, Ishida T, Senda T (2007) Molecular mechanism of the redox-dependent interaction between NADH-dependent ferredoxin reductase and Rieske-type [2Fe-2S] ferredoxin. J Mol Biol 373:382–400

Steiner RA, Kalk KH, Dijkstra BW (2002) Anaerobic enzyme substrate structures provide insight into the reaction mechanism of the copper-dependent quercetin 2, 3-dioxygenase. Proc Natl Acad Sci 99:16625–16630

Throne-Holst M, Wentzel A, Ellingsen TE, Kotlar H-K, Zotchev SB (2007) Identification of novel genes involved in long-chain n-alkane degradation by *Acinetobacter* sp. strain DSM17874. Appl Environ Microbiol 73:3327–3332

Tropel D, Meyer C, Jouanneau Y (2002) Ferredoxin-mediated reactivation of the chlorocatechol 2, 3-dioxygenase from *Pseudomonas putida* GJ31. Arch Microbiol 177:345–351

Vaillancourt FH, Bolin JT, Eltis LD (2006) The ins and outs of ring-cleaving dioxygenases. Crit Rev Biochem Mol Biol 41:241–67

van Beilen JB, Smits TH, Roos FF, Brunner T, Balada SB, Röthlisberger M, Witholt B (2005) Identification of an amino acid position that determines the substrate range of integral membrane alkane hydroxylases. J Bacteriol 187:85–91

van Beilen JB, Funhoff EG, van Loon A, Just A, Kaysser L, Bouza M, Holtackers R, Röthlisberger M, Li Z, Witholt B (2006) Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. Appl Environ Microbiol 72:59–65

van Beilen JB, Funhoff EG (2007) Alkane hydroxylases involved in microbial alkane degradation. Appl Microbiol Biotechnol 74:13–21

van Berkel WJ, Kamerbeek NM, Fraaije MW (2006) Flavoprotein monooxygenases, a diverse class of oxidative biocatalysts. J Biotechnol 124:670–689

Yamaguchi M, Fujisawa H (1982) Subunit structure of oxygenase component in benzoate-1, 2-dioxygenase system from Pseudomonas arvilla C-1. J Biol Chem 257:12497–12502