Non-heme iron oxygenases

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Our understanding of the biological significance and chemical properties of non-heme iron oxygenases has increased dramatically in recent years. New group members have emerged from genome sequences and biochemical analyses. Spectroscopic and crystallographic studies have provided critical insights into catalysis. Self-hydroxylation reactions, commonplace in these proteins, reveal important features of metallocenter reactivity.

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Abbreviations

ACCO	1-aminocyclopropane-1-carboxylate oxidase
αKG	α-ketoglutarate (or 2-oxoglutarate)
CAS	clavaminate synthase
Δ9D	stearoyl-acyl carrier protein Δ^9 desaturase
DAOCS	deacetoxycephalosporin C synthase
EPR	electron paramagnetic resonance
HIF	hypoxia-inducible factor
HPPD	4-hydroxyphenylpyruvate dioxygenase
IPNS	isopenicillin *N* synthase
MMO	methane monooxygenase
MPC	metapyrocatechase (or catechol 2,3-dioxygenase)
PCD	protocatechuate dioxygenase
R2	ribonucleotide reductase subunit
ROO	rubredoxin:oxygen oxidoreductase
TauD	taurine/αKG dioxygenase
TfdA	2,4-dichlorophenoxyacetic acid/αKG dioxygenase

Introduction

In the four years since oxygen-activating non-heme iron enzymes were reviewed here [1], significant advances have increased our understanding of these versatile catalysts. Solomon *et al.* [2••] carried out a comprehensive review of the field two years ago, whereas we focus our discussion on five themes arising out of recent work: the rapid growth in number of enzymes recognized as being in this group, significant new structures derived from X-ray crystallography studies, the multitude of self-hydroxylation reactions among the non-heme iron oxygenases, similarities and differences within the dinuclear enzymes, and the development of tools to probe Fe(II) sites. Readers of this Opinion may also value more detailed treatments of selected topics as found in recent reviews on α-ketoglutarate (αKG)-dependent dioxygenases [3•,4], aromatic amino acid hydroxylases [5], oxygenase mechanisms [6], structure/function aspects of the broader family of non-heme iron proteins [7••], and geometric and electronic features of these enzymes [2••].

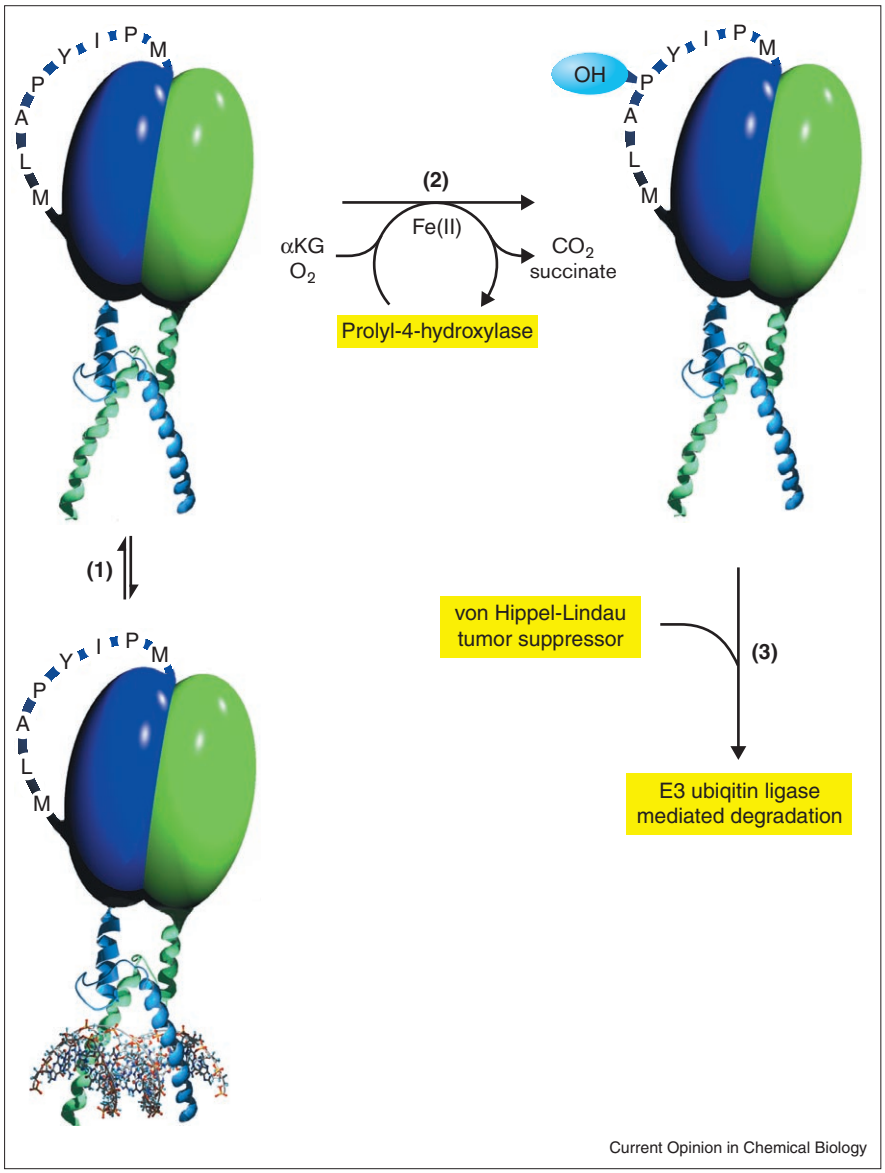
Expansion of the non-heme iron oxygenase family

The number of known and suspected non-heme iron oxygenases and related enzymes is increasing rapidly because of intense biochemical investigation and, more significantly, the wealth of sequence data becoming available from genome-sequencing efforts. For example, the recently sequenced plant *Arabidopsis thaliana* contains 64 open reading frames encoding potential Fe(II)- and αKG-dependent dioxygenases [3•]. Sequence profile searches suggest the DNA repair enzyme AlkB (unrelated to alkane monooxygenase, another non-heme iron enzyme using the same genetic abbreviation), the extracellular matrix protein leprecan, and many additional proteins are members of this enzyme group [8]. Biochemical characterizations have newly revealed additional αKG dioxygenases in plants, animals, microorganisms (e.g. [9–11]), and even a virus [12]. Particularly noteworthy are reports [13••,14–16] describing a conserved family of prolyl-4-hydroxylases involved in cellular oxygen sensing (Figure 1). In the presence of oxygen, Fe(II) and αKG these enzymes specifically hydroxylate a particular prolyl residue in one subunit of the hypoxia-inducible factor (HIF), a eukaryotic transcription factor. This modification stimulates binding by additional protein components that target that subunit of the transcription factor for proteolysis. Another notable example of a mononuclear non-heme iron oxygenase is 4-hydroxymandelic acid synthase, required for biosynthesis of the vancomycin antibiotics [17]. The di-iron carboxylate family of oxygenases similarly is expanding and now probably includes ubiquinol (alternative) oxidase in plant mitochondria [18], Coq7 involved in ubiquinone biosynthesis [19], a carotenoid desaturase [20], Crd1 needed for photosystem I accumulation in copper deficiency and hypoxia [21], and other putative new members. Continued sequence and biochemical analyses are certain to identify many additional representatives of the oxygen-activating non-heme iron enzymes (see also Update).

Significant new structures

Recent crystallographic investigations have extended our knowledge regarding catalysis by the αKG-dependent dioxygenase family [4]. For example, the structures of clavaminate synthase (CAS) with bound *N*-α-acetyl-L-arginine or proclavaminic acid (Figure 2a,b) reveal how the different substrates are uniquely positioned for the separate hydroxylation and oxidative cyclization/desaturation reactions catalyzed by the enzyme [22••]. Additional studies with a mutant form of deacetoxycephalosporin C synthase (DAOCS) illustrate the structure (Figure 2c) of a possible product complex in which αKG is replaced by succinate and unhydrated CO_2 [23]. In very elegant studies, the structures of a product complex and a possible reaction intermediate (Figure 2d,e) were inferred for the related enzyme isopenicillin *N* synthase

Figure 1



Oxygen sensing by eukaryotic cells involves specific hydroxylation of one HIF subunit by the non-heme iron enzyme prolyl-4-hydroxylase. HIF is a heterodimeric transcription factor formed by interaction of HIFα (shown in blue) with one of several other transcription factors such as the aryl hydrocarbon receptor nuclear translocator (in green). The heterodimer binds to specific sites on DNA (step **1**) to regulate transcription of nearby genes. In the presence of sufficient oxygen, αKG, and Fe(II), a particular prolyl residue in HIF is hydroxylated by a prolyl hydroxylase, an αKG-dependent dioxygenase (step **2**). This modification leads to binding of the von Hippel–Lindau tumor suppressor protein and ubiquitin ligase (step **3**), resulting in proteasome-mediated proteolysis [13••,14–16]. This figure is based on a HIF–DNA model determined for the 59-residue DNA-binding fragments of the heterodimer [80].

(IPNS) [24••]. IPNS crystals were obtained with bound substrate (L-α-aminoadipoyl-L-cysteinyl-D-valine) or substrate analogue (L-α-aminoadipoyl-L-cysteinyl-L-*S*-methyl cysteine), exposed to high pressures of oxygen, frozen, and structurally characterized to reveal enzyme with bound bicyclic or monocyclic product structures. Another member of this class of enzymes, proline 3-hydroxylase, was also structurally characterized in the absence of substrate [25]. All αKG-dioxygenase family members reveal a common 'jellyroll' fold with three iron ligands derived from a conserved His-X-Asp/Glu-X_n-His motif (where the values observed for n divide these enzymes into three distinct groups) [4,26•].

Exciting structural results also were achieved with 4-hydroxyphenylpyruvate dioxygenase (HPPD) and various ring-cleaving dioxygenases. HPPD is mechanistically related to the αKG-dependent dioxygenases; however, its structure [27•] possesses the same fold as one class of extradiol ring-cleaving enzymes that includes catechol 2,3-dioxygenase (also known as metapyrocatechase [MPC]) [28]. These enzymes coordinate Fe(II) via two histidines and one glutamic acid (Figure 2f,g) in a sequence motif distinct from that observed in the αKG-dependent dioxygenases. LigAB, a protocatechuate 4,5-dioxygenase (4,5-PCD), is the first structurally defined representative of an unrelated class of extradiol dioxygenases [29•]. The Fe(II) center of this enzyme (Figure 2h) is bound by two histidines and one glutamic acid, but the metal ligation involves a unique fold with a novel pattern of coordinating residues. Still another new structural class of ring-cleaving dioxygenases was defined by analysis of homogentisate dioxygenase (Figure 2i) [30•], a protein needed to prevent

alkaptonuria in humans. In contrast to the two-histidine-one-carboxylate ligand set used to coordinate Fe(II) in the above enzymes, the intradiol dioxygenases (such as the recently reported 3,4-PCD [31] and catechol 1,2-dioxygenase [32] from *Acinetobacter* sp. ADP1) utilize two histidines and two tyrosines to coordinate their Fe(III) sites (Figure 2j,k). Substrate binding leads to displacement of one tyrosine ligand that is then available to participate in catalysis. Oxygen is unlikely to bind to the oxidized metal site in the intradiol dioxygenases, but rather is thought to react directly with substrate [2••].

We highlight reports describing crystal structures of five other oxygen-activating non-heme iron enzymes; most provide fresh insight into previously described mono nuclear and dinuclear structures, but one example represents a novel dinuclear structure. Refinement and cyclic averaging of the naphthalene 1,2-dioxygenase structure (Figure 2l) revealed unexpected electron density for a flat aromatic compound at the Fe(II) active site [33] indicating an indole–dioxygen adduct. This structure supports a proposal involving iron-bound peroxide attack on the substrate. The crystal structure of the purple oxidized form of lipoxygenase (Figure 2m) revealed Fe(III) complexed to a lipid hydroperoxide [34•]. Human phenylalanine hydroxylase was structurally characterized in the presence of 7,8-dihydro-L-biopterin (Figure 2n), revealing a 6.1 Å distance between the metal and oxidized cofactor [35]. This distance shrinks to 5.9 Å in the catalytically active, reduced enzyme [36]. Methane monooxygenase (MMO) hydroxylase (Figure 3a) was examined with its di-iron site in three oxidation states: Fe(II)Fe(II), Fe(II)Fe(III) and Fe(III)Fe(III) [37]. Upon oxidation, one iron atom of the di-iron core and its glutamic acid ligand shifted significantly, and this process is thought to have mechanistic implications in this and related enzymes. Additional studies with the same enzyme reported the structures of the methanol and ethanol product complexes [38] and the putative substrate-binding cavities deduced from the xenon and halogenated alkane-binding sites [39]. Finally, the structure was defined for rubredoxin:oxygen oxidoreductase (ROO), a protein that allows *Desulfovibrio gigas* to survive brief exposure to oxygen [40••]. In addition to a flavodoxin-like domain, ROO has a domain containing a novel di-iron center in a Zn-β-lactamase-like fold (Figure 3b). Homologues of this protein are encoded within genomes of a wide range of bacteria and archaea, suggesting an important physiological function.

Self-hydroxylation reactions

Non-heme iron oxygenases have been found to catalyze several types of post-translational modification reactions of their amino acid side chains. For example, mass spectrometry was used to demonstrate the αKG-dependent hydroxylation of Trp112, adjacent to a metal ligand, in 2,4-dichlorophenoxyacetic acid/αKG dioxygenase (TfdA) [41]. Modification of aromatic side chains also occurs in selected mutants of the metal-binding subunit of ribo-nucleotide reductase subunit (R2). For example, Phe208 becomes hydroxylated at the *meta* position in the Glu238Ala variant, whereas a Tyr208 variant forms dihydroxyphenylalanine at this position [42,43]. *Meta* hydroxylation of Phe300 at the active site of tyrosine hydroxylase has been noted for enzyme incubated with a large excess of 7,8-dihydropterin, dithiothreitol, and Fe(II) [44,45]. Similar reactions are likely to occur in HPPD to account for the characteristic blue color of oxidized enzyme (diagnostic of phenolate coordination of Fe(III)). This conjecture is based on structural studies (Figure 2f) that reveal the absence of tyrosine, but presence of several phenylalanine, at the active site [27•]. The ability of a non-heme iron site to catalyze such reactions in an enzyme is not surprising given the analogous chemistry reported for an αKG-dependent dioxygenase mimic [46].

The physiological relevance of the self-hydroxylation reactions described above remains to be determined. It is possible that the modifications arise from spurious side reactions carried out by the activated oxygen species formed at the active site. Because the modifications do not abolish activity (e.g. modified TfdA [41] appears to be active), it is possible that their formation protects the enzymes from more damaging oxidative reactions such as cleavage of the peptide backbone. Regardless of the possible roles for such modifications, analysis of their formation may aid in identification of activated enzyme intermediates. For example, the αKG-dependent self-modification reactions occur more slowly than substrate hydroxylation, so it may be possible to detect intermediates in the absence of substrate. Thus, studies to examine the chemistry of side chain modification may enhance our understanding of the chemistry involved in catalysis.

Di-iron enzymes

With the exception of the Zn-β-lactamase-type fold in ROO [40••], the di-iron carboxylate enzymes possess similar four-helix bundle three-dimensional structures (Figure 3). Not surprisingly, they also share many spectroscopic intermediates related to their oxygen-activating activities. For example, a blue peroxodiferric intermediate has been characterized in the ferroxidase reaction of ferritin [47], stearoyl-acyl carrier protein Δ^9 desaturase (Δ9D) [48], R2, and MMO. The Fe–Fe distance (2.53 Å) of the ferritin center is much shorter than typically observed in the di-iron carboxylate centers, suggesting why it eliminates peroxide as a product [49]. In other cases, the peroxodiferric species converts to higher valent intermediates including Fe(IV)Fe(IV) species (such as Q in MMO) or Fe(IV)Fe(III) species (such as X in R2) [2••], as discussed below. Several of these proteins also generate oxo-bridged diferric centers, but again slight differences exist. Specifically, resonance Raman methods indicate that the source of the oxo bridge is water in Δ9D versus O_2 in R2 [50]. Subtle changes in the protein structure appear to tune the metallocenter properties and reactivity to dictate the reaction catalyzed. As a vivid illustration of this control, mutations were designed into R2 to change it into a self-hydroxylating monooxygenase reminiscent of MMO activity [43].

Figure 2

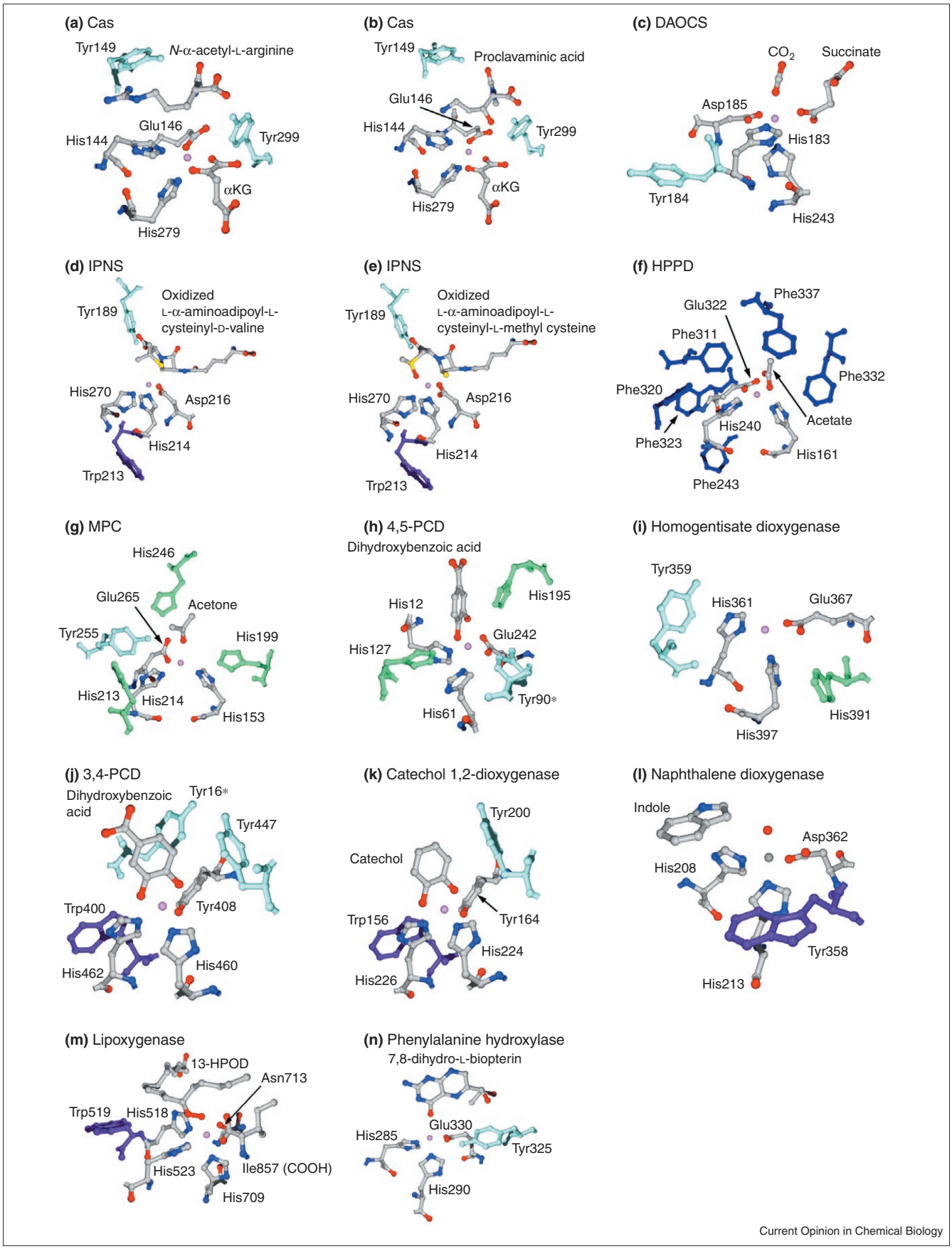


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Figure 2 legend

Structures of the active sites of mononuclear non-heme iron enzymes. Structures (and their PDB codes) are **(a)** CAS with bound *N*-α-acetyl-L-arginine (1DRY) [22••], **(b)** CAS with bound proclavaminic acid (1DRT) [22••], **(c)** DAOCS with bound CO_2 and succinate (1E5H) [23], **(d)** IPNS with bound isopenicillin generated by oxygen exposure of the substrate (1QJE) [24••], **(e)** IPNS with the product derived from in crystal oxidation of L-α-aminoadipoyl-L-cysteinyl-D-valine (1QJF) [24••], **(f)** HPPD with bound acetate (1CJX) [27•], **(g)** MPC with bound acetone (1MPY) [28], **(h)** 4,5-PCD with bound dihydroxybenzoate (1B4U) [29•], **(i)** homogentisate dioxygenase (1EY2) [30•], **(j)** 3,4-PCD with bound dihydroxybenzoate (1EOB) [31], **(k)** catechol 1,2-dioxygenase with bound catechol (1DLT) [32], **(l)** naphthalene dioxygenase with bound indole (1EG9) [33], **(m)** lipoxygenase (1E5D) [34•], and **(n)** phenylalanine hydroxylase with bound 7,8-dihydro-L-biopterin (1DMW) [35]. The residue numbers are shown for ligands and, in selected cases, nearby histidine (green), tyrosine (light blue), phenylalanine (dark blue), or tryptophan (purple) residues. An asterisk next to the ligand number indicates that it derives from another subunit than that for the other residues shown. HPOD is (9*Z*,11*E*)-13(*S*)-hydroperoxy-9,11-octadecadienoic acid.

A wide range of approaches has been used to investigate catalytic intermediates of di-iron oxygenases, with much recent attention focused on MMO (reviewed in [51]). Kinetic studies suggest the presence of two distinct dioxygen adducts of this enzyme, only the second of which exhibits the aforementioned blue absorption (λ_{max} = 700–725 nm; ε = 1800–2500 M^{-1} cm^{-1}) [52,53]. The blue chromophore directly converts to the high-valent intermediate Q, a yellow (λ_{max} ~425 nm, ε ~ 8000 M^{-1} cm^{-1}) species thought to possess a di-μ-oxo di-Fe(IV) 'diamond core' structure. Various spectroscopic analyses as well as density functional calculations [54,55] allow the formulation of detailed structural models of each of these species. Q hydroxylates MMO substrates by reactions that exhibit several anomalous features. For example, the reaction rates vary linearly with substrate concentrations. Furthermore, these rates do not correlate to C–H bond energies of the substrates. In addition, methane oxidation exhibits a very large deuterium kinetic isotope effect. Finally, Arrhenius or Eyring plots show non-linear temperature effects for methane, but not for CD_4 or other substrates [53,56•]. These results led Brazeau and Lipscomb [56•] to propose a mechanism involving two successive steps in which Q-like species bind two substrate molecules with different activation parameters. This two-step mechanism is suggested to be compatible with the above anomalous results, and is certain to stimulate further studies.

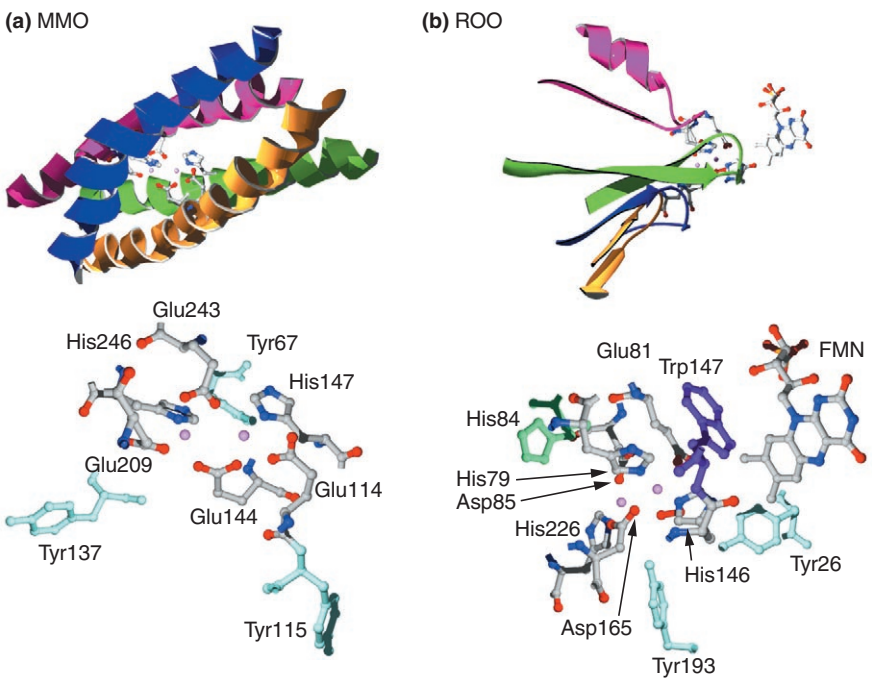
Additional studies using radical clock methods have tested for the intermediacy of a substrate radical during catalysis by di-iron oxygenases. These studies can be confounded by steric effects leading to preferred orientations of the substrate analogues at the active site, misidentification of trace products, and numerous other concerns; thus, it is not surprising to find inconsistencies in the literature. In one recent MMO study, the absence of rearranged products during oxidation of a series of substituted cyclopropanes was thought to rule out a radical intermediate [57]. Subsequent MMO studies with other cyclopropanes did detect rearrangements, but these could be accounted for by cationic or radical intermediate species [58,59]. Oxidation of methylcubane by MMO was shown to yield small amounts of cubylmethanol and methyl cubanols, but the major species was an unidentified rearranged product leading to the suggestion of a radical intermediate in the reaction [60]. Later studies identified this species as homocubanol, a compound derived from cationic rearrangement [58]. The most definitive studies were those examining MMO-catalyzed oxidation of norcarane. All carbon atoms are similarly accessible in this compound and the products derived from radical and cationic rearrangements clearly differ. The results suggest formation of an initial substrate radical intermediate (of at least 20 ps lifetime) that undergoes oxygen rebound, intramolecular rearrangement followed by oxygen rebound, or oxidation to a cationic intermediate that reacts with hydroxide [61•]. Analogous studies carried out with alkane monooxygenase also indicate the intermediacy of a carbon-centered substrate radical, in that case with a lifetime of ~ 1 ns [62•].

Analysis of oxygen activation by ribonucleotide reductase continues to yield new insights as revealed by two areas of research. In R2 of *Escherichia coli*, formation of the high-valent intermediate X was previously proposed to involve electron transfer from the near-surface residue Trp48. This tryptophanyl cation radical has now been confirmed based on its absorption at 560 nm and its $g = 2$ EPR (electron paramagnetic resonance) signal [63]. Substitution of Trp48 by phenylalanine causes a diversion of the electron transfer chemistry to generate a radical at Tyr122 [64]. The resulting diradical (X-tyrosyl radical pair) was extensively characterized and may serve as a benchmark for understanding other radical/paramagnet systems. The yeast *Saccharomyces cerevisiae* contains two R2-like proteins, only one of which (Y2) develops a diferric center and tyrosyl radical. The second R2 homologue, Y4, lacks two histidines and a glutamic acid that function as metal ligands in other di-iron systems. Y4 is needed for formation of the di-iron center and the tyrosyl radical in Y2, but Y4 does not bind iron or help to fold Y2 [65]. Both Y2 and Y4 form homodimers, but together they form a heterodimer that has been crystallographically characterized [66]. The precise role of Y4 in ribonucleotide reductase activation in yeast and the extent of similarity between the yeast and bacteria systems are unclear.

Tools for probing Fe(II) states

Fe(II) centers are often spectroscopically 'silent'; thus, their analysis in non-heme iron proteins can represent a formidable challenge. Kinetic methods can provide K_d

Figure 3



Structures of the active sites of dinuclear non-heme iron enzymes. Structures (and their PDB codes) of the regions surrounding the di-iron sites are shown for **(a)** the diferrous form of MMO (1FYZ) [37] and **(b)** ROO (1E5D) [40••], comparing both (top) the secondary structures and (bottom) the residues at the active sites. Nearby histidine (green), tyrosine (light blue), or tryptophan (purple) residues are highlighted in addition to the metal ligands.

values and Fe(II) dissociation rates, as nicely illustrated in a recent study of 1-aminocyclopropane-1-carboxylate oxidase (ACCO) [67]. Such studies reveal that mononuclear sites often bind the metal ion only very weakly (e.g. K_d is around the micromolar range for ACCO), raising the unanswered question of how the enzymes function within the cell where the free Fe(II) concentrations are likely to be much lower. Useful insights into the properties of an Fe(II) center can be obtained by substituting the metal with Cu(II), which is more amenable to EPR spectroscopic methods (e.g. [26•,68,69]); however, the results must be interpreted with caution because the metals differ significantly in their coordination properties. Alternatively, a paramagnetic center is formed with the native Fe(II) site upon binding nitric oxide, an oxygen analogue. A variety of spectroscopic approaches have been used to investigate proteins containing NO-bound centers (e.g. TfdA [68], ACCO [70], and MMO [71]). Mössbauer analysis does not require that the iron site be paramagnetic, and has been of great utility in identifying intermediates in the non-heme iron enzymes [43,71]. Arguably, one of the most powerful spectroscopic tools to study the Fe(II) sites of non-heme iron proteins is variable temperature magnetic circular dichroism spectroscopy [72]. Recent examples of its use include studies of CAS [73], phenylalanine hydroxylase [74], reduced 3,4-PCD [75], and R2 [76], revealing important features of active site coordination and changes induced by substrate binding. Finally, the Fe(II) sites of αKG-dependent dioxygenases are unique in forming a diagnostic chromophore in the absence of oxygen and presence of αKG (e.g. in taurine/αKG dioxygenase (TauD) [77•] and TfdA [68]). This weak chromophore (λ_{max} ~530 nm, ε ~250 M^{-1} cm^{-1}), also studied by resonance Raman spectroscopy [78], is perturbed upon binding of the substrate and shown to be sensitive to the coordination number (decreasing from six to five upon substrate binding). As indicated by these examples, a variety of methods have been successfully applied to study Fe(II) sites in non-heme iron proteins.

Conclusions

Although still under-investigated compared with heme enzymes, the study of non-heme iron oxygenases is a vigorous field with the medical, ecological and biotechnological importance of these enzymes becoming better realized. The total number of non-heme iron enzymes and the number of structurally characterized examples are increasing rapidly. Numerous tools are in place to characterize all redox states of these enzymes, and mechanistic understanding will continue to be refined. In particular, the effects and importance of self-hydroxylation reactions in this group of enzymes will be further examined. Another important direction for future studies involves the use of site-directed mutagenesis to specifically alter the reactivity and substrate specificity of these enzymes (e.g. [43,79]). The field has come far in the past four years, and the pace is unlikely to slacken in the future.

Update

Significant advances recently were reported for two classes of ferrous ion-dependent oxygenases. Carotenoid cleavage

dioxygenases play critical roles in the synthesis of a variety of chemical compounds including retinoids and the plant hormone abscisic acid. Although no metallocenter studies have yet been reported for this group of enzymes, representative genes encoding these enzymes have now been cloned and expressed from plants, insects, and vertebrates, including humans (e.g. [81–85]). Similarly, the gene encoding *myo*-inositol oxygenase, which catalyzes the first committed step in the pathway of *myo*-inositol catabolism, has been cloned and expressed from pig kidney [86].

Detailed spectroscopic and electronic structure studies also were recently reported for the ferric enzyme protocatechuate 3,4-dioxygenase [87]. These results highlight features associated with the two tyrosinate ligands, one of which dissociates during catalysis while the other exhibits a strong *trans* influence on substrate activation.

Acknowledgements

We sincerely thank the many investigators who provided reprints and preprints of their work.

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