The bioinorganic chemistry of iron in oxygenases and supramolecular assemblies

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The bioinorganic chemistry of iron is central to life processes. Organisms must recruit iron from their environment, control iron storage and trafficking within cells, assemble the complex, iron-containing redox cofactors of metalloproteins, and manage a myriad of biochemical transformations by those enzymes. The coordination chemistry and the variable oxidation states of iron provide the essential mechanistic machinery of this metabolism. Our current understanding of several aspects of the chemistry of iron in biology are discussed with an emphasis on the oxygen activation and transfer reactions mediated by heme and nonheme iron proteins and the interactions of amphiphilic iron siderophores with lipid membranes.

That an iron-containing enzyme mediated the activation and transfer of molecular oxygen into its substrate was first demonstrated by Hayashi et al. (1) in the 1950s. It was shown, in some of the first mechanistically informative oxygen isotopic measurements, that both of the inserted oxygen atoms in the conversion of catechol to cis-muconic acid derived from O2 and not water. These findings challenged the then-firmly held view that oxygen in biomolecules was derived exclusively from water via hydration processes. The biosynthesis of cholesterol and its precursor, lanosterol, from the hydrocarbon squalene were also shown to derive their oxygen functionality from molecular oxygen (2). Here, a single oxygen atom derived from molecular oxygen while the other was transformed to water. Later, the prostaglandins were shown to derive from the incorporation of two molecules of oxygen to form, initially, an alkyl hydroperoxide-endoperoxide. Thus, what appeared at first to be an obscure process of bacteria and fungi became recognized as a major theme of aerobic metabolism in higher plants and animals. The subsequent search for “active oxygen species” and efforts to elucidate and understand the molecular mechanisms of oxygen activation and transfer have been richly rewarding. The roles of iron in these wonderfully varied processes have been a major force in the development of both bioinorganic chemistry and chemical catalysis over the past three decades. Novel and unusual iron redox chemistry has appeared as our understanding of biological iron acquisition, transport and storage, and enzymatic oxidation strategies has developed. The goal of this perspective is to discuss several aspects of the current state of bioinorganic chemistry relating to iron and our understanding of the chemical pathways and mechanisms by which iron–oxygen systems function.

Oxygen Activation by Heme Proteins

The heme-containing metalloenzymes cytochrome P450 (3), chloroperoxidase (CPO, refs. 4 and 5), NO synthase (NOS, ref. 6), and their relatives catalyze a host of crucial biological oxidation reactions. Highly specific P450s are involved in the selective oxygenations of steroid and prostaglandin biosynthesis. Myeloperoxidase, which is a CPO, is an integral part of the immune response, and NOS is the source of the highly regulated signal transducer NO. Certain fungal CPOs and bacterial P450s have been genetically engineered for large-scale biotransformations (7–10). The active sites of these three protein families, known in detail from a number of x-ray crystal structures (4, 11–13), are remarkably similar. All three have an iron-protoporphyrin IX center coordinated to a cysteine thiolate. All of them are oxidoreductases that activate molecular oxygen (O2), in the cases of P450 and NOS, or hydrogen peroxide in the case of CPO, at the iron center and incorporate one of the oxygen atoms into a wide variety of biological substrates, with concurrent transformation of the other oxygen atom to H2O. All three are proposed to initiate their chemistry through the oxidation of a resting iron(III) state (1) to a reactive oxoiron(IV)–porphyrin cation radical intermediate (2) (Fig. 1). A depiction of the CPO active site derived from the crystal structure of this protein from Calderomyces fumago is shown in Fig. 5, which is published as supporting information on the PNAS web site, www.pnas.org. The structure, biochemistry, molecular biology, and the chemistry of cytochrome P450 and related model systems have been extensively reviewed (14–18).

Our understanding of the mechanism of action of these heme proteins comes from the direct observation of intermediates in the catalytic cycle through a variety of spectroscopic techniques, the use of diagnostic substrates with mechanistically revealing rearrangements during oxidation, and the parallel development of the chemistry of synthetic metalloporphyrins. The principal features of the consensus mechanism of cytochrome P450 (19) are as outlined in Scheme 1: binding of substrate to the enzyme, sometimes accompanied by a spin-state change of the iron, to afford an enzyme-substrate adduct; reduction of the ferric cytochrome P450 by an associated reductase with an NADPH-derived electron to the ferrous cytochrome P450; binding of molecular oxygen to the ferrous heme to produce a ferrous cytochrome P450–oxygen complex; and, finally, oxygen atom transfer from this iron-oxo intermediate to the bound substrate to form the oxygenated product complex. Product dissociation completes the cycle.

There were a number of important realizations in the course of elucidating this mechanism. That hydrogen peroxide, alkyl hydroperoxides, periodate, and iodosylbenzene were also functional with cytochrome P450 suggested that the chemistry of “oxygen activation” was the two-electron reduction of molecular oxygen to hydrogen peroxide and that, in analogy to the peroxidases, the active oxygen species was a ferryl (or oxene) complex Fe=O, formally iron(V). It was shown that a synthetic oxoiron(IV)porphyrin cation radical species could be formed at low temperature by the oxidation of an iron(III) precursor with peroxyacids (9 → 10) (20). Intermediate 10 did have the requisite reactivity to transfer an oxygen atom to hydrocarbon substrates. It is this oxygen atom transfer from the oxygen donor to the substrate complex 8 that has been termed oxygen rebound. Such an iron-oxo species (compound I) has been observed for the CPO of C. fumago (21), but the active species of cytochrome P450 has remained elusive. Very recently, it has been shown that an intermediate with the spectral properties similar to those of CPO com-

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compound I and the model iron porphyrin systems is formed upon the oxidation of Cyp119, a thermostable cytochrome P450, with a peroxyacid, analogous to the model systems (22). Consistent with the high reactivity expected for P450 compound I, this intermediate decayed with a rate constant of 29 e−1 at 4°C. Interestingly, similar experiments with P450cam, the camphor oxidizing enzyme from Pseudomonas putida, resulted in an iron(IV)-protein tyrosine radical species, presumably via a one-electron oxidation of Tyr-96, which is only 9.4 Å from the iron center (21).

There has been much discussion in the field about the oxygen transfer process 6 → 7 → 8. Specifically, the hydroperoxo iron(III) complex 6 has also been suggested to effect substrate oxygenations based on observed changes in product ratios and loss of hydrogen peroxide (uncoupling) upon P450 active site mutations (23). An important recent advance has been the development of cryo-spectroscopic studies by Hoffman and colleagues (24) that have allowed the step-wise interrogation of intermediates depicted in Scheme 1. Thus, the injection of an electron into complex 5 via γ-radiation, followed by thermal annealing of the sample has produced EPR and electron nuclear double resonance evidence for the formation of first a hydrogen-bonded hydroperoxo species and then the iron–hydroperoxo complex 6. Although no ferryl intermediate 7 was observed, the product alcohol was found to be formed with its oxygen atom coordinated to the iron center and with the substrate-derived proton attached to the product alcohol as depicted in structure 8 (Scheme 1). This arrangement has important mechanistic implications because, if a ferryl species (7) is the immediate precursor of the product complex 8, then coordination of the product oxygen would be a necessary consequence. By contrast, if the hydroperoxo species 6 were the source of the electrophilic oxygen, then water would be coordinated to iron rather than the product alcohol. A product complex such as 8 could also be the source of cationic rearrangement products that are sometimes observed during P450 oxygenations.

Significant recent advances in computational approaches to the study of biological catalysis, and the applications of these techniques to the cytochrome P450 mechan- ism have also been illuminating. Thus, Shaik and colleagues (25) have presented the results of a density functional theory analysis of the reactivity of hydroperoxo iron(III) complexes such as 6. The protonation and heterolytic O–O bond cleavage of 6 to afford a ferryl species analogous to 7 was found to proceed with little energetic barrier, in accord with earlier experimental results for the oxidation of an iron(III) porphyrin 9 to an iron(IV)porphyrin cation radical species 10 with a peroxyacid (26). Further, the oxygen transfer from 7 to ethylene to form an epoxide proceeded with only a low barrier. It was concluded that the density functional theory (DFT) calculations exclude a hydroperoxoiron(III) intermediate such as 6 as a reactive, electrophilic oxidant. Several modes of oxygen transfer from the hydroperoxide intermediate encountered exceedingly high barriers for reaction. The lowest energy of these was an interaction of the substrate ethylene with the proximal, iron-bound oxygen of the Fe(III)-OOH ensemble.

Nucleophilic reactions of a hydroperoxy iron(III) intermediate 6, as have been suggested by Akhtar et al. (27), Cole and Robinson (28), and Vaz et al. (29) for the deoxygenation reactions characteristic of the P450 aromatase, do seem to be suggested by the significant basicity of the distal, hydroxylc oxygen found in the calculations for the Fe(III)-OOH group. This mode of reactivity is highly analogous to the reactions of enzymes such as cyclohexanone monoxygenase that proceed through a flavin 4a-hydroperoxide (30). Here, only electron-deficient olefins react to afford epoxides even thought the flavin hydroperoxide is $2 \times 10^5$ times more reactive than a simple alkyl hydroperoxide (31). The hydroxylation of a C—H bond does seem to require the full formation of a reactive ferryl intermediate as in 11. This applies both for the reductive activation of dioxygen and the very revealing cases of alkyl hydroperoxide isomerization catalyzed by P450 in which the alkyl group (R) occupies the substrate-binding cavity (32, 33). For P450s in which the proton relay system has been disrupted by active-site mutations, one would expect that particularly reactive substrates could interact with the proximal oxygen earlier in this reaction profile as shown in 12. Although similar atomic trajectories and electronic charge redistributions are followed in each case, the former (11) is analogous to the S81 reaction in organic chemistry, generating a discreet ferryl intermediate, whereas the latter (12) is Sn2-like, requiring assistance from the electron-rich substrate. Indeed, in a recent report by Jin et al. (34), mutation of the conserved active site threonine-252 to alanine in P450cam was shown to disable camphor hydroxylation while maintaining some reactivity for more reactive olefinic substrates. Similarly, two reactive intermediates, as suggested by Voz et al. (35) for the reactions of a thioether substrate, and also for model porphyrin systems described by Nam et al. (36), could reasonably derive from a mechanistic spectrum of this type. An important precedent for this behavior is seen in the reactions of peroxyacids with model Fe(III) porphyrins. Thus, Machii, Watanabe, and Morishima (37, 38) have shown that the iron-coordinated peroxyacid 9 reacted with olefins at the iron-coordinated oxygen atom in nonpolar solvents to give epoxides but would not react with saturated hydrocarbons. By contrast, the same oxoiron(IV)porphyrin cation radical, 10, was formed with a variety of peroxyacids in more polar media. This effect is also seen in model compounds with a thiolate ligand to iron (39). The protein-derived hydrogen bonds to the axial thiolate ligand to iron in P450cam have been shown to affect the O–O bond cleavage (40).

**Mechanisms and Molecular Trajectories for Hydroxylation by Cytochrome P450**

Among all of the varied reactions mediated by cytochrome P450 none has captured the imagination of chemists more.

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**Fig. 1.** Iron(III) protoporphyrin IX with a cysteinate as the axial ligand (1), which is typical of cytochrome P450, CPO, and NOS enzymes. The active oxygen species of these proteins and related heme enzymes is an oxoiron(IV)porphyrin cation radical (2), often called compound I.

**Scheme 1.** Consensus catalytic cycle for oxygen activation and transfer by cytochrome P450.

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mediate radical could be trapped in a sub-
radical at the heme active site. The inter-
rearrangements observed were consistent
arrangements that are known to accom-
observations of a variety of molecular re-
view. A nonconcerted pathway for C
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that can form the product alcohol coordinated to the ferric, resting
form of the protein (8).

2.4 \times 10^9 \text{s}^{-1} \) at room temperature by
using laser flash photolysis techniques
(45). Thus, a rate constant of \( k_{OH} =
1.7 \times 10^{10} \text{M}^{-1}\text{s}^{-1} \) was estimated for the
rebound process. Radical clocks with very
fast rearrangement times were shown to
produce less rearrangement than slower
clocks in the P450-mediated hydroxy-
ations, however. The results led Newcomb
et al. (46) to question whether a radical
pathway existed because the apparent life-
times revealed by these probes were in the
range of 100 fs, too short to represent a
bona fide intermediate. Thus, either there
was something wrong with the clocks or
there were unrecognized subtleties in rad-
cal rebound mechanism. Several sugges-
tions have been considered to resolve this
dilemma and the question is still an area
of active experiment and debate. As
shown in Scheme 2, the transition state
for hydrogen abstraction will position the
active oxygen only a few tenths of an A
farther from the hydroxylated carbon
atom than the transition state for the ulti-
mate C-O bond formation. Thus, the
extent of radical rearrangement might be
expected to depend critically on the tight-
ness of the radical cage and the ensemble
of steric and electronic forces experienced
by the incipient radical within the cage.
Even the molecular makeup of the active
site will depend on how the substrate fills
the site, leaving room for movement of
amino acid side chains in the vicinity of
the substrate or allowing additional water
molecules into the active-site area. The
extent of rearrangement detected by a
particular probe may simply reflect a fac-
cile molecular trajectory from the hydrogen
abstraction transition state to the hydroxy-
lation transition state in this variable envi-
ronment. For substrates with a very strong
C-H bond and a small steric size, both
effects would push the reaction coordinate
toward a tighter radical cage. Indeed, it
has been shown that the effective lifetime
of a radical intermediate can even be af-
fected by the stereochemistry of the hy-
drogen abstraction event (19). Here, the
pro-R hydrogen of ethyl benzene was hy-
droxylated with nearly complete retention
of configuration at carbon whereas the
pro-S hydrogen underwent significant
racemization.

For a reaction that involves a paramag-
etic iron-oxo intermediate and proceeds
to produce paramagnetic radical inter-
mediates, it is likely that spin-orbit coupling
effects and the spin states of reacting in-
termediates may offer another significant
consideration (47). Schwarz et al. (48) first
suggested that the unusually slow reaction of FeO^{+} with hydrogen in the gas phase
was caused by spin conservation effects
that were imposed on these intermolec-
ular encounters. Detailed density functional
theory calculations on this simplest iron-
C—O bond. By contrast, the low-spin trajectory could proceed to products without encountering this barrier. This two-state hypothesis could provide a way out of the mechanistic dilemma presented by the radical clock results because the apparent timing of the clocks would depend on the relative importance of the high- and low-spin pathways.

Is it possible to support this hypothesis by experiment? There is suggestive data in hand in the comparative behavior of P450 and a ruthenium porphyrin model system. Thus, evidence for short-lived substrate radicals has been presented recently for the oxidation of the mechanistically diagnostic probe molecule norcarane by cytochrome P450 (50). An alternate interpretation of similar data, involving unusual behavior of the probe molecule at the active site, has also been presented (51).

In all known cases of reactions involving a radical intermediate, this norcarane probe produces a product derived from the 3-cyclohexenylmethyl radical, as the major rearrangement product. The rate constant for the radical rearrangement of the 2-norcarnaryl radical has been found to be $2 \times 10^8$ s$^{-1}$. By contrast, for reactions proceeding through discreet carbocations, rearrangement leads instead to 3-cycloheptenol as the major rearrangement product. The extent of observed rearrangement with a panel of P450 enzymes leads to a radical lifetime in the picosecond to nanosecond regime, certainly long enough to be considered an intermediate. A consistent timing was found for several similar probes. Smaller amounts of cation-derived products were also observed and attributed to a competing electron transfer oxidation of the incipient radical, a well-precedent process. By contrast, the hydroxylation of norcarane with a ruthenium porphyrin catalyst that proceeds through a reactive oxoruthenium(V) porphyrin intermediate, afforded no detectable rearrangement. Density functional theory calculations on the ruthenium mediated hydroxylation show that the low-spin reaction trajectory is preferred throughout, in accord with general expectations for the behavior of second row transition metals (52).

Thus, the data for the iron and ruthenium porphyrin systems is in accord with the predictions of theory that a radical rebound process is viable for iron, which has an accessible high-spin state but not for ruthenium that is always low-spin.

Other, more exotic factors such as nonstochastic behavior (53) and tunneling effects (54) could also be involved in causing the mistiming of events during C—H bond hydroxylation. Indeed, a carbenic ring-expansion reaction was very recently found to have a large quantum tunneling effect that significantly affected the observed rate (55).

High-level calculations indicated that a thermal, over-the-barrier, process and quantum tunneling of carbon were still competitive even at room temperature. Applied to C—H hydroxylation by a reactive oxidant, this situation could give the appearance of multiple oxidants and non-Arrhenius behavior. Thus, for a step-wise reaction via the caged radical intermediate in Scheme 2, a spectrum of apparent lifetimes, perhaps dependent on vibrational state, might be observed for rebound through transition state R to intermediate 8 (Scheme 2).

The nonheme diiron hydroxylases, such as methane monoxygenase (56) and AlkB, the $\omega$-hydroxylase from P. putida, have also yielded to similar structural, spectroscopic, and mechanistic probes. Interestingly, there are striking similarities between the consensus mechanism for the heme and nonheme iron proteins. For methane monoxygenase (MMO) the resting enzyme has both iron centers in the ferric state. Reduction and binding of oxygen again produces a peroxo intermediate, which is oxidized to a reactive species, compound Q, that has been characterized as a bis-$\mu$-oxo-iron(IV) intermediate. Both AlkB (57) and MMO (51, 58) have been interrogated recently with the diagnostic probe norcarane and both have shown the radical rearrangement product, hydroxymethylcyclohexene. For the histidine-rich hydroxylase AlkB, the results were particularly striking because fully 15% of the product was indicative of the radical rearrangement pathway. A significant aspect of this work was that it was performed on whole cells and clones into which the AlkB genes had been introduced. With MMO, it was possible to show that it was the reactive intermediate Q that was interacting with the substrate probe. Thus, mechanistically informative biochemistry can be obtained from this type of biological screen.

Iron in Supramolecular Assemblies

The self-assembled and highly ordered nature of phospholipid membranes is a central structural and functional motif in biology. Many of the iron-containing oxygenases, such as cytochrome P450s, mitochondrial NOS (59–67), and AlkB, are membrane bound. Synthetic phospholipid vesicles have presented the opportunity to construct novel supramolecular assemblies and elucidate the membrane-binding properties of biomolecules. For example, a synthetic multiheme assembly has been described that recruited cytochrome c to the outer surface of phospholipid vesicles (Fig. 3, ref. 68). The structure of the construct has been probed by observing electron transfer between cytochrome c and the redox centers embedded within a...
A very interesting class of iron-binding siderophores has amphiphilic properties, having a polar head group containing the iron binding site and one or two hydrophobic side chains reminiscent of a phospholipid. The first of these to be discovered were the exochelins and mycobactins of *Mycobacterium tuberculosis* (69), rhizobactin 1021 from a terrestrial, nitrogen-fixing symbiont (70, 71), and acinetoferrin (72) from the pathogens *Acinetobacter haemoliticus* and *Acineto- bacter baumanii*. The amphiphilic marinobactins and acquachelins have been discovered more recently in marine bacteria (73), indicating that such structures are widely distributed in nature.

Current interest in the iron-uptake strategies of pathogenic organisms stems from their increasing antibiotic resistance and the rising numbers of difficult-to-treat infections in humans (74, 75). Our interest in iron and membrane dynamics has led us to investigate how the amphiphilic nature of these compounds may be advantageous to the organisms.

The molecular architecture of Fe-mycobactin J, Fe-rhizobactin 1021, and Fe-marinobactin E, as depicted in Fig. 4, can be seen to be remarkably similar. Fig. 4 also depicts the dynamic interactions of marinobactin E and its iron complex with phospholipid vesicles that have recently been elucidated (76). Rates of iron chelation could be measured by stopped-flow spectroscopy by observing the UV absorption of the iron complex. The observed chelation rate decreased as the lipid concentration increased, indicating that nearly all of the siderophore molecules were associated with the vesicles and that the iron acquisition by free marinobactin (k₁ in Fig. 4) is insignificant in comparison to the membrane-bound process k₂. Iron acquisition by a membrane-associated siderophore would be one way to mitigate losses of the siderophore molecules caused by diffusion.

Because the iron(III) complex of marinobactin is paramagnetic, it was expected that binding of Fe-marinobactin to lipid membranes could be detected by NMR line-broadening techniques. The choline methyl groups of the inner and outer membrane leaflets of small unilamellar vesicles are spectroscopically distinguishable by proton NMR because of the membrane curvature. As shown in Fig. 4 (traces A and B), the addition of Fe-marinobactin E to a lipid vesicle suspension caused a dramatic broadening of the 1H-choline resonance corresponding to the outer membrane leaflet, whereas the inner membrane choline resonance was relatively unaffected, indicating rapid binding of the siderophore to the lipid phase.

Mixed vesicle experiments were designed to see whether Fe-marinobactin molecules could reversibly dissociate from the lipid. Here, Fe-marinobactin E was first bound to a suspension of small unilamellar vesicles composed of deuterated choline N-methyls. The NMR 1H-resonance of the choline N-methyl of proteo-dimyristoylphosphatidylcholine, but without the iron(III)-siderophore, was monitored and used as an indicator of the redistribution of the Fe-Mₑ molecules. Broadening of the outer membrane leaflet choline resonance within 5 min indicated a fast redistribution of Fe-marinobactin E from one membrane population to the other with a dissociation rate, k⁻¹_, Fe-Mₑ, of 4.4 × 10⁻³ s⁻¹ (Fig. 4).

The lipid partition coefficient of Fe-marinobactin E was determined by this technique to be 2.3 × 10² M⁻¹, ~50 times smaller than that of the iron-free siderophore.

The interaction of the marinobactins and other amphiphilic siderophores with
phospholipid vesicles is of interest for elucidating the molecular mechanisms involved in these unusual amphiphilic siderophores. The membrane-binding properties of marinobactin E are very similar to those of the lipopeptide, surfactin, a detergent-like peptide of similar overall constitution. As can be seen in Fig. 4, apo-marinobactin E has an extended molecular shape that folds into a more compact, spherical head group arrangement upon iron binding. Further, two of the iron-binding ligands are immediately adjacent to the point of attachment of the hydrophobic fatty acid appendage. This arrangement is of interest for elucidating the mode of interaction of the iron-binding ligands are lost. This suggests that the amphiphilic marine aquachelins have recently been shown by Butler and colleagues (78) to be photoactive. The iron-hydroxamate chromophore absorbs visible light and the resulting ligand-to-metal charge-transfer chemistry causes an internal reduction of the iron(III) to iron(II) and oxidation of the organic portion of the siderophore. Significantly, this photochemical redox transformation causes a cleavage of the molecular species of the iron-binding side chain and two of the iron-binding ligands are lost. This could have important implications for how iron is recycled in a marine environment that is critically short of iron near the surface.

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